Riitta Paakkanen

Major Histocompatibility Complex Genes

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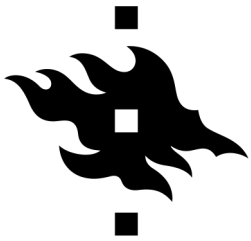
Method of Analysis, Association with Acute Coronary Syndromes and Effect on Immune Reactions

Academic Dissertation



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To my grandmother

"The saddest aspect of life right now is that science gathers knowledge faster than society gathers wisdom."

- Isaac Asimov

ABSTRACT

Coronary atherosclerosis is a leading cause of death and disability in the developed world and is emerging as such also in developing countries. Despite various efforts, its pathophysiology is not fully understood. Atherosclerosis is a chronic, inflammatory, lifestyle-disease that is also influenced by genetic factors. Coronary atherosclerosis manifests as silent, chronic and acute forms. The acute forms, acute coronary syndromes (ACS) are an important cause of death due to coronary atherosclerosis. Clinically, two different classes of ACS are distinguished. Depending on electrocardiography, patients are divided into those with ST-segment elevation and those without it (STEMI and NSTE-ACS, respectively). STEMI and NSTE-ACS differ in mortality, patient population and clinical management.

The major histocompatibility complex (MHC) region is a collection of genes on chromosome 6 that are associated with immune response. The genes in this region are usually inherited as a strongly linked genetic combination, a haplotype. Certain alleles and haplotypes of the MHC region have been linked with coronary atherosclerosis.

The aims of this Thesis were to develop a novel quantitative polymerase chain reaction (qPCR) method for genetic complement component *C4* analyses, to confirm the role of MHC genes and haplotypes in different clinical forms of ACS and to evaluate the possible mechanisms through which the association of MHC genes/haplotypes and ACS could be mediated.

A qPCR method with a novel concentration range approach and SYBR® Green dye was developed (I). The method was validated by applying it to over 1600 patient samples with available C4 protein data and by analysing 129 samples that were also assessed with other methods for *C4* gene analysis. Selected MHC genes/alleles (*C4* copy number, *C4* mutation, *HLA-DRB1*01* and *HLA-B*35*) were studied in patients with STEMI (III), whereas in patients with NSTE-ACS, the C4 variation was studied (II and IV). The control population consisted of cases without significant coronary atherosclerosis, a general population sample (III) and healthy blood donors (IV). The haplotype frequencies were computationally constructed using the Bayesian method. Possible inflammatory mechanisms linking MHC and ACS were evaluated by assessing autoantibodies to heat shock protein 60 (HSP60, enzyme-linked immunoassay), the level of high-sensitivity C-reactive protein (hsCRP, immunochemical assay) and by reanalysing the response to macrolide treatment in relation to MHC.

The results indicated that the concentration range and SYBR® Green labelling can be used to reliably assess *C4* copy number variation and mutation (I). In addition, we found that the assessment of *C4* mutation can increase the frequency of diagnosed *C4* deficiencies by nearly twenty percentages.

The presence of C4 deficiency was not increased in patients with either form of ACS, but the power was too low to exclude a weak association (III and IV). *DRB1*01* was not associated with STEMI, but a haplotype with *DRB1*01*, with neither *C4* deficiency nor *B*35* was more frequently detected in patients suffering from STEMI than in CAD-free controls (III). However, if only men were studied, both *DRB1*01* alone and the *DRB1*01*-haplotype were significantly associated with ACS.

The MHC was shown to associate with alterations in the studied inflammatory parameters. C4 deficiency segregated patients that benefitted from macrolide treatment in secondary prevention of cardiovascular disease (II). C4 deficiency was also associated with elevated HSP60 IgA autoantibody level, which, in turn, was shown to be associated with recurrent cardiovascular end points (IV). *C4* deficiency was also increased in patients suffering from recurrent infections (I). The *DRB1*01*-haplotype that was increased in STEMI associated with higher hsCRP levels in patients and in controls (III).

In summary, MHC genetics can be assessed by concentration range and SYBR® Green labelled qPCR. The MHC region encloses a *DRB1*01*-related haplotype that associates with ACS. The genetic polymorphism in MHC may be linked with ACS by affecting the inflammatory responses. These data are observational and thus do not indicate causality. However, these data might help to elucidate the complex interplay of inflammatory reactions in ACS.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

I. Paakkanen R, Vauhkonen H, Eronen K, Järvinen A, Seppänen M, Lokki M-L. Copy number analysis of complement *C4A*, *C4B* and *C4A* silencing mutation by real-time quantitative polymerase chain reaction. PLoS ONE 7(6): e38813, 2012.

II. Paakkanen R, Palikhe A, Seppänen M, Nieminen M S, Vauhkonen H, Saikku P, Lokki M-L, Sinisalo J. Beneficial effect of clarithromycin in patients with acute coronary syndrome and complement C4 deficiencies. Scand Cardiovasc J. 43:395–401, 2009.

III. Paakkanen R, Lokki M-L, Seppänen M, Tierala I, Nieminen M S, Sinisalo J. Proinflammatory *HLA-DRB1*01*-haplotype predisposes to ST-elevation myocardial infarction. Atherosclerosis 221:461–466, 2012.

IV. Paakkanen R, Sinisalo J, Palikhe A, Saikku P, Leinonen M, Paldanius M, Nieminen M S, Lokki M-L. Elevated serum human heat shock protein 60 IgA associates with coronary artery disease. Submitted.

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ABBREVIATIONS

ACS	acute coronary syndrome
C	complement component
C4	complement component 4
C4A	acidic isotype of complement component 4
C4B	basic isotype of complement component 4
CAD	coronary artery disease
CI	confidence interval
CNV	copy number variation
Cpn	*Chlamydophila pneumoniae*
CRP	C-reactive protein
Ct	threshold cycle
CTins	CT insertion mutation
DC	dendritic cell
DNA	deoxyribonucleic acid
GWAS	genome-wide association study
HDL	high-density lipoprotein
HLA	human leukocyte antigen
HR	hazard ratio
hsCRP	high-sensitivity C-reactive protein
HSP	heat shock protein
HWE	Hardy-Weinberg equilibrium
Ig	immunoglobulin (classes A, G, E and M)
IHD	ischemic heart disease
IL-	interleukin
LDL	low-density lipoprotein particle
MHC	major histocompatibility complex
MI	myocardial infarction
NSTE-ACS	non-ST-elevation acute coronary syndrome
NSTEMI	non-ST-elevation myocardial infarction
OR	odd's ratio
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
sHSP	soluble heat shock protein
SLE	systemic lupus erythematosus
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
STEMI	ST-segment elevation myocardial infarction
UAP	unstable angina pectoris

1. INTRODUCTION

Atherosclerosis is a chronic inflammatory disease that causes significant mortality and morbidity worldwide. It affects median-sized arteries of the body. The pathognomonic feature of atherosclerosis is the presence of an atheromatous plaque, an atheroma, which includes both thickening of the innermost layer of the artery and the formation a soft lipid core ("athero" = porridge/paste, "scleros" = hardening in Greek). Coronary artery disease (CAD) is a subtype of atherosclerosis, affecting the arteries supplying the heart [1].

The clinical spectrum of coronary atherosclerosis is wide. Coronary atherosclerosis may be silent or manifest as stable cardiac pain on exercise, acute coronary syndrome (ACS), rhythm disturbance, heart failure or sudden cardiac death. Whereas coronary atherosclerosis is defined as the presence of any atheroma in the coronary vasculature, in the clinical use, CAD is denoted as the presence of a significantly large atheroma that may cause hemodynamic limitation of blood flow (>50% obstruction). Therefore the term "obstructive CAD" is commonly used. A patient with typical symptoms/signs of obstructive CAD has ischemic hear disease (IHD). The relationship between IHD and obstructive CAD is not straightforward. IHD may sometimes be present independently of obstructive CAD and the treatment of obstruction does not necessarily resolve the symptoms of IHD [2]. Atherosclerotic plaques are estimated to be present in coronary arteries in over 70% of population of 16 to 60 years of age [3], whereas the prevalence of IHD is less than 15% in Western countries [4, 5].

In ACS, the atheroma is abruptly disrupted and superimposed by thrombus, leading to limitation of blood flow. The mechanisms leading to ACS (a composite of myocardial infarction [MI], with apparent tissue necrosis and unstable angina pectoris [UAP], in which no necrosis is detected) are not fully understood [6]. It has been stated that the majority of MI is attributable to modifiable risk factors [7]. However, some patients with MI present without classical risk factors, whereas some patients with risk factors do not develop MI [8, 9, 10,11]. The genetic variation [12] and unaccounted environmental factors such as infection [13] or autoimmune reactions [14] are factors that may contribute to these "unexplained" cases.

The major histocompatibility complex (MHC) is a gene-rich region in chromosome 6. It encompasses over 200 genes that are involved in, e.g., inflammatory reactions [15, 16]. Genetic variation in the MHC has been linked with various disease conditions [17-19] including CAD, MI and IHD [20-27]. The possible mechanisms of association have not been elucidated.

This Thesis was aimed to develop and validate a novel genetic assay for complement component *C4* and to confirm the association of four CAD-related MHC genes and alleles, *HLA-B*35* [23], deficiencies of complement component *C4A* and *C4B* [21, 28-30] and *HLA-DRB1*01* [23, 31, 32] and their combination in ACS. In addition, the effect of these genetic markers on inflammatory level, autoimmune antibodies and on antibiotic response

was assessed in order to identify possible pathologic mechanism mediating the association between MHC and ACS.

2. REVIEW OF THE LITERATURE

2.1 ACUTE CORONARY SYNDROME (ACS)

ACS is a common cause of mortality and increasing health costs worldwide. It is estimated that every 25 seconds, an American suffers a coronary event [5]. In Finland, there were over 20 000 cases of MI in 2009 (http://www.thl.fi/cvdr). In Finns over 30 years of age, 7% of males and 1% of females have survived MI [4].

2.1.1 CLINICAL PRESENTATION

During ACS, the atherosclerotic plaque is disrupted and superimposed by thrombus. This leads to acute luminal obstruction of the coronary artery and impaired blood flow to the myocardium the vessel supplies. The inadequate supply of oxygen results in ischemia, which in turn causes the symptoms, the plaque disruption itself being symptomless. The most common symptoms include retrosternal pain, which radiates into left arm, neck, jaws or epigastrium. In classical ACS, ischemic pain resembles to that experienced in IHD. However, it usually lasts for over 20 minutes. In contrast to IHD, the ischemia may result on minimal exercise or even at rest and may be dynamic in nature. The pain may be intermittent and accompanied by diaphoresis, nausea, abdominal pain, syncope and dyspnoea. The uncommon symptoms include epigastric pain, indigestion, stabbing chest pain and pleuritic chest pain. The symptoms may be more varied especially in women, in older individuals and in patients with diabetes or heart failure [33, 34].

ACS involving cardiac cell damage, indicated by release of cardiac biomarkers, is defined as MI, whereas in UAP, no significant cardiac cell damage is detected [6, 33]. For clinicians, the diagnosis of ACS is demanding as the absence of any of the hallmarks of ACS, i.e. symptoms, electrocardiographic changes or cardiac biomarker leak does not exclude ACS [35-37]. The symptoms may be so mild that the patient does not know to have suffered from ACS. As much as 20% of MIs may go unrecognized [35]. Besides coronary atherosclerosis, ischemia may also result from other factors such as endothelial dysfunction, embolism and anaemia. Depending on the causative agent, five different types of MI have been classified **(Table 1)** [6, 33]. Presumably, these classifications may be extended to ACS as well.

The clinical presentation of ACS is not uniform between different patient groups. Differences between age groups, ethnicity, gender and socioeconomic status are evident in the incidence, presentation, management or outcomes after ACS [7, 38, 39]. Women, for example, usually present ten years later than men and have increased short-term mortality compared with men [39]. The mortality rates are decreasing in men, but remain constant in young women [38]. The risk factor burden seems to associate with a larger risk in men, but

hypertension and diabetes seem to be stronger risk factors in women than in men [7, 10]. However, as women are underrepresented in most studies in CAD, these factors are hard to evaluate.

Table 1
Clinical classification of different types of myocardial infarction

Type	Definition	Possible causative agents
Type 1	Spontaneous infarction due to primary coronary event	Plaque rupture, erosion, fissuring and dissection
Type 2	Secondary infarction due to ischemic imbalance	Endothelial dysfunction, coronary artery spasm, embolism, arrhythmias, anemia, respiratory failure, hypotension and hypertension
Type 3	Cardiac death	
Type 4a	PCI	
Type 4b	Stent thrombosis	
Type 5	CABG	

Abbreviations; PCI, percutaneous coronary intervention; CABG, coronary bypass by grafting.
Adapted from Thygesen et al. [33].

ACS is associated with high mortality. One estimate states that an American dies of ACS every minute [5]. Because a significant portion of ACS-related mortality is believed to occur before obtaining cardiac markers or electrocardiography, the exact number of ACS-related mortality cannot be given. According to Finnish data, almost one third of incident MI leads to death before reaching a hospital, half within 28 days and a little over half within 1 year. However, these figures vary substantially in different age groups and gender [40]. In some countries, the figures have been reported to be lower [5]. In the developed countries, the incidence and mortality of MI is declining [38, 40, 41]. This trend might not continue as only a fraction of adults and adolescent have optimal cardiovascular health [42] and less than half of patients with CAD adhere to the medical secondary prevention [43]. The success of preventive measures in these populations will set the future trend on cardiovascular epidemiology.

Different clinical entities of ACS can be distinguished. Depending on the presence of cardiac cell damage, the cases are divided into MI and UAP. Depending on the electrocardiographic findings, ACS is divided into cases with prolonged (>20 minutes) ST-segment elevation (STE-ACS) and into those without it (non-STE-ACS, i.e. NSTE-ACS, **Figure 1**). As most cases with STE-ACS show cardiac cell damage, the ST-elevation myocardial infarction (STEMI), these terms are interchangeably used. Based on cardiac biomarkers, NSTE-ACS is divided into non-ST elevation myocardial infarction (NSTEMI) and UAP. At presentation these two entities may not be differentiated from each other [6].

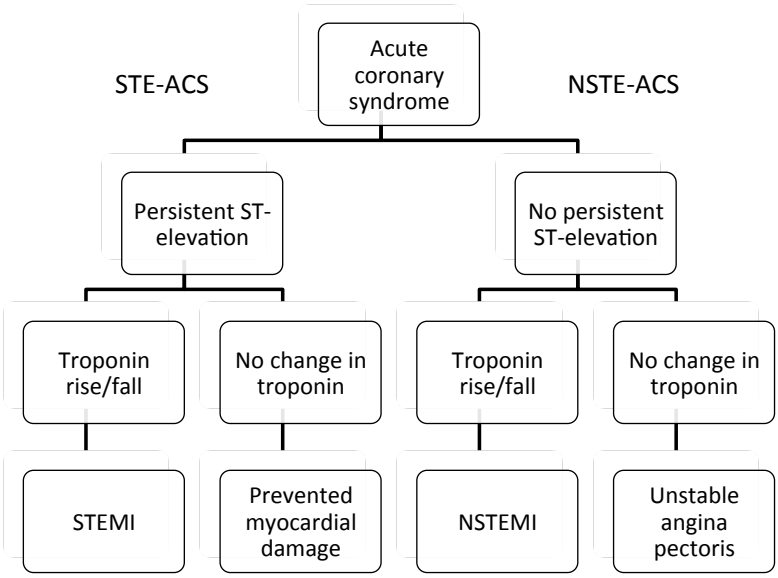

Figure 1. Classification of acute coronary syndrome. Based on the cardiac biomarker leak (for example troponin) and electrocardiography, they are grouped into infarctions with or without ST-segment elevation (STEMI and NSTEMI) or unstable angina pectoris. Adapted from Hamm et al. and Nikus et al. [34, 44]

In the clinical use, the most important distinction is made between STEMI and NSTE-ACS. STEMI is though to associate with complete occlusion of a coronary artery. It is less common than NSTE-ACS and is more often treated invasively due to the higher early mortality rate [6]. The invasive treatments include percutaneous coronary intervention and coronary artery bypass by grafting. Patients with STEMI are usually younger, have less comorbidity and are more often males than patients with NSTEMI [45]. Currently, the relative rate of STEMI is declining, whereas the relative incidence of NSTEMI is increasing [45]. These changes are due to changes in the definition of MI, decreased incidence and mortality [36, 38, 40, 46]. The population trends in the incidence of UAP are controversial [38].

At the local level, the underlying difference between STEMI and NSTE-ACS is the duration and the extent of the coronary artery occlusion (**Figure 2**). However, this view is simplistic as various factors in oxygen content, coronary anatomy, coronary blood flow and coronary blood demand, modulate the clinical picture of ACS (**Table 1**). Plaque rupture (70 vs. 47%), thin-cap atheroma and red thrombus are more often detected in STEMI than in NSTE-ACS [47]. The site of plaque rupture (pre- vs. post-stenosis) may also vary between STEMI and NSTE-ACS [47].

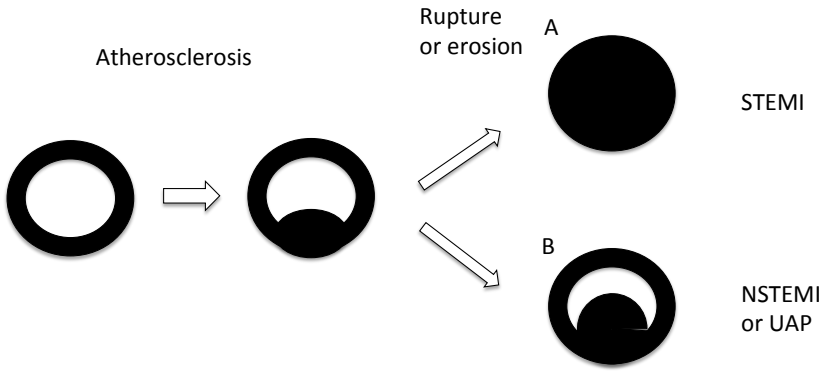

Figure 2. Acute coronary syndromes. Plaque rupture or erosion may lead to total occlusion of the coronary artery, usually causing STEMI (A). However, in the presence of collateral circulation or spontaneous thrombolysis, the electrocardiography may correspond to NSTEMI or UAP. If the occlusion by thrombus is partial and possibly accompanied by distal embolization, the resulting clinical picture is most often NSTEMI or UAP (B).

2.1.2 PATHOPHYSIOLOGY

2.1.2.1 Pathophysiology of coronary atherosclerosis

Even though the first observations linking inflammation with atherosclerosis were published in the beginning of the 19th century, the two scientists that published their work in the mid-19th century, Carl Rokitansky and Rudolph Virchow are usually accounted for discovering the inflammatory nature of atherosclerosis. Rokitansky suggested that the initiating step of atherosclerosis was caused by the accumulation of blood-derived products in the arterial wall, and inflammation was the reaction to the accumulated lipids. Rudolph Virchow, in turn, suggested that the inflammatory reactions were the preliminary step. After these researchers, inflammation in atherosclerosis was forgotten for almost one hundred years [48]. In the 1970's, three prevailing viewing points, endothelial injury, smooth muscle cell (SMC) growth and the lipid hypothesis were combined under the "response to injury" hypothesis by Russell Ross [49]. Evolving from this hypothesis, atherosclerosis is currently seen as an inflammatory disease including various cell types and lipoprotein metabolism, which is affected by genes and risk factors (such as aging, smoking, diabetes mellitus, hypertension, dyslipidaemia and family history of early CAD [MI before 55 years of age in males or before 65 years of age in females]). Although the inflammatory reactions are accepted to be involved in all stages of atherosclerosis progression, the debate on the initiating step is still on-going [50].

An atherosclerotic plaque may present as uni- or multifocal changes in an individual. It is usually found in predicted locations, predominantly in the proximal portions and in the branching points of the arteries. The atherosclerotic plaque forms in the innermost, intimal layer of arteries through various stages during several years or even decades. The possible causative/initiating elements are altered lipoprotein particles, cholesterol crystals and endothelial injury [51-53].

The earliest stage of atherosclerosis is called the fatty streak, where cholesterol is accumulated within inflammatory cells, the foam cells. It is formed when blood-derived lipoprotein particles are retained in the intima, undergo biochemical modification and are subsequently internalized by macrophages. The following step is the formation of a fibrofatty atheroma, which includes a necrotic core of extracellular lipids and dead cells underneath a fibrous cap. It is formed by amplification of local inflammation, recruitment of inflammatory cells and SMCs, death of foam cells and accumulation of lipid-rich core. The last stage, the complicated atheroma has features of neovascularization, repeated injury, growth, calcification and remodelling (**Figure 3**) [6].

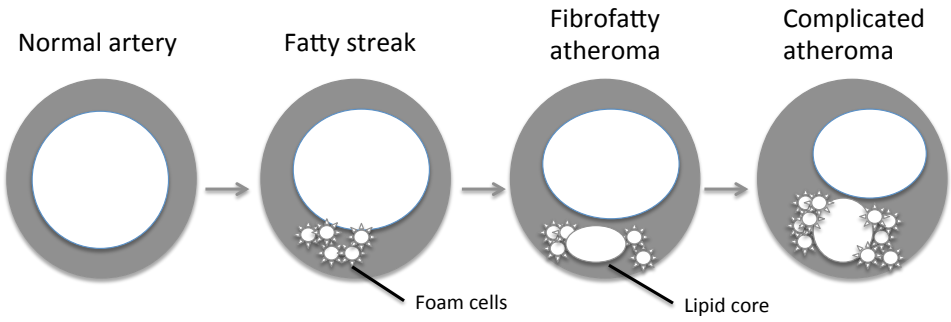

Figure 3. Simplified presentation of the formation of an atheroma. A fatty streak is present in young individuals and consists mainly of macrophages, T-cells and lipoprotein particles (cholesterol). A fibrofatty atheroma is formed when a lipid-, cholesterol crystal- and cellular debris- containing necrotic core is detectable under a fibrous cap consisting of smooth muscle cells and extracellular matrix. The complicated atheroma continues to grow due to inflammation, intraplaque hemorrhage from infiltrating microvessels (neovascularization), and plaque erosion and rupture. The atheroma undergoes dynamic changes in size of the lipid core, thickness of the fibrous cap or in the level of internal or external remodelling (growth inwards or outwards) [50, 54].

Almost all inflammatory cell types have been identified in atherosclerotic plaques. The most important cells are monocytes/macrophages, T-cells, neutrophils, mast cells, dendritic cells (DCs) and B-cells. The cellular subtypes may exhibit important variations in mediating either proatherogenic or atheroprotective effects. For example, regulatory T-cells, B-1-cells and myeloid DCs have been stated to depress atherosclerotic changes,

whereas CD8+T-cells, plasmocytic DCs and B-2-cells cause progression of an atherosclerotic plaque [52, 53, 55]. The role of inflammatory cells residing outside the plaque, in the adventitia and/or perivascular adipose tissue, are also gaining interest [56]. Various cytokines, produced mainly by T-cells and macrophages have been identified as pro-atherogenic (interleukin [IL-] 1, 2 and 18, macrophage colony-stimulating factor, tumor necrosis factor alpha and interferon gamma) as anti-atherogenic (IL-10) or as having dual function (IL-4, IL-6, granulocyte macrophage colony-stimulating factor) [57]. Complement components are present in the plaque in a spatial fashion. The inner intima harbours classical and alternative pathway components, whereas the deeper layers harbour also the end product, the membrane-attack complex [58]. Other blood-derived molecules such as immunoglobulins (Igs), C-reactive protein (CRP) and hemosiderin may be present in the atheroma as well [52, 53, 55].

2.1.2.2 Pathophysiology of ACS

ACS is one of the possible outcomes of an atherosclerotic plaque (**Figure 4**). Atherosclerosis and superimposing thrombus, atherotrombosis, causes the majority of ACS, although other causes, both due to atherosclerosis and independent of it, have also been identified **(Table 1)**. In atherothrombosis, the atherosclerotic plaque is either ruptured, where the fibrous cap is partly absent and the lipid core and vascular wall become in contact with blood. As these structures are very prothrombotic, a superimposing thrombus is formed. Another cause of atherothrombosis is the superficial erosion of the atheroma, where the fibrous cap is still intact, but the endothelial/intimal structure is damaged (**Table 2**) [6]. The third cause, the calcified nodule has also been suggested, but data regarding it is scarce and it may be more important in the carotid atherosclerosis [59]. Atherothrombosis may instantly cause ACS, but in some cases, the ischemic symptoms develop several days after the thrombus formation [60]. Atherothrombosis does not always lead to ACS but may result in plaque growth (**Figures 2** and **4**) [61].

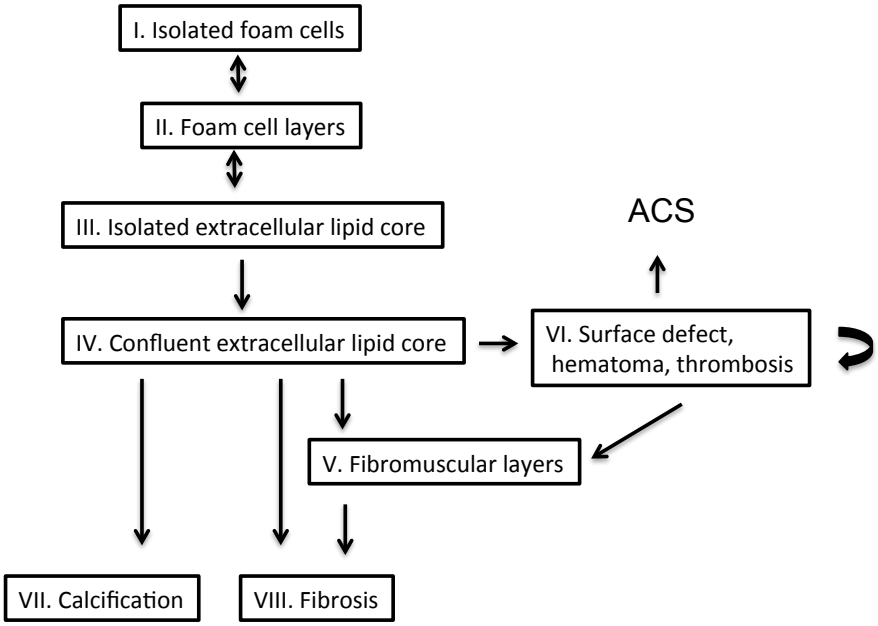

Figure 4. A proposition for the natural history of atheromatous plaques. Regression until type III is possible, but after this point, at the formation of a lipid core, the atherosclerotic plaque is formed and may lead to different plaque types (V–VIII). Surface disruption or erosion, hemorrhage and/or thrombosis (VI) may result in plaque growth or acute coronary syndromes (ACS). Modified from Stary [62].

Table 2

Rupture, erosion and calcified nodule in different forms of ACS and CAD

Clinical presentation	Rupture	Erosion	Nodule	Reference
Sudden cardiac death	55–60%	30–35%	2–7%	59
MI	73 %	23 %	.	63
STEMI	70 %	.	.	47
NSTE–ACS	47 %	.	.	47
Stable CAD	27 %	.	.	64

"." stands for not reported.

In lack of animal models for spontaneous plaque rupture and erosion, the current knowledge on local cellular and molecular mechanisms leading to ACS are based on human histopathology findings [52]. The plaque characteristics associated with plaque rupture have conventionally been called as features of "vulnerable plaque", although a vulnerable plaque, a plaque that is prone to cause ACS, in its true sense, should also cover characteristics of plaque erosion. The plaque characteristics that are associated with rupture are a thin fibrous cap, large lipid core and an abundance of macrophages within the cap, low number of SMC, intraplaque haemorrhage, spotty plaque calcification, large plaque size and external remodelling [59, 65, 66]. Various markers of inflammation, including

serum high-sensitivity C-reactive protein (hsCRP), associate with the aforementioned plaque characteristics [65].

The plaque characteristics associated with erosion are calcification, eccentric, pathological intimal thickening and fibrous cap atheroma. In plaque erosion, there is minimal inflammation, and most often the necrotic core is minimal. As erosion is less common and is linked with several plaque characteristics, the data concerning the pathophysiology of erosion is scarce. Extracellular matrix composition, coronary vasospasm and myeloperoxidase are linked with coronary plaque erosion [54, 59, 67, 68].

The initiating factor for plaque disruption (erosion or rupture) is not known. Various internal, external, systemic and local factors that are associated with ACS have been described, but it is not known, how specific they are to ACS instead of being mere markers for CAD. The most probable mechanism of ACS is a network of various factors that converge in thrombosis-prone and/or rupture-prone state, which, when occurring simultaneously, may result in ACS (**Figure 5**).

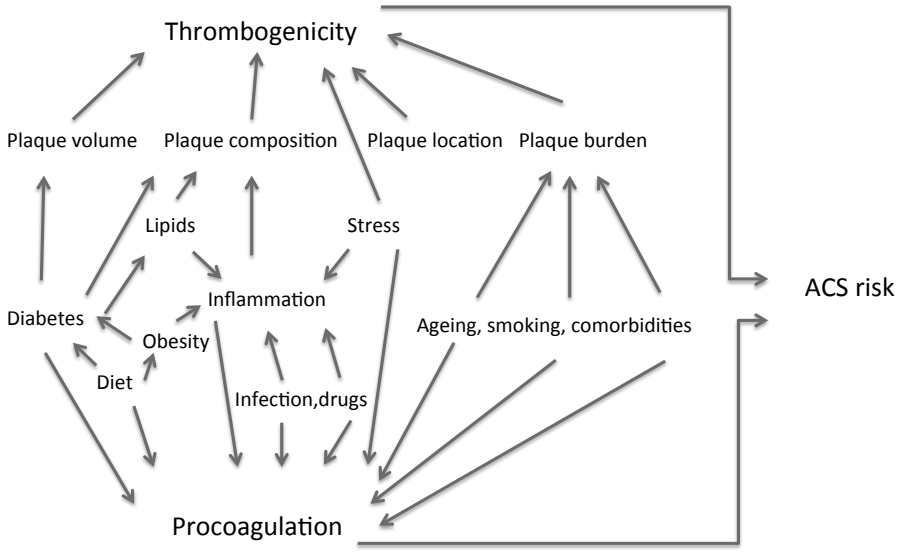

Figure 5. An example of a network of factors affecting the risk of ACS. Adapted from Arbab-Zadeh et al. [68]. Note that genetic variation might influence the presence of a certain risk factor of the relationship/effect of different risk factors.

2.1.3 GENETICS

The research focusing on ACS is extensive. Its particular challenges are the recent change in the definition of MI, rending the comparisons between old and new data problematic and the difficulty of clinical diagnosis of ACS, possibly omitting certain patient groups (the elderly, women and patients with UAP) from clinical studies. Currently, the majority of research involves only men, patients with MI (not UAP) and of cases of European descent.

The genetic factors are important in the pathogenesis of CAD/ACS. Family history of premature MI is a risk factor for IHD and MI [69, 70]. In addition, family history of any MI (all MI and non-premature MI) is also a risk factor for MI, although the risk is generally lower [7, 69, 70]. This increased risk may be attributed to shared environmental factors such as unhealthy health habits, shared genetic factors that lead to ACS or shared genetic factors that affect the risk factor prevalence and thus affect ACS intermediately [71, 72]. However, epidemiological data has shown that family history of MI is independent of traditional risk factors [7, 69, 70, 73]. Furthermore, a recent adoption study showed that genes transmit a stronger risk for CAD than the shared environment does [12]. Also supporting the role of genetic contribution of ACS, a large twin study showed that heritability (h2) of death due to CAD is 0.38 (95% confidence interval [CI]=0.26–0.50) for women and 0.57 (95%CI=0.45–0.69) for men. In addition, in men, the risk attribution for monozygotic twins was significantly higher than for dizygotic twins [74]. However, the heritability of CAD can vary between different phenotypes, age and ethnicity [75, 76].

Candidate gene studies have associated polymorphism of various genes with CAD/MI. However, these results have not been reliably replicated. The known candidate genes encompass mainly genes within pathways that are involved in the pathogenesis of CAD/MI, such as lipoprotein metabolism, risk factors and inflammatory processes [77].

Following the somewhat disappointing results of candidate gene studies, the results of genome-wide association studies (GWAS) were much anticipated. These studies were based on testing as many as one million single nucleotide polymorphisms (SNPs) simultaneously and comparing these between cases and controls. Since 2007, the hypothesis-free GWAS have confirmed at least 46 SNPs that associate with CAD/MI. These SNP associations remain enigmatic because they are usually present in non-coding sequences, cause relatively low risk addition, are frequently found in healthy populations, have pleiotropic effects with other conditions besides CAD/MI and most often do not form any known network of protein-protein interaction, co-expression network or biological pathway. The known risk SNPs attribute only for 6% of estimated heritability (0.4) for CAD. In the future, novel strategies in the genetic research are warranted [77-80].

The major histocompatibility complex (MHC) region on chromosome 6 includes genes that affect the immune responses and host defence. The genetic variation in this region is extensive and consequently, the earlier SNP assays did not cover this region in sufficient

detail [17]. Denser MHC-coverage has now been obtained and there is increasing evidence linking the MHC region with CAD/ACS both in GWAS and candidate gene studies, emphasising the role of inflammation in CAD/ACS (See Section 2.2.1).

Most genetic studies have been conducted with a combination of CAD and MI. Lately, concern was raised that the genetic background is different in these conditions [81]. However, a replication study could not confirm that different clinical manifestations were more strongly associated with different phenotypes, but the power to discriminate between different phenotypes was low [82]. It has been suggested that the majority of CAD-related genes increase the likelihood of CAD and thereby, indirectly the risk of ACS [75].

2.1.4 PREDICTION

Currently, the prediction ACS by plaque characteristics [83], genetic risk score [84-87] or biomarkers [88] do not add significantly to the standard risk assessment of traditional risk factors or algorithms done by SCORE, FINRISK, Framingham risk score in the general population. However, these unconventional strategies might be useful in patients with low or moderate risk [89]. Given the imperfect predictive value of classical risk factors [8, 9, 10,11] there is an impelling need for understanding the pathophysiologic event that results in CAD and ACS. This knowledge would help to better identify high-risk patients and hopefully lead to the development of novel pharmacotherapeutic approaches.

In the future, the so-called -omics (genomics, metabolomics, proteomics) and systems biology approaches are expected to enlighten the pathophysiology of CAD and ACS. The improved imaging modalities will hopefully help to determine the natural history of the atherosclerotic plaque. The prevention of CAD might be targeted to the prevention of risk factor emergence, through the "primordial prevention". Novel therapeutic approaches include stem cell therapies, new treatment devices, deoxyribonucleic acid (DNA) targeting, pharmacogenomics and personalized medicine [90]. Various different treatment strategies against inflammatory reactions are also under way (see Section 2.3.1).

2.2 THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

The MHC region was discovered in the 1950's. It was named for being the major cluster of genes controlling the allograft outcome after transplantation. The MHC region is located in chromosome 6 (6p21.3) and it contains over 220 genes. It is divided into three classes from I to III (**Figure 6**). The surrounding genome is rather conserved and it, together with the MHC region, is denoted as the extended MHC. The extended MHC covers over 420 genes. The MHC region is extremely rich in polymorphism (number of different alleles), has strong linkage disequilibrium, which may differ in length between regions, alleles, and populations [17]. The MHC region has been linked with over 100 autoimmune, infectious and inflammatory diseases. Until lately, GWAS did not often sufficiently cover the MHC region, but new methods do so into much greater detail. In humans, MHC classes I and II are called the human leukocyte antigen (HLA). Throughout the Thesis, this nomenclature is applied.

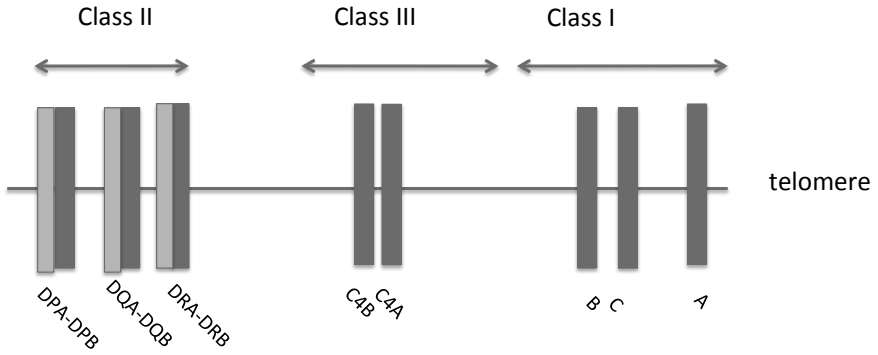

Figure 6. Simplified presentation of the organization of the MHC region. The class II MHC genes encode for both α and β subunits of classical HLA II molecules HLA-DP, -DQ and –DR (DPA and DPB, DQA and DQB, DRA and DRB for α and β subunits, respectively). In addition to the protein-coding genes, various pseudogenes are also present. Class III MHC is a collection of non-related genes between classes I and II. The class I MHC harbors the genes encoding for the α subunit of classical HLA I molecules HLA-A, -B and -C. For detailed structure of C4 gene region, see **Figure 11**. Based on Marsh et al. [91].

Immune reactions against foreign HLA I and II molecules are still amongst the major determinants of transplant outcome. These reactions include both humoral and cellular immune reactions. It is currently not known how big a mismatch is safe for transplantations. It seems that the organ in question as well as the type of transplantation (solid organ vs. blood marrow) affect the rate of acceptable mismatches. Autoantibodies to non-self HLA may arise from multiple pregnancies or blood transfusions [92].

The nomenclature in the HLA is somewhat complex. Different HLA alleles are numerically separated, whereas colons separate the different information about the

structure. The first numbers group all alleles with a given allele family, corresponding to the old serological groups. The second numbers gather the alleles differing in their amino acid sequence. The third numbers differentiate alleles having synonymous mutations and the last digits segregate nucleotide changes in introns or in the untranslated regions (**Figure 7**) [93].

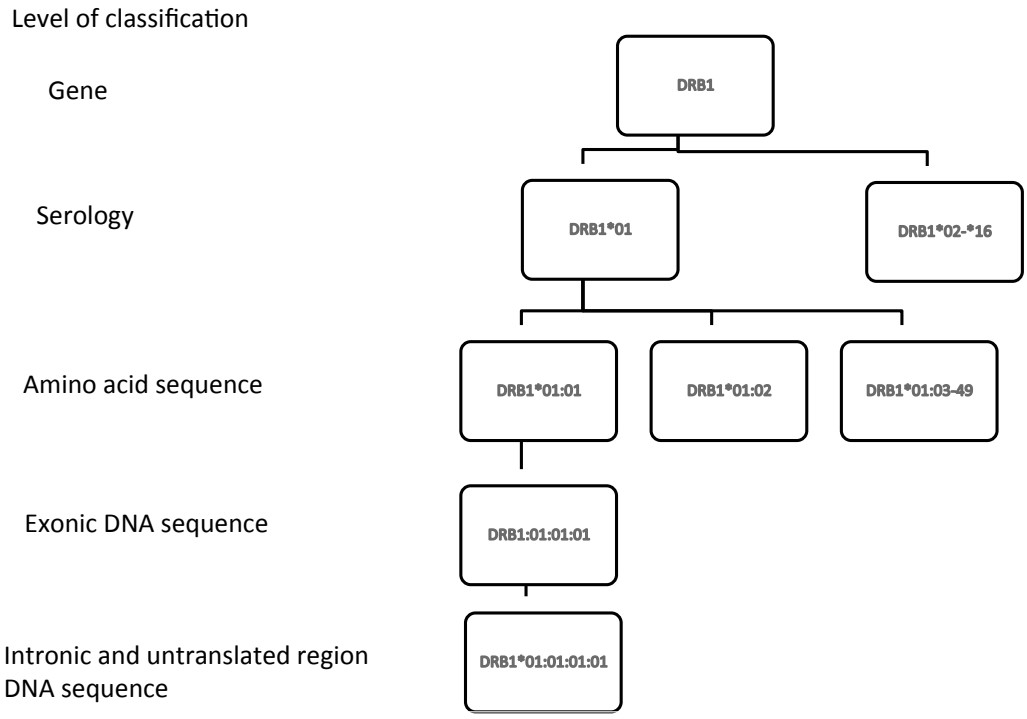

Figure 7. Nomenclature and classification of HLA-alleles, with *DRB1*01* as an example. Based on Robinson [93].

2.2.1 MHC GENES IN CORONARY ARTERY DISEASE (CAD)

Many studies have identified both susceptible and protective factors for CAD in the MHC. Lymphotoxin alpha SNPs, complement component *C4* deficiency and *DRB1*01* allele are amongst the most studied factors in the MHC (**Table 3**). In addition, the MHC region has been also linked with blood lipids [94], large-artery atherosclerosis [95] and cardiovascular mortality in patients with autoimmune conditions [96, 97 98, 99]. The MHC region may also be linked with endothelial dysfunction [100].

Table 3

Recent studies reporting MHC genes in CAD

	Phenotype	Comment	Reference
Genome-wide association studies			
HLA II SNP	CAD/MI	Susceptible	27
HLA I SNP	CAD/MI	Meta-analysis, susceptible	26
HLA II SNP	MI	Susceptible	101
Candidate gene studies			
HLA-G SNPs	CAD	Susceptible	25
LTA (*TNF-B*) SNPs	CAD/MI	Conflicting data	23, 27, 30, 102, 103
TNF-α (308A) SNP	CAD/MI	Conflicting data	104
Factor B	CAD	No association	28
C4 deficiency	CAD	Conflicting data	See **Table 10**
C2 deficiency	MI	Complete deficiency	105
HSP70 SNP	CAD/MI/IHD	Susceptible	24
HFE SNP	CAD/MI	Conflicting data	106
HLA I and/or II alleles screened			
*HLA-DRB1*01*	CAD	Susceptible	See **Table 7**
*HLA-DRB1*04*	CAD	Susceptible	32
HLA-B	Premature MI	Susceptible	20
*HLA-DRB1*15*	CAD/MI	Protective	23
*DRB1*07*	MI	Protective	31
*HLA-DRB1*12:02:01*	CAD	Protective	107
HLA-haplotypes			
*DRB1*01-B*35*	CAD/MI	Susceptible	23
*DRB1*07-DQA1*02-DQB1*02*	MI	Protective	31
*DRB1*0101-DQA1*01-DQB1*05*	MI	Susceptible	31

Abbreviations; SNP, single nucleotide polymorphism; LTA, lymphotoxin alpha; TNF, tumor necrosis factor; HSP, heat shock protein; HFE, high serum Fe, for hemochromatosis.

2.2.2 MHC I

HLA I molecules are formed from two subunits, the α chain (aka. the heavy chain) and the β_2-microglobulin (**Figure 8**). Class I HLA genes encode for the α chain, whereas β_2-microglobulin is encoded in chromosome 15. The classical HLA I (aka. class Ia) gene products are components of HLA-A, -B and -C. In addition to these, the class I MHC region harbors also non-classical HLA I-genes (HLA-E, HLA-F and HLA-G), pseudogenes (HLA-J, -K, -L), structurally HLA I-like genes (hemochromatosis and MHC class I chain-related genes A and B) and approximately 50 additional genes that are not

related in form of function with HLA I or II. The remaining HLA-like proteins (for example CD1) are encoded in other regions of the genome [91].

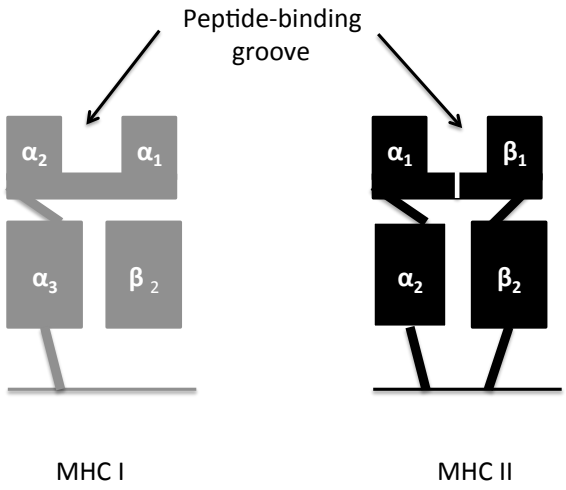

Figure 8. HLA I and II molecules. The subunits 1–3 in α chain of MHC I correspond to exons 2–4 encoded in HLA I, and β_2 is for B_2-microglobulin.The subunits 1–2 in MHC II correspond to exons 2 and 3 in either A or B gene, both in the HLA II. The peptide fragments are anchored within the peptide-binding groove by peptide-binding pockets, of which there are six on HLA I and five on HLA II. Each pocket is formed from various amino acid residues on the HLA molecule [91].

The HLA I molecules are cell-surface glycoproteins present in all nucleated cells. The classical HLA I molecules activate CD8+ killer T-cells through the interaction of peptide-HLA I complex, costimulatory molecules and the αβ T-cell receptor. This enables the screening of cellular function (protein synthesis), leading to killing of infected or altered cells by the CD8+ killer T-cells [91]. Certain HLA I molecules inactivate natural killer cells through killer immunoglobulin-like receptor (namely the HLA-A, -B and C) and CD94/NKG2A receptor (HLA-E) [108]. The HLA I molecules may affect antigen-presenting cell function by interacting with the inhibitory leukocyte immunoglobulin-like receptor as well [109].

The HLA I molecules present mainly peptides derived from the intracellular compartment. Peptides from extracellular proteins can be presented in HLA I by cross-presentation and possibly also by acquiring membranes directly from other cells [110]. In addition, soluble HLA I molecules circulating in blood are stable, increase in inflammatory diseases and may have immunoregulatory functions [111]. Non-immunological functions such as neuronal development have recently been described for HLA I molecules [112].

Over 5000 HLA class I alleles have been identified [93]. The majority of the allelic variation resides in exons 2 and 3, leading to amino acid polymorphisms in the peptide-binding region between α_1 and α_2 subunits and possibly affecting the selection of peptides presented to CD8+ T-cells (**Figure 8**). The peptide repertoire of certain HLA I allele is becoming unraveled and is very polymorphic, tissue specific, affected by the presence of different proteases and surprisingly restricted. Factors such as medication, infection, metabolic changes and cancer affect the peptide repertoire [113, 114].

*2.2.2.1 HLA-B*35*

At least 205 different *B*35* alleles coding for different proteins have been described [93]. The frequency of *B*35* varies amongst different geographical areas, even within Finland [115]. *B*35* is a part of ancestral haplotype 35.2, a collection of HLA-genes that are usually inherited together and often seen in different populations. Besides *B*35*, the 35.2 ancestral haplotype includes *A*03, C*04, C4B* null allele, *DRB1*01:01* and *DQB*05:01* [116]. A haplotype otherwise like the 35.2, but including *A*11* has also been described [117].

*B*35* has been associated with infectious and autoimmune conditions (**Table 4**). Other HLA-alleles have also been associated with these conditions and no definite conclusions on *B*35* in disease pathology have yet been made. In addition, *B*35* has been involved in increased apoptotic signaling [118] and altered endothelial cell function [119]. *B*35* has been associated with CAD only as a part of haplotype together with a HLA II allele *DRB1*01* [23].

Table 4
Recent disease associations of *HLA-B*35*

Condition	Association	Reference
Infections		
HIV	Accelerated progression	120-122
Chlamydophila pneumoniae	Positive serology	123
Hepatitis B	Positive surface antigen	124
Hepatitis C	Chronic infection	125
Autoimmune/inflammatory		
Pulmonary hypertension	In patients with systemic sclerosis	126
Presentation of leprosy	Association with tuberculoid form	127

2.2.3 MHC II

The HLA II molecules are cell-surface glycoproteins formed from non-covalent joining of α and β chains, encoded by corresponding HLA II genes (for example HLA-DRA and HLA-DRB, for α and β chains, respectively). In contrast to HLA I, HLA II molecules are entirely encoded by the MHC region and the peptide-binding pocket is determined by both α and β chains (**Figure 8**). Classical HLA II-molecules (HLA-DR, -DQ and -DP) are involved in T-cell activation, whereas the non-classic molecules (HLA-DM and -DO) are participating in intracellular MHC II assembly. Other genes in MHC II region are mainly involved in the function of HLA I molecules (transporter associated with antigen processing) [91].

The genetic polymorphism is the most prevalent in the peptide-binding pocket and promoter region, but for HLA-DRA, the gene coding for α chain is largely invariant. HLA- DR β chain is encoded by HLA-DRB1, -3, -4 and -5 genes, all of which have allelic variation. HLA-DR genes can be divided into haplogroups that vary in the presence of additional DRB genes and pseudogenes (**Table 5**). As a result, humans may have from one to four different HLA-DR molecules on their cellular surface [15, 91].

HLA II molecules are present on professional antigen-presenting cells, including DCs, monocytes, macrophages and B-cells. Upon stimulation, various other cell types such as endothelial cells, SMCs and T-cells. Usually, HLA II molecules present peptides from extracellular origin, but in some cases, also intracellular peptides are presented. The best-known function of HLA II is the activation of T-cell receptors on helper CD4+ T-cells. HLA I and II molecules are also involved in T-cell priming and selection in thymus [15, 16].

Table 5

Major HLA-DR haplogroups

Haplogroup	DRB gene	DRB1 alleles	Pseudogene	Additional DRB	Pseudogene
DR1	DRB1	**01, *10*	DRB6		DRB9
DR8	DRB1	**08*			DRB9
DR51	DRB1	**15, *16*	DRB6	DRB5	DRB9
DR52	DRB1	**03, *11, *12, *13, *14*	DRB2	DRB3	DRB9
DR53	DRB1	**04, *07, *09*	DRB7	DRB4	DRB9

Adapted from Handunnethi et al. [128].

The role of HLA II molecules beyond T-cell activation is largely unknown, but HLA II has also been associated with intracellular cross-talk with Toll-like receptor-signaling [129] and regulatory signal transduction/receptor function[130,131]. The role of HLA II molecules on non-antigen-presenting cells are not understood, but they might be take part in attenuation of inflammatory reactions [132] or promotion of T-cell activation [133]. In addition to the membrane-bound HLA II, soluble HLA II molecules are detected in blood and other body fluids [134]. Soluble HLA II may be elevated in disease conditions [133, 135, 136] and possibly elicit or enhance T-cell activation [137]. The expression of HLA II molecules is tightly regulated and includes chromatin modification, cis- and trans-acting regulatory elements and long-range controlling [138, 139].

*2.2.3.1 HLA-DRB1*01*

*HLA-DRB1*01* is one of the 16 allele groups of the DRB1 gene. *DRB1*01* is divided into 49 different protein-coding alleles (*DRB1*01:01-DRB1*01:49*), which, in turn are divided into 30 alleles, differing by exon sequence, but not by amino acid structure (**Figure 7**) [93]. *DRB1*01* is more frequent in Europe and Eastern Asia than in other geographical areas. In worldwide populations, the frequency of *DRB1*01* ranges from 0 to 0.24. Of the protein-coding allele groups, *DRB1*01:01* and **01:02* are the most common. In Finland, the most common protein-coding allele is *DRB1*01:01*, covering half of the *DRB1*01* [140]. In Swedish population, the frequency of *DRB1*01:01* is almost two times higher than the frequency of *DRB1*01:02* [31]. Our recent data suggests that the proportion of *DRB1*01:01* in Finland might be significantly higher than reported earlier (Eronen et al, unpublished data). The frequency of *DRB1*01:01* also varies by geographical area, ranging from 0 to 0.19. The highest frequency is found in Eastern Europe. For *DRB1*01:02*, the frequencies range from 0 to 0.12, being the highest in Africa. The *DRB1*01:01* allele can be divided into 22 subtypes having synonymous mutations [140].

*DRB1*01* has been linked with various inflammatory and infectious conditions, but most of these associations have also been described for other HLA-alleles as well. Overall, the associations are not very strong and they are not *DRB1*01*-specific. These associations usually cover autoimmune conditions and viral infections, both susceptibility to symptomatic infection and resistance (**Table 6**).

The most studied disease association of *DRB1*01* is its association together with some subtypes of *DRB1*04* and *DRB1*10* with rheumatoid arthritis. These alleles are collectively called the “shared epitope” alleles for their sequence similarity. It has been suggested that interaction with smoking, serotonin receptors, activation of cell surface molecules independent of the presented peptide or the distinctive structure on the rim of DRB molecule mediate the possible effect, but causality has not been shown between the shared epitope alleles and rheumatoid arthritis [141-143].

Table 6
Recent reported disease associations of *HLA-DRB1*01*

	Association	Reference
Infection		
Sindbis virus	Associated with symptomatic infection	144
Human immunodeficiency virus	Associated with resistance	145
Hepatitis C	Associated with increased viral clearance	146
Recurrent lymphocytic meningitis	Association	147
Periodontitis	Association	148
Ebstein-Barr virus	Associated with symptomatic infection	149
Inflammation		
Rheumatoid arthritis	Association	150
Juvenile idiopathic arthritis	Association	151
Autoimmune hepatitis	Association	152
Henoch-Schönlein purpura	Association	153
Other		
Coronary artery disease	Association	See **Table 6**
Chagas disease	Protective from complications	154
Allergy	Mugwort pollen and cockroach	155, 156
Lung cancer	Death to lung cancer in patients with rheumatoid arthritis	97
Hypersensitivity	Hypersensitivity to nevirapine	157, 158
Non–Hodgin lymphoma	Association	159

There are various peptides that have been shown to bind to *DRB1*01:01* in either peptide-binding assays or from naturally presented peptides enlisted in the immune epitope database (www.immuneepitope.org). There are at least 45 peptides have been eluted from tissue *DRB1*01*, but these data or based on two individuals or on cell cultures [160, 161]. The eluted peptides include apolipoprotein E and B, tubulin and transferrin receptor. For *DRB1*01:02*, only cell line eluates has been characterized [160]. These eluates included mosty different peptide fragments between DRB1*01:01 and *01:02, although some overlap was detected.

*2.2.3.2 DRB1*01 in CAD*

*DRB1*01* has been linked with different forms of CAD/MI in four independent materials and three different ethnic groups with odd's ratios (ORs) from 1.2 to 4.7 (**Table 7**). In three populations, no association was found, but these studies were performed in either in

populations with low *DRB1*01* frequency in the general population [107] or with small sample size [162]. Other MHC genes have also been linked with CAD (see Section 2.2.1)

Table 7
Studies assessing *DRB1*01* in CAD

	Frequency[a]	N[a]	Phenotype	OR	Population	Reference
Association						
**01:01*	0.09/0.07	1188/1191	MI	1.24 (1.00–1.53)	Swedish	31
**01*	0.22/0.07	166/150	CAD/MI/IHD	4.71 (not reported)	Chinese	32
**01*	0.21/0.10	100/74	NSTE-ACS	2.36 (1.25–4.44)	Finnish	23
1	0.13/0.07	115/90	CAD	2.37 (1.33–4.25)	Finnish	23
No association						
1	0.16/0.15	50/50	CAD		Swedish	162
1	0.14/0.08	50/48	premature CAD		Swedish	162
**01:01:01*	0.009/0.00	219/208	CAD		Chinese	107
**01:01:02*	0.002/0.00	219/208	CAD		Chinese	107

[a] cases/controls.

2.2.4 COMPLEMENT COMPONENT C4

Class III MHC is a gene rich region between MHC I and II that harbors at least 75 genes, 35 of which are expressed (**Figure 6**) [163]. The C4 protein is first synthesized as a single-chain pre-protein, which undergoes cleavage into three subunits as well as biochemical modifications including glycosylation and sulfation. After secretion, this molecule is further cleaved. The biochemical modifications are not uniform, leading to the presence of different C4 molecules in plasma. The presence of various protein forms is the reason for pre-treating C4 proteins by neuraminidase and carboxyl peptidase B and thus reducing the protein variation prior to immunophenotyping (**Figure 9**) [164]. C4 is secreted by various cell types including macrophages, fibroblasts and activated platelets, but liver is the major source of serum C4 protein [165, 166]. The serum levels of total C4 vary from 0.13 to 0.54 (g/L) depending on age, body mass index, *C4* gene number, surrounding genes, consumption, gene length and circadian rhythm [167 168-171]. The surrounding HLA region may also affect the C4 level, but the extent to which the surrounding HLA modulates the effect of *C4* copy number variation (CNV) is not known [172]. C4 may be differentially expressed in different inflammatory cell subsets [173]. C4b binds to complement receptor 1 on blood cells, B-cells, DCs and monocytes and functions as an opsonin. The other function of C4b is the formation of C3 convertase together with C2, ultimately leading to membrane-attack complex formation[164]. Complement C4 is a part of classical and lectin complement activation pathways although a C4-independent activation route in lectin pathway has been described (**Figure 10**).

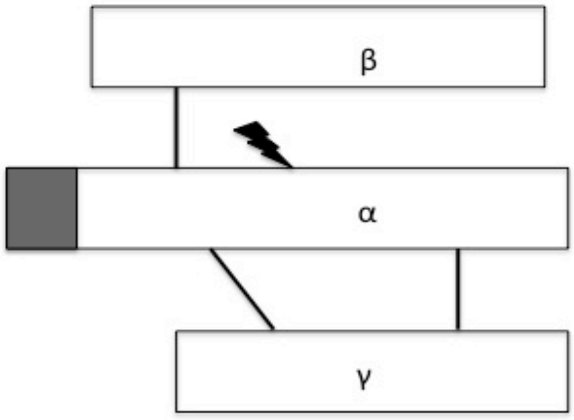

Figure 9. A simplified schematic presentation of the complement component C4. The protein is formed from three peptide chains, α, β and γ. These subunits are linked through disulphide bonds (lines). One of the most important structures of C4 is the internal thioester bond (the lightning symbol), located in the α chain. The anaphylatoxin cleaved from C4 is also located in the α chain (dark box on the left end of α chain). The amino acid variation leading to the different allotypes (C4A and C4B protein isoforms) is located near to the thioester bond region. Modified from Atkinson and Yu [164].

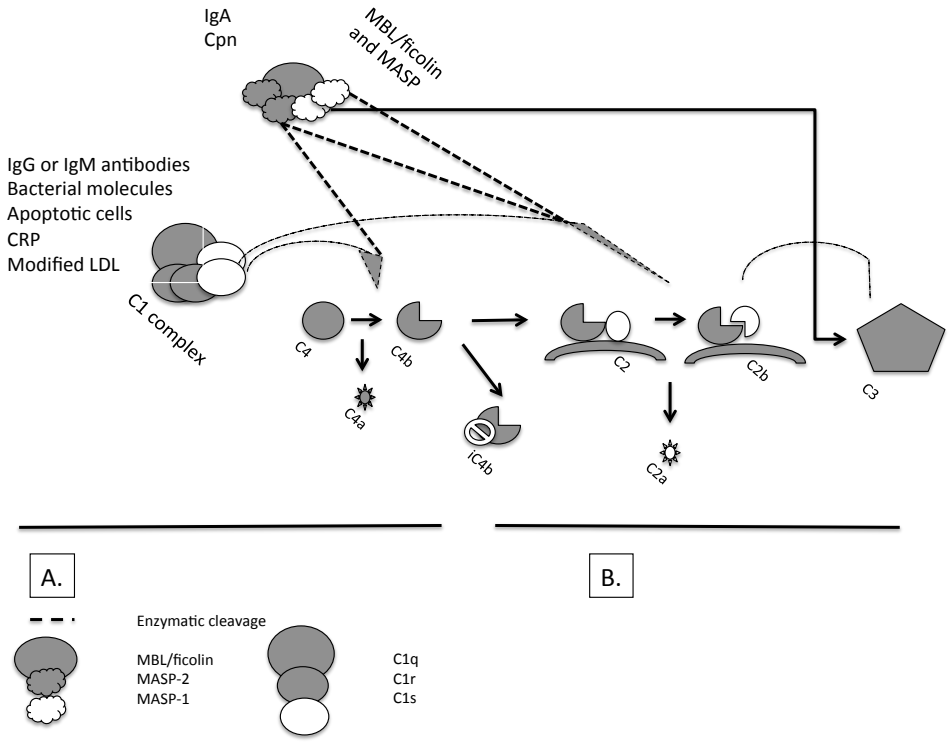

Figure 10. Complement component C4 in the complement cascade. (**A**). The classical pathway is activated when C1q binds to distinct structures on pathogens or damaged cells directly or through different molecules (for example IgG, IgM and CRP). Subsequently the proteases of the C1 complex, C1r and C1s are activated, C1r cleaving first C1s, which cleave C4 into C4a, anaphylatoxin and C4b. The lectin pathway is activated through mannose-binding lectin and ficolins recognizing carbohydrate patterns. The MASP-proteases are subsequently activated. MASP-1 cleaves only C2, whereas MASP-2 cleaves both C4 and C2. MASP-1 is though to amplify the lectin pathway, but a C4- and C2-independent activation pathways may also be functioning (continuous line). (**B**). The cleavage of C4 uncovers an internal thioester bond in C4b, which can covalently bind to adjacent carbohydrate or protein stuctures by forming ester or amide bonds, respectively. If a suitable target is not found, the C4b is inactivated into iC4b. The surface-bound C4b binds to C2, which is cleaved into C2a and C2b by MASP 1 or 2 or C1s. The resulting C4bC2b is a C3 convertase that cleaves C3 [164, 174-176 177].

C4A (acidic C4) and *C4B* (basic C4) genes are distinguished from each other by five nucleotides. The protein isoforms have differences in their biological actions, electrophoretic mobility, activated tertiary structures and disease associations (**Table 8**). Contradictory findings have been reported on the binding of complement receptors [178]. However, the exact binding profiles and the extent to which different isotypes or allotypes affect the function of classical and lectin pathways is not known. Alternative splicing and glycosylation might also affect the biological responses [164].

Table 8
Characteristics of complement C4A and C4B

	C4A (acidic)	C4B (basic)	Reference
Synonyms	C4F (fast)	C4S (slow)	
Number of alleles	At least 14	At least 17	179
Most common allotype	C4A 3	C4B 1	180
Structure			
Number of exons	41	41	181
AA in positions 1120-1125	PCPVLD	LSPVIH	182
Length	Usually long (21kb)	Long (21 kb) or short (14.6 kb)	180
Rh/Ch antigen	Usually Rodgers	Usually Chido	180
Function			
Specificity	Amino groups	Hydroxyl groups	183
Hemolytic activity	low	high	184
Deficiency			
Association with HLA	TNFα-308A, CYP21A2 656, 861 and 2761, LTA+252G,	TNFa-238A, HLA-DRB1*01, LTA +633C, CYP21A2 1106, 1113, 1559 and 2209	23, 28, 30
Association with haplotypes	limited	various	185, 186

Abbreviations; TNFα, tumor-necrosis factor alpha; CYP, cytochrome P450; LTA, lymphotoxin alpha.

The genetic variation of *C4* is the greatest in the whole complement cascade. Genes encoding *C4* are embedded in the RCCX module, a gene cluster consisting of functional and non-functional genes (**Figure 11**). The RCCX module consists of serine-threonine kinase, C4 (A or B), steroid 21-hydrolase (CYP21) and tenascin-X. In each chromosome, one to four RCCX modules may be seen (mono-, bi-, tri- and quadrimodular chromosomes). At least 20 different possible RCCX haplotypes and 17 diploid combinations can be present. In almost half of the cases, two *C4* genes are present in both chromosomes [180]. The CNV of total *C4* is mainly between 2 and 6, but eight copies have been reported [164]. The CNV of *C4A* varies between zero and five, and between zero and four in *C4B* [186-192]. *C4* genes may harbour also silencing mutations [193].

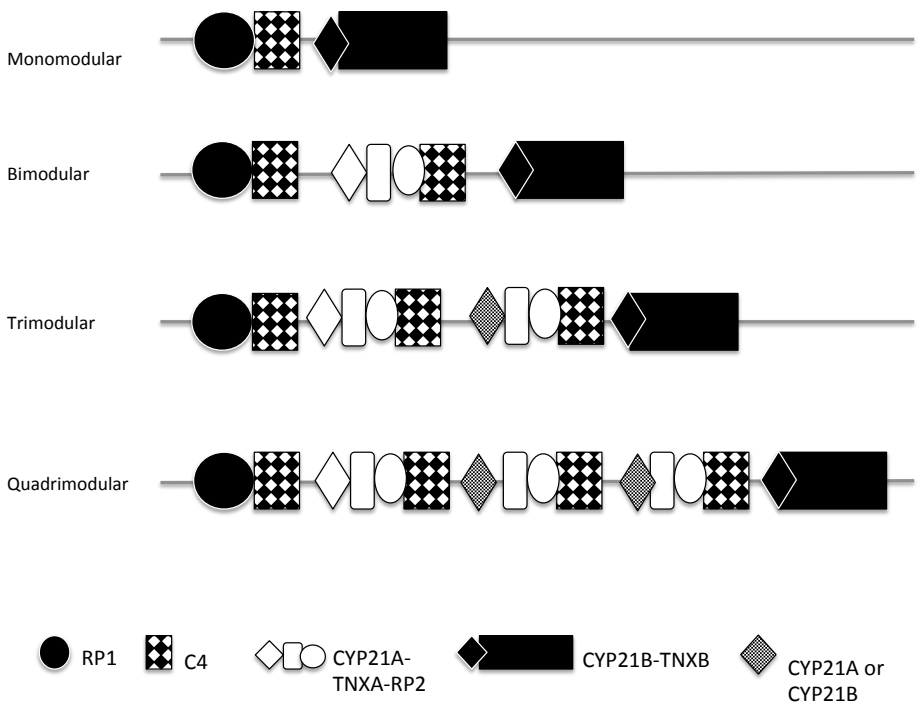

Figure 11. The RCCX-structure. The duplications of CYP21 (CYP21A), TNX (TNXA) and RP (RP2) are pseudogenes (in white). In trimodular and quadrimodular structures, the duplicated CYP21 can be either CYP21A (aka. CYP21A1P) or CYP21B (aka. CYP21A2). Due to recombination events, CYP21A can be found also in monomodular structures and TNXA can be transformed into TNXB-like functional gene. A C4 gene in RCCX may be short or long and encode C4A or C4B. The duplicated C4 genes are usually functional, but may also have silencing mutations. Adapted from Szilagyi et al. [185] and Saxena et al. [192].

2.2.4.1 C4 deficiency

C4 deficiency is historically defined as a low amount of C4A or C4B proteins. This may result from genetic factors (gene deletion, gene conversion or silencing mutations) or increased consumption (**Figure 12**). C4 deficiencies are classified into complete (absence of all functional *C4* genes) and partial (lack of *C4A* or *C4B* gene[s]). Partial deficiencies are divided into homozygous (no *C4A* or *C4B*) or heterozygous deficiencies (one copy of *C4A* or *C4B* missing and one left). Due to the low frequency of complete and partial homozygous deficiencies, *C4* deficiency is usually modelled as the presence of any *C4A* or *C4B* deficiency, thus overestimating the frequency of heterozygous partial *C4* deficiency. *C4* deficiency due to low CNV for *C4A* is 11–22%, whereas for *C4B* it is 21–41% in the general population [186, 189-191]. In addition to the low amount of protein, some hemolytically inactive *C4* alleles have been described [194, 195]. These, together with some drugs, such as hydralazine and isoniazid can inhibit the function of C4 and cause a deficiency of functional C4 proteins [196].

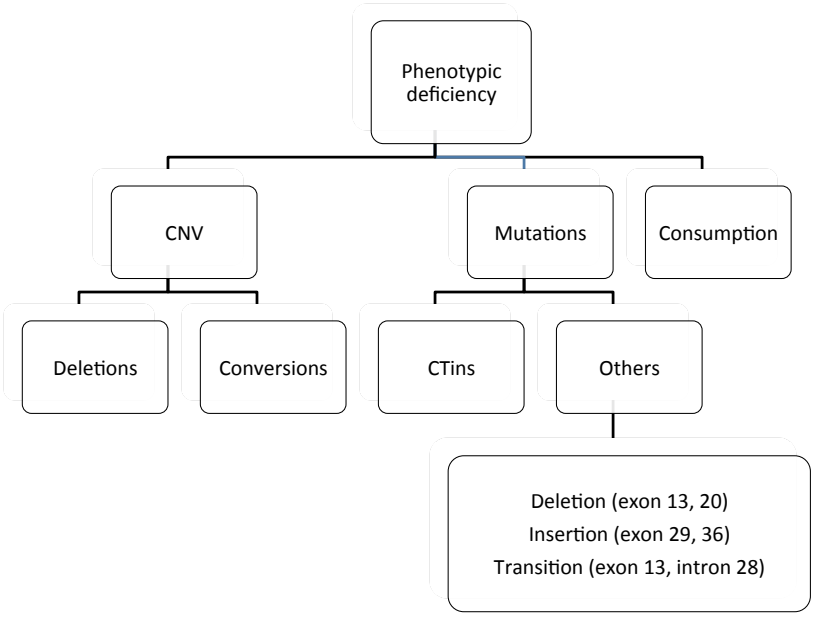

Figure 12. C4 deficiency.

The relationship between the total numbers of *C4* genes, the number of *C4A* and *C4B* genes and C4 deficiency is complex (**Table 9**). Low CNV (less than 2 copies of C4A or C4B) causes part, but not all phenotypic C4 deficiencies. CNV records deletions and conversions, but not the silencing mutations (**Figure 12**). The most common mutation

leading to *C4A* silencing is a two-nucleotide CT insertion in exon 29 (*CTins*), codon 1213 and is virtually absent in *C4B* [197].

In the literature, 28 individuals with complete C4 deficiency have been identified. All of them have developed systemic lupus erythematosus (SLE, n=17), a lupus-like disorder (n=5) or kidney disease (n=6). Other diseases are rrepeated and invasive infections (meningitis, osteomyelitis, otitis media, respiratory tract infection), recorded in seven patients. Five of these patients have died between the ages of 2 and 25. One individual was initially recorded healthy, but later developed haematuria and invasive infection [198]. Even in the presence of complete C4 deficiency, there is at least one monomodular RCCX-structure, where the C4 gene is mutated into an inactive form [164].

Table 9
Relationship between C4 CNV, number of C4 genes and C4 deficiency

Number of C4 genes	Possible C4A/C4B copy number combination[a]	Possible C4 deficiency (A, B or both)[b]
2	0/2	A
	1/1	A, B
	2/0	(A), B
3	0/3	A
	1/2	A
	2/1	(A), B
	3/0	B
4	0/4	A
	4/0	B
	2/2	.
	3/1	(A), B
	4/0	B
5	1/4	A
	2/3	(A)
	3/2	.
	4/1	B

[a] Assuming the presence of mono- to quadrimodular genotypes

[b] (A) in case of *CTins* silencing C4A.

Various infectious and autoimmune diseases have been associated with (partial) *C4* deficiency (**Table 10**). It is not known, whether the *C4* deficiency itself or the linkage with the surrounding MHC are linked with SLE. Recent data suggests that *C4A* deficiency is not independently associated with SLE, but for *C4B*, the role of surrounding MHC is less important [199]. On the other hand, however, partial deficiency of C4 can be very common in the general population that never develop SLE (see above).

C4 deficiency has not been associated with renal transplant outcome [188]. Recently, disease

associations have been extended beyond inflammatory and infectious diseases. The disease associations have also challenged the traditional dogma that links *C4A* deficiency with immune complex diseases and *C4B* deficiency with increased susceptibility to infections [166].

The effect of the total number of functional *C4* genes has only seldom been determined (**Table 10**). *CTins* has been rarely assessed in case-control settings in screening both cases and controls. One study with children surviving from meningococcal meningitis showed a two times higher, but statistically insignificant difference in *CTins* [190]. *CTins* has not been shown to be associated with SLE [199].

Table 10
Complement component C4 deficiencies in different diseases

Condition	C4A deficiency	C4B deficiency	C4A or B deficiency	Total C4 CNV <4	Method	Reference
Infectious						
Upper respiratory tract infections						
Chronic or recurrent rhinosinusitis	Associated	Ns	Associated	.	qPCR	191
Chronic or recurrent rhinosinuitis	Ns	Associated	Associated	.	qPCR	200
Recurrent respiratory infections	Associated	Ns	Ns	.	qPCR and immunophenotyping	201
Severe chronic periodontitis	Ns	Ns	Associated	.	qPCR	202
Pulmonary tuberculosis	Associated	Ns	.	.	PCR	203
Meningitis	.	Conflicting data	.	.	MPLA and immunophenotyping	190
HSV complications	Ns	Ns	Inverse association	.	qPCR and immunophenotyping	204
Leprosy	Ns	Associated	.	.	Immunophenotyping	205
Inflammatory						
Sarcoidosis	Associated	Inverse association	.	.	qPCR	206
Coronary artery disease	Associated	Associated	.	.	qPCR and immunophenotyping	See **Table 11**
Systemic lupus erythematous	Associated	Associated	.	.	Paralog ratio test	199
Autism	Ns	Associated	.	.	Immunophenotyping and qPCR	207, 208
Grave's disease	Inverse association	Inverse association	.	Inverse association	qPCR	209
Seropositive rheumatoid arthritis	Ns	Associated	.	Ns	Southern blot	210

Table 10 (continued)

Complement component C4 deficiencies in different diseases

Condition	C4A deficiency	C4B deficiency	C4A or B deficiency	Total C4 CNV <4	Method	Reference
Henoch-Shönlein purpura	Ns	Associated	Associated	.	Immunophenotyping	211
Other						
Renal cell carcinoma survival	.	.	Associated	Ns	RFLP	212
Capillary leak syndrome	Associated	Ns	.	.	Immunophenotyping	213

"." Denotes not reported, "Associated" denotes susceptibility of C4 deficiency for the condition of question and "Inverse association" decreased incidence of C4 deficiency in the condition in question.

Abbreviations; total CNV, copy number variation of all C4 genes in total (A and B added together <4); Ns, not significant; qPCR, quantitative polymerase-chain reaction; PCR, polymerase-chain reaction; MPLA, multiple ligation probe analysis; RFLP, restriction fragment length polymorphism.

2.2.4.2 C4 deficiency in CAD

Interest in the role of C4 in ACS was aroused by the observation that the frequency of C4B deficiency was lower in increasing age [214]. This led to the hypothesis that diseases with high mortality, such as MI, could account for the observed decrease in the frequency of C4B deficiency. Since then, the finding of the decreased frequency of C4B deficiency in the elderly has been replicated [215].

The role of complement C4 deficiency in CAD is controversial (**Table 11**). C4B deficiency has been associated with STEMI [21] and with CAD in patients undergoing GABG [28], but was not increased in patients with history of MI [28]. *C4A* deficiency and tumor necrosis factor alpha -308A SNP have been associated with CAD and history of MI [28]. In the study with a positive association with C4B deficiency and STEMI was seen only when patients were compared against with controls of over 60 years, in whom the frequency of C4 was previously shown to be decreased when comparing with younger population [21, 216]. In addition, in this study, the serum C4 allotyping was performed directly after the diagnosis of STEMI, why the increased complement consumption might bias the results (See section 2.2.5). On the other hand, no association was found with C4 deficiency and premature MI or MI during follow-up [217, 218]. The possibly increased mortality after MI might explain the discrepant findings, but these data are based on less than 30 patients [21, 30].

Subgroup analyses have linked C4B deficiency with CAD/MI in smokers, but the raw data was not given in all studies and the patients were selected by two characteristics and may not thus be generalized (**Table 11**) [29, 21, 30]. One study reported association of C4 deficiency and CAD among current smokers over 50 years in Icelandic population. However, the age dichotomization was performed on the basis of controls, in which a sharp decrease in the frequency of *C4B* deficiency in smokers was seen after 50 years of age. A confirmatory analysis of a different ethnic group was performed, but the number of cases versus controls was very small. In addition, the interaction term was not tested [29]. In another study, the role of *C4B* deficiency in smoking was tested also on one-year mortality after MI. An association was seen in the whole population and in ever smokers [30]. The interaction term was significant, but the number of current smokers was small and thus the comparison was made between ever vs. never smokers.

C4 deficiency might be linked with CAD by functioning as a marker for the real CAD-predisposing genetic variation. *C4A* deficiency associates with certain SNP markers in introns in the MHC III cluster (r^2 from 0.44 to 0.6) and with *DRB1*03* (r^2 from 0.03 to 0.049). For C4B, the linkage is consistently lower, but *DRB1*01* and SNP in the central region have been described (**Table 7**). Of the described markers, *DRB1*01* has been associated with CAD (**Table 6**), whereas the role of others C4-deficiency linked markers is not known.

Table 11
Studies addressing C4 deficiency and CAD

N (cases/ controls)	Isotype deficiency	Phenotype	OR (95%CI)	Reference
Association				
181/737	C4B	STEMI	N.A.	21
28/153	C4B	Mortality from STEMI	N.A.	21
24/118	C4B[a]	Mortality from MI	4.13 (1.65–10.37)	30
318/248	C4B	>70% stenosis in patients with CABG	1.5 (1.0–2.1)	28
318/248	C4A and TNFα-308A	>70% stenosis in patients with GABG	2.2 (1.3–3.8)	28
158/162	C4A	anamnestic MI in patients with CABG	1.9 (1.2–3.1)	28
No association				
100/164	C4A or C4B	STEMI in young patients	Ns	215
118/134	C4A or C4B	STEMI during follow-up	Ns	215
100/90	C4A or C4B	STEMI in young patiente	Ns	217
Subgroup analyses				
24/50	C4B[a]	First MI in smokers in >50 years	22.66 (2.45–206.59)	29
23/50	C4B[a]	History of MI in smokers >50 years	Ns	29
19/50	C4B[a]	Angina pectoris, >50 years and smoker	30.07 (1.93–469.15)	29
130/17	C4B[a]	CAD and >50 years and smoking	8.90 (1.12–70.98)	29
N.A.	C4B	Men over 60 years	7.57 (3.31–17.2)	21
N.A.	C4B[a]	Survival after acute MI in ever smokers	3.50 (1.38–8.87)	30

Abbreviations; N.A, not available; Ns, not significant; GABG, coronary artery bypass by grafting; TNFα, tumor-necrosis factor alpha; OR, odd's ratio; 95%CI, 95% confidence interval).

[a] Genotypic analysis.

2.2.4.3 Complement in CAD

The role of the complement system in CAD is complex. The complement system may be connected with CAD locally or systemically and by the induction of too strong or alternatively by insufficient activation as well [58]. It is not known, what is sufficient, but not too extensive complement activation for optimal cardiovascular health. In human studies, genetic variation causing low phenotypic levels in classical and in lectin pathways, namely *C4, C2* and mannose-binding lectin deficiencies, have been associated with CAD/MI [22, 185, 219]. Animal models have shown that knocking out *C1q* [220] and mannose-binding lectin increase plaque size, suggesting that early classical and lectin

pathway activation is protective of atherosclerosis. Animal models also indicate that the activation of alternative pathway and the presence of membrane-attack complex may be atherogenic [58]. These data are not conclusive, and on the other hand, in the deficiency of C1 inhibitor, the cause of hereditary angioedema, the risk of atherosclerosis might also be increased [221]. To complicate things further, serum mannose-binding lectin levels have been proposed to have an U-shaped association with CAD [222]. For C4, very high serum levels have been associated with MI [223]. Also low C4 levels, transcribed in the serum C3/C4 ratio have also been linked with cardiovascular disease [224].

In addition to the pathogenesis of CAD [225], the complement system may have an important role in the ischemia-reperfusion injury and tissue repair after MI [226]. Pexelizumab, an antibody preventing the cleavage of C5 has been studied in MI and with coronary artery bypass by grafting in preventing the ischemia-reperfusion damage. The results in coronary artery bypass surgery have been positive [227], but for STEMI, the administration of pexelizumab after two hours after the onset of cardiac pain may be too late to prevent terminal complement activation [228]. The role of complement in ACS is difficult to assess as the decreased serum levels might be caused by increased consumption in the acute setting, thus reflecting a large infarction. On the other hand, complement components are induced in the acute setting and increased serum levels might also reflect large acute tissue damage.

The complement participates in tissue homeostasis and lipoprotein metabolism, has interconnection with coagulation and bradykinin systems, modulates the innate and adaptive immune reactions and participates in host defense against microbes and abnormal cells in more distant locations [229, 230]. Given the complexity of the pathogenesis of CAD, it seems highly likely that also the systemic reactions of complement are important [52, 53].

2.2.5 Methods for MHC gene analyses

The analysis of MHC I and II alleles was originally based on serology (complement-dependent cytotoxicity assay). From the 1980's onwards, restriction fragment length polymorphism, reference standard conformational analysis and later polymerase chain reaction (PCR) methods have been used in the HLA-allele analyses. The PCR methods include sequence-specific oligonucleotide probe, sequence-specific primer amplification, single-strand conformation polymorphism, sequence-based typing and microarray applications (**Table 12**). These methods are usually applied for the analysis of an individual's HLA alleles. Sequence-specific primer amplifications are also used for the detection of a single HLA-allele of interest, such as *B*27* for ankylosing spondylitis and *B*15:02* for abacavir hypersensitivity [231]. Lately, the SNP panels are becoming more intensely mapped with MHC, rending the imputation of HLA-alleles into large sample

sizes [26, 27]. According to our experience, the imputation may need a reference population of the same genetic background in order to accurately determine the HLA-alleles.

Various methods for *C4* genetic analyses are available (**Table 13**). *C4* CNV was first indirectly assessed by immunophenotyping the plasma C4 proteins [232]. Restriction enzymes with southern blotting was amongst the first genetic methods for assessing RCCX module composition and *C4* CNV [233]. Subsequently various PCR-based methods were developed [234]. Newer methods include paralog ratio test [186] and multiple ligation probe analysis [190]. SNP variation has not been linked with *C4* CNV status and thus the *C4* genetic status is not recorded in GWAS [199].

There are also methods for detecting the deletion of *C4A* [235], the presence of *C4A/C4B* genes [236], the *C4A* to *C4B* gene dosage and *C4* module number [233]. *CTins* has been detected by sequencing or direct PCR [237], with multiple ligation probe analysis [190] and paralog ratio test, but it has not been reported with quantitative polymerase chain reaction (qPCR). SNPs have not been associated with *CTins* presence [197].

Table 12

Comparison of assays for genetic HLA-allele analyses

Method	Principle	Through output	Resolution	Cost
Imputation	SNP haplotypes are inferred as HLA-alleles based on reference individuals with in-depth sequence and HLA-allele data.	High	High	High
RFLP	Differentially digested DNA fragments are separated by electrophoresis.	Low	Low	Not commonly used
RSCA	DNA is hybridized to probes and the mismatches are differentiated by electrophoretic mobility.	Low	Low	Not commonly used
PCR				
SSO	PCR amplicon is hybridized with a specific oligonucleotide probes.	High	Medium	Low
SSP	Several PCR reactions specific for a certain alleles.	Low	Low, medium or high	High
SSCP	Amplified DNA is run on a gel. Different alleles differ by secondary structures and thus by migration patterns.			
SBT	Amplified DNA is sequenced by primers.	Low	High	High
Microarrays	Amplified DNA is bound to specific probes and detected by lazer.	Low	High	High
Next-generation sequencing	DNA or PCR fragments are separated, immobilized by adaptors and analyzed individually.	High	High	High

Abbreviations; RFLP, restriction fragment length polymorphism; RSCA, reference standard conformational analysis; PCR, polymerase chain reaction; SSO, sequence-specific oligonucleotide probe; SSP, sequence-specific primer amplification; SSCP, single-strand conformation polymorphism; SBT, sequence-based typing. Data adapted from Smith [231], Davies at al.,[26] and Takeuchi et al.[27].

Table 13
Methods for detecting C4 copy number variation (CNV)

Method	Description	Advantage	Disadvantage	Reference
Restriction fragment length polymorphism and Southern	"Golden standard". Digestion of genomic DNA by restriction enzyme are run in a gel, subsequently transferred into membranes and hybridized with selected probes.	No extensive hardware need. All components of RCCX module can be assessed.	Laborious, time-consuming (1 week), demands large amounts of DNA, high cost per sample, need for radioactive labeling. CNV is indirectly assessed by adding results of different assays that fit together.	187
Quantitative polymerase-chain reaction (qPCR) with TaqMan	Upon binding to target sequence the TaqMan probe emits fluorescence. The amount of fluorescence is normalized to a reference gene and is compared to the fluorescence of dilution series of a control. The results are adjusted to control samples with a calibration curve.	Specific, high through output, the possibility of detecting multiple PCR product in a single run, automated CNV calling.	Inaccurate with elevated CNV levels, relatively expensive, assumes the equal amplification of target and control sequences. The inaccuracy can be circumvented by addition of controls of known CNV.	234
qPCR with TaqMan	The amount of *C4* genes is estimated directly by the ratio of reference and target genes.	Automated, use of large concentration range.	Unbalanced amplification of reference and target sequences.	189
Paralog ratio test	Near-identical PCR pairs amplify target and reference sequences and the ratio results in CNV. PCR amplicons are digested by using restriction enzymes and subsequently separated by electrophoresis. The amount is assessed by comparing target and reference genes.	Information of total *C4* genes and *C4A* to *C4B* ratio. Sample quality may not be so important.	The amount of *C4* genes is first assessed comparing to reference gene. The amount of *C4A* and *C4B* genes assessed by comparing the ratio between these and dividing the whole *C4* CNV by these. Unbalanced amplification of reference and target genes is plausible.	186
Multiplex PCR with capillary electrophoresis	Multiplex PCR produces four different amplicons; *C4A*, *C4B*, *C4* and reference control gene. The sense primers are fluorescently labeled.	Simple, one-tube multiplex PCR.	Unequal amplification of target and reference genes has to be corrected by a mathematical formula.	189
Multiplex Ligation-probe Amplification	Multiplex PCR uses synthetic two- or three segmented probes that are ligated, when associated with their target sequences. The PCR reaction is performed on these ligated probes.	Multiplex PCR allows simultaneous characterization of *C4* CNV, *C4A* and *C4B* CNV, *CTins* and length variants. All probes have identical primer ends that ensure comparable amplification.	The algorithm for assessing *C4* CNV is not reported and the development of commercial kit is under way. Commercial probes are not available. Unbalanced amplification may be present.	190

2.3 INFLAMMATION, AUTOIMMUNITY AND INFECTION IN ACS

2.3.1 INFLAMMATION

Atherosclerosis is considered to be an inflammatory disease [50]. This is emphasized by the abundance of inflammatory cells, cytokines and inflammatory markers within the plaque. Inflammatory features have also been associated with the plaques that are ruptured [65]. Even though inflammatory features within the eroded plaques are not as prominent, inflammation has been suggested to be involved with plaque erosions as well [238] (see Sections 2.1.2.1 and 2.1.2.2). In addition, inflammation may also alter the lipoprotein metabolism and the thrombogeneity of blood, favouring ACS [52, 68]. However, ACS is caused by other factors than plaque rupture and erosion (**Table 1**) and inflammation is not the only factor in the pathogenesis of ACS (**Figure 5**) [68].

CRP is an unspecific marker of the systemic inflammatory level [239]. CRP is present in atherosclerotic plaques. High serum levels of low-grade inflammation (serum hsCRP) have also been associated with MI [240], with plaque characteristics that are prone to rupture [65] and predictive of future MI [241]. Elevated serum hsCRP levels have been shown to have similar correlation over time with other measurable cardiovascular risk parameters [241]. CRP itself may cause various pro-inflammatory biological responses [242] but it is currently believed that elevation in hsCRP reflects the inflammatory state and is not causative by itself [243-245]. In addition, hsCRP is neither sensitive nor specific in predicting MI [240].

The causality of inflammation on the pathogenesis of ACS has not been shown, although current data supports this assumption. Inflammatory reactions may also be involved in tissue repair after atherogenic injury and be advantageous in certain conditions [246]. In the future, imaging techniques and novel therapeutic strategies will help to identify the role of inflammatory processes in CAD and ACS. Treatment options against proinflammatory factors include the increase of high-density lipoprotein (HDL) by HDL-mimetics, inhibition of lipoprotein-associated phospholipase A_2 by darapladip, inhibition of matrix metalloproteinases by tetracyclines, inhibition of thrombin by melagatran, inhibition of cytokines (IL-18, IL-12, IL-23), blocking chemokine or cytokine receptors (CCR2, IL-1-receptor, MIF, CCR5), directly targeting specific interactions in immune cells (CCL5-CXCL4), inducing tolerance by vaccination, antigen-loaded DCs or mucosal immunization and selective B-cell depletion by antibodies [53, 54, 247, 248].

2.3.2 INFECTION

Infection was first associated with atherosclerosis in animal models [249, 250]. In human studies, conditions such as *Chlamydophila pneumoniae* (*Cpn*) infection, acute respiratory and urinary infection and chronic periodontal infection have been associated with

increased risk for MI [251-253]. In addition, various infective pathogens have been associated with CAD/IHD/MI in different levels of evidence (**Table 14**). Infection could be involved in coronary atherosclerosis at increasing the systemic inflammatory rate, inducing autoimmunity (through for example molecular mimicry), by direct infection of the cells in atheroma [254], by activating platelets [255], by production of immunomodulatory agents [256], by activation of DC [257] or by direct damage to the cells by toxins [258]. On the other hand, cross-reactive antibody production might also be protective [259].

No infectious agent has been shown to cause atherosclerosis. There is still debate concerning the methodology of pathogen detection and the role of one versus several pathogens, named the pathogen burden hypothesis. Evidence exists that influenza vaccination decreases mortality, but this does not suggest causality. More studies are needed to replicate the role of influenza virus in ACS [254, 260].

Table 14
Examples of infections in CAD

Infectious agent	Sero-epidemiology	Immuno-histochemistry[a]	Electron microscopy[a]	Nucleic acids[a]	In situ hybridization[a]	Viable micro-organisms[a]
Chlamydophila pneumoniae	+	+	+	+	+	+
Mycoplasma pneumoniae	+		+		+	
Porphyromonas gingivalis	-	+		+		-
Aggregatibacter actinomycetemcomitans	-			+		
Cytomegalovirus	+	+				
Herpes simplex viruses	+	+	+	+	+	
Hepatitis virus B	+					
Hepatitis virus C	+					
Helicobacter pylori				+		
Fungi				+		

Adapted from Lockhart et al. [254] and Rosenfeld and Campbell [13].
[a] Detected within atherosclerotic plaque.

2.3.2.1 Chlamydophila pneumoniae (Cpn)

Of the studied microbes, *Cpn* has the strongest evidence of associating with coronary atherosclerosis (**Table 14**). The mechanisms through which *Cpn* infection may be pathogenic include the infection of endothelial cells, macrophages and SMCs. When infected, these cells undergo changes in surface composition and cellular function. In addition, the release of chlamydial heat shock protein 60 (HSP60) may be proinflammatory [246].

However, all large-scale antibiotic treatments for targeting *Cpn* have failed to show benefit, regardless of the serological status of *Cpn* (**Table 15**). As these antimicrobial

agents have not shown to efficiently eradicate the chronic forms of *Cpn* and they do not inhibit the possibility of reinfection, it is not possible to assess the effect of *Cpn* in ACS/CAD based on these data. Therefore new strategies for treating chronic *Cpn* infection have been suggested. These include targeting the important molecules in replication, survival and infective properties of *Cpn* as well as host pathways that are though to mediate the adverse inflammatory responses caused by *Cpn* [261].

Table 15
Randomized clinical trials for secondary prevention of CAD/ACS

Reference	n=	Treatment	Daily dosage	Duration	Follow-up (yrs)	Outcome	Result
Stable CAD/MI survivor							
262	60	AZ	500 mg	3d	1.5	Death, ACS	Beneficial[a]
263	4373	CF	500 mg	14d	3	Death, ACS	Ns
264	302	AZ	500 mg[b]	3mo	2	Death, ACS, stroke, revasc	Ns[a]
265	7747	AZ	600 mg[c]	3mo	2.5	Death, ACS, revasc	Ns[2]
266	4012	AZ	600 mg weekly	12mo	3.9	Death, ACS, revasc	Ns
ACS							
267	325	AZ	500 mg	7d	1	Acs	Beneficial
268	202	RX	150 mg x2	1mo	0.5	Death, ACS	Ns
269	872	RX	300 mg	1.5mo	1	Death, ACS, stroke	Ns
270	4162	GF	400 mg[d]	3mo	2	Death, ACS, stroke, revasc	Ns
271	148	CF	500 mg	3mo	3	Death, ACS, stroke	Beneficial
272	84	RX	150 mg x 2	1mo			
PCI						Death, ACS, revasc	
273	1010	RX	300 mg	1mo	1	Restenosis, MI	Ns
Meta-analysis							
274	19217	macrolides				Death or ACS	Ns
275	25271	all antibiotics				Death	Increased risk
276	25009	all antibiotics				Death or ACS or composite[e]	Ns

Abbreviations; AZ, azithromycin; CF, clarithromycin; RX, roxithromycin; GF, gemfibrozile; revasc, revascularization; PCI, percutaneous coronary intervention; d, days; mo, months; ns, no treatment effect.

[a] High *Cpn* titers.

[b] for three days, subsequently one per month.

[c] for 3 days, then 600 mg weekly.

[d] daily for 2 weeks, then 10-day course each month.

[e] Subgroups; stable/unstable, *Cpn* infection and type of antibiotic.

2.3.2.2 Macrolides in ACS

Macrolides are a collection of antimicrobial molecules sharing a macrocyclic lactone ring of variable size. Macrolides were discovered in 1952, isolated from *Streptomyces erythraea*. The most common macrolides are erythromycin, clarithromycin, rozithromycin (14-atom lactone ring) and azithromycin (15-atom lactone ring). Macrolides affect a moderately broad range of aerobic and anaerobic, mostly gram-positive bacteria. They are used for common respiratory tract and skin infections [277].

The 14- and 15-membered ring macrolides have pleiotropic effects, discovered in the 1980's when patients with diffuse panbronchiolitis, were reported to experience amelioration during erythromycin treatment [278]. The beneficial effect is thought to result from antimicrobial and anti-inflammatory actions, alone or combined (**Table 16**). Macrolides are used as maintenance treatment for chronic inflammatory pulmonary diseases such as cystic fibrosis, chronic sinusitis and bronchiectasis, but evidence for macrolide use on these conditions is not solid [279]. The treatment of CAD by macrolides was not beneficial and some studies even reported adverse effects (**Table 15**).

Table 16
The effects of macrolides

Effect	Mechanism
Antimicrobial	
Gram-positive cocci, limited gram-negative activity, possibly intracellular pathogens	Inhibition of protein synthesis Direct lysis Attenuation of biofilm formation Attenuation of bacterial communication (quorum sensing) Decreased adherence Impaired mobility Decreased production of bacterial toxins Increased intracellular concentration Cell surface alteration
Anti-inflammatory	
cytokines	Decreased synthesis/secretion of proinflammatory cytokines Increased release of anti-inflammatory cytokines
macrophages	Promotion of phagocytosis of apoptotic cells Increased differentiation Enhanced function
Neutrophils	Reduction to chemokines Stimulation of exocytosis Increased apoptosis
Endothelium	Decreased adherence molecule expression
T cells	Increased apoptosis of activated cells Increased Th1/Th2 ratio
Dendritic cells	Increase of CD80 costimulatory molecule Inhibition of IL-6 and IL-2 production
B-cells	Contradictory findings

Most of the data is derived from *in vitro* or animal models and is by no means conclusive [277, 280].

For clarithromycin, the reported adverse effects are gastrointestinal adverse reactions (9%), rash (0.5–6%), hepatotoxicity, cardiotoxicity and ototoxicity. Cardiotoxicity is related to QT-interval elongation and cytochrome P450 inhibition of other proarrythmogenic drugs [279]. In addition, clarithromycin may subject to rhabdomyolysis in interaction with statins [281]. Sudden cardiac death has been associated with erythromycin, clarithromycin and azithromycin use [282-284]. Currently antimicrobial agents such as tetracyclines are under investigation for their anti-inflammatory mechanisms for the treatment of CAD [285].

2.3.3 AUTOIMMUNITY

Autoimmune reactions mainly directed against native and oxidated low-density lipoprotein lipase (LDL) and heat shock proteins are involved in the pathogenesis of CAD, possibly in an Ig-class specific way. Different Ig classes differ in their ability for example in activating complement [53 286]. The risk of ACS is also increased in autoimmune rheumatic conditions, due to inflammatory factors, traditional risk factors and treatment [287].

2.3.3.1 Heat shock protein 60 (HSP60)

HSP60 (also referred as HSPD) is a mitochondrial protein. The gene for HSP60 is located in chromosome 2. HSP60 is present in various cell types and is also found in cytosol, cell surface and in soluble form HSP60 (sHSP60). Bacteria have also HSP60, which shares great sequence homology with human HSP60 [288, 289]. In atherosclerotic plaques, cell surface HSP60 is seen on endothelial cells, macrophages, foam cells and sometimes on SMCs, but only rarely on normal arterial intima [290]. Cell surface HSP60 is a target for HSP60 antibodies [291, 292]. HSP60 on cell surfaces may be involved in cellular signaling, membrane transport and immune signaling [288].

sHSP60 may originate from secretion or release from stressed or damaged cells. sHSP60 is present in healthy individuals, but high levels of sHSP60 have been associated with CAD [293] and with the extent and severity of CAD [294]. Risk factors such as infection, smoking, oxLDL, blood pressure and drugs lead to increase in HSP60 expression levels [295]. The levels of sHSP60 may vary among different ethnic groups [296]. An individual's sHSP60 level has been shown to remain stable over 5 years (r=0.40) [297]. sHSP60 is independent [293, 298] or weakly correlated with HSP60 antibody levels (r=0.26) [297], suggesting that antibodies are not involved in the elimination of sHSP60.

Increased plasma HSP60 is associated with MI [293]. Extracellular HSP60, or fragments of it, activate innate and adaptive immune cells. The effect might be pro- or anti-inflammatory, depending on the concentration and type of the receptor engaged. HSP60

may be a self-antigen, a foreign antigen, a carrier of functional molecules and a ligand for Toll-like receptor signaling [289].

The role of HSP60 in immunity is complex (**Figure 13**). HSP60 itself is an immune-modulating molecule, and antibodies to HSP60 are present in all individuals. Antibodies to bacterial HSP are though to result in resolution of infection, but elevated levels of HSP60 antibodies are linked with various autoimmune-like disorders such as type 1 diabetes mellitus, rheumatoid arthritis, multiple sclerosis, Bechet's disease, SLE, inflammatory bowel disease and vasculitis [289, 299].

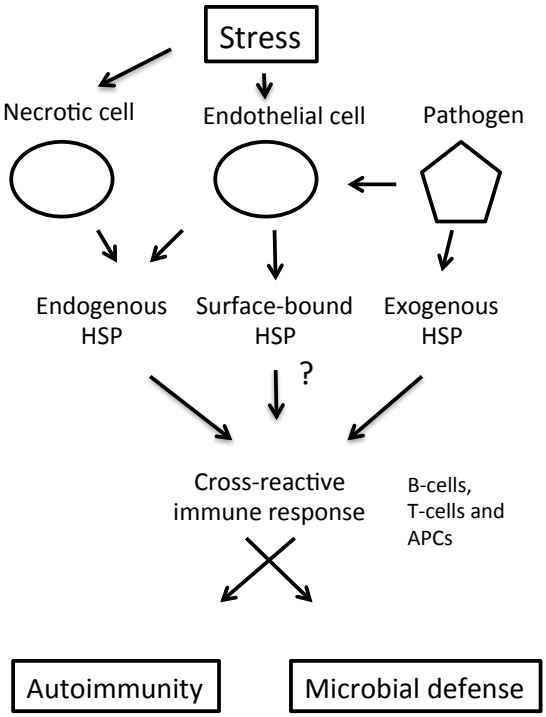

Figure 13. Different aspects of HSP60 immunity in atherosclerosis. Various stressors and infection increase the amount of surface-bound HSP and free endogenous HSP, which may have inflammatory properties. Exogenous HSP results from infection. Immune responses against bacterial HSP60 are usually involved in infection and vaccination. These antibodies cross-react with human HSP60. Autoimmunity to HSP60 is present in all individuals and it may be involved in inflammatory signaling. Modified from Alard et al. [300] and Quintana et al. [289].

High levels of IgG antibodies against human HSP60 have been associated with CAD/MI[293, 301-303]. Human HSP60 IgA, but not IgG HSP60, antibodies have been shown to predict future MI [304, 305]. Animal models have shown that HSP60 antibodies are causative in atherosclerosis [306]. The origin or the biological response to elevated HSP60 autoantibodies is not known, but all individuals have antibodies against HSP60. They might result from infection, elevated sHSP60 or be genetically determined as a part of

natural antibody repertoire [14, 293]. HSP60-reacting antibodies could be proatherogenic by causing endothelial cell apoptosis [307], thrombus formation [308] or complement- and cell-mediated lysis of (endothelial) cells [292]. HSP60 antibodies may also modulate or potentiate immune reactions [309].

T-cell immunity to HSP60 might also be important. T-cell reactivity in atherosclerotic plaques, but not in peripheral blood has been reported [291]. In animal models, tolerization to mycobacterial HSP60 has been shown to reduce plaque size [310, 311].

Antibodies to bacterial HSP60 are also linked with CAD/ACS [303]. However, these antibodies may have different epitope specificity and different biological properties than the antibodies against human HSP60 [303]. The HSP60 autoantibody levels remain constant over time, suggesting constant production or genetic control [312]. The anti-human HSP60 autoantibodies might be bacterial HSP60 antibodies that cross-react with human HSP60, or a part of natural antibody repertoire. Alternatively they might arise from recognition of modified human HSP60 or in a T-cell independent manner by stimulation of B-cells [289].

3.3.3.2 Other HSPs

HSPs 70, 27 and 90 have also been found in atherosclerotic plaques. The role of sHSP70 and HSP70 antibodies in CAD is controversial [313, 314], but HSP70 immunology might be involved in ischemia-reperfusion injury [314, 315]. sHSP27 has not been shown to associate with future cardiovascular events [316]. sHSP90 and HSP90 antibodies are associated with carotid atherosclerosis [317], but their role in CAD is not known.

3. AIMS OF THE STUDY

The aims of this Thesis were

I. To develop a novel qPCR method for genetic *C4* analyses (I).

II. To confirm the role of MHC genes and haplotypes in two clinically different ACS populations (II, III, IV).

III. To identify the possible effect of ACS-related MHC genes on inflammatory and infectious reactions by studying
    a. the response to secondary prevention trial of macrolide treatment (II),
    b. hsCRP as a marker for the general inflammatory level (III),
    c. the level of autoantibodies to HSP60 (IV).

4. MATERIALS AND METHODS

4.1 STUDY MATERIALS

The qPCR method was applied on three different materials (I; **Table 17**). The patient samples consisted of all of our HLA-Laboratory's samples between November 2004 and December 2009 that had both C4 qPCR and immunophenotyping results (n=1648). Sixty per cent of these samples were sent from the Division of Infectious Diseases in Helsinki University Central Hospital, representing mainly infection prone patients (personal communication, A. Järvinen, Chief Physician). Genomic DNA from cell lines of consanguineous subjects (n=48) was purchased from the International Histocompatibility Working Group Cell Bank (Seattle, WA). Two samples (IHW09038 and 09102) were not available. The *C4* CNV status has been previously published [234]. HapMap samples (n=89) were obtained from Coriell Cell Repositories. The C4 genetic status was assessed by southern blotting and paralog ratio test [186]. Method validation was performed on seven samples from our Laboratory and on six cell bank samples. The validation was performed by replicating the analysis from two different dilutions and in various independent runs.

The NSTE-ACS population (II, IV) was initially collected for a prospective randomized, placebo controlled, double blind secondary prevention trial of ACS between September 1998 and December 2000. The samples were collected from nine hospitals in different parts of Finland (Southern n=91, Central n=20 and Western n=37) [271]. The population consisted of cases suffering form UAP (n=43) or NSTEMI (n=105) according to the definition of MI at the time (**Table 17**). A serum sample for C4 analysis was available from 144 patients (97.3%). The secondary prevention was daily oral administration of clarithromycin (500 mg) or placebo for three months (85 days). During the average follow-up of 555 days (range 138 to 924 days), recurrent cardiovascular events (death, stroke, myocardial infarction or UAP) were recorded. Controls were age- and sex matched blood donors (n=74) that can be regarded as healthy since the presence of CAD, stroke, hypertension, hypercholesterolemia, diabetes mellitus were considered as contraindications for blood donation (IV) [23].

The STEMI patients (III; n=203) were initially recruited for an open, nonrandomized study comparing thrombolysis and primary percutaneous coronary intervention between April 2004 and April 2005 [318] (**Table 17**). Patients receiving thrombolysis underwent coronary angiography within 180 minutes after lytic therapy. There was no exclusion by age- or comorbidity. For the genetic analyses, samples were available from 162 cases (80%). Death before sample collection (n=17), no treatable lesion (n=6), lack of sample (n=17) and coronary artery bypass by grafting (n=1) were the reasons for not obtaining a sample. Controls were age- and sex matched cases without angiographic evidence of CAD (stenosis <50%), no evidence of cardiac biomarker leak, no anamnestic MI and no CAD-related intervention (n=319). The controls underwent coronary angiography and were selected from the COROGENE-cohort [319]. A reference population (n=150) that consisted of healthy subjects undergoing a health survey before accepting a new

occupational post was included [191]. One reference population sample was excluded due to degraded DNA.

Age matching was done with ≤ 5 years difference in both studies. Baseline characteristics were drawn from hospital records and from patient questionnaires. Background information was not available from the NSTE-ACS controls or the reference population.

Table 17
Patient populations

Study	Number of subjects	Samples available	Inclusion criteria	Exclusion criteria
Patients				
I	1648	1648	Genetic and phenotypic C4 analysis.	None.
II, IV	148	144/143[a]	Accelerating or prolonged anginal pain with minimal effort/exertion <48hours before randomization and transient ST-elevation, new ST-segment depression or T-wave inversion (transient or persistent) or cardiac biomarker leak.	Lack of consent, previous STEMI within 48 hours, ongoing antibiotic therapy, STEMI or thrombolysis within 48 hours, PCI or GABG performed in past six/three months or these planned, age<18 or >90 years.
III	203	166	Prolonged anginal pain accompanied with a novel ST-elevation. The onset of pain ≤12 hours.	Lack of consent, LBBB, contraindication for anticoagulants, blood pressure >160/110 after initial treatment or violation of time constraints.
Controls				
I	50	48	All available IHWG samples.	None.
I	89	81	Samples with concordant result in southern blotting and paralog-ratio test.	None.
III	319	319	Age- and sex matching, <50% stenosis in angiography.	Lack of consent, anamnestic ACS, PCI or GABG, current cardiac enzyme elevation.
III	150	149	Participation to a health survey before accepting a novel occupational post.	Lack of consent.
IV	74	74	Consecutive age- and sex matched blood donors.	None in addition to contraindications for blood donation.

Abbreviations; STEMI, ST-elevation myocardial infarction, PCI, percutaneous coronary intervention; GABG, coronary bypass by grafting; LBBB, left bundle-branch block; IHWG, International Histocompatibility Working Group.

[a] In Study II/IV, respectively

Definitions:

ST-segment elevation or depression >0.1mV in at least two extremity leads or >0.2mV in precordial leads.

Proloned anginal pain >20 minutes

T-inversion >0.3mV in at least three extremity or precordial leads (excluding V1).

4.2 METHODS

The studied parameters varied within different populations and are summarised in **Table 18**. The details of each analysis are described below.

Table 18

Laboratory analyses

Population	HLA markers	Serum parameters
Infection-prone patients (I)	C4 CNV	_
NSTE–ACS (II, IV)	HLA-A, HLA-B and HLA-DRB1 alleles, C4 allotypes and four LTA SNPs	hsCRP, anti-hHSP 60, cardiac biomarkers, pro-BNP, lipids, leukocyte count, antibodies to *Cpn,* C3 and C4 concentrations
Controls (IV)	HLA-A, HLA-B and HLA-DRB1 alleles, C4 allotypes and four LTA SNPs	Antibodies to *Cpn*
STEMI (III)	*HLA-B*35, HLA-DRB1*01* and *C4* CNV	hsCRP, cardiac biomarkers and lipids
Controls (III)	*HLA-B*35, HLA-DRB1*01* and *C4* CNV	hsCRP, cardiac biomarkers and lipids
Population sample (III)	HLA-A, HLA-B and HLA-DRB1 alleles, C4 allotypes and *C4* CNV	hsCRP

Abbreviations; CNV, copy number variation; LTA, lymphotoxin alpha; hsCRP, high sensitivity C-reactive protein; hHSP, human heat shock protein; BNP brain natriuretic peptide; *Cpn*, *Chlamydophila pneumoniae*; C3, C4, complement components 3 and 4.
Definitions:
Cardiac biomarkers; troponin T, troponin I or creatine kinase MB-mass.
Lipids; total cholesterol, low-density lipoprotein lipase, high-density lipoprotein lipase and triglycerides.

4.2.1 DNA ANALYSES

4.2.1.1 Isolation

Genomic DNA was isolated using the Manufacturers' instructions. For cases, NucleoSpin ® QuickPure (Macherey-Nagel, GmbH & Co. KG, Düren, Germany; I, III) or Genta Puregene Kit (Qiagen, Vienna Austria; II, IV) were used. For controls, Autopure LS® with Puregene (Qiagen; III), Gentra Puregene (Qiagen) or salting out (II, IV) [320] were used. For the reference population sample, Gentra Puregene Kit (Qiagen; III) was used.

4.2.1.2. Primers

The primer sequences and detailed information on the qPCR protocols are given in **Table 19**.

Table 19
Annealing temperature, master mix, amplicon size and analysis threshold for each qPCR

qPCR run	Specificity	Primer sequence 5'–3'	SYBR® Green Mix	Annealing temperature (ºC)	Analysis threshold
HLA-B*35_1	*B*35_f*	GTCCGAGGACGGAGCCCCG	ABsolute™	60	0.05
	*B*35_r*	GTAGCCGCGCAGGTTCCGC			
HLA-B*35_2	*B*35_f*	CCGCTTCATCGCAGTGGGC	ABsolute™	60	0.05
	*B*35_r*	GTGTTGGTCTTGAAGATCT			
HLA-DRB1*01	*DRB1*01_f*	CTTGTGGCAGCTTAAGTTTGAAT	Brilliant®	55	0.05
	*DRB1*01_r*	GCATCTTTCCAGCAACCG			
C4A	*C4A_f*	AGGACCCCTGTCCAGTGTTAGAC	ABsolute™	55	0.03
	C4_r	CACTCTCTGCTTCAATGGCT			
C4B	*C4B_f*	AGGACCTCTCTCCAGTGATACA	Brilliant®	57	0.03
	C4_r	CACTCTCTGCTTCAATGGCT			
CTins	*Ctins_f*	CTCTTCTCCCTGCCTTCCT	Brilliant®	57	0.1
	Ctins_r	GCTCTGAGAACCAGTGACTGAGAG			
Beta-actin	*Beta-actin_f*	GCACTCTTCCAGCCTTCC	ABsolute™	60	0.05
	Beta-actin_r	GCGCTCAGGAGGAGCAAT			

HLA-B*35 is first assessed by HLA-B*35_1 qPCR run accompanied by melt-analysis. Positive samples are entered in HLA-B*35_2 run, which differentiates real and false positive signals.
Abbreviations; f forward primer; r, reverse primer.

4.2.1.3. Real-time qPCR

The qPCR method was developed in our Laboratory. Genomic DNA was diluted to 50 ng/µl in sterile water, and, after assessing purity (A260/A280 >1.7) by spectrophotometer (NanoDrop ® ND-1000, NanoDrop Technologies, Wilmington, DE, precision 0.1 ng/µl), the sample was adjusted to 10 ng/µl (between 8.0 and 14.0 ng/µl). The primers for C4 (Sigma Genosys, Heverhill, UK) were selected based on published sequences [181, 193, 197, 237]. Two SYBR® Green Master Mixes (ABsolute™ qPCR SYBR® Green Mix, AB-1159, ABgene, Epsom, UK and Brilliant SYBR® Green QPRC Master Mix, Staratagene, AH

Diagnostics, Skärholmen, Sweden) with modified *Taq* polymerase having hot start capability were used (**Table 19**).

Real-time qPCR was performed with Rotor-Gene 3000 (Qiagen). The qPCR program was as follows: Hot start at +95°C for 15 minutes, followed by 30 three-step cycles (15 seconds at +95°C, 45 seconds at annealing temperature and 45 seconds at +72°C). The annealing temperatures were optimized for each run (**Table 19**). Samples with known *C4* CNV (from 0 to 3 in *C4A* and *C4B* runs and from 0 to 1 in *CTins* run, patient samples with consistent immunophenotyping and qPCR results) served as controls. For *DRB1*01* and *B*35*, controls with 0, 1 and 2 copies were used (III).

Data were analysed with Rotor-Gene software v 6.0 (Qiagen). The primary data was normalized according to the Manufacturer's instructions. Run validity was ensured by controls and adequate standard curves (R^2 >0.8). Prior to the *C4* CNV analyses, the concentration comparability between samples and controls was assured by the amplification of a housekeeping gene (beta-actin) in parallel with standard dilutions of 8, 10 and 14 ng/µl. Samples outside this range were discarded. The sample's concentration was determined as comparable (9–11 ng/µl, assuming control concentration 10 ng/µl), lower or higher than the control's concentration (**Figure 14**). The threshold level of fluorescence was set to separate different CNVs by one threshold cycle (Ct) value. Ct is the number of cycles at which the sample's trace exceeds the arbitrary threshold. Ct values >26 were considered as outliers. The use of concentration range was used to prevent the false interpretation of CNVs by rounding (**Table 20**).

Table 20

Prevention of false interpretation of copy number variation (CNV) by the use of concentration range

Real CNV	False CNV interpretation	X-fold change in the amount of DNA needed for false interpretation	Concentration for the false interpretation (ng/ul)[a]	Action by beta-actin run[b]
1	2	2.0	20.0	discard
2	1	0.5	5.0	discard
	3	1.5	15.0	discard
	4	2.0	20.0	discard
3	1	0.3	3.0	discard
	2	0.6	6.0	discard
	4	1.3	13.0	re-dilute
4	1	0.25	2.5	discard
	2	0.5	5.0	discard
	3	0.75	7.5	discard
	5	1.25	12.5	re-dilute

[a] Result of X-fold change in the sample concentration of 10 ng/ul.

[b] Beta-actin inclusion range is 8-14 ng/ul. Samples within this range, but differing from the control's concentration are used, but rounded up or down to compensate for the difference.

The determination of CNV was performed by visual inspection, superimposing the sample's trace on the controls' traces. Samples with comparable concentration with the controls were recorded as having the CNV of the closest trace (Ct difference <0.4; **Figure 14**). Samples with lower or higher concentration than the controls, but within the concentration range, were rounded up or down, respectively. The analysis program calculates linear standard curve equation, which is formed from controls' Ct-values and logarithmic transformation of a given concentration (with beta-actin analyses) or CNV (with *C4* analyses). The unknown sample's DNA quantity is calculated from the equation of standard curve with the obtained Ct value. This value was used as a "second opinion".

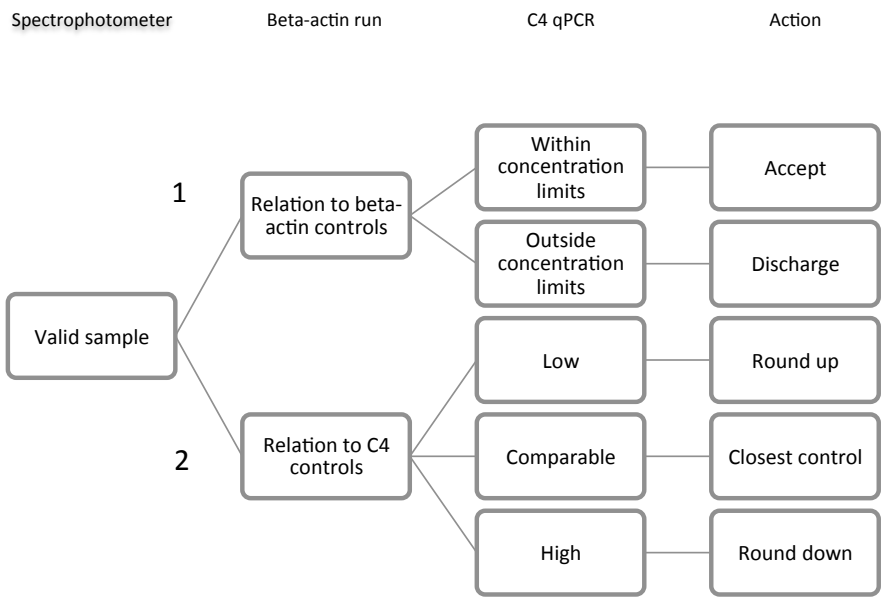

Figure 14. Sample quality check-up and analysis workflow. The sample validity is first assessed by assuring sample purity by spectrophotometer and subsequently in beta-actin run (1). If the sample quality is adequate, the sample's relation to C4 controls is also assessed (2).

4.2.1.3 HLA analyses

The presence of *B*35* and *DRB1*01* was assessed with the qPCR procedure described above (III) except for population sample, which was assessed by commercial kit (SSP for *HLA-B* and *HLA-DRB,* Qiagen; **Table 19**). HLA-A, HLA-B and HLA-DRB1 genotypes were determined by commercial kits, following the Manufacturer's instructions (II, IV). Four lymphotoxin alpha SNPs were assessed by PCR (II, IV). These data on HLA in Studies II and IV were available from a previous study by our group [23]. All analyses were performed at our HLA-Laboratory accredited by European Federation for Immunogenetics.

4.2.2 SERUM ANALYSES

Carboxypeptidase B (Roche Diagnostics Gmbh, Mannheim, Germany) and neuraminidase (Type IV Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) treated

serum samples were separated by electrophoresis and subsequently stained with polyclonal anti-C4 antibody (DiaSorin Inc., Stillwater, MN) (I, II, IV) according to Sim and Cross [232]. The number of *C4* genes was estimated from the relative intensities of protein bands.

IgA and IgG antibodies to human recombinant HSP60 (Stressgen, Victoria BC, Canada) were detected with enzyme-linked immunoassay. The patients' values had been previously published [312]. For the Study IV, the results were adjusted to commercially available sample (Sandoglobulin; Sandoz, Basel, Switzerland) as 100 EIU.

Microimmunofluorescence was used to assess the IgA and IgG antibodies against the elementary bodies of the Kajaani 6 strain of *Cpn*. Immune complexes against *Cpn* were recorded with polyethylene glycol precipitation. Dissociated IgG was measured from dissociated immune complexes by microimmunofluorescence. The results have been previously published [123]. Serum C3 and C4 concentrations were measured by nephelometry [224]. Creatine kinase MB mass, Troponin T (Roche Diagnostics, GmbH Mannheim, Germany; immunochemiluminometric, accredited method), propeptide of B-type N-terminal natriuretic peptide (Roche Diagnotics; immunochemiluminometric method), total cholesterol, HDL, triglycerides (Roche Diagnostics; enzymatic, [colorimetric for total cholesterol], accredited method) and hsCRP (Orion Diagnostica, Espoo, Finland; photometric, immunochemic, accredited method) were measured according to the laboratory standards of the accredited Laboratory Services of the Hospital District of Helsinki and Uusimaa (HUSLAB, Helsinki, Finland). LDL cholesterol was calculated from the Friedewald formula [321].

4.2.3 STATISTICAL ANALYSES

4.2.3.1 General statistical analyses

All statistical analyses were performed with SPSS, version 12.0.1 (II) and 18.0.3 (I, III-IV; IBM, New York, NY). Univariate analyses were performed with the appropriate statistic (Chi-square test, Fisher's exact test, Student's t-test and Mann-Whitney U-test). The continuous variables that did not follow normal distribution were log-transformed, if the transformation resulted in normal distribution. hsCRP values above 10.0 mg/l were excluded in order to eliminate acute infectious and inflammatory reactions (III).

In studies with qPCR typing of *C4*, *CTins* was reduced from the total *C4A* CNV (I, III). Complement *C4* genes were assessed as the presence of *C4A* deficiency (<2 copies), *C4B* deficiency (<2 copies) or these combined (<2 copies of either C4A or C4B; I-IV). HLA-alleles and haplotypes were assessed as population frequencies (number of alleles/2*number of patients; III) and as marker positive cases (II-IV). HLA-haplotypes with population frequency <5% in all study materials were summed as "else" (III).

The continuous variables were used as such and as dichotomized to high and low levels. The cut-off values were based on the suggested values, when available (**Table 21**).

The association between different genotyping results was assessed with Cohen's kappa (I). Correlation (r^2) was measured with parametric or nonparametric methods (IV). ORs with their 95%CIs were assessed by Chi-square statistics or by binary logistic regression. Multivariate analysis was done using binary logistic (III, IV), or Cox regression (II, IV). The multivariate adjustment was made with the statistically important covariates identified in the conditional forward modelling that were subsequently assessed with the enter-model (III) or with available background characteristics, regardless of their statistical effect on the dependent variable (II, IV). Interaction terms were studied (II–IV). Kaplan-Meier test was used to analyse recurrent cardiovascular events between different patient groups during the follow-up (II, IV). P-value <0.05 was considered statistically significant. The p-value was corrected for multiple comparisons in Study IV, but not in Study III. Hazard ratios (HR) were assessed by Cox regression (II, IV).

Table 21
Cut-off values of continuous variables

	Study	Cut-off value	Cut-off point and reference
hsCRP	III	3.0 mg/L in women and 2.5 mg/L in men	HUSLAB reference value above 97.5th percentile, based on healthy blood donors
Cpn serology	II, IV		
IC		2	Median [322]
IgA		40	4th quartile [322]
IgG		128	4th quartile [322]
HSP60	IV		
IgA	cases vs. controls	22.98 and 44.4EIU	Tertile
IgG	cases vs. controls	50.33 and 87.85 EIU	Tertile
IgA	follow-up	40.57 EIU	Median
IgG	follow-up	71.56 EIU	Median

4.2.3.2 Power calculation

The observed power with α=0.05 for survival with presence of C4 deficiency using cases receiving clarithromycin as cases and those receiving placebo as controls was 63% (http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize; II). Power calculation with α=0.05 was assessed for case-control setting with the following settings; risk allele frequency=0.15, prevalence=0.03, OR=2.37, D'=1.0 with *DRB1*01* as the risk marker (http://pngu.mgh.harvard.edu/~purcell/gpc/; III) [4, 23].

4.2.3.3 Haplotype construction (III)

The diploid *C4* copy numbers were split into gene frequency data, based on other gene markers (Eronen et al, submitted). For gene copy numbers zero, one and three, the splitting was straightforwardly done as gene frequencies of 0/0, 0/1 and 2/1, between the two chromosomes, respectively. From 394 cases with two *C4A* genes, all except 46 were assessed as equally distributed between the two chromosomes. For *C4B*, all cases with two genes were distributed equally. All cases with four *C4A* genes were equally distributed, whereas no cases with four *C4B* genes were detected.

Haplotypes were constructed using a Bayesian algorithm, PHASE (version 2.1) according to the Manufacturer's instructions [323]. The estimates of population haplotype frequencies were also drawn from PHASE and the 95%CI was assessed as ±1.96 standard error of frequency estimate, SE(f). Haplotypes were entered to Arlequin (version 3.5) [324] for analyses of linkage disequilibrium, Hardy-Weinberg equilibrium (HWE) and differences in haplotype frequencies between populations (exact population differentiation). The tests were performed according to the Manufacturer's instructions.

4.3 ETHICAL CONSIDERATIONS

The patients included in the studies gave an informed patient consent and the study protocols were approved by the Ethics Committee, Department of Medicine, Hospital District of Helsinki and Uusimaa (II–IV). The Ethics Committee waived the need for committee's approval and patient consent for Study I. This study used a patient material that was retrospectively and anonymously analysed as frequency data from our Diagnostic Laboratory's results without patient identification, any interventions or contacts during the study. The results were commanded on clinical grounds from different institutions and used to guide patient care as seen suitable by the treating clinician. The clinical studies (II–IV) were performed during 1998–2000 and 2004–2005. Therefore no clinical trial number exists.

5. RESULTS

5.1 qPCR METHOD

5.1.1 SPECIFICITY

The specificity was assessed by computer search of published genomic sequence and ascertained by the melt-analyse of the PCR products (I). The primers did not recognize any other identical sequences and no PCR products of similar size, even when primer mismatches were allowed, were found. Melt-analyses did not reveal unspecific amplicons for *C4A* or *C4B* assays, whereas some unspecific amplification was detected for *CTins* assay. The unspecific amplification in *CTins* run did not exceed the Ct value and thus did not result in false positive recording of *CTins*. Negative controls and empty wells were devoid of any recordable PCR products, indicating the absence of primer-dimers and contamination.

5.1.2 RELIABILITY

Internal reliability was tested using two different dilutions, five (for internal validation samples) or eight (for external validation samples) independent runs and various replications within a run (I). The internal (n=7) and external (n=6) validation samples showed concordant results in all replications, showing no inter-assay variation between different dilutions or different runs. However, as the CNV is manually determined, the numerical data on the reliability reflects mainly the quality of sample processing and data recording.

External reliability was evaluated by concordance rate between different methods (I). It was high and essentially similar between different methods, ranging from 95.7 to 98.8% for *C4A* analysis and from 97.2 to 100.0% for *C4B* analysis (**Table 22**). *CTins* was not validated by an independent analysis. However, the *CTins* primer sequence is in parallel with the recent sequencing results [197, 237] and all four patient samples with two *CTins* copies had no trace of plasma C4A in immunophenotyping.

Table 22

Concordance (%) for C4 qPCR

Method	*C4A* qPCR	*C4B* qPCR
Immunophenotyping	95.7 (1436/1500)	97.2 (1499/1542)
TaqMan qPCR	97.9 (47/48)	100 (48/48)
Southern blot and paralog ratio test[a]	98.8 (80/81)	100 (81/81)

[a] Concordant results from paralog ratio test and Southern blotting.

The qPCR method was applied for patients with STEMI (III). The genetic tests showed that *C4A* CNV was not in HWE in patients and in controls, whereas for *C4B*, HWE was not attained in the reference population sample ($p=0.002$, $p<0.001$ and $p=0.0416$, respectively). As the most common *C4* status is the presence of two *C4A* and two *C4B* genes and the presence of homozygous deficiency is very rare, the violation of HWE does not question the reliability of the results but rather reflects the rarity of the *C4A* deficiency.

5.1.3 CTINS IN C4 DEFICIENCY

The described qPCR method was used to assess the presence of *CTins*, the most common mutation leading to *C4A* silencing [193]. Although the frequency of *CTins* was low (3–6%), it resulted in *C4A* deficiency in 72–80% of its carriers. This corresponds to 18–20% of *C4A* deficiencies and 3–5% of all cases (I, unpublished observation) (**Table 23**). It is presumed that all *CTins* mutations are present in *C4A* because the presence of *CTins* in *C4B* has been reported only rarely [193, 197, 237, 325].

Table 23

Frequency of CT insertion (*CTins*)

	CTins	*CTins* leading to C4A deficiency			
	n (%)	n	Of *CTins*	Of C4A deficiency	Of all cases
Recurrent infections (n=1618)	105 (6.4%)	76	72.4%	19.9 %	4.7 %
STEMI (n=162)[a]	5 (3.1%)	4	80.0%	18.2 %	2.5 %
No CAD (n=316)[a]	15 (4.7%)	12	80.0%	18.2 %	3.8 %

[a] Unpublished data

5.2 MHC IN ACS

5.2.1 C4 DEFICIENCY IN ACS

The association between MHC and NSTE-ACS has been published [23]. However, this study assessed only the MHC haplotypes. When the frequencies of C4 deficiencies in patients with NSTE-ACS and controls were compared, no statistically significant difference was seen (IV; **Table 24**). In addition, C4 deficiency (A and B combined or alone) was not associated with anamnestic MI, anamnestic coronary intervention or cardiovascular end points during the follow-up of patients with NSTE-ACS (II). *C4* deficiency was not associated with STEMI (III; **Table 24**).

Table 24
C4 deficiency in ACS

C4 deficiency	STEMI (III)			NSTE–ACS (IV)		
	Patients	Controls		Patients	Controls	
	n=162	n=316	p=	n=144	n=74	p=
C4A	13.6 (22)	20.7 (66)	0.057	29.2 (42)	25.7 (19)	0.587
C4B	50.0 (81)	46.4 (148)	0.454	39.6 (57)	28.4 (21)	0.102
C4A or C4B	61.1 (99)	64.3 (205)	0.498	66.0 (95)	52.7 (39)	0.057

Data is expressed as % (n).

5.2.2 MHC IN STEMI

When patients with STEMI and controls without CAD were compared, the allele frequency of *DRB1*01* was higher in patients, but statistical significance was not attained (22.84%, 74/324 vs. 17.71%, 113/638, respectively; p=0.057; III). However, when patients with STEMI were compared with the reference population, the frequency of *DRB1*01* was significantly increased in STEMI (22.84%, 74/324 vs. 14.77%, 44/298, respectively; p=0.010; **Table 25**). When the STEMI population was pooled with NSTE-ACS and obstructive CAD from a previous study [23], *DRB1*01* was more frequently recorded in all patients than in controls (22.0%, 166/754 vs. 15.0%, 166/1108, respectively; p<0.001). *B*35* was not associated with STEMI (data not shown).

Table 25
Allele frequency of *DRB1*01* in ACS (III)

Study population	Patients	Controls	Reference population	p[a]	p[b]
*DRB1*01*					
STEMI	22.84 (74/324)	17.71 (113/638)	14.7 (44/298)	0.057	0.010
CAD[c]	22.0 (166/754)	15.0 (166/1108)		<0.001	
*DRB1*01*-haplotype[d]					
STEMI	5.86 (19/324)	2.82 (18/638)	2.68 (8/298)	0.002	0.052
ACS[e]	5.34 (28/524)	2.80 (22/786)		0.019	

Data is expressed as % (n[cases]/n[controls]).
[a] Patients vs. controls
[b] Patients vs. reference population
[c] Patients with STEMI, NSTE-ACS and obstructive CAD combined
[d] *DRB1*01*, no *C4* deficiency nor *HLA-B*35*
[e] Patients with STEMI and NSTEMI combined

5.2.3 MHC HAPLOTYPE IN ACS

Overall, the haplotype frequencies were similar across all three populations (p=0.075). However, a haplotype with *DRB1*01* with neither *C4A/C4B* deficiency nor *B*35* (the 1,1,1,0-haplotype, respectively), was more frequent in patients (5.86%, 19/324) than in controls (2.82%, 18/638; p=0.020) but the frequency was only borderline higher than in the reference population (2.68%, 8/298; p=0.052; III; **Table 25**). After multivariate adjustment, the presence of the 1,1,1,0-haplotype doubled the risk for STEMI, but only between cases and controls (11.1%, 18/162 in cases vs. 5.3%, 18/319 in controls, OR=2.69, 95%CI=1.21-5.96; p=0.015, adjusted for LDL and HDL).

When STEMI and NSTEMI [23] patients were combined, the frequency of the 1,1,1,0-haplotype was higher in patients than in controls (5.34%, 28/524 vs. 2.80%, 22/786; p=0.019; III). The baseline characteristics that were associated with the haplotype were lack of family history of CAD and presence of arterial hypertension (p=0.007 and p=0.012, respectively). Previous MI was twice as common in haplotype carriers, but the difference was not statistically significant (p=0.143). Such differences were detected only in the STEMI population.

*5.2.4 DRB1*01 HAPLOTYPE IN MEN*

Genders were assessed separately. In males, both *DRB1*01* and the 1,1,1,0-haplotype were associated with STEMI (III; **Table 26**). *DRB1*01* was more common in STEMI than in controls or in the reference population sample (25.00%, 59/236 in patients,

17.74%, 83/468 in controls and 10.20%, 10/98 in the reference population; p<0.05 for both comparisons). For the 1,1,1,0-haplotype, the results were similar (6.78%, 16/236 in patients, 2.35%, 11/468 in controls and 1.02%, 1/98 in reference population; p<0.05 for both comparisons). However, the interaction term between gender and the haplotype was not significant, and the statistical power in women was too small to exclude the effect of the haplotype. The results were similar when previous material was combined.

Table 26

*DRB1*01* in ACS in males (III)

Study population	Patients	Controls	Reference population	p[a]	p[b]
*DRB1*01*					
STEMI	25.00 (59/236)	17.74 (83/468)	10.20 (10/98)	0.023	0.002
CAD	22.32 (133/596)	14.71 (125/850)		<0.001	
*DRB1*01*-haplotype					
STEMI	6.78 (16/236)	2.35 (11/468)	1.01 (1/98)	0.004	0.029
ACS	5.59 (21/376)	2.12 (12/566)		0.005	

Data is expressed as % (n[cases]/n[controls]).

[a] Patients vs. controls

[b] Patients vs. reference population

5.3 MHC IN INFECTION, INFLAMMATION AND AUTOIMMUNITY

5.3.1 ANTIMICROBIAL TREATMENT AND INFECTION

Due to the small number of end points and marker positive cases of *DRB1*01* and *B*35* in the NSTE-ACS population [23], the outcome of the clarithromycin trial was reanalysed only in relation to the patients' C4 deficiency status (II). We studied whether C4 deficiency affects the antimicrobial treatment effect in preventing cardiovascular end points in a *post hoc* analysis [271].

In cases with C4 deficiency, clarithromycin was associated with lower end point frequency (18.8%, 9/48 vs. 40.4%, 19/47 for clarithromycin vs. placebo use, respectively, HR=0.34, 95%CI=0.13–0.86; p=0.021; II). The event-free survival was also better (Kaplan-Meier log rank test; p=0.015). The clarithromycin treatment was the only baseline characteristic that was associated with decreased number of end points (Cox regression, HR=0.15, 95%CI=0.041–0.52; p=0.003). In cases without C4 deficiency, no treatment effect was seen. However, the interaction term between C4 deficiency and clarithromycin was not significant. The power was too low to exclude the advantage of clarithromycin in patients without C4 deficiency. Although the beneficial effect seemed

to account for the C4B deficiency, due to the small number of patients, the effects of different isotype deficiencies (C4A vs. C4B) could not be reliably estimated.

C4A deficiency was more common in infection prone patients than in general population (24%, 381/1618 vs. 16%, 24/149, OR=1.60, 95%CI=1.02–2.52; p=0.039; I). *Cpn* infection markers did not associate with C4, C4A or C4B deficiency or change during antimicrobial treatment (II).

5.3.2 hsCRP

In patients with STEMI, hsCRP data were available at a steady state only from 86 patients and were further selected to values below 10.0 mg/l resulting in available cases in 50.0% (81/162) in included patients, 88.7% (283/319) in controls and 96.6% (144/149) in the reference population (III). The selection of values below 10.0 mg/L excludes cases with acute infection or inflammation and is commonly used, but it also reduces the amount of available samples.

The 1,1,1,0-haplotype carriers had higher hsCRP levels than non-carriers in patients (median [IQR] 3.37 [5.27] vs. 1.14 [1.65] mg/L; p=0.019) and controls (median [IQR] 2.90 [3.03] vs. 1.21 [1.73] mg/L; p=0.009; III). In conditional forward binary regression, the 1,1,1,0-haplotype was the only factor to associate with elevated hsCRP levels (levels in the top 2.5% of a healthy reference sample) in patients (OR=5.16, 95%CI=1.29–20.54; p=0.020), whereas in controls the 1,1,1,0-haplotype and body mass index were identified as significant factors (body mass index-adjusted OR=4.18, 95%CI=1.42–12.35; p=0.01; III). Neither C4 deficiency, *DRB1*01* nor *B*35* alone were associated with the elevation in hsCRP-level (II, III). The numbers in different subgroups became too small to assess the effect of 1,1,1,0-haplotype on hsCRP levels in males.

5.3.3 Autoantibodies

HSP60 IgA antibodies were associated with ACS (see section 5.4). C4 deficiency was associated with elevated HSP60 IgA antibodies in patients with STEMI (r^2=0.21, p=0.011), but not in controls (IV). The HSP60 IgG was not correlated with C4 deficiency. *DRB1*01* or *DRB1*01-B*35*-haplotype were not associated with significant alteration in HSP60 IgA.

5.4 HSP60 AUTOANTIBODIES IN ACS

After establishing the correlation between C4 deficiency and HSP60 IgA, we wanted to see whether the HSP60 IgA was significantly associated with NSTE-ACS (IV).

5.4.1 PATIENTS WITH NSTE-ACS

Serum HSP60 IgA level was higher in patients than in controls (median [IQR] 40.56 [37.96] vs. 19.72 [16.53]; p<0.001; at admission). The results were similar at all four time points, also after correcting for multiple comparisons. The ORs for NSTE-ACS increased with increasing HSP60 IgA levels (middle vs. low tertile, OR=2.36 95%CI=1.20–4.62; p=0.013 and high vs. low tertile, OR=15.32, 95%CI=5.83–40.08; p<0.001). Adjusting for possible confounders (age, gender, *B*35*, *DRB1*01*, positive *Cpn* serology) did not change the results (data not shown).
The association between HSP60 IgG levels and NSTE-ACS was not as clear. In only half of the four time points, the IgG levels were significantly higher in patients and increasing levels did not translate into increased OR.

5.4.2 PATIENTS WITH CARDIOVASCULAR END POINTS

During the follow-up, 31.65% of patients (44/139) had a cardiovascular end point (ACS, death, stroke or refractory angina). In the initial study, clarithromycin treatment was associated with significant reduction in the number of end points [271]. Therefore the treatment groups were analyzed separately. In the placebo group, patients with end points had higher HSP60 IgA levels at admission (median [IQR] 56.90 [43.18] vs. 37.08 [26.89], p=0.035). Baseline characteristics or cardiac biomarkers did not differ in patients with or without end points. In the clarithromycin group, no differences were found. HSP60 IgG was not different in patients with and without end points in either treatment group.

5.4.3 PREDICTION OF CARDIOVASCULAR END POINTS

HSP60 IgA was dichotomized according to median for assessing its predictive role on recurrent end points. Patients with HSP60 IgA levels above median, belonging to the placebo group had the worst event-free survival, when compared to the other groups (Kaplan-Meier logrank; p<0.01 for all; **Table 25**). There was a significant interaction between HSP60 IgA level and study treatment on the occurrence of end points (p=0.026). In patients on placebo, elevated HSP60 IgA was associated with future end points, independently of background risk factors (adjusted HR=3.16, 95%CI=1.35–7.36;

p=0.008), whereas clarithromycin was protective only in the elevated HSP60 IgA group (HR=0.23, 95%CI=0.09–0.61; p=0.003). Adjustment was performed for age, gender, smoking, body-mass index, diabetes mellitus, hypertension and hypercholesterolemia. In patients on clarithromycin, HSP60 IgA had no effect of end points. Similarly, in patients with low HSP60 IgA, clarithromycin did not prevent end points.

Table 25
Recurrent cardiovascular end points, grouped by randomization and HSP60 IgA levels

Randomization	HSP60 IgA	End points % (n/n)[a]	Kaplan-Meier p=
Placebo	High	55.9 (19/34)	Reference
Placebo	Low	26.5 (9/34)	0.006
Clarithromycin	High	17.1 (6/35)	0.001
Clarithromycin	Low	25.0 (9/36)	0.009

[a] patients with/without end points.

5.4.4 FACTORS AFFECTING HSP60 AUTOANTIBODIES

HSP60 IgA level in samples taken 12 months apart were strongly correlated (r^2 =0.66). C4 deficiency, IgA antibodies to *Cpn* (IV) and periodontal pathogens [312] correlated weakly with HSP60 IgA. HSP60 IgA levels were not correlated with baseline characteristics, cardiac enzymes or serum levels of C4 or C3 except for hypertension, which had negative correlation with HSP60 IgA.

6. DISCUSSION

6.1 qPCR METHOD

6.1.1 VALIDATION AND APPLICABILITY

CNV is an important form of structural variation of the genome. In addition to small insertions and deletions (1–50 base pairs) and large chromosomal arrangements (>1 mega base pairs), CNV contributes to the structural differences in genomes between individuals. Regions that are susceptible to CNV cover all chromosomes, approximately 12% of the genome, exceeding the area affected by SNPs [326]. CNV causes loss or gain of genomic sequence, is usually present as segmental duplication that reflects to the diploid copy number of the locus. The genes that are subjected to CNV are often involved in immune and environmental pathways, suggesting that the CNV might be involved in adaptive selection in human populations. Microarrays and sequencing are used for the genome-wide detection or discovery of CNVs [327]. For validation of a locus-specific CNV, different methods are applied [328].

Low C4 CNV can be transcribed as C4 deficiency (**Table 9** on p. 30). C4 deficiency has been linked with various inflammatory conditions and the associations are expanding to processes that are not classically seen as such, such as autism [166, 207, 208]. Many of the disease association studies of C4 deficiency have been performed on small sample size and need to be replicated in large-scale materials. As GWAS cannot be used for C4 CNV analysis [186, 197, 199], there is a need for high-throughput genetic analyses for C4 CNV.

Various methods for analyzing *C4* CNV have been developed. Each of these has their its advantages and disadvantages (see Section 2.2.5). One of the major advantages of our method it the use of qPCR. Real-time qPCR operates in a closed system, which saves time and lowers the risk of contamination. In addition, it is extremely sensitive and demands only minute amounts of DNA. The disadvantages are the limited amount of probes or assessed genes per run [328], whereas in multiple ligation probe analysis [190], various different genetic regions can be covered within a single run. Another limitation is the extreme sensitivity to small amounts of DNA, which induces high risk of contamination. Possibly underlying the sensitivity and the laboriousness of qPCR run optimization, Chung et al. reported technical difficulties in setting up a *C4* qPCR analysis [233]. The rate of concordance with southern blotting is essentially comparable with paralog ratio test [186], qPCR with TaqMan probing [234] and with our method (I). Therefore the optimal method for each laboratory depends on the requirements, sample size, knowhow and existing hardware, to name a few.

Previously, two methods using real-time qPCR for *C4* CNV typing have been published. Both of them use the TaqMan® chemistry, but differ in qPCR program and data analysis [189, 234]. Contrary to these, we have chosen SYBR® Green, the unspecific dye for our *C4* analyses. Compared with TaqMan®, SYBR® Green is less expensive and can be used

with all real-time machines. In addition, the imbalances of amplification between reference and control gene may subject qPCR to errors in TaqMan®, but in our method, the assays are performed in separate runs that are individually optimized for efficiency (I). However, the TaqMan® analyses may also be performed in separate runs, whereby the advantage of SYBR® Green would be diminished.

The disadvantage of our method is it's extreme sensitivity to DNA quality, amount and concentration. We experienced difficulties in comparability of samples isolated with different methods (I). Further optimization might thus be needed for example differently isolated DNA samples. For TaqMan® *C4* qPCR assays, these issues have not been reported as problematic. In addition, the unspecific detection of any double-stranded DNA by SYBR® Green subjects the method to errors in unspecific replication and for primer-dimers. Therefore, proper primer design and validation is essential. The use of concentration range also means that our method requires three separate runs for *C4* CNV determination, whereas with TaqMan the analyses can be performed in one [189] or two [234] runs. Furthermore, the data is manually analyzed, which is laborious compared to automated techniques. Finally, the use of a stringent concentration range increases the time to sample handling and dilution.

Recently, a qPCR method with SYBR® Green detection for total *C4* CNV was described [172]. However, no primers, qPCR cycling conditions or data assay methods were provided. Our method differs from the previous qPCR methods with SYBR® Green labeling in that we perform normalization by selecting only samples with comparable concentration for analyses. To the best of my knowledge, this has not been described before. The idea itself is not new, but it is a simple way of performing absolute quantification of CNV by comparing Ct values of known CNV and an unknown sample. The small differences in the amount of input DNA are corrected by rounding up or down manually, whereas the previous methods rely on mathematical formulae [329, 330]. We have applied the described method on over 2000 patient and research samples (I, III)[144, 147, 201, 206]. We are also using the same methodology for HLA-allele assays (III).

The most important limitations in Study I are the lacking confirmatory assay for *CTins* qPCR run (discussed in section 5.1.3) and the use of immunophenotyping as a confirmatory method for *C4* CNV analysis. Immunophenotyping itself is subjected to many errors and the serologic levels of C4 proteins may vary according to increased production or increased consumption, in cases of low quality gels and in subjects with homozygous C4 allotypes. In addition, the immunophenotyping was performed in our Laboratory in parallel with genotyping. Although the two assays are performed independently, the result of genotypic assay might have caused the reanalysis of phenotypic data in cases with clearly discrepant results more often than in cases with concordant results. This might have enhanced the concordance of the two methods. However, as the samples were not initially intended for method validation, but for the diagnosis of patient samples, it seems unlikely that the results would be greatly biased. In addition, the concordance rates between other, independent *C4* CNV assays were essentially similar.

6.1.2. CTINS

Along with the mere copy number count, CNV may be linked with pathologic states through the detailed structure of the CNV region. For example mutations may alter the function of the remaining or replicated regions [327]. *C4* region is an example of the importance of mutation detection. Although low CNV of *C4* may lead to C4 deficiency the silencing mutations may lead to decreased number of functional *C4* gene copies (See 2.2.4.2).

CTins is the most common mutation leading to *C4A* silencing [193]. We assessed the presence of *CTins* in parallel with *C4* CNV analyses by a similar qPCR. In our material, *CTins* was present in 3–6% of the studied patient populations (I, unpublished data). In previous studies, *CTins* has been recorded in 2–6% in general European populations, but is only rarely present in the Asian population [190, 191, 199, 237, 331]. We found that the carrier status of *CTins* resulted in *C4A* deficiency in ¾ of the cases. If *CTins* had not been assessed, the rate of genetically determined *C4A* deficiency would have been 20% lower (I). This corresponds with the majority of previous data, where *CTins* was shown to account for 10–30% of *C4A* deficiencies in the general population [190, 202, 332]. However, in a study by Blanchong et al., the frequency of *C4A* deficiency caused by *CTins* was only 5% [180]. The most probable explanation for this variation is the frequency of *CTins* across different populations [197].

The golden standard for assessing phenotypic deficiencies is immunophenotyping, but it is subjected to errors. By reducing the amount of *CTins* copies from the number of functional *C4A* genes, the rate of "phenotypic" *C4A* deficiencies can be estimated. The comparison of phenotypic vs. genetic (or low CNV-attributed) deficiency might robustly help to differentiate, whether a disease association would be due to lower phenotypic C4 level or linkage with the surrounding genes. The lack of *CTins* assessment in most of the genetic *C4* disease association studies might cause a significant bias in comparing the frequency of C4A deficiency between the old studies with phenotypic data and the more recent studies with CNV data. Therefore, discrepancies seen with C4A deficiency should be interpreted in keeping the analysis method and the presence of *CTins* in mind. In addition to *CTins*, other silencing mutations have been characterized. The frequency of these mutations is though to be low [164].

In including *CTins* in disease studies, it is important to recognize that *CTins* is strongly associated with *DRB1*13* [197]. This allele should be accounted for when assessing the role of *C4A* deficiencies with *CTins,* especially in cases of positive associations.

6.2 MHC IN ACS

6.2.1 C4 DEFICIENCY IN ACS

No association between any C4 deficiency (A or B) and ACS was detected (II–IV). In addition, the frequency of *CTins* was not increased in patients with STEMI (unpublished observation). However, the statistical power was not sufficient to detect small differences between cases and controls, and thus a possible role of C4 deficiency in ACS cannot be excluded based on the present data. For the independent analyses of C4A and C4B deficiency the results were similar in ACS (III, IV) but for recurrent cardiovascular events, reliable individual analysis of the two isotypes could not be performed due to small number of patients (II). In a previous analysis of recurrent cardiovascular events, C4A deficiency was associated with recurrent cardiovascular end points with patients in NSTE-ACS [224]. However, these data were derived from the clinical intervention trial (used in Studies II and IV) without taking into account the study randomization and thus the results must be interpreted with caution.

The presented data is supported by earlier, small-scale studies performed in Sweden, where no association with MI with any C4 deficiency was seen [215, 217]. On the other hand, mortality of MI has been shown to be increased in patients with C4B deficiency in Icelandic and Hungarian populations [21, 30]. A positive association between C4B deficiency and MI has been reported with a Hungarian population, but the timing of phenotypic C4 measurement and the control population selection may have biased the results [21] (see section 2.2.4.3). In another Hungarian study, C4A and C4B deficiencies were also associated with obstructive CAD, but only C4A deficiency was associated with a history of MI [28]. These data were derived from patients undergoing GABG and were thus selected. In addition to our Study (III), only one study has used genetic analyses [30].

One possible explanation for the discrepant findings is differences in the frequency of C4 deficiency. In Hungarian population, the frequency of C4 deficiency is lower than that seen in Sweden and Finland. Alternatively, C4 deficiency might be a risk factor for MI in Hungarian but not in Scandinavian populations. Another possibility is that the risk of C4 is mediated only in high-risk groups such as in smokers [29, 30]. However, we did not identify any significant role of smoking on the association between C4 deficiency and MI (unpublished observation). On the other hand, smoking has been described to be associated with *HLA-DRB1*01*, which in turn is associated with C4B deficiency [23].

The available data does not allow one to conclude the role of C4 deficiency in ACS or CAD as no high–powered genetic study with unbiased patient and control selection exists and the large-scale GWAS cannot be used to estimate the role of *C4* in MI as no tag SNP for *C4* exists [186]. In the future, a follow-up study with genetic *C4* analysis is needed to identify the role of C4 deficiency in ACS. It would be interesting to perform a GWAS in parallel with C4 CNV analysis to see the possible effect of C4 deficiency in relation to

other genetic risk factors for MI. If only patients who have survived ACS are selected, the high case mortality of MI might bias the results significantly [333]. In addition, if patients with ACS are analysed in during follow-up, ischemia-reperfusion damage may bias the results. Extensive complement activation is thought to aggravate the ischemia-reperfusion damage [334], and if *C4* deficiency would limit the reperfusion injury, the follow-up data might show a "false" beneficial effect of C4 deficiency on outcome. The controls might be either cases without ACS or patients with CAD, not developing ACS, depending on the presented hypothesis.

*6.2.2 DRB1*01 IN ACS*

*DRB1*01* has been linked with CAD and MI in previous studies [23, 31, 32]. In Study III, however, the association between *DRB1*01* and ACS was not consistent. The frequency of *DRB1*01* was not significantly different between patients with STEMI and controls without significant obstructive CAD. However, *DRB1*01* was significantly higher in patients with STEMI than in the reference population representing general population and when all patients with CAD from the current and a previous study [23] were compared with all controls (III). On the other hand, when only male patients were compared, *DRB1*01* was more common in patients than in controls or in the reference population. As the power of this study was adequate to detect over two times increased risk of STEMI, the lack of association does not exclude an association with lower OR.

This discrepancy in the comparisons between cases and controls and cases and the reference population might be due to the biased selection of controls. All of them were not healthy as they all had symptoms of signs for undergoing coronary angiography. Some of them had atherosclerotic changes in their arteries (<50% stenosis). These subtle atherosclerotic changes might lead to ACS in the future. The absence of significant stenosis is not the best way to select a CAD-free control population. More sensitive imaging modalities for excluding CAD should have been applied to exclude the presence of CAD in the control material. Due to the limited availability of these imaging modalities, these were not performed.

Previous data shows that *DRB1*01* is associated with various inflammatory conditions and mostly viral infections [144, 145, 147, 149, 150, 153, 157, 159]. These studies suggest that *DRB1*01* or genetic alterations linked to it might be involved in aberrant inflammatory processes.

Our primary data on NSTE-ACS and heart transplantation patients implied that the *DRB1*01-B*35* haplotype predisposes to NSTE-ACS and obstructive CAD [23]. However, in the present study, no association with *DRB1*01-B*35* haplotype and STEMI was found. Instead, the haplotype with *DRB1*01* but without *B*35* (the 1,1,1,0-halotype) was increased in STEMI compared with CAD-free controls (III). On the other hand, the difference in the frequency of 1,1,1,0-haplotype was not significant when comparing the

reference population and patients with STEMI. Although the CAD-free control population was biased, the difference was still significant after accounting for possible confounders. Compared with the previous haplotype study, Study III had more patients and controls than the NSTE-ACS [23] and importantly, the control material was characterized in more detail.

The haplotypes in both studies were constructed with similar protocols. One explanation for the differences in NSTE-ACS and STEMI populations is that the genetic pathophysiology might differ between STEMI and NSTE-ACS. STEMI and NSTE-ACS do differ in clinical presentation, thrombus appearance, clinical management, patient's characteristics and possibly in pathophysiology [47, 59, 335]. Another explanation is that the differences in the gene frequency of the background population bias the results. The STEMI population and controls were all collected from the capital area. NSTE-ACS patients were derived from different parts of Finland, but the controls came from the capital area. The geographical differences in the frequency of *B*35* are significant within Finland [115]. The third explanation is that the lower survival in STEMI could result in lower frequency of *B*35* [333]. Perhaps correspondingly, the inclusion rate of STEMI was also lower than for the NSTE-ACS study. However, as the *B*35* frequency was similar in patients and controls, strong negative selection of the *DRB1*01-B*35* or *B*35* seems unlikely.

The most important limitations of Study III were the possible bias in the control population selection and the lacking background information in the reference population. In addition, the number of females was too low to reliably assess the role of MHC in females. Furthermore, the p-value was not corrected for testing multiple haplotypes against cases and controls. The results may have thus resulted in chance after performing multiple comparisons. To assess the reliability of the haplotype comparisons further, we compared the 95%CIs between the populations. No overlap was seen between the patients with STEMI and the controls or the reference population. The population frequency estimates account for the presence of multiple haplotypes [323]. The association between hsCRP and the 1,1,1,0-haplotype supports the hypothesis that the observed difference in frequencies is a true one (see Section 6.3.2).

In the future, the allelic variation linking *DRB1*01* and CAD should be elucidated, preferably with in-depth analysis of the surrounding MHC. If an atherogenic peptide or a set of peptides would be identified, the presentation of these might be modifiable [336]. Based on structure or function, *DRB1*01* shares common features with other DRB types. The functional grouping of HLA II has been shown to have great overlap in the bound peptides [337] and thus, testing for different supergroups in CAD does not seem an interesting approach. The "shared epitope" alleles (*DRB1*01:01, DRB1*04:01* and *DRB1*04:04* in Europeans) were not associated with CAD [31]. One way to assess the role of *DRB1*01* would be to test whether the allele groups that have been associated with disease conditions or aberrant immune responses might have an association with CAD.

The mechanisms how HLA II allelic variation might cause disease are not fully understood and due to the tight linkage disequilibrium in the MHC region, the effect of surrounding genes is hard to assess. DQA1 or DQB1 alleles have not been associated with CAD independently of *DRB1*01* [31, 32]. The effects of HLA II molecules can possibly cover a large amount of inflammatory cells and a variety of functions and no single mechanism is likely to explain the biological function of *DRB1*01* in CAD.

6.3 Mechanisms linking MHC and ACS

6.3.1 C4 deficiency and antimicrobial treatment

In previous studies, both partial homozygous and heterozygous C4 deficiency has been associated with increased presence of immune complexes, which, in turn have been associated with MI and CAD [217, 218, 338, 339]. C4 deficiency has also been linked with various inflammatory and infectious conditions [191, 199, 201, 210, 211]. Increased inflammation and infection have been associated with coronary atherosclerosis, although the causality remains to be shown [52, 65]. Thus, although hypothetical, there is a biologically valid model linking C4 deficiency and CAD/ACS.

The role of C4 deficiency in recurrent infections (I) or *Cpn* infection (II) could not be reliably assessed. This was due to the shortcomings in patient characterization (I) or to weak correlation between serology and the presence of *Cpn* in atheromas [340]. However, we found that only patients with a C4 deficiency benefitted from clarithromycin treatment, also after accounting for possible confounders (II). The results imply that C4 deficiency subjects its carriers to infectious or pro-inflammatory condition. In such a condition, the antimicrobial or anti-inflammatory effect of clarithromycin, or a combination of these two, might be beneficial in preventing recurrent cardiovascular manifestations [277]. The limitations in the antimicrobial study are the small number of patients and the lack of complete information on factors that might be associated with future end points, for example the GRACE and the TIMI risk scores [34] (II).

Another possible explanation for the observation is the unequal subgrouping, which might cause a selection bias. The initial study showed a beneficial effect of clarithromycin treatment in the whole population [271]. Consequently, if the size of the selected subgroup is large enough, one is bound to have a significant effect of clarithromycin. The number of patients with C4 deficiency was almost two times larger than that of those without C4 deficiency (95 vs. 49). In addition, the non-significant interaction term between C4 deficiency and clarithromycin treatment might reflect unequal selection (i.e. lack of real association) or alternatively the small sample size.

The observed result may have also been raised by chance, by performing multiple *post hoc* analyses. Study II is the second *post hoc* study performed on the initial material. The first *post hoc* analysis assessed the role of periodontal infection on the antimicrobial response [341]. Contrary to expectations, the lack of positive infection markers was associated with a beneficial effect of clarithromycin treatment. Interestingly, the number of patients without periodontal infection exceeded the number of patients with markers of periodontal infection by two. Therefore the unequal subgrouping discussed above may have biased the results of the first *post hoc* analysis as well. Alternatively, the periodontal infection exerts too strong an infectious or inflammatory stimuli, where the use of clarithromycin is too weak to show any benefit [341].

Unfortunately, the number of patients in different subgroups became too small to compare the effects of C4 deficiency and periodontal infection on the antimicrobial response. However, C4 deficiency has not been reported to be associated with periodontal infection and thus the results might be independent of each other [148].

To the best of my knowledge, this was the first study assessing the possible pharmacogenomics factor in secondary prevention of CAD. Our material is one of the three small-scale trials with positive findings [262, 267, 271]. The first published study showed an advantage in cases with elevated *Cpn* IgG titers [262]. One study reported benefit from the use of antimicrobial agents in acute MI, but not UAP, independently of bacterial infection markers [267]. The frequency of C4 deficiency in Finland is higher than in North America, where the majority of the large-scale studies have been performed [202, 342]. The pharmacogenomic effect of C4 deficiency might explain the lack of association in secondary antimicrobial prevention studies [274-276].

The subgroup analyses of previous studies have not consistently identified any population benefitting from antimicrobial treatment. Although currently the data shows no benefit of secondary antimicrobial trials for treating CAD [274-276] and some have indicated even increased mortality [275], no novel trials should be conducted at this point. However, the existing large-scale antimicrobial trials could be reanalyzed in relation to C4 status. If our results are replicated, the pharmacogenomic effect of C4 deficiency might be helpful in directing personalized treatment to patients.

*6.3.2 DRB1*01 AND INFLAMMATION*

Supporting the importance of the *DRB1*01*-related 1,1,1,0-haplotype in STEMI, we found an association between the haplotype and increased hsCRP levels both in patients and in controls (III). *DRB1*01* alone did not affect hsCRP levels in these populations. *DRB1*01* seemed to be more important in male patients, but due to the small amount of data, the effect of *DRB1*01* on hsCRP in males could not be assessed.

This is the first publication demonstrating that there could be an inflammation-related mechanisms linking *DRB1*01* and ACS. In previous papers, the association with hsCRP and *DRB1*01* was not tested [23, 31, 32]. Hypothetically, the observed data could mean that the 1,1,1,0-haplotype predisposes to plaque rupture by increased inflammatory reactions. This assumption is based on the data, by which increased serum hsCRP has been associated with the plaque type that is considered prone to rupture [65]. Plaque rupture has also been shown to be more common in patients with STEMI than with NSTE-ACS, possibly explaining the lack of association in NSTE-ACS patients [23, 47]. However, in addition to plaque rupture, also other mechanisms leading to MI have been described. As we do not have data on the underlying cause of STEMI (plaque rupture vs. erosion, primary or secondary MI) [33], the theory above has to be approached critically.

High hsCRP has also been associated with increased risk of MI/CAD. The assessment of hsCRP is not routinely recommended, but it may be beneficial for individuals with moderate classical risk factors [89, 241, 244, 245]. hsCRP levels have been shown to associate with various cardiovascular risk factors, dietary patterns and multiple genetic variation. These facts render hsCRP a robust measure of inflammation [244, 343]. In addition, elevated hsCRP is not seen in all patients with MI [240]. Based on our current data, it is impossible to say, whether the 1,1,1,0-haplotype is linked with STEMI, with underlying CAD or whether it results from chance.

The limitations of Study III are the lacking data on hsCRP from almost half of the patients (discussed in Section 5.3.3). In addition, the p-value in the present or previous work was not correct for multiple comparisons. However, when the 95%CI of population frequency estimates of the 1,1,1,0-haplotype was compared between different populations, no overlap was seen. These frequencies were derived from PHASE, which accounts for the presence of multiple haplotypes [323]. As we did not have information of the genetic variation of CRP-related genes and lifestyle factors, the true effect of the 1,1,1,0-haplotype on hsCRP levels remains unknown. Larger materials, with data available on multiple inflammatory markers and genetic variation of CRP and surrounding MHC are warranted to confirm this finding.

Taken together, the available data shows than *DRB1*01* is associated with CAD. The new data presented in the Thesis could mean that *DRB1*01* might harbor a subtype, defined by the surrounding MHC genes, that is associated with increased inflammation and with ACS. Based on the current data, the relative importance of *DRB1*01* vs. 1,1,1,0-haplotype cannot be reliably estimated.

Besides the presented peptides, *DRB1*01* might be pathogenic by surface expression level. The surface expression level of *DRB1*01* is not known, but the transcription level is believed to be low [128]. Assuming that *DRB1*01* has a biological effect, it would be interesting to see whether the conditioning on factors affecting its expression, such as statin, vitamin D, estrogen and various cytokines would affect the association between *DRB1*01* and CAD/ACS. The frequency of the 1,1,1,0-haplotype was too low to perform such calculations, but interestingly, no association with STEMI was seen in women (III).

One could speculate that estrogen, a known suppressor for HLA II expression [128, 344], exerts cardioprotection by lowering the expression of a proatherogenic HLA-molecule. This might also explain the lack of association with the 1,1,1,0-haplotype and hsCRP levels in the population sample as well as the lack of significance in population frequencies of 1,1,1,0-haplotype comparing patients with STEMI and reference population, which included an increased frequency of premenopausal women. The small number of included females prevents drawing any conclusions.

6.3.3 C4 DEFICIENCY AND AUTOIMMUNITY TO HSP60

C4 deficiency was associated with increased IgA autoantibody to HSP60 (IV). HSP60 IgA, in turn, was associated with ACS and had a significant interaction with clarithromycin treatment (discussed in Section 6.4). On the other hand, no association between C4 deficiency and hsCRP (II, III) or *Cpn* infection serology status (II) was detected. These data are not sufficient to exclude the association between C4 deficiency and inflammatory or infectious conditions due to the shortcomings in patient characterization, lack of association between serological and intracellular *Cpn* infection status (discussed in Section 5.2.3) and the robustness of hsCRP (discussed in Section 5.3.3) as inflammatory marker.

The possible mechanisms linking C4 deficiency and elevated IgA levels against HSP60 are not known. However, it can be hypothesized that C4 deficiency might cause elevation in HSP60 antibody levels either through lower phenotypic levels of C4 or by linkage to the surrounding HLA genes, which would be the true causative agent. The observed data does not support the role of low C4 concentration in associating with IgA levels as no correlation with serum C4 and HSP60 IgA was seen.

C4 deficiency might lead to higher amount of infections. Infections, in turn, could lead to increased amount of HSP60 IgA by increasing the amount of cross-reactive antibodies. C4 deficiency might also cause deficient immune clearance, which could lead to increased amount of human HSP60 and eventually to increased autoantibody formation. However, these mechanisms are merely speculative and the association between C4 deficiency and HSP60 IgA needs first to be replicated. Interestingly, C4 deficiency has been associated with conditions having high IgA production such as Henoch–Schönlein purpura and IgA nephropathy [211, 345, 346].

6.4 HSP60 IGA AND ACS

In studies assessing antibodies against oxidated LDL, IgG antibodies have been shown to be associated with CAD/IHD, whereas IgM antibodies are linked with decreased

prevalence of CAD [53]. Lack of association between HSP60 IgG and cardiovascular disease during follow-up has been reported on a two occasions [304, 305]. Our data support the observation that IgA and IgG may have different roles in ACS [304]. We showed that HSP60 IgA, but not IgG, was associated with NSTE-ACS (IV). Even though the number of patients in different subgroups were small and the control population lacked background information, the association between elevated serum HSP60 IgA and NSTE-ACS seen when comparing patients and controls could be replicated when comparing patients with and without end points. The results were not affected by the assessment of HSP60 IgA as a continuous or as a categorized variable or the adjustment for possible confounders. Elevated HSP60 IgA was independent of CAD-related genes (*DRB1*01* and *DRB1*01-B*35* haplotype) [23] and risk factors.

IgA autoantibodies have rarely been assessed in CAD/ACS. There are some previous publications reporting associations with IgA and CAD, however. The relative fractions of IgA are elevated in CAD-related HSP65 antibodies [292], IgA antibodies to oxidized LDL, cardiolipin and B2-microglobulin are associated with CAD/ACS [347, 348] and elevated serum IgA antibody level predict future CVD [349].

IgA antibody has subclass (1 and 2), size (monomeric and polymeric) and biochemical (glycosylation) variation. The specific receptor for IgA is FCαRi (aka. CD89), which is expressed on DCs, Kupffer cells, eosinophils, basophils, monocytes, macrophages and neutrophils. FCαRi may elicit either pro- or anti-inflammatory functions depending on IgA structure, cytokine milieu, genetic alteration of the IgA receptor and possibly also on the level of IgA glycosylation [286, 350].

The biological effects of HSP60-specific IgA are not known. It would seem that the risk attribution of HSP60 IgA is not related to ischemia-reperfusion damage, as the cardiac enzymes did not correlate with HSP60 IgA levels. The target of HSP60 IgA is present in plasma, on stressed endothelial cells and within the cells of an atheromatous plaque [14]. The possible effects of HSP60 IgA thus cover a wide range or possible reactions, possibly involving various different cell types.

Besides the mere association with ACS (discussed above), the association of elevated HSP60 IgA on secondary cardiovascular disease during follow-up was only seen in patients treated with placebo. In patients randomized to receive three months of clarithromycin, the end points were independent of HSP60 IgA. The interaction term was significant. The clarithromycin treatment seems to be beneficial only in what seemed to be high-risk patients (e.g. those with high HSP60 IgA).

There are at least four possible explanations for the observed results. First, human and bacterial HSP60 have been shown to cross-react [14]. HSP60 IgA has shown mild-to moderate correlation with IgA levels against *Cpn* and periodontal bacteria [304, 312, 351]. The elevated levels of HSP60 IgA might thus be a caused by increased amount of infection. In this group, clarithromycin might have resulted in beneficial treatment effect by reducing the infection burden. However, neither periodontal infection [341] nor positive *Cpn* serology

[276] have been shown to associate with beneficial treatment effect of macrolides. In addition, the serological markers of *Cpn* (II) and oral bacteria remained unchanged by clarithromycin [312]. Macrolides affect a moderately broad range of mostly gram-positive bacteria [277] and the beneficial effect of clarithromycin might thus result from reduction of various infectious pathogens. Previous data indicates that clarithromycin does not decrease the HSP60 IgA level [312].

HSP60 expression is induced by various CAD-predisposing factors [295]. The increased amount of autologous HSP60 might in turn lead to increased production of autoantibodies. The second explanation is that the elevated HSP60 IgA is a result of a pro-inflammatory condition. In this inflammatory state, the anti-inflammatory properties of clarithromycin [277] might be beneficial in reducing recurrent cardiovascular end points.

The third explanation is that HSP60 IgA in itself is pro-inflammatory. The moderate to strong correlation of HSP60 IgA across 12 months indicates constant production. The constant production could be affected by genetic or environmental factors. However, no risk factors for CAD were seen to explain the elevation in HSP60 IgA. Of the genetic factors studied, only C4 deficiency, but not *DRB1*01* was associated with alteration in the HSP60 IgA.

The fourth explanation is that the results are caused by mere chance. As in any medical study, a certain amount of chance is operating. However, the association between HSP60 IgA and CAD could be replicated in two different settings and after adjusting for confounders and multiple comparisons. In contrast to the earlier studies of the same material (II), [341], patients and controls were evenly distributed by choosing the median as a cut-off point for HSP60 IgA in assessing the future cardiovascular end points. This reduced the possibility of selection bias, but not that of chance.

There is one positive and one negative association study reported for HSP60 IgA and CAD. The positive study was a nested case-control study of middle-aged dyslipidemic men who developed MI during the follow-up. It showed that levels above median at inclusion predicted future CVD [304], especially in conjunction with other risk factors [352]. The study with no apparent association was a case-control study, in which HSP60 IgA was initially associated with obstructive CAD [351], but when patients and MI-free controls were compared, no difference was seen [353].

More studies have to be conducted to replicate the observed results in a larger patient material treated with optimal medical treatment. If the association is shown to really exist, the epitope specificity, cross-reactivity and biological modifications should be determined to assess the potential consequences resulting from elevated HSP60 IgA levels. HSP60 is not the only heat shock protein involved in CAD/ACS. It would be interesting to see, how the combination of HSP antibody repertoire is linked with CAD/ACS and whether the HSP genes or their polymorphism affect these associations.

7. SUMMARY AND CONCLUSIONS

The first aim of this Thesis was the validation of a novel qPCR method for *C4* analyses. The results showed that the new concentration range approach and SYBR® Green labeling can be used in real-time qPCR analyses for *C4* CNV determination and mutation detection. In addition, the analysis of patient data showed that the assessment of *CTins* can significantly increase the number of *C4A* deficiencies and as it can be easily assessed, *CTins* determination should be included in disease association studies (I).

The second aim was to ascertain the role of selected MHC genes and alleles in different clinical forms of ACS. We were able to confirm the role of a *DRB1*01*-haplotype in STEMI, whereas *DRB1*01* alone was associated with STEMI only in males (III). Contrary to previous data, no effects of other MHC markers were detected. However, due to small sample size and inadequate power, the role of C4 deficiency could not be reliably excluded (III, IV).

The third aim was to assess the possible biological mechanisms, through which the MHC could be linked with ACS. It was observed that the MHC region could be involved in the pathogenesis of ACS/CAD by increasing inflammatory, infectious and autoimmune states. This was shown by the association with *DRB1*01*-haplotype and elevated hsCRP levels (III), C4 deficiency and the beneficial effect of antimicrobial prevention of ACS (II) and the association between C4 deficiency and elevated levels of HSP60 IgA, an autoantibody that was discovered to be linked with ACS (IV).

In conclusion, the MHC region is associated with ACS and there are possible biological mechanisms mediating this association. These findings may be important in understanding the pathogenesis of ACS, but larger materials with more in-depth genetic analyses are warranted to replicate these findings and to address the possible causality.

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9. REFERENCES

1. Robbins Basic Pathology. Kumar VA, A. Fausto, N. Mitchell, R. *Saunders, Philadelphia.* **2007**; 339-379.

2. Marzilli M, Merz CN, Boden WE, Bonow RO, Capozza PG, Chilian WM, DeMaria AN, Guarini G, Huqi A, Morrone D, Patel MR, Weintraub WS. *Obstructive Coronary Atherosclerosis and Ischemic Heart Disease: An Elusive Link!* J Am Coll Cardiol. 60:951-956, **2012**.

3. Nemetz Pn. RVL, Ransom J. E., Bailey K. R., Edwards W. D., Leibson C. L. *Recent Trends in the Prevalence of Coronary Disease: A Population-Based Autopsy Study of Nonnatural Deaths*. Arch Intern Med. 168:264-270, **2008**.

4. Aromaa A, Koskinen S. *Health and Functional Capacity in Finland. Baseline Result of the Health 2000 Examination Survey*. Publications of the National Public Health Institute B3/2002. **2002.**

5. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Soliman EZ, Sorlie PD, Sotoodehnia N, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. *Heart Disease and Stroke Statistics--2012 Update: A Report from the American Heart Association*. Circulation. 125:e2-e220, **2012**.

6. Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine. Bonow RM, Zipes, Libby, P. *Elsevier, Philadelphia, PA.* **2011**; 1049-1270.

7. Yusuf S, Hawken S, Ôunpuu S, Dans T, Avezum A, Lanas F, McQueen M, Budaj A, Pais P, Varigos J, Lisheng L. *Effect of Potentially Modifiable Risk Factors Associated with Myocardial Infarction in 52 Countries (the INTERHEART Study): Case-Control Study*. The Lancet. 364:937-952, **2004**.

8. Greenland P, Knoll MD, Stamler J, Neaton JD, Dyer AR, Garside DB, Wilson PW. *Major Risk Factors as Antecedents of Fatal and Nonfatal Coronary Heart Disease Events*. JAMA. 290:891-897, **2003**.

9. Canto JG, Kiefe CI, Rogers WJ, Peterson ED, Frederick PD, French WJ, Gibson CM, Pollack CV, Jr., Ornato JP, Zalenski RJ, Penney J, Tiefenbrunn AJ, Greenland P. *Number of Coronary Heart Disease Risk Factors and Mortality in Patients with First Myocardial Infarction*. JAMA. 306:2120-2127, **2011**.

10. Berry JD, Dyer A, Cai X, Garside DB, Ning H, Thomas A, Greenland P, Van Horn L, Tracy RP, Lloyd-Jones DM. *Lifetime Risks of Cardiovascular Disease*. N Engl J Med. 366:321-329, **2012**.

11. Folsom AR, Yatsuya H, Nettleton JA, Lutsey PL, Cushman M, Rosamond WD. *Community Prevalence of Ideal Cardiovascular Health, by the American Heart Association Definition, and Relationship with Cardiovascular Disease Incidence*. J Am Coll Cardiol. 57:1690-1696, **2011**.

12. Sundquist K, Winkleby M, Li X, Ji J, Hemminki K, Sundquist J. *Familial [Corrected] Transmission of Coronary Heart Disease: A Cohort Study of 80,214 Swedish Adoptees Linked to Their Biological and Adoptive Parents*. Am Heart J. 162:317-323, **2011**.

13. Rosenfeld ME, Campbell LA. *Pathogens and Atherosclerosis: Update on the Potential Contribution of Multiple Infectious Organisms to the Pathogenesis of Atherosclerosis*. Thromb Haemost. 106:858-867, **2011**.

14. Grundtman C, Kreutmayer SB, Almanzar G, Wick MC, Wick G. *Heat Shock Protein 60 and Immune Inflammatory Responses in Atherosclerosis*. Arterioscler Thromb Vasc Biol. 31:960-968, **2011**.

15. Klein J, Sato A. *The HLA System. First of Two Parts*. N Engl J Med. 343:702-709, **2000**.

16. Klein J, Sato A. *The HLA System. Second of Two Parts*. N Engl J Med. 343:782-786, **2000**.

17. Vandiedonck C, Knight JC. *The Human Major Histocompatibility Complex as a Paradigm in Genomics Research*. Brief Funct Genomic Proteomic. 8:379-394, **2009**.

18. Blackwell JM, Jamieson SE, Burgner D. *HLA and Infectious Diseases*. Clin Microbiol Rev. 22:370-385, Table of Contents, **2009**.

19. Trowsdale J. *The MHC, Disease and Selection*. Immunol Lett. 137:1-8, **2011**.

20. Stone PH, Sherrid MV, Cohn KE. *Correlation of HLA Types in Premature Coronary Artery Disease: An Attempt to Define Independent Genetic Risk Factors*. Chest. 79:381-385, **1981**.

21. Kramer J, Rajczy K, Hegyi L, Fulop T, Mohacsi A, Mezei Z, Keltai M, Blasko G, Ferenczy E, Anh-Tuan N. *C4B*Q0 Allotype as Risk Factor for Myocardial Infarction*. BMJ. 309:313-314, **1994**.

22. Jönsson Gr, Truedsson L, Sturfelt G, Oxelius V-A, Braconier JH, Sjöholm AG. *Hereditary C2 Deficiency in Sweden*. Medicine (Baltimore). 84:23-34, **2005**.

23. Palikhe A, Sinisalo J, Seppanen M, Valtonen V, Nieminen MS, Lokki ML. *Human MHC Region Harbors Both Susceptibility and Protective Haplotypes for Coronary Artery Disease*. Tissue Antigens. 69:47-55, **2007**.

24. He M, Guo H, Yang X, Zhou L, Zhang X, Cheng L, Zeng H, Hu FB, Tanguay RM, Wu T. *Genetic Variations in HSPA8 Gene Associated with Coronary Heart Disease Risk in a Chinese Population*. PLoS One. 5:e9684, **2010**.

25. Boiocchi C, Bozzini S, Zorzetto M, Pelissero G, Cuccia M, Falcone C. *Association between Two Polymorphisms in the HLA-G Gene and Angiographic Coronary Artery Disease*. Mol Med Report. 5:1141-1145, **2012**.

26. Davies RW, Wells GA, Stewart AF, Erdmann J, Shah SH, Ferguson JF, Hall AS, Anand SS, Burnett MS, Epstein SE, Dandona S, Chen L, Nahrstaedt J, Loley C, Konig IR, Kraus WE, Granger CB, Engert JC, Hengstenberg C, Wichmann HE, Schreiber S, Tang WH, Ellis SG, Rader DJ, Hazen SL, Reilly MP, Samani NJ, Schunkert H, Roberts R, McPherson R. *A Genome-Wide Association Study for Coronary Artery Disease Identifies a Novel Susceptibility Locus in the Major Histocompatibility Complex*. Circ Cardiovasc Genet. 5:217-225, **2012**.

27. Takeuchi F, Yokota M, Yamamoto K, Nakashima E, Katsuya T, Asano H, Isono M, Nabika T, Sugiyama T, Fujioka A, Awata N, Ohnaka K, Nakatochi M, Kitajima H, Rakugi H, Nakamura J, Ohkubo T, Imai Y, Shimamoto K, Yamori Y, Yamaguchi S, Kobayashi S, Takayanagi R, Ogihara T, Kato N. *Genome-Wide Association Study of Coronary Artery Disease in the Japanese*. Eur J Hum Genet. 20:333-340, **2012**.

28. Szalai C, Füst G, Duba J, Kramer J, Romics L, Prohászka Z, Cászár A. *Association of Polymorphisms and Allelic Combinations in the Tumour Necrosis Factor-Alpha-Complement MHC Region with Coronary Artery Disease*. J Med Genet. 39:46-51, **2002**.

29. Arason GJ, Kramer J, Blaskó B, Kolka R, Thorbjornsdottir P, Einarsd´óttir K, Sigfúsdottir A, Sigurdarson ST, Sigurdsson G, Rónai Z, Prohászka Z, Sasvári-Székely M, Bödvarsson S, Thorgeirsson G, Füst G. *Smoking and a Complement Gene Polymorphism Interact in Promoting Cardiovascular Disease Morbidity and Mortality*. Clin Exp Immunol. 149:132-138, **2007**.

30. Blaskó B, Kolka R, Thorbjornsdottir P, Sigurdarson ST, Sigurdsson G, Rónai Z, Sasvári-Székely M, Bödvarsson S, Thorgeirsson G, Prohászka Z, Kovacs M, Füst G, Arason GJ. *Low Complement C4B Gene Copy Number Predicts Short-Term Mortality after Acute Myocardial Infarction*. Int Immunol. 20:31-37, **2008**.

31. Björkbacka H, Lavant EH, Fredrikson GN, Melander O, Berglund G, Carlson JA, Nilsson J. *Weak Associations between Human Leucocyte Antigen Genotype and Acute Myocardial Infarction*. J Intern Med. 268:50-58, **2010**.

32. Sun W, Cui Y, Zhen L, Huang L. *Association between HLA-DRB1, HLA-DRQB1 Alleles, and CD4(+)CD28(Null) T Cells in a Chinese Population with Coronary Heart Disease*. Mol Biol Rep. 38:1675-1679, **2011**.

33. Thygesen K, Alpert JS, Jaffe AS, Simoons ML, Chaitman BR, White HD, Thygesen K, Alpert JS, White HD, Jaffe AS, Katus HA, Apple FS, Lindahl B, Morrow DA, Chaitman BR, Clemmensen PM, Johanson P, Hod H, Underwood R, Bax JJ, Bonow RO, Pinto F, Gibbons RJ, Fox KA, Atar D, Newby LK, Galvani M, Hamm CW, Uretsky BF, Gabriel Steg P, Wijns W, Bassand JP, Menasche P, Ravkilde J, Ohman EM, Antman EM, Wallentin LC, Armstrong PW, Simoons ML, Januzzi JL, Nieminen MS, Gheorghiade M, Filippatos G, Luepker RV, Fortmann SP, Rosamond WD, Levy D, Wood D, Smith SC, Hu D, Lopez-Sendon JL, Robertson RM, Weaver D, Tendera M, Bove AA, Parkhomenko AN, Vasilieva EJ, Mendis S, Bax JJ, Baumgartner H, Ceconi C, Dean V, Deaton C, Fagard R, Funck-Brentano C, Hasdai D, Hoes A, Kirchhof P, Knuuti J, Kolh P, McDonagh T, Moulin C, Popescu BA, Reiner Z, Sechtem U, Sirnes PA, Tendera M, Torbicki A, Vahanian A, Windecker S, Morais J, Aguiar C, Almahmeed W, Arnar DO, Barili F, Bloch KD, Bolger AF, Botker HE, Bozkurt B, Bugiardini R, Cannon C, de Lemos J, Eberli FR, Escobar E, Hlatky M, James S, Kern KB, Moliterno DJ, Mueller C, Neskovic AN, Pieske BM, Schulman SP, Storey RF, Taubert KA, Vranckx P, Wagner DR. *Third Universal Definition of Myocardial Infarction*. Eur Heart J. 9:620-33, **2012**.

34. Hamm CW, Bassand JP, Agewall S, Bax J, Boersma E, Bueno H, Caso P, Dudek D, Gielen S, Huber K, Ohman M, Petrie MC, Sonntag F, Uva MS, Storey RF, Wijns W, Zahger D, Bax JJ, Auricchio A, Baumgartner H, Ceconi C, Dean V, Deaton C, Fagard R, Funck-Brentano C, Hasdai D, Hoes A, Knuuti J, Kolh P, McDonagh T, Moulin C, Poldermans D, Popescu BA, Reiner Z, Sechtem U, Sirnes PA, Torbicki A, Vahanian A, Windecker S, Windecker S, Achenbach S, Badimon L, Bertrand M, Botker HE, Collet JP, Crea F, Danchin N, Falk E, Goudevenos J, Gulba D, Hambrecht R, Herrmann J, Kastrati A, Kjeldsen K, Kristensen SD, Lancellotti P, Mehilli J, Merkely B, Montalescot G, Neumann FJ, Neyses L, Perk J, Roffi M, Romeo F, Ruda M, Swahn E, Valgimigli M, Vrints CJ, Widimsky P. *ESC Guidelines for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation: The Task Force for the Management of Acute Coronary Syndromes (ACS) in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC)*. Eur Heart J. 32:2999-3054, **2011**.

35. Boland LL, Folsom AR, Sorlie PD, Taylor HA, Rosamond WD, Chambless LE, Cooper LS. *Occurrence of Unrecognized Myocardial Infarction in Subjects Aged 45 to 65 Years (the ARIC Study)*. Am J Cardiol. 90:927-931, **2002**.

36. Thygesen K, Alpert JS, White HD, Jaffe AS, Apple FS, Galvani M, Katus HA, Newby LK, Ravkilde J, Chaitman B, Clemmensen PM, Dellborg M, Hod H, Porela P, Underwood R, Bax JJ, Beller GA, Bonow R, Van der Wall EE, Bassand JP, Wijns W, Ferguson TB, Steg PG, Uretsky BF, Williams DO, Armstrong PW, Antman EM, Fox KA, Hamm CW, Ohman EM, Simoons ML, Poole-Wilson PA, Gurfinkel EP, Lopez-Sendon JL, Pais P, Mendis S, Zhu JR, Wallentin LC, Fernandez-Aviles F, Fox KM, Parkhomenko AN, Priori SG, Tendera M, Voipio-Pulkki LM, Vahanian A, Camm AJ, De Caterina R, Dean V, Dickstein K, Filippatos G, Funck-Brentano C, Hellemans I, Kristensen SD, McGregor K, Sechtem U, Silber S, Tendera M, Widimsky P, Zamorano JL, Morais J, Brener S, Harrington R, Morrow D, Lim M, Martinez-Rios MA, Steinhubl S, Levine GN, Gibler WB, Goff D, Tubaro M, Dudek D, Al-Attar N. *Universal Definition of Myocardial Infarction*. Circulation. 116:2634-2653, **2007**.

37. Gallagher S, Jones DA, Anand V, Mohiddin S. *Diagnosis and Management of Patients with Acute Cardiac Symptoms, Troponin Elevation and Culprit-Free Angiograms*. Heart. 98:974-981, **2012**.

38. Ruff CT, Braunwald E. *The Evolving Epidemiology of Acute Coronary Syndromes*. Nat Rev Cardiol. 8:140-147, **2011**.

39. Claassen M, Sybrandy KC, Appelman YE, Asselbergs FW. *Gender Gap in Acute Coronary Heart Disease: Myth or Reality?* World J Cardiol. 4:36-47, **2012**.

40. Lehto HR, Lehto S, Havulinna AS, Ketonen M, Lehtonen A, Kesaniemi YA, Airaksinen KJ, Salomaa V. *Sex Differences in Short- and Long-Term Case-Fatality of Myocardial Infarction*. Eur J Epidemiol. 26:851-861, **2011**.

41. Smolina K, Wright FL, Rayner M, Goldacre MJ. *Long-Term Survival and Recurrence after Acute Myocardial Infarction in England, 2004 to 2010*. Circ Cardiovasc Qual Outcomes. 5:532-540, **2012**.

42. Ford ES. *Ideal Cardiovascular Health: Start Young, Finish Strong*. Circulation. 125:1955-1957, **2012**.

43. Jakevicius CA, Mamdani M, Tu Jv. *Adherence with Statin Therapy in Elderly Patients with and without Acute Coronary Syndrome.* JAMA. 288:462-467, **2002.**

44. Nikus KE, Koponen H. Koukkunen H, Laukkala T, Porela P, Pulkki K, Puurunen M, Salomaa V, Tierala I, Valli J, Voipio-Pulkki L-M, Kinnula M, Ukkonen H. *Käypä Hoito Suositus: Sydäninfarktin Diagnostiikka*. **2009**.

45. Yeh RW, Sidney S, Chandra M, Sorel M, Selby JV, Go AS. *Population Trends in the Incidence and Outcomes of Acute Myocardial Infarction*. N Engl J Med. 362:2155-2165, **2010**.

46. Smolina K, Wright FL, Rayner M, Goldacre MJ. *Determinants of the Decline in Mortality from Acute Myocardial Infarction in England between 2002 and 2010: Linked National Database Study*. BMJ. 344:d8059, **2012**.

47. Ino Y, Kubo T, Tanaka A, Kuroi A, Tsujioka H, Ikejima H, Okouchi K, Kashiwagi M, Takarada S, Kitabata H, Tanimoto T, Komukai K, Ishibashi K, Kimura K, Hirata K, Mizukoshi M, Imanishi T, Akasaka T. *Difference of Culprit Lesion Morphologies between ST-Segment Elevation Myocardial Infarction and Non-ST-Segment Elevation Acute Coronary Syndrome: An Optical Coherence Tomography Study*. JACC Cardiovasc Interv. 4:76-82, **2011**.

48. Inflammation and Atherosclerosis. Wick GG, Grundtman C. *Springer, Wien.* **2011**; 1-19.

49. Ross R, Glomset JA. *Atherosclerosis and the Arterial Smooth Muscle Cell: Proliferation of Smooth Muscle Is a Key Event in the Genesis of the Lesions of Atherosclerosis*. Science. 180:1332-1339, **1973**.

50. Ross R. *Atherosclerosis--an Inflammatory Disease*. N Engl J Med. 340:115-126, **1999**.

51. Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, Abela GS, Franchi L, Nunez G, Schnurr M, Espevik T, Lien E, Fitzgerald KA, Rock KL, Moore KJ, Wright SD, Hornung V, Latz E. *NLRP3 Inflammasomes Are Required for Atherogenesis and Activated by Cholesterol Crystals*. Nature. 464:1357-1361, **2010**.

52. Hansson GK, Hermansson A. *The Immune System in Atherosclerosis*. Nat Immunol. 12:204-212, **2011**.

53. Weber C, Noels H. *Atherosclerosis: Current Pathogenesis and Therapeutic Options*. Nat Med. 17:1410-1422, **2011**.

54. Ylä-Herttuala S, Bentzon JF, Daemen M, Falk E, Garcia-Garcia HM, Herrmann J, Hoefer I, Jukema JW, Krams R, Kwak BR, Marx N, Naruszewicz M, Newby A, Pasterkamp G, Serruys PW, Waltenberger J, Weber C, Tokgozoglu L. *Stabilisation of Atherosclerotic Plaques. Position Paper of the European Society of Cardiology (ESC) Working Group on Atherosclerosis and Vascular Biology*. Thromb Haemost. 106:1-19, **2011**.

55. Van Vre EA, Van Brussel I, Bosmans JM, Vrints CJ, Bult H. *Dendritic Cells in Human Atherosclerosis: From Circulation to Atherosclerotic Plaques*. Mediators Inflamm. 2011:941396, **2011**.

56. Campbell KA, Lipinski MJ, Doran AC, Skaflen MD, Fuster V, McNamara CA. *Lymphocytes and the Adventitial Immune Response in Atherosclerosis*. Circ Res. 110:889-900, **2012**.

57. Kleemann R, Zadelaar S, Kooistra T. *Cytokines and Atherosclerosis: A Comprehensive Review of Studies in Mice*. Cardiovasc Res. 79:360-376, **2008**.

58. Speidl WS, Kastl SP, Huber K, Wojta J. *Complement in Atherosclerosis: Friend or Foe?* J Thromb Haemost. 9:428-440, **2011**.

59. Virmani R, Burke AP, Farb A, Kolodgie FD. *Pathology of the Vulnerable Plaque*. J Am Coll Cardiol. 47:C13-18, **2006**.

60. Rittersma SZ, van der Wal AC, Koch KT, Piek JJ, Henriques JP, Mulder KJ, Ploegmakers JP, Meesterman M, de Winter RJ. *Plaque Instability Frequently Occurs Days or Weeks before Occlusive Coronary Thrombosis: A Pathological Thrombectomy Study in Primary Percutaneous Coronary Intervention*. Circulation. 111:1160-1165, **2005**.

61. Jackson SP. *Arterial Thrombosis--Insidious, Unpredictable and Deadly*. Nat Med. 17:1423-1436, **2011**.

62. Stary HC. *Natural History and Histological Classification of Atherosclerotic Lesions: An Update*. Arterioscler Thromb Vasc Biol. 20:1177-1178, **2000**.

63. Kubo T, Imanishi T, Takarada S, Kuroi A, Ueno S, Yamano T, Tanimoto T, Matsuo Y, Masho T, Kitabata H, Tsuda K, Tomobuchi Y, Akasaka T. *Assessment of Culprit Lesion Morphology in Acute Myocardial Infarction: Ability of Optical Coherence Tomography Compared with Intravascular Ultrasound and Coronary Angioscopy*. J Am Coll Cardiol. 50:933-939, **2007**.

64. Hong MK, Mintz GS, Lee CW, Kim YH, Lee SW, Song JM, Han KH, Kang DH, Song JK, Kim JJ, Park SW, Park SJ. *Comparison of Coronary Plaque Rupture between Stable Angina and Acute Myocardial Infarction: A Three-Vessel Intravascular Ultrasound Study in 235 Patients*. Circulation. 110:928-933, **2004**.

65. Choi SY, Mintz GS. *What Have We Learned About Plaque Rupture in Acute Coronary Syndromes?* Curr Cardiol Rep. 12:338-343, **2010**.

66. Fishbein MC. *The Vulnerable and Unstable Atherosclerotic Plaque*. Cardiovasc Pathol. 19:6-11, **2010**.

67. Lindstedt KA, Leskinen MJ, Kovanen PT. *Proteolysis of the Pericellular Matrix: A Novel Element Determining Cell Survival and Death in the Pathogenesis of Plaque Erosion and Rupture*. Arterioscler Thromb Vasc Biol. 24:1350-1358, **2004**.

68. Arbab-Zadeh A, Nakano M, Virmani R, Fuster V. *Acute Coronary Events*. Circulation. 125:1147-1156, **2012**.

69. Assmann G, Cullen P, Schulte H. *Simple Scoring Scheme for Calculating the Risk of Acute Coronary Events Based on the 10-Year Follow-up of the Prospective Cardiovascular Munster (PROCAM) Study*. Circulation. 105:310-315, **2002**.

70. Sivapalaratnam S, Boekholdt SM, Trip MD, Sandhu MS, Luben R, Kastelein JJ, Wareham NJ, Khaw KT. *Family History of Premature Coronary Heart Disease and Risk Prediction in the Epic-Norfolk Prospective Population Study*. Heart. 96:1985-1989, **2010**.

71. Watkins H, Farrall M. *Genetic Susceptibility to Coronary Artery Disease: From Promise to Progress*. Nat Rev Genet. 7:163-173, **2006**.

72. Clinical Cardiogenetics. Baars HF, van der Smagt JJ, Doevendans PA . *Springer-Verlag, London.* **2011;** 369-383.

73. Lloyd-Jones DM, Nam BH, D'Agostino RB, Sr., Levy D, Murabito JM, Wang TJ, Wilson PW, O'Donnell CJ. *Parental Cardiovascular Disease as a Risk Factor for Cardiovascular Disease in Middle-Aged Adults: A Prospective Study of Parents and Offspring*. JAMA. 291:2204-2211, **2004**.

74. Zdravkovic S, Wienke A, Pedersen NL, Marenberg ME, Yashin AI, De Faire U. *Heritability of Death from Coronary Heart Disease: A 36-Year Follow-up of 20 966 Swedish Twins*. J Intern Med. 252:247-254, **2002**.

75. Diemert P, Schunkert H. *Genomic Reflections of Coronary Artery Disease*. Circ Cardiovasc Genet. 4:7-8, **2011**.

76. Franceschini N, Carty C, Buzkova P, Reiner AP, Garrett T, Lin Y, Vockler JS, Hindorff LA, Cole SA, Boerwinkle E, Lin DY, Bookman E, Best LG, Bella JN, Eaton C, Greenland P, Jenny N, North KE, Taverna D, Young AM, Deelman E, Kooperberg C, Psaty B, Heiss G. *Association of Genetic Variants and Incident Coronary Heart Disease in Multiethnic Cohorts: The PAGE Study*. Circ Cardiovasc Genet. 4:661-672, **2011**.

77. Lusis AJ. *Genetics of Atherosclerosis*. Trends Genet. 28:267-275, **2012**.

78. Ho E, Bhindi R, Ashley EA, Figtree GA. *Genetic Analysis in Cardiovascular Disease: A Clinical Perspective*. Cardiol Rev. 19:81-89, **2011**.

79. Davison LJ, Wallace C, Cooper JD, Cope NF, Wilson NK, Smyth DJ, Howson JM, Saleh N, Al-Jeffery A, Angus KL, Stevens HE, Nutland S, Duley S, Coulson RM, Walker NM, Burren OS, Rice CM, Cambien F, Zeller T, Munzel T, Lackner K, Blankenberg S, Fraser P, Gottgens B, Todd JA, Attwood T, Belz S, Braund P, Cambien F, Cooper J, Crisp-Hihn A, Diemert P, Deloukas P, Foad N, Erdmann J, Goodall AH, Gracey J, Gray E, Gwilliams R, Heimerl S, Hengstenberg C, Jolley J, Krishnan U, Lloyd-Jones H, Lugauer I, Lundmark P, Maouche S, Moore JS, Muir D, Murray E, Nelson CP, Neudert J, Niblett D, O'Leary K, Ouwehand WH, Pollard H, Rankin A, Rice CM, Sager H, Samani NJ, Sambrook J, Schmitz G, Scholz M, Schroeder L, Schunkert H, Syvannen AC, Tennstedt S, Wallace C. *Long-Range DNA Looping and Gene Expression Analyses Identify Dexi as an Autoimmune Disease Candidate Gene*. Hum Mol Genet. 21:322-333, **2012**.

80. Deloukas P, Kanoni S, Willenborg C, Farrall M, Assimes TL, Thompson JR, Ingelsson E, Saleheen D, Erdmann J, Goldstein BA, Stirrups K, Konig IR, Cazier JB, Johansson A, Hall AS, Lee JY, Willer CJ, Chambers JC, Esko T, Folkersen L, Goel A, Grundberg E, Havulinna AS, Ho WK, Hopewell JC, Eriksson N, Kleber ME, Kristiansson K, Lundmark P, Lyytikäinen LP, Rafelt S, Shungin D, Strawbridge RJ, Thorleifsson G, Tikkanen E, Van Zuydam N, Voight BF, Waite LL, Zhang W, Ziegler A, Absher D, Altshuler D, Balmforth AJ, Barroso I, Braund PS, Burgdorf C, Claudi-Boehm S, Cox D, Dimitriou M, Do R, Doney AS, Mokhtari NE, Eriksson P, Fischer K, Fontanillas P, Franco-Cereceda A, Gigante B, Groop L, Gustafsson S, Hager J, Hallmans G, Han BG, Hunt SE, Kang HM, Illig T, Kessler T, Knowles JW, Kolovou G, Kuusisto J, Langenberg C, Langford C, Leander K, Lokki ML, Lundmark A, McCarthy MI, Meisinger C, Melander O, Mihailov E, Maouche S, Morris AD, Muller-

Nurasyid M, Nikus K, Peden JF, Rayner NW, Rasheed A, Rosinger S, Rubin D, Rumpf MP, Schafer A, Sivananthan M, Song C, Stewart AF, Tan ST, Thorgeirsson G, Schoot CE, Wagner PJ, Wells GA, Wild PS, Yang TP, Amouyel P, Arveiler D, Basart H, Boehnke M, Boerwinkle E, Brambilla P, Cambien F, Cupples AL, de Faire U, Dehghan A, Diemert P, Epstein SE, Evans A, Ferrario MM, Ferrieres J, Gauguier D, Go AS, Goodall AH, Gudnason V, Hazen SL, Holm H, Iribarren C, Jang Y, Kahonen M, Kee F, Kim HS, Klopp N, Koenig W, Kratzer W, Kuulasmaa K, Laakso M, Laaksonen R, Lee JY, Lind L, Ouwehand WH, Parish S, Park JE, Pedersen NL, Peters A, Quertermous T, Rader DJ, Salomaa V, Schadt E, Shah SH, Sinisalo J, Stark K, Stefansson K, Tregouet DA, Virtamo J, Wallentin L, Wareham N, Zimmermann ME, Nieminen MS, Hengstenberg C, Sandhu MS, Pastinen T, Syvänen AC, Hovingh GK, Dedoussis G, Franks PW, Lehtimäki T, Metspalu A, Zalloua PA, Siegbahn A, Schreiber S, Ripatti S, Blankenberg SS, Perola M, Clarke R, Boehm BO, O'Donnell C, Reilly MP, Marz W, Collins R, Kathiresan S, Hamsten A, Kooner JS, Thorsteinsdottir U, Danesh J, Palmer CN, Roberts R, Watkins H, Schunkert H, Samani NJ. *Large-Scale Association Analysis Identifies New Risk Loci for Coronary Artery Disease*. Nat Genet. **2012**.

81. Reilly MP, Li M, He J, Ferguson JF, Stylianou IM, Mehta NN, Burnett MS, Devaney JM, Knouff CW, Thompson JR, Horne BD, Stewart AF, Assimes TL, Wild PS, Allayee H, Nitschke PL, Patel RS, Myocardial Infarction Genetics Consotrium, Wellcome Trust Case Control Consortium, Martinelli N, Girelli D, Quyyumi AA, Anderson JL, Erdmann J, Hall AS, Schunkert H, Quertermous T, Blankenberg S, Hazen SL, Roberts R, Kathiresan S, Samani NJ, Epstein SE, Rader DJ. *Identification of ADAMTS7 as a Novel Locus for Coronary Atherosclerosis and Association of ABO with Myocardial Infarction in the Presence of Coronary Atherosclerosis: Two Genome-Wide Association Studies*. Lancet. 377:383-392, **2011**.

82. Kitsios GD, Dahabreh IJ, Trikalinos TA, Schmid CH, Huggins GS, Kent DM. *Heterogeneity of the Phenotypic Definition of Coronary Artery Disease and Its Impact on Genetic Association Studies*. Circ Cardiovasc Genet. 4:58-67, **2011**.

83. Jang IK. *Near Infrared Spectroscopy: Another Toy or Indispensible Diagnostic Tool?* Circ Cardiovasc Interv. 5:10-11, **2012**.

84. Ripatti S, Tikkanen E, Orho-Melander M, Havulinna AS, Silander K, Sharma A, Guiducci C, Perola M, Jula A, Sinisalo J, Lokki ML, Nieminen MS, Melander O, Salomaa V, Peltonen L, Kathiresan S. *A Multilocus Genetic Risk Score for Coronary Heart Disease: Case-Control and Prospective Cohort Analyses*. Lancet. 376:1393-1400, **2010**.

85. Paynter NP, Chasman DI, Pare G, Buring JE, Cook NR, Miletich JP, Ridker PM. *Association between a Literature-Based Genetic Risk Score and Cardiovascular Events in Women*. JAMA. 303:631-637, **2010**.

86. Thanassoulis G, Peloso GM, Pencina MJ, Hoffmann U, Fox CS, Cupples LA, Levy D, D'Agostino RB, Hwang SJ, O'Donnell CJ. *A Genetic Risk Score Is Associated with Incident Cardiovascular Disease and Coronary Artery Calcium: The Framingham Heart Study*. Circ Cardiovasc Genet. 5:113-121, **2012**.

87. Vaarhorst AA, Lu Y, Heijmans BT, Dolle ME, Bohringer S, Putter H, Imholz S, Merry AH, van Greevenbroek MM, Jukema JW, Gorgels AP, van den Brandt PA, Muller M, Schouten LJ, Feskens EJ, Boer JM, Slagboom PE. *Literature-Based Genetic Risk Scores for Coronary Heart Disease: The Cardiovascular Registry Maastricht (CAREMA) Prospective Cohort Study*. Circ Cardiovasc Genet. 5:202-209, **2012**.

88. Lindahl B. *Are There Really Biomarkers of Vulnerable Plaque?* Clin Chem. 58:151-153, **2012**.

89. Greenland P, Alpert JS, Beller GA, Benjamin EJ, Budoff MJ, Fayad ZA, Foster E, Hlatky MA, Hodgson JM, Kushner FG, Lauer MS, Shaw LJ, Smith SC, Jr., Taylor AJ, Weintraub WS, Wenger NK, Jacobs AK. *2010 ACCF/AHA Guideline for Assessment of Cardiovascular Risk in Asymptomatic Adults: A Report of the American College of Cardiology*

Foundation/American Heart Association Task Force on Practice Guidelines. Circulation. 122:e584-636, **2010**.

90. Braunwald E. *The Rise of Cardiovascular Medicine*. Eur Heart J. 33:838-845, 845a, **2012**.

91. The HLA Facts Book. Marsh SGE, Parham P, Barber LD. *Academic Press, London* **2000**; 3-73.

92. The HLA Complex in Biology and Medicine. Mehra NK, Kaur G, McCluskey J, Christiansen FT, Claas FHJ. *JP Medical Publishers Ltd, New Delhi, India.* **2010**; 465-471.

93. Robinson J, Mistry K, McWilliam H, Lopez R, Parham P, Marsh SG. *The IMGT/HLA Database*. Nucleic Acids Res. 39:D1171-1176, **2011**.

94. Teslovich TM, Musunuru K, Smith AV, Edmondson AC, Stylianou IM, Koseki M, Pirruccello JP, Ripatti S, Chasman DI, Willer CJ, Johansen CT, Fouchier SW, Isaacs A, Peloso GM, Barbalic M, Ricketts SL, Bis JC, Aulchenko YS, Thorleifsson G, Feitosa MF, Chambers J, Orho-Melander M, Melander O, Johnson T, Li X, Guo X, Li M, Shin Cho Y, Jin Go M, Jin Kim Y, Lee JY, Park T, Kim K, Sim X, Twee-Hee Ong R, Croteau-Chonka DC, Lange LA, Smith JD, Song K, Hua Zhao J, Yuan X, Luan J, Lamina C, Ziegler A, Zhang W, Zee RY, Wright AF, Witteman JC, Wilson JF, Willemsen G, Wichmann HE, Whitfield JB, Waterworth DM, Wareham NJ, Waeber G, Vollenweider P, Voight BF, Vitart V, Uitterlinden AG, Uda M, Tuomilehto J, Thompson JR, Tanaka T, Surakka I, Stringham HM, Spector TD, Soranzo N, Smit JH, Sinisalo J, Silander K, Sijbrands EJ, Scuteri A, Scott J, Schlessinger D, Sanna S, Salomaa V, Saharinen J, Sabatti C, Ruokonen A, Rudan I, Rose LM, Roberts R, Rieder M, Psaty BM, Pramstaller PP, Pichler I, Perola M, Penninx BW, Pedersen NL, Pattaro C, Parker AN, Pare G, Oostra BA, O'Donnell CJ, Nieminen MS, Nickerson DA, Montgomery GW, Meitinger T, McPherson R, McCarthy MI, McArdle W, Masson D, Martin NG, Marroni F, Mangino M, Magnusson PK, Lucas G, Luben R, Loos RJ, Lokki ML, Lettre G, Langenberg C, Launer LJ, Lakatta EG, Laaksonen R, Kyvik KO, Kronenberg F, Konig IR, Khaw KT, Kaprio J, Kaplan LM, Johansson A, Jarvelin MR, Janssens AC, Ingelsson E, Igl W, Kees Hovingh G, Hottenga JJ, Hofman A, Hicks AA, Hengstenberg C, Heid IM, Hayward C, Havulinna AS, Hastie ND, Harris TB, Haritunians T, Hall AS, Gyllensten U, Guiducci C, Groop LC, Gonzalez E, Gieger C, Freimer NB, Ferrucci L, Erdmann J, Elliott P, Ejebe KG, Doring A, Dominiczak AF, Demissie S, Deloukas P, de Geus EJ, de Faire U, Crawford G, Collins FS, Chen YD, Caulfield MJ, Campbell H, Burtt NP, Bonnycastle LL, Boomsma DI, Boekholdt SM, Bergman RN, Barroso I, Bandinelli S, Ballantyne CM, Assimes TL, Quertermous T, Altshuler D, Seielstad M, Wong TY, Tai ES, Feranil AB, Kuzawa CW, Adair LS, Taylor HA, Jr., Borecki IB, Gabriel SB, Wilson JG, Holm H, Thorsteinsdottir U, Gudnason V, Krauss RM, Mohlke KL, Ordovas JM, Munroe PB, Kooner JS, Tall AR, Hegele RA, Kastelein JJ, Schadt EE, Rotter JI, Boerwinkle E, Strachan DP, Mooser V, Stefansson K, Reilly MP, Samani NJ, Schunkert H, Cupples LA, Sandhu MS, Ridker PM, Rader DJ, van Duijn CM, Peltonen L, Abecasis GR, Boehnke M, Kathiresan S. *Biological, Clinical and Population Relevance of 95 Loci for Blood Lipids*. Nature. 466:707-713, **2010**.

95. Holliday EG, Maguire JM, Evans TJ, Koblar SA, Jannes J, Sturm JW, Hankey GJ, Baker R, Golledge J, Parsons MW, Malik R, McEvoy M, Biros E, Lewis MD, Lincz LF, Peel R, Oldmeadow C, Smith W, Moscato P, Barlera S, Bevan S, Bis JC, Boerwinkle E, Boncoraglio GB, Brott TG, Brown RD, Jr., Cheng YC, Cole JW, Cotlarciuc I, Devan WJ, Fornage M, Furie KL, Gretarsdottir S, Gschwendtner A, Ikram MA, Longstreth WT, Jr., Meschia JF, Mitchell BD, Mosley TH, Nalls MA, Parati EA, Psaty BM, Sharma P, Stefansson K, Thorleifsson G, Thorsteinsdottir U, Traylor M, Verhaaren BF, Wiggins KL, Worrall BB, Sudlow C, Rothwell PM, Farrall M, Dichgans M, Rosand J, Markus HS, Scott RJ, Levi C, Attia J. *Common Variants at 6p21.1 Are Associated with Large Artery Atherosclerotic Stroke*. Nat Genet. 44:1147-1151, **2012**.

96. Gonzalez-Gay MA, Gonzalez-Juanatey C, Lopez-Diaz MJ, Pineiro A, Garcia-Porrua C, Miranda-Filloy JA, Ollier WE, Martin J, Llorca J. *Hla-DRB1 and Persistent Chronic Inflammation Contribute to Cardiovascular Events and Cardiovascular Mortality in Patients with Rheumatoid Arthritis*. Arthritis Rheum. 57:125-132, **2007**.

97. Mattey DL, Thomson W, Ollier WE, Batley M, Davies PG, Gough AK, Devlin J, Prouse P, James DW, Williams PL, Dixey J, Winfield J, Cox NL, Koduri G, Young A. *Association of DRB1 Shared Epitope Genotypes with Early Mortality in Rheumatoid Arthritis: Results of Eighteen Years of Followup from the Early Rheumatoid Arthritis Study*. Arthritis Rheum. 56:1408-1416, **2007**.

98. Farragher TM, Goodson NJ, Naseem H, Silman AJ, Thomson W, Symmons D, Barton A. *Association of the HLA-DRB1 Gene with Premature Death, Particularly from Cardiovascular Disease, in Patients with Rheumatoid Arthritis and Inflammatory Polyarthritis*. Arthritis Rheum. 58:359-369, **2008**.

99. Söderlund J, Forsblom C, Ilonen J, Thorn LM, Waden J, Parkkonen M, Groop PH. *HLA Class II Is a Factor in Cardiovascular Morbidity and Mortality Rates in Patients with Type 1 Diabetes*. Diabetologia. **2012**.

100. Gonzalez-Juanatey C, Testa A, Garcia-Castelo A, Garcia-Porrua C, Llorca J, Vidan J, Hajeer AH, Ollier WE, Mattey DL, Gonzalez-Gay MA. *HLA-DRB1 Status Affects Endothelial Function in Treated Patients with Rheumatoid Arthritis*. Am J Med. 114:647-652, **2003**.

101. Shiffman D, Kane JP, Louie JZ, Arellano AR, Ross DA, Catanese JJ, Malloy MJ, Ellis SG, Devlin JJ. *Analysis of 17,576 Potentially Functional SNPs in Three Case-Control Studies of Myocardial Infarction*. PLoS One. 3:e2895, **2008**.

102. Ozaki K, Ohnishi Y, Iida A, Sekine A, Yamada R, Tsunoda T, Sato H, Sato H, Hori M, Nakamura Y, Tanaka T. *Functional SNPs in the Lymphotoxin-Alpha Gene That Are Associated with Susceptibility to Myocardial Infarction*. Nat Genet. 32:650-654, **2002**.

103. Koch W, Hoppmann P, Michou E, Jung V, Pfeufer A, Mueller JC, Gieger C, Wichmann HE, Meitinger T, Schomig A, Kastrati A. *Association of Variants in the BAT1-NFKBIL1-LTA Genomic Region with Protection against Myocardial Infarction in Europeans*. Hum Mol Genet. 16:1821-1827, **2007**.

104. Zhang HF, Xie SL, Wang JF, Chen YX, Wang Y, Huang TC. *Tumor Necrosis Factor-Alpha G-308a Gene Polymorphism and Coronary Heart Disease Susceptibility: An Updated Meta-Analysis*. Thromb Res. 127:400-405, **2011**.

105. Jönsson G, Truedsson L, Sturfelt G, Oxelius VA, Braconier JH, Sjöholm AG. *Hereditary C2 Deficiency in Sweden: Frequent Occurrence of Invasive Infection, Atherosclerosis, and Rheumatic Disease*. Medicine (Baltimore). 84:23-34, **2005**.

106. Porto I, Leone AM, Crea F, Andreotti F. *Inflammation, Genetics, and Ischemic Heart Disease: Focus on the Major Histocompatibility Complex (MHC) Genes*. Cytokine. 29:187-196, **2005**.

107. Liu B, Xiong L, Tian C, Zhou Q, Zhong Y, Li A, Liu SM. *HLA-DRB1*12:02:01 Plays a Protective Role against Coronary Artery Disease in Women of Southern Han Chinese Descent*. Hum Immunol. 73:122-126, **2012**.

108. Thielens A, Vivier E, Romagne F. *Nk Cell MHC Class I Specific Receptors (KIR): From Biology to Clinical Intervention*. Curr Opin Immunol. 24:239-245, **2012**.

109. Willcox BE, Thomas LM, Bjorkman PJ. *Crystal Structure of HLA-A2 Bound to LIR-1, a Host and Viral Major Histocompatibility Complex Receptor*. Nat Immunol. 4:913-919, **2003**.

110. Wakim LM, Bevan MJ. *Cross-Dressed Dendritic Cells Drive Memory CD8+ T-Cell Activation after Viral Infection*. Nature. 471:629-632, **2011**.

111. Tabayoyong WB, Zavazava N. *Soluble HLA Revisited*. Leuk Res. 31:121-125, **2007**.

112. Datwani A, McConnell MJ, Kanold PO, Micheva KD, Busse B, Shamloo M, Smith SJ, Shatz CJ. *Classical MHCI Molecules Regulate Retinogeniculate Refinement and Limit Ocular Dominance Plasticity*. Neuron. 64:463-470, **2009**.

113. Chen A, Shen Y, Xia M, Xu L, Pan N, Yin Y, Miao F, Shen C, Xie W, Zhang J. *Expression of the Nonclassical HLA Class I and MICA/B Molecules in Human Hepatocellular Carcinoma*. Neoplasma. 58:371-376, **2011**.

114. de Verteuil D, Granados DP, Thibault P, Perreault C. *Origin and Plasticity of MHC I-Associated Self Peptides*. Autoimmun Rev. 11:627-635, **2012**.

115. Sirén MK, Sareneva H, Lokki ML, Koskimies S. *Unique HLA Antigen Frequencies in the Finnish Population*. Tissue Antigens. 48:703-707, **1996**.

116. Dawkins R, Leelayuwat C, Gaudieri S, Tay G, Hui J, Cattley S, Martinez P, Kulski J. *Genomics of the Major Histocompatibility Complex: Haplotypes, Duplication, Retroviruses and Disease*. Immunol Rev. 167:275-304, **1999**.

117. Ahmad T, Neville M, Marshall SE, Armuzzi A, Mulcahy-Hawes K, Crawshaw J, Sato H, Ling KL, Barnardo M, Goldthorpe S, Walton R, Bunce M, Jewell DP, Welsh KI. *Haplotype-Specific Linkage Disequilibrium Patterns Define the Genetic Topography of the Human MHC*. Hum Mol Genet. 12:647-656, **2003**.

118. Salazar G, Colombo G, Lenna S, Antonioli R, Beretta L, Santaniello A, Scorza R. *HLA-B35 Influences the Apoptosis Rate in Human Peripheral Blood Mononucleated Cells and HLA-Transfected Cells*. Hum Immunol. 68:181-191, **2007**.

119. Lenna S, Townsend DM, Tan FK, Kapanadze B, Markiewicz M, Trojanowska M, Scorza R. *HLA-B35 Upregulates Endothelin-1 and Downregulates Endothelial Nitric Oxide Synthase Via Endoplasmic Reticulum Stress Response in Endothelial Cells*. J Immunol. 184:4654-4661, **2010**.

120. Hendel H, Caillat-Zucman S, Lebuanec H, Carrington M, O'Brien S, Andrieu JM, Schachter F, Zagury D, Rappaport J, Winkler C, Nelson GW, Zagury JF. *New Class I and II HLA Alleles Strongly Associated with Opposite Patterns of Progression to Aids*. J Immunol. 162:6942-6946, **1999**.

121. Gao X, O'Brien TR, Welzel TM, Marti D, Qi Y, Goedert JJ, Phair J, Pfeiffer R, Carrington M. *HLA-B Alleles Associate Consistently with HIV Heterosexual Transmission, Viral Load, and Progression to AIDS, but Not Susceptibility to Infection*. AIDS. 24:1835-1840, **2010**.

122. Carrington M, Nelson GW, Martin MP, Kissner T, Vlahov D, Goedert JJ, Kaslow R, Buchbinder S, Hoots K, O'Brien SJ. *HLA and HIV-1: Heterozygote Advantage and B*35-Cw*04 Disadvantage*. Science. 283:1748-1752, **1999**.

123. Palikhe A, Lokki ML, Saikku P, Leinonen M, Paldanius M, Seppanen 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. 15:55-59, **2008**.

124. Kim J-H, Pyo C-W, Koh DK, Hur JK, Kang JH, Kim T-G. *Alteration of the Influences of HLA Classes I and II Alleles on the Perinatal Hepatitis B Virus Infection after Immunoprophylaxis in Korean Children*. Hepatol Res. 35:118-126, **2006**.

125. Yoon SK, Han JY, Pyo CW, Yang JM, Jang JW, Kim CW, Chang UI, Bae SH, Choi JY, Chung KW, Sun HS, Choi HB, Kim TG. *Association between Human Leukocytes Antigen Alleles and Chronic Hepatitis C Virus Infection in the Korean Population*. Liver Int. 25:1122-1127, **2005**.

126. Grigolo B, Mazzetti I, Meliconi R, Bazzi S, Scorza R, Candela M, Gabrielli A, Facchini A. *Anti-Topoisomerase II Autoantibodies in Systemic Sclerosis - Association with Pulmonary Hypertension and HLA-B35*. Clin Exp Immunol. 121:539-543, **2000**.

127. Franceschi DS, Tsuneto LT, Mazini PS, Sacramento WS, Reis PG, Rudnick CC, Clementino SL, Sell AM, Visentainer JE. *Class-I Human Leukocyte Alleles in Leprosy Patients from Southern Brazil*. Rev Soc Bras Med Trop. 44:616-620, **2011**.

128. Handunnetthi L, Ramagopalan SV, Ebers GC, Knight JC. *Regulation of Major Histocompatibility Complex Class II Gene Expression, Genetic Variation and Disease*. Genes Immun. 11:99-112, **2010**.

129. Liu X, Zhan Z, Li D, Xu L, Ma F, Zhang P, Yao H, Cao X. *Intracellular MHC Class II Molecules Promote TLR-Triggered Innate Immune Responses by Maintaining Activation of the Kinase BTK*. Nat Immunol. 12:416-424, **2011**.

130. Mehindate K, al-Daccak R, Dayer JM, Kennedy BP, Kris C, Borgeat P, Poubelle PE, Mourad W. *Superantigen-Induced Collagenase Gene Expression in Human IFN-Gamma-Treated Fibroblast-Like Synoviocytes Involves Prostaglandin E2. Evidence for a Role of Cyclooxygenase-2 and Cytosolic Phospholipase A2*. J Immunol. 155:3570-3577, **1995**.

131. Al-Daccak R, Mooney N, Charron D. *MHC Class II Signaling in Antigen-Presenting Cells*. Curr Opin Immunol. 16:108-113, **2004**.

132. Kreisel D, Richardson SB, Li W, Lin X, Kornfeld CG, Sugimoto S, Hsieh CS, Gelman AE, Krupnick AS. *Cutting Edge: MHC Class II Expression by Pulmonary Nonhematopoietic Cells Plays a Critical Role in Controlling Local Inflammatory Responses*. J Immunol. 185:3809-3813, **2010**.

133. Lahat N, Bitterman H, Weiss-Cerem L, Rahat MA. *Hypoxia Increases Membranal and Secreted HLA-DR in Endothelial Cells, Rendering Them T-Cell Activators*. Transpl Int. 24:1018-1026, **2011**.

134. Aultman D, Adamashvili I, Yaturu K, Langford M, Gelder F, Gautreaux M, Ghali GE, McDonald J. *Soluble HLA in Human Body Fluids*. Hum Immunol. 60:239-244, **1999**.

135. Adamashvili I, Kelley RE, Pressly T, McDonald JC. *Soluble HLA: Patterns of Expression in Normal Subjects, Autoimmune Diseases, and Transplant Recipients*. Rheumatol Int. 25:491-500, **2005**.

136. Minagar A, Adamashvili I, Kelley RE, Gonzalez-Toledo E, McLarty J, Smith SJ. *Saliva Soluble HLA as a Potential Marker of Response to Interferon-Beta 1a in Multiple Sclerosis: A Preliminary Study*. J Neuroinflammation. 4:16, **2007**.

137. Gansuvd B, Hagihara M, Higuchi A, Ueda Y, Tazume K, Tsuchiya T, Munkhtuvshin N, Kato S, Hotta T. *Umbilical Cord Blood Dendritic Cells Are a Rich Source of Soluble HLA-DR: Synergistic Effect of Exosomes and Dendritic Cells on Autologous or Allogeneic T-Cell Proliferation*. Hum Immunol. 64:427-439, **2003**.

138. Choi NM, Majumder P, Boss JM. *Regulation of Major Histocompatibility Complex Class II Genes*. Curr Opin Immunol. 23:81-87, **2011**.

139. Paul P, van den Hoorn T, Jongsma ML, Bakker MJ, Hengeveld R, Janssen L, Cresswell P, Egan DA, van Ham M, Ten Brinke A, Ovaa H, Beijersbergen RL, Kuijl C, Neefjes J. *A Genome-Wide Multidimensional RNAi Screen Reveals Pathways Controlling MHC Class II Antigen Presentation*. Cell. 145:268-283, **2011**.

140. Gonzalez-Galarza FF, Christmas S, Middleton D, Jones AR. *Allele Frequency Net: A Database and Online Repository for Immune Gene Frequencies in Worldwide Populations*. Nucleic Acids Res. 39:D913-919, **2011**.

141. Raychaudhuri S, Sandor C, Stahl EA, Freudenberg J, Lee HS, Jia X, Alfredsson L, Padyukov L, Klareskog L, Worthington J, Siminovitch KA, Bae SC, Plenge RM, Gregersen PK, de Bakker PI. *Five Amino Acids in Three HLA Proteins Explain Most of the Association between MHC and Seropositive Rheumatoid Arthritis*. Nat Genet. 44:291-296, **2012**.

142. Seddighzadeh M, Korotkova M, Kallberg H, Ding B, Daha N, Kurreeman FA, Toes RE, Huizinga TW, Catrina AI, Alfredsson L, Klareskog L, Padyukov L. *Evidence for Interaction between 5-Hydroxytryptamine (Serotonin) Receptor 2a and MHC Type II Molecules in the Development of Rheumatoid Arthritis*. Eur J Hum Genet. 18:821-826, **2010**.

143. Padyukov L, Silva C, Stolt P, Alfredsson L, Klareskog L. *A Gene-Environment Interaction between Smoking and Shared Epitope Genes in HLA-DR Provides a High Risk of Seropositive Rheumatoid Arthritis*. Arthritis Rheum. 50:3085-3092, **2004**.

144. Sane J, Kurkela S, Lokki ML, Miettinen A, Helve T, Vaheri A, Vapalahti O. *Clinical Sindbis Alphavirus Infection Is Associated with HLA-DRB1*01 Allele and Production of Autoantibodies*. Clin Infect Dis. 55:358-363, **2012**.

145. MacDonald KS, Fowke KR, Kimani J, Dunand VA, Nagelkerke NJD, Blake Ball T, Oyugi J, Njagi E, Gaur LK, Brunham R, Wade J, Luscher MA, Krausa P, Rowland-Jones S, Ngugi E, Bwayo JJ, Plummer FA. *Influence of HLA Supertypes on Susceptibility and Resistance to Human Immunodeficiency Virus Type 1 Infection*. J Infect Dis. 181:1581-1589, **2000**.

146. Thio CL, Thomas DL, Goedert JJ, Vlahov D, Nelson KE, Hilgartner MW, O'Brien SJ, Karacki P, Marti D, Astemborski J, Carrington M. *Racial Differences in HLA Class II Associations with Hepatitis C Virus Outcomes*. J Infect Dis. 184:16-21, **2001**.

147. Kallio-Laine K, Seppänen M, Aittoniemi J, Kautiainen H, Seppälä I, Valtonen V, Färkkila M, Kalso E, Lokki ML. *HLA-DRB1*01 Allele and Low Plasma Immunoglobulin G1 Concentration May Predispose to Herpes-Associated Recurrent Lymphocytic Meningitis*. Hum Immunol. 71:179-181, **2010**.

148. Palikhe A, Lokki ML, 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. 71:530-537, **2008**.

149. Ramagopalan SV, Meier UC, Conacher M, Ebers Gc, Giovannoni G, Crawford DH, McAulay KA. *Role of the HLA System in the Association between Multiple Sclerosis and Infectious Mononucleosis*. Arch Neurol. 68:469-472, **2011**.

150. Ruyssen-Witrand A, Constantin A, Cambon-Thomsen A, Thomsen M. *New Insights into the Genetics of Immune Responses in Rheumatoid Arthritis*. Tissue Antigens. 80:105-118, **2012**.

151. Pazar B, Gergely P, Jr., Nagy ZB, Gombos T, Pozsonyi E, Rajczy K, Balogh Z, Sevcic K, Orban I, Szodoray P, Poor G. *Role of HLA-DRB1 and PTPN22 Genes in Susceptibility to Juvenile Idiopathic Arthritis in Hungarian Patients*. Clin Exp Rheumatol. 26:1146-1152, **2008**.

152. Amarapurkar DN, Patel ND, Amarapurkar AD, Kankonkar SR. *HLA Genotyping in Type-I Autoimmune Hepatitis in Western India*. J Assoc Physicians India. 51:967-969, **2003**.

153. Amoli MM, Thomson W, Hajeer AH, Calvino MC, Garcia-Porrua C, Ollier WE, Gonzalez-Gay MA. *HLA-DRB1*01 Association with Henoch-Schonlein Purpura in Patients from Northwest Spain*. J Rheumatol. 28:1266-1270, **2001**.

154. del Puerto F, Nishizawa JE, Kikuchi M, Roca Y, Avilas C, Gianella A, Lora J, Velarde FU, Miura S, Komiya N, Maemura K, Hirayama K. *Protective Human Leucocyte Antigen Haplotype, HLA-DRB1*01-B*14, against Chronic Chagas Disease in Bolivia*. PLoS Negl Trop Dis. 6:e1587, **2012**.

155. Donfack J, Tsalenko A, Hoki DM, Parry R, Solway J, Lester LA, Ober C. *HLA-DRB1*01 Alleles Are Associated with Sensitization to Cockroach Allergens*. J Allergy Clin Immunol. 105:960-966, **2000**.

156. Jahn-Schmid B, Fischer GF, Bohle B, Fae I, Gadermaier G, Dedic A, Ferreira F, Ebner C. *Antigen Presentation of the Immunodominant T-Cell Epitope of the Major Mugwort Pollen Allergen, Art V 1, Is Associated with the Expression of HLA-DRB1 *01*. J Allergy Clin Immunol. 115:399-404, **2005**.

157. Martin AM, Nolan D, James I, Cameron P, Keller J, Moore C, Phillips E, Christiansen FT, Mallal S. *Predisposition to Nevirapine Hypersensitivity Associated with HLA-DRB1*0101 and Abrogated by Low CD4 T-Cell Counts*. AIDS. 19:97-99, **2005**.

158. Vitezica ZG, Milpied B, Lonjou C, Borot N, Ledger TN, Lefebvre A, Hovnanian A. *HLA-DRB1*01 Associated with Cutaneous Hypersensitivity Induced by Nevirapine and Efavirenz*. AIDS. 22:540-541, **2008**.

159. Conde L, Halperin E, Akers NK, Brown KM, Smedby KE, Rothman N, Nieters A, Slager SL, Brooks-Wilson A, Agana L, Riby J, Liu J, Adami HO, Darabi H, Hjalgrim H, Low HQ, Humphreys K, Melbye M, Chang ET, Glimelius B, Cozen W, Davis S, Hartge P, Morton LM, Schenk M, Wang SS, Armstrong B, Kricker A, Milliken S, Purdue MP, Vajdic CM, Boyle P, Lan Q, Zahm SH, Zhang Y, Zheng T, Becker N, Benavente Y, Boffetta P, Brennan P, Butterbach K, Cocco P, Foretova L, Maynadie M, de Sanjose S, Staines A, Spinelli JJ, Achenbach SJ, Call TG, Camp NJ, Glenn M, Caporaso NE, Cerhan JR, Cunningham JM, Goldin LR, Hanson CA, Kay NE, Lanasa MC, Leis JF, Marti GE, Rabe KG, Rassenti LZ, Spector LG, Strom SS, Vachon CM, Weinberg JB, Holly EA, Chanock S, Smith MT, Bracci PM, Skibola CF. *Genome-Wide Association Study of Follicular Lymphoma Identifies a Risk Locus at 6p21.32*. Nat Genet. 42:661-664, **2010**.

160. Verreck FA, van de Poel A, Drijfhout JW, Amons R, Coligan JE, Konig F. *Natural Peptides Isolated from Gly86/Val86-Containing Variants of HLA-DR1, -DR11, -DR13, and -DR52*. Immunogenetics. 43:392-397, **1996**.

161. Fissolo N, Haag S, de Graaf KL, Drews O, Stevanovic S, Rammensee HG, Weissert R. *Naturally Presented Peptides on Major Histocompatibility Complex I and II Molecules Eluted from Central Nervous System of Multiple Sclerosis Patients*. Mol Cell Proteomics. 8:2090-2101, **2009**.

162. Jonasson L, Eriksson T, Dahlén GH, Lindblom B. *Lipoprotein(a) and HLA-DRB1 and -DQB1 Genes in Coronary Artery Disease*. Atherosclerosis. 133:111-114, **1997**.

163. Yang Z, Yu CY. *Organizations and Gene Duplications of the Human and Mouse MHC Complement Gene Clusters*. Exp Clin Immunogenet. 17:1-17, **2000**.

164. Systemic Lupus Erythematosus. Lahita G,. Buyon J, Koike T. *Elsevier, London, UK*. **2011**; 21-45.

165. Coppinger JA, Cagney G, Toomey S, Kislinger T, Belton O, McRedmond JP, Cahill DJ, Emili A, Fitzgerald DJ, Maguire PB. *Characterization of the Proteins Released from Activated Platelets Leads to Localization of Novel Platelet Proteins in Human Atherosclerotic Lesions*. Blood. 103:2096-2104, **2004**.

166. Mougey R. *A Review of the Chido/Rodgers Blood Group*. Immunohematology. 26:30-38, **2010**.

167. Ulgiati D, Subrata LS, Abraham LJ. *The Role of SP Family Members, Basic Kruppel-Like Factor, and E Box Factors in the Basal and IFN-Gamma Regulated Expression of the Human Complement C4 Promoter*. J Immunol. 164:300-307, **2000**.

168. McGreal EP, Hearne K, Spiller OB. *Off to a Slow Start: Under-Development of the Complement System in Term Newborns Is More Substantial Following Premature Birth*. Immunobiology. 217:176-186, **2012**.

169. Reis ES, Lange T, Kohl G, Herrmann A, Tschulakow AV, Naujoks J, Born J, Kohl J. *Sleep and Circadian Rhythm Regulate Circulating Complement Factors and Immunoregulatory Properties of C5a*. Brain Behav Immun. 25:1416-1426, **2011**.

170. Ritchie RF, Palomaki GE, Neveux LM, Navolotskaia O. *Reference Distributions for Complement Proteins C3 and C4: A Comparison of a Large Cohort to the World's Literature*. J Clin Lab Anal. 18:9-13, **2004**.

171. Wu YL, Higgins GC, Rennebohm RM, Chung EK, Yang Y, Zhou B, Nagaraja HN, Birmingham DJ, Rovin BH, Hebert LA, Yu CY. *Three Distinct Profiles of Serum Complement C4 Proteins in Pediatric Systemic Lupus Erythematosus (SLE) Patients: Tight Associations of Complement C4 and C3 Protein Levels in SLE but Not in Healthy Subjects*. Adv Exp Med Biol. 586:227-247, **2006**.

172. Gojobori T, Yang X, Sun J, Gao Y, Tan A, Zhang H, Hu Y, Feng J, Qin X, Tao S, Chen Z, Kim S-T, Peng T, Liao M, Lin X, Zhang Z, Tang M, Li L, Mo L, Liang Z, Shi D, Huang Z, Huang X, Liu M, Liu Q, Zhang S, Trent JM, Zheng SL, Xu J, Mo Z. *Genome-Wide Association Study for Serum Complement C3 and C4 Levels in Healthy Chinese Subjects*. PLoS Genetics. 8:e1002916, **2012**.

173. Luo C, Chen M, Madden A, Xu H. *Expression of Complement Components and Regulators by Different Subtypes of Bone Marrow-Derived Macrophages*. Inflammation. 35:1448-1461, **2012**.

174. Atkinson JP, Frank MM. *Bypassing Complement: Evolutionary Lessons and Future Implications*. J Clin Invest. 116:1215-1218, **2006**.

175. Selander B, Martensson U, Weintraub A, Holmstrom E, Matsushita M, Thiel S, Jensenius JC, Truedsson L, Sjoholm AG. *Mannan-Binding Lectin Activates C3 and the Alternative Complement Pathway without Involvement of C2*. J Clin Invest. 116:1425-1434, **2006**.

176. Ricklin D, Hajishengallis G, Yang K, Lambris JD. *Complement: A Key System for Immune Surveillance and Homeostasis*. Nat Immunol. 11:785-797, **2010**.

177. Schwaeble WJ, Lynch NJ, Clark JE, Marber M, Samani NJ, Ali YM, Dudler T, Parent B, Lhotta K, Wallis R, Farrar CA, Sacks S, Lee H, Zhang M, Iwaki D, Takahashi M, Fujita T, Tedford CE, Stover CM. *Targeting of Mannan-Binding Lectin-Associated Serine Protease-2 Confers Protection from Myocardial and Gastrointestinal Ischemia/Reperfusion Injury*. Proc Natl Acad Sci. 108:7523-7528, **2011**.

178. Clemenza L, Isenman DE. *The C4A and C4B Isotypic Forms of Human Complement Fragment C4b Have the Same Intrinsic Affinity for Complement Receptor 1 (CR1/CD35)*. J Immunol. 172:1670-1680, **2004**.

179. Mauff G, Luther B, Schneider PM, Rittner C, Stradmann-Bellinghausen B, Dawkins R, Moulds JM. *Reference Typing Report for Complement Component C4*. Exp Clin Immunogenet. 15:249-260, **1998**.

180. Blanchong CA, Zhou B, Rupert KL, Chung EK, Jones KN, Sotos JF, Zipf WB, Rennebohm RM, Yung Yu C. *Deficiencies of Human Complement Component C4A and C4B and Heterozygosity in Length Variants of RP-C4-CYP21-TNX (RCCX) Modules in Caucasians. The Load of RCCX Genetic Diversity on Major Histocompatibility Complex-Associated Disease*. J Exp Med. 191:2183-2196, **2000**.

181. Yu CY. *The Complete Exon-Intron Structure of a Human Complement Component C4A Gene. DNA Sequences, Polymorphism, and Linkage to the 21-Hydroxylase Gene*. J Immunol. 146:1057-1066, **1991**.

182. Yu CY, Belt KT, Giles CM, Campbell RD, Porter RR. *Structural Basis of the Polymorphism of Human Complement Components C4A and C4B: Gene Size, Reactivity and Antigenicity*. EMBO J. 5:2873-2881, **1986**.

183. Law SK, Dodds AW, Porter RR. *A Comparison of the Properties of Two Classes, C4A and C4B, of the Human Complement Component C4*. EMBO J. 3:1819-1823, **1984**.

184. Carroll MC, Fathallah DM, Bergamaschini L, Alicot EM, Isenman DE. *Substitution of a Single Amino Acid (Aspartic Acid for Histidine) Converts the Functional Activity of Human Complement C4B to C4A*. Proc Natl Acad Sci. 87:6868-6872, **1990**.

185. Szilagyi A, Fust G. *Diseases Associated with the Low Copy Number of the C4B Gene Encoding C4, the Fourth Component of Complement*. Cytogenet Genome Res. 123:118-130, **2008**.

186. Fernando MM, Boteva L, Morris DL, Zhou B, Wu YL, Lokki ML, Yu CY, Rioux JD, Hollox EJ, Vyse TJ. *Assessment of Complement C4 Gene Copy Number Using the Paralog Ratio Test*. Hum Mutat. 31:866-874, **2010**.

187. Yang Y, Chung EK, Wu YL, Savelli SL, Nagaraja HN, Zhou B, Hebert M, Jones KN, Shu Y, Kitzmiller K, Blanchong CA, McBride KL, Higgins GC, Rennebohm RM, Rice RR, Hackshaw KV, Roubey RA, Grossman JM, Tsao BP, Birmingham DJ, Rovin BH, Hebert LA, Yu CY. *Gene Copy-Number Variation and Associated Polymorphisms of Complement Component C4 in Human Systemic Lupus Erythematosus (SLE): Low Copy Number Is a Risk Factor for and High Copy Number Is a Protective Factor against SLE Susceptibility in European Americans*. Am J Hum Gene. 80:1037-1054, **2007**.

188. Wahrmann M, Dohler B, Ruhenstroth A, Haslacher H, Perkmann T, Exner M, Rees AJ, Bohmig GA. *Genotypic Diversity of Complement Component C4 Does Not Predict Kidney Transplant Outcome*. J Am Soc Nephrol. 22:367-376, **2011**.

189. Szilagyi A, Blaskó B, Szilassy D, Füst G, Sasvári-Székely M, Ronai Z. *Real-Time PCR Quantification of Human Complement C4A and C4B Genes*. BMC genet. 7:1, **2006**.

190. Wouters D, van Schouwenburg P, van der Horst A, de Boer M, Schooneman D, Kuijpers TW, Aarden LA, Hamann D. *High-Throughput Analysis of the C4 Polymorphism by a Combination of MLPA and Isotype-Specific Elisa's*. Mol Immunol. 46:592-600, **2009**.

191. Seppänen M, Suvilehto J, Lokki ML, Notkola IL, Järvinen A, Jarva H, Seppala I, Tahkokallio O, Malmberg H, Meri S, Valtonen V. *Immunoglobulins and Complement Factor C4 in Adult Rhinosinusitis*. Clin Exp Immunol. 145:219-227, **2006**.

192. Saxena K, Kitzmiller KJ, Wu YL, Zhou B, Esack N, Hiremath L, Chung EK, Yang Y, Yu CY. *Great Genotypic and Phenotypic Diversities Associated with Copy-Number Variations of Complement C4 and RP-C4-CYP21-TNX (RCCX) Modules: A Comparison of Asian-Indian and European American Populations*. Mol Immunol. 46:1289-1303, **2009**.

193. Barba G, Rittner C, Schneider PM. *Genetic Basis of Human Complement C4A Deficiency. Detection of a Point Mutation Leading to Nonexpression*. J Clin Invest. 91:1681-1686, **1993**.

194. Anderson MJ, Milner CM, Cotton RG, Campbell RD. *The Coding Sequence of the Hemolytically Inactive C4A6 Allotype of Human Complement Component C4 Reveals That a Single Arginine to Tryptophan Substitution at Beta-Chain Residue 458 Is the Likely Cause of the Defect*. J Immunol. 148:2795-2802, **1992**.

195. McLean RH, Niblack G, Julian B, Wang T, Wyatt R, Phillips JA, 3rd, Collins TS, Winkelstein J, Valle D. *Hemolytically Inactive C4B Complement Allotype Caused by a Proline to Leucine Mutation in the C5-Binding Site*. J Biol Chem. 269:27727-27731, **1994**.

196. Sim E, Gill EW, Sim RB. *Drugs That Induce Systemic Lupus Erythematosus Inhibit Complement Component C4*. Lancet. 2:422-424, **1984**.

197. Boteva L, Imagen, Wu YL, Cortes-Hernandez J, Martin J, Vyse TJ, Fernando MM. *Determination of the Loss of Function Complement C4 Exon 29 CT Insertion Using a Novel Paralog-Specific Assay in Healthy UK and Spanish Populations*. PloS one. 6:e22128, **2011**.

198. Lipsker D, Hauptmann G. *Cutaneous Manifestations of Complement Deficiencies*. Lupus. 19:1096-1106, **2010**.

199. Boteva L, Morris DL, Cortes-Hernandez J, Martin J, Vyse TJ, Fernando MM. *Genetically Determined Partial Complement C4 Deficiency States Are Not Independent Risk Factors for SLE in UK and Spanish Populations*. Am J Hum Genet. 90:445-456, **2012**.

200. Suvilehto. *Academic Dissertation*. **2009**.

201. Kainulainen L, Peltola V, Seppänen M, Viander M, He Q, Lokki ML, Ruuskanen O. *C4A Deficiency in Children and Adolescents with Recurrent Respiratory Infections*. Hum Immunol. 73:498-501, **2012**.

202. Seppänen M, Lokki ML, Notkola IL, Mattila K, Valtonen V, Nieminen A, Vesanen M, Asikainen S, Meri S. *Complement and C4 Null Alleles in Severe Chronic Adult Periodontitis*. Scand J Immunol. 65:176-181, **2007**.

203. Senbagavalli P, Kumar N, Kaur G, Mehra NK, Geetha ST, Ramanathan VD. *Major Histocompatibility Complex Class III (C2, C4, Factor B) and C3 Gene Variants in Patients with Pulmonary Tuberculosis*. Hum Immunol. 72:173-178, **2011**.

204. Seppänen M, Meri S, Notkola IL, Seppälä IJ, Hiltunen-Back E, Sarvas H, Lappalainen M, Välimaa H, Palikhe A, Valtonen VV, Lokki ML. *Subtly Impaired Humoral Immunity Predisposes to Frequently Recurring Genital Herpes Simplex Virus Type 2 Infection and Herpetic Neuralgia*. J Infect Dis. 194:571-578, **2006**.

205. de Messias IJ, Santamaria J, Brenden M, Reis A, Mauff G. *Association of C4B Deficiency (C4B*Q0) with Erythema Nodosum in Leprosy*. Clin Exp Immunol. 92:284-287, **1993**.

206. Wennerström A, Pietinalho A, Vauhkonen H, Lahtela L, Palikhe A, Hedman J, Purokivi M, Värkki E, Seppanen M, Lokki ML, Selroos O. *HLA-DRB1 Allele Frequencies and C4 Copy Number Variation in Finnish Sarcoidosis Patients and Associations with Disease Prognosis*. Hum Immunol. 73:93-100, **2012**.

207. Odell D, Maciulis A, Cutler A, Warren L, McMahon WM, Coon H, Stubbs G, Henley K, Torres A. *Confirmation of the Association of the C4B Null Allelle in Autism*. Hum Immunol. 66:140-145, **2005**.

208. Mostafa GA, Shehab AA. *The Link of C4B Null Allele to Autism and to a Family History of Autoimmunity in Egyptian Autistic Children*. J Neuroimmunol. 223:115-119, **2010**.

209. Liu YH, Wan L, Chang CT, Liao WL, Chen WC, Tsai Y, Tsai CH, Tsai FJ. *Association between Copy Number Variation of Complement Component C4 and Graves' Disease*. J Biomed Sci. 18:71, **2011**.

210. Rigby WF, Wu YL, Zan M, Zhou B, Rosengren S, Carlson C, Hilton W, Yu CY. *Increased Frequency of Complement C4B Deficiency in Rheumatoid Arthritis*. Arthritis Rheum. 64:1338-1344, **2012**.

211. Stefansson TV, Kolka R, Sigurdardottir SL, Edvardsson VO, Arason G, Haraldsson A. *Increased Frequency of C4B*Q0 Alleles in Patients with Henoch-Schönlein Purpura*. Scand J Immunol. 61:274-278, **2005**.

212. Zafar GI, Grimm EA, Wei W, Johnson MM, Ellerhorst JA. *Genetic Deficiency of Complement Isoforms C4A or C4B Predicts Improved Survival of Metastatic Renal Cell Carcinoma*. J Urol. 181:1028-1034; discussion 1034, **2009**.

213. Zhang S, Wang S, Li Q, Yao S, Zeng B, Ziegelstein RC, Hu Q. *Capillary Leak Syndrome in Children with C4A-Deficiency Undergoing Cardiac Surgery with Cardiopulmonary Bypass: A Double-Blind, Randomised Controlled Study*. Lancet. 366:556-562, **2005**.

214. Kramer J, Rajczy K, Füst G. *Low Incidence of Null Alleles of the Fourth Component of Complement (C4) in Elderly People*. Immunol Lett. 20:83-85, **1989**.

215. Nityanand S, Hamsten A, Lithell H, Holm G, Lefvert AK. *C4 Null Alleles and Myocardial Infarction*. Atherosclerosis. 143:377-381, **1999**.

216. Kramer J, Fulop T, Rajczy K, Nguyen AT, Füst G. *A Marked Drop in the Incidence of the Null Allele of the B Gene of the Fourth Component of Complement (C4B*Q0) in Elderly Subjects: C4B*Q0 as a Probable Negative Selection Factor for Survival*. Hum Genet. 86:595-598, **1991**.

217. Lefvert AK, Hamsten A, Holm G. *Association between Circulating Immune Complexes, Complement C4 Null Alleles, and Myocardial Infarction before Age 45 Years*. Arterioscler Thromb Vasc Biol. 15:665-668, **1995**.

218. Nityanand S, Truedsson L, Mustafa A, Bergmark C, Lefvert AK. *Circulating Immune Complexes and Complement C4 Null Alleles in Patients in Patients Operated on for Premature Atherosclerotic Peripheral Vascular Disease*. J Clin Immunol. 19:406-413, **1999**.

219. Best LG, Davidson M, North KE, MacCluer JW, Zhang Y, Lee ET, Howard BV, DeCroo S, Ferrell RE. *Prospective Analysis of Mannose-Binding Lectin Genotypes and Coronary Artery Disease in American Indians: The Strong Heart Study*. Circulation. 109:471-475, **2004**.

220. Bhatia VK, Yun S, Leung V, Grimsditch DC, Benson GM, Botto MB, Boyle JJ, Haskard DO. *Complement C1q Reduces Early Atherosclerosis in Low-Density Lipoprotein Receptor-Deficient Mice*. Am J Pathol. 170:416-426, **2007**.

221. Demirturk M, Polat N, Guz G, Gurdal A, Altun I, Gelincik A, Toz B, Oflaz H, Colakoglu B, Dal M, Buyukozturk S. *There Is an Increased Risk of Atherosclerosis in Hereditary Angioedema*. Int Immunopharmacol. 12:212-216, **2012**.

222. Troelsen LN, Garred P, Christiansen B, Torp-Pedersen C, Christensen IJ, Narvestad E, Jacobsen S. *Double Role of Mannose-Binding Lectin in Relation to Carotid Intima-Media Thickness in Patients with Rheumatoid Arthritis*. Mol Immunol. 47:713-718, **2010**.

223. Engstrom G, Hedblad B, Janzon L, Lindgarde F. *Complement C3 and C4 in Plasma and Incidence of Myocardial Infarction and Stroke: A Population-Based Cohort Study*. Eur J Cardiovasc Prev Rehabil. 14:392-397, **2007**.

224. Palikhe A, Sinisalo J, Seppänen M, Haario H, Meri S, Valtonen V, Nieminen MS, Lokki ML. *Serum Complement C3/C4 Ratio, a Novel Marker for Recurrent Cardiovascular Events*. Am J Cardiol. 99:890-895, **2007**.

225. Oksjoki R, Kovanen PT, Mäyränpää MI, Laine P, Blom AM, Meri S, Pentikäinen MO. *Complement Regulation in Human Atherosclerotic Coronary Lesions. Immunohistochemical Evidence That C4B-Binding Protein Negatively Regulates the Classical Complement Pathway, and That C5B-9 Is Formed Via the Alternative Complement Pathway*. Atherosclerosis. 192:40-48, **2007**.

226. Trendelenburg M, Theroux P, Stebbins A, Granger C, Armstrong P, Pfisterer M. *Influence of Functional Deficiency of Complement Mannose-Binding Lectin on Outcome of Patients with Acute ST-Elevation Myocardial Infarction Undergoing Primary Percutaneous Coronary Intervention*. Eur Heart J. 31:1181-1187, **2010**.

227. Testa L, Van Gaal WJ, Bhindi R, Biondi-Zoccai GG, Abbate A, Agostoni P, Porto I, Andreotti F, Crea F, Banning AP. *Pexelizumab in Ischemic Heart Disease: A Systematic Review and Meta-Analysis on 15,196 Patients*. J Thorac Cardiovasc Surg. 136:884-893, **2008**.

228. Martel C, Granger CB, Ghitescu M, Stebbins A, Fortier A, Armstrong PW, Bonnefoy A, Theroux P. *Pexelizumab Fails to Inhibit Assembly of the Terminal Complement Complex in Patients with ST-Elevation Myocardial Infarction Undergoing Primary Percutaneous Coronary Intervention. Insight from a Substudy of the Assessment of Pexelizumab in Acute Myocardial Infarction (APEX-AMI) Trial*. Am Heart J. 164:43-51, **2012**.

229. Lewis RD, Perry MJ, Guschina IA, Jackson CL, Morgan BP, Hughes TR. *CD55 Deficiency Protects against Atherosclerosis in ApoE-Deficient Mice Via C3a Modulation of Lipid Metabolism*. Am J Pathol. 179:1601-1607, **2011**.

230. Bovenberg SA, Alipour A, Elte JW, Rietveld AP, Janssen JW, van de Geijn GJ, Njo TN, van Mechelen R, Hervas SM, Cabezas MC. *Cell-Mediated Lipoprotein Transport: A Novel Anti-Atherogenic Concept*. Atheroscler Suppl. 11:25-29, **2010**.

231. The HLA Complex in Biology and Medicine. A Resource Book. Mehra NK, Kaur G, McCluskey J, Christiansen FT, CLaas FH. *JB Medical Publishers Ltd, New Delhi, India.* **2010.** 175-188.

232. Sim E, Cross SJ. *Phenotyping of Human Complement Component C4, a Class-III HLA Antigen*. Biochem J. 239:763-767, **1986**.

233. Chung EK, Yang Y, Rupert KL, Jones KN, Rennebohm RM, Blanchong CA, Yu CY. *Determining the One, Two, Three, or Four Long and Short Loci of Human Complement C4 in a Major Histocompatibility Complex Haplotype Encoding C4A or C4B Proteins*. Am J Hum Gene. 71:810-822, **2002**.

234. Wu YL, Savelli SL, Yang Y, Zhou B, Rovin BH, Birmingham DJ, Nagaraja HN, Hebert LA, Yu CY. *Sensitive and Specific Real-Time Polymerase Chain Reaction Assays to Accurately Determine Copy Number Variations (CNVs) of Human Complement C4A, C4B, C4-Long, C4-Short, and RCCX Modules: Elucidation of C4 CNVs in 50 Consanguineous Subjects with Defined HLA Genotypes*. J Immunol. 179:3012-3025, **2007**.

235. Grant SF, Kristjansdottir H, Steinsson K, Blondal T, Yuryev A, Stefansson K, Gulcher JR. *Long PCR Detection of the C4A Null Allele in B8-C4AQ0-C4B1-DR3*. J Immunol Methods. 244:41-47, **2000**.

236. Barba GM, Braun-Heimer L, Rittner C, Schneider PM. *A New PCR-Based Typing of the Rodgers and Chido Antigenic Determinants of the Fourth Component of Human Complement*. Eur J Immunogen. 21:325-339, **1994**.

237. Ittiprasert W, Kantachuvesiri S, Pavasuthipaisit K, Verasertniyom O, Chaomthum L, Totemchokchyakarn K, Kitiyanant Y. *Complete Deficiencies of Complement C4A and C4B Including 2-Bp Insertion in Codon 1213 Are Genetic Risk Factors of Systemic Lupus Erythematosus in Thai Populations*. J Autoimmun. 25:77-84, **2005**.

238. Lindstedt KA, Kovanen PT. *Proteolysis of Pericellular Matrix: A Process Linking Inflammation to Plaque Destabilization and Rupture*. Arterioscler Thromb Vasc Biol. 24:2205-2206, **2004**.

239. Armani A, Becker RC. *The Biology, Utilization, and Attenuation of C-Reactive Protein in Cardiovascular Disease: Part I*. Am Heart J. 149:971-976, **2005**.

240. Cristell N, Cianflone D, Durante A, Ammirati E, Vanuzzo D, Banfi M, Calori G, Latib A, Crea F, Marenzi G, De Metrio M, Moretti L, Li H, Uren NG, Hu D, Maseri A. *High-Sensitivity C-Reactive Protein Is within Normal Levels at the Very Onset of First ST-Segment Elevation Acute Myocardial Infarction in 41% of Cases: A Multiethnic Case-Control Study*. J Am Coll Cardiol. 58:2654-2661, **2011**.

241. Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, Lowe GD, Pepys MB, Gudnason V. *C-Reactive Protein and Other Circulating Markers of Inflammation in the Prediction of Coronary Heart Disease*. N Engl J Med. 350:1387-1397, **2004**.

242. Bisoendial RJ, Boekholdt SM, Vergeer M, Stroes ES, Kastelein JJ. *C-Reactive Protein Is a Mediator of Cardiovascular Disease*. Eur Heart J. 31:2087-2091, **2010**.

243. Anand SS, Yusuf S. *C-Reactive Protein Is a Bystander of Cardiovascular Disease*. Eur Heart J. 31:2092-2096, **2010**.

244. Kaptoge S, Di Angelantonio E, Lowe G, Pepys MB, Thompson SG, Collins R, Danesh J. *C-Reactive Protein Concentration and Risk of Coronary Heart Disease, Stroke, and Mortality: An Individual Participant Meta-Analysis*. Lancet. 375:132-140, **2010**.

245. Wensley F, Gao P, Burgess S, Kaptoge S, Di Angelantonio E, Shah T, Engert JC, Clarke R, Davey-Smith G, Nordestgaard BG, Saleheen D, Samani NJ, Sandhu M, Anand S, Pepys MB, Smeeth L, Whittaker J, Casas JP, Thompson SG, Hingorani AD, Danesh J. *Association between C Reactive Protein and Coronary Heart Disease: Mendelian Randomisation Analysis Based on Individual Participant Data*. BMJ. 342:d548, **2011**.

246. Chen GY, Nunez G. *Sterile Inflammation: Sensing and Reacting to Damage*. Nat Rev Immunol. 10:826-837, **2010**.

247. Lahoute C, Herbin O, Mallat Z, Tedgui A. *Adaptive Immunity in Atherosclerosis: Mechanisms and Future Therapeutic Targets*. Nat Rev Cardiol. 8:348-358, **2011**.

248. Camici PG, Rimoldi OE, Gaemperli O, Libby P. *Non-Invasive Anatomic and Functional Imaging of Vascular Inflammation and Unstable Plaque*. Eur Heart J. 33:1309-1317, **2012**.

249. Gilbert AL, G. *Arterites Infectieuses Experimentales*. CR HEBD Seances.583-584, **1889**.

250. Fabricant CG, Fabricant J, Litrenta MM, Minick CR. *Virus-Induced Atherosclerosis*. J Exp Med. 148:335-340, **1978**.

251. Saikku P, Leinonen M, Mattila K, Ekman MR, Nieminen MS, Mäkelä PH, Huttunen JK, Valtonen V. *Serological Evidence of an Association of a Novel Chlamydia, TWAR, with Chronic Coronary Heart Disease and Acute Myocardial Infarction*. Lancet. 2:983-986, **1988**.

252. Mattila KJ, Nieminen MS, Valtonen VV, Rasi VP, Kesaniemi YA, Syrjala SL, Jungell PS, Isoluoma M, Hietaniemi K, Jokinen MJ. *Association between Dental Health and Acute Myocardial Infarction*. BMJ. 298:779-781, **1989**.

253. Smeeth L, Thomas SL, Hall AJ, Hubbard R, Farrington P, Vallance P. *Risk of Myocardial Infarction and Stroke after Acute Infection or Vaccination*. N Engl J Med. 351:2611-2618, **2004**.

254. Lockhart PB, Bolger AF, Papapanou PN, Osinbowale O, Trevisan M, Levison ME, Taubert KA, Newburger JW, Gornik HL, Gewitz MH, Wilson WR, Smith SC, Jr., Baddour LM. *Periodontal Disease and Atherosclerotic Vascular Disease: Does the Evidence Support an Independent Association?: A Scientific Statement from the American Heart Association*. Circulation. 125:2520-2544, **2012**.

255. Cox D, Kerrigan SW, Watson SP. *Platelets and the Innate Immune System: Mechanisms of Bacterial-Induced Platelet Activation*. J Thromb Haemost. 9:1097-1107, **2011**.

256. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, Feldstein AE, Britt EB, Fu X, Chung YM, Wu Y, Schauer P, Smith JD, Allayee H, Tang WH, DiDonato JA, Lusis AJ, Hazen SL. *Gut Flora Metabolism of Phosphatidylcholine Promotes Cardiovascular Disease*. Nature. 472:57-63, **2011**.

257. Niessner A, Sato K, Chaikof EL, Colmegna I, Goronzy JJ, Weyand CM. *Pathogen-Sensing Plasmacytoid Dendritic Cells Stimulate Cytotoxic T-Cell Function in the Atherosclerotic Plaque through Interferon-Alpha*. Circulation. 114:2482-2489, **2006**.

258. Kloft N, Neukirch C, Bobkiewicz W, Veerachato G, Busch T, von Hoven G, Boller K, Husmann M. *Pro-Autophagic Signal Induction by Bacterial Pore-Forming Toxins*. Med Microbiol Immunol. 199:299-309, **2010**.

259. Binder CJ, Horkko S, Dewan A, Chang MK, Kieu EP, Goodyear CS, Shaw PX, Palinski W, Witztum JL, Silverman GJ. *Pneumococcal Vaccination Decreases Atherosclerotic Lesion Formation: Molecular Mimicry between Streptococcus Pneumoniae and Oxidized LDL*. Nat Med. 9:736-743, **2003**.

260. Johnstone J, Loeb M, Teo KK, Gao P, Dyal L, Liu L, Avezum A, Cardona-Munoz E, Sleight P, Fagard R, Yusuf S. *Influenza Vaccination and Major Adverse Vascular Events in High-Risk Patients*. Circulation. 126:278-286, **2012**.

261. Deniset JF, Pierce GN. *Possibilities for Therapeutic Interventions in Disrupting Chlamydophila Pneumoniae Involvement in Atherosclerosis*. Fundam Clin Pharmacol. 24:607-617, **2010**.

262. Gupta S, Leatham EW, Carrington D, Mendall MA, Kaski JC, Camm AJ. *Elevated Chlamydia Pneumoniae Antibodies, Cardiovascular Events, and Azithromycin in Male Survivors of Myocardial Infarction*. Circulation. 96:404-407, **1997**.

263. Jespersen CM, Als-Nielsen B, Damgaard M, Hansen JF, Hansen S, Helo OH, Hildebrandt P, Hilden J, Jensen GB, Kastrup J, Kolmos HJ, Kjoller E, Lind I, Nielsen H, Petersen L, Gluud C. *Randomised Placebo Controlled Multicentre Trial to Assess Short Term Clarithromycin for Patients with Stable Coronary Heart Disease: CLARICOR Trial*. BMJ. 332:22-27, **2006**.

264. Muhlestein JB, Anderson JL, Carlquist JF, Salunkhe K, Horne BD, Pearson RR, Bunch TJ, Allen A, Trehan S, Nielson C. *Randomized Secondary Prevention Trial of Azithromycin in Patients with Coronary Artery Disease : Primary Clinical Results of the Academic Study*. Circulation. 102:1755-1760, **2000**.

265. O'Connor CM, Dunne MW, Pfeffer MA, Muhlestein JB, Yao L, Gupta S, Benner RJ, Fisher MR, Cook TD, Investigators in the WS. *Azithromycin for the Secondary Prevention of Coronary Heart Disease Events: The WIZARD Study: A Randomized Controlled Trial*. JAMA. 290:1459-1466, **2003**.

266. Grayston JT, Kronmal RA, Jackson LA, Parisi AF, Muhlestein JB, Cohen JD, Rogers WJ, Crouse JR, Borrowdale SL, Schron E, Knirsch C, Investigators A. *Azithromycin for the Secondary Prevention of Coronary Events*. N Engl J Med. 352:1637-1645, **2005**.

267. Stone AF, Mendall MA, Kaski JC, Edger TM, Poloniecki J, Camm AJ, Northfield TC. *Effect of Treatment for Chlamydia Pneumoniae and Helicobacter Pylori on Markers of Inflammation and Cardiac Events in Patients with Acute Coronary Syndromes: South Thames Trial of Antibiotics in Myocardial Infarction and Unstable Angina (STAMINA)*. Circulation. 106:1219-1223, **2002**.

268. Gurfinkel E, Bozovich G, Beck E, Testa E, Livellara B, Mautner B. *Treatment with the Antibiotic Roxithromycin in Patients with Acute Non-Q-Wave Coronary Syndromes. The Final Report of the ROXIS Study*. Eur Heart J. 20:121-127, **1999**.

269. Zahn R, Schneider S, Frilling B, Seidl K, Tebbe U, Weber M, Gottwik M, Altmann E, Seidel F, Rox J, Hoffler U, Neuhaus KL, Senges J. *Antibiotic Therapy after Acute Myocardial Infarction: A Prospective Randomized Study*. Circulation. 107:1253-1259, **2003**.

270. Cannon CP, Braunwald E, McCabe CH, Grayston JT, Muhlestein B, Giugliano RP, Cairns R, Skene AM, Pravastatin or Atorvastatin E, Infection Therapy-Thrombolysis in Myocardial Infarction Investigators. *Antibiotic Treatment of Chlamydia Pneumoniae after Acute Coronary Syndrome*. N Engl J Med. 352:1646-1654, **2005**.

271. Sinisalo J, Mattila K, Valtonen V, Anttonen O, Juvonen J, Melin J, Vuorinen-Markkola H, Nieminen MS, Clarithromycin in Acute Coronary Syndrome Patients in Finland Study Group. *Effect of 3 Months of Antimicrobial Treatment with Clarithromycin in Acute Non-Q-Wave Coronary Syndrome*. Circulation. 105:1555-1560, **2002**.

272. Leowattana W, Bhuripanyo K, Singhaviranon L, Akaniroj S, Mahanonda N, Samranthin M, Pokum S. *Roxithromycin in Prevention of Acute Coronary Syndrome Associated with Chlamydia Pneumoniae Infection: A Randomized Placebo Controlled Trial*. J Med Assoc Thai. 84 Suppl 3:S669-675, **2001**.

273. Neumann F, Kastrati A, Miethke T, Pogatsa-Murray G, Mehilli J, Valina C, Jogethaei N, da Costa CP, Wagner H, Schomig A. *Treatment of Chlamydia Pneumoniae Infection with Roxithromycin and Effect on Neointima Proliferation after Coronary Stent Placement (ISAR-3): A Randomised, Double-Blind, Placebo-Controlled Trial*. Lancet. 357:2085-2089, **2001**.

274. Andraws R, Berger JS, Brown DL. *Effects of Antibiotic Therapy on Outcomes of Patients with Coronary Artery Disease: A Meta-Analysis of Randomized Controlled Trials*. JAMA. 293:2641-2647, **2005**.

275. Gluud C, Als-Nielsen B, Damgaard M, Fischer Hansen J, Hansen S, Helo OH, Hildebrandt P, Hilden J, Jensen GB, Kastrup J, Kolmos HJ, Kjoller E, Lind I, Nielsen H, Petersen L, Jespersen CM. *Clarithromycin for 2 Weeks for Stable Coronary Heart Disease: 6-Year Follow-up of the Claricor Randomized Trial and Updated Meta-Analysis of Antibiotics for Coronary Heart Disease*. Cardiology. 111:280-287, **2008**.

276. Song Z, Brassard P, Brophy JM. *A Meta-Analysis of Antibiotic Use for the Secondary Prevention of Cardiovascular Diseases*. Can J Cardiol. 24:391-395, **2008**.

277. Altenburg J, de Graaff CS, van der Werf TS, Boersma WG. *Immunomodulatory Effects of Macrolide Antibiotics - Part 1: Biological Mechanisms*. Respiration. 81:67-74, **2011**.

278. Kudoh S, Uetake T, Hagiwara K, Hirayama M, Hus LH, Kimura H, Sugiyama Y. *[Clinical Effects of Low-Dose Long-Term Erythromycin Chemotherapy on Diffuse Panbronchiolitis]*. Nihon Kyobu Shikkan Gakkai Zasshi. 25:632-642, **1987**.

279. Altenburg J, de Graaff CS, van der Werf TS, Boersma WG. *Immunomodulatory Effects of Macrolide Antibiotics - Part 2: Advantages and Disadvantages of Long-Term, Low-Dose Macrolide Therapy*. Respiration. 81:75-87, **2011**.

280. Tateda K, Ishii Y, Kimura S, Horikawa M, Miyairi S, Yamaguchi K. *Suppression of Pseudomonas Aeruginosa Quorum-Sensing Systems by Macrolides: A Promising Strategy or an Oriental Mystery?* J Infect Chemother. 13:357-367, **2007**.

281. Strandell J, Bate A, Hagg S, Edwards IR. *Rhabdomyolysis a Result of Azithromycin and Statins: An Unrecognized Interaction*. Br J Clin Pharmacol. 68:427-434, **2009**.

282. Ray WA, Murray KT, Meredith S, Narasimhulu SS, Hall K, Stein CM. *Oral Erythromycin and the Risk of Sudden Death from Cardiac Causes*. N Engl J Med. 351:1089-1096, **2004**.

283. Gluud C, Als-Nielsen B, Damgaard M, Fischer Hansen J, Hansen S, Helo OH, Hildebrandt P, Hilden J, Jensen GB, Kastrup J, Kolmos HJ, Kjoller E, Lind I, Nielsen H, Petersen L, Jespersen CM, Group CT. *Clarithromycin for 2 Weeks for Stable Coronary Heart Disease: 6-Year Follow-up of the CLARICOR Randomized Trial and Updated Meta-Analysis of Antibiotics for Coronary Heart Disease*. Cardiology. 111:280-287, **2008**.

284. Ray WA, Murray KT, Hall K, Arbogast PG, Stein CM. *Azithromycin and the Risk of Cardiovascular Death*. N Engl J Med. 366:1881-1890, **2012**.

285. Ohshima S, Fujimoto S, Petrov A, Nakagami H, Haider N, Zhou J, Tahara N, Osako MK, Fujimoto A, Zhu J, Murohara T, Edwards DS, Narula N, Wong ND, Chandrashekhar Y, Morishita R, Narula J. *Effect of an Antimicrobial Agent on Atherosclerotic Plaques: Assessment of Metalloproteinase Activity by Molecular Imaging*. J Am Coll Cardiol. 55:1240-1249, **2010**.

286. Daha NA, Banda NK, Roos A, Beurskens FJ, Bakker JM, Daha MR, Trouw LA. *Complement Activation by (Auto-) Antibodies*. Mol Immunol. 48:1656-1665, **2011**.

287. Symmons DP, Gabriel SE. *Epidemiology of CVD in Rheumatic Disease, with a Focus on RA and SLE*. Nat Rev Rheumatol. 7:399-408, **2011**.

288. Cappello F, Conway de Macario E, Di Felice V, Zummo G, Macario AJ. *Chlamydia Trachomatis Infection and Anti-HSP60 Immunity: The Two Sides of the Coin*. PLoS pathogens. 5:e1000552, **2009**.

289. Quintana FJ, Cohen IR. *The HSP60 Immune System Network*. Trends Immunol. 32:89-95, **2011**.

290. Andrie RP, Bauriedel G, Braun P, Hopp HW, Nickenig G, Skowasch D. *Prevalence of Intimal Heat Shock Protein 60 Homologues in Unstable Angina and Correlation with Anti-Heat Shock Protein Antibody Titers*. Basic Res Cardiol. 106:657-665, **2011**.

291. Benagiano M, D'Elios MM, Amedei A, Azzurri A, van der Zee R, Ciervo A, Rombola G, Romagnani S, Cassone A, Del Prete G. *Human 60-kDa Heat Shock Protein Is a Target Autoantigen of T Cells Derived from Atherosclerotic Plaques*. J Immunol. 174:6509-6517, **2005**.

292. Schett G, Xu Q, Amberger A, Van der Zee R, Recheis H, Willeit J, Wick G. *Autoantibodies against Heat Shock Protein 60 Mediate Endothelial Cytotoxicity*. J Clin Invest. 96:2569-2577, **1995**.

293. Zhang X, He MA, Cheng L, Zhou L, Zeng H, Wang J, Wang F, Chen Y, Hu FB, Wu T. *Joint Effects of Antibody to Heat Shock Protein 60, Hypertension, and Diabetes on Risk of Coronary Heart Disease in Chinese*. Clin Chem. 54:1046-1052, **2008**.

294. Mandal K, Afzal AR, Brecker SJ, Poloniecki J, Xu Q, Jahangiri M. *Association of Serum Soluble Heat Shock Protein 60 with Toll-Like Receptor 4 Polymorphism and Severity of Coronary Artery Disease*. Heart. 92:683-685, **2006**.

295. Grundtman C, Wick G. *The Autoimmune Concept of Atherosclerosis*. Curr Opin Lipidol. 22:327-334, **2011**.

296. Shamaei-Tousi A, Stephens JW, Bin R, Cooper JA, Steptoe A, Coates AR, Henderson B, Humphries SE. *Association between Plasma Levels of Heat Shock Protein 60 and Cardiovascular Disease in Patients with Diabetes Mellitus*. Eur Heart J. 27:1565-1570, **2006**.

297. Xiao Q, Mandal K, Schett G, Mayr M, Wick G, Oberhollenzer F, Willeit J, Kiechl S, Xu Q. *Association of Serum-Soluble Heat Shock Protein 60 with Carotid Atherosclerosis*. Stroke. 36:2571-2576, **2005**.

298. Pockley AG, Bulmer J, Hanks BM, Wright BH. *Identification of Human Heat Shock Protein 60 (HSP60) and Anti-HSP60 Antibodies in the Peripheral Circulation of Normal Individuals*. Cell Stress Chaperones. 4:29-35, **1999**.

299. Alard JE, Dueymes M, Youinou P, Jamin C. *HSP60 and Anti-HSP60 Antibodies in Vasculitis: They Are Two of a Kind*. Clin Rev Allergy Immunol. 35:66-71, **2008**.

300. Alard JE, Dueymes M, Youinou P, Jamin C. *Modulation of Endothelial Cell Damages by Anti-HSP60 Autoantibodies in Systemic Autoimmune Diseases*. Autoimmun Rev. 6:438-443, **2007**.

301. Burian K, Kis Z, Virok D, Endresz V, Prohaszka Z, Duba J, Berencsi K, Boda K, Horvath L, Romics L, Fust G, Gonczol E. *Independent and Joint Effects of Antibodies to Human Heat-Shock Protein 60 and Chlamydia Pneumoniae Infection in the Development of Coronary Atherosclerosis*. Circulation. 103:1503-1508, **2001**.

302. Hoshida S, Nishino M, Tanouchi J, Kishimoto T, Yamada Y. *Acute Chlamydia Pneumoniae Infection with Heat-Shock-Protein-60-Related Response in Patients with Acute Coronary Syndrome*. Atherosclerosis. 183:109-112, **2005**.

303. Okada T, Ayada K, Usui S, Yokota K, Cui J, Kawahara Y, Inaba T, Hirohata S, Mizuno M, Yamamoto D, Kusachi S, Matsuura E, Oguma K. *Antibodies against Heat Shock Protein 60 Derived from Helicobacter Pylori: Diagnostic Implications in Cardiovascular Disease*. J Autoimmun. 29:106-115, **2007**.

304. Huittinen T, Leinonen M, Tenkanen L, Manttari M, Virkkunen H, Pitkanen T, Wahlstrom E, Palosuo T, Manninen V, Saikku P. *Autoimmunity to Human Heat Shock Protein 60, Chlamydia Pneumoniae Infection, and Inflammation in Predicting Coronary Risk*. Arteriosclerosis, Thrombosis & Vascular Biology. 22:431-437, **2002**.

305. Veres A, Fust G, Smieja M, McQueen M, Horvath A, Yi Q, Biro A, Pogue J, Romics L, Karadi I, Singh M, Gnarpe J, Prohaszka Z, Yusuf S, Heart Outcomes Prevention Evaluation (HOPE) Study Investigators. *Relationship of Anti-60 kDa Heat Shock Protein and Anti-Cholesterol Antibodies to Cardiovascular Events*. Circulation. 106:2775-2780, **2002**.

306. Foteinos G, Afzal AR, Mandal K, Jahangiri M, Xu Q. *Anti-Heat Shock Protein 60 Autoantibodies Induce Atherosclerosis in Apolipoprotein E-Deficient Mice Via Endothelial Damage*. Circulation. 112:1206-1213, **2005**.

307. Bason C, Corrocher R, Lunardi C, Puccetti P, Olivieri O, Girelli D, Navone R, Beri R, Millo E, Margonato A, Martinelli N, Puccetti A. *Interaction of Antibodies against Cytomegalovirus with Heat-Shock Protein 60 in Pathogenesis of Atherosclerosis*. Lancet. 362:1971-1977, **2003**.

308. Dieude M, Gillis MA, Theoret JF, Thorin E, Lajoie G, Levine JS, Merhi Y, Rauch J. *Autoantibodies to Heat Shock Protein 60 Promote Thrombus Formation in a Murine Model of Arterial Thrombosis*. J Thromb Haemost. 7:710-719, **2009**.

309. Yokota S, Minota S, Fujii N. *Anti-HSP Auto-Antibodies Enhance HSP-Induced Pro-Inflammatory Cytokine Production in Human Monocytic Cells Via Toll-Like Receptors*. Int Immunol. 18:573-580, **2006**.

310. Maron R, Sukhova G, Faria A-M, Hoffmann E, Mach F, Libby P, Weiner HL. *Mucosal Administration of Heat Shock Protein-65 Decreases Atherosclerosis and Inflammation in Aortic Arch of Low-Density Lipoprotein Receptor-Deficient Mice*. Circulation. 106:1708-1715, **2002**.

311. Jing H, Yong L, Haiyan L, Yanjun M, Yun X, Yu Z, Taiming L, Rongyue C, Liang J, Jie W, Li Z, Jingjing L. *Oral Administration of Lactococcus Lactis Delivered Heat Shock Protein 65 Attenuates Atherosclerosis in Low-Density Lipoprotein Receptor-Deficient Mice*. Vaccine. 29:4102-4109, **2011**.

312. Alfakry H, Paju S, Sinisalo J, Nieminen MS, Valtonen V, Saikku P, Leinonen M, Pussinen PJ. *Periodontopathogen- and Host-Derived Immune Response in Acute Coronary Syndrome*. Scand J Immunol. 74:383-389, **2011**.

313. Zhu J, Quyyumi AA, Wu H, Csako G, Rott D, Zalles-Ganley A, Ogunmakinwa J, Halcox J, Epstein SE. *Increased Serum Levels of Heat Shock Protein 70 Are Associated with Low Risk of Coronary Artery Disease*. Arterioscler Thromb Vasc Biol. 23:1055-1059, **2003**.

314. Zhang X, Xu Z, Zhou L, Chen Y, He M, Cheng L, Hu FB, Tanguay RM, Wu T. *Plasma Levels of HSP70 and Anti-HSP70 Antibody Predict Risk of Acute Coronary Syndrome*. Cell Stress Chaperones. 15:675-686, **2010**.

315. Dybdahl B, Slørdahl SA, Waage A, Kierulf P, Espevik T, Sundan A. *Myocardial Ischaemia and the Inflammatory Response: Release of Heat Shock Protein 70 after Myocardial Infarction*. Heart. 91:299-304, **2005**.

316. Kardys I, Rifai N, Meilhac O, Michel J-B, Martin-Ventura JL, Buring JE, Libby P, Ridker PM. *Plasma Concentration of Heat Shock Protein 27 and Risk of Cardiovascular Disease: A Prospective, Nested Case-Control Study*. Clin Chem. 54:139-146, **2008**.

317. Businaro R, Profumo E, Tagliani A, Buttari B, Leone S, D'Amati G, Ippoliti F, Leopizzi M, D'Arcangelo D, Capoano R, Fumagalli L, Salvati B, Riganò R. *Heat-Shock Protein 90: A Novel Autoantigen in Human Carotid Atherosclerosis*. Atherosclerosis. 207:74-83, **2009**.

318. Tierala I, Nikus KC, Sclarovsky S, Syvänne M, Eskola M, the HAAMU Study Group. *Predicting the Culprit Artery in Acute ST-Elevation Myocardial Infarction and Introducing a New Algorithm to Predict Infarct-Related Artery in Inferior ST-Elevation Myocardial Infarction: Correlation with Coronary Anatomy in the HAAMU Trial*. J Electrocardiol. 42:120-127, **2009**.

319. Vaara S, Nieminen MS, Lokki ML, Perola M, Pussinen PJ, Allonen J, Parkkonen O, Sinisalo J. *Cohort Profile: The COROGENE Study*. Int J Epidemiol. **2011**.

320. Miller SA, Dykes DD, Polesky HF. *A Simple Salting out Procedure for Extracting DNA from Human Nucleated Cells*. Nucleic Acids Res. 16:1215, **1988**.

321. Friedewald WT, Levy RI, Fredrickson DS. *Estimation of the Concentration of Low-Density Lipoprotein Cholesterol in Plasma, without Use of the Preparative Ultracentrifuge*. Clin Chem. 18:499-502, **1972**.

322. Huittinen T, Leinonen M, Tenkanen L, Virkkunen H, Mänttari M, Palosuo T, Manninen V, Saikku P. *Synergistic Effect of Persistent Chlamydia Pneumoniae Infection, Autoimmunity, and Inflammation on Coronary Risk*. Circulation. 107:2566-2570, **2003**.

323. Stephens M, Smith NJ, Donnelly P. *A New Statistical Method for Haplotype Reconstruction from Population Data*. Am J Hum Gene. 68:978-989, **2001**.

324. Excoffier L, Laval G, Schneider S. *Arlequin (Version 3.0): An Integrated Software Package for Population Genetics Data Analysis*. Evol Bioinform Online. 1:47-50, **2005**.

325. Lokki ML, Circolo A, Ahokas P, Rupert KL, Yu CY, Colten HR. *Deficiency of Human Complement Protein C4 Due to Identical Frameshift Mutations in the C4A and C4B Genes*. J Immunol. 162:3687-3693, **1999**.

326. Redon R, Ishikawa S, Fitch KR, Feuk L, Perry GH, Andrews TD, Fiegler H, Shapero MH, Carson AR, Chen W, Cho EK, Dallaire S, Freeman JL, Gonzalez JR, Gratacos M, Huang J, Kalaitzopoulos D, Komura D, MacDonald JR, Marshall CR, Mei R, Montgomery L, Nishimura K, Okamura K, Shen F, Somerville MJ, Tchinda J, Valsesia A, Woodwark C, Yang F, Zhang J, Zerjal T, Zhang J, Armengol L, Conrad DF, Estivill X, Tyler-Smith C, Carter NP, Aburatani H, Lee C, Jones KW, Scherer SW, Hurles ME. *Global Variation in Copy Number in the Human Genome*. Nature. 444:444-454, **2006**.

327. Girirajan S, Campbell CD, Eichler EE. *Human Copy Number Variation and Complex Genetic Disease*. Annu Rev Genet. 45:203-226, **2011**.

328. Gouas L, Goumy C, Veronese L, Tchirkov A, Vago P. *Gene Dosage Methods as Diagnostic Tools for the Identification of Chromosome Abnormalities*. Pathol Biol (Paris). 56:345-353, **2008**.

329. Weksberg R, Hughes S, Moldovan L, Bassett AS, Chow EW, Squire JA. *A Method for Accurate Detection of Genomic Microdeletions Using Real-Time Quantitative PCR*. BMC Genomics. 6:180, **2005**.

330. D'Haene B, Vandesompele J, Hellemans J. *Accurate and Objective Copy Number Profiling Using Real-Time Quantitative PCR*. Methods. 50:262-270, **2010**.

331. Man XY, Luo HR, Li XP, Yao YG, Mao CZ, Zhang YP. *Polymerase Chain Reaction Based C4AQ0 and C4BQ0 Genotyping: Association with Systemic Lupus Erythematosus in Southwest Han Chinese*. Ann Rheum Dis. 62:71-73, **2003**.

332. Sullivan KE, Kim NA, Goldman D, Petri MA. *C4A Deficiency Due to a 2 Bp Insertion Is Increased Patients with Systemic Lupus Erythematosus*. J Rheumatol. 26:2144-2147, **1999**.

333. Williams P, Pendyala L, Superko R. *Survival Bias and Drug Interaction Can Attenuate Cross-Sectional Case-Control Comparisons of Genes with Health Outcomes. An Example of the Kinesin-Like Protein 6 (KIF6) Trp719arg Polymorphism and Coronary Heart Disease*. BMC Med Gen. 12:42, **2011**.

334. Trouw LA, Okroj M, Kupreishvili K, Landberg G, Johansson B, Niessen HW, Blom AM. *C4B-Binding Protein Is Present in Affected Areas of Myocardial Infarction During the Acute Inflammatory Phase and Covers a Larger Area Than C3*. PLoS One. 3:e2886, **2008**.

335. Alfonso F, Virmani R. *New Morphological Insights on Coronary Plaque Rupture: Bridging the Gap from Anatomy to Clinical Presentation?* JACC Cardiovasc Interv. 4:83-86, **2011**.

336. Call MJ. *Small Molecule Modulators of MHC Class Ii Antigen Presentation: Mechanistic Insights and Implications for Therapeutic Application*. Mol Immunol. 48:1735-1743, **2011**.

337. Greenbaum J, Sidney J, Chung J, Brander C, Peters B, Sette A. *Functional Classification of Class II Human Leukocyte Antigen (HLA) Molecules Reveals Seven Different Supertypes and a Surprising Degree of Repertoire Sharing across Supertypes*. Immunogenetics. 63:325-335, **2011**.

338. Arason GJ, Kolka R, Hreidarsson AB, Gudjonsson H, Schneider PM, Fry L, Arnason A. *Defective Prevention of Immune Precipitation in Autoimmune Diseases Is Independent of C4A*Q0*. Clin Exp Immunol. 140:572-579, **2005**.

339. Burut DF, Karim Y, Ferns GA. *The Role of Immune Complexes in Atherogenesis*. Angiology. 61:679-689, **2010**.

340. Puolakkainen M, Kuo CC, Shor A, Wang SP, Grayston JT, Campbell LA. *Serological Response to Chlamydia Pneumoniae in Adults with Coronary Arterial Fatty Streaks and Fibrolipid Plaques*. J Clin Microbiol. 31:2212-2214, **1993**.

341. Paju S, Pussinen PJ, Sinisalo J, Mattila K, Dogan B, Ahlberg J, Valtonen V, Nieminen MS, Asikainen S. *Clarithromycin Reduces Recurrent Cardiovascular Events in Subjects without Periodontitis*. Atherosclerosis. 188:412-419, **2006**.

342. Yang Y, Chung EK, Zhou B, Blanchong CA, Yu CY, Fust G, Kovacs M, Vatay A, Szalai C, Karadi I, Varga L. *Diversity in Intrinsic Strengths of the Human Complement System: Serum C4 Protein Concentrations Correlate with C4 Gene Size and Polygenic Variations, Hemolytic Activities, and Body Mass Index*. J Immunol. 171:2734-2745, **2003**.

343. Shen J, Ordovas JM. *Impact of Genetic and Environmental Factors on hsCRP Concentrations and Response to Therapeutic Agents*. Clin Chem. 55:256-264, **2009**.

344. Lukaszewicz AC, Faivre V, Payen D. *Is Monocyte HLA-DR Expression Monitoring a Useful Tool to Predict the Risk of Secondary Infection?* Minerva Anestesiol. 76:737-743, **2010**.

345. Kohsaka T, Jin DK, Abe J. *The Significance of C4 Locus II Deletion in IgA Nephropathy and Henoch-Schönlein Nephritis and It's Correlation with Other HLA Genes*. Nihon rinsho.Japanese journal of clinical medicine. 50:3064-3071, **1992**.

346. Abe J, Kohsaka T, Tanaka M, Kobayashi N. *Genetic Study on HLA Class II and Class III Region in the Disease Associated with IgA Nephropathy*. Nephron. 65:17-22, **1993**.

347. Wu R, Nityanand S, Berglund L, Lithell H, Holm G, Lefvert AK. *Antibodies against Cardiolipin and Oxidatively Modified LDL in 50-Year-Old Men Predict Myocardial Infarction*. Arterioscler Thromb Vasc Biol. 17:3159-3163, **1997**.

348. Artenjak A, Lakota K, Frank M, Cucnik S, Rozman B, Bozic B, Shoenfeld Y, Sodin-Semrl S. *Antiphospholipid Antibodies as Non-Traditional Risk Factors in Atherosclerosis Based Cardiovascular Diseases without Overt Autoimmunity. A Critical Updated Review*. Autoimmun Rev. **2012**.

349. Kovanen PT, Mänttari M, Palosuo T, Manninen V, Aho K. *Prediction of Myocardial Infarction in Dyslipidemic Men by Elevated Levels of Immunoglobulin Classes A, E, and G, but Not M*. Arch Intern Med. 158:1434-1439, **1998**.

350. Monteiro RC. *The Role of IgA and IgA Fc Receptors as Anti-Inflammatory Agents*. J Clin Immunol. 30 Suppl 1:S61-64, **2010**.

351. Pesonen E, El-Segaier M, Persson K, Puolakkainen M, Sarna S, Ohlin H, Pussinen PJ. *Infections as a Stimulus for Coronary Occlusion, Obstruction, or Acute Coronary Syndromes*. Therapeutic advances in cardiovascular disease. 3:447-454, **2009**.

352. Kervinen H, Huittinen T, Vaarala O, Leinonen M, Saikku P, Manninen V, Mänttari M. *Antibodies to Human Heat Shock Protein 60, Hypertension and Dyslipidemia. A Study of Joint Effects on Coronary Risk*. Atherosclerosis. 169:339-344, **2003**.

353. Pussinen PJ, Sarna S, Puolakkainen M, Ohlin H, Sorsa T, Pesonen E. *The Balance of Serum Matrix Metalloproteinase-8 and Its Tissue Inhibitor in Acute Coronary Syndrome and Its Recurrence*. Int J Cardiol. **2012**.