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Association analysis between genes involved in the inflammatory response, mean plasma levels and variability of CRP in post myocardial infarction survivors

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Contents

SUMMARY

Atherosclerosis is a complex disease and still the leading cause of death and illness in developed countries. There is convincing evidence that atherosclerosis has typical features of an inflammatory disease. Local inflammation in the artery wall is accompanied by a systemic low-grade inflammatory response, which can be measured by a variety of inflammatory biomarkers. The largest database so far has been accumulated for C-reactive protein (CRP), an acute phase reactant synthesized in the liver. Although several studies have investigated the effect of *CRP* polymorphisms on CRP concentrations, the impact of genetic variants still requires refinement owing to a number of factors including the influence of innate immunity genes on CRP concentrations and on the variability within individuals.

This thesis had two main aims. The first was to test the hypothesis that genetic variation in innate immunity genes could affect CRP plasma concentrations in a high-risk group of myocardial infarction (MI) survivors, while the second was to assess the genetic impact of the within-subject variability as a marker of the individual response.

1,003 MI survivors were recruited in scope of the AIRGENE study and CRP was measured repeatedly every 4-6 weeks. Thirteen candidate genes were selected including the *CRP* gene itself, the three chains of fibrinogen, the cytokines *IL-6*, *TNFα*, *LTA*, *IL-10* and *IL-18*, *TLR4*, a signalling receptor, as well as three components of the NF-κB transcription factor (*NFKB1*, *RELA* and *NFKBIA*). Within those gene loci 114 polymorphisms were selected and genotyped using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and their influence on CRP plasma concentration was assessed. The minor alleles of two variants, both located in the *CRP* gene itself, were strongly associated with lower CRP concentrations in MI survivors. However, none of the other investigated variants was associated with CRP levels. The overall contribution of these two *CRP* polymorphisms to phenotypic variance was calculated to be 4%. Compared to the observed heritability estimates of 30-50%, it is still

likely that other genetic variants outside the *CRP* gene locus contribute to the regulation of CRP concentrations.

Regarding intra-individual variability, the minor alleles of several variants in the selected candidate genes were significantly associated with either increased or decreased individual variability of CRP concentrations, even after excluding outlying observations. Genetic influences on the inflammatory cascade regulation may cause some individuals to have a more vigorous response than others, but within the range of overall population variability. A SNP in a candidate gene not associated with CRP mean concentrations might still be associated with its intra-individual variability, since this implies a short time effect which could be rapidly compensated. However, since this genetic influence on the intra-individual variability of CRP concentrations was demonstrated for the first time, other studies with repeated measurements of CRP are essential to confirm these associations.

ZUSAMMENFASSUNG

Atherosklerose ist eine komplexe Erkrankung und die häufigste Todes- und Krankheitsursache in den Industrieländern. Bei der Atherosklerose handelt es sich nach heutigem Kenntnisstand um eine entzündliche Erkrankung, bei der ein lokaler entzündlicher Prozess in der Gefäßwand von einer systemischen Immunantwort begleitet wird. Diese Immunantwort kann durch von einer Reihe von Entzündungsmarkern gemessen werden, von denen das C-reaktive Protein (CRP), das in der Leber als Reaktion auf entzündliche Stimuli gebildet wird, das am besten untersuchte ist. Der Einfluss von *CRP* Polymorphismen auf den CRP-Spiegel wurde bereits von mehreren Studien untersucht, Forschungsbedarf besteht jedoch noch im Hinblick auf den Einfluss von Polymorphismen in Genen der Immunantwort, sowie deren Assoziation mit der intra-individuellen Variabilität des CRP-Spiegels.

Die vorliegende Arbeit hatte zwei Ziele. Zum einen sollte untersucht werden, ob Polymorphismen in Genen der Immunantwort den CRP-Spiegel in einer Hochrisikopopulation beeinflussen, zum anderen sollte die genetische Komponente der intra-individuellen Variabilität analysiert werden.

Im Rahmen der AIRGENE Studie wurden dafür 1003 Herzinfarktüberlebende rekrutiert, bei denen CRP wiederholt im Abstand von 4-6 Wochen gemessen wurde. In dreizehn Genen der Immunantwort wurden 114 Polymorphismen mittels MALDI-TOF Massenspektrometrie genotypisiert und ihr Einfluss auf den CRP-Spiegel analysiert. Die untersuchten Kandidatengene umfassten das *CRP* Gen selbst, sowie die Gene für die drei Ketten des Fibrinogens, die Zytokine *IL-6*, *TNFα*, *LTA*, *IL-10* und *IL-18*, *TLR4*, einen Signalrezeptor, sowie drei Komponenten des NF-κB Transkriptionsfaktors (*NFKB1*, *RELA* und *NFKBIA*). Von den analysierten 114 Polymorphismen waren die seltenen Allele zweier Polymorphismen, beide im *CRP* Gen selbst lokalisiert, stark mit niedrigeren CRP Konzentrationen assoziiert. Ihr Beitrag zur Gesamtvarianz des CRP-Spiegels betrug 4%.

Verglichen mit der genetischen Komponente der CRP Konzentrationen, die auf 30-50% geschätzt wird, ist es wahrscheinlich, dass andere als die hier untersuchten genetischen Varianten außerhalb des Gens für CRP zur Regulation der CRP Konzentration beitragen.

Das zweite Ziel der vorliegenden Arbeit war es, die genetische Komponente der intra-individuellen Variabilität zu untersuchen. Änderungen in der systemischen Immunbalance, verursacht durch genetische Varianten, könnten bei suszeptiblen Individuen zu einer stärkeren Immunantwort als Antwort auf umweltbedingte Reize führen. Diese Antwort würde sich jedoch trotzdem innerhalb der Varianz des Populationsmittelwertes bewegen. In der vorliegenden Arbeit konnte gezeigt werden, dass verschiedene Allele in Polymorphismen der selektierten Kandidatengene mit Änderungen der individuellen Variabilität des CRP-Spiegels assoziiert sind. Genetische Varianten, die nicht mit dem Populationsmittelwert des CRP-Spiegels assoziiert sind, könnten dennoch mit Änderungen der individuellen Variabilität assoziiert sein, da es sich dabei um relativ schnell kompensierbare Kurzzeiteffekte handelt. Da in der vorliegenden Arbeit dieser Zusammenhang das erste Mal gezeigt wurde, sind weitere Studien mit wiederholten CRP Messungen notwendig, um diese Ergebnisse zu bestätigen.

LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme
AMELX	amelogenin
ARE	AU-rich elements
BMI	body mass index
C	complement component
CAD	coronary artery disease
CD32	FcRγIIa receptor
CD64	FcRγI receptor
CFH	complement factor H
CHD	coronary heart disease
CHOICE	Choices for Healthy Outcomes in Caring for ESRD
CI	confidence interval
COX-2	cyclooxygenase-2
CRP	C-reactive protein
CV	coefficient of variation
CVD	cardiovascular disease
ddNTP	dideoxynucleotides
dNTP	deoxynucleotides
EDTA	ethylenediamine tetraacetic acid
EM	expectation-maximization
ESRD	end-stage renal disease
FGA	fibrinogen, alpha polypeptide chain
FGB	fibrinogen, beta polypeptide chain

FGG	fibrinogen, gamma polypeptide chain
GYG2	glycogenin
HbA1c	glycosylated haemoglobin
HDL	high-density lipoprotein
HMCS	human–mouse conserved segment
hME	homogenous MassExtend
HMG	3-Hydroxy-3-methylglutaryl
HWE	Hardy-Weinberg-equilibrium
IL-1	interleukin-1
IL-10	interleukin-10
IL-18	interleukin-18
IL-6	interleukin-6
LD	linkage disequilibrium
LDL	low-density lipoprotein
LPS	lipopolysaccharides
LTA	lymphotoxin alpha
m/z	mass-to-charge ratio
MALDI	matrix-assisted laser desorption/ionisation
MI	myocardial infarction
MS	mass spectrometry
NCBI	National Center for Biotechnology
NF-κB	nuclear factor-kappa B
NHANES III	Third National Health and Nutrition Examination Survey
NHLBI	National Heart, Lung, and Blood Institute
NT-proBNP	N-terminal proB-type natriuretic peptide
OD	optical density

PCR	polymerase chain reaction
PPAR	peroxisome proliferator-activated receptor
PRINCE	Pravastatin Inflammation/CRP Evaluation
RBC	red blood cell
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
SNP	single nucleotide polymorphism
SOP	standardized operating procedure
TBE	tris-borate-EDTA
TFBS	transcription factor-binding site
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TOF	time-of-flight
UTR	untranslated region
UV	ultraviolet

1. Introduction

1.1. General Introduction

Atherosclerosis is a common and complex disease, and still the leading cause of death and illness in developed countries. Its complex aetiology includes both environmental and genetic components.[1] In recent years the essential role of inflammation in the pathogenesis of atherosclerosis and in development of acute coronary syndromes has been established.[2] Once considered a passive process of lipid accumulation, atherosclerosis is now widely accepted as an active process of vascular cell activation, inflammation, and thrombosis.[3] Inflammation has a fundamental role in almost every step of atherosclerosis, from adhesion and migration of monocytes to the intima, transformation of monocytes to macrophages and then foam cells, to fatty streak formation and maturation, and eventually fibrous cap thinning and plaque rupture leading to acute ischemic events.

However, inflammation in atherosclerosis is difficult to assess. There are no imaging techniques and arterial biopsy is not a practical way to study inflammation processes.[4] Therefore inflammatory biomarkers provide an interesting tool for measuring indirectly the inflammatory response and are able to provide new insights into several important atherosclerotic processes. The largest database for a single biomarker in this context has so far been accumulated for C-reactive protein (CRP). More than 25 prospective studies have shown a strong and consistent association between elevated CRP concentrations and various cardiovascular endpoints.[5-8] Evidence from *in-vitro* and clinical studies suggest that CRP might also have a causal role in the pathophysiology of atherogenesis, but this still represents a controversial issue.

The inter-individual variability of CRP concentrations has been extensively studied and was reported to be triggered by several factors including environmental factors and medication. Additionally, it has been shown that CRP concentrations have a strong genetic component.

Genetic research on CRP can therefore add to knowledge about the mechanisms of involvement in disease processes and the use of CRP as a biomarker. Although several studies have investigated the effect of single nucleotide polymorphisms (SNPs) on CRP concentrations, their impact still requires refinement owing to a number of factors including the variability within individuals, the influence of other genes in the inflammatory pathways and its use in subpopulations, since most studies have been limited to the general population.

The multicenter epidemiological study "Air Pollution and Inflammatory Response in Myocardial Infarction Survivors: Gene-Environment Interaction in a High Risk Group" (AIRGENE) was set up to study the role of air pollution in eliciting inflammation in myocardial infarction (MI) survivors recruited in six European cities. In addition, the study was designed to assess the role of candidate gene polymorphisms hypothesized to lead to a modification of the short-term effects of ambient air pollution. Therefore, inflammatory biomarkers were measured repeatedly every 4-6 weeks. Within the scope of the AIRGENE study, the aim of this thesis was to assess the influence of genetic polymorphisms in innate immunity genes on changes in CRP concentrations in a high risk group of MI survivors and to determine its within-subject variability as a marker of the individual response.

1.2. Inflammation in atherosclerosis

In the last decades, inflammation has been shown to play a key role in atherosclerosis, which before was considered to be a lipid storage disease, with lipid deposits formed on the surface of arteries until they restricted and blocked the blood supply, resulting in a cardiovascular event, such as MI or stroke.[2] Inflammation participates in atherosclerosis from its development to the final step of thrombotic complications. The initial step in atherosclerosis is the recruitment of leukocytes to the nascent atherosclerotic lesion. Normally, blood leukocytes adhere poorly to endothelial cells, which form the inner surface of the artery wall. When the endothelial monolayer becomes inflamed, it expresses adhesion molecules that

allow leukocytes to adhere. Additionally, proinflammatory cytokines provide a chemotactic stimulus to adherent leukocytes to migrate into the artery intima, the layer underneath the endothelium.[2;9;10]

The development of the fatty streak starts when these monocytes penetrate the endothelial lining and enter the intima of the vessel wall. Within the intima, monocytes mature into macrophages, exhibit increased expression of scavenger receptors, and engulf modified lipoproteins. Cholesterol esters accumulate in the cytoplasm, and the macrophages become foam cells characteristic of atherosclerotic lesions. T lymphocytes join macrophages in the intima during lesion evolution and secrete cytokines and growth factors that can promote the migration and proliferation of smooth muscle cells. Macrophages also multiply and release several growth factors and cytokines, thus amplifying and sustaining proinflammatory signals.[2;9;10]

Inflammation is also central to the progression from fatty streak to complex plaque. As the plaque evolves, T cells also secrete cytokines that inhibit the production of collagen by smooth muscle cells and that stimulate macrophages to express collagen-degrading enzymes. This weakens the fibrous cap, which is formed of collagen and protects the blood from contact with the lipid core. Physical disruption of the atherosclerotic plaque causes most acute coronary syndromes, resulting in thrombus formation and sudden expansion of the lesion. The blood flow through the affected artery may become compromised or even completely blocked leading to acute ischemic events.[2;9;10]

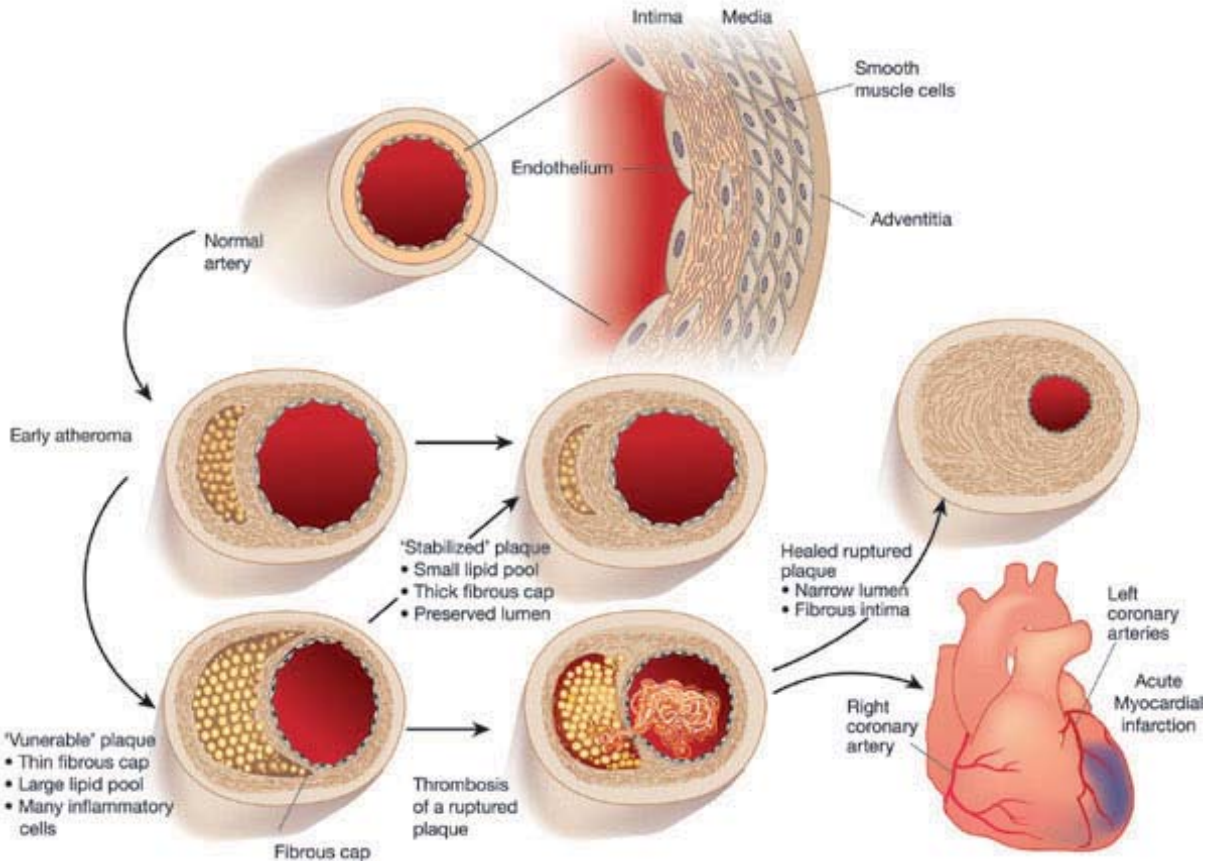

Figure 1: Development of atherosclerosis. Adhesion and migration of monocytes to the intima, transformation to macrophages and then foam cells, fatty streak formation and maturation, and eventually fibrous cap thinning and plaque rupture leading to acute ischemic events (Figure adapted from Libby *et al.*).[2]

1.3. C-reactive protein

CRP was the first acute phase protein to be described, named for its capacity to precipitate the somatic C-polysaccharide of *Streptococcus pneumoniae*.[11] CRP is an acute phase protein produced in the liver when stimulated by certain inflammatory molecules. CRP is also known to be a marker of tissue damage due to its potent ability to induce complement. CRP belongs of the pentraxin family of plasma proteins.[11] Pentraxins share a Ca^{2+}-dependent ligand-binding activity as well as sequence and structural homology. The evolutionary conservation of CRP and its upregulation in response to inflammatory mediators suggest that CRP has an

important role in host defense.[12] In response to tissue damage or during the acute phase, levels of CRP may increase by as much as 10000-fold from usual concentrations of less than 1 mg/l.[11] Following an inflammatory stimulus, concentrations of CRP are rising by about 6 h and having the highest peak at about 48 h.[13] The half-life of CRP in plasma is 19 h, therefore the main determinant of the plasma concentration is the synthesis rate, reflecting the inflammatory stimulus.[14]

1.3.1. Molecular structure of CRP

Human CRP is composed of 5 identical, 21,500-molecular weight subunits. It contains an 18-amino acid signal sequence and a mature protein of 206 amino acids.[15] The protomers are non-covalently associated in an annular configuration with cyclic pentameric symmetry.[16]

1.3.2. CRP gene expression

The gene coding for CRP (*CRP)* is a relatively small with two exons and a single intron, spanning about 2 kb. It is located on chromosome 1q21-23. A single non-functional pseudogene is found close to the authentic *CRP* gene, and typical promoter sequences are located upstream of the cap region (104 nucleotides from the start of the signal peptide). CRP expression is principally controlled at the level of transcription. *In vitro* studies in hepatocyte cell lines have identified some of the intra-cellular signalling pathways and have shown that secretion is more efficient during an acute phase response.[17;18] Synthesis of CRP is mainly regulated by interleukin-6 (IL-6), which in turn is upregulated by other inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα).[19] Plasma CRP is principally produced by hepatocytes. However, other sites for CRP synthesis have been suggested.[11;20] Small amounts of CRP can also be produced locally,[21] for example in atherosclerotic lesions by smooth muscle cells and monocytic cells.[22;23] CRP has also been

detected on the surface of about 4% of normal blood lymphocytes and it has been demonstrated that this CRP is produced by the lymphocytes themselves.[24]

1.4. Biological functions of CRP and their implications for atherosclerosis

CRP contributes to a large number of biological pathways. CRP is involved in complement activation, cell adhesion and recruitment, the expression of regulatory cytokines and apoptosis. All these mechanisms are part of the process of inflammation. CRP may thus contribute to the development of the atherosclerotic lesion via a direct pro-inflammatory effect. However, CRP also exerts activity in protective mechanisms. Thereby CRP is capable of maintaining a certain balance in the inflammatory process and stability of the atherosclerotic lesion.

1.4.1. Complement activation

Activation of the complement system is the classical biological function of CRP.[25] Human CRP binds with highest affinity to phosphocholine residues, as well as a variety of other autologous and extrinsic ligands. Autologous ligands include plasma lipoproteins,[26] damaged cell membranes,[27] a number of different phospholipids, small nuclear ribonucleoprotein particles,[28;29] and apoptotic cells.[30] Extrinsic ligands include many components of micro-organisms as well as plant products.[31;32] When human CRP is ligand-bound, it is recognized by complement component 1q (C1q) and potently activates the classical complement pathway, engaging C3, the main adhesion molecule of the complement system, and the terminal membrane attack complex, C5-C9.[11;33;34] Activation of this membrane attack complex is characterized by elevated levels of component C5a,[35] which itself exerts potent chemotactic and pro-inflammatory effects and its plasma levels have been associated with increased cardiovascular risk in patients with advanced atherosclerosis.[36]

On the other hand, CRP is also involved in the inhibition of complement activation through interaction with complement factor H (CFH). The complex of CRP and CFH interferes with the activity of C3b, and thus will prevent formation of the membrane attack complex.[37] In addition, CRP is involved in the process of apoptosis.[38] It binds to apoptotic cells in a Ca^{2+}-dependent manner and initiates the classical pathway of complement but protects the cells from assembling the terminal membrane attack complex. Furthermore, CRP enhances opsonization and phagocytosis of apoptotic cells by macrophages associated with the expression of anti-inflammatory molecules.

The CRP-mediated complement activation regulates the inflammatory reaction, as it promotes the removal of debris from tissues and the deleterious effects of complement activation in patients with cardiovascular disease (CVD).[39] However, since complement activation also leads to the production of a variety of pro-inflammatory molecules, this mechanism might also amplify the inflammatory status in the entire body as well as in the atherosclerotic plaque. Therefore, the direct interaction between CRP and complement can both activate and inhibit inflammation in atherosclerotic lesions.

1.4.2. Effects on the endothelium and monocyte recruitment

Endothelial dysfunction is one of the early abnormalities in atherosclerosis, characterized by upregulation of adhesion molecules on the endothelial surface leading to migration of monocytes into the vessel wall. *In vitro* experiments have shown, that CRP contributes to an arterial pro-inflammatory and pro-atherosclerotic phenotype by inducing the expression of cytokines, adhesion molecules and chemoattractant chemokines in endothelial cells, vascular smooth muscle cells and monocytes.[40] In addition, CRP increases proliferation and migration of vascular smooth muscle cells.[41] Via these processes, CRP induces platelet adhesion to endothelial cells and stimulates endothelial cell dysfunction and the recruitment of monocytes and T-lymphocytes towards the endothelial wall.

CRP also appears to be directly involved in the recruitment of monocytes into the vessel wall and their development into foam cells. The deposition of CRP in the arterial wall precedes monocyte infiltration and direct involvement of CRP in recruitment of blood monocytes has been demonstrated *in vitro*, suggesting that CRP is chemotactic for human blood monocytes.[42] However, these cell culture experiments have been questioned in recent years owing to the difficulty of completely removing contaminants like lipopolysaccharides (LPS) or azides from the culture when using commercially available CRP. Additional problems are the lack of controls in some studies, and the high concentrations of CRP required to achieve these changes.[25]

1.4.3. Interaction with lipids

There are several interactions between lipids and CRP. CRP binds to enzymatically degraded LDL and thus enhances complement activation, which may be relevant to the development at early stages of the atherosclerotic lesion, when low concentrations of degraded LDL are present.[43] The majority of foam cells show positive staining for CRP. It was demonstrated that native LDL that was incubated with CRP was taken up by macrophages via macropinocytosis. Therefore, foam cell formation in human atherogenesis might be caused in part by uptake of CRP-opsonized native LDL.[44]

1.4.4. Interaction with cell surface receptors

CRP binds to several receptors on human monocytes; to FcRγIIa (CD32) with high affinity and to FcRγI (CD64) with lower affinity increasing phagocytosis and the release of inflammatory cytokines.[45;46] The Fc receptors have been described to mediate the effect of CRP on human aortic endothelial cells.[47] FcRγIIa is known as the putative CRP receptor for leukocytes.[48] The binding of CRP to a receptor suggests its direct involvement in cell-

mediation and opsonization. However, the downstream effects of CRP binding have not yet been elucidated.

1.5. CRP in the pathogenesis of atherosclerosis

The mechanism underlying the relationship between CRP and atherosclerosis is complex and has not yet been fully elucidated, but there are strong indications that CRP also contributes directly to the progression of atherosclerosis.[42;49;50] The *in vitro* experiments described above have shown, that CRP promotes a number of proinflammatory cellular effects like monocyte recruitment, activation of complement, and stimulation of cellular adhesion molecule and cytokine expression.[49;51;52] Furthermore, CRP has been identified within atherosclerotic plaques.[53;54] It is produced in smooth muscle cells and macrophages in the atheroma.[43;53] Although this is supportive evidence for a direct role of CRP on plaque inflammation or local synthesis of CRP by activated vascular cells, the presence of CRP in plaque does not prove that it is a central player in plaque destabilization.

Results from studies with transgenetically bred mice that produce human CRP have produced conflicting results. Investigation of a CRP 'knock-in' mouse model reported that male mice, but not females, developed larger atherosclerotic lesions in CRP+ compared with CRP^{-} mice.[55] However, other reports of mice that express human CRP failed to demonstrate any relationship between the degree of atherosclerosis and the presence of human CRP.[56;57] In addition, mouse models with CRP are difficult to interpret as CRP does not appear to be an acute phase reactant in mice and concentrations of CRP generated in CRP+ mice are much higher than the concentrations associated with coronary artery disease in humans. Another study, infusing CRP into seven healthy humans provoked an immediate inflammatory response.[58] Because commercial CRP was used, however, the possibility remains that it was contaminated. Another study repeated the experiment in mice with several CRP preparations, showing that only commercially available CRP created a robust response.[59] To summarize,

there is evidence for a causal role of CRP in the pathogenesis of atherosclerosis, but it still remains a controversial issue requiring additional research.

1.6. CRP in the prediction of cardiovascular risk

Although CRP may not be directly involved in the pathogenesis of atherosclerosis, its role as a predictor of cardiovascular risk is well established. More than 25 prospective studies have shown a strong and consistent association between elevated CRP concentrations and the risk of future cardiovascular events.[8;60-62]

A meta-analysis including 11 prospective studies reported a 2-fold higher relative risk (95% CI, 1.6 to 2.5) for major coronary events between the upper and lower tertiles of CRP independent of clinical risk assessment or lipid profiles. A more recent update in form of a meta-analysis of 22 prospective studies published between 1996 and 2003 that examined CRP as a predictor for CVD events provides a modestly attenuated estimate of the relative risk.[61] Participants in these studies had a mean age at entry of 57 years and were followed for a mean of 12 years. Almost all of the studies adjusted for smoking and at least some other coronary heart disease (CHD) risk factors. For the highest compared with the lowest tertile of CRP, the combined multivariable-adjusted odds ratio for CHD was 1.6 (95% CI, 1.5 to 1.7). However, absence of a relationship between CRP and risk of MI has been reported as well, especially after comprehensive adjustment for established risk factors.[63] Nevertheless, most clinical studies report that CRP is consistently, although weakly, associated with cardiovascular disease even after considering other cardiovascular risk factors such as age, smoking, obesity, diabetes, hypercholesterolemia and hypertension. Therefore, the American Heart Association has recommended that CRP may be measured at the physician's discretion in asymptomatic people with an intermediate risk of coronary heart disease (Class IIa recommendation) to optimize the global assessment of cardiovascular risk. Patients can be categorized using CRP-

based risk categories of low (<1 mg/L), average (2 to 3 mg/L), and high (>3 mg/L) on the basis of the average of 2 measurements taken optimally at least 2 weeks apart.[64]

1.7. Factors influencing CRP concentrations

The CRP response to inflammatory stimuli is non-specific and is triggered by many factors including environmental and genetic factors as well as medication.[64] When analysing the effect of polymorphisms on CRP concentrations, it is therefore essential to consider these factors as potential confounders in the statistical analysis.

1.7.1. Clinical and environmental correlates

Age and gender both affect CRP concentrations. CRP tends to increase with age and females have slightly higher circulating concentrations.[65] Increased body mass index (BMI) has also been shown to be strongly associated with higher CRP values.[66] CRP levels are also influenced by several life-style factors. Weight loss predictably leads to lower baseline CRP concentrations,[67;68] as does moderate alcohol intake,[69;70] and there is an association between exercise and reductions in CRP production.[71;72] Other associations with elevated baseline CRP values include periodontal disease, smoking, consumption of coffee, and stress.[73;74]

Higher values have also found to be associated with many features of insulin resistance or the metabolic syndrome[75-77] and diabetes.[78] CRP production predicts the development of type 2 diabetes independently of traditional risk factors.[79]

1.7.2. Medication

3-Hydroxy-3-methylglutaryl (HMG) CoA-reductase inhibitors (statins) are the most extensively studied agents proven to lower CRP levels.[80-82] Statins reduced the concentration of CRP by 13% to 50% compared with placebo in 13 controlled studies.[83] Moreover, in statin-treated patients, the achieved level of CRP correlates with subsequent risk of major coronary

events independently of LDL.[82] Such an effect may reflect direct hepatocyte interactions, and/or a reduction in atherosclerotic plaque inflammation.[84] Activators of peroxisome proliferator-activated receptor (PPAR) alpha have been shown to directly suppress IL-1-induced but not IL-6-induced expression of CRP in cell culture.[85] Other CRP-lowering drugs include Cyclooxygenase-2 (COX-2) inhibitors,[86] platelet inhibitors,[87] hypolipidemic agents,[88] antidiabetic agents,[89] β-adrenoreceptor antagonists[90] and antioxidants[91]. Aspirin has also been reported to lower CRP levels, but results are conflicting.[92;93] Oral contraceptive use and systemic post-menopausal hormone replacement therapy are associated with significantly raised baseline CRP concentrations without any sign of tissue-damaging inflammation.[94-96] The effect of inhibitors of the renin-angiotensin system on CRP concentrations like angiotensin converting enzyme (ACE) inhibitors or angiotensin II receptor blockers is still ambiguous,[93] while the use of calcium channel antagonists or diuretics did not show any influence on CRP concentration in several studies.[93]

1.7.3. Genetic factors

Heritability studies have shown that CRP is not only influenced by environmental but also by genetic factors.[97;98] Heritability estimates attempt to define the proportion of a trait attributable to genetics compared to environmental factors. A study of 2,163 individuals from the National Heart, Lung, and Blood Institute (NHLBI) family heart study reported a heritability estimate of 0.40.[99] Another study of 588 individuals in 98 British Caucasian families showed that CRP has a heritability estimate of 0.39.[100] In a sample of Japanese Americans, heritability measurements have also been relatively high, with estimates of 0.30.[101]

These results are supported by twin studies. In a monozygotic twin study, the within-pair correlation coefficient for baseline CRP was 0.40.[102] Similarly, a study of monozygotic and dizygotic female twins estimated the heritability of CRP concentrations to be 0.52.[103] The

degree of heritability of baseline CRP concentrations suggests that genetic factors contribute significantly, but since environmental factors can aggregate within families, the exact genetic contribution to CRP levels remains elusive. In addition, several studies reported associations between SNPs in the *CRP* gene and the inter-individual variability in CRP levels, but results are conflicting.[97;98;104-114]

1.8. Hypotheses

Besides the *CRP* gene itself, there may be polymorphisms in other genes acting upstream of CRP productions, that may influence baseline CRP concentrations. So far, only few studies investigated the effect of polymorphisms in genes involved in the inflammatory cascade on CRP concentrations, and most of these studies have been restricted to a small number of polymorphisms.[100;115-118] Variants in innate immunity genes may either directly influence baseline CRP levels, changes in CRP concentrations in response to medication, or changes in its magnitude in response to environmental stimuli. Subtle impairment might cause imbalances between pro- and anti-inflammatory factors and change the level of inflammatory proteins, including CRP. Therefore, genes of the innate immunity system are good candidates for potential effects on CRP levels.

Therefore, the first hypothesis of this thesis was that genetic variation in innate immunity genes could affect the inter-individual variability of baseline CRP concentrations in a high-risk group of MI survivors. To test this hypothesis, thirteen candidate genes were selected. Selected genes included the *CRP* gene itself, fibrinogen (*FGA*, *FGB* and *FGG*), the cytokines *IL-6*, *TNFα*, lymphotoxin alpha (*LTA*), interleukin-10 (*IL-10*) and interleukin-18 (*IL-18*), the signalling receptor toll-like receptor (TLR) 4 (*TLR4*), as well as three components of the nuclear factor-kappa B (*NF-κB*) complex (*NFκB1*, *NFκBIA* and *RELA*). The influence of polymorphisms in these candidate genes on the inter-individual changes in CRP concentrations was analysed (Figure 2A).

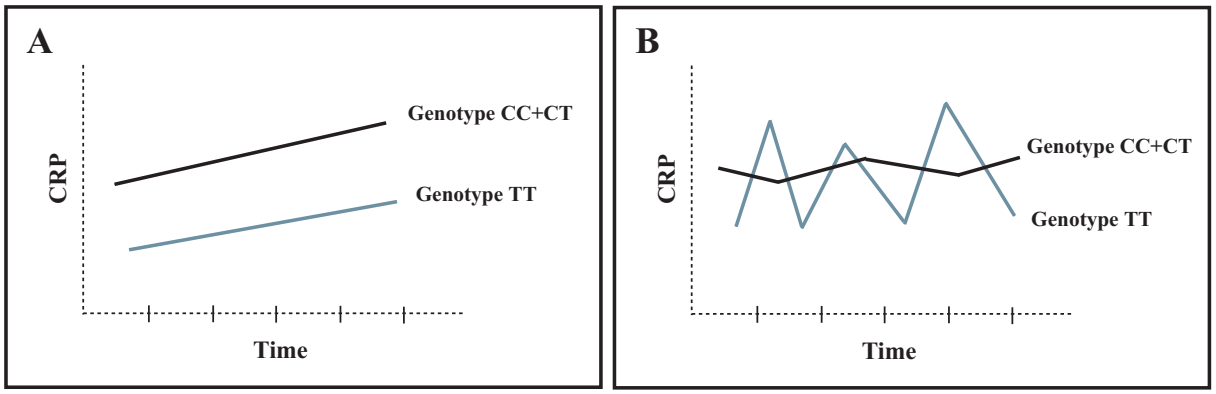

Figure 2: Hypotheses of the present thesis. A: Subjects having a certain genotype constitutively express higher levels of CRP compared to subjects not having this genotype. B: External stimuli induce a short-term increase in CRP concentrations in subjects having a certain genotype compared to subjects not having this genotype.

The second hypothesis of this thesis was that genetic variants influence the within-subject variability as a marker of the individual response. Theoretically, external stimuli might induce an increase in CRP at different time points, but within the range of overall population variability, which would not change the overall distribution of CRP values measured. However, no changes in CRP concentrations would be observed in subjects without the inducible genotype, while they would display the same overall mean. Therefore, in the present thesis, the external stimuli was considered as a random unknown component and the variability attributable to genetic components was assessed, based on the rationale that it seems unlikely that genotype and absence of random environmental factors coincide. A SNP in a candidate gene not associated with mean CRP levels might still be associated with intra-individual variability of CRP, since this implies a short-term effect which could be rapidly compensated (Figure 2B).

1.9. Aims

This thesis had two main aims. The first was to test the hypothesis that genetic variation in innate immunity genes could affect the inter-individual variability of baseline CRP

concentrations in a high-risk group of MI survivors, while the second was to assess the genetic impact of the within-subject variability as a marker of the individual response.

2. Study population

2.1. Study design

The multicenter longitudinal study "Air Pollution and Inflammatory Response in Myocardial Infarction Survivors: Gene-Environment Interaction in a High Risk Group" (AIRGENE) was conducted between May 2003 and July 2004 in six European cities. The study was designed to assess inflammatory responses in association with ambient air pollution concentrations in a large cohort of MI survivors and to determine the role of gene variants by assessing gene-environment interactions.

Study centers included Athens (Greece, 3.1 million inhabitants), Augsburg (Germany; 0.5 million inhabitants), Barcelona (Spain; 1.5 million inhabitants), Helsinki (Finland; 0.5 million inhabitants), Rome (Italy; 2.7 million inhabitants), and Stockholm (Sweden; 1.0 million inhabitants). The design was chosen to include a variety of geographical conditions. At each location, the goal was to recruit 200 MI survivors resulting in a study population of 1,200 post-MI patients. In each subject six repeated clinical examinations were scheduled: one every 4 weeks. Therefore, a total of 7,200 clinical examinations were anticipated.

Study participants were identified in population registries of patients with MI (Augsburg ,[119] Barcelona, Stockholm) or in administrative data-bases of hospital admissions (Athens, Helsinki, Rome). They were contacted either directly or through their hospitals, depending on the national ethical requirements. MI was defined based on the recommendation by the European Society of Cardiology/American College of Cardiology Committee.[120] A detailed description of the inclusion and exclusion criteria is listed below.

Inclusion criteria:

(1) Survival of a MI between 3 months and 6 years before entry into the study, corresponding to a MI in the years between 1997 and 2003.

(2) Age between 35 and 80 years.

Exclusion criteria:

(1) A MI and/or interventional procedure (percutaneous transluminal coronary angioplasty, bypass surgery) less than 3 months before the beginning of the study.

(2) Not resident in the study area.

(3) An extended period of absence from the study area planned during the study period.

(4) Any major illness preventing patients from complying with the study protocol.

(5) Chronic inflammatory diseases and/or anti-inflammatory medication modifying the biomarkers considered in the study.

(6) Only one or no valid blood sample available per patient.

Blood samples were excluded when patients had a cold/flu, urinary tract infection, gastro-intestinal infection, respiratory infection, a surgery or a major dental procedure in the three days before the clinical visit, since these could have severely altered the concentrations of CRP and other inflammatory markers. Preferably, currently non-smoking MI survivors were recruited. Ex-smokers had to have quit three months before the start of the study to be considered as non-smokers. However, light current smokers were accepted in Southern European centers because it was not possible for these centers to completely avoid recruiting current regular smokers in a reasonable time frame.

Altogether, 1,003 MI survivors were recruited, who fulfilled the inclusion and none of the exclusion criteria and had at least two valid, repeated blood samples taken (Table 1). Overall, 5,813 CRP plasma samples were collected to determine CRP concentrations. Altogether the

success rate was 96.6% of the scheduled six blood samples within the 1,003 patients. The average number of repeated visits per study subject ranged from 4.5 in Athens to 6.6 in Barcelona, as for Barcelona one additional visit was conducted.

Table 1: Study description.

Center	Patients recruited at baseline	Patients fulfilling study criteria	Excluded blood samples	Usable blood samples	Blood samples: mean per patient
Helsinki	212	195	7	1155	5.9
Stockholm	207	197	4	1168	5.9
Augsburg	213	200	7	1144	5.7
Rome	163	134	115	741	5.5
Barcelona	183	169	11	1119	6.6
Athens	151	108	111	486	4.5
All Centers	1129	1003	255	5813	5.8

2.2. Field study

All partners received approval of the study protocol by their local human subject committees. Informed consent was obtained from all patients at the first clinical visit after a detailed description of the study protocol.

2.2.1. Clinical characterisation at baseline

The health status of each patient was assessed at the first visit at the local study center. The cohorts were characterised with respect to their cardiovascular risk factor profile. This part was crucial in order to describe similarities and differences of the cohorts recruited in the 6 locations. The protocol included a history of CHD and other co-morbidities. A baseline questionnaire assessed regular exercise, smoking history, environmental tobacco smoke exposures, socio-economic status, and alcohol intake. All medication taken was recorded including brand name, dose, and intake pattern. Clinical measurements included a blood pressure measurement, the

determination of the BMI, and a resting 12-lead electrocardiogram. A blood serum sample was drawn to determine serum lipids including total cholesterol and glycosylated haemoglobin (HbA1c). Ethylenediamine tetraacetic acid (EDTA)-plasma samples were collected to assess CRP and N-terminal proB-type natriuretic peptide (NT-proBNP).

2.2.2. Repeated clinical visits

At each clinical visit, EDTA-plasma samples were collected to determine the concentrations of CRP and other inflammatory markers. A short questionnaire was administered, collecting time-varying factors such as smoking. A seven-day recall on medication intake was obtained. If patients suffered from acute infections such as a cold or influenza during the three days before the scheduled visit, examinations were postponed or blood samples were excluded from the analyses.

2.2.3. Quality assurance in scope of the field study

A study manual was developed describing the methods of the study including standardized operating procedures (SOPs) for specific parts of the field study. Questionnaires were translated into the different languages by the local partners. A two-day training session instructed the investigators from the centers on the implementation of the SOPs in the field. Before the field phase, the personnel who conducted the examinations were trained locally on the basis of the study manual.

The progress of the field study, including patient recruitment, training of the study personnel and the number of clinical visits were continuously monitored. At each study center a site-visit was conducted by a scientist of the coordinating center (Helmholtz Zentrum München, Neuherberg, Germany) at the beginning of the field phase. During this site-visit, the study components were

assessed based on a questionnaire, deviations were discussed with the local investigators and procedures were altered if necessary.

2.3. Study description

A detailed description of the study participants stratified by city is presented in the appendix under point 7.1. All variables were formally tested for heterogeneity between cities (*p*-value). More women participated in the Nordic centers Stockholm and Helsinki compared to the other centers. Participants had a similar age range, however, a higher proportion of young men were recruited in Athens. Regarding BMI, 70 to 80% of all participants were overweight or obese. The proportion of obese subjects ranged between 23% and 37% in the different cities. Only in Helsinki, Stockholm and Augsburg it was possible to completely avoid recruiting current regular smokers and still reach the recruitment goals in a reasonable time frame. Also, a history of smoking was more prevalent in the Southern European centers among current non-smokers, particularly Athens and Barcelona. Stockholm showed the lowest prevalence of HbA1c equal or greater than 6.5%. Self-assessed health status ranged between “good” and “average”, but was best in Athens. The proportion of patients with first MI and the proportion of patients reporting a history of hypertension were similar across centers. A higher proportion of patients in Rome reported a history of respiratory disease, but frequency of symptoms at baseline and the use of medication to treat respiratory diseases were not more frequent in Rome. Therefore, the self-reported history may reflect the emphasis on respiratory diseases at Columbus hospital in Rome, where the patients had their baseline visit.

Regarding medication, significant differences were observed between cities. As expected, the majority of patients were treated with lipid-lowering medication, mainly statins. The highest proportion of lipid-lowering medication use was observed in Augsburg and Stockholm. A remarkably high proportion of subjects were taking anti-inflammatory and antirheumatic agents for systemic use and nitrates in Helsinki. The smallest proportion of patients obtaining

medication was seen in Athens. Subjects from Barcelona were most often treated with analgesics and anti-infectives for systemic use. The proportion of participants taking ACE-inhibitors was highest in Rome. Table 2 describes the medication use of the study participants.

Table 2: Treatment of 1,003 MI survivors from 6 European cities.

Treatment [%]	**Helsinki** N=195	**Stockholm** N=197	**Augsburg** N=200	**Rome** N=134	**Barcelona** N=169	**Athens** N=108
ACE-inhibitors	50.20	50.70	72.10	80.50	58.50	51.80
Analgesics*	34.80	39.50	10.60	25.30	64.40	2.70
Anti-infectives*	21.00	22.30	14.50	19.40	35.50	1.80
Anti-inflammatory and antirheumatic agents*	38.90	14.20	25.00	21.60	25.40	3.70
Diuretics	23.00	24.80	43.50	32.80	23.00	10.10
Lipid lowering agents	85.10	88.80	90.00	82.80	86.30	74.00
Nitrates	80.00	26.30	18.50	26.10	37.20	37.00

*including only medication for systemic use

3. Material

3.1. Devices

Centrifuge:	Sigma 4K15C (Sigma Laborzentrifugen, Osterode, Germany) Rotanta 46 RS (Hettich, Tuttlingen, Germany) Mikrozentrifuge (NeoLab, Heidelberg, Germany) Centrifuge 5417R (Eppendorf AG, Hamburg, Germany)
Gel electrophoresis documentation system:	UVT-40 M Transilluminator (Herolab, Wiesloch, Germany) E.A.S.Y. 429 K Camera (Herolab, Wiesloch, Germany)
Gel electrophoresis device:	Bio-Rad Power Pac 300/3000 (BIO-RAD Laboratories, Munich, Germany)
Gel tray:	Sub-Cell GT Systems (BIO-RAD Laboratories, Munich, Germany)
Mass spectrometer:	Bruker™ Autoflex (Sequenom, Hamburg, Germany) BIFLEX II-TOF (Bruker Franzen Analytik, Bremen, Germany)
Nanodispenser:	Mass Array™ Nanodispenser (Sequenom, Hamburg, Germany) Spectro Point™ Nanoliter Pipetting Systems (Sequenom, Hamburg, Germany)
PCR cycler:	DNA Engine DYAD™ (MJ Research, South San Francisco, USA) DNA Engine Tetrad (MJ Research, South San Francisco, USA)
Photometer:	Genios® Flourescence Plate Reader (Tecan AG, Crailsheim, Germany)
Pipetting robots:	Multimek 96 Automated 96-Channel Pipettor (Beckman Coulter, Fullerton, USA) Temo (Tecan AG, Crailsheim, Germany) Genesis RSP 150 Work Station (Tecan AG, Crailsheim, Germany)

Scale:	572 Präzisionswaage (Schott Duran, Mainz, Germany)
Shaker:	Roto-Shake Genie (Scientific Industries, NewYork, USA)
Thermal mixer:	Thermomixer Comfort (Eppendorf AG, Hamburg, Germany)
Ultrapure water purification system:	Milli-Q (Millipore GmbH, Schwalbach, Germany)
Vortex:	MS2 Minishaker (IKA-Labortechnik, Staufen, Germany)

3.2. Software

3.2.1. Software for MALDI-TOF mass spectrometry and pipetting robots

Gemini 3.2 (Visual Basic Programme)	Tecan, Crailsheim, Germany
Normalisation WorklistMaker (Visual Basic Programme)	Tecan, Crailsheim, Germany
Xflour4	Tecan, Crailsheim, Germany

3.2.2. Software for assay design and sequence analysis

SpectroDESIGNER	Sequenom, Hamburg, Germany
Vector NTI Suite 7	www.informaxinc.com

3.2.3. Statistical software

JLIN	www.genepi.com.au/jlin
Haploview	www.broad.mit.edu/mpg/haploview
R 2.4.0	www.r-project.org
SAS 9.1	SAS Institute Inc., Cary, USA

3.2.4. Online databases and programs for SNP selection and sequence analysis

Ensembl	www.ensembl.org
Innate Immunity Program for Genomic Applications (IIPGA)	http://innateimmunity.net
International HapMap Project	www.hapmap.org
National Center for Biotechnology Information	www.ncbi.nlm.nih.gov
MatInspector	www.genomatix.de
PromoterInspector	www.genomatix.de
SeattleSNPs Program for Genomic Applications	http://pga.gs.washington.edu
UCSC Genome Browser	http://genome.ucsc.edu/cgi-bin/hgGateway
UTRScan	www.ba.itb.cnr.it/BIG/UTRScan

3.3. Buffer, solutions and reagents

3.3.1. Solutions for DNA extraction

RBC lysis buffer (pH 7.4)	NH_4Cl (155 mM) $KHCO_3$ (20 mM) Na_2EDTA (0.1 mM)
SE buffer (pH 8.0)	NaCl (75 mM) Na_2EDTA (25 mM)
NaCl solution (saturated)	NaCl (~6M)
TE buffer (pH 8.0)	Tris/HCl (10 mM) EDTA (1mM)
SDS solution	SDS (20%)

3.3.2. Buffers, solutions and reagents for agarose gel electrophoresis

6x Loading Dye Solution	MBI Fermentas, St. Leon-Rot, Germeny
Agarose ultra pure	GibcoBRL, Eggenstein, Germany

DNA agarose	Biozym, Oldendorf, Germany
Ethidium bromide	Biomol, Hamburg, Germany
pUC Mix Marker VIII	MBI Fermentas, St. Leon-Rot, Germeny
Tris-Borate-EDTA buffer (TBE)	Sigma-Aldrich, Osterode, Germany

3.3.3. Buffers and reagents for PCR

dNTP mix (25mM)	MBI Fermentas, St. Leon-Rot, Germeny
MgCl2 (25mM)	Qiagen GmbH, Hilden, Germany
PCR-Buffer with MgCl2 (10x)	Qiagen GmbH, Hilden, Germany

3.3.4. Buffers and reagents for SNP detection

3-Point Calibrant	Sequenom, Hamburg, Germany
hME MassEXTENT Nucleotide Tri-Mix Pack	Sequenom, Hamburg, Germany
hME buffer	Sequenom, Hamburg, Germany
Spectro CLEAN	Sequenom, Hamburg, Germany

3.4. Enzymes

HotStar Taq DNA polymerase	Qiagen, Hilden, Germany
Proteinase K	Merck, Darmstadt, Germany
Shrimp Alkaline Phosphatatse (SAP)	Amersham, Freiburg, Germany
Thermosequenase	Amersham, Freiburg, Germany

3.5. Primer

All primers were ordered by Metabion (Martinsried, Germany). Extension primers were purified by high performance liquid chromatography. Used primers are listed under point 7.2.

4. Methods

4.1. SNP selection

SNPs are variations of single base pairs, mostly biallelic, with, per definition, a minor allele frequency greater than 1%. They are found approximately every 300 bp in the human genome.[121]

There are two main approaches in selecting genetic markers in association studies of complex diseases. The first is a functional or candidate gene approach, where SNPs are selected in genes that may plausibly play a relevant role in the process, pathway or disease under investigation. Those genes can be completely sequenced to capture the complete variation at the locus, or SNPs can be chosen from public databases and literature.

In database and literature searches, preferably SNPs are taken that are proposed to influence or alter gene expression or protein function. This can happen by a variety of mechanisms. They may lead to an amino acid exchange in the corresponding protein, where functions like DNA binding or catalytic activity are altered or abolished. Additionally, SNPs may change the reading frame shift or introduce stop codons, all with consequences for insufficient or prematurely truncated peptides.[120;122] Polymorphisms may also affect transcription, RNA processing, stability and translation. SNPs in known promoter motifs can lead to changes in mRNA levels of the protein itself and downstream targets. mRNA splicing mutants are most commonly found at the beginning and end of the donor and acceptor consensus splice sequence and cause either exon skipping or utilization of cryptic splice sites resulting in the absence of normally spliced mRNA.[120;122] Finally, RNA cleavage-polyadenylation mutants can occur in the AAUAAA sequence upstream of the polyadenylation sites.[120;122] The 5' untranslated regions (UTRs) are proposed to play a role in controlling mRNA translation while sequence variants in the 3' UTR control RNA cleavage, stability, export and intracellular localization.[120;122;123]

The second approach is an indirect or positional one, in which markers in a particular region or the whole genome are systematically screened, on the basis that they may be in linkage disequilibrium (LD) with disease related functional variants.[120;124] For the second approach, the efficiency of the selection can be improved by recognizing the redundancy between near-by markers through the presence of LD. Due to the great amount of SNPs and the appropriate LD information stored in public databases, a subset of SNPs, called tag SNPs, can be selected for genotyping and analysis with minimal loss of information.[125;126]

In this thesis, a combined approach was used. SNPs were chosen on the basis of positional and functional aspects to enhance the chance of detecting associations. A literature and database search was performed to include DNA variants previously showing an association with CRP concentrations or cardiovascular endpoint and related phenotypes. Additionally, all up to then known tag SNPs were selected on the basis of density (if possible, distance <5 kb), frequency (<5%) and level of validation. Following the functional approach, SNPs were chosen if they occur in regions of known importance like described above, for example occurrence in hypothetical transcription factor-binding sites (TFBS) or human–mouse conserved segments (HMCS).

For SNP selection, the National Center for Biotechnology (NCBI) SNP database dbSNP Build 124, the international HapMap project database, the Innate Immunity Program for Genomic Applications database and the SeattleSNPs Program for Genomic Applications were used.

4.2. DNA extraction

The method for DNA extraction used in this thesis is slightly modified based on Miller[127] and involves salting out of the cellular proteins by dehydration and precipitation with a saturated sodium chloride (NaCl) solution.

Genomic DNA was extracted from 9 ml frozen EDTA anticoagulated blood for all centers except for Barcelona, where the extraction was done from 1 ml buffy coats instead. DNA

obtained from this technique yielded average quantities from 300-600 µg of genomic DNA for 9 ml EDTA blood and 150-400 µg of genomic DNA for the 1 ml buffy coat samples. DNA samples were stored at temperatures from 4°C (short-term storage) down to -80°C (long-term storage).

4.2.1. DNA extraction from EDTA blood

The frozen EDTA samples (9 ml) were resuspended in 50 ml polypropylene tubes with 30 ml red blood cell (RBC) lysis buffer to separate the erythrocytes from the rest of the cells with intact nuclei. After centrifugation at 2,500 rpm the supernatant was discarded. The cell lysates were mixed with 25 µl protease K solution, 5 ml SE buffer and 250 µl of 20% sodium dodecyl sulfate (SDS) and digested overnight at 55°C. After digestion was complete, 3 ml of saturated NaCl together with 5 ml SDS buffer was added to each tube and shaken vigorously, followed by centrifugation at 3,500 rpm for 15 minutes. The precipitated protein pellet was discarded and 13 ml of absolute isopropanol was added to the supernatant containing the DNA. The tubes were inverted several times until the DNA precipitated. Afterwards, the DNA was washed using 10 ml of 70% ethyl alcohol and then dried at room temperature. The DNA was dissolved in 1.2 ml TE buffer.

4.2.2. DNA extraction from buffy coats

Buffy coats (1 ml) of nucleated cells obtained from anticoagulated blood were resuspended in 15 ml polypropylene tubes with 4 ml RBC lysis buffer for erylysis followed by centrifugation at 2,500 rpm. The cell lysates were mixed with 6.5 µl protease K solution, 1.3 ml SE buffer and 63 µl of 20% SDS and digested overnight at 55°C. The remaining proteins were removed by salt precipitation, adding 0.75 ml of saturated NaCl together with 1.25 ml SDS buffer and centrifugation at 3,500 rpm. The pellet was solved in the supernatant and precipitated by

addition of 3.4 ml 100% isopropanol. The DNA pellet was washed with 2.5 ml 70% ethyl alcohol and the dried pellet was dissolved in 0.6 ml TE buffer.

4.3. DNA concentration measurement

Spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength. DNA absorbs ultraviolet (UV) light very efficiently. The nitrogenous bases in nucleotides have an absorption maximum at about 260 nm. In contrast to nucleic acids, proteins have a UV absorption maximum of 280 nm, due mostly to the aromatic residues. The ratio of the absorbance at 260 nm and the absorbance at 280 nm gives an estimate of the protein contamination of the DNA sample. Concentration and quality of the DNA samples were measured with the photometer GeniOS (Tecan, Crailsheim, Germany) following the manufacturer`s protocol and using standard settings. TE buffer was used for DNA dilution (1:20) and for the reference. The optical density (OD) $_{260}$ / OD $_{280}$ ratios of the extracted DNA samples were consistently 1.7-2.0, demonstrating good deproteinization during DNA extraction.

4.4. Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate DNA fragments by size. The size of the separated fragments can be estimated by comparison with known fragments (DNA standards). Nucleic acids migrate to the anode in electric fields due to the natural negative charge carried on their phosphodiester backbone. Shorter fragments move faster than longer ones. Depending on the size of the analysed DNA fragments, gels containing 0.8-3% agarose were made, dissolving agarose in tris-borate-EDTA (TBE) buffer by heating in the microwave. Loading buffers are added with the DNA in order to visualize it and sediment it in the gel well. After the separation is completed, the fractions of DNA fragments of different length are

visualized using ethidium bromide that intercalates with DNA and fluoresces under UV light (266 nm).

4.5. Polymerase Chain Reaction

With the introduction of the polymerase chain reaction (PCR),[128] it became possible to exponentially amplify specific DNA regions and thus generate billion copies of the fragment to be analysed. A PCR reaction starts with a denaturing step where samples are heated to 95°C. Prior to the first cycle, an additional denaturation step of several minutes is used to disentangle the complex structure of the template DNA. For annealing, the temperature is lowered. This allows the excess of primers to anneal to their complementary sequences. The primers are usually only 18 to 25 base pairs long and designed to bracket the DNA region to be amplified. The annealing temperature of this stage depends on the primers and is usually 5°C below their melting temperature. During the following elongation step, the temperature is raised to 72°C, the optimum temperature of the Taq polymerase, a thermostable polymerase from Thermus aquaticus. The Taq polymerase attaches at each priming site and uses the 3'OH ends of the primers and the provided deoxynucleotides (dNTPs) to catalyse the synthesis of the new DNA strands. A final elongation step is frequently used after the last cycle to ensure that any remaining single stranded DNA is completely copied. The amplification consists of 30-45 cycles denaturation, annealing and elongation. The master mix used for standard PCRs is shown in Table 3. Thermal cycling was performed on PCR DNA Engine Tetrad (BIO-RAD Laboratories, Munich, Germany). The temperature conditions for a standard PCR are presented in Table 4. PCR in scope of the homogenous MassExtend (hME) assay, including master mixes and conditions is described under 4.6.2. Primers are listed under point 7.2.

Table 3: Master mix for standard PCR.

Reagent	Volume
Nanopure water	11.3 µl
PCR-Buffer with $MgCl_2$ (10x)	2.0 µl
dNTPs (2mM)	2.0 µl
MgCl2 (25mM)	1.5 µl
Forward PCR primer (10 µM)	1.0 µl
Reverse PCR primer (10 µM)	1.0 µl
genomic DNA (50ng/µl)	1.0 µl
Taq DNA-polymerase (1U/µl)	0.2 µl

Table 4: Temperature conditions for standard PCR.

Step	Temperature [°C]	Time	Number of cycles
Denaturation	95	15 min	1x
Denaturation	95	30 sec	
Annealing	X	30 sec	35x
Elongation	72	1 min	
Final elongation	72	10 min	1x

4.6. SNP detection via MALDI-TOF mass spectrometry

The introduction of the **M**atrix-**A**ssisted **L**aser **D**esorption/**I**onization **T**ime-**O**f-**F**light **M**ass **S**pectrometry (MALDI-TOF MS) by Karas and Hillenkamp[129] has offered a solution for fast and accurate genotyping of SNPs in a high-throughput manner. During MALDI-TOF mass spectrometry, the sample is staggered with a 100 to 1,000 fold excess of matrix, co-crystallised on a sample plate and irradiated with an intensive laser pulse for a few nanoseconds in the high vacuum chamber of the mass spectrometer.[129;130] With the development of this method, the molecular weight of DNA fragments in a mass range of 1,000 – 9,000 Dalton can be determined with an accuracy of 0.1 to 0.01%, making it valuable for SNP analysis.[131-134] A widely-used and well developed method for high-throughput

genotyping of SNPs is the hME assay.[132] In this method, allele-specific extension products are generated by primer extension and can be distinguished by their molecular weight using mass spectrometry.

4.6.1. hME assay

The hME assay is based upon the annealing of an oligonucleotide primer (extension primer) adjacent to the polymorphic site of interest. The addition of a DNA polymerase (Thermosequenase), plus a mixture of nucleotides and terminators, allows the extension of the primer through the polymorphic site. The resultant mass of the primer extension product is then analysed and used to determine the sequence of the nucleotides at the polymorphic site. To analyse a SNP by MALDI-TOF mass spectrometry, the genomic sequence around the SNP has to be amplified, generating PCR products of approximately 100 bp which are afterwards used as a template for primer extension.

4.6.2. Polymerase chain reaction in scope of the hME assay

The PCR was carried out in a 384-well plate format with 5µl genomic DNA (1ng/µl) that was dried overnight at room temperature.

Table 5: PCR master mix for 1 to 6 primer pairs.

Reagent	Volume
PCR-Buffer with $MgCl_2$ (10x)	0.6 µl
$MgCl_2$ (25mM)	0.24µl
dNTPs (2mM)	0.6 µl
Forward PCR primer (100mM)	0.01 µl
Reverse PCR primer (100mM)	0.01 µl
HotStar Taq® (5U/µl)	0.02 µl-0.05 µl
Nanopure water	Fill up to 6 µl

Table 6: PCR master mix for more than 6 primer pairs.

Reagent	Volume
PCR-Buffer with $MgCl_2$ (10x)	0.75 µl
$MgCl_2$ (25mM)	0.39µl
dNTPs (2mM)	1.5 µl
Forward PCR primer (100mM)	0.01 µl
Reverse PCR primer (100mM)	0.01 µl
HotStar Taq® (5U/µl)	0.06 µl
Nanopure water	Fill up to 6 µl

Two types of master mixes were used since the concentrations in the mixes had to be adapted to the number of reactions carried out in one well (multiplex level). The reaction volume of 6 µl was pipetted using the pipette station Genesis RSP 150 workstation (Tecan, Crailsheim, Germany). PCR primers and extension primers were designed using Assay Design 2.0 software (Sequenom, Hamburg, Germany), minimizing chances for overlapping peaks in spectra. The software also considers potential unwanted intra- and inter-primer interactions in order to avoid non-template extensions. PCR primers with the same masses as the extension products would interfere with the following MALDI-TOF analysis as they are not removed from the reaction. Therefore each PCR primer consists of a 10-mer tag (5`-ACGTTGGATG-3`), on its 5`end additionally to the primer sequence. The tag increases the masses of unused PCR primers to keep them outside the mass range of analytical peaks. PCR and extension primers are listed in the appendix under point 7.2. Thermal cycling was performed on PCR DNA Engine Tetrad (BIO-RAD Laboratories, Munich, Germany) following the conditions shown in Table 7.

Table 7: Temperature conditions for a 384-well plate format PCR.

Step	Temperature [°C]	Time	Number of cycles
Denaturation	94	15 min	1x
Denaturation	94	20 sec	
Annealing	56	30 sec	45x
Elongation	72	60 sec	
Final elongation	72	10 min	1x

4.6.3. Shrimp alkaline phosphatase reaction in scope of the hME assay

After the PCR reaction remaining dNTPs have to be deactivated with the shrimp alkaline phosphatase (SAP). This step is necessary to avoid the incorporation of the remaining dNTPs during the primer extension reaction, leading to other than the specified extension products and thus problems with the correct detection of the alleles. The enzyme deactivates dNTPs by splitting off the phosphate group. The master mix for the SAP reaction is shown in Table 8.

Table 8: SAP reaction master mix.

Reagent	Volume
SAP buffer (10x)	0.170 µl
Nanopure water	1.530 µl
SAP enzyme (1U/µl)	0.300 µl

The SAP master mix was dispensed using the pipetting robot Multimek 96 (Beckman Coulter, Fullerton, USA). The reaction was performed in two steps on PCR DNA Engine Tetrad (BIO-RAD Laboratories, Munich, Germany). In the first step, the reaction mix was incubated 20 minutes at 37°C, the optimum temperature for the enzyme. After that, the enzyme has to be denatured at 85°C for 10 minutes to not disturb the primer extension amplification reaction.

4.6.4. Primer adjustment in scope of the hME assay

The extension primers may not have comparable heights in the mass spectrum. Variations in peak height may derive from inconsistent oligonucleotide quality or concentration and different desorption/ionization behaviour in MALDI-TOF MS. Thus they have to be adjusted prior to the primer extension reaction to harmonize peak heights (intensities) in the mass spectrum. An assay with a very low intensity will systematically fail when applied to samples as part of a multiplex. For primer adjustment a mix of all required primers in one assay is prepared with a final concentration of 9 µM of each primer in the mix. 1 µl of the primer mix is then pipetted into a well of a microplate and 24 µl nanopure water is added to obtain a 360 nM dilution of the primer mix. 3 mg ion exchanger (SpectroClean^{TM}, Sequenom, Hamburg, Germany) is added to remove ions and the sample was dispensed to a silicon chip with Spectro Point Nanoliter Pipetting Systems™ (Sequenom, Hamburg, Germany). Spectra are analysed and peaks with less than 50% of the height of the highest peak are adjusted using MassARRAY Typer software 3.0.1. (Sequenom, Hamburg, Germany). Primer adjustment was performed for all assays with a plexing level above six.

4.6.5. Primer extension reaction in scope of the hME assay

To initiate the primer extension reaction, the extension primers, Thermosequenase and a mixture of one dNTP and three dideoxynucleotides (ddNTPs) are added to the hME reaction. The extension primer is hybridized directly to the polymorphic site of interest. Nucleotide mixes are selected to maximize mass differences for all potential extension products. Appropriate dNTPs are incorporated through the polymorphic site of interest until a single ddNTP is incorporated and the reaction terminates. Since the termination point and the number of nucleotides is allele specific, the mass difference of the generated extension products can be detected via MALDI-TOF MS analysis. The reaction volume of 2 µl was

pipetted with the pipetting robot Multimek 96 (Beckman Coulter, Fullerton, USA). Similarly to the PCR reaction, two types of primer extension mixes were used since the concentrations in the mixes had to be adapted to the multiplex level.

Table 9: Primer extension mix for 1 to 6 primer pairs.

Reagent	Volume
Nanopure water	1.728 µl
hME Extend mix (containing buffer and d/ddNTPs)	0.200 µl
Extension primers	0.054 µl
Thermosequenase	0.018 µl

Table 10: Primer extension mix for more than 6 primer pairs.

Reagent	Volume
Nanopure water	0.760 µl
hME Extend mix (containing buffer and d/ddNTPs)	0.200 µl
Adjusted primer mix	1.000 µl
Thermosequenase	0.040 µl

Thermal cycling was performed on PCR DNA Engine Tetrad (BIO-RAD Laboratories, Munich, Germany) following the conditions presented in Table 11.

Table 11: Temperature conditions for primer extension.

Step	Temperature [°C]	Time	Number of cycles
Denaturation	94	2 min	1x
Denaturation	94	5 sec	
Annealing	52	5 sec	75x
Elongation	72	10 sec	

After the extension reaction, the samples were purified with ion exchanger (Spectro Clean, Sequenom, Hamburg, Germany) to remove extraneous salts that interfere with MALDI-TOF MS analysis.

4.6.6. MALDI-TOF mass spectrometry

Following the primer extension reaction, 1–2 nl from the ion removed sample is transferred to a silicon chip with Spectro Point Nanoliter Pipetting Systems™ (Sequenom Hamburg, Germany). The chip matrix consists of 3-hydroxypicolinic acid, which is specially well applicable for DNA analysis.[135-137] Functions of the matrix include absorbing the applied laser energy and supporting, respectively inducing, the ionisation of the analyte molecule. Additionally the matrix should prevent a photolytic damage of the analyte and avoid interaction of analyte molecules with each other or with the sample carrier.[138]

The silicon chip contains 384 matrix spots consisting of 3-hydroxypicolinic acid for the samples and 10 matrix spots for the calibrant. The calibrant, a mix of three oligonucleotides with known masses is also applied onto the chip for calibrating the analysis system. After loading, he chip is transferred to a metallic sample carrier and put in the vacuum lock of the MassARRAY™ mass spectrometer. Measurement of the samples and calibration of the system is done automatically with standard setting.

The transfer of laser energy to sample molecules in the matrix generates mainly single charged molecule ions that trespass into the gas phase.[138] Under high vacuum conditions, the matrix crystals are irradiated with nanosecond duration laser pulses, leading to formation of a plume of volatilized matrix and analyte as well as charge transfer from matrix ions to analyte molecules. After electric field-induced acceleration in the mass spectrometer source region, the gas phase ions travel through a field-free region at a velocity inversely proportional to their mass-to-charge ratios (m/z), until they hit the detector.[131;139] Ions with low m/z values are faster than ions with higher m/z values and reach the detector earlier. The TOF-analyser

measures exactly the time until the ions hit the detector.[131] The resulting time-resolved spectrum is translated into a mass spectrum upon calibration. These mass spectra were further processed and analysed by the software Spectrotyper (Sequenom, Hamburg, Germany) for baseline correction and peak identification.

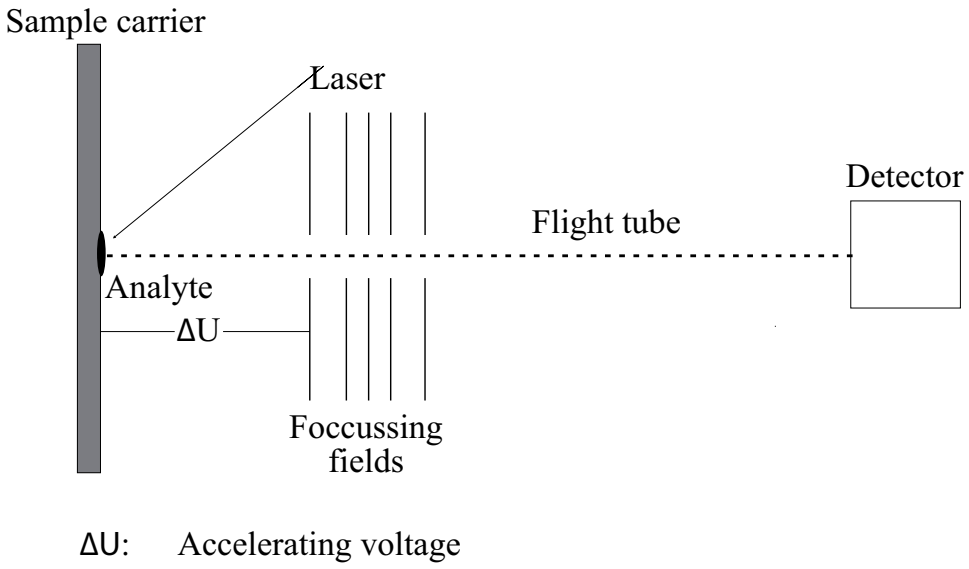

Figure 3. Principles of MALDI-TOF.

4.6.7. Quality assurance during genotyping

To assure that samples do not get mixed up and that no contamination occurs, 8 asymmetric negative as well as 8 positive controls are used on each measurement. Negative controls usually consist of distilled water and show no reaction at all, while the positive controls consist of well defined DNA. In addition, 30% of the samples were double checked. The discrepancy rate was 0.18%. Samples can also get mixed up during recruitment. As another step in quality assurance, sex determination was performed with validated genotyping assays. The assays are detecting DNA variants between the genes for Amelogenin (*AMELX*) and Glycogenin (*GYG2*) on the X chromosome and their homologous pseudogenes on the Y

chromosome. If the assay result did not match the patient information stored in the database, the sample was excluded from further analysis. Nine samples had to be excluded in the present thesis.

4.7. Measurement of C-reactive protein and other blood markers

Measurement of CRP was carried out by the Department of Internal Medicine II-Cardiology at the University of Ulm. Whole blood was drawn after a 12 h fasting in all patients. To obtain plasma samples, EDTA-blood was centrifuged at 2,500 rpm for 20 minutes, aliquoted and frozen immediately at -80°C for further analysis. No specimen inadvertently thawed during storage. CRP was determined by an immunonephelometric method (Dade Behring N Latex High Sensitivity CRP mono assay) on a Behring Nephelometer II analyser. The detection limit was 0.17 mg/l, and the measurement range was 0.175-1.100 mg/l, according to the manufacturer's instruction. Coefficients of variation (CV) were between 3.5% and 8.0% for a CRP concentration of 2.4 mg/l and between 3.2% and 5.8% for a CRP-concentration of 13.5 mg/l. To control the reliability of the laboratory tests, blind duplicates were sampled. These samples were assigned a special identification number but otherwise were to be treated like all other samples. For Athens, no duplicates were available. Results for the original and the duplicate blood sample were compared in up to 35 samples. The CV for the original compared with the duplicate blood sample was ranging from 1.5 in Barcelona to 4.1 in Stockholm. Total cholesterol and high-density lipoprotein (HDL) cholesterol were measured by routine enzymatic methods (CHOD-PAP; Boehringer Mannheim, Mannheim, Germany). Finally, NT-proBNP and HbA1c were measured by routine methods.

4.8. Statistical methods

All analyses unless otherwise noted were performed using the statistical packages SAS version 9.1 (SAS Institute Inc., Cary, USA) or R version 2.4.0 (www.r-project.org).

Haplotype reconstruction was performed by Martina Müller (Helmholtz Zentrum München, Neuherberg, Germany) using R version 2.4.0 (www.r-project.org).

4.8.1. Hardy-Weinberg-Equilibrium

Each SNP was tested for deviations from Hardy-Weinberg-equilibrium (HWE)[140;141] by means of a chi-square test or Fisher's exact test depending on allele frequency. SNPs showing deviations from HWE were checked for indication of genotyping errors, but where otherwise not excluded from further analysis, because the present study is not a random population sample, but a highly selected group of MI survivors, in which SNPs can violate HWE because of the selection process.

4.8.2. Descriptive statistics and selection of covariates

A phenotypic univariate description as well as distribution plots were performed for all variables. Genotype description was done for all polymorphisms including genotype frequencies, allele frequencies, missing data and genotyping success rates.

Confounder models were built to identify the time-invariant patient characteristics associated with the mean levels of CRP to allow the assumption of a normally distributed random patient intercept. Variables considered to impact the average CRP concentrations were selected with respect to literature and biological plausibility and included for example sex, age, diabetes, smoking, physical activity and alcohol consumption. All decisions on goodness-of-fit were based on Akaike's Information Criterion. The final model included city, sex, BMI, age, packyears of smoking, systolic blood pressure, total cholesterol number of MIs, self-assesed health status, HbA1c, NT-proBNP, chronic obstructive pulmonary disease, chronic bronchitis, and hypertension. NT-proBNP needed to be log-transformed while age was included as a cubic function. This model was respectively notated as "main effect model".

In addition, a sensitivity analysis was performed including medication data. Similarly to the selection of time-invariant patient characteristics, medication variables considered to impact the average CRP concentrations were selected with respect to literature and plausibility. Accessorily to all covariates of the main effect model, the final model included ACE-inhibitors, analgesics for systemic use, lipid lowering medication (mainly statins), anti-infectives for systemic use, anti-inflammatory and antirheumatic agents for systemic use, diuretics and nitrates.

4.8.3. General modelling strategy

Data were analysed using mixed effects models with random patient effects to account for the clustered data structure (SAS Proc MIXED). To model correlations between the repeated measures in each patient, compound symmetry structure for the covariance matrix was assumed as the half-life of CRP is much shorter than the intervals between the visits. This assumption was also verified in a model assuming an exponentially decaying correlation with time in the errors. As the estimated correlation between two measurements was then found to be 0.64 for two visits four weeks apart compared to 0.62 for two visits 20 weeks apart, a compound symmetry structure was subsequently used.

The residuals of the model were tested for normal distribution by the Kolmogorov-Smirnov test and distribution plots. CRP needed to be log-transformed to fulfil the model assumptions of residual normality. All analyses were done crude and with the final main effect model. To avoid bias resulting from population stratification, stratified analysis was performed for all major results. Partial r^2 was analysed with SAS Proc REG using the mean CRP concentrations of each patient.

For association analysis, the global significance level of 5% was corrected for the number of independent tests following the Bonferroni procedure (0.05/(64×2)=0.0004). The number of independent tests was calculated as the number of effective loci obtained through spectral

decomposition of the correlation matrix of all SNPs analysed times two for testing for inter- and intra-individual variability.[142]

To assess differences in intra-individual variability, mean and variance were then modelled simultaneously allowing for different error variances between genotype groups. Homogeneity of variances was tested using the asymptotic chi-square distribution of the restricted likelihood ratio test statistic, comparing the two models with equal and unequal variances. Finally, a sensitivity analysis was performed, excluding outlying subject variances according to Cochran's test, and outlying patient mean values using Reed's criterion.[143]

4.8.4. Linkage disequilibrium

The linkage disequilibrium analysis[144] reveals a possible co-segregation and the non-random association of alleles across two ore more linked polymorphic loci due to lacking recombination events. As measures for pairwise LD between each pair of SNP loci, Lewontin´s disequilibrium coefficient D´ and the squared correlation coefficient r^2 were estimated using the JLIN v1.0 software (http://www.genepi.com.au/jlin).

4.8.5. Haplotype reconstruction

Haplotype reconstruction was performed within blocks of high D' using expectation-maximization (EM) algorithm haplo.em[145] as it is available within the R software library haplo.stats. To avoid large reconstruction errors resulting from missing data, haplotype estimation is based only on patients from whom all genotypes were available. In order to avoid bias resulting from possible population stratification, haplotype probabilities were estimated for each center separately. The number of copies of best-guess haplotypes per subjects (i.e., the haplotype pair with the highest probability) was calculated for each haplotype variable and haplotype frequencies over the complete sample were evaluated. Haplotypes with frequencies <5% in all centers were collected into a separate group of rare

haplotypes (“haplo rare”). Haplotypes were analysed categorically, with the most common haplotype used as the reference.

5. Results

5.1. Description of genotyped polymorphisms

As depicted in the introduction, CRP concentrations have been shown to have a strong genetic component. The observed heritability may not only arise from the *CRP* itself, but may also involve genes acting upstream in the inflammatory cascade. To investigate the association between genetic variants involved in the regulation of the inflammatory pathways and inter- as well as intra-individual variability of CRP concentrations in a high risk group of patients with a history of MI, thirteen candidate genes were selected. A detailed literature and database search was performed to include genes previously showing an association with CRP concentrations or cardiovascular endpoints and related phenotypes. Selected candidate genes included the *CRP* gene itself, the three chains of fibrinogen, the cytokines *IL-6*, *TNFα*, *LTA*, *IL-10* and *IL-18*, *TLR4*, a signalling receptor, and three genes for components of the transcription factor NF-κB.

Within these candidate genes, polymorphisms, mainly biallelic SNPs, where selected as described under point 4.1. The genotyping of the selected polymorphisms was then performed using the hME assay, a method where allele-specific extension products are generated by primer extension and can be distinguished by their molecular weight using mass spectrometry. For polymorphisms with valid genotyping assays, sufficient genotyping success rates and allele frequencies greater than 1%, a phenotypic univariate description was performed prior to the association analysis. Altogether 114 SNPs within the selected candidate genes were successfully genotyped and included in the final dataset used for analysis. All polymorphisms were additionally analysed by center and tested for heterogeneity between centers. These detailed information on observed genotype frequencies by center and HWE statistics are presented under point 7.3, but will also be summarized at the end of each chapter.

5.1.1. Polymorphisms within the CRP gene

The most obvious candidate gene to have an influence on CRP concentrations is the *CRP* itself. Six biallelic and one triallelic polymorphism where selected from public databases. Since there is 50-80% region-specific homology between *CRP* and its pseudogene, primers were aligned to the gene cluster in order to eliminate false results from genotyping duplicated genomic regions. The positions of the selected SNPs are shown in Figure 4, while their characteristics are summarized in Table 12.

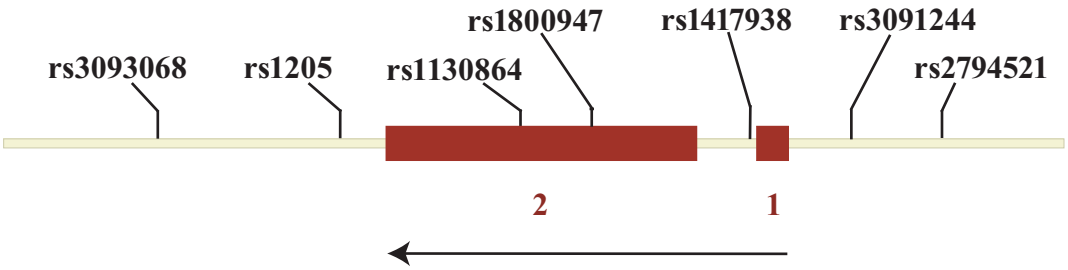

Figure 4: True to scale structure of the *CRP* gene on chromosome 1q21-23 (2 kb). Exons and appropriate exon numbers are shown in red while the direction of transcription is indicated by an arrow.

Table 12: Characteristics of the *CRP* variants.

dbSNP identifier	Exchange	Chromosome position	Nucleotide position	Gene location	Minor allele frequency [%]
rs2794521	T > C	157951720	-717	5´Upstream	26.38
rs3091244	G > A	157951289	-286	5´Upstream	32.39
rs3091244	G > T	157951289	-286	5´Upstream	6.94
rs1417938	T > A	157950810	89	Intronic	32.36
rs1800947	C > G	157950062	837	Leu184Leu, HMCS	6.33
rs1130864	G > A	157949715	1184	3´UTR	32.49
rs1205	C > T	157948857	2042	3´Flanking	33.22
rs3093068	G > C	157947988	2911	3´Flanking	6.78

All seven selected polymorphisms were successfully genotyped with an average genotyping success rate of 99.4% (98.9-99.9%). Additionally, no deviations from HWE were observed

and frequencies did not differ significantly between European cities except for rs1205 (p=0.04).

5.1.2. Polymorphisms within the fibrinogen gene cluster

The three chains of fibrinogen are encoded by different genes, denoted *FGA*, *FGB* and *FGG* that are grouped in a cluster of approximately 65 kb. Within the fibrinogen gene locus, *FGA* and *FGG* reside on the same DNA strand and are transcribed in the same direction (3´ to 5´), whereas *FGB* is on the opposite strand and transcribed in reverse order. Eleven SNPs were selected in the gene coding for the α-chain of fibrinogen. One of them, rs6051 in exon 5, which is leading to a silent amino-acid exchange at position 357 (Thr357Thr) turned out to be monomorphic in the present study population. Two other SNPs had a frequency of less than 1% and were therefore excluded from association analysis. One of them, rs2070033, is located in exon 6 and is leading to a silent amino-acid exchange at position 862 (Pro862Pro), while the other one, rs2070034, is located in a transcription factor binding site in the untranslated region of exon 6. The positions of the remaining SNPs are shown in Figure 5.

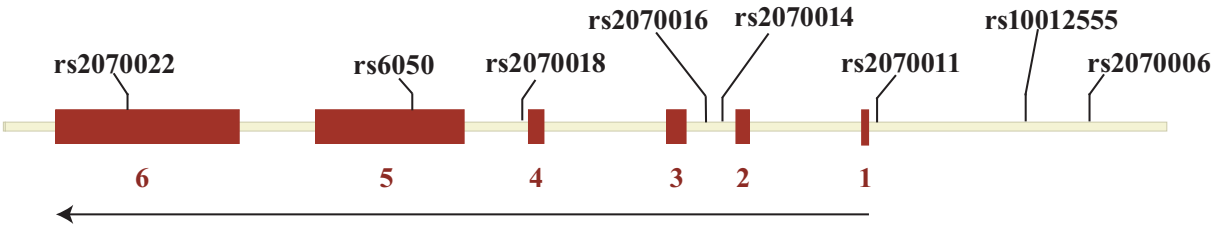

Figure 5: True to scale structure of the *FGA* gene on chromosome 4q28 (8 kb). Exons and appropriate exon numbers are shown in red while the direction of transcription is indicated by an arrow.

The remaining eight polymorphisms had an average genotyping success rate of 97.7% (94.7-99.6%). Their basic characteristics, including chromosome position and minor allele

frequencies for each SNP are shown in Table 13. One SNP in the *FGA*, rs2070011, fails HWE criteria (p=0.02). Regarding differences between cities, genotype frequencies significantly differed between centers for rs6050 (p=0.01), rs2070016 (p=0.04), rs2070011 (p=0.01) and rs2070006 (p=0.01).

Table 13: Characteristics of the *FGA* variants.

dbSNP identifier	Exchange	Chromosome position	Nucleotide position	Gene location	Minor allele frequency [%]
rs2070006	C > T	155733316	-2027	5´Upstream	39.24
rs10012555	T > C	155732726	-1438	5´Upstream	12.50
rs2070011	C > T	155731347	-58	5´Upstream	38.99
rs2070014	C > T	155729916	1374	Intronic	16.37
rs2070016	A > G	155729764	1526	Intronic	15.39
rs2070018	A > G	155728077	3213	Intronic	12.44
rs6050	T > C	155727040	4250	Ala331Thr	26.42
rs2070022	G > A	155724398	6892	3´UTR	16.67

To systematically cover *FGB*, coding for the beta chain of the fibrinogen molecule, eight SNPs were selected from public databases and literature. For one SNP, rs2227439 located in the 3´flanking region of the gene, the assay failed. The positions of the remaining SNPs are shown in Figure 6.

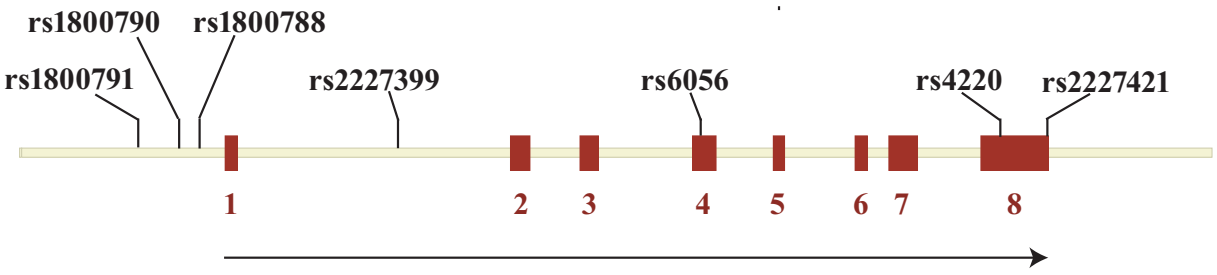

Figure 6: True to scale structure of the *FGB* gene on chromosome 4q28 (8 kb). Exons and appropriate exon numbers are shown in red while the direction of transcription is indicated by an arrow.

The selected polymorphisms had an average genotyping success rate of 99.6 % (99.4-99.7%). None of them showed departures from HWE. Genotype frequencies significantly differed between cities for rs1800791, rs1800790, rs1800788, rs2227399 and rs2227421 (p<0.03). The characteristics of the selected SNPs are presented in Table 14.

Table 14: Characteristics of the *FGB* variants.

dbSNP identifier	Exchange	Chromosome position	Nucleotide position	Gene location	Minor allele frequency [%]
rs1800791	G > A	155702759	-854	5´Upstream	14.75
rs1800790	G > A	155703158	-455	5´Upstream	20.21
rs1800788	C > T	155703364	-249	5´Upstream	21.65
rs2227399	T > G	155705303	1683	Intronic	20.44
rs6056	C > T	155708271	4651	Ser189Ser, HMCS	18.67
rs4220	G > A	155711209	7589	Lys478Arg, HMCS, TFBS	18.59
rs2227421	A > C	155711674	8054	3´UTR	31.76

Eight SNPs were selected in the *FGG* gene. rs2066870, leading to an amino-acid exchange (His140Tyr) in exon 5 turned out to be monomorphic in the AIRGENE study. For the intronic rs2066864, no valid assay could be established. The positions of the six SNPs that were successfully genotyped are shown in Figure 7.

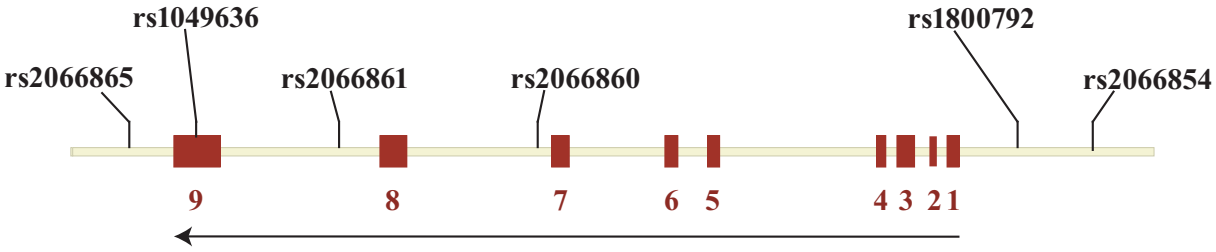

Figure 7: True to scale structure of the *FGG* gene on chromosome 4q28 (9 kb). Exons and appropriate exon numbers are shown in red while the direction of transcription is indicated by an arrow.

The *FGG* variants had an average genotyping success rate of 99.4 % (98.6-99.7%). No deviations from HWE were observed. The characteristics of the selected SNPs, including chromosome position and minor allele frequency for each SNP are shown in Table 15.

Table 15: Characteristics of the *FGG* variants.

dbSNP identifier	Exchange	Chromosome position	Nucleotide position	Gene location	Minor allele frequency [%]
rs2066854	T > A	155754631	-1420	5´Upstream	24.80
rs1800792	T > C	155753858	-647	5´Upstream	44.78
rs2066860	C > T	155748924	4288	Intronic	3.70
rs2066861	C > T	155746886	6326	Intronic	24.55
rs1049636	A > G	155745420	7792	3´UTR	29.88
rs2066865	G > A	155744726	8486	3´Flanking	24.60

Four out of six polymorphisms significantly differed between European cites, including rs2066865, rs2066861, rs1800792 and rs2066854 (p<0.01).

5.1.3. Polymorphisms within the IL-6 gene

Ten polymorphisms were selected to cover the *IL-6* gene. One of them, rs2069830, located in exon 2 and leading to an amino-acid exchange at position 32 (Ser32Pro) turned out to be monomorphic**.** A second polymorphism, rs2069860, located in exon 5 and also leading to an amino-acid exchange at position 162 (Val162Asp) had a frequency of less than 1% and was thus excluded from association analysis. The remaining eight polymorphisms (Figure 8) had an average genotyping success rate of 99.3 % (97.9-99.8%). rs2069827 fails HWE (p=0.04). Six polymorphisms significantly differed in their frequencies across European cities (p<0.01). The characteristics of the *IL-6* variants are shown in Table 16.

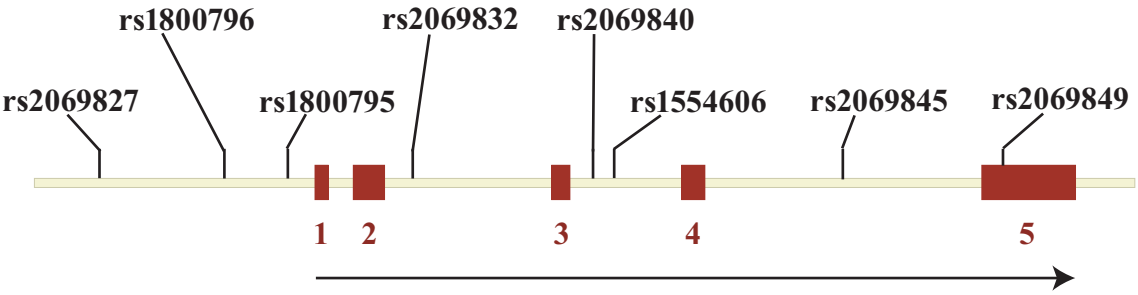

Figure 8: True to scale structure of the *IL-6* gene on chromosome 7p21 (5 kb). Exons and appropriate exon numbers are shown in red while the direction of transcription is indicated by an arrow.

Table 16: Characteristics of the *IL-6* variants.

dbSNP identifier	Exchange	Chromosome position	Nucleotide position	Gene location	Minor allele frequency [%]
rs2069827	G > T	22731981	-1362	5´Upstream	11.40
rs1800796	G > C	22732771	-572	5´Upstream	5.56
rs1800795	G > C	22733170	-174	5´Upstream, HMCS	40.68
rs2069832	G > A	22733958	613	Intronic	41.04
rs2069840	C > G	22735097	1752	Intronic	31.15
rs1554606	G > T	22735232	1887	Intronic	43.19
rs2069845	A > G	22736674	3329	Intronic	43.44
rs2069849	C > T	22737681	4336	Phe201Phe	2.50

5.1.4. Polymorphisms within the IL-10 gene

Six SNPs were selected in the *IL-10* itself. Additionally, another nine SNPs where selected to systematically tag the *IL-10* promoter region, which is reported to be very large, spanning about 6 kb.[146-148] For three SNPs (rs1554286 in intron 3, rs1800896 and rs6703630, both in the promoter region) no valid assay could be established or the assay failed. The positions of the remaining SNPs are shown in Figure 9.

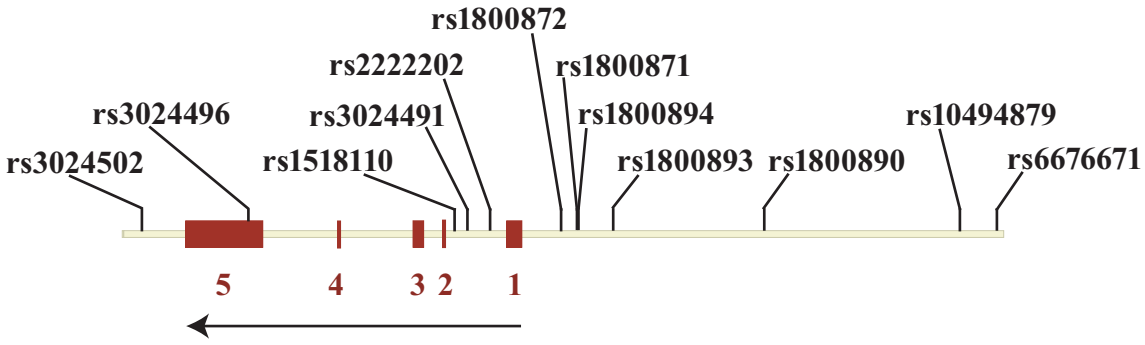

Figure 9: True to scale structure of the *IL-10* gene on chromosome 1q31-32 (5 kb). Exons and appropriate exon numbers are shown in red while the direction of transcription is indicated by an arrow.

The polymorphisms in the *IL-10* had an average genotyping success rate of 98.9% (97.0-100.0%). Table 17 summarizes the characteristics of the selected SNPs.

Table 17: Characteristics of the *IL-10* variants.

dbSNP identifier	Exchange	Chromosome position	Nucleotide position	Gene location	Minor allele frequency [%]
rs6676671	T > A	205019371	-6968	5´Upstream	34.20
rs10494879	C > G	205018827	-6424	5´Upstream	36.63
rs1800890	A > T	205015988	-3585	5´Upstream	34.15
rs1800893	C > T	205013790	-1387	5´Upstream	44.95
rs1800894	C > T	205013289	-851	5´Upstream	4.28
rs1800871	G > A	205013257	-819	5´Upstream	23.94
rs1800872	G > T	205013030	-592	5´Upstream	23.90
rs2222202	G > A	205012004	434	Intronic	44.67
rs3024491	C > A	205011669	769	Intronic, HMCS	44.62
rs1518110	C > A	205011484	954	Intronic, HMCS	21.41
rs3024496	A > G	205008487	3951	3´UTR	44.86
rs3024502	C > T	205006933	5504	3´Flanking	43.58

Seven polymorphisms significantly differed in their frequencies across centers (p<0.02). Remarkably, a big proportion of SNPs failed HWE, including rs3024496 (p=0.03), rs3024491 (p=0.04), rs1800890 (p=0.04), rs10494879 (p=0.02) and rs6676671 (p=0.03). Polymorphisms

in high linkage disequilibrium with each other, consistently both failed HWE, indicating that the deviations from HWE are not due to genotyping errors.

5.1.5. Polymorphisms within the TNF gene cluster

Nine polymorphisms were successfully genotyped in the *TNFα* gene (Figure 10). Table 18 summarizes their characteristics. The polymorphisms within the *TNFα* had an average genotyping success rate of 99.5% (99.1-100.0%). Two SNPs, rs1799724 (p=0.03) and rs3091257 (p<0.01), showed deviations from HWE.

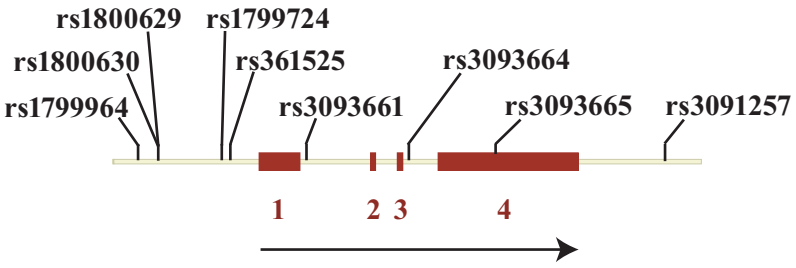

Figure 10: True to scale structure of the *TNFα* gene on chromosome 6p21.3 (3 kb). Exons and appropriate exon numbers are shown in red while the direction of transcription is indicated by an arrow.

Table 18: Characteristics of the *TNFα* variants.

dbSNP identifier	Exchange	Chromosome position	Nucleotide position	Gene location	Minor allele frequency [%]
rs1799964	T > C	31650287	-1031	5´Upstream	21.61
rs1800630	C > A	31650455	-863	5´Upstream, TFBS	17.20
rs1799724	C > T	31650461	-857	5´Upstream, TFBS	12.42
rs1800629	G > A	31651010	-308	5´Upstream, TFBS	12.88
rs361525	G > A	31651080	-238	5´Upstream	4.11
rs3093661	G > A	31651737	240	Intronic	3.02
rs3093664	A > G	31652621	1124	Intronic, TFBS	7.38
rs3093665	A > C	31653370	1873	3´UTR	2.85
rs3091257	G > A	31654829	3332	3´Flanking	7.44

In addition, several SNPs showed differences in their frequencies including rs1799724 (p<0.01), rs1800629 (p=0.02), rs361525 (p=0.02), rs3093664 (p=0.03), rs3093665 (p<0.01) and rs3091257 (p<0.01).

To systematically tag the *LTA*, eight polymorphisms where systematically selected. One polymorphism, rs3093544, located in the untranslated region of exon 4, had a frequency of less than 1% and was thus excluded from analysis. The positions of the remaining SNPs are shown in Figure 11 while their characteristics are shown in Table 19. The *LTA* variants had an average genotyping success rate of 99.5% (98.9-99.8%). No departures from HWE were observed. Regarding genotype frequencies, significant differences between European cities were observed for rs1041981, rs909253, rs2239704 and rs1800683 (p<0.05).

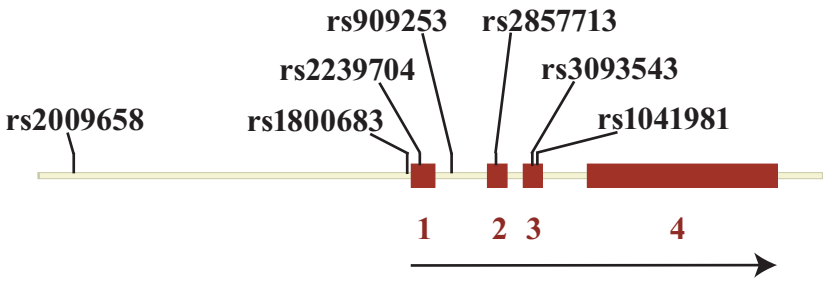

Figure 11: True to scale structure of the *LTA* gene on chromosome 6p21.3 (2 kb). Exons and appropriate exon numbers are shown in red while the direction of transcription is indicated by an arrow.

Table 19: Characteristics of the *LTA* variants.

dbSNP identifier	Exchange	Chromosome position	Nucleotide position	Gene location	Minor allele frequency [%]
rs2009658	C > G	31646223	-1816	5´Upstream	17.64
rs1800683	G > A	31648050	10	5´UTR, HMCS	28.11
rs2239704	C > A	31648120	80	5´UTR	41.56
rs909253	A > G	31648292	252	5´UTR	28.22
rs2857713	T > C	31648535	495	Arg13Cys, HMCS, TFBS	30.48
rs3093543	A > C	31648736	696	Pro51His, HMCS	6.88
rs1041981	C > A	31648763	723	Thr60Asn, HMCS	28.36

5.1.6. Polymorphisms within the IL-18 gene

Eleven SNPs where selected in the *IL-18* gene. For rs360723, located in the untranslated region between exon 1 and 2 of the gene, no valid assay could be established. The intronic rs5744263 had a frequency less than 1% and was therefore excluded from association analysis. The positions of the remaining nine SNPs are shown in Figure 12 while their characteristics are summarized in Table 20. The polymorphisms within the *IL-18* had an average genotyping success rate of 99.0% (97.1-99.9%). All of the genotyped SNPs fulfilled HWE criteria. Frequencies did not differ between cities except for rs2043055 (p=0.03).

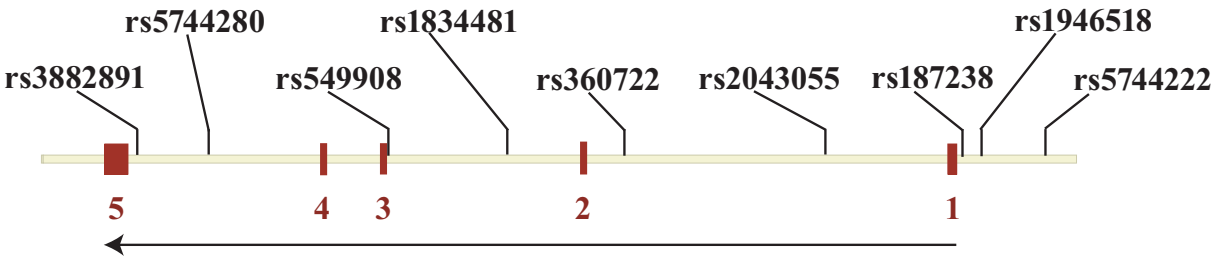

Figure 12: True to scale structure of the *IL-18* gene on chromosome 11q22.2-22.3 (21 kb). Exons and appropriate exon numbers are shown in red while the direction of transcription is indicated by an arrow.

Table 20: Characteristics of the *IL-18* variants.

dbSNP identifier	Exchange	Chromosome position	Nucleotide position	Gene location	Minor allele frequency [%]
rs5744222	G > T	111542224	-2163	5´Upstream	23.29
rs1946518	G > T	111540668	-607	5´Upstream, TFBS	43.08
rs187238	C > G	111540198	-137	5´Upstream, TFBS	27.74
rs2043055	A > G	111536834	3228	5´UTR	34.77
rs360722	G > A	111531913	8149	5´UTR	14.07
rs1834481	C > G	111529037	11025	Intronic	22.65
rs549908	T > G	111526126	13936	Ser35Ser	30.60
rs5744280	G > A	111521724	18338	Intronic	32.32
rs3882891	T > G	111519971	20091	Intronic	44.81

5.1.7. Polymorphisms within the TLR4 gene

Eleven SNPs where selected to systematically cover the *TLR4*. One of them, rs5030710, located in exon 3 and leading to a silent amino-acid exchange at position 105 (Ser105Ser) had a frequency less than 1% in the AIRGENE study population and was therefore excluded from association analysis. The positions of the remaining ten SNPs are shown in Figure 13 while their characteristics are summarized in Table 21.

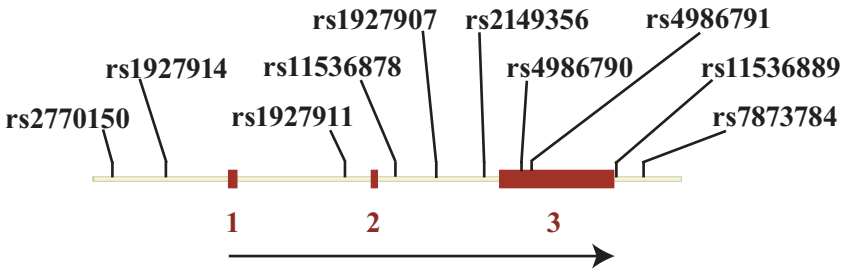

Figure 13: True to scale structure of the *TLR4* gene on chromosome 9q32-33 (12 kb). Exons and appropriate exon numbers are shown in red while the direction of transcription is indicated by an arrow.

Table 21: Characteristics of the *TLR4* variants.

dbSNP identifier	Exchange	Chromosome position	Nucleotide position	Gene location	Minor allele frequency [%]
rs2770150	A > G	119502960	-3612	5´Upstream	25.50
rs1927914	A > G	119504546	-2026	5´Upstream	34.89
rs1927911	G > A	119509875	3304	Intronic	27.10
rs11536878	C > A	119511374	4803	Intronic	11.09
rs1927907	C > T	119512585	6014	Intronic	16.42
rs2149356	G > T	119514020	7449	Intronic, TFBS	33.09
rs4986790	A > G	119515123	8552	Gly299Asp	5.88
rs4986791	C > T	119515423	8852	Ile399Thr, HMCS	6.56
rs11536889	G > C	119517952	11381	3´UTR	14.35
rs7873784	G > C	119518757	12186	3´Flanking	13.38

The *TLR4* variants had an average genotyping success rate of 98.3% (95.0-99.7%). Table 21 summarizes the characteristics of the selected SNPs. All SNPs fulfilled HWE. Frequencies

differed significantly between cities for all selected SNPs (p<0.04), except for rs2770150, rs4986791 and rs11536889.

5.1.8. Polymorphisms within genes coding for components of the NF-κB complex

Due to the large size of *NFKB1*, 19 SNPs and one deletion/insertion polymorphism where selected. For rs980455, located in the promoter region of the gene, rs4648050, next to an exon/intron boundary and rs1609993 which is leading to a silent amino-acid exchange at position 381 (Ala381Ala), the assay failed. The positions of the remaining SNPs are shown in Figure 14. Polymorphisms in the *NFKB1* had an average genotyping success rate of 99.1% (95.3-99.7%). Genotype frequencies significantly differed between cities only for rs230521 (p=0.01). Additionally, similar to *IL-10*, several SNPs showed deviations from HWE, including rs28362491 (p=0.02), rs230521 (p<0.01), rs230498 (p=0.02), rs1801 (p=0.01), rs3774956 (p=0.01), rs1020759 (p=0.02), rs3774964 (p=0.01) and rs11722146 (p=0.03). Polymorphisms in high linkage disequilibrium with each other, consistently both failed HWE, indicating that these deviations are not due to genotyping errors.

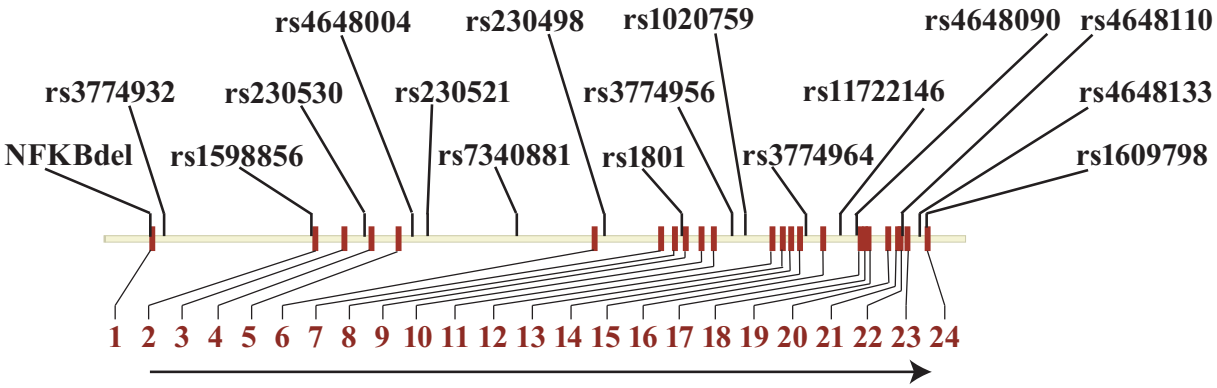

Figure 14: True to scale structure of the *NFKB1* gene on chromosome 4q23-q24 (156 kb). Exons and appropriate exon numbers are shown in red while the direction of transcription is indicated by an arrow.

Table 22: Characteristics of the *NFKB1* variants.

dbSNP identifier	Exchange	Chromosome position	Nucleotide position	Gene location	Minor allele frequency [%]
rs28362491	ATTG > -	103641187..103641190	-24515	5´Upstream	37.53
rs3774932	G > A	103643223	-22483	5´UTR	47.64
rs1598856	G > A	103665145	-561	5´UTR	47.79
rs230530	A > G	103673010	7305	Intronic	46.88
rs4648004	A > G	103680136	14432	Intronic	36.43
rs230521	G > C	103682357	16652	Intronic	37.19
rs7340881	C > T	103695601	29896	Intronic	15.50
rs230498	G > A	103708639	42934	Intronic	31.78
rs1801	G > C	103720092	54387	Intronic	32.55
rs3774956	C > T	103727564	61859	Intronic	38.16
rs1020759	C > T	103729549	63844	Intronic	38.33
rs3774964	A > G	103738525	72820	Intronic	34.27
rs11722146	G > A	103743667	77962	Intronic	26.70
rs4648090	G > A	103746106	80401	Intronic	13.93
rs4648110	T > A	103752867	87162	Intronic	19.90
rs4648133	T > C	103755459	89754	Intronic	27.30
rs1609798	C > T	103756488	90783	Intronic	27.96

Seven polymorphisms where selected to ensure an appropriate coverage of *RELA*. Their positions are shown in Figure 15.

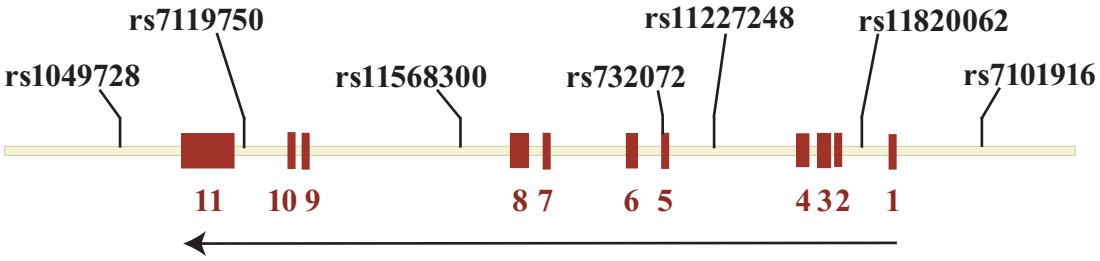

Figure 15: True to scale structure of the *RELA* gene on chromosome 11q12-q13 (8 kb). Exons and appropriate exon numbers are shown in red while the direction of transcription is indicated by an arrow.

RELA polymorphisms had an average genotyping success rate of 99.6% (99.4-99.8%). All SNPs fulfilled HWE criteria. Three SNPs, rs1049728, rs11568300 and rs732072 significantly

differed between cities in their observed genotype frequencies (p<0.02). Table 23 summarizes the characteristics of the selected SNPs.

Table 23: Characteristics of the *RELA* variants.

dbSNP identifier	Exchange	Chromosome position	Nucleotide position	Gene location	Minor allele frequency [%]
rs7101916	C > T	65187936	-1057	5´Upstream	11.24
rs11820062	T > C	65186512	368	Intronic	47.55
rs11227248	C > T	65184754	2126	Intronic, TFBS	34.03
rs732072	G > A	65184144	2736	Intronic	8.73
rs11568300	C > G	65181743	5137	Intronic, TFBS	36.76
rs7119750	C > T	65179167	7713	Intronic	11.50
rs1049728	G > C	65177693	9187	3´Flanking, HMCS	6.81

Within *NFKBIA*, nine SNPs where selected from public databases. For two of them, the intronic rs2233411 and rs10782383, leading to a silent amino-acid exchange (Ala102Ala), no valid assay could be established. The positions of the remaining polymorphisms are shown in Figure 16.

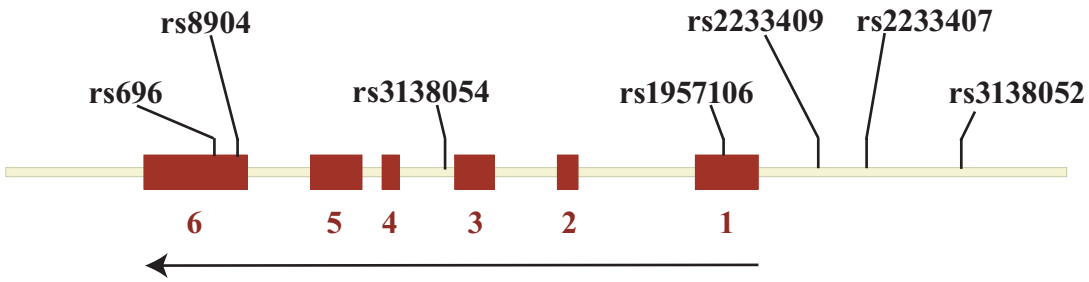

Figure 16: True to scale structure of the *NFKBIA* gene on chromosome 14q13 (4 kb). Exons and appropriate exon numbers are shown in red while the direction of transcription is indicated by an arrow.

The polymorphisms within the *NFKBIA* had an average genotyping success rate of 98.9% (94.9-99.8%). All SNPs fulfilled HWE criteria. Two SNPs, rs2233407 and rs1957106 significantly differed between cities regarding their genotype frequencies (p<0.01). Table 24 summarizes the characteristics of the selected SNPs.

Table 24: Characteristics of the *NFKBIA* variants.

dbSNP identifier	Exchange	Chromosome position	Nucleotide position	Gene location	Minor allele frequency [%]
rs3138052	T > C	34944772	-1171	5´Upstream	28.23
rs2233407	T > A	34944274	-673	5´Upstream	5.89
rs2233409	G > A	34944021	-420	5´Upstream, HMCS	22.75
rs1957106	G > A	34943521	81	Asp27Asp, HMCS, TBBS	31.38
rs3138054	C > T	34942058	1544	Intronic, TFBS	16.65
rs8904	G > A	34940968	2634	3´UTR, HMCS	40.44
rs696	C > T	34940844	2758	3´UTR	39.80

5.2. Repeated measurements of CRP concentrations

CRP concentrations were analysed in 5,813 blood samples from 1,003 MI patients. The highest CRP concentrations were observed in Barcelona, while the lowest were observed in Helsinki. Mean CVs were between 59.3 and 52.0 with Barcelona showing the highest and Stockholm the lowest values. CRP displayed a skewed distribution and was thus log-transformed. Mean CRP concentrations, mean CV, geometric mean and geometric standard deviation stratified by city is shown in Table 25.

Table 25: Repeated measurements of CRP levels (mg/l) based on 5,813 blood samples collected from 1,003 myocardial infarction survivors from 6 European cities.

City	Blood samples	Number of patients	Mean	Mean CV* [%]	Geometric mean	Geometric standard deviation
Helsinki	1155	195	1.98	53.6	1.18	2.60
Stockholm	1168	197	2.86	52.0	1.41	3.03
Augsburg	1144	200	2.26	56.8	1.18	2.83
Rome	741	134	2.56	52.3	1.34	2.90
Barcelona	1119	169	3.52	59.3	1.98	2.69
Athens	486	108	2.52	55.9	1.34	2.88

*CVs were calculated for the repeated measurements of the individuals.

5.3. Association of *CRP* variants with CRP plasma concentrations

Seven SNPs were genotyped in the *CRP* gene. Association analysis was performed using mixed effects models with random patient effects. To model the genetic effects, an unconstrained approach was used. Therefore SNPs were classified into three groups with the most common homozygous genotype for each polymorphism as the reference category. For two SNPs (rs1800947 and rs3093068) the homozygotes for the minor allele were pooled with the heterozygotes in order to avoid conclusions from low numbers. rs3091244 was analysed assuming an dose-dependent effect for the major allele as shown in previous studies and numbers where too low for unconstrained modeling.[98]

All analyses were done crude (Table 26) and with the final main effect model adjusting for potential confounders including city, sex, BMI, age, packyears of smoking, systolic blood pressure, total cholesterol, number of MIs, health status, HbA1c, NT-proBNP, chronic obstructive pulmonary disease, chronic bronchitis and hypertension (Table 27). To correct for multiple testing, the significance level was reduced to $\alpha=0.0004$, corresponding to an overall significance level of $\alpha=0.05$.

In both, the crude and the main effect model, the minor alleles of two *CRP* variants, rs1800947 and rs1205, were associated with significant lower CRP concentrations. MI survivors' homozygote or heterozygote for the G allele of rs1800947 showed a 35% decrease in their geometric mean compared to homozygotes of the C allele. For rs1205, homozygote carriers of the T allele had a 38% lower geometric mean while heterozygotes had a 24% lower geometric mean compared to homozygotes of the T allele. Adjusting for covariables did not change these results, however, marginal lower values were observed (Table 27).

Additionally, a trend to higher CRP concentrations was observed for the minor alleles of rs3093068, the triallelic rs3091244 and rs2794521. However, these effects were not reaching the level of statistical significance, especially after adjusting for covariates.

Table 26: Association analysis of *CRP* SNPs with CRP plasma concentrations (crude analysis). CRP concentration is given as the geometric mean with the appropriate geometric standard deviation in brackets. Bold characters indicate significances after correction for multiple testing.

Allelic Variant	Plasma samples (Number of patients)	CRP concentration	% change of geometric mean [95% CI]	*p*-value
rs1205				
TT	621 (105)	1.02 (2.78)	-38.39 [-49.08; -25.46]	**2×10^{-8}**
TC	2643 (451)	1.26 (2.88)	-24.44 [-32.84; -15.00]	
CC	2513 (439)	1.66 (2.77)	Reference	
rs3093068				
CC+CG	738 (132)	1.73 (2.94)	33.40 [12.93; 57.59]	7×10^{-4}
GG	4987 (856)	1.35 (2.83)	Reference	
rs1800947				
GG+GC	722 (123)	0.94 (2.73)	-34.88 [-45.09; -22.76]	**8.6×10^{-7}**
CC	5009 (865)	1.47 (2.85)	Reference	
rs1130864				
AA	592 (102)	1.40 (2.51)	11.40 [-8.42; 35.52]	0.135
AG	2560 (438)	1.47 (2.81)	12.55 [-0.19; 26.91]	
GG	2585 (448)	1.30 (2.99)	Reference	
rs1417938				
AA	597 (103)	1.42 (2.52)	11.97 [-7.83; 36.03]	0.181
TA	2569 (440)	1.46 (2.80)	11.10 [-1.39; 25.18]	
TT	2621 (455)	1.31 (2.99)	Reference	
rs2794521				
CC	359 (63)	1.67 (2.77)	25.73 [-0.85; 59.45]	0.043
TC	2296 (399)	1.49 (2.97)	12.91 [0.35; 27.04]	
TT	3116 (533)	1.28 (2.77)	Reference	
rs3091244				
TT+AT+AA	849 (150)	1.52 (2.55)	28.90 [8.44; 53.22]	0.005
GT+GA	2817 (482)	1.46 (2.89)	17.59 [3.90; 33.07]	
GG	2097 (362)	1.25 (2.94)	Reference	

Coefficients for the clinical covariates revealed that higher BMI was associated with higher concentrations of CRP ($p<10^{-23}$). Concentrations of total cholesterol ($p<10^{-5}$) and NT-proBNP ($p<10^{-5}$) had a similar effect, although the correlation was weaker. Additionally, smoking was significantly associated with CRP concentrations ($p<10^{-5}$).

Table 27: Association analysis of *CRP* SNPs with CRP plasma concentrations (main effect model). CRP concentration is given as the geometric mean with the appropriate geometric standard deviation in brackets. Bold characters indicate significances after correction for multiple testing.

Allelic Variant	Plasma samples (Number of patients)	CRP concentration	% change of geometric mean [95% CI]	*p*-value
rs1205				
TT	621 (105)	1.02 (2.78)	-34.36 [-44.58; -22.25]	**7×10^{-8}**
TC	2643 (451)	1.26 (2.88)	-20.85 [-28.69; -12.15]	
CC	2513 (439)	1.66 (2.77)	Reference	
rs3093068				
CC+CG	738 (132)	1.73 (2.94)	23.29 [6.48; 42.76]	0.005
GG	4987 (856)	1.35 (2.83)	Reference	
rs1800947				
GG+GC	722 (123)	0.94 (2.73)	-33.06 [-42.41; -22.20]	**1.8×10^{-7}**
CC	5009 (865)	1.47 (2.85)	Reference	
rs1130864				
AA	592 (102)	1.40 (2.51)	6.60 [-10.16; 26.49]	0.257
AG	2560 (438)	1.47 (2.81)	9.19 [-1.76; 21.36]	
GG	2585 (448)	1.30 (2.99)	Reference	
rs1417938				
AA	597 (103)	1.42 (2.52)	5.96 [-10.59; 25.59]	0.275
TA	2569 (440)	1.46 (2.80)	8.89 [-1.95; 20.92]	
TT	2621 (455)	1.31 (2.99)	Reference	
rs2794521				
CC	359 (63)	1.67 (2.77)	20.77 [-1.68; 48.34]	0.020
TC	2296 (399)	1.49 (2.97)	14.28 [2.85; 26.98]	
TT	3116 (533)	1.28 (2.77)	Reference	
rs3091244				
TT+AT+AA	849 (150)	1.52 (2.55)	19.95 [3.10; 39.55]	0.011
GT+GA	2817 (482)	1.46 (2.89)	15.86 [3.93; 29.15]	
GG	2097 (362)	1.25 (2.94)	Reference	

Covariates and *CRP* polymorphisms explained 27% of the inter-individual variability in CRP concentrations. BMI explained the biggest proportion (11%), followed by smoking (3%). rs1205, rs1800947, total cholesterol and NT-proBNP explained each 2%. All other covariates contributed less than 1%. None of the SNP×covariate interaction terms introduced into the main effect model turned out to be significant.

Since population stratification can be a problem in genetic association studies, the results for rs1800947 and rs1205 were stratified by city (Figure 17) and formally tested for heterogeneity. Testing for heterogeneity reveals, that differences between the stratified estimates were not statistically significant (p>0.1).

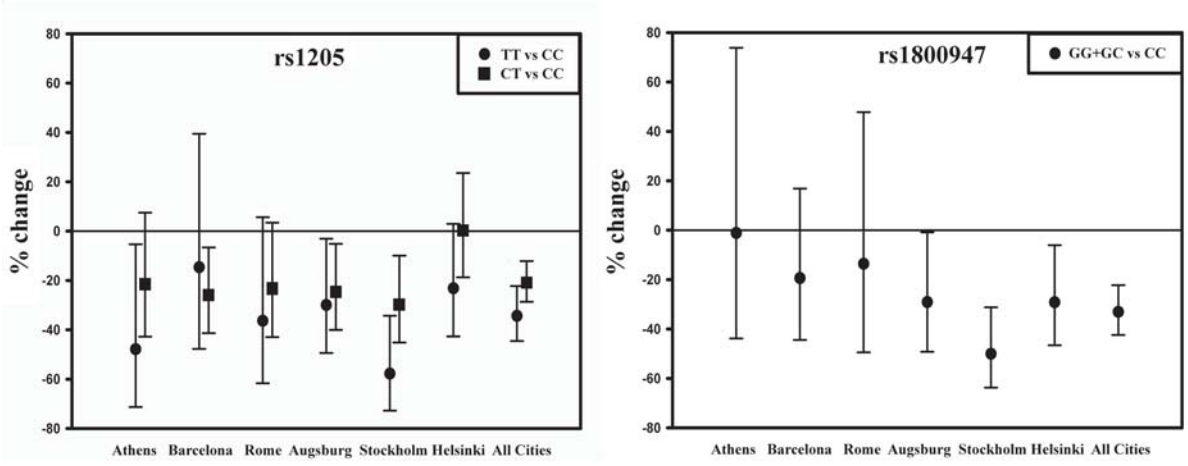

Figure 17: Association results stratified by city for rs1205 and rs1800947. The % change of the geometric mean of CRP is given together with 95 percent CIs for the minor allele versus the major allele and for the heterozygotes versus the major allele. For rs1800947 the minor allele homozygotes and heterozygotes were pooled due to low numbers.

5.4. Association of SNPs involved in the inflammatory pathways with CRP plasma concentrations

Heritability of CRP concentrations may not only arise from the *CRP* gene itself, but may also involve pro- and anti-inflammatory pathways. To investigate the association between genetic variants acting upstream in the inflammatory cascade with differences in CRP concentrations, 106 polymorphisms and one deletion/insertion variant were analysed in the selected candidate genes. However, none of those polymorphisms was associated with significant differences in CRP concentrations after correction for multiple testing. A trend was observed for the minor

allele of a *NFKBIA* variant, rs696 (β=-0.18; *p*=0.04), located in the untranslated region of exon 6. Detailed information on all results is listed under point 7.3.

5.5. Association of *CRP* haplotypes with CRP plasma concentrations

Since only *CRP* variants were significantly associated with CRP concentrations, haplotype reconstruction was restricted to polymorphisms of the *CRP* gene. One highly preserved LD block covering the whole gene was observed with D´>0.95 (Figure 18). High correlation was detected between rs1417938, rs1130864 and the A allele of rs3091244 (r^2>0.99). The T allele of the rs3091244 was additionally correlated with the C allele of the rs3093068 (r^2>0.84).

Haplotype reconstruction showed that only for two patients the best guess haplotype probabilities ranged between 80% and 90%, whereas all other best guess haplotype pairs occurred with probabilities greater than 95%. This indicates that no substantial loss is expected due to the modelling of best guess haplotypes only. Due to the nearly complete correlation with rs1417938, rs1130864 was excluded from haplotype reconstruction.

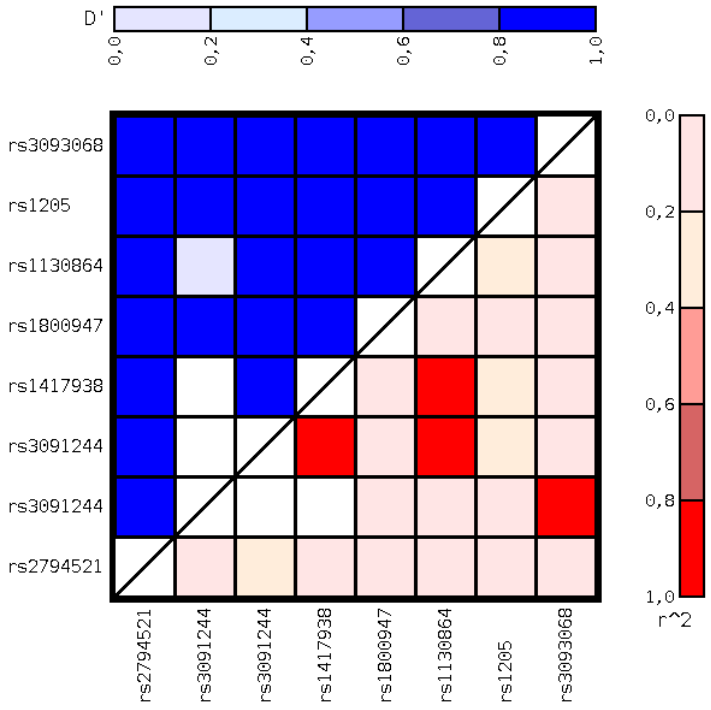

Figure 18: Pairwise LD *D´*and r^2 plots of seven SNPs across the *CRP* gene.

Haplotypes showed a homogeneous distribution for nearly all cities, except for Helsinki (Figure 19).

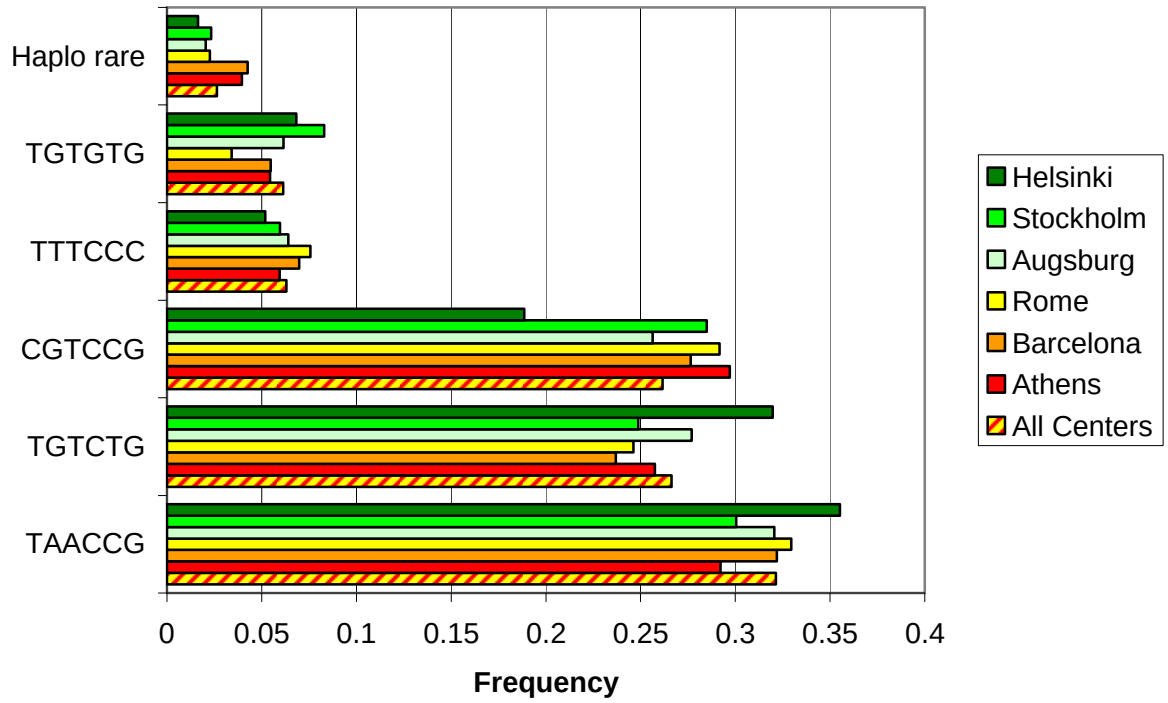

Figure 19: Distribution of haplotype frequencies across 6 European Cities.

Three major haplotypes were observed with frequencies ranging from 26.16% to 32.15%. The haplotype carrying the major alleles at all loci, had a frequency of only 1.24% and was thus pooled into the group "haplo rare". Results of the association analysis with *CRP* haplotypes are shown in Table 28. The findings of the single SNP analysis were supported by the haplotype analysis. H5, the haplotype containing the minor alleles of both, rs1205 and rs1800947, was significantly associated with lower CRP concentrations ($p<10^{-6}$). Additionally, a trend to lower CRP concentrations was observed for H2, carrying the minor allele of rs1205.

Table 28: Association analysis of *CRP* haplotypes with CRP plasma concentrations. Bold characters indicate significance after correction for multiple testing with a significance level of α=0.0004. SNPs are shown in the following order (1-6): rs2794521, rs3091244, rs1417938, rs1800947, rs1205 and rs3093068.

Haplotype	SNP						Frequency [%]*	% change of geometric mean† [95% CI]	*p*-value†
	1	2	3	4	5	6			
H1	T	A	A	C	C	G	32.15 [29.21-35.52]	Reference	Reference
H2	T	G	T	C	T	G	26.63 [23.64-31.97]	-13.06 [-21.18; -4.11]	0.003
H3	C	G	T	C	C	G	26.16 [18.85-29.70]	6.18 [-3.73; 17.12]	0.232
H4	T	T	T	C	C	C	6.3 [5.19-7.58]	15.03 [-1.67; 34.55]	0.079
H5	T	G	T	G	T	G	6.14 [3.41-8.29]	-33.63 [-43.27; -22.37]	**4×10^{-7}**
Haplo rare							2.63 [1.64-4.24]	19.72 [-3.50; 48.53]	0.113

*mean and range in brackets, †main effect model

5.6. Analysis of intra-individual variability

Substantial between-subject and within subject-variability was observed for CRP in all cities. The proportion of subjects whose CRP levels were always above 3 mg/l varied between cities (6% in Athens, 14% Barcelona, 10% Rome, 7% Augsburg, 7% Helsinki and 11% Stockholm). Similarly, the proportion of subjects whose CRP concentrations varied between below and above 3 mg/l differed between centers (40% in Athens, 52% Barcelona, 38% Rome, 39% Augsburg, 32% Helsinki and 38% Stockholm). Within-person variability for CRP was high in the repeated visits, even in cities that indicated very low variability in blind duplicate samples.

The differences in the variability within an individual as a marker of the individual response may be influenced by genetic polymorphisms. To assess differences in intra-individual

variability, mean and variance were modelled simultaneously allowing for different error variances between genotype groups. Homogeneity of variances was then tested comparing the two models with equal and unequal variances. Significant results are summarized in Table 29.

The minor alleles of several variants were significantly associated with either greater or smaller intra-individual variability. The minor alleles of rs3774964 *(NFKB1*), rs2043055 (*IL18*), rs2070011 and rs2070006 (both *FGA*), rs11227248 (*RELA*), rs2794521 (*CRP*), rs1800796 (*IL6*) and rs1800894 (*IL10*) were associated with greater intra-individual variability after correction for multiple testing while rs732072 (*RELA*), rs2070016 (*FGA*), rs1130864 and rs1417938 (both *CRP*), rs1049636 (*FGG*), rs187238 (*IL18*), rs2857713 (*LTA*), rs1800890 and rs6676671 (both *IL10*), rs4648004 (*NFKB1*), rs1927907 (*TLR4*) and rs3093661, rs3093664 and rs361525 (*TNFA*) were associated with smaller intra-individual variability. For rs1800788, located in the *FGB* gene, being heterozygote was associated with greater intra-individual variability while homozygotes for the minor allele showed smaller values.

For rs2069827 within the *IL6* gene, being heterozygote was associated with smaller while being homozygote was associated with greater intra-individual variability. However, in addition to rs1417938 and rs1130864 in the *CRP* gene, rs2070006 and rs2070011 in the *FGA* gene and rs1800890 and rs6676671 in the *IL10* gene were highly correlated (r^2>0.95).

Table 30: Significant results of the analysis of intra-individual variability.

Gene	dbSNP identifier	Exchange (X > Y)	Ratio of intra-individual variability for XY compared to reference (XX)	Ratio of intra-individual variability for YY compared to reference (XX)	*p*-value
CRP	rs2794521	T > C	1.11	1.36	2.5×10^{-4}
CRP	rs1417938	T > A	0.99	0.71	1.4×10^{-5}
CRP	rs1130864	G > A	0.98	0.70	6.1×10^{-6}
FGA	rs2070016	A > G	0.84	0.54	1.1×10^{-6}
FGA	rs2070006	C > T	0.96	1.31	8.3×10^{-7}
FGA	rs2070011	C > T	0.93	1.31	1.2×10^{-7}
FGB	rs1800788	C > T	1.26	0.82	3.2×10^{-8}
FGG	rs1049636	A > G	1.08	0.71	4.8×10^{-7}
IL10	rs1800890	A > T	0.97	0.74	2.9×10^{-5}
IL10	rs6676671	T > A	0.98	0.74	2.8×10^{-5}
IL10	rs1800894*	C > T	1.42		1.2×10^{-6}
IL18	rs2043055	A > G	1.18	1.25	6.8×10^{-5}
IL18	rs187238	C > G	0.89	0.71	6.4×10^{-5}
IL6	rs1800796*	G > C	1.32		1.8×10^{-5}
IL6	rs2069827	G > T	0.62	1.50	2.6×10^{-19}
LTA	rs2857713	T > C	0.96	0.71	2.5×10^{-5}
NFKB1	rs4648004	A > G	1.04	0.80	2.6×10^{-4}
NFKB1	rs3774964	A > G	1.20	1.04	1.8×10^{-4}
RELA	rs732072	G > A	0.97	0.40	3.7×10^{-4}
RELA	rs11227248	C > T	0.97	1.36	8.7×10^{-7}
TLR4	rs1927907	C > T	0.80	0.99	3.2×10^{-5}
TNFA	rs361525*	G > A	0.70		1.2×10^{-5}
TNFA	rs3093661*	G > A	0.64		3.7×10^{-6}
TNFA	rs3093664*	A > G	0.71		3.7×10^{-8}

*heterozygotes and minor allele homozygotes were pooled for analysis

5.7. Sensitivity analysis

CRP concentrations are also triggered by medication use. To assess if and how the main results of this thesis are sensitive to model choice a sensitivity analysis was performed including medication data in addition to the covariates of the main effect model. In general, a sensitivity analysis is the varying of model input parameters and observing the relative change in model response.

Medication variables considered to impact the average CRP concentrations included ACE-inhibitors, analgesics for systemic use, lipid lowering medication, anti-infectives for systemic use, anti-inflammatory and antirheumatic agents for systemic use, diuretics and nitrates.

5.7.1. Association of CRP variants with CRP plasma concentrations including medication

The minor alleles of two variants within the *CRP*, rs1800947 and rs1205, were significantly associated with lower CRP concentrations in the main effect model. The result of the sensitivity analysis in comparison to the main effect model for those two SNPs is summarized in Table 30.

Table 30: Sensitivity analysis including medication data in comparison to main effect models for rs1205 and rs1800947.

SNP	Genotype	Estimate*	*p*-value*	Estimate†	*p*-value†
rs1205	TT	-0.484	7×10^{-8}	-0.416	5.3×10^{-8}
	TC	-0.280		-0.241	
	CC	Reference			
rs1800947	GG+GC	-0.429	1.8×10^{-7}	-0.395	2.7×10^{-7}
	CC	Reference			

* main effect model; † main effect model including medication

Introducing medication data did not change the observed estimates exceedingly, however, slightly lower estimates where observed. Additionally, covariates where hardly ever affected. Like in the main effect model, covariates associated with CRP concentrations were BMI ($p<10^{-23}$), total cholesterol ($p<10^{-4}$), NT-proBNP ($p<10^{-4}$) and smoking ($p<10^{-5}$) after applying the significance level of $\alpha=0.0004$. Regarding medication, only treatment with analgesics ($p=4.4\times10^{-7}$) and anti-inflammatory and antirheumatic agents ($p=3.5\times10^{-4}$) was significantly associated with lower CRP concentrations.

5.7.2. Analysis of intra-individual variability including medication

The variability within an individual, the magnitude of pending around its own mean can be triggered by extreme and outlying values wrongly suggesting an association. Therefore, a sensitivity analysis was performed, excluding outlying subject variances according to Cochran's test, and outlying patient mean values using Reed's criterion. Results of the sensitivity analysis are summarized in Table 31.

Table 31: Sensitivity analysis of intra-individual variability.

Gene	dbSNP identifier	Exchange (X > Y)	Ratio of intra-individual variability for XY compared to reference (XX)	Ratio of intra-individual variability for YY compared to reference (XX)	*p*-value
CRP	rs2794521	T > C	1.02	1.80	1.2×10^{-12}
CRP	rs1417938	T > A	0.92	0.62	1.2×10^{-8}
CRP	rs1130864	G > A	0.91	0.63	1.2×10^{-8}
FGA	rs2070016	A > G	0.90	0.59	0.001
FGA	rs2070006	C > T	0.87	1.14	3.5×10^{-5}
FGA	rs2070011	C > T	0.83	1.07	1.2×10^{-5}
FGB	rs1800788	C > T	1.00	0.59	6.8×10^{-6}
FGG	rs1049636	A > G	0.97	0.72	1.8×10^{-4}
IL10	rs1800890	A > T	1.07	0.96	0.135
IL10	rs6676671	T > A	1.10	0.97	0.071
IL10	rs1800894*	C > T	1.30		4.5×10^{-4}
IL18	rs2043055	A > G	1.19	0.89	5.0×10^{-6}
IL18	rs187238	C > G	0.98	0.66	1.8×10^{-5}
IL6	rs1800796*	G > C	1.30		7.0×10^{-5}
IL6	rs2069827	G > T	0.65	1.48	7.8×10^{-16}
LTA	rs2857713	T > C	0.97	0.65	8.8×10^{-8}
NFKB1	rs4648004	A > G	0.77	0.63	1.9×10^{-13}
NFKB1	rs3774964	A > G	0.94	1.05	0.223
RELA	rs732072	G > A	1.06	0.39	3.0×10^{-4}
RELA	rs11227248	C > T	3.74	3.15	3.8×10^{-8}
TLR4	rs1927907	C > T	0.71	1.14	2.3×10^{-11}
TNFA	rs361525*	G > A	0.86		0.061
TNFA	rs3093661*	G > A	0.60		6.7×10^{-7}
TNFA	rs3093664*	A > G	0.80		3.5×10^{-4}

*heterozygotes and minor allele homozygotes were pooled for analysis

After excluding extreme values, rs361525 (*TNFα*), rs2070016 (*FGA*), rs3774964 (*NFKB1*) and the two correlated SNPs rs1800890 and rs6676671 in the *IL10* gene failed to obtain statistical significance. Interestingly, the effects of variants in the *CRP* gene itself became even clearer, while for others, like for variants in the fibrinogen genes, the effect was weakened.

6. Discussion

To test the hypothesis whether genetic variation in innate immunity genes affect CRP concentrations, 114 polymorphisms within thirteen candidate genes were selected and their influence on the inter-individual as well as the intra-individual changes in CRP concentrations was analysed in a sample of 1,003 MI survivors using a repeated measurement design. Within those genes, only two polymorphisms, rs1800795 and rs1205, both located in the *CRP* gene itself were found, where the minor alleles were strongly associated with lower concentrations of CRP. These results were supported by the haplotype analysis. Each SNP explained 2% of the inter-individual variability of CRP concentrations. Regarding intra-individual variability, the minor alleles of several variants of innate immunity genes were significantly associated with changes in the individual variability of CRP concentrations.

6.1. Human genetic association studies

Epidemiological studies demonstrate that inherited factors play a major role in the development and prognosis of several complex traits. The challenge of human genetic association studies is to identify patterns of polymorphisms that vary systematically between individuals with different states of a certain trait or disease. Thereby, the effects of these alleles can be risk-enhancing or protective.[149]

However, genetic association studies have often produced contradictory evidence, relating to problems with experimental design, statistical analysis, study size, power and replication. These major methodological flaws may explain the lack of consistent evidence for an effect from polymorphisms. Thousands of associations between candidate gene polymorphisms and the risk of outcome have so far been published. However, in many cases it has proved difficult to reproduce initial results. In a meta-analysis of 370 studies examining 36 genetic associations for various disease outcomes the correlation between the strength of association

reported in the first publication was only poorly related to that in subsequent studies, a phenomenon known as „winners curse". In addition, the effect tended to diminish in second and subsequent reports.[150] Additionally, a comprehensive review of 166 possible genetic associations that had been studied at least three times found only six which had been consistently replicated.[151]

Therefore a number of research bodies have published guidelines and checklists on the conduct of genetic association studies.[152-155] Attributes of reliable genetic association studies include a plausible biological context, low *p*-values, rigorous phenotyping and genotyping as well as appropriate statistical analysis.

The most likely explanation for the poor replication of the studies identified is the frequent publication of false-positive associations. The majority of probably true positive reports have found only very modest effects of the candidate gene with odds ratios often less than 2. This means that genetic association studies need to test thousands of individuals to have a reasonable chance of finding an effect. It has been calculated that even a *p*-value of 0.001 in a study of a highly probable candidate gene in 200 cases and 200 controls would still be more likely to be a false-positive result.[156] Therefore, adequate study size and study power are essential. Other fundamental issues are the significant threshold of a true association, especially in light of multiple-hypothesis testing, technical reliability, the problem of population stratification and admixture and the difficulty in precisely defining and measuring the phenotype of interest.

6.2. Genotyping and description of polymorphisms

Technical problems with genotyping methods may also lead to false-positive and inconsistent results. In this thesis, 114 polymorphisms were successfully genotyped in the selected candidate genes. For ten SNPs, the assay design failed or no valid assay could be established. During SNP selection, preferably validated polymorphisms with a frequency >5% were

selected from public databases. Nonetheless, three SNPs turned out to be completely monomorphic and six SNPs had a minor allele frequency of less than 1%.

To assure that samples do not get mixed up during genotyping 30% of the samples were double checked, demonstrating a very low discrepancy rate (0.18%). Since samples can also get mixed up during recruitment, sex determination was performed with validated genotyping assays. The assay result did not match the patient information stored in the database for nine samples, which were excluded from further analysis. However, differences could only be detected between sexes, mixed up samples of the same sex would not be detected by this method.

6.2.1. Deviations from Hardy-Weinberg equilibrium

Deviations from HWE can be due to inbreeding, population stratification or selection. Apparent deviations from HWE can also arise because of a mutated PCR-primer site, duplicated regions in the genome or because of a tendency to miscall heterozygotes as homozygotes. Therefore, testing for HWE can be an important data quality check. However, deviations from HWE can also be a symptom of disease association, especially in cases.[157]

Altogether, seventeen SNPs showed deviations from HWE, without any indication of a genotyping error. They were not excluded from further analysis, because the present study is a highly selected group of MI survivors, in which SNPs can violate HWE because of the selection process. Remarkably, HWE deviations were not randomly distributed, but were mainly restricted to two genes, *IL-10* and *NFKB1*. Thirteen out of the seventeen SNPs showing departures from HWE were located in one of those two genes. Polymorphisms in high linkage disequilibrium with each other, consistently both failed HWE. These observations can be due to chance, but it is also possible that they reflect population substructure, admixture or selection at this two loci. In addition, the possibility remains that duplicated regions were genotyped although PCR primers were blasted against the genome.

Interestingly, in a previous study genotyping nine candidate genes, including *NFKB1* and *TLR4*, HWE departures were also restricted to the *NFKB1* gene.[116]

6.2.2. Distribution of genotype frequencies and population stratification

Population stratification is one of the major problems in human genetic association studies, especially when analysing data from multicenter studies. Population stratification refers to differences in allele frequencies between centers due to systematic differences in ancestry rather than association of genes with disease,[158] leading to both false positive and false negative results.[159] If subjects of different centers are genetically different at a variety of loci and the prevalence of the trait of interest is higher in one of these centers, the center with the highest prevalence of the trait will be over-represented and thus will be "associated" with this trait. For example a higher allele frequency of a certain allele in Barcelona, the center with the highest CRP values, may lead to a false positive association of this allele with higher CRP concentrations. From an epidemiological point of view, this is an example of confounding.

A study analysing 75 markers to infer relationships between populations from four continents found the greatest differentiation along continental lines. However, they noted that within continents, the branches also divided groups along the pre-existing ethnic lines, although these differences were smaller than across continents.[160] Therefore in this thesis, genotype frequencies were also analysed stratified by city and tested for differences in their frequencies between cities. Nearly half of the polymorphisms genotyped (53 out of 114 polymorphisms) showed significant differences in their genotype frequencies between European cities.

There are several approaches of dealing with population stratification. It has been argued that in case-control studies, the effects of stratification can be eliminated simply by carefully matching cases and controls according to self-reported ancestry and geographical origin.[158] Another method of quantitatively estimating the amount of stratification is the method of Genomic Control.[161] This method examines the distribution of association statistics between

unlinked genetic variants typed in different centers or in cases and controls. The statistic at a candidate allele being tested for association can then be compared with the genome-wide distribution of statistics for markers that are probably unrelated to the trait of interest to assess whether the candidate allele stands out. The method of Genomic Control performs well, but it is limited in applicability to single-SNP analyses, and can be conservative in extreme settings. Additionally, it causes additional costs. Another option is to use structured association methods. These approaches are based on the idea of attributing the genomes of study individuals to hypothetical subpopulations, and testing for association that is conditional on this subpopulation allocation.[149] These theoretical constructs only imperfectly reflect reality. Another possibility are null SNPs, that can mitigate the effects of population structure when included as covariates in regression analyses.[149]

To avoid false positive results due to population stratification, in this thesis the simple method of stratification was used. To stratify a sample, subjects are grouped into relatively homogeneous subgroups and these strata are then analysed separately. In case of population stratification, results would systematically differ between strata. Therefore, main results of this thesis were analysed stratified by center and differences between strata were formally tested for heterogeneity.

6.3. Repeated measurements of CRP concentrations

A further problem in human genetic association studies is the difficulty in precisely measuring and defining the phenotype of interest. This may lead to a marked heterogeneity in patient populations which may explain some of the variation in findings.

Therefore, the present study is based on a common protocol and SOPs applied in all European cities. Site visits were conducted to ensure uniform procedures in all centers. The analyses of the inflammatory markers were done in one central laboratory and blinded duplicate samples

monitored the variability of procedures within centers (CVs ranging from 1.5 to 4.1). However, no duplicate samples were available from Athens.

6.4. Statistical analysis

The AIRGENE study applies a repeated measurement design in a potentially susceptible subgroup. CRP has a half-life of approximately 19 hours. Therefore, the repeated measurements taken once every 4 weeks can be regarded as being uncorrelated over time, but more similar within individuals than between individuals. To account for this special study design, data were analysed using mixed effects models with random patient effects. To model correlations between the repeated measures in each patient compound symmetry structure for the covariance matrix was used.

In epidemiologic association studies, it is also important to give appropriate attention to confounding variables. A confounding factor is an independent variable that distorts the association between another independent variable and the trait under study, as it is related to both. Therefore, detailed adjustment of potential confounders was performed.

6.4.1. Choice of genetic model

There is no generally accepted answer to the question of how to model genetic effects. Since it is not known what proportion of predisposing variants function dose-dependent, dominant, recessive or even over-dominant and also the degree of penetrance is not clear, an unconstrained model was used in this thesis, making no such assumptions. However, the lack of assumptions is paid with a minimal loss of power, due to the additional degree of freedom in statistical analysis.

6.4.2. Problem of multiple testing

One fundamental problem of human genetic association studies is that the genome is so large that patterns that are suggestive of a causal polymorphism could well arise by chance. To help distinguish causal from spurious signals, correction for multiple testing is essential. The frequentist paradigm of controlling the overall type-1 error rate sets a significance level α, mostly 5%, and all the tests that are planned should together generate no more than the probability α of a false positive. In simple settings the frequentist approach gives a practical solution for n SNP´s tested, the Bonferroni correction: α`≈ α/n. However, for tightly linked SNPs, the Bonferroni correction may be too conservative. A practical alternative, following the Bonferroni procedure, is to correct the global significance level of 5% for the number of independent tests, which can be calculated as the number of effective loci. In this thesis, the number of independent tests was calculated to be 64, times two for testing for inter- and intra-individual variability, leading to a significance level of α=0.0004, that was applied for all analyses.

6.5. Association of polymorphisms with CRP concentrations and variability

Altogether, 114 polymorphisms in thirteen candidate genes were genotyped and their influence on mean CRP concentrations as well as on its intra-individual variability was assessed. The observed heritability of CRP concentrations may result from polymorphisms at the *CRP* locus, but may also be influenced from upstream inflammatory pathways, which may involve pro- and anti-inflammatory genes. However, only a small number of studies investigating the impact of inflammatory genes on mean CRP concentrations and most of them were restricted to only one or two polymorphisms without consideration of the patterns of variation at the locus at a whole and the sample size was often small.

The second aim of this thesis was to investigate whether polymorphisms influence the intra-individual variability, a marker of the individual response. High within-subjects variability

has raised the question whether genetically determined subsets of the population may have an altered response to certain stimuli. An emerging hypothesis is that genetic influences on the inflammatory cascade regulation may cause some individuals to have a more vigorous response than others. A SNP in a candidate gene not associated with CRP mean concentrations might still be associated with its intra-individual variability, since this implies just a short time effect which could be rapidly compensated. However, the impact of genetic variation on variability of CRP has so far not been studied and therefore.

6.5.1. C-reactive protein

Seven SNPs were investigated within the *CRP* gene in 1,003 MI survivors. The minor alleles of two SNPs, rs1800947 and rs1205, were strongly associated with lower concentrations of CRP. Each SNP explained 2% of the inter-individual variability in CRP concentrations. Additionally, the effect remained stable after multivariable adjustment. These results are consistent with some[105-107;110] but not all pervious studies.[115] Table 32 summarizes the results of large epidemiologic studies conducted since 2005, mostly from the general population, restricted to SNPs investigated in this thesis. A recently published study found the minor alleles of rs1800947 and rs1205 being significantly associated with decreased risk of cardiovascular disease mortality.[114]

rs1800947, in the literature often described as 1059 G/C polymorphism in exon 2, is located in a segment with evolutionary conservation which suggests functional importance of this sequence although the SNP is leading to a silent amino acid exchange (Leu184Leu). Stratification by city as described in Figure 17 showed that the effect for the minor allele of the rs1800947 seemed to be stronger in Northern and Central Europe. However, differences between centers seen in stratified analysis were not statistically significant for both SNPs.

Table 32: Large (n > 500) epidemiologic studies investigating the association between *CRP* polymorphisms and CRP levels since 2005, restricted to SNPs investigated in this thesis. In case of multi-ethnicity, results and numbers are given for the sample of European descent.

First author	Study population	Study design (study size)	dbSNP	Effect of MA* on CRP levels
Suk *et al.*	PRINCE	Cohort (n=2,397)	rs1800947	↓
			rs1417938	↑
Davey Smith *et al.*	British Women's Heart and Health Study	Cross-sectional (n=3,529)	rs1800947	↓
Kovacs *et al.*	MI survivors	Case-control	rs2794521	↔
		(n=357/383)	rs1800947	↔
		Case only (n=208)	rs1130864	↔
			rs3091244	↑
Miller *et al.*	Women's Health Study	Cohort (n = 717)	rs2794521	↔
	PRINCE	Cohort (n = 1,110)	rs3091244	↑
	Physicians' Health Study	Cohort (n = 509)	rs1417938	↑
			rs1800947	↓
			rs1130864	↑
			rs1205	↓
Kardys *et al.*	Rotterdam Study	Cohort (n=6,658)	rs1130864	↑
			rs1205	↓
			rs3093068	↑
Kathiresan *et al.*	Framingham Heart Study	Cohort (n=1,640)	rs3091244	↑
			rs1417938	↔
			rs1130864	↔
			rs1205	↓
			rs1800947	↔
Crawford *et al.*	NHANES III	Cross-sectional (n=7,159)	rs3091244	↔
			rs1417938	↔
			rs1205	↔
			rs1800947	↓
Lange *et al.*	Cadiovascular Health Study	Cohort (n=3,941)	rs1417938	↑
			rs1800947	↓
			rs1205	↓
Eklund *et al.*	Cardiovascular Risk in Young Finns Study	Cohort (n=2,283)	rs2794521	↑
			rs3091244	↑
			rs1800947	↓
			rs1130864	↑
			rs1205	↓
Zhang *et al.*	CHOICE	Cohort (n=504)	rs1417938	↔
			rs1205	↓

*Minor allele

rs1205 demonstrated a dose-dependent effect with each copy of the minor allele increasing the difference in CRP concentrations. rs1205 is located in the 3`flanking region or the 3`UTR, depending on the transcript, a region possible controlling RNA cleavage, stability, export and intracellular localization. The frequency of the rs1205 tends to differ between populations. Using the HapMap Catalogue, frequencies of the minor allele of this SNP are ranging from 0.15% to 0.73% in different populations. In the present European sample, a frequency for the minor allele ranging from 5% in Barcelona to 15% in Helsinki ($p<0.05$) was observed.

Recently published studies found a probably functional triallelic polymorphism (rs3091244) in the promoter of the *CRP* gene associated with CRP plasma concentrations.[98] This finding was not replicated in this thesis after correction for multiple testing, possibly due to the selected population of MI survivors.

The strength of LD within the *CRP* gene was reflected by restricted haplotype diversity. Five haplotypes with a frequency >5% were observed. Similar to the association results of the single SNPs, haplotype H5, tagged by SNPs rs1205 and rs1800947, were associated with the lowest plasma concentrations of CRP. This is consistent with the result of a recently published mendelian randomisation study.[110] Additionally, a population-based study showed that this haplotype was associated with the lowest concentrations of CRP.[106] Two other studies also found H2 and H5 being significantly associated with lower CRP concentrations, however, after correction for multiple testing, the significant result for H2 could not be replicated.[107;108]

Regarding intra-individual variability, the minor allele of rs2794521 was associated with greater, while the minor alleles of the two correlated SNPs rs1417938 and rs1130864 was associated with decreased intra-individual variability. After excluding outlying observations in sensitivity analysis, the effect of these *CRP* SNPs became even stronger.

6.5.2. Fibrinogen gene cluster

Human Fibrinogen is, like CRP, an acute phase reactant synthesized in the liver which is composed of three pairs of nonidentical polypeptide chains linked by disulfide bonds. Its role as a predictor of cardiovascular risk has been established by several epidemiologic studies.[60;162] Moreover, fibrinogen is crucial in the process of thrombus formation and may have a direct role in atherogenesis and thrombogenesis by acting as a bridging molecule for many types of cell-cell adhesion events critical for atherogenesis.[163] It also seems to be chemotactic for smooth muscle cells[164] and to affect the stability of atheromatous plaque.[165] Fibrinogen is a mediator of platelet aggregation and a determinant of plasma viscosity[166], it plays a central role in the coagulation cascade with a critical impact in the formation of fibrin clots following the rupture of an atherosclerotic plaque.[167] Several SNPs in all three fibrinogen genes have been associated with variation in plasma fibrinogen levels.[168;169]

Altogether 21 SNPs were investigated in the fibrinogen gene cluster. None of them had an influence on mean concentrations of CRP, but the minor alleles of the correlated SNPs rs2070011 and rs2070006, both in the gene coding for the alpha chain were associated with greater intra-individual variability while rs2070016 in the *FGA* and rs1049636 in the *FGG* were associated with decreased variability. The effect for rs1800788, located in the *FGB* was less clear. Being heterozygotes was associated with increased intra-individual variability while homozygotes for the minor allele showed decreased variability.

However, when excluding extreme values the effect for rs2070016 in the *FGA* failed to obtain significance. Therefore further examinations have to clarify the role of this SNP. For rs1800788 in the *FGB* gene, the picture became more distinct, with the heterozygotes showing no difference at all while homozygotes for the minor allele showed even smaller intra-individual variability. Therefore, the effect of being heterozygote for this SNP seen in the main effect model was likely only due to extreme values.

6.5.3. Interleukin-6

IL-6 is a pro-inflammatory multifunctional cytokine that plays a central role in host defense due to its wide range of immune and haematopoietic activities and its potent ability to induce the acute phase response. IL-6 is synthesized predominately by activated lymphocytes[3] but also by other activated vascular cells, and it is released into the bloodstream in minute quantities. CRP and IL-6 form an inflammatory axis. CRP, like fibrinogen, is synthesized in the liver particularly in response to IL-6 and is released in much higher concentrations, thus amplifying the inflammatory signal of IL-6.[170] Additionally, plasma concentrations of IL-6 are related to subsequent atherosclerotic events in some studies.[171]

IL-6 induces *CRP* gene expression in the liver during the acute phase response. There may be multiple genetic influences on CRP concentrations. The *CRP* promoter contains several IL-6 response elements.[172] Although no evidence exists, genetic variation within this response could affect transcription factor binding. Genetic variation that affects IL-6 expression could also indirectly influence CRP plasma concentrations by varying IL-6 levels.

Eight SNPs were investigated in the *IL-6* gene. None of them was associated with inter-individual variability after correction for multiple testing. Most studies determining the relationship of *IL-6* variants on CRP levels focused on two SNPs in the promoter region of the *IL-6* gene, rs1800795 and rs1800796. A study of CRP concentrations among 588 post-menopausal women from 98 British families found an association with the minor allele of rs1800795, with individuals carrying these variants having higher CRP concentrations.[100] The two promoter variants were also studied in relation to CRP concentrations in a cohort of 495 postmenopausal women where a significant effect was observed for the minor allele of rs1800796 but not for rs1800795.[173] Other studies have shown only weak effects of those two polymorphisms with CRP concentrations. A study of 92 individuals from the general population found the CC genotype of rs1800795 to be associated with lower levels of CRP.[174]

Another study observed an association of rs1800795 with IL-6 but not with CRP concentrations in 111 patients with multi-vessel coronary artery disease.[175] Similar to the results observed in this thesis, no association between the minor allele of rs1800795 and differences in CRP concentrations was observed in 160 patients with CHD.[117]

Although no effect was observed for inter-individual variability of CRP concentrations, rs1800796 was associated with significantly increased variability. The effect remained stable after excluding outlying observations. The greatest effect on the intra-individual variability of CRP concentrations was observed for rs2069827 within the *IL-6* promoter. Being heterozygote was associated with smaller while being homozygote was associated with greater intra-individual variability. Excluding outlying observations did not affect this result.

6.5.4. Interleukin-10

IL-10, also known as human cytokine synthesis inhibitory factor, is an anti-inflammatory cytokine produced by activated lymphocytes and monocytes. IL-10 inhibits the production of several proinflammatory cytokines and also induces release of the IL-1 receptor antagonist.[176] Direct inhibition of inflammatory gene expression by IL-10 is modulated in part by AU-rich elements (ARE) present in the 3' untranslated region of sensitive genes. IL-10 effectively destabilizes the mRNA of genes with ARE motifs.[177] One way of down-regulating the acute phase reaction is releasing IL-10 by the Kupffer cells which results in suppression of local IL-6 production.[178] By this mechanism, IL-10 might affect CRP production.

IL-10 serum levels have been shown to be associated with improved systemic endothelial vasoreactivity in patients with elevated CRP levels, demonstrating that the balance between pro- and anti-inflammatory mediators is a major determinant of endothelial function in patients with coronary artery disease (CAD).[179] Additionally, it has been shown that IL-10 blocks atherosclerotic events *in vitro* and *in vivo*.[180] Several studies reported associations between *IL-10* polymorphisms and IL-10 concentrations.[181-183] Lack of association between

IL-10 polymorphisms and CAD was observed in a case-control study of Japanese individuals.[184] Similarly, no association was observed between *IL-10* polymorphisms and risk of CAD or MI in a German case-control study.[185]

None of the SNPs investigated in the *IL-10* gene was associated with differences in mean CRP levels. The minor alleles of the correlated SNPs rs1800890 and rs6676671 were associated with decreased variability, while the minor allele of rs1800894 in the promoter region of the *IL-10* was associated with increased variability. However, in sensitivity analysis, only a borderline effect for rs1800894 remained while rs1800890 and rs6676671 completely failed to obtain significance. Therefore, future studies investigating the effect of *IL-10* polymorphisms on the variability of CRP are warranted.

6.5.5. TNF gene cluster

Tumor necrosis factor alpha and lymphotoxin alpha are multifunctional proinflammatory cytokines that effect lipid metabolism, coagulation, insulin resistance, and endothelial function and are involved in apoptosis and cell survival.[186]

Both cytokines belong to the TNF family of ligands. All members of this family consist of three polypeptide chains made up of three identical subunits. TNFα and lymphotoxin-α share two receptors (the 55-kd and 75-kd TNF receptors) with similar affinity. Binding to the 55-kd receptor is linked to activation of the caspase pathway, resulting in programmed cell death, while cell activation through activation of the NF-κB system is mainly mediated by the 75-kd receptor.[187]

None of the polymorphisms in the TNF cluster was associated with mean CRP concentrations in this thesis. A previous study assessed the relationship of two SNPs within *TNFα*, rs1800628 and rs361525 with CRP concentrations. Median CRP levels were significantly higher among carriers of the minor allele of rs1800628, but the study size ($n < 300$) was small.[188] This association was replicated in a cohort of 684 Brazilian from the general

population, but only in particular age groups.[115] In a former study, the minor allele of rs1800628 was associated with elevated TNFα production, suggesting that the effect of rs1800628 is mediated through the increase in TNFα levels.[189]

The G allele of rs909253 in the LTA gene was found to be associated with increased levels of CRP in one case-control study.[118] Polymorphisms in the *LTA* gene have additionally been shown to be associated with MI. By means of a large-scale, case–control association study using 92,788 gene-based SNP markers, a candidate locus on 6p21 associated with susceptibility to MI was identified in 94 Japanese patients.[190] Genotyping of relevant SNPs revealed that the LTA polymorphisms rs1800683, rs909253 and rs1041981 were significantly associated with a high risk of MI.[190] *In-vitro* functional analyses indicated that rs1041981 affected a twofold increase in induction of several cell-adhesion molecules in vascular smooth muscle cells of human coronary artery.[190] 1041981 was also positively associated with CAD in a powerful trios families study[191] and further linked to the extent of coronary atherosclerosis.[192] rs909253 on the other hand, enhanced the transcriptional level of LTA 1.5-fold.[190] These findings suggest a link between LTA and the pathogenesis of MI. However, other case-control studies could not replicate the association between rs909253 or rs1041981 with MI.[193;194] In addition, *TNFα* or *LTA* polymorphisms were not associated with CAD in a German case-control study.[185]

The minor alleles of rs2857713 (*LTA*) and of rs3093661, rs3093664 and rs361525 (*TNFα*) were associated with a smaller intra-individual variability. These results remained stable in sensitivity analysis except for rs361525, were the result did not reach significance when excluding outlying values.

6.5.6. Interleukin-18

IL-18 was originally described as an interferon-γ inducing factor.[195] It is a proinflammatory cytokine that induces the expression of other proinflammatory cytokines and adhesion

molecules.[196] IL-18 is capable of modulating the immune response by costimulating Th1 cell development and natural killer cell activation.[197] IL-18 also increases Fas ligand on natural killer cells and thus cytotoxicity.[198] Additionally, IL-18 enhances expression of macrophage colony-stimulating factor, decreases the production of IL-10 and induces smooth muscle cell proliferation and migration.[199-201] IL-18 mediates its effects through a widely expressed receptor. Expression of IL-18 is induced by several proinflammatory cytokines including IL-6 and TNFα.[202] Moreover, CRP induces the production of IL-18 in endothelial cells and levels of CRP were shown to be positively correlated with IL-18 in patients with unstable angina pectoris.[203;204]

IL-18 has been localized to human atherosclerotic lesions,[202;205] and circulating IL-18, which is increased in acute coronary syndromes,[206] has been shown to predict future cardiovascular events.[206] Additionally, a positive correlation between serum IL-18 levels and carotid intima-media thickness has been demonstrated.[207] Genetic association studies have shown that variations of the *IL-18* gene consistently influence circulating levels of IL-18 and clinical outcomes in patients with coronary artery disease, which supports the hypothesis of a causal role of IL-18 in atherosclerosis and its complications.[208]

In this thesis, polymorphisms in the *IL-18* were associated with intra- but not with inter-individual variability. The minor allele of rs2043055 was associated with greater intra-individual variability after correction for multiple testing while the promoter polymorphism rs187238 was associated with smaller intra-individual variability. The results for both SNPs remained significant in sensitivity analysis.

6.5.7. Toll-like receptor 4

TLRs are members of the IL-1 receptor family,[209] an evolutionary conserved signalling system against invading pathogens. Due to their ability to recognize microbial components, mammalian TLRs are among the most important components of the innate immunity

pathway.[210] The first described and best-known member of this family is TLR4,[209] identified as the signalling receptor for LPS.[211] Besides LPS, TLR4 interacts with endogenous ligands such as heat shock proteins, fibronectin, fibrinogen,[212] and minimally modified LDL.[213] TLR4 ligation activates several intracellular signalling pathways, with the TLR4/ NF-κB pathway being the most important one,[214] leading to the synthesis and release of inflammatory cytokines and other costimulatory molecules that provide a link to adaptive immunity.[215]

TLR4 have been found to be expressed in both human and mouse atherosclerotic lesions.[216;217] Expression has mainly been located to endothelial cells and macrophages within the lesion. Also, patients with acute coronary syndromes show increased TLR4 expression on circulating monocytes compared with control patients.[218;219] Additionally, oxidized LDL induces upregulation of TLR4 expression in macrophages *in vitro* and might therefore contribute to the TLR4-dependent inflammatory process in the arterial wall.[217]

To date several SNPs have been identified in the *TLR4* gene. Two of them, the congregated Asp299Gly and the Thr399Ile have been intensively studied and the rare allele 299Gly has been shown to block LPS-mediated signalling and cause hyporesponsiveness to LPS.[220] The 299Gly allele has also been associated with both decreased risk for atherosclerosis and acute coronary events.[221;222] Studies have also failed, however, to show an association between the same polymorphism and carotid atherosclerosis and stroke,[223;224] and this polymorphism has even been connected to an increased risk for MI.[225] Although it seems as if the minor alleles of *TLR4* polymorphisms in some studies may be associated with decreased atherosclerosis risk, results are inconsistent.

None of the investigated SNPs in the gene for TLR4 were associated with differences in CRP concentrations. Similar, a recently published study systematically investigated polymorphisms in nine innate immunity genes, including *TLR4*, without detecting differences in CRP concentrations.[116] Regarding intra-individual variability, the minor allele of rs1927907 was

associated with smaller intra-individual variability even after excluding extreme values in sensitivity analysis.

6.5.8. NF-κB complex

Nuclear factor-kappa B (NF-κB) is a transcription factor that controls the transcription of several genes, such as cytokines, chemokines, adhesion molecules, acute phase proteins, regulators of apoptosis, and cell proliferation.[226] The NF-κB family of transcription factors consisting of 5 members: p65 (RelA), c-Rel, RelB, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100). The proteins can form different complexes of either homodimers or heterodimers. All members share a Rel homology domain, mediating dimerization, association with inhibitory proteins, and DNA binding, whereas only the first three contain a transcriptional activation domain. The transcriptional activity of NF-κB is stimulated upon phosphorylation of its p65 subunit.[227]

The NF-κB complex is inhibited by I-kappa-B proteins (NF-κBIA or NF-κBIB), which inactivate NF-κB by trapping it in the cytoplasm. Phosphorylation of serine residues on the I-kappa-B proteins by kinases marks them for destruction via the ubiquitination pathway, thereby allowing activation of the NF-κB complex. Activated NF-κB complex translocates into the nucleus where it can activate specific target genes through selective binding to the NF-κB consensus sequence.

NF-κB is activated by a wide variety of stimuli such as cytokines, oxidant-free radicals, inhaled particles, ultraviolet irradiation, and bacterial or viral products.[228;229] Activated NF-κB was demonstrated in human atherosclerotic lesions.[230] Activation of NF-κB was also shown in smooth muscle cells, macrophages, and endothelial cells. Moreover, hypercholesterolemia was shown to induce activated NF-κB in the vessel wall in a pig model for atherosclerosis.[231] Using mice, another study reported increased levels of components of the NF-κB system in regions prone to develop atherosclerosis, also indicative for a role of NF-κB in

atherosclerosis.[232] NF-κB is thought to contribute to the different stages in atherosclerosis development, mainly by regulating the transcription of several genes with an established role in atherosclerosis. CRP has been indicated to activate NF-κB in smooth muscle cells.[233] Therefore, CRP has been suggested to mediate proliferation and activation of vascular smooth muscle cells, causing the accumulation of these cells in the vascular intima, which is a key event in the development of arterial lesions. NF-κB might influence CRP concentrations either directly or indirectly by varying cytokine levels at baseline or in the acute phase.

None of the SNPs investigated in the genes coding for p65 (RelA), NF-κB1 or NF-κBIA were associated with constitutive changes in the expression of CRP. Similar, a recently published study systematically investigated polymorphisms in nine innate immunity genes, including *NFKB1*, without detecting differences in CRP concentrations.[116]

The minor alleles of rs3774964 *(NFKB1*) and rs11227248 (*RELA*) were associated with greater intra-individual variability while rs732072 (*RELA*) and rs4648004 (*NFKB1*) were associated with decreased variability. However, the effect of rs3774964 *(NFKB1*) may only be due to outlying observations, since after excluding extreme values; the effect did not obtain significance. In contrast, the effect of rs4648004 (*NFKB1*) became even more pronounced.

6.5.9. Comparison with results from the general population

Compared to the general population, no differences were observed regarding associations of polymorphisms with mean CRP concentrations. The two *CRP* variants associated with lower plasma levels of CRP in MI survivors have also been shown to be associated with decreased concentrations in the general population.[98;105;106;111]

However, differences were observed regarding the non-genetic correlates of CRP concentrations. In this thesis, covariates and *CRP* polymorphisms explained 27% of the inter-individual variability in CRP concentrations with BMI explaining the biggest proportion (11%), followed by smoking (3%), total cholesterol (2%) and NT-proBNP (2%). In recent

published study systematically investigating the correlates of CRP concentrations in the general population, age was found to be the main determinant and also treatment with lipid-lowering medication was found to be important.[98] These differences may be due to the characteristics of the MI survivors recruited in the AIRGENE study, since most of them where old and the majority was treated with statins or other systemic medication, making it difficult to clearly assess an effect from these variables.

Since the genetic component of the intra-individual variability was analysed for the first time, one can just hypothesise on the influence of this special population. However, it is likely that these effects are more pronounced in a population at high-risk than in the general population since they may probably be more sensitive to certain environmental stimuli.

6.6. Strengths and limitations

The participants of the AIRGENE study represent a highly selected group of MI survivors of European descent. Therefore replication in other populations is needed before results can be generalized, especially since the effects on the intra-individual variability were demonstrated for the first time. Additionally, the majority of these patients was treated with statins or other systemic medication, which is known to be associated with changes in plasma CRP concentrations.[98] However, detailed adjustment for clinical covariates has been performed, including a sensitivity analysis with medication data. Population stratification can also be a confounding factor in genetic association studies. Therefore, main results of this thesis were analysed stratified by center and tested for heterogeneity.

The repeated measurements represent a major advantage of the present work, particularly because of the high intra-individual variability. A recent article on the effect of single measurements in the studies of biomarkers, has shown that for CRP using only a single measurement could modify the regression coefficient by up to 40%.[234] Strengths of the study

also include a relatively large sample size, rigorous phenotyping and the systematic selection of polymorphisms.

6.7. Conclusions and perspectives

The minor alleles of two variants were strongly associated with differences in CRP concentrations in MI survivors. Both were located in the *CRP* gene itself. Although no contribution of polymorphisms in innate immunity genes to the inter-individual variation in CRP concentrations was observed, other polymorphisms or variation in other genes involved in the inflammatory cascade may be associated with differences in CRP concentrations.

The overall contribution of the two *CRP* polymorphisms to phenotypic variance appears to be modest (~ 4%). Compared to the observed heritability estimates of 30-50%, it is still likely that other genetic variants outside the *CRP* gene locus contribute to the regulation of CRP concentrations. Therefore, whole genome approaches may provide an opportunity to identify those variants.

Additionally, the hypothesis that genetically determined subgroups have an altered response to certain environmental stimuli was investigated. Genetic influences on the inflammatory cascade regulation may cause some individuals to have a more vigorous response than others, but within the range of overall population variability. The minor alleles of several variants in selected candidate genes were observed to be significantly associated with either increased or decreased individual variability of CRP concentrations even after excluding outlying observations. A SNP in a candidate gene not associated with CRP mean concentrations might still be associated with its intra-individual variability, since this implies just a short time effect which could be rapidly compensated. However, since this genetic influence on the intra-individual variability of CRP concentrations was demonstrated for the first time, other studies with repeated measurements of CRP are essential to confirm these associations. In addition, these results may provide the basis for further functional approaches.

7. Appendix

7.1. Description of the study participants stratified by city

	Helsinki N=195	Stockholm N=197	Augsburg N=200	Rome N=134	Barcelona N=169	Athens N=108	All Centers N=1003	*p*-value
Sex = male [%]	68.7	70.6	82.0	86.6	83.4	87.0	78.6	<0.0001#
Age [yrs]*	64.6 (45-78)	64.0 (38-76)	61.9 (39-76)	62.7 (39-79)	62.1 (37-81)	54.7 (38-75)	62.2 (37-81)	<0.0001**
Body Mass Index*	28.6 (19.1-48.9)	27.6 (17.5-43.2)	28.7 (19.1-48.4)	27.7 (19.0-39.4)	28.8 (19.3-43.5)	28.8 (20.9-46.4)	28.4 (17.5-48.9)	0.0039††
Packyears (cigarettes only)*	9.1 (0-65.0)	12.2 (0-73.8)	15.1 (0-205.2)	21.8 (0-171.8)	28.1 (0-192.3)	35.6 (0-174.0)	18.7 (0-205.2)	<0.0001‡‡
Total cholesterol [mg/dl]*	182.2 (91.1-291.9)	173.4 (96.7-324.7)	181.0 (107.0-316.0)	190.3 (120.0-321.0)	193.2 (119.0-390.0)	195.4 (92.0-293.0)	184.5 (91.1-390.0)	<0.0001††
Systolic Blood Pressure [mmHg]*	139.9 (93-209)	137.6 (97-196)	128.4 (84-198)	134.7 (95-188)	129.5 (81-196)	136.1 (100-190)	134.3 (81-209)	<0.0001††
HbA1c > 6.5% [%]†	15.9	6.6	9.5	8.2	10.7	14.8	11.1	0.010#
Health status = Bad or very bad [%]‡	5.1	5.0	9.0	7.4	8.2	5.5	6.8	<0.0001#
log(BNP) [pg/ml]*	5.4 (2.38-8.24)	5.2 (2.64-8.22)	5.4 (2.52-9.14)	5.4 (2.95-8.73)	5.2 (2.42-8.86)	4.8 (2.40-8.49)	5.2 (2.38-9.14)	0.0013††
First MI [%]	81.5	85.8	87.5	87.3	86.4	80.6	85.0	0.37#
No COPD /chronic bronchitis [%]§	70.8	69.4	79.5	84.3	72.2	86.1	76.0	0.007#
Self-reported history [%]‖								
Hypertension	51.3	49.7	51.0	55.2	46.2	54.6	51.0	0.73#
Chronic bronchitis	2.0	1.5	8.0	13.4	11.2	6.4	6.7	<0.0001#

* mean and range in brackets, † Indication for diabetes: HbA1c ≥ 6.5%, ‡ Scale 1 ("excellent") to 5 ("very bad"), § at least some indication of COPD/chronic bronchitis, ‖ ever physician diagnosed, # Chi-Square-Test, ** Kruskal-Wallis-Test , †† ANOVA, ‡‡ Median-Test

7.2. Primers used for genotyping

Gene	rs Number	1st Primer	2nd Primer	Extension primer
CRP	rs1130864	ACGTTGGATGATCTCCAAGATCTGTCCAAC	ACGTTGGATGCTGGGAGCTCGTTAACTATG	AAATTCTGATTCTTTTGGACC
CRP	rs1205	ACGTTGGATGCAGTAGCCATCTTGTTTGCC	ACGTTGGATGTTTGTCAATCCCTTGGCTCC	TTGCCACATGGAGAGAGACT
CRP	rs1417938	ACGTTGGATGTTCATGCAGTCTTAGACCCC	ACGTTGGATGTCATGCTTTTGGCCAGACAG	CCCCATACCTCAGATCAAA
CRP	rs1800947	ACGTTGGATGGAAATGTGAACATGTGGGAC	ACGTTGGATGAGGACATTAGGACTGAAGGG	ATGTGGGACTTTGTGCT
CRP	rs2794521	ACGTTGGATGCTGAGAAAATGTGTCCATGC	ACGTTGGATGTCCTGTGTCCAAGTATTCTC	AAAAAACCAAACACCGC
CRP	rs3091244	ACGTTGGATGTGAAGTAGGTGTTGGAGAGG	ACGTTGGATGCTTTGGCTATCTATCCTGCG	GCACCCAGATGGCCACT
CRP	rs3093068	ACGTTGGATGGCTCAAAGGGTTGCTATAAG	ACGTTGGATGATAAGAGCTTACTATGTGCC	CATGCAAGCAAAGCACTTACTA
FGA	rs10012555	ACGTTGGATGGTTGGTTAGAGGGCACAAAG	ACGTTGGATGCTATAGTCACCATGCTGCAC	AGAGGGCACAAAGTTTGAG
FGA	rs2070006	ACGTTGGATGTCTAGCAAAGAACCCAAGAC	ACGTTGGATGTCTGCTTCCTACATGAAGCC	AACCCAAGACTTAGTAAACATTCA
FGA	rs2070011	ACGTTGGATGTGTCTACACAGGACAAAGCC	ACGTTGGATGGCACTCCAGCTGAAAGAAAG	GGATGGGAACTAGGAGTGGC
FGA	rs2070014	ACGTTGGATGAACAGTGCTCTTGGGAGTAG	ACGTTGGATGACCTGATTGGTCCCAGAAAC	GAGTAGGTTCCTTTTCTTTATTTG
FGA	rs2070016	ACGTTGGATGGAGAAACTCTGGAATGAGGG	ACGTTGGATGAACCAGGCTCCTGAGTATTG	GGGTCCACTTAGCCATA
FGA	rs2070018	ACGTTGGATGGTGCATAACTATCGCCTTCC	ACGTTGGATGGACTGGAGGTAAGTATGTGG	CCTTTTCCCTCTACTCA
FGA	rs2070022	ACGTTGGATGACTTTTTGCTTTCCAATGGC	ACGTTGGATGCTCTCTGCAACCTTGAAGAC	GCATTATGATTTTGGGTGGTAT
FGA	rs6050	ACGTTGGATGCAGTACTTCCAGTTCCAGAG	ACGTTGGATGGACTGCAACCTGGAAACCTG	AGAGTTCCAGCTTCCAG
FGB	rs1800788	ACGTTGGATGTAATTGGACAAAATGATGGG	ACGTTGGATGGGAGCTTGTGTAGTTTCCAC	GACAAAATGATGGGAAGTTAGG
FGB	rs1800790	ACGTTGGATGGCTTATGTTTTCTGACAATG	ACGTTGGATGGTCTAAAACAAAAGATAAACAC	ATTCTATTTCAAAAGGGGC
FGB	rs1800791	ACGTTGGATGCACACAAGTGAACAGACAAG	ACGTTGGATGTCACAGACTAAATGAGGCCC	AATTTTGTGGCTTGTGG
FGB	rs2227399	ACGTTGGATGAGCTATACATCCTTTGGAGC	ACGTTGGATGCAAGAGAGCTCAGTTGTTGC	ACAAACGACATGATTTGGA
FGB	rs2227421	ACGTTGGATGCTCATTGAATGTGTGGATTAC	ACGTTGGATGGATCTGTCAAAGAAAACTTCC	AAACATTAACTTATTGCAACAGAG
FGB	rs4220	ACGTTGGATGGAAGGGCCTGATCTTCATAC	ACGTTGGATGTGGCACAGATGATGGTGTAG	GGCCTGATCTTCATACTCATCTTC
FGB	rs6056	ACGTTGGATGACGAAGCACACGAAGGTTAG	ACGTTGGATGCCTCAGAACTGGAAAAGCAC	ACGAAGGTTAGTTGGGATATT
FGG	rs1049636	ACGTTGGATGTTTCTGAAACTTTGTGGGTC	ACGTTGGATGCTCACTTTACCCTGAGGATG	AAACTTTGTGGGTCAATAGAA
FGG	rs1800792	ACGTTGGATGATGCCCACCTTCAGACAAAG	ACGTTGGATGCCTCTGTGTCAACCATGTTC	CTCAAAAGCTCCCTGAG
FGG	rs2066854	ACGTTGGATGATGCCGTAGCCACAGAAATC	ACGTTGGATGCCTCAGATAGCATTTGTAGC	AGTTTACAATCTAATGCAGTGG
FGG	rs2066860	ACGTTGGATGGCAGCCTGACAAATGCAAAC	ACGTTGGATGTTTACCTTTTTGGATTGCCC	TTTCTCCAGGAGTCTGTTT
FGG	rs2066861	ACGTTGGATGCAGTGAACAGGTGTTTGGAG	ACGTTGGATGCTCAAGCTTTCACAAACCCC	GTGTTTGGAGGAACAGAATAAA
FGG	rs2066865	ACGTTGGATGCCTTTTGTTTCCTAAGACTAG	ACGTTGGATGCTGATGGTTGCCCAATTGTA	TCCTAAGACTAGATACATGGTA
IL6	rs1554606	ACGTTGGATGATGTTTAAAACTCCCACAGG	ACGTTGGATGGGCAGCCAGAGAGGGAAAAG	TCCTGGGAAAGGTACTC
IL6	rs1800795	ACGTTGGATGGATTGTGCAATGTGACGTCC	ACGTTGGATGAGCCTCAATGACGACCTAAG	AATGTGACGTCCTTTAGCAT

Gene	rs Number	1st Primer	2nd Primer	Extension primer
IL6	rs1800796	ACGTTGGATGACGCCTTGAAGTAACTGCAC	ACGTTGGATGTCTTCTGTGTTCTGGCTCTC	GCAGTTCTACAACAGCC
IL6	rs2069827	ACGTTGGATGAAATGCCCAACAGAGGTCAC	ACGTTGGATGGGTGTTACCTTCACAATCGG	CAACAGAGGTCACTGTTTTATC
IL6	rs2069832	ACGTTGGATGGCCGACTAGACTGACTTCTG	ACGTTGGATGGGATTTCAGACCCATTCTGC	CTTTGCTGGTGTCAGGA
IL6	rs2069840	ACGTTGGATGAGGCAGCAACAAAAAGTGGG	ACGTTGGATGCTGTCCAAGAATAAACTGCC	AAATTTCATGAGGAGGCCAA
IL6	rs2069845	ACGTTGGATGATGGTGGGTCTATGGAAAGG	ACGTTGGATGCACCATCCCTTTAGGATCTG	GTCCTCTTTACACCACC
IL6	rs2069849	ACGTTGGATGGCAGGACATGACAACTCATC	ACGTTGGATGCCATGCTACATTTGCCGAAG	CGCAGCTTTAAGGAGTT
IL10	rs10494879	ACGTTGGATGCTTCCTTGCCAATCTATGTTC	ACGTTGGATGGTGATGATGGAAATGGAGAC	TATCACCAGAACCTCCA
IL10	rs1518110	ACGTTGGATGTGATTTTTTTGGGCCAGAGC	ACGTTGGATGATGCGGTCTTTTTGATGCCC	TGGGCCAGAGCCAATTT
IL10	rs1800871	ACGTTGGATGTAGTGAGCAAACTGAGGCAC	ACGTTGGATGGTACAGTAGGGTGAGGAAAC	AAACTGAGGCACAGAGAT
IL10	rs1800872	ACGTTGGATGTCCTCAAAGTTCCCAAGCAG	ACGTTGGATGAAAGGAGCCTGGAACACATC	AGACTGGCTTCCTACAG
IL10	rs1800890	ACGTTGGATGTGATTTCCCAGTACATCCCC	ACGTTGGATGCAAGCCCAGATGCATAGTAG	ACATCCCCCACTGGAAAAAT
IL10	rs1800893	ACGTTGGATGTATAGAGTGGCAGGGCCAAG	ACGTTGGATGTGCCTGCCATTCCAGTTTAG	CCTCCTGCACCTAGGTCA
IL10	rs1800894	ACGTTGGATGTCACCTGTACAAGGGTACAC	ACGTTGGATGTCTATGTGCTGGAGATGGTG	CAAGGGTACACCAGTGC
IL10	rs2222202	ACGTTGGATGAAATGTGCAGGAAACCTGCC	ACGTTGGATGAAAGTATAGAGCGCCAGCAG	TGCCTTATAAAGTAAATGCGTTCT
IL10	rs3024491	ACGTTGGATGAAGGGTTGACATAGGTGTCC	ACGTTGGATGAGGGAAAGCAGAAGCTTGAC	AGGTGTCCCTTAAAGCC
IL10	rs3024496	ACGTTGGATGCCCCTTGAGAAACCTTATTG	ACGTTGGATGTCGTTCACAGAGAAGCTCAG	AAACCTTATTGTACCTCTCT
IL10	rs3024502	ACGTTGGATGAATTGCTGGGATTACAGGCG	ACGTTGGATGAATCCTCAGGATAACCCAGG	GGCGTGAGCCACCACACCCG
IL10	rs6676671	ACGTTGGATGTAAATAGTGTGGGCTCCCAG	ACGTTGGATGGGTGCATAAAATGGGTCCAG	CAGCCTCCATACCAGTG
IL18	rs1834481	ACGTTGGATGCTGGAAGTAGAGGAGATAGG	ACGTTGGATGATGCCATGGTTTCTTTCAGC	CAAGAAGACCACATTCAGAG
IL18	rs187238	ACGTTGGATGACAGAGCCCCAACTTTTACG	ACGTTGGATGGCAGAGGATACGAGTACTTC	CCAACTTTTACGGAAGAAAA
IL18	rs1946518	ACGTTGGATGTATCAGATGCAAGCCACACG	ACGTTGGATGCTCCCCAAGCTTACTTTCTG	CGGATACCATCATTAGAATTTTAT
IL18	rs2043055	ACGTTGGATGGTGTTGATTTTGAATTCTTC	ACGTTGGATGGGATGAGAACACACTACTTG	ACAATTCTTCTTGTTAGTTCTCA
IL18	rs360722	ACGTTGGATGACCATGTCTCAAGATCTCTG	ACGTTGGATGTAGTAGTACTTGTGACTCTG	TCTCAAGATCTCTGCAATAA
IL18	rs3882891	ACGTTGGATGATTCTCCCTGTCATGGGATG	ACGTTGGATGGAGAGGAGAGAAGTAAGCTG	TATTCAGTTCCAGGCCTCTA
IL18	rs549908	ACGTTGGATGAATGTTTATTGTAGAAAACC	ACGTTGGATGGTCAATGAAGAGAACTTGGTC	TTATTGTAGAAAACCTGGAATC
IL18	rs5744222	ACGTTGGATGCAGATTGCTATTGTTCTGGG	ACGTTGGATGTAGGCCACATAATACTAGCC	TGTTCTGGGACAGTGTTT
IL18	rs5744280	ACGTTGGATGGAGCGAGACTCCGTTTCAAA	ACGTTGGATGATTGCCTGTTCTACCTATCC	GAAAGTTACAGTTGAAAGC
LTA	rs1041981	ACGTTGGATGTTACCAATGAGGTGAGCAGC	ACGTTGGATGTGTTGGCCTCACACCTTCAG	GTGAGCAGCAGGTTTGAGG
LTA	rs1800683	ACGTTGGATGTATAAAGGGACCTGAGCGTC	ACGTTGGATGTAGTCCAAAGCACGAAGCAC	GTCCGGGCCCAGGGGCTCC
LTA	rs2009658	ACGTTGGATGAGCTCCAACCCCTCTAACAC	ACGTTGGATGTAATACCAACTTGTCACCTC	ACTCTCCAAGTAAAATCACAT
LTA	rs2239704	ACGTTGGATGGTGCTTCGTGCTTTGGACTA	ACGTTGGATGATCCAGGCAGCAGGTGCAG	GCTTTGGACTACCGCCC

Gene	rs Number	1st Primer	2nd Primer	Extension primer
LTA	rs2857713	ACGTTGGATGCTCTTTCTCTGCAGGTTCTC	ACGTTGGATGAGAAGGAGGAGGTGTAGGGT	TCTCTTCCTCCCAAGGGTG
LTA	rs3093543	ACGTTGGATGTTACCAATGAGGTGAGCAGC	ACGTTGGATGTGTTGGCCTCACACCTTCAG	GCAAGATGCATCTTGGGG
LTA	rs909253	ACGTTGGATGAGACAGAGAGAGACAGGAAG	ACGTTGGATGACTCTCCATCTGTCAGTCTC	GAAGGGAACAGAGAGGAA
NFKB1	rs28362491	ACGTTGGATGATGACTCTATCAGCGGCACT	ACGTTGGATGTAGGGAAGCCCCCAGGAAG	GCGTTCCCCGACCATTG
NFKB1	rs1020759	ACGTTGGATGGGGATGACCTTTAAGTGGAG	ACGTTGGATGCTGCAGTGAACAGTAATGAG	CAGATTTATAATTTCCCCATC
NFKB1	rs11722146	ACGTTGGATGGAGACTCTCCAGTGTGTTAG	ACGTTGGATGGAGAGTCCCCAAATCAGAAG	ACAAAAACCCTGAAACC
NFKB1	rs1598856	ACGTTGGATGTCACCTCTTTTTGTGCTGCG	ACGTTGGATGATAGTGTACCAGGCTCTGTC	TTTGTGCTGCGTTATCCAT
NFKB1	rs1609798	ACGTTGGATGACATCCTCACAGTATGTCCC	ACGTTGGATGTCACTGTCATGACTGCTCAC	GTATCTTCTGCCCTTCC
NFKB1	rs1801	ACGTTGGATGCTGCGGTATGAGTCTGTATC	ACGTTGGATGCTCTGTGCTCCAAGTACAAC	AACCTTTCACTTCCCTTCAAA
NFKB1	rs230498	ACGTTGGATGCCTGCATTTCAAAGCTCTCC	ACGTTGGATGCGTGTCTCCTGTTGTATGTC	TCTCCTAGGTCAATCAGGAA
NFKB1	rs230521	ACGTTGGATGGAAGTCTACTCAAATCCTGC	ACGTTGGATGAGCCTACACCTATGCGAAAC	CTACTCAAATCCTGCATTTTCTA
NFKB1	rs230530	ACGTTGGATGTGGACATACAAGCATTCTCC	ACGTTGGATGAAAGGCATATGGTGGTTCTC	TTAGCACCAAACATCTTAATTT
NFKB1	rs3774932	ACGTTGGATGTTAAACTGCATTCGTGTTTG	ACGTTGGATGGGATTCATCTCTAGAAAGCAC	CTTTAAAAGGGAAACTTAAAACT
NFKB1	rs3774956	ACGTTGGATGACACATACAGGTAACCTGGG	ACGTTGGATGCTTCTCCAGAAATATGTCTTC	TTCTCTGTGTTAGCAAAAACTG
NFKB1	rs3774964	ACGTTGGATGGGCTGCTGTCTCTTGTTATT	ACGTTGGATGAAAGCCACCTGGCTTCTTTC	CTCTTGTTATTTTTATAGGCTTT
NFKB1	rs4648004	ACGTTGGATGGAAAACAAGGGAGAGAGAGG	ACGTTGGATGAAAAAGGTAAGTGCTGGGCC	AGAGAGGCTCACTGGTCA
NFKB1	rs4648090	ACGTTGGATGTCAGCATAGAATCATGGCAC	ACGTTGGATGAGGCTATGTTTTCACTGAAG	CATGGCACTTTTGTTAATTG
NFKB1	rs4648110	ACGTTGGATGGGTAACACCTTACCTTACAG	ACGTTGGATGAAGGGAAGGAGATTATGCCC	TTGACTTTACTCTGTTAACATC
NFKB1	rs4648133	ACGTTGGATGGAAGAGCTTCCACAGCAATG	ACGTTGGATGTGTGTGCCTAGCAGTGTTCG	TTTAAGAAAGCAAACGAGTA
NFKB1	rs7340881	ACGTTGGATGGTACTGGTTCTGTTCTAGGG	ACGTTGGATGAGGGCATATCTTCGTCTCTC	AGAAAATTAAATAGTGCCTTTGTC
NFKBIA	rs1957106	ACGTTGGATGCGACGGGCTGAAGAAGGAG	ACGTTGGATGGTACTCCTCGTCTTTCATGG	AGAAGGAGCGGCTACTGGA
NFKBIA	rs2233407	ACGTTGGATGAGTTCCTTTGCTGCAAAGAG	ACGTTGGATGAAGGACGCACTGTGGTTAGG	TTTGCTGCAAAGAGCCTGGT
NFKBIA	rs2233409	ACGTTGGATGTGCACCCTGTAATCCTGTCC	ACGTTGGATGCGACGACCCCAATTCAAATC	TCCTGTCCCTCTGCAAGTGA
NFKBIA	rs3138052	ACGTTGGATGGAAAGTAGTTGCCAGGCATC	ACGTTGGATGCAGGACAAGAGAGCAAGGAG	TCCTGCGGGAGGCTCAAGG
NFKBIA	rs3138054	ACGTTGGATGTGCCTGGACTCCTTAAGTTG	ACGTTGGATGACAATGGTATGTCTGCCTCC	CACCTGCCCCTTCTCCA
NFKBIA	rs696	ACGTTGGATGAAGCAACAAAATGAGGGCTG	ACGTTGGATGGTGTACTTATATCCACACTGC	AATAAGACGTTTTGGGC
NFKBIA	rs8904	ACGTTGGATGAGTCCATGTTCTTTCAGCCC	ACGTTGGATGTTCAGCTGCCCTATGATGAC	TCTTTCAGCCCCTTTGC
RELA	rs1049728	ACGTTGGATGTCTAGCCAGCTTGGCAACAG	ACGTTGGATGTCTCCAGTCAGGAGGCATAG	GTTCAGAGTAGAAAGAGCAAGA
RELA	rs11227248	ACGTTGGATGGGACTGAAACTGTTACTCTG	ACGTTGGATGCCCGACACTGTGTCTCTTAA	AAACTGTTACTCTGACAGCA
RELA	rs11568300	ACGTTGGATGATGTGAAATGGAGGTGTGCG	ACGTTGGATGAGCTTCAATGCCACCTTCTC	AGAGAAGCACGGGGGCACA
RELA	rs11820062	ACGTTGGATGGCATCTGATTCAGTTTCCTC	ACGTTGGATGTCAGGGCCTGTTGTACTTTC	GTGGGGCGTGCCCTCCCTCA

Gene	rs Number	1st Primer	2nd Primer	Extension primer
RELA	rs7101916	ACGTTGGATGTCCCCCAAGACCAAGCCCAA	ACGTTGGATGCCTGCAGTGGAGCATCCTC	CCCACCCCAGGCCTCAT
RELA	rs7119750	ACGTTGGATGTCTTCATGGTGCTCAGGCTC	ACGTTGGATGGGGTAAGAGGAAGGAGATAG	CTCTAGGGAGTCACTGC
RELA	rs732072	ACGTTGGATGCAACAACAACCCCTTCCAAG	ACGTTGGATGTGAGGGAGATGCAGGAAAGG	AACCTGCCTGCCACACC
TLR4	rs11536878	ACGTTGGATGTTCTTGACTACCCACCACAG	ACGTTGGATGTAGCGACATATAACAGTAGG	CCCCTCTTCTGAAAAATACAATTA
TLR4	rs11536889	ACGTTGGATGACCCCATTAATTCCAGACAC	ACGTTGGATGCTGTTTCCTGTTGGGCAATG	TCTCAATGATAACATCCACT
TLR4	rs1927907	ACGTTGGATGGGGTATCCAGTGGATTGAAG	ACGTTGGATGGTAGACCACCTCTCCCTTTT	GAATTACATAAGAGACATTGTTTG
TLR4	rs1927911	ACGTTGGATGGATGTCCAGACCTTCCTTAG	ACGTTGGATGCATCACTTTGCTCAAGGGTC	TTTGACAACTGCATTCTTTT
TLR4	rs1927914	ACGTTGGATGACAGTAGAACTATCTAGGAC	ACGTTGGATGGGAAAGTAGCAAGTGCAATG	GTAGAACTATCTAGGACTTAGCAT
TLR4	rs2149356	ACGTTGGATGGTTGTTAGTTGGTAGCCAAG	ACGTTGGATGACATTAAGAGTATCTGTGAC	ATCAATTTCAGAGATACGAAA
TLR4	rs2770150	ACGTTGGATGTTTCCTGGGAAATGAATGGG	ACGTTGGATGACACATGGTCTGCCTTCTGG	AATCAAAGACATCTAGGTTCTATG
TLR4	rs4986790	ACGTTGGATGAGCATACTTAGACTACTACC	ACGTTGGATGCACACTCACCAGGGAAAATG	TACTTAGACTACTACCTCGATG
TLR4	rs4986791	ACGTTGGATGACACCATTGAAGCTCAGATC	ACGTTGGATGAGGTTGCTGTTCTCAAAGTG	CAGATCTAAATACTTTAGGCTG
TLR4	rs7873784	ACGTTGGATGGCTCTAAAGATCAGCTGTAT	ACGTTGGATGATGAGAGGTACCCTCTTAAC	TCAGCTGTATAGCAGAGTTC
TNFA	rs1799724	ACGTTGGATGTCCTGGAGGCTCTTTCACTC	ACGTTGGATGCTATGGAAGTCGAGTATGGG	TCTACATGGCCCTGTCTTC
TNFA	rs1799964	ACGTTGGATGTACATGTGGCCATATCTCCC	ACGTTGGATGGGGAAGCAAAGGAGAAGCTG	CAGACCCTGACTTTTCCTTC
TNFA	rs1800629	ACGTTGGATGGATTTGTGTGTAGGACCCTG	ACGTTGGATGGGTCCCCAAAAGAAATGGAG	GAGGCTGAACCCCGTCC
TNFA	rs1800630	ACGTTGGATGCAGCAATGGGTAGGAGAATG	ACGTTGGATGCTCTACATGGCCCTGTCTTC	CGAGTATGGGGACCCCC
TNFA	rs3091257	ACGTTGGATGAGGATCTCACTATGTTGCCC	ACGTTGGATGATCCTACCACTTTGCAAGGC	ACTATGTTGCCCAGGTTG
TNFA	rs3093661	ACGTTGGATGTTCATCCACTCTCCCACCCA	ACGTTGGATGATCAGCGCACATCTTTCACC	CAAGGGGAAATGGAGAC
TNFA	rs3093664	ACGTTGGATGAAGTTCTGCCTACCATCAGC	ACGTTGGATGGTAAGAGCTCTGAGGATGTG	CATCAGCCGGGCTTCAA
TNFA	rs3093665	ACGTTGGATGAGTTCTAAGCTTGGGTTCCG	ACGTTGGATGTATTACCCCCTCCTTCAGAC	TTTGAGCCAGAAGAGGT
TNFA	rs361525	ACGTTGGATGATCAAGGATACCCCTCACAC	ACGTTGGATGGGGTCCTACACACAAATCAG	CCCCATCCTCCCTGCTC

7.3. Supplemental result tables

Table I: Frequencies stratified by city including HWE statistics.

Gene	SNP	Genotype	Helsinki (N=195)	Stockholm (N=197)	Augsburg (N=200)	Rome (N=134)	Barcelona (N=169)	Athens (N=108)	All Centers (N=1003)	*p*-value	HWE (*p*-value)
CRP	rs3093068	GG	0.880	0.882	0.873	0.858	0.838	0.856	0.866	0.8126*	0.31
		CG	0.120	0.113	0.127	0.134	0.162	0.144	0.132		
		CC	0.000	0.005	0.000	0.007	0.000	0.000	0.002		
CRP	rs1205	CC	0.392	0.406	0.460	0.511	0.450	0.461	0.441	0.0382†	0.52
		CT	0.454	0.508	0.400	0.398	0.491	0.461	0.453		
		TT	0.155	0.086	0.140	0.090	0.059	0.078	0.106		
CRP	rs1130864	GG	0.421	0.492	0.465	0.451	0.420	0.479	0.453	0.4958†	0.72
		AG	0.441	0.406	0.434	0.429	0.509	0.448	0.443		
		AA	0.138	0.102	0.101	0.120	0.071	0.073	0.103		
CRP	rs1800947	CC	0.859	0.832	0.884	0.925	0.887	0.891	0.876	0.1418*	0.42
		CG	0.141	0.168	0.106	0.075	0.113	0.109	0.122		
		GG	0.000	0.000	0.010	0.000	0.000	0.000	0.002		
CRP	rs1417938	TT	0.426	0.492	0.465	0.451	0.432	0.471	0.461	0.4926†	0.83
		AT	0.436	0.406	0.435	0.421	0.497	0.462	0.441		
		AA	0.138	0.102	0.100	0.128	0.071	0.067	0.103		
CRP	rs3091244	AG	0.393	0.371	0.390	0.376	0.438	0.423	0.396	0.9390*	0.73
		GG	0.361	0.396	0.375	0.346	0.325	0.375	0.364		
		AA	0.136	0.102	0.100	0.128	0.071	0.067	0.103		
		GT	0.068	0.086	0.090	0.098	0.107	0.087	0.089		
		AT	0.042	0.041	0.045	0.045	0.059	0.048	0.046		
		TT	0.000	0.005	0.000	0.008	0.000	0.000	0.002		

*=Fisher's exact test, †=Chi-Square-Test

Gene	SNP	Genotype	Helsinki (N=195)	Stockholm (N=197)	Augsburg (N=200)	Rome (N=134)	Barcelona (N=169)	Athens (N=108)	All Centers (N=1003)	*p*-value	HWE (*p*-value)
CRP	rs2794521	TT	0.655	0.500	0.555	0.485	0.500	0.466	0.536	0.0505†	0.37
		CT	0.309	0.429	0.370	0.440	0.435	0.476	0.401		
		CC	0.036	0.071	0.075	0.075	0.065	0.058	0.063		
FGA	rs2070022	GG	0.611	0.685	0.704	0.746	0.710	0.777	0.697	0.2019*	0.57
		AG	0.342	0.274	0.271	0.231	0.266	0.214	0.273		
		AA	0.047	0.041	0.025	0.022	0.024	0.010	0.030		
FGA	rs6050	TT	0.438	0.602	0.560	0.493	0.625	0.618	0.553	0.0128†	0.05
		CT	0.443	0.343	0.370	0.418	0.310	0.275	0.365		
		CC	0.119	0.054	0.070	0.090	0.065	0.108	0.082		
FGA	rs2070018	AA	0.764	0.791	0.785	0.784	0.710	0.816	0.772	0.3952*	0.11
		AG	0.220	0.199	0.175	0.201	0.266	0.165	0.206		
		GG	0.016	0.010	0.040	0.015	0.024	0.019	0.021		
FGA	rs2070016	AA	0.763	0.682	0.729	0.767	0.713	0.641	0.719	0.0440*	0.71
		AG	0.221	0.303	0.236	0.203	0.281	0.291	0.255		
		GG	0.016	0.015	0.035	0.030	0.006	0.068	0.025		
FGA	rs2070014	CC	0.640	0.689	0.698	0.754	0.711	0.770	0.702	0.4346*	0.48
		CT	0.312	0.269	0.276	0.231	0.265	0.220	0.268		
		TT	0.048	0.041	0.026	0.015	0.024	0.010	0.030		
FGA	rs2070011	CC	0.269	0.429	0.410	0.361	0.401	0.485	0.386	0.0114†	0.02
		CT	0.511	0.454	0.426	0.474	0.451	0.327	0.448		
		TT	0.220	0.117	0.164	0.165	0.148	0.188	0.165		
FGA	rs10012555	TT	0.750	0.804	0.776	0.777	0.717	0.798	0.769	0.6134*	0.38
		CT	0.239	0.186	0.193	0.208	0.259	0.181	0.213		
		CC	0.011	0.010	0.031	0.015	0.024	0.021	0.019		

*=Fisher's exact test, †=Chi-Square-Test

Gene	SNP	Genotype	Helsinki (N=195)	Stockholm (N=197)	Augsburg (N=200)	Rome (N=134)	Barcelona (N=169)	Athens (N=108)	All Centers (N=1003)	*p*-value	HWE (*p*-value)
FGA	rs2070006	CC	0.259	0.426	0.400	0.346	0.396	0.476	0.378	0.0084†	0.23
		CT	0.539	0.457	0.440	0.481	0.450	0.330	0.458		
		TT	0.202	0.117	0.160	0.173	0.154	0.194	0.164		
FGB	rs1800791	GG	0.621	0.716	0.758	0.769	0.740	0.845	0.730	0.0086*	0.45
		AG	0.338	0.249	0.222	0.216	0.237	0.155	0.245		
		AA	0.041	0.036	0.020	0.015	0.024	0.000	0.025		
FGB	rs1800790	GG	0.701	0.610	0.692	0.575	0.651	0.515	0.636	0.0244*	1.00
		AG	0.253	0.354	0.263	0.403	0.308	0.447	0.324		
		AA	0.046	0.036	0.045	0.022	0.041	0.039	0.039		
FGB	rs1800788	CC	0.487	0.650	0.625	0.647	0.690	0.680	0.622	0.0079†	0.14
		CT	0.426	0.299	0.320	0.316	0.280	0.252	0.322		
		TT	0.087	0.051	0.055	0.038	0.030	0.068	0.055		
FGB	rs2227399	TT	0.696	0.604	0.685	0.575	0.651	0.515	0.633	0.0141*	1.00
		GT	0.257	0.365	0.260	0.403	0.308	0.447	0.327		
		GG	0.047	0.030	0.055	0.022	0.041	0.039	0.040		
FGB	rs6056	CC	0.708	0.640	0.698	0.624	0.675	0.549	0.659	0.1407*	0.60
		CT	0.262	0.335	0.256	0.361	0.296	0.412	0.310		
		TT	0.031	0.025	0.045	0.015	0.030	0.039	0.031		
FGB	rs4220	GG	0.706	0.646	0.695	0.627	0.675	0.549	0.660	0.1841*	0.53
		AG	0.268	0.328	0.260	0.358	0.296	0.412	0.310		
		AA	0.026	0.026	0.045	0.015	0.030	0.039	0.030		
FGB	rs2227421	AA	0.631	0.454	0.415	0.383	0.381	0.431	0.457	<.0001†	0.27
		AC	0.349	0.439	0.490	0.511	0.500	0.422	0.450		
		CC	0.021	0.107	0.095	0.105	0.119	0.147	0.094		

*=Fisher's exact test, †=Chi-Square-Test

Gene	SNP	Genotype	Helsinki (N=195)	Stockholm (N=197)	Augsburg (N=200)	Rome (N=134)	Barcelona (N=169)	Athens (N=108)	All Centers (N=1003)	*p*-value	HWE (*p*-value)
FGG	rs2066865	GG	0.464	0.614	0.553	0.567	0.661	0.637	0.576	0.0020†	0.15
		AG	0.433	0.340	0.377	0.388	0.292	0.245	0.354		
		AA	0.103	0.046	0.070	0.045	0.048	0.118	0.069		
FGG	rs1049636	AA	0.414	0.503	0.518	0.507	0.485	0.602	0.496	0.2115†	0.49
		AG	0.466	0.395	0.391	0.425	0.406	0.350	0.409		
		GG	0.120	0.103	0.091	0.067	0.109	0.049	0.094		
FGG	rs2066861	CC	0.464	0.614	0.555	0.567	0.665	0.627	0.576	0.0027†	0.20
		CT	0.438	0.340	0.375	0.388	0.287	0.255	0.355		
		TT	0.098	0.046	0.070	0.045	0.048	0.118	0.068		
FGG	rs2066860	CC	0.964	0.904	0.910	0.910	0.952	0.913	0.927	0.1151*	1.00
		CT	0.036	0.091	0.090	0.090	0.048	0.087	0.072		
		TT	0.000	0.005	0.000	0.000	0.000	0.000	0.001		
FGG	rs1800792	CT	0.436	0.503	0.513	0.504	0.461	0.412	0.475	0.0001†	0.25
		TT	0.456	0.269	0.286	0.278	0.293	0.275	0.315		
		CC	0.108	0.228	0.201	0.218	0.246	0.314	0.209		
FGG	rs2066854	TT	0.456	0.614	0.550	0.564	0.661	0.631	0.573	0.0013†	0.15
		AT	0.436	0.340	0.380	0.391	0.292	0.252	0.356		
		AA	0.108	0.046	0.070	0.045	0.048	0.117	0.070		
IL6	rs2069827	GG	0.621	0.812	0.800	0.866	0.856	0.864	0.792	<.0001*	0.04
		GT	0.308	0.173	0.190	0.134	0.138	0.136	0.188		
		TT	0.072	0.015	0.010	0.000	0.006	0.000	0.020		
IL6	rs1800796	GG	0.907	0.929	0.910	0.881	0.834	0.863	0.891	0.0787*	1.00
		CG	0.093	0.066	0.090	0.112	0.160	0.137	0.106		
		CC	0.000	0.005	0.000	0.007	0.006	0.000	0.003		

*=Fisher's exact test, †=Chi-Square-Test

Gene	SNP	Genotype	Helsinki (N=195)	Stockholm (N=197)	Augsburg (N=200)	Rome (N=134)	Barcelona (N=169)	Athens (N=108)	All Centers (N=1003)	*p*-value	HWE (*p*-value)
IL6	rs1800795	CG	0.492	0.569	0.490	0.418	0.426	0.320	0.468	<.0001†	0.39
		GG	0.223	0.208	0.333	0.493	0.444	0.650	0.360		
		CC	0.285	0.223	0.177	0.090	0.130	0.029	0.172		
IL6	rs2069832	AG	0.492	0.569	0.495	0.398	0.426	0.333	0.468	<.0001†	0.32
		GG	0.225	0.208	0.320	0.496	0.444	0.637	0.356		
		AA	0.283	0.223	0.186	0.105	0.130	0.029	0.176		
IL6	rs2069840	CC	0.585	0.497	0.432	0.481	0.399	0.337	0.466	0.0003†	0.34
		CG	0.354	0.457	0.472	0.436	0.470	0.500	0.444		
		GG	0.062	0.046	0.095	0.083	0.131	0.163	0.090		
IL6	rs1554606	GG	0.220	0.179	0.305	0.455	0.404	0.577	0.329	<.0001†	0.52
		GT	0.482	0.554	0.497	0.433	0.452	0.404	0.479		
		TT	0.298	0.267	0.198	0.112	0.145	0.019	0.191		
IL6	rs2069845	AA	0.211	0.173	0.300	0.459	0.408	0.592	0.364	<.0001†	0.40
		AG	0.485	0.556	0.495	0.436	0.444	0.388	0.477		
		GG	0.304	0.270	0.205	0.105	0.148	0.019	0.195		
IL6	rs2069849	CC	0.974	0.934	0.945	0.955	0.941	0.951	0.950	0.5592†	1.00
		CT	0.026	0.066	0.055	0.045	0.059	0.049	0.050		
		TT	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
IL10	rs3024502	CC	0.307	0.272	0.274	0.398	0.396	0.406	0.330	0.0002†	0.19
		CT	0.450	0.436	0.503	0.481	0.500	0.436	0.469		
		TT	0.243	0.292	0.223	0.120	0.104	0.158	0.201		
IL10	rs3024496	AA	0.301	0.272	0.278	0.402	0.353	0.398	0.323	0.0110†	0.03
		AG	0.451	0.431	0.490	0.477	0.461	0.427	0.457		
		GG	0.249	0.297	0.232	0.121	0.186	0.175	0.220		

*=Fisher's exact test, †=Chi-Square-Test

Gene	SNP	Genotype	Helsinki (N=195)	Stockholm (N=197)	Augsburg (N=200)	Rome (N=134)	Barcelona (N=169)	Athens (N=108)	All Centers (N=1003)	*p*-value	HWE (*p*-value)
IL10	rs1518110	CC	0.686	0.650	0.628	0.552	0.568	0.573	0.617	0.2717*	0.93
		AC	0.284	0.305	0.332	0.403	0.379	0.350	0.336		
		AA	0.031	0.046	0.041	0.045	0.053	0.078	0.046		
IL10	rs3024491	AC	0.455	0.437	0.492	0.489	0.450	0.427	0.460	0.0123†	0.04
		CC	0.304	0.269	0.279	0.391	0.367	0.398	0.324		
		AA	0.241	0.294	0.228	0.120	0.183	0.175	0.216		
IL10	rs2222202	AG	0.456	0.431	0.497	0.474	0.467	0.427	0.461	0.0081†	0.05
		GG	0.301	0.272	0.276	0.406	0.353	0.398	0.323		
		AA	0.244	0.297	0.226	0.120	0.180	0.175	0.216		
IL10	rs1800872	GG	0.604	0.599	0.588	0.537	0.544	0.553	0.575	0.8358†	0.54
		GT	0.365	0.355	0.352	0.403	0.396	0.369	0.371		
		TT	0.031	0.046	0.060	0.060	0.059	0.078	0.053		
IL10	rs1800871	GG	0.607	0.600	0.589	0.537	0.542	0.553	0.576	0.8767†	0.66
		AG	0.356	0.354	0.350	0.403	0.398	0.369	0.369		
		AA	0.037	0.046	0.061	0.060	0.060	0.078	0.055		
IL10	rs1800894	CC	0.921	0.923	0.918	0.939	0.904	0.882	0.916	0.6613*	1.00
		CT	0.079	0.077	0.082	0.061	0.096	0.108	0.083		
		TT	0.000	0.000	0.000	0.000	0.000	0.010	0.001		
IL10	rs1800893	CC	0.305	0.262	0.276	0.398	0.347	0.379	0.318	0.0214†	0.08
		CT	0.453	0.446	0.492	0.474	0.479	0.437	0.465		
		TT	0.242	0.292	0.231	0.128	0.174	0.184	0.217		
IL10	rs1800890	AA	0.416	0.386	0.390	0.530	0.500	0.558	0.449	0.0015†	0.04
		AT	0.437	0.406	0.485	0.403	0.393	0.346	0.419		
		TT	0.147	0.208	0.125	0.067	0.107	0.096	0.132		

*=Fisher's exact test, †=Chi-Square-Test

Gene	SNP	Genotype	Helsinki (N=195)	Stockholm (N=197)	Augsburg (N=200)	Rome (N=134)	Barcelona (N=169)	Athens (N=108)	All Centers (N=1003)	*p*-value	HWE (*p*-value)
IL10	rs10494879	CC	0.380	0.335	0.365	0.515	0.479	0.529	0.419	0.0001†	0.02
		CG	0.464	0.421	0.487	0.409	0.402	0.346	0.430		
		GG	0.156	0.244	0.147	0.076	0.118	0.125	0.151		
IL10	rs6676671	TT	0.415	0.391	0.390	0.530	0.497	0.558	0.449	0.0022†	0.03
		AT	0.441	0.401	0.480	0.403	0.391	0.346	0.417		
		AA	0.144	0.208	0.130	0.067	0.112	0.096	0.133		
IL18	rs3882891	GT	0.518	0.462	0.485	0.493	0.482	0.615	0.501	0.1049†	0.70
		TT	0.262	0.320	0.355	0.343	0.274	0.221	0.301		
		GG	0.221	0.218	0.160	0.164	0.244	0.163	0.198		
IL18	rs5744280	GG	0.560	0.487	0.438	0.402	0.488	0.392	0.471	0.0703†	0.11
		AG	0.314	0.406	0.454	0.439	0.423	0.490	0.414		
		AA	0.126	0.107	0.108	0.159	0.089	0.118	0.116		
IL18	rs549908	TT	0.481	0.477	0.513	0.507	0.391	0.515	0.478	0.2516†	0.71
		GT	0.412	0.411	0.392	0.440	0.527	0.408	0.431		
		GG	0.107	0.112	0.095	0.052	0.083	0.078	0.091		
IL18	rs1834481	CC	0.564	0.569	0.565	0.627	0.645	0.667	0.598	0.2051*	0.93
		CG	0.400	0.376	0.355	0.343	0.296	0.313	0.352		
		GG	0.036	0.056	0.080	0.030	0.059	0.020	0.050		
IL18	rs360722	GG	0.705	0.758	0.774	0.762	0.753	0.611	0.736	0.0610*	0.89
		AG	0.258	0.232	0.221	0.208	0.235	0.368	0.245		
		AA	0.037	0.010	0.005	0.031	0.012	0.021	0.019		
IL18	rs2043055	AA	0.538	0.452	0.405	0.391	0.429	0.376	0.440	0.0251†	0.09
		AG	0.333	0.431	0.470	0.398	0.464	0.485	0.427		
		GG	0.128	0.117	0.125	0.211	0.107	0.139	0.134		

*=Fisher's exact test, †=Chi-Square-Test

Gene	SNP	Genotype	Helsinki (N=195)	Stockholm (N=197)	Augsburg (N=200)	Rome (N=134)	Barcelona (N=169)	Athens (N=108)	All Centers (N=1003)	*p*-value	HWE (*p*-value)
IL18	rs187238	CC	0.510	0.508	0.555	0.552	0.450	0.558	0.519	0.2732†	0.69
		CG	0.397	0.406	0.360	0.396	0.503	0.375	0.407		
		GG	0.093	0.086	0.085	0.052	0.047	0.067	0.074		
IL18	rs1946518	GG	0.292	0.355	0.380	0.361	0.325	0.225	0.330	0.0698†	0.37
		GT	0.497	0.452	0.450	0.436	0.446	0.637	0.476		
		TT	0.210	0.193	0.170	0.203	0.229	0.137	0.193		
IL18	rs5744222	GG	0.560	0.556	0.565	0.624	0.625	0.676	0.592	0.1501*	0.60
		GT	0.389	0.388	0.350	0.346	0.298	0.304	0.351		
		TT	0.052	0.056	0.085	0.030	0.077	0.020	0.057		
LTA	rs2009658	CC	0.728	0.675	0.680	0.633	0.646	0.692	0.678	0.7282*	0.91
		CG	0.246	0.289	0.285	0.352	0.311	0.288	0.291		
		GG	0.026	0.036	0.035	0.016	0.043	0.019	0.030		
LTA	rs1800683	GG	0.462	0.401	0.575	0.537	0.575	0.549	0.511	0.0255†	0.58
		AG	0.451	0.487	0.365	0.388	0.365	0.402	0.413		
		AA	0.087	0.112	0.060	0.075	0.060	0.049	0.076		
LTA	rs2239704	CA	0.446	0.421	0.528	0.463	0.467	0.524	0.471	0.0066†	0.36
		CC	0.421	0.426	0.271	0.381	0.314	0.243	0.350		
		AA	0.133	0.152	0.201	0.157	0.219	0.233	0.179		
LTA	rs909253	AA	0.456	0.401	0.575	0.537	0.574	0.549	0.510	0.0224†	0.59
		AG	0.456	0.487	0.365	0.388	0.367	0.402	0.414		
		GG	0.087	0.112	0.060	0.075	0.059	0.049	0.076		
LTA	rs2857713	TT	0.443	0.528	0.523	0.432	0.485	0.558	0.493	0.6163†	0.14
		CT	0.448	0.376	0.367	0.447	0.414	0.365	0.403		
		CC	0.108	0.096	0.111	0.121	0.101	0.077	0.104		

*=Fisher's exact test, †=Chi-Square-Test

Gene	SNP	Genotype	Helsinki (N=195)	Stockholm (N=197)	Augsburg (N=200)	Rome (N=134)	Barcelona (N=169)	Athens (N=108)	All Centers (N=1003)	*p*-value	HWE (*p*-value)
LTA	rs3093543	AA	0.881	0.868	0.895	0.821	0.861	0.832	0.865	0.2790*	0.62
		AC	0.119	0.132	0.095	0.179	0.133	0.168	0.132		
		CC	0.000	0.000	0.010	0.000	0.006	0.000	0.003		
LTA	rs1041981	CC	0.464	0.403	0.575	0.519	0.565	0.553	0.508	0.0474†	0.64
		AC	0.448	0.485	0.365	0.406	0.375	0.388	0.414		
		AA	0.088	0.112	0.060	0.075	0.060	0.058	0.077		
NFKB1	rs28362491	Ins/Ins	0.359	0.372	0.374	0.481	0.429	0.495	0.407	0.1983†	0.02
		Ins/Del	0.503	0.459	0.439	0.376	0.405	0.388	0.436		
		Del/Del	0.138	0.168	0.187	0.143	0.167	0.117	0.157		
NFKB1	rs3774932	AG	0.515	0.482	0.485	0.504	0.497	0.495	0.496	0.7907†	0.90
		GG	0.253	0.315	0.301	0.218	0.266	0.291	0.276		
		AA	0.232	0.203	0.214	0.278	0.237	0.214	0.228		
NFKB1	rs1598856	AG	0.521	0.480	0.485	0.492	0.491	0.500	0.494	0.8271†	0.85
		GG	0.247	0.316	0.295	0.227	0.269	0.284	0.275		
		AA	0.232	0.204	0.220	0.280	0.240	0.216	0.230		
NFKB1	rs230530	AA	0.258	0.333	0.300	0.216	0.284	0.294	0.284	0.6913†	0.95
		AG	0.511	0.477	0.485	0.515	0.497	0.500	0.496		
		GG	0.232	0.190	0.215	0.269	0.219	0.206	0.220		
NFKB1	rs4648004	AA	0.358	0.462	0.407	0.398	0.386	0.462	0.409	0.7621†	0.54
		AG	0.484	0.410	0.467	0.451	0.476	0.423	0.454		
		GG	0.158	0.128	0.126	0.150	0.139	0.115	0.137		
NFKB1	rs230521	GG	0.358	0.365	0.395	0.557	0.438	0.505	0.419	0.0056†	<0.00
		CG	0.503	0.452	0.430	0.296	0.354	0.379	0.416		
		CC	0.140	0.183	0.175	0.148	0.208	0.117	0.165		

*=Fisher's exact test, †=Chi-Square-Test

Gene	SNP	Genotype	Helsinki (N=195)	Stockholm (N=197)	Augsburg (N=200)	Rome (N=134)	Barcelona (N=169)	Athens (N=108)	All Centers (N=1003)	*p*-value	HWE (*p*-value)
NFKB1	rs7340881	CC	0.768	0.701	0.735	0.707	0.737	0.592	0.716	0.1142*	0.81
		CT	0.227	0.274	0.235	0.271	0.234	0.359	0.259		
		TT	0.005	0.025	0.030	0.023	0.030	0.049	0.025		
NFKB1	rs230498	GG	0.474	0.451	0.460	0.541	0.473	0.524	0.481	0.5809†	0.02
		AG	0.428	0.426	0.379	0.361	0.420	0.369	0.401		
		AA	0.098	0.123	0.162	0.098	0.107	0.107	0.118		
NFKB1	rs1801	GG	0.467	0.442	0.440	0.530	0.473	0.524	0.472	0.4035†	0.01
		CG	0.431	0.437	0.385	0.366	0.419	0.350	0.404		
		CC	0.103	0.122	0.175	0.104	0.108	0.126	0.124		
NFKB1	rs3774956	CC	0.358	0.354	0.382	0.481	0.401	0.500	0.401	0.2197†	0.01
		CT	0.489	0.467	0.427	0.376	0.431	0.356	0.433		
		TT	0.153	0.179	0.191	0.143	0.168	0.144	0.166		
NFKB1	rs1020759	CC	0.351	0.350	0.376	0.485	0.399	0.500	0.397	0.1392†	0.02
		CT	0.500	0.467	0.437	0.373	0.429	0.356	0.437		
		TT	0.149	0.183	0.188	0.142	0.173	0.144	0.166		
NFKB1	rs3774964	AA	0.399	0.413	0.415	0.530	0.473	0.539	0.450	0.2838†	0.01
		AG	0.461	0.439	0.445	0.358	0.391	0.324	0.413		
		GG	0.140	0.148	0.140	0.112	0.136	0.137	0.137		
NFKB1	rs11722146	GG	0.531	0.548	0.490	0.590	0.592	0.587	0.550	0.5040†	0.03
		AG	0.387	0.376	0.388	0.336	0.349	0.317	0.364		
		AA	0.082	0.076	0.122	0.075	0.059	0.096	0.086		
NFKB1	rs4648090	GG	0.814	0.736	0.765	0.709	0.728	0.683	0.746	0.2170*	0.15
		AG	0.170	0.228	0.199	0.276	0.254	0.288	0.228		
		AA	0.015	0.036	0.036	0.015	0.018	0.029	0.025		

*=Fisher's exact test, †=Chi-Square-Test

Gene	SNP	Genotype	Helsinki (N=195)	Stockholm (N=197)	Augsburg (N=200)	Rome (N=134)	Barcelona (N=169)	Athens (N=108)	All Centers (N=1003)	*p*-value	HWE (*p*-value)
NFKB1	rs4648110	TT	0.728	0.619	0.655	0.639	0.625	0.544	0.644	0.1688*	0.77
		AT	0.251	0.330	0.295	0.331	0.333	0.398	0.315		
		AA	0.021	0.051	0.050	0.030	0.042	0.058	0.041		
NFKB1	rs4648133	TT	0.531	0.543	0.485	0.575	0.568	0.567	0.540	0.7508†	0.05
		CT	0.387	0.381	0.393	0.351	0.361	0.337	0.372		
		CC	0.082	0.076	0.122	0.075	0.071	0.096	0.088		
NFKB1	rs1609798	CC	0.482	0.505	0.482	0.571	0.592	0.596	0.529	0.1953†	0.08
		CT	0.420	0.413	0.397	0.361	0.349	0.298	0.381		
		TT	0.098	0.082	0.121	0.068	0.059	0.106	0.090		
NFKBIA	rs696	CC	0.383	0.369	0.288	0.368	0.408	0.408	0.366	0.1442†	0.47
		CT	0.415	0.497	0.551	0.429	0.444	0.456	0.469		
		TT	0.202	0.133	0.162	0.203	0.148	0.136	0.164		
NFKBIA	rs8904	AG	0.405	0.497	0.553	0.437	0.456	0.394	0.467	0.1204†	0.35
		GG	0.395	0.365	0.286	0.349	0.394	0.408	0.361		
		AA	0.200	0.137	0.161	0.214	0.150	0.197	0.172		
NFKBIA	rs3138054	CC	0.736	0.680	0.650	0.731	0.716	0.709	0.701	0.4749*	0.25
		CT	0.233	0.294	0.320	0.216	0.260	0.243	0.266		
		TT	0.031	0.025	0.030	0.052	0.024	0.049	0.033		
NFKBIA	rs1957106	GG	0.358	0.477	0.432	0.571	0.506	0.559	0.471	0.0017†	0.88
		AG	0.477	0.416	0.467	0.376	0.423	0.373	0.429		
		AA	0.166	0.107	0.101	0.053	0.071	0.069	0.100		
NFKBIA	rs2233409	GG	0.603	0.614	0.555	0.582	0.629	0.637	0.601	0.8419†	0.53
		AG	0.345	0.345	0.380	0.336	0.323	0.314	0.344		
		AA	0.052	0.041	0.065	0.082	0.048	0.049	0.055		

*=Fisher's exact test, †=Chi-Square-Test

Gene	SNP	Genotype	Helsinki (N=195)	Stockholm (N=197)	Augsburg (N=200)	Rome (N=134)	Barcelona (N=169)	Athens (N=108)	All Centers (N=1003)	*p*-value	HWE (*p*-value)
NFKBIA	rs2233407	TT	0.800	0.863	0.915	0.910	0.946	0.913	0.887	0.0003*	0.77
		AT	0.179	0.137	0.085	0.090	0.054	0.087	0.109		
		AA	0.021	0.000	0.000	0.000	0.000	0.000	0.004		
NFKBIA	rs3138052	TT	0.565	0.548	0.455	0.485	0.533	0.544	0.521	0.5919†	0.39
		CT	0.368	0.365	0.445	0.403	0.396	0.388	0.395		
		CC	0.067	0.086	0.100	0.112	0.071	0.068	0.084		
RELA	rs1049728	GG	0.796	0.883	0.860	0.866	0.929	0.923	0.871	0.0191*	0.13
		CG	0.194	0.107	0.125	0.127	0.071	0.077	0.121		
		CC	0.010	0.010	0.015	0.007	0.000	0.000	0.008		
RELA	rs7119750	CC	0.821	0.797	0.797	0.761	0.751	0.779	0.787	0.4559*	0.16
		CT	0.179	0.178	0.183	0.209	0.225	0.212	0.195		
		TT	0.000	0.025	0.020	0.030	0.024	0.010	0.018		
RELA	rs11568300	CC	0.287	0.413	0.383	0.500	0.479	0.471	0.411	0.0019†	0.08
		CG	0.497	0.439	0.485	0.343	0.402	0.431	0.440		
		GG	0.215	0.148	0.133	0.157	0.118	0.098	0.149		
RELA	rs732072	GG	0.897	0.873	0.850	0.746	0.834	0.760	0.838	0.0015*	0.17
		AG	0.103	0.112	0.130	0.231	0.166	0.022	0.151		
		AA	0.000	0.015	0.020	0.022	0.000	0.010	0.011		
RELA	rs11227248	CC	0.464	0.429	0.470	0.440	0.387	0.408	0.436	0.2327†	0.94
		CT	0.464	0.449	0.415	0.485	0.458	0.427	0.449		
		TT	0.072	0.122	0.115	0.075	0.155	0.165	0.115		
RELA	rs11820062	CT	0.477	0.472	0.530	0.444	0.408	0.500	0.473	0.1057†	0.09
		TT	0.218	0.294	0.260	0.316	0.343	0.324	0.287		
		CC	0.306	0.234	0.210	0.241	0.249	0.176	0.240		

*=Fisher's exact test, †=Chi-Square-Test

Gene	SNP	Genotype	Helsinki (N=195)	Stockholm (N=197)	Augsburg (N=200)	Rome (N=134)	Barcelona (N=169)	Athens (N=108)	All Centers (N=1003)	*p*-value	HWE (*p*-value)
RELA	rs7101916	CC	0.821	0.797	0.815	0.761	0.754	0.779	0.791	0.3899*	0.16
		CT	0.179	0.178	0.165	0.209	0.228	0.212	0.192		
		TT	0.000	0.025	0.020	0.030	0.018	0.010	0.017		
TLR4	rs2770150	AA	0.601	0.577	0.515	0.602	0.524	0.510	0.557	0.2703†	0.93
		AG	0.347	0.371	0.420	0.308	0.423	0.382	0.378		
		GG	0.052	0.052	0.065	0.090	0.054	0.108	0.066		
TLR4	rs1927914	AA	0.314	0.345	0.445	0.520	0.444	0.455	0.410	0.0069†	0.11
		AG	0.541	0.513	0.460	0.386	0.457	0.495	0.480		
		GG	0.144	0.142	0.095	0.094	0.099	0.051	0.110		
TLR4	rs1927911	GG	0.451	0.415	0.545	0.586	0.554	0.674	0.520	0.0032†	0.15
		AG	0.467	0.497	0.405	0.353	0.387	0.304	0.416		
		AA	0.082	0.087	0.050	0.060	0.060	0.022	0.064		
TLR4	rs11536878	CC	0.870	0.731	0.803	0.813	0.725	0.825	0.792	0.0056*	0.34
		AC	0.119	0.259	0.192	0.157	0.251	0.155	0.193		
		AA	0.010	0.010	0.005	0.030	0.024	0.019	0.015		
TLR4	rs1927907	CC	0.550	0.627	0.701	0.752	0.819	0.800	0.694	<.0001*	0.64
		CT	0.397	0.333	0.294	0.233	0.169	0.190	0.281		
		TT	0.053	0.040	0.005	0.016	0.013	0.010	0.024		
TLR4	rs2149356	GG	0.328	0.365	0.460	0.568	0.482	0.636	0.448	<.0001†	0.72
		GT	0.523	0.497	0.445	0.328	0.421	0.307	0.440		
		TT	0.149	0.137	0.095	0.104	0.098	0.057	0.112		
TLR4	rs4986790	AA	0.820	0.908	0.889	0.946	0.885	0.881	0.885	0.0374*	1.00
		AG	0.175	0.087	0.111	0.054	0.109	0.119	0.112		
		GG	0.005	0.005	0.000	0.000	0.006	0.000	0.003		

*=Fisher's exact test, †=Chi-Square-Test

Gene	SNP	Genotype	Helsinki (N=195)	Stockholm (N=197)	Augsburg (N=200)	Rome (N=134)	Barcelona (N=169)	Athens (N=108)	All Centers (N=1003)	*p*-value	HWE (*p*-value)
TLR4	rs4986791	CC	0.814	0.893	0.865	0.918	0.880	0.863	0.870	0.1094*	0.31
		CT	0.186	0.102	0.135	0.082	0.114	0.137	0.128		
		TT	0.000	0.005	0.000	0.000	0.006	0.000	0.002		
TLR4	rs11536889	GG	0.794	0.766	0.760	0.657	0.714	0.670	0.737	0.0610*	0.90
		CG	0.201	0.218	0.225	0.291	0.268	0.301	0.243		
		CC	0.005	0.015	0.015	0.052	0.018	0.029	0.020		
TLR4	rs7873784	GG	0.827	0.682	0.746	0.754	0.705	0.748	0.743	0.0314*	0.13
		CG	0.173	0.308	0.244	0.216	0.283	0.233	0.244		
		CC	0.000	0.010	0.010	0.030	0.012	0.019	0.012		
TNFA	rs1799964	TT	0.707	0.624	0.596	0.537	0.592	0.663	0.621	0.0880†	0.23
		CT	0.257	0.315	0.359	0.418	0.325	0.288	0.325		
		CC	0.037	0.061	0.045	0.045	0.083	0.048	0.053		
TNFA	rs1800630	CC	0.744	0.670	0.685	0.642	0.675	0.673	0.685	0.6378*	0.82
		CA	0.236	0.299	0.280	0.343	0.284	0.308	0.287		
		AA	0.021	0.030	0.035	0.015	0.041	0.019	0.028		
TNFA	rs1799724	CC	0.868	0.837	0.735	0.761	0.775	0.598	0.777	<.0001*	0.03
		CT	0.122	0.138	0.245	0.231	0.207	0.333	0.201		
		TT	0.011	0.026	0.020	0.007	0.018	0.069	0.022		
TNFA	rs1800629	GG	0.762	0.665	0.814	0.805	0.750	0.808	0.762	0.0194*	0.32
		AG	0.212	0.289	0.176	0.188	0.244	0.173	0.218		
		AA	0.026	0.046	0.010	0.008	0.006	0.019	0.020		
TNFA	rs361525	GG	0.964	0.924	0.910	0.880	0.887	0.962	0.921	0.0242*	0.23
		AG	0.036	0.076	0.090	0.113	0.101	0.038	0.076		
		AA	0.000	0.000	0.000	0.008	0.012	0.000	0.003		

*=Fisher's exact test, †=Chi-Square-Test

Gene	SNP	Genotype	Helsinki (N=195)	Stockholm (N=197)	Augsburg (N=200)	Rome (N=134)	Barcelona (N=169)	Athens (N=108)	All Centers (N=1003)	*p*-value	HWE (*p*-value)
TNFA	rs3093661	GG	0.963	0.949	0.920	0.910	0.935	0.971	0.940	0.2234*	0.60
		AG	0.037	0.051	0.080	0.082	0.065	0.029	0.059		
		AA	0.000	0.000	0.000	0.007	0.000	0.000	0.001		
TNFA	rs3093664	AA	0.918	0.848	0.875	0.776	0.870	0.846	0.861	0.0250*	0.10
		AG	0.082	0.137	0.110	0.209	0.130	0.144	0.130		
		GG	0.000	0.015	0.015	0.015	0.000	0.010	0.009		
TNFA	rs3093665	AA	0.990	0.964	0.965	0.872	0.935	0.885	0.944	<.0001*	0.56
		AC	0.010	0.036	0.035	0.120	0.065	0.115	0.055		
		CC	0.000	0.000	0.000	0.008	0.000	0.000	0.001		
TNFA	rs3091257	GG	0.845	0.769	0.884	0.925	0.874	0.942	0.864	0.0008*	<0.00
		AG	0.130	0.210	0.106	0.075	0.120	0.048	0.123		
		AA	0.026	0.021	0.010	0.000	0.006	0.010	0.013		

*=Fisher's exact test, †=Chi-Square-Test

Table II: Detailed association results of polymorphisms in candidate genes involved in the inflammatory pathways and CRP concentrations in 1,003 MI survivors.

Gene	SNP	Genotype	Frequency	Estimate	Standard Error	*p*-value
FGA	rs2070022	AA	0.030	0.051	0.170	0.77
		AG	0.273	-0.030	0.066	0.64
		GG	0.697	Reference		
FGA	rs6050	CC	0.082	0.078	0.111	0.48
		CT	0.365	0.092	0.064	0.15
		TT	0.553	Reference		
FGA	rs2070018	GG	0.021	-0.057	0.202	0.78
		AG	0.206	0.127	0.072	0.08
		AA	0.772	Reference		
FGA	rs2070016	GG	0.025	-0.010	0.188	0.96
		AG	0.255	-0.035	0.067	0.61
		AA	0.719	Reference		
FGA	rs2070014	TT	0.030	0.017	0.173	0.92
		CT	0.268	-0.009	0.067	0.89
		CC	0.702	Reference		
FGA	rs2070011	TT	0.165	0.147	0.086	0.09
		CT	0.448	0.063	0.064	0.32
		CC	0.386	Reference		
FGA	rs10012555	CC	0.019	-0.034	0.219	0.88
		CT	0.213	0.123	0.072	0.09
		TT	0.769	Reference		
FGA	rs2070006	TT	0.164	0.149	0.086	0.08
		CC	0.378	0.063	0.063	0.32
		CT	0.458	Reference		
FGB	rs1800791	AA	0.025	0.052	0.185	0.78
		AG	0.245	-0.027	0.067	0.69
		GG	0.730	Reference		
FGB	rs1800790	AA	0.039	0.279	0.150	0.06
		AG	0.324	0.007	0.062	0.92
		GG	0.636	Reference		
FGB	rs1800788	TT	0.055	0.032	0.128	0.80
		CT	0.322	0.055	0.063	0.38
		CC	0.622	Reference		
FGB	rs2227399	GG	0.040	0.277	0.149	0.06
		GT	0.327	0.018	0.062	0.77
		TT	0.633	Reference		

Gene	SNP	Genotype	Frequency	Estimate	Standard Error	*p*-value
FGB	rs6056	TT	0.031	0.210	0.168	0.21
		CT	0.310	-0.005	0.063	0.94
		CC	0.659	Reference		
FGB	rs4220	AA	0.030	0.168	0.171	0.33
		AG	0.310	-0.004	0.063	0.96
		GG	0.660	Reference		
FGB	rs2227421	CC	0.094	-0.117	0.104	0.26
		AC	0.450	-0.031	0.061	0.61
		AA	0.457	Reference		
FGG	rs2066865	AA	0.069	0.058	0.116	0.62
		AG	0.354	-0.037	0.062	0.55
		GG	0.576	Reference		
FGG	rs1049636	GG	0.094	0.137	0.103	0.18
		AG	0.409	0.015	0.061	0.80
		AA	0.496	Reference		
FGG	rs2066861	TT	0.068	0.067	0.117	0.57
		CT	0.355	-0.039	0.062	0.53
		CC	0.576	Reference		
FGG	rs2066860	TT + CT	0.073	-0.179	0.111	0.11
		CC	0.927	Reference		
FGG	rs1800792	CC	0.209	-0.086	0.081	0.29
		CT	0.475	-0.095	0.066	0.15
		TT	0.315	Reference		
FGG	rs2066854	AA	0.070	0.077	0.116	0.51
		AT	0.356	-0.033	0.062	0.60
		TT	0.573	Reference		
IL6	rs2069827	TT	0.020	-0.355	0.206	0.08
		GT	0.188	-0.072	0.074	0.33
		GG	0.792	Reference		
IL6	rs1800796	CC + CG	0.109	-0.103	0.093	0.27
		GG	0.891	Reference		
IL6	rs1800795	CC	0.172	-0.095	0.085	0.26
		GG	0.360	-0.052	0.064	0.41
		CG	0.468	Reference		
IL6	rs2069832	AA	0.176	-0.074	0.085	0.39
		GG	0.356	-0.030	0.065	0.65
		AG	0.468	Reference		
IL6	rs2069840	GG	0.090	0.055	0.105	0.60
		CG	0.444	0.048	0.061	0.43
		CC	0.466	Reference		

Gene	SNP	Genotype	Frequency	Estimate	Standard Error	*p*-value
IL6	rs1554606	TT	0.191	-0.035	0.083	0.68
		GG	0.329	-0.030	0.066	0.65
		GT	0.479	Reference		
IL6	rs2069845	GG	0.195	-0.043	0.083	0.60
		AA	0.364	-0.031	0.066	0.64
		AG	0.477	Reference		
IL6	rs2069849	TT + CT	0.050	0.102	0.132	0.44
		CC	0.950	Reference		
IL10	rs3024502	TT	0.201	0.009	0.083	0.92
		CC	0.330	0.041	0.067	0.54
		CT	0.469	Reference		
IL10	rs3024496	GG	0.220	-0.004	0.080	0.96
		AA	0.323	0.036	0.067	0.59
		AG	0.457	Reference		
IL10	rs1518110	AA	0.046	-0.253	0.139	0.07
		AC	0.336	0.060	0.062	0.33
		CC	0.617	Reference		
IL10	rs3024491	AA	0.216	-0.011	0.081	0.89
		CC	0.324	0.035	0.067	0.60
		AC	0.460	Reference		
IL10	rs2222202	AA	0.216	-0.002	0.080	0.98
		GG	0.323	0.031	0.066	0.64
		AG	0.461	Reference		
IL10	rs1800872	TT	0.053	-0.167	0.131	0.20
		GT	0.371	0.062	0.061	0.31
		GG	0.575	Reference		
IL10	rs1800871	AA	0.055	-0.203	0.130	0.12
		AG	0.369	0.059	0.061	0.34
		GG	0.576	Reference		
IL10	rs1800894	TT + CT	0.084	0.182	0.105	0.08
		CC	0.916	Reference		
IL10	rs1800893	TT	0.217	0.013	0.081	0.88
		CC	0.318	0.071	0.067	0.29
		CT	0.465	Reference		
IL10	rs1800890	TT	0.132	-0.045	0.091	0.62
		AT	0.419	-0.006	0.062	0.93
		AA	0.449	Reference		
IL10	rs10494879	GG	0.151	-0.046	0.087	0.60
		CC	0.419	0.034	0.063	0.59
		CG	0.430	Reference		

Gene	SNP	Genotype	Frequency	Estimate	Standard Error	*p*-value
IL10	rs6676671	AA	0.133	-0.043	0.090	0.63
		AT	0.417	0.012	0.062	0.84
		TT	0.449	Reference		
IL18	rs3882891	GG	0.198	0.132	0.083	0.11
		GT	0.501	0.096	0.066	0.15
		TT	0.301	Reference		
IL18	rs5744280	AA	0.116	-0.167	0.095	0.08
		AG	0.414	0.006	0.062	0.93
		GG	0.471	Reference		
IL18	rs549908	GG	0.091	-0.020	0.105	0.85
		GT	0.431	0.024	0.061	0.70
		TT	0.478	Reference		
IL18	rs1834481	GG	0.050	-0.178	0.134	0.18
		CG	0.352	0.026	0.061	0.67
		CC	0.598	Reference		
IL18	rs360722	AA	0.019	0.278	0.220	0.21
		AG	0.245	0.106	0.068	0.12
		GG	0.736	Reference		
IL18	rs2043055	GG	0.134	-0.139	0.090	0.12
		AG	0.427	0.026	0.062	0.67
		AA	0.440	Reference		
IL18	rs187238	GG	0.074	-0.062	0.113	0.58
		CG	0.407	0.040	0.060	0.51
		CC	0.519	Reference		
IL18	rs1946518	TT	0.193	0.076	0.083	0.36
		GG	0.330	0.082	0.066	0.21
		GT	0.476	Reference		
IL18	rs5744222	TT	0.057	-0.141	0.126	0.26
		GT	0.351	0.015	0.062	0.81
		GG	0.592	Reference		
LTA	rs2009658	GG	0.030	-0.241	0.170	0.16
		CG	0.291	0.045	0.064	0.48
		CC	0.678	Reference		
LTA	rs1800683	AA	0.076	-0.055	0.112	0.62
		AG	0.413	-0.074	0.060	0.22
		GG	0.511	Reference		
LTA	rs2239704	AA	0.179	0.069	0.084	0.41
		CC	0.350	0.102	0.064	0.11
		CA	0.471	Reference		

Gene	SNP	Genotype	Frequency	Estimate	Standard Error	*p*-value
LTA	rs909253	GG	0.076	-0.058	0.112	0.61
		AG	0.414	-0.074	0.060	0.22
		AA	0.510	Reference		
LTA	rs2857713	CC	0.104	-0.119	0.099	0.23
		CT	0.403	0.070	0.061	0.25
		TT	0.493	Reference		
LTA	rs3093543	CC + AC	0.135	0.048	0.085	0.57
		AA	0.865	Reference		
LTA	rs1041981	AA	0.077	-0.043	0.111	0.70
		AC	0.414	-0.064	0.061	0.29
		CC	0.508	Reference		
NFKB1	rs28362491	Del/Del	0.157	-0.083	0.086	0.33
		Ins/Ins	0.407	-0.006	0.063	0.92
		Ins/Del	0.436	Reference		
NFKB1	rs3774932	AA	0.228	0.099	0.082	0.23
		GG	0.276	0.081	0.069	0.24
		AG	0.496	Reference		
NFKB1	rs1598856	AA	0.230	0.091	0.082	0.27
		GG	0.275	0.080	0.069	0.24
		AG	0.494	Reference		
NFKB1	rs230530	GG	0.220	0.083	0.082	0.31
		AA	0.284	0.068	0.068	0.32
		AG	0.496	Reference		
NFKB1	rs4648004	GG	0.137	-0.023	0.091	0.80
		AA	0.409	0.066	0.063	0.29
		AG	0.454	Reference		
NFKB1	rs230521	CC	0.165	-0.065	0.086	0.45
		CG	0.416	-0.019	0.065	0.77
		GG	0.419	Reference		
NFKB1	rs7340881	TT	0.025	0.150	0.186	0.42
		CT	0.259	-0.069	0.066	0.30
		CC	0.716	Reference		
NFKB1	rs230498	AA	0.118	-0.079	0.094	0.40
		AG	0.401	-0.004	0.062	0.94
		GG	0.481	Reference		
NFKB1	rs1801	CC	0.124	-0.052	0.092	0.57
		CG	0.404	0.003	0.062	0.96
		GG	0.472	Reference		
NFKB1	rs3774956	TT	0.166	-0.076	0.085	0.37
		CC	0.401	0.012	0.064	0.85
		CT	0.433	Reference		

Gene	SNP	Genotype	Frequency	Estimate	Standard Error	*p*-value
NFKB1	rs1020759	TT	0.166	-0.059	0.084	0.48
		CC	0.397	0.001	0.063	0.99
		CT	0.437	Reference		
NFKB1	rs3774964	GG	0.137	-0.007	0.089	0.94
		AG	0.413	-0.039	0.062	0.53
		AA	0.450	Reference		
NFKB1	rs11722146	AA	0.086	0.025	0.106	0.81
		AG	0.364	-0.016	0.062	0.80
		GG	0.550	Reference		
NFKB1	rs4648090	AA	0.025	-0.068	0.186	0.71
		AG	0.228	-0.064	0.069	0.36
		GG	0.746	Reference		
NFKB1	rs4648110	AA	0.041	0.023	0.147	0.88
		AT	0.315	-0.055	0.063	0.38
		TT	0.644	Reference		
NFKB1	rs4648133	CC	0.088	0.048	0.105	0.65
		CT	0.372	-0.016	0.062	0.80
		TT	0.540	Reference		
NFKB1	rs1609798	TT	0.090	0.071	0.105	0.50
		CT	0.381	-0.017	0.061	0.78
		CC	0.529	Reference		
NFKBIA	rs696	TT	0.164	-0.179	0.086	0.04
		CC	0.366	-0.099	0.064	0.12
		CT	0.469	Reference		
NFKBIA	rs8904	AA	0.172	-0.155	0.087	0.07
		GG	0.361	-0.060	0.066	0.36
		AG	0.467	Reference		
NFKBIA	rs3138054	TT	0.033	-0.152	0.162	0.35
		CT	0.266	0.038	0.066	0.56
		CC	0.701	Reference		
NFKBIA	rs1957106	AA	0.100	-0.058	0.101	0.57
		AG	0.429	-0.004	0.061	0.95
		GG	0.471	Reference		
NFKBIA	rs2233409	AA	0.055	-0.083	0.129	0.52
		AG	0.344	0.035	0.062	0.57
		GG	0.601	Reference		
NFKBIA	rs2233407	AA + AT	0.113	0.028	0.091	0.76
		TT	0.887	Reference		
NFKBIA	rs3138052	CC	0.084	-0.044	0.107	0.68
		CT	0.395	0.009	0.061	0.88
		TT	0.521	Reference		

Gene	SNP	Genotype	Frequency	Estimate	Standard Error	*p*-value
RELA	rs1049728	CC + CG	0.129	0.118	0.086	0.17
		GG	0.871	Reference		
RELA	rs7119750	TT	0.018	-0.230	0.216	0.29
		CT	0.195	-0.014	0.073	0.85
		CC	0.787	Reference		
RELA	rs11568300	GG	0.149	0.003	0.087	0.97
		CC	0.411	-0.064	0.063	0.31
		CG	0.440	Reference		
RELA	rs732072	AA	0.011	-0.245	0.276	0.37
		AG	0.151	-0.033	0.081	0.68
		GG	0.838	Reference		
RELA	rs11227248	TT	0.115	0.106	0.096	0.27
		CC	0.436	-0.013	0.061	0.83
		CT	0.449	Reference		
RELA	rs11820062	CC	0.240	-0.040	0.080	0.61
		TT	0.287	-0.093	0.068	0.17
		CT	0.473	Reference		
RELA	rs7101916	TT	0.017	-0.258	0.222	0.25
		CT	0.192	-0.026	0.073	0.73
		CC	0.791	Reference		
TLR4	rs2770150	GG	0.066	-0.174	0.120	0.15
		AG	0.378	-0.059	0.061	0.33
		AA	0.557	Reference		
TLR4	rs1927914	GG	0.110	0.137	0.099	0.17
		AA	0.410	-0.018	0.062	0.77
		AG	0.480	Reference		
TLR4	rs1927911	AA	0.064	0.167	0.122	0.17
		AG	0.416	0.032	0.060	0.60
		GG	0.520	Reference		
TLR4	rs11536878	AA	0.015	-0.169	0.239	0.48
		AC	0.193	0.109	0.073	0.14
		CC	0.792	Reference		
TLR4	rs1927907	TT	0.024	0.104	0.194	0.59
		CT	0.281	-0.019	0.066	0.77
		CC	0.694	Reference		
TLR4	rs2149356	TT	0.112	0.122	0.098	0.21
		GT	0.440	-0.019	0.062	0.76
		GG	0.448	Reference		
TLR4	rs4986790	GG + AG	0.115	-0.099	0.091	0.28
		AA	0.885	Reference		

Gene	SNP	Genotype	Frequency	Estimate	Standard Error	*p*-value
TLR4	rs4986791	TT + CT	0.130	-0.051	0.086	0.55
		CC	0.870	Reference		
TLR4	rs11536889	CC	0.020	0.070	0.208	0.74
		CG	0.243	0.037	0.068	0.58
		GG	0.737	Reference		
TLR4	rs7873784	CC	0.012	-0.233	0.268	0.38
		CG	0.244	0.078	0.068	0.25
		GG	0.743	Reference		
TNFA	rs1799964	CC	0.053	-0.202	0.130	0.12
		CT	0.325	0.088	0.063	0.16
		TT	0.621	Reference		
TNFA	rs1800630	AA	0.028	-0.271	0.175	0.12
		CA	0.287	0.056	0.064	0.38
		CC	0.685	Reference		
TNFA	rs1799724	TT	0.022	0.233	0.197	0.24
		CT	0.201	0.095	0.073	0.19
		CC	0.777	Reference		
TNFA	rs1800629	AA	0.020	-0.204	0.206	0.32
		AG	0.218	-0.050	0.070	0.48
		GG	0.762	Reference		
TNFA	rs361525	AA + AG	0.079	0.048	0.107	0.65
		GG	0.921	Reference		
TNFA	rs3093661	AA + AG	0.060	-0.016	0.123	0.89
		GG	0.940	Reference		
TNFA	rs3093664	GG + AG	0.139	0.043	0.083	0.61
		AA	0.861	Reference		
TNFA	rs3093665	CC + AC	0.056	0.075	0.125	0.55
		AA	0.944	Reference		
TNFA	rs3091257	AA	0.013	-0.198	0.254	0.44
		AG	0.123	-0.053	0.088	0.55
		GG	0.864	Reference		

Table III: Analysis of intra-individual variability of CRP concentrations: Results for polymorphisms in candidate genes involved in the inflammatory pathways in 1,003 MI survivors.

Gene	dbSNP identifier	Exchange (X > Y)	Ratio of intra-individual variability for XY compared to reference (XX)	Ratio of intra-individual variability for YY compared to reference (XX)	*p*-value
CRP	rs1205	C > T	0.96	0.94	0.549
CRP	rs3093068*	G > C	1.00		1.000
CRP	rs1800947*	C > G	0.89		0.065
CRP	rs1130864	G > A	0.98	0.70	6.1×10^{-6}
CRP	rs1417938	T > A	0.99	0.71	1.4×10^{-5}
CRP	rs2794521	T > C	1.11	1.36	2.5×10^{-4}
CRP	rs3091244	G > A > T	0.97	0.79	0.001
FGA	rs2070022	G > A	0.90	0.77	0.012
FGA	rs6050	T > C	1.19	1.12	4.1×10^{-4}
FGA	rs2070018	A > G	1.20	0.83	0.001
FGA	rs2070016	A > G	0.84	0.54	1.1×10^{-6}
FGA	rs2070014	C > T	0.93	0.64	0.002
FGA	rs2070011	C > T	0.93	1.31	1.2×10^{-7}
FGA	rs10012555	T > C	1.20	0.83	0.001
FGA	rs2070006	C > T	0.96	1.31	8.3×10^{-7}
FGB	rs1800791	G > A	0.90	0.91	0.344
FGB	rs1800790	G > A	0.93	0.76	0.024
FGB	rs1800788	C > T	1.26	0.82	3.2×10^{-8}
FGB	rs2227399	T > G	0.94	0.88	0.223
FGB	rs6056	C > T	0.92	0.80	0.050
FGB	rs4220	G > A	0.92	0.83	0.064
FGB	rs2227421	A > C	0.95	1.21	0.005
FGG	rs2066865	G > A	1.09	1.01	0.165
FGG	rs1049636	A > G	1.08	0.71	4.8×10^{-7}
FGG	rs2066861	C > T	1.10	1.01	0.091
FGG	rs2066860*	C > T	1.05		0.527
FGG	rs1800792	T > C	0.96	1.09	0.064
FGG	rs2066854	T > A	1.10	1.00	0.086
IL6	rs2069827	G > T	0.62	1.50	2.6×10^{-19}
IL6	rs1800796*	G > C	1.32		1.8×10^{-5}
IL6	rs1800795	G > C	1.05	1.06	0.497
IL6	rs2069832	G > A	1.07	1.09	0.223

*heterozygotes and minor allele homozygotes were pooled for analysis

Gene	dbSNP identifier	Exchange (X > Y)	Ratio of intra-individual variability for XY compared to reference (XX)	Ratio of intra-individual variability for YY compared to reference (XX)	*p*-value
IL6	rs2069840	C > G	1.01	1.12	0.317
IL6	rs1554606	G > T	0.98	1.02	0.741
IL6	rs2069845	A > G	1.07	1.03	0.387
IL6	rs2069849*	C > T	0.70		4.3×10^{-4}
IL10	rs3024502	C > T	1.00	0.99	0.951
IL10	rs3024496	A > G	1.04	1.00	0.638
IL10	rs1518110	C > A	1.01	0.89	0.449
IL10	rs3024491	C > A	0.98	0.94	0.577
IL10	rs2222202	G > A	1.03	1.00	0.741
IL10	rs1800872	G > T	0.96	0.93	0.549
IL10	rs1800871	G > A	0.90	0.94	0.071
IL10	rs1800894*	C > T	1.42		1.2×10^{-6}
IL10	rs1800893	C > T	0.98	0.97	0.861
IL10	rs1800890	A > T	0.97	0.74	2.9×10^{-5}
IL10	rs10494879	C > G	0.92	0.81	0.003
IL10	rs6676671	T > A	0.98	0.74	2.8×10^{-5}
IL18	rs3882891	T > G	1.01	0.87	0.021
IL18	rs5744280	G > A	1.13	1.25	0.001
IL18	rs549908	T > G	0.86	0.81	0.001
IL18	rs1834481	C > G	0.93	0.80	0.037
IL18	rs360722	G > A	1.16	0.68	0.001
IL18	rs2043055	A > G	1.18	1.25	6.8×10^{-5}
IL18	rs187238	C > G	0.89	0.71	6.4×10^{-5}
IL18	rs1946518	G > T	1.02	0.90	0.086
IL18	rs5744222	G > T	0.94	0.86	0.135
LTA	rs2009658	C > G	0.97	0.94	0.705
LTA	rs1800683	G > A	0.94	1.23	0.002
LTA	rs2239704	C > A	1.00	1.16	0.024
LTA	rs909253	A > G	0.94	1.23	0.003
LTA	rs2857713	T > C	0.96	0.71	2.5×10^{-5}
LTA	rs3093543*	A > C	0.82		0.007
LTA	rs1041981	C > A	0.95	1.22	0.006
NFKB1	rs28362491	ATTG > -	1.05	1.09	0.287
NFKB1	rs3774932	G > A	0.90	0.84	0.010
NFKB1	rs1598856	G > A	0.90	0.84	0.008
NFKB1	rs230530	A > G	0.93	0.81	0.002
NFKB1	rs4648004	A > G	1.04	0.80	2.6×10^{-4}
NFKB1	rs230521	G > C	1.08	1.09	0.183

*heterozygotes and minor allele homozygotes were pooled for analysis

Gene	dbSNP identifier	Exchange (X > Y)	Ratio of intra-individual variability for XY compared to reference (XX)	Ratio of intra-individual variability for YY compared to reference (XX)	*p*-value
NFKB1	rs7340881	C > T	1.07	1.25	0.105
NFKB1	rs230498	G > A	1.08	1.14	0.082
NFKB1	rs1801	G > C	1.14	1.15	0.007
NFKB1	rs3774956	C > T	1.10	1.08	0.091
NFKB1	rs1020759	C > T	1.15	1.13	0.007
NFKB1	rs3774964	A > G	1.20	1.04	1.8×10^{-4}
NFKB1	rs11722146	G > A	1.15	1.06	0.007
NFKB1	rs4648090	G > A	1.20	1.03	0.002
NFKB1	rs4648110	T > A	1.08	1.15	0.122
NFKB1	rs4648133	T > C	1.10	1.05	0.100
NFKB1	rs1609798	C > T	1.11	0.98	0.035
NFKBIA	rs696	C > T	0.91	0.92	0.086
NFKBIA	rs8904	G > A	0.93	0.93	0.273
NFKBIA	rs3138054	C > T	0.98	1.05	0.861
NFKBIA	rs1957106	G > A	1.02	0.98	0.779
NFKBIA	rs2233409	G > A	0.94	0.87	0.192
NFKBIA	rs2233407*	T > A	0.88		0.069
NFKBIA	rs3138052	T > C	0.92	0.79	0.007
RELA	rs1049728*	G > C	1.06		0.403
RELA	rs7119750	C > T	0.93	0.72	0.058
RELA	rs11568300	C > G	0.96	1.11	0.078
RELA	rs732072	G > A	0.97	0.40	3.7×10^{-4}
RELA	rs11227248	C > T	0.97	1.36	8.7×10^{-7}
RELA	rs11820062	T > C	0.97	0.99	0.779
RELA	rs7101916	C > T	0.93	0.65	0.016
TLR4	rs2770150	A > G	1.01	0.97	0.951
TLR4	rs1927914	A > G	1.03	0.83	0.013
TLR4	rs1927911	G > A	0.97	0.78	0.021
TLR4	rs11536878	C > A	1.19	0.71	0.001
TLR4	rs1927907	C > T	0.80	0.99	3.2×10^{-5}
TLR4	rs2149356	G > T	1.07	0.92	0.052
TLR4	rs4986790*	A > G	1.15		0.030
TLR4	rs4986791*	C > T	1.16		0.014
TLR4	rs11536889	G > C	0.92	1.58	0.001
TLR4	rs7873784	G > C	1.15	0.84	0.008
TNFA	rs1799964	T > C	0.95	0.86	0.212
TNFA	rs1800630	C > A	0.98	1.00	0.905
TNFA	rs1799724	C > T	1.07	0.93	0.350

*heterozygotes and minor allele homozygotes were pooled for analysis

Gene	dbSNP identifier	Exchange (X > Y)	Ratio of intra-individual variability for XY compared to reference (XX)	Ratio of intra-individual variability for YY compared to reference (XX)	*p*-value
TNFA	rs1800629	G > A	1.12	0.82	0.021
TNFA	rs361525*	G > A	0.70		1.2×10^{-5}
TNFA	rs3093661*	G > A	0.64		3.7×10^{-6}
TNFA	rs3093664*	A > G	0.71		3.7×10^{-8}
TNFA	rs3093665*	A > C	0.83		0.046
TNFA	rs3091257	G > A	1.08	1.05	0.427

*heterozygotes and minor allele homozygotes were pooled for analysis

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7.5. List of publications

Publications included in this thesis:

Kolz M, Koenig W, Muller M, Andreani M, Greven S, Illig T, Khuseyinova N, Panagiotakos D, Pershagen G, Salomaa V, Sunyer J, Peters A. DNA variants, plasma levels and variability of C-reactive protein in myocardial infarction survivors: results from the AIRGENE study. Eur Heart J. 2007.

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Sunyer J, Pistelli R, Plana E, Andreani M, Baldari F, **Kolz M**, Koenig W, Pekkanen J, Peters A, Forastiere F. Systemic inflammation, genetic susceptibility and lung function. Eur Respir J 2008, in press.

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