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On The Genetic Variation of Interleukin-6 in Health and Coronary Heart Disease

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Ge mig gåvan att acceptera
vad som inte kan ändras,
mod att ändra vad som bör ändras
och visdom nog att skilja
det ena från det andra

Franciscus av Assisi

To Knodden

Abstract

There is increasing evidence that inflammation plays an important role in the development of atherosclerosis and coronary heart disease (CHD). Prospective studies on healthy individuals and on patients with unstable angina pectoris or non-Q-wave myocardial infarction have shown that individuals with high interleukin-6 (IL-6) concentrations have an increased risk of myocardial infarction and death due to cardiovascular disease. The mechanisms responsible for triggering and sustaining elevation of IL-6 in healthy subjects and patients with CHD are largely unknown and remain to be defined. Thrombosis underlies most of the acute manifestations of CHD including myocardial infarction. The causes of thrombosis are not fully clear, but an important mechanism might be the connection between inflammation and coagulation. The present research program was set up to investigate the genetic variation of IL-6 in vivo in health and CHD and connection between inflammation and coagulation.

In ***study I*** 222 patients with ST-elevation myocardial infarction were included. They were genotyped for -174 G>C single nucleotide polymorphism (SNP) of the IL-6 gene. Plasma IL-6 concentration was measured at admission and after 48 hrs. This study showed that patients with IL-6 above the median at admission had an increased risk for CHD death or a new myocardial infarction, whereas the genotype did not influence CHD risk or plasma IL-6 levels.

In ***study II and III,*** the effect of an inflammatory stimulus on circulating IL-6 and factor VIIa (FVIIa) concentrations depending on genotype was investigated in forty healthy subjects challenged vaccination with Salmonella typhii vaccine. The study subjects were genotyped for the -174 G>C SNP of the IL-6 gene and for the Arg353Gln SNP of the FVII gene. The results demonstrates that the response differed according to genotype, indicating that IL-6 and FVIIa are influenced by genetic variation.

In ***study IV*** the influence of environmental factors and IL-6 genotype on IL-6 concentration was investigated**.** Three hundred eighty-seven patients with their first myocardial infarction before the age of 60 and matched healthy controls were enrolled. Antibodies against different pathogens were examined. Patients and controls were genotyped for the -174 G>C SNP of the IL-6 gene. Plasma IL-6 concentrations were significantly higher in patients compared to healthy controls when measured 3 months after the acute event. Furthermore, patients who were homozygous for the G-allele had higher IL-6 levels compared to those being hetereo- or homozygotes for the C-allele. In healthy controls no such genotype-phenotype association was found. We were not able to show any association between -174 G>C and risk of CHD. Neither a single, nor a number of antibodies against multiple pathogens differed between patients and healthy controls and no associations between these and circulating IL-6 concentration were indicated.

Conclusion: This thesis demonstrates that circulating IL-6 concentration is influenced by genetic variation of the IL-6 gene in vivo both in health and CHD. However, we found no association between the -174 G>C genotype of the IL-6 gene and CHD. Nonetheless, our results showed that patients with myocardial infarction and a plasma IL-6 concentration above the median at admission had an increased cardiovascular risk, which supports the importance of IL-6 as a risk marker in CHD.

Key words: atherosclerosis, coronary heart disease, factor VIIa, genetics, inflammation, interleukin-6, pathogen burden, polymorphism.

Contents

List of Original Papers

This thesis is based on the following original articles, which will be referred to by their Roman numerals

I Bennermo M, Held C, Green F, Strandberg LE, Ericsson CG, Hansson LO, Watkins H, Hamsten A, Tornvall P. Prognostic value of plasma interleukin-6 concentrations and the -174 G > C and -572 G > C promoter polymorphisms of the interleukin-6 gene in patients with acute myocardial infarction treated with thrombolysis.
Atherosclerosis. 2004;174:157-63.

II Bennermo M, Held C, Stemme S, Ericsson CG, Silveira A, Green F, Tornvall P. Genetic predisposition of the interleukin-6 response to inflammation: implications for a variety of major diseases?
Clin Chem. 2004;50:2136-40.

III Bennermo M, Held C, Ericsson CG, Silveira A, Hamsten A, Tornvall P.
Genotype-specific increase in plasma concentrations of activated coagulation factor VII in response to experimental inflammation. A link between infection and acute myocardial infarction?
Submitted

IV Bennermo M, Nordin M, Lundman P, Boqvist S, Held C, Samnegård A, Ericsson CG, Silveira A, Hamsten A, Tornvall P.
Genetic and environmental influences on circulating interleukin-6 concentrations in patients with a recent myocardial infarction. A case-control study
Manuscript

List of Abbreviations

ACS	acute coronary syndrome
AP-1	activating protein 1
AUC	area under the curve
BMI	body mass index
CABG	coronary artery bypass grafting
CAD	coronary artery disease
CD40L	CD40 ligand
CHD	coronary heart disease
CMV	cytomegalovirus
CPN	chlamydia pneumoniae
CRP	C-reactive protein
CVD	cardiovascular disease
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
FFA	free fatty acid
FVII	coagulation factor VII
FVIIa	activated coagulation factor VII
HDL	high density lipoprotein
HSV	herpes simplex viruse
HPY	Helicobacter pylorii
IL	interleukin
INF-γ	interferon-γ
LDL	low density lipoprotein
LPL	lipoprotein lipase
MCP-1	monocyte chemoattractant protein
M-CSF	macrophage colony-stimulating factor
MMP	matrix metalloproteinase
MRE	multiple response element
NIDDM	non-insulin-dependent-diabetes-mellitus
NF-κβ	nuclear factor kappa β
NO	nitric oxide
PAI-1	plasminogen activator inhibitor-1
PCR	polymerase chain reaction
PSGL-1	P-selectin glycoprotein-1
ROS	reactive oxygen species
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
TF	tissue factor
TG	triglyceride
TNF	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule-1
VIIa	activated factor VII
vWF	von Willebrand factor

Introduction

Cardiovascular disease (CVD) is the major cause of morbidity and mortality in the industrialized world, although the death rate has declined during the last decade. This decline is thought to be due, in part, to a decreasing prevalence of CVD risk factors and advent of new therapies[1]. Despite the decline, it is expected that by 2020 CVD will be the largest cause of disease burden worldwide, since demographic and lifestyle changes are resulting in an "epidemiological transition" from perinatal and infectious diseases to non-communicable diseases such as CVD in the developing world.

Coronary heart disease (CHD) causes the majority of the death in CVD[1]. The incidence of CHD is related to multiple genetic and environmental risk factors. Not all of the CHD incidence can be attributed to traditional CHD risk factors such as age, diabetes mellitus, family history, gender, hypercholesterolemia, hypertension, obesity and smoking. Additional risk factors have been found to be associated with CHD such as low levels of high-density lipoprotein cholesterol (HDL), hypercoagulability, hyperinsulinemia, impaired fibrinolysis, physical inactivity and psycho-social factors. Several of these factors are also associated with each other. One such cluster of risk factors is the metabolic syndrome consisting of abdominal obesity, dyslipidemia, hypertension and impaired glucose tolerance[2].

Conventional risk factors account for a large part of the attributable risk of CHD. In search for new risk factors, inflammation has been suggested as a novel risk factor[3].

During the past decade research has shown that inflammation plays a key role in the development of atherosclerosis. Inflammatory markers such as C-reactive protein (CRP), fibrinogen and interleukin-6 (IL-6) have been shown to be associated with an increased risk of CHD[4-10]. There is also increasing evidence that inflammation induces a procoagulant state and that this might be another link to CHD[11].

This thesis focuses on IL-6 as a risk factor for CHD, the regulation of IL-6 and the connection between inflammation and coagulation in healthy subjects and patients with CHD.

Inflammation and atherosclerosis

Atherosclerotic lesions occur principally in large- and medium-sized arteries. CHD, peripheral arterial disease and stroke are the most prevalent manifestations of CVD, the underlying cause being atherosclerosis.
Established risk factors, such as raised levels of low density lipoprotein (LDL) and decreased HDL cholesterol levels, smoking, hypertension and increased glucose concentrations all serve to activate inflammatory cells and promote their entry into the arterial wall via several pathways[3].

The process of atherosclerosis begins at sites of arterial inflammation in the vessel wall. Activated dysfunctional or injured endothelial cells express chemokines and pro-inflammatory cytokines, such as IL-6[12], which cause monocytes to transform into macrophages. Macrophages start taking up modified LDL particles (oxLDL), via the scavenger receptor, thereby converting macrophages into foam cells. Areas rich in foam cells form a fatty streak that is the precursor of the atherosclerotic plaque. Subsequently, smooth muscle cells (SMC) migrate from the media to the intima and release fibrous elements that contribute to the development of the fibrous plaque.
When foam cells die, their lipid content becomes part of the necrotic core of the lesion. At this stage, while the atherosclerotic lesion grows towards the adventitia, the arterial lumen

is still unchanged. As the inflammatory process continues additional monocytes/macrophages and lymphocytes accumulate in the lesion and release proteases, chemokines, cytokines and growth factors. The growing lesion acquires a fibrous cap and starts to intrude into the lumen. Finally, the growing mature plaque forms a stenosis that limits blood flow leading to ischemia, and, if the plaque ruptures, thrombosis and an acute coronary event will ensue[13].

Leukocytes adhere poorly to the healthy endothelium. However, when the endothelial monolayer becomes inflamed, it expresses cell adhesion molecules that bind cognate ligands on leukocytes. Selectins mediate a rolling interaction with the inflamed luminal endothelium, whereas integrins interact with vascular adhesion molecules (VCAM-1) and mediate firmer leukocyte attachment to the endothelium. Pro-inflammatory cytokines, expressed within the atheroma, provides a chemotactic stimulus to the adherent leukocytes, directing their migration into the intima. Inflammatory mediators such as monocyte colony stimulating factor (M-CSF) and macrophage chemoattractant factor (MCP-1) can augment the expression of macrophage scavenger receptors, stimulating the uptake of oxLDL and formation of foam cells. M-CSF and other mediators produced in plaques promote replication of macrophages within the intima as well. T-lymphocytes join macrophages in the intima during lesion evolution. Leukocytes and resident vascular wall cells secrete cytokines and growth factors that promote migration and proliferation of SMCs.

Medial SMCs express enzymes, matrix metalloproteinaseses (MMPs), that can degrade the elastin and collagen in response to inflammatory stimulation. Degradation of the arterial extracellular matrix permits penetration of the SMCs through the elastic laminae and collagenous matrix of the growing plaque. Inflammatory mediators can inhibit collagen synthesis and evoke the expression of MMPs by foam cells within the intimal lesion. These alterations in extracellular matrix metabolism induce a thinning of the fibrous cap, rendering it weak and susceptible to rupture. Cross-talk between T-lymphocytes and macrophages stimulates expression of the potent procoagulant tissue factor (TF)[14-16]. Thus, when a plaque ruptures TF induced by the inflammatory signaling triggers the coagulation cascade leading to thrombus formation, a key event in most acute complications of atherosclerosis.

Traditional cardiovascular risk factors work, in part, by undermining the endogenous defenses of the vascular endothelium and contribute to its dysfunctional state. Hypercholesterolemia promotes increased formation of oxLDL and foam cells, reduces intracellular concentrations of nitric oxide (NO) and increase reactive oxygen species (ROS)[17]. Angiotensin II, a vasoconstrictor associated with hypertension, opposes the action of NO, stimulates production of ROS, increases the expression of the proinflammatory cytokines IL-6 and MCP-1, and up regulates VCAM-1 on endothelial cells[18-20].
Other inflammatory markers, such as elevated CRP, can also promote endothelial dysfunction by decreasing the production and bioavailability of NO[21]. Furthermore, CRP potently up regulates nuclear factor-κβ (NF-κβ)[22], a key nuclear factor facilitating transcription of numerous proinflammatory genes. Synthesis of many cytokines such as IL-1β, IL-6, IL-8 and tumor necrosis factor-α (TNF-α), is mediated by NF-κβ, as is the expression of cyclooxygenase.
Accumulating evidence has established correlative and causative links between chronic inflammation and insulin resistance. In obesity, when adiposity reaches a certain threshold, cytokines are released from adipocytes that induce widespread macrophage activation and infiltration and impair adipocyte insulin sensitivity.

Inflammation and thrombosis

It has been suggested that inflammation with subsequent thrombus formation provides a potential explanation for the substantial percentage of patients who suffer an acute coronary event without evidence of traditional risk factors for atherosclerosis[5,6,23,24]. Many serious clinical manifestations of coronary atherosclerosis, such as unstable angina pectoris, acute myocardial infarction and sudden death, result

from thrombosis, usually occurring on a disrupted atherosclerotic plaque. Inflammatory cells may contribute to both plaque disruption and subsequent thrombosis[25]. Plaques prone to rupture have large lipid-rich cores with evidence of cap-thinning and active inflammation. As mentioned previously, local effects of inflammatory cells may cause degradation of the fibrous cap leading to plaque disruption and thrombus formation. It is assumed that many plaque ruptures occur sub clinically and may contribute to the growth of the atherosclerotic plaque, whereas if the thrombus is large or occlusive, it will result in an acute coronary event.

Thrombogenic risk factors (e.g. PAI) may modulate the degree of thrombogenicity and thereby determine the growth of the plaque and the occurrence of CHD[26-27]. TF is expressed in the exposed intima and activates factor VII (FVII) which in turn activates factors IX and X. Collagen in the exposed intima binds von Willebrand factor (vWF), which mediates platelet adherence by binding to the glycoprotein Ib/V/IX platelet surface receptor complex under high shear stress conditions. The vWF itself is the carrier protein for factor VIII, an essential component of the amplifying mechanism of factor X to Xa conversion. Furthermore, platelets activated by adhesion aggregate to each other through the glycoprotein IIb/IIIa receptor and its ligands, vWF and fibrinogen. Activated platelets then release PAI-1, which locally inhibits the fibrinolytic system[28].

Inflammation may promote thrombosis by acting both locally and systemically. Local mechanisms include cytokine mediated expression of TF by endothelial cells and macrophages. Indirectly, inflammation may act locally to induce thrombosis by weakening the fibrous cap of the atheromatous plaque, a process that might result in plaque rupture with subsequent release of TF.

Inflammation can affect systemic hemostatic activity by IL-6 mediated stimulation of hepatocytes to produce acute phase reactants. These include certain coagulation and antifibrinolytic factors, such as fibrinogen and PAI-1, which both induce a prothrombotic state[28].
It has become apparent that expression of TF on endothelial cells, monocytes and SMCs is regulated, not only by pro-inflammatory cytokines, including TNF-α and IL-1. In addition to initiating coagulation, interaction of TF with the adhesion molecule P-selectin, has been demonstrated to accelerate the rate and extent of fibrin formation and deposition in plaque. P-selectin is expressed on activated platelets and endothelium and serves as the receptor for the endogenous ligand, P-selectin glycoprotein-1 (PSGL-1), that is expressed on various types of leukocytes. In addition to mediating transient interactions between endothelial cells and leukocytes, P-selectin has been reported to mediate adherence of platelets to monocytes and neutrophils via the specific interaction with PSGL-1. P-selectin is rapidly cleaved off the surface of the platelet membrane and appears in the circulation as a soluble form, which has been reported to be elevated in patients with the acute coronary syndrome[29].

CD40 and CD40Ligand are expressed on a number of cells, including the B-lymphocytes and T-lymphocytes, vascular endothelial cells, and smooth muscle cells, and are also expressed by platelets binding to the receptor CD40 on the cell surface of the leukocytes. An enhanced CD40L and CD40 interaction promotes prothrombotic activity by enhancing TF expression in macrophages and through the direct regulation of endothelium procoagulant activity[30]. Ox-LDL induces TF expression in macrophages and decreases the anticoagulant activity of the endothelium by interfering with thrombomodulin expression and inactivating the TF pathway inhibitor[31]. TF expression is upregulated in circulating and endothelium-adherent monocytes. Accordingly, TF activity has been found to be increased in coronary tissue of culprit lesions in patients with unstable angina pectoris[32-34].

Recently, several studies have indicated that an increased concentration of circulating CRP is associated with an increased risk of future CHD[6,35]. Cermak and coworkers showed that CRP induced secretion of tissue factor from monocytes[36]. Recently, however, Paffen and

coworkers showed that CRP does not induce tissue factor directly in these cells. However, the results of that study suggest that CRP can induce tissue factor indirectly, probably through crosstalk between cells[37]. In a study on mice it has been shown that high CRP concentration leads to an increased risk of thrombosis[38]. Other acute phase reactants have also been shown to be associated with an increased risk of CHD. Fibrinogen concentrations have been found to predict CHD independently[15,39]. Furthermore, PAI-1 was shown to predict re-infarction in survivors of a first infarct myocardial infarction. It is now accepted that platelets might promote an inflammatory response. Studies have shown that activated platelets may mediate the homing of leukocytes by interaction with the subendothelial matrix under shear stress that do not allow neutrophil adhesion[40,41]. Platelets from patients with unstable angina pectoris are characterized by decreased intracellular sCD40L concentrations as well as by decreased release of sCD40L[42].

Expression of procoagulant factors by inflammatory cells in the unstable plaque, in particular TF, might initiate activation of coagulation. The generation of thrombin will activate platelets and subsequently this will result in the formation of a platelet-rich fibrin thrombus.

The occurrence of systemic activation of the coagulation cascade in combination with micro-vascular failure will contribute to multiple organ dysfunctions in sepsis[43]. Conversely, both coagulation and inflammation systems closely interact, whereby coagulation may substantially modulate the inflammatory activity.

The immune system

The principal purpose of the immune system is to protect against infectious agents. There are several lines of defense.
The first line consists of physical barriers like the skin.
The second line is the innate immune system where macrophages are pivotal. They express a limited number of highly conserved pattern recognition receptors such as scavenger receptors and Toll-like receptors and Macrophages use these in the search for foreign antigen. Vertebrates have a third line of defense, which is often referred to as the specific or adaptive immune system. It is made up of B- and T-lymphocytes and it is characterized by ability to continuously change and adapt in response to invasion. Thus, adaptive immunity is specific but much slower than innate immunity.
The humoral immune response, which is orchestrated by B-cells produces a number of highly specific antibodies (immunoglobulins) i.e. soluble B-cell receptors. These immunoglobulins recognize microbes and tag them in order to facilitate uptake and destruction by macrophages directly or indirectly by the complement system.
The cellular immune response consists of T-cells which have highly specific and diverse receptors that recognize antigen when presented to them by cells, such as the dendritic cells, macrophages or B-cells.
T-cells are either of T-helper or T-killer type.T-killer cells recognize surface structures and kill potentially harmful cells that have been infected by virus or otherwise transformed. Activated T-helper cells produce large amounts of cytokines to signal, to activate or to recruit other cells, such as neuthrophils. Thus T-helper cells initiate a local inflammatory response. T-helper cells also provide signals that are essential for differeniation and activation of B-cells. T-helper cells can also be divided into different subpopulations, based on the production of functionally distinct cytokine profiles. The major subpopulations are denoted TH-1 and TH-2 cells. TH-1 cells produce IL-2, IL-12, TNF-α and interferon-γ (INF-γ). TH-2 cells produce IL-4, IL-5, IL-6 and IL-10. TH-1 cells activate T-killer cells and TH-2 cells activate B-cells. The two types of T-helper cells regulate each other. TH-1 cells are down regulated by interleukin-10 and TH-2 cells are inhibited by INF-γ[44,45].

Cytokines

Cytokines are small, soluble protein molecules that serve as messengers in cell-cell communications. Many cytokines are produced by more than one cell type and act on a variety of target

cells at different stages of cellular differentiation and proliferation. Their action is usually an effect on nearby cells, and they therefore function in a predominantly paracrine fashion. They might also act at a distance (endocrine) or have effects on their cell of origin (autocrine)[46].

Cytokines mediate their information through binding to specific receptors expressed on the surface of the target cell, thereby triggering complex intracellular signalling events that control gene expression required for the cellular response. Each cytokine has many overlapping functions and since each function is potentially mediated by more than one cytokine, it is not easy to classify these molecules. However, functionally inflammatory cytokines may be grouped into pro-inflammatory, such as IL-1 and IL-6 or anti-inflammatory, such as IL-10.

Genetic variation

A family history of CVD is a strong risk marker for myocardial infarction with a relative risk of 3.4 for men with two or more affected parents or siblings[47]. Results from a cohort of 20.000 Swedish twins showed that the relative contribution to the risk of death from CHD due to genetic effects is strongest at young age, but the risk attributed to genetic factors remains high up to the age of 75[48]. Another twin study showed that the contribution of heritable factors to death from CHD is 50-60% among males and 30-55% among females[49-50].

The mechanisms for the hereditary behind CHD is not clear, but it is likely that the disease is influenced by a large number of genetic variants[51]. Some may be potent enough to exert a direct impact on the disease phenotype; others increase the risk by affecting an intermediate phenotype such as hypercholesterolemia. CHD is more likely to be caused by genetic variants when they occur in combination with other genetic variants or with particular environmental or metabolic factors[52].

The small inter-individual differences in base pairs of the genome are called polymorphisms. A polymorphism is a common, inherited variation in DNA sequence and is distinguished from rare variations in that the least abundant allele is required to have a frequency of 1% or more. The frequencies of polymorphic alleles may vary within or between populations.

There are also several different types of polymorphisms. A single nucleotide polymorphism (SNP) is a difference between the DNA sequences of an individual base pair. Deletions or duplications of base pairs are other types of polymorphisms. Tandem repeats are characterized by a core sequence that consists of a variable number of identical repeated sequences and can be divided into two categories based on the repeat length. Human leukocyte antigen, hemoglobin, chemokines receptors, immunoglobulin and blood group antigens are examples of highly polymorphic human genes.

The most common form of polymorphism is the SNP. It can be a substitution, insertion, or deletion of a single base pair. SNPs are highly abundant and are estimated to occur at an average rate of one per 1000 base pairs in the human genome. Thus it estimated that the human genome contains roughly 11 million SNPs. Genetic polymorphisms account for traits and for varied susceptibility to complex diseases (e.g. atherosclerosis).
Many SNPs are located in sites of the genome where they have no apparent effect on gene function (silent SNPs). Others occur in promoter or encoding regions of a gene and alter the level or structure and thus the function of a protein. SNPs in the promoter region of a gene might change the binding affinity of a transcription factor (e.g. NF-κβ). This changes the rate of transcription in response to a stimulus and ultimately leads to altered protein levels.
SNPs in coding regions may alter the amino acid sequence and the structure of the protein. Interestingly enough, even SNPs in regions other than the promoter or exon can alter the stability of transcribed mRNA and consequently alter efficiency of gene translation.
SNPs tend to occur in multiple in genes during meiosis. The unit of a group of SNPs that are linked together is called an SNP haplotype or simply a haplotype. Thus, in studies of the genetics of complex diseases, the units of genetic

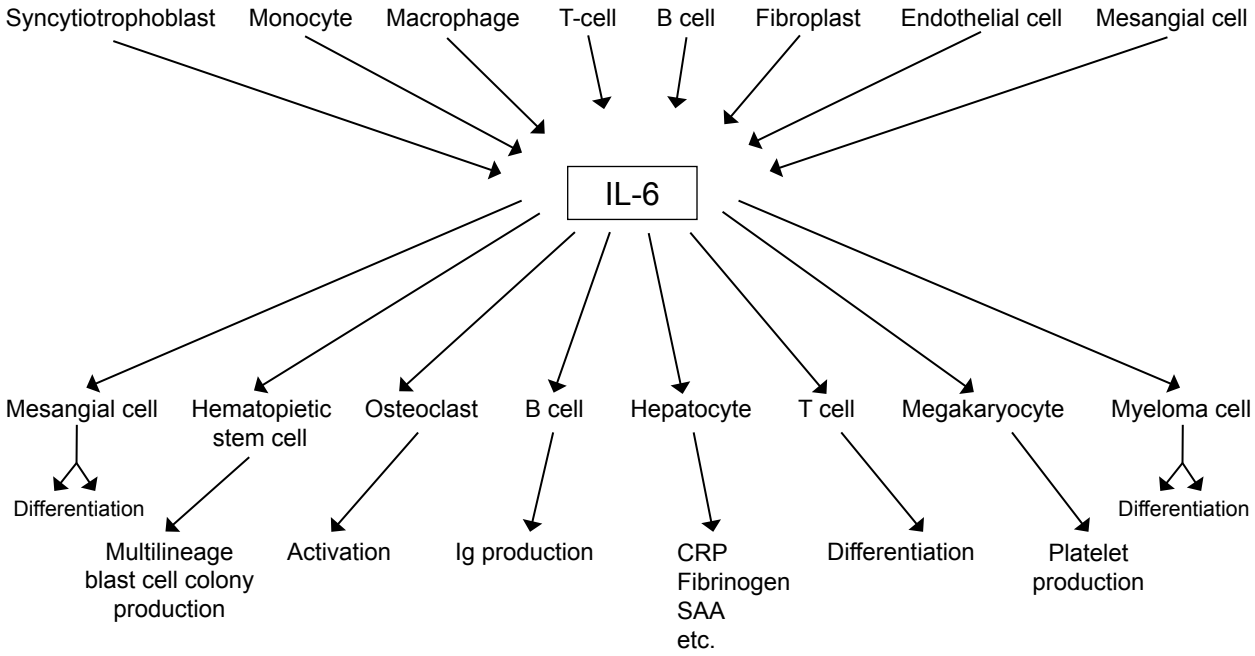

Figure 1. General effects on and of Interleukin-6.

information which are compared are often haplotypes. Genetic association studies are often case-control or cohort studies. In such studies, the genotype is associated with a protein level, an intermediate clinical phenotype for example atherosclerosis and/or with clinical outcome such as myocardial infarction or death.

The majority of sequences of the human genome are constant and do not vary between individuals. SNPs are interspersed along the sequence and are sources of variability between individuals. Thus, SNPs are in sequence with large parts of the genome that are not variable, but the SNPs are linked to other SNPs along the genome, a phenomenon known as linkage disequilibrium. Thus, if a study shows an association between an SNP and an adverse outcome, it is not clear whether the reported SNP is the causal SNP or is merely in linkage disequilibrium with the causal SNP. The advantage of analysis using haplotypes is that there is no need to start from the premise of a candidate SNP. It is merely assumed that the haplotype might contain a causal SNP within it.

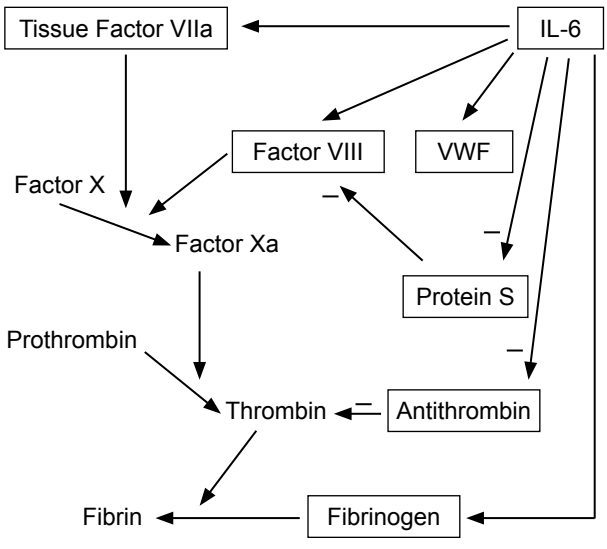

Figure 2. Pathways by which IL-6 influences the coagulation cascade.

Interleukin-6

IL-6 is a multi-functional circulating cytokine (figure 1) that plays a central role in the host defense due to its wide range of immune and hematopoietic activities, as well as its potent ability to induce an acute phase response[53].
It is normally tightly regulated, and is expressed at low levels except during infection, trauma and other forms of stress. Under these circumstances, a strongly enhanced IL-6 expression contributes to a cascade of events typical of inflammation, including leukocytosis, thrombocytosis, lymphocyte activation and acute phase protein synthesis.
Apart from its hematologic, immune, and hepatic effects, it also exerts many endocrine and

metabolic actions. Specifically, it is a potent stimulator of the hypothalamic-pituitary-adrenal axis under tight negative control of glucocorticoids, but is stimulated by catecholamines. It has also become apparent that in the quiescent state, IL-6 gene expression is kept low by a complex network that involves estrogen and testosterone. After menopause or andropause, loss of the inhibiting sex steroids results in elevated IL-6 levels, which accounts for certain of the phenotypic changes of advanced age, particularly those that resemble chronic inflammatory diseases. The age-associated rise in IL-6 has been linked to lymphoproliferative disorders, multiple myeloma, neoplasias, rheumatoid arthritis, dementia, and postmenopausal osteoporosis.

Circulating IL-6 levels are currently also regarded as a diagnostic marker for tumor progression and prognosis in various forms of cancers[54]. Hence, selective interference with IL-6 activation may offer therapeutic benefits.
After interaction with its receptor, present on a great variety of cells, IL-6 promotes a wide range of activities including viral inhibition and enhanced proliferation of hematopoietic progenitors[55].
Furthermore, selective interference with IL-6 activity may induce release of acute-phase reactant from hepatocytes[56], a feature shared with other cytokines, collectively known as the IL-6 cytokine family.
Hepatic stimulation by IL-6 results in the production of CRP, a useful surrogate marker of IL-6 stimulation[57].

The role of IL-6 in hemostasis is mediated through a series of different effects on endothelial cells, hepatocytes and leukocytes with promotion of synthesis of coagulation factors such as factor VIII, fibrinogen and TF (figure 2)[36,58,59]. In addition, IL-6 contributes to hemostasis by enhancing platelet production and altering platelet function and enhancing thrombin-induced platelet activation[60].

IL-6 expression in different cell types is regulated in response to a number of stimuli including endotoxins, IL-1, TNF-α, IL-4 and IFN-γ[61,62]. Different cell types might respond differently to these stimuli. Indeed, IL-6 expression might

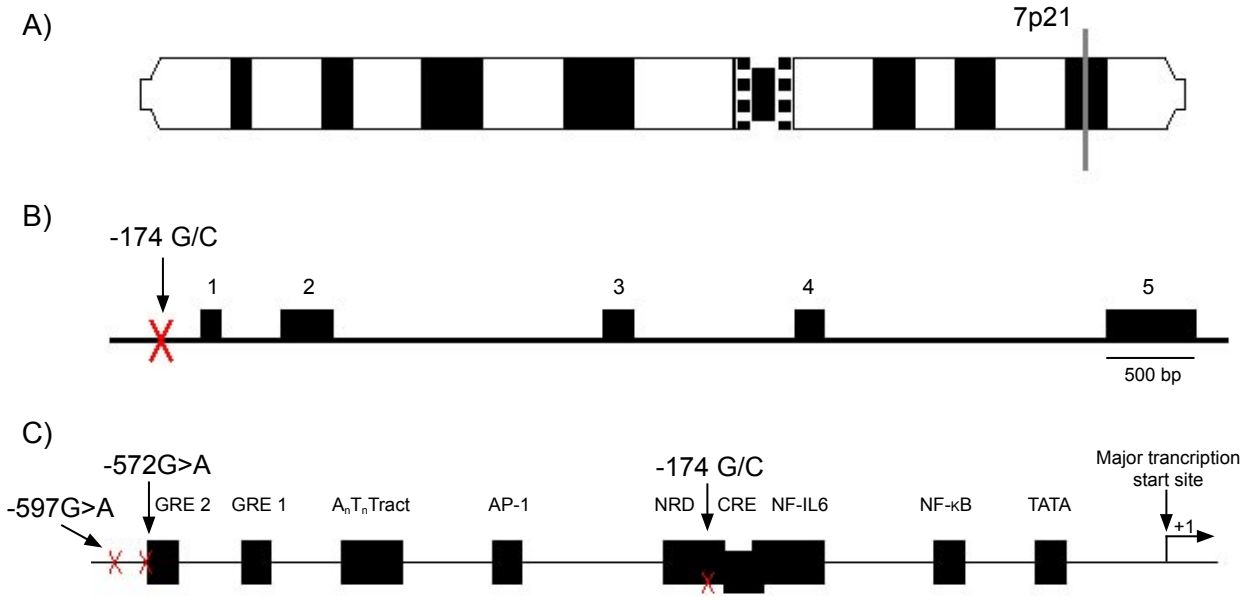

Figure 3. The human IL-6 gene structure. The marked crosses show where the polymorphisms are located.
A. The human IL-6 gene is located at chromosome 7p21.
B. It consists 5 exons and 4 introns.
C. Schematic presentation of the promoter region of the IL-6 gene from -600 to +1 identifying the 4 SNP´s -174 G>C, -373 AnTn tract, -572 G>C and -597 G>A. The transcriptional factor binding sites: GRE (glucocorticoid responsive element, AP-1 (activation protein 1), NRD (negatively regulatory domain), CRE (cAMP responsive element).

be promoted by some cytokines in certain cell types, whereas the same cytokine might inhibit IL-6 expression in other cell types[62].

Molecular biology of interleukin-6

Human IL-6 is a single glycoprotein chain with a molecular mass of about 26 kDa[63,64]. The gene for IL-6, mapped to chromosome 7p21[65] (figure 3A), consists of four introns and five exons[66] (figure 3B), and has three transcriptional initiation sites. Its transcriptional regulation is fairly complex[67].

Several transcription factors including (NF-κB), nuclear factor IL-6 (NF-IL-6), activator protein-1 (AP-1) and the multiple response element (MLE) mediate the activation of the IL-6 promoter, whereas steroids control elements that suppress the activity of the IL-6 promoter.

The IL-6 promoter has several recognition sites for transcription factors: a glucocorticoid-responsive element (GRE), an NFκ-B-binding site, an activating (AP-1)-binding site, a c-fos serum-responsive element (SRE) homology, a c-fos retinoblastoma control element homology, an NF-IL-6 and a cAMP-responsive element have all been identified within the conserved region of the IL-6 promoter[67-69](figure 3C). In addition to the complexity derived from the organization of the IL-6 promoter, alternative splicing of human IL-6 mRNA increases the phenotypic variability of IL-6[70].

IL-6 exerts its broad range of action through the IL-6 receptor, a transmembrane receptor not directly involved in signal transduction. Instead, activation of the receptor by IL-6 induces homodimerization of another transmembrane receptor (gp130), that initiates the transduction cascade[71] .

The IL-6 receptor has a second soluble form that consists of the extracellular domain of the membrane receptor. IL-6 can also activate gp130 through this soluble form, even on cells that lack the IL-6 receptor on their membranes[72].

Genetics of IL-6

The involvement of IL-6 in many biologic functions is paralleled by genetic associations between allelic variants of IL-6 and several physiological and pathophysiological conditions.

Table 1. Summarized of studies analyzed the genotype-phenotype association of the -174G>C promoter polymorphism of the IL-6 gene.

Study	Underlying disease	n	Genotype associated with increased IL-6
Basso et al.[144]	Moderately hypercholesterolemic men (controls)	281	None
Bonafe et al.[145]	Elderly healthy people	269	GG
Brull et al.[117]	Patients undergoing first-time CABG	127	C
Burzotta et al. [118]	Patients before CABG[3]	111	GG
Fernandez-Real et al.[84]	Healthy volunteers	59	GG and GC
Fishman et al.[114]	Healthy volunteers	102	GG and GC
Jenny et al.[146]	Population-based sample (>65 years)	1424	CC
Jones et al. [115]	Patients with abdominal aortic aneurism	231	CC
Kilpinen et al.[116]	Healthy newborns after vaginal delivery	50	CC
Nauck et al. [80]	Patients undergoing coronary angiography	942	None
Schlüter et al. [122]	Sepsis	50	None

Table 2. Summarized results of studies evaluating the association of the IL-6 −174 G/C polymorphism with CAD and CAD mortality.

	Design	Disease phenotype	High risk allele	Outcome
Brull[117]	Prospective	CABG	C	Higher IL-6 levels postoperative
Burzotta[118]	Prospective	CABG	G	Longer stay in hospital
Flex[147]	Case.control	Perifery artery disaese and controls	G	OR for PAOD: 4.6
Georges[78]	Case-control	MI and control	C	OR for MI: 1.34
Humphries[79]	Prospective	CAD and healthy	C	RR for CAD:1.54
Jones[115]	Prospective	Abdominal aortic aneurysm	C	RR for CVD mortality: 3.41
Nauck[80]	Case-control	MI and CAD	none	No association with MI or CAD

Recent experimental work has identified the presence of four polymorphisms in the IL-6 gene promoter: -597 G>A, -572 G>C and -174 G>C and a fourth polymorphism located at position -373 with varying numbers of As and Ts[73] (figure 3C).

The -174 G>C polymorphism has been reported as functionally important since it influences the transcription rate of the gene and plasma concentrations of circulating IL-6. However, the results have been conflicting and table 1 summarizes the results of association studies regarding -174 G>C genotype and IL-6 polymorphisms.

Interleukin-6 and coronary heart disease

Prospective studies on healthy individuals and patients with unstable angina pectoris or non-Q-wave myocardial infarction have shown that plasma levels of IL-6 in the upper quartile of the range considered normal are predictive of an increased risk of death or future myocardial infarction[74,75] and the role of IL-6 in coronary heart disease and acute coronary syndrome (ACS) has been discussed during the last decade[9,76]. The role of IL-6 in the development of ACS[77] is shown in figure 4.

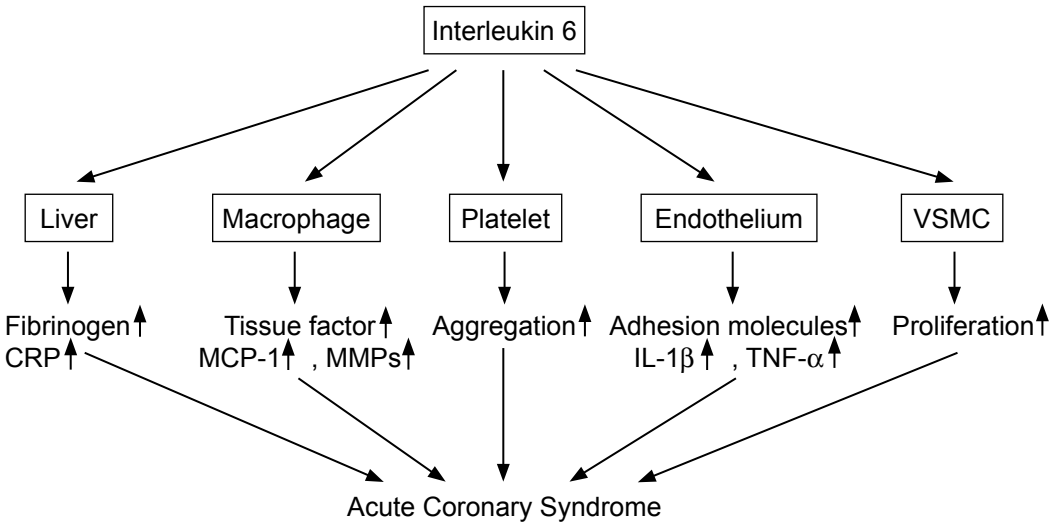

Figure 4. Interleukin-6´s role in acute coronary syndrome.

It has been suggested that the IL-6 -174 G>C promoter polymorphism might be a strong predictor of CHD. However, previous studies have come to conflicting results regarding which allele is the risk allele. Georges and coworkers reported an association between the G allele and myocardial infarction[78], whereas Humphries and coworkers found that the C allele was associated with an increased risk of CHD as well as with increased systolic blood pressure[79].

In contrast, Nauck and coworkers did not find any significant association between the -174 G>C polymorphism and myocardial infarction in a case-control study of CHD patients with angiographically documented CAD[80]. In table 2, these and other studies are summarized.

Interleukin-6 and cardiovascular risk factors

Age

Several studies indicate that IL-6 gene expression, as well as tissue and circulating levels of the cytokine, increase with age. The mechanism for the increase in IL-6 has not been fully explained. One potential mechanism is a reduced influence of the inhibiting sex steroids on IL-6 expression. The age-associated rise in IL-6 has been linked to Alzheimer disease, cardiovascular disease, osteoporosis and type 2 diabetes mellitus[81].

Hyperlipidemia

In rats, IL-6 inhibits the activity of lipoprotein lipase in adipocytes and induces increases in hepatic triglyceride secretion. In humans, IL-6 infusion leads to increased plasma free fatty acid and triglyceride concentrations[82]. Moreover, elevations of post-glucose load free fatty acids, fasting triglycerides and very low density lipoprotein cholesterol, have been linked to increased circulating IL-6 concentration[83].

Hypertension

In recent studies, blood pressure was a significant and independent predictor of circulating IL-6 concentrations in women, but not in men[84-85], but all studies have not been concordant[86].
IL-6 stimulates the central nervous system and the sympathetic nervous system, which may result in hypertension[87,88]. Infusion of IL-6 resulted in increased heart rate in healthy women and increased norepinephrine levels and heart rate in women with fibromyalgia[89]. However, other mechanisms behind the associations between IL-6 and hypertension cannot be excluded.
IL-6 might increase in concert with the modification of the redox state in the vascular wall in chronic hypertension, as occurs in some hypertensive animal models[90].
Furthermore, IL-6 is a well-characterized inducer of fibrinogen which is a major determinant of blood viscosity and this might be a reason for hypertension[91].
Finally, IL-6 might produce hypertension via effects on angiotensinogen expression, resulting in increased concentrations of angiotensin II, a potent vasoconstrictor[92].

Obesity

In humans, IL-6 is secreted from adipose tissue under non-inflammatory conditions. Studies of the dynamics of circulating IL-6 concentrations in humans showed that IL-6 increases postprandially, in parallel to glucose and insulin levels[93]. These findings indicate that IL-6 might modulate adipose tissue glucose metabolism in the fed state.

It has been calculated that one third of the total circulating IL-6 under unstimulated conditions originates from adipose tissue[94]. Positive associations between different measures of obesity and plasma IL-6 levels have been described in both men and postmenopausal women[84,85,93]. Furthermore, abdominal arterial IL-6 concentration is also associated with BMI[95]. The relationship is not straightforward, however. One study showed that plasma IL-6 levels were higher in obese patients with sleep apnea, but not in obese controls, compared to healthy subjects with normal weight[96]. In another study, the relationship between BMI and IL-6 was only observed in postmenopausal women and this relationship was lost among women receiving hormone replacement[85]. In fact, estrogens are well-known inhibitors of IL-6 expression[97].

Insulin resistance and diabetes mellitus

Plasma IL-6 levels are elevated in patients with type 2 diabetes mellitus (NIDDM), with features of the insulin resistance syndrome[98]. Recent studies have shown a connection between NIDDM and the -174 G>C polymorphism of the IL-6 gene[99-100].

Circulating IL-6 concentration has been described to predict the development of type 2 diabetes mellitus irrespective of the amount of body fat.
IL-6 and CRP are associated with increased BMI, hyperglycemia, insulin resistance, and overt type 2 diabetes mellitus.
One interpretation is that type 2 diabetes mellitus and the insulin resistance syndrome lead to a chronic acute-phase response with IL-6 derived from unsuppressed adipose or immune tissue secretion. In fact, this hypothesis is supported by the findings of increased blood concentrations of markers of the acute-phase response, including CRP, sialic acid and cortisol in these conditions[98]. However, the opposite could also be true. Accordingly, increased concentrations of IL-6 and markers of the acute-phase response are perhaps counteracting hyperglycemia and insulin resistance when inflammation is chronic or uncontrolled and when an inflammatory challenge becomes overwhelming.
The failure to achieve the desirable effect results in worsening of hyperglycemia and insulin resistance.

Recent studies have shown that circulating IL-6 is associated with insulin action in human subjects[84]. In men, circulating IL-6 levels were associated with insulin sensitivity after controlling for BMI, absolute fat mass or percentual fat mass[84]. Both insulin resistance and insulin secretion seemed to contribute to circulating IL-6.
All of these findings are strengthened by the recent description of IL-6 receptors in human adipocytes[101] and by the demonstration that IL-6 impairs insulin signaling in primary mouse hepatocytes and the human hepatocarcinoma cell line, HepG2[102].
Plasma IL-6 concentrations and insulin sensitivity relationships seem to occur in parallel to increases in plasma free fatty acids. Furthermore, IL-6 levels in serum and subcutaneous adipose tissue are reduced after weight loss in obese women[103].

Smoking

Several studies have shown that smokers have higher circulating concentrations of IL-6 compared with non-smokers. Smoking, presumably because of its inflammatory impact on lung tissue, promotes leukocytosis that induces increases in the levels of circulating IL-6[104].

Factor VII

FVII is a vitamin K-dependent glycoprotein that, once bound to TF, is converted to its active form, FVIIa.
The TF-FVIIa complex is able to convert Factor X to Xa, ultimately giving rise to a fibrin clot (figure 2).
The Northwick Park Heart Study made the original association between elevated FVII levels and myocardial infarction[105].

An Arg(R) 353 to Gln(Q) polymorphism in the FVII gene is an important determinant of plasma FVII levels and the R-allele is associated with a 20% to 25% reduction in plasma FVII levels. Hunault and coworkers found that the mechanism of this reduction in FVII levels was reduced secretion of FVII and that there is an association between the Arg353Gln polymorphism of FVII and triglycerides[106].

Two case-control studies found a relationship between the Arg353Gln polymorphisms and myocardial infarction in patients with familiar cardiovascular disease[107] and in patients with CHD[108].
he ArgArg genotype of the R353Q polymorphism was associated with the highest risk.
In contrast, Doggen and coworkers performed a larger study on patients with myocardial infarction and found that patients with the Arg-allele of the Arg353Gln polymorphism, despite having higher levels of FVII, had a lower risk of myocardial infarction[109].
Other studies of patients with myocardial infarction have found no association between myocardial infarction and the Arg353Gln polymorphism or plasma elevations of FVII. Thus,

although there is a genetic basis for variations in plasma levels of FVII, the majority of clinical epidemiology studies do not support an association between the Arg353Gln polymorphism and myocardial infarction.

Hypothesis and Aims

Hypothesis

Concentrations of circulating Interleukin-6 are influenced by genetic variation and this is of importance regarding the risk for coronary heart disease.

Aims

- to investigate the importance of circulating interleukin-6 concentrations and Interleukin-6 genotype for the risk for coronary heart disease
- to determine the importance of interleukin-6 genotype for interleukin-6 response to myocardial infarction
- to assess the interleukin-6 response to experimental inflammation in vivo according to genotype
- to assess the factor VIIa response to experimental inflammation in vivo according to genotype
- to evaluate whether pathogen burden or interleukin-6 genotype are associated with circulating interleukin-6 concentrations in patients with a previous myocardial infarction and healthy controls

Material and Methods

Study Cohorts

Study I

During a period from September 1991 to March 1995, 222 of 380 eligible (58%) patients with ST-elevation myocardial infarction were included in this study. All patients were admitted to one coronary care unit (CCU) which serves a population of 310 000 patients in the central and northern areas of Stockholm.

The inclusion criteria were patients below the age of 75 years treated with thrombolysis because of typical symptoms of acute myocardial infarction with duration of less than 12 h on admission to hospital and with electrocardiographic findings of ST-segment elevation in at least one standard or two adjacent precordial leads or bundle-branch block.

The reasons for not including 158 patients were: (i) inclusion breaks during holidays or periods of overcrowding at the Coronary Care Unit (n = 107); (ii) cardiogenic shock at admission (n = 11); (iii) patients living outside the Stockholm metropolitan area (n = 10); (iv) unwillingness to participate in the study (n = 6); and (v) alcohol abuse (n = 2). The reasons the remaining 22 patients were excluded could not be determined.

Comparison of the two patient groups (patients included/not included in the study), showed no statistically significant differences were found regarding age, sex, smoking or a history of hypertension. However, a history of previous myocardial infarction (16 vs. 34%, $p< 0.001$) and diabetes mellitus (12 vs. 20%, $p< 0.05$) was less common in the study group compared with the patient group not included in the study.

At baseline, revascularization procedures had been performed in six of the 222 patients in the study group.

Patients received thrombolytic treatment with either streptokinase (89%) or front–loaded recombinant tissue-type plasmingen activator (11%). DNA was available from 208 patients. Basic characteristics are described in table 4.

Study II and III

Forty healthy subjects were recruited from a cohort of 392 healthy men and women recruited from the general population. These healthy individuals were controls to patients with their first myocardial infarction before the age of 60 years in study IV. The first 250 subjects were genotyped for the promoter polymorphisms -174 G>C and -572 G>C of the IL-6 gene.

Based on homozygosity for the G or C allele of the -174 SNP and the G allele of the -572 SNP, 70 subjects were invited to participate in the present study of which 40 subjects agreed. Exclusion criteria were: treatment for hyperli-

Table 3. Study cohorts.

Study no	No of subjects	Sex (M/F)	Age (years)	
I	222	170/38	61±8.8	Patients with myocardial infarction
II	38	30/8	58±5.3	Healthy subjects
III	40	32/8	58±5.3	Healthy subjects
IV	378 378	308/56 309/55	54±3.4 54±3.2	Patients with myocardial infarction and Sex and aged matched healthy controls

Values are presented as number or means± S.D

Table 4. Baseline characteristics of the study group.

Age (years)	61 ± 9
Female	45 (22)
Family history of CHD	124 (60)
Smoking history	
Present smoker	86 (41)
Former smoker	64 (31)
Never smoked	58 (28)
Hypertension#	63 (30)
Diabetes mellitus#	21 (10)
Heart failure#	5 (2)
Angina Pectoris#	87 (42)
Previous Myocardial Infarction#	32 (15)
Medication at admission	
Acetylsalicylic acid	23 (11)
Cardioselective beta-blocker	46 (22)
Diuretic	19 (9)
Calcium antagonist	17 (8)
ACE inhibitor	14 (7)
Long-acting nitrates	20 (10)
Statins	0 (0)

Values are presented as means ± SD or number of subjects in group (%).
CHD = coronary heart disease,
ACE = angiotensin-converting enzyme
# based on a previous medical history given by the patients.

Table 5. Clinical and metabolic characteristics of the study subjects in study II and III.

	n=40
Sex (M/F)	32/8
Smoking, present	5
Age, years	57 ± 5
Waist-hip ratio	0.9 ± 0.1
Systolic blood pressure, mmHg	133 ± 10
Diastolic blood pressure, mmHg	82 ± 6
Total cholesterol, mmol/l	5.6 ± 0.8
HDL cholesterol, mmol/l	1.5 ± 0.3
LDL cholesterol, mmol/l	3.5 ± 0.8
Plasma triglycerides, mmol/l	1.3 ± 0.5
Plasma glucose, mmol/l	5.4 ± 0.5
Platelet count x 10^9	232 ± 48
White blood cells x 10^9 0 hour	5.9 ± 1.5
White blood cells x 10^9 6 hour	8.7[1] ± 2.0

Values are expressed as number or mean ± S.D.
LDL; low-density-lipoprotein, HDL; high density lipoprotein.
[1] $p<0.001$ between 0 and 6 hours

pidemia, hypertension, ongoing postmenopausal substitution therapy, use of acetyl salicylic acid and infection at the time of investigation.

Study IV

Between January 1996 and December 2000, a total of 387 consecutive patients below the age of 60 years from the northern Stockholm metropolitan area suffering from their first myocardial infarction and diagnosed according to national criteria, were enrolled in a clinical research program targeting mechanisms underlying premature CHD. All patients had been admitted to the coronary care units of three hospitals.
A total of 755 patients were considered, of whom 433 patients entered the study resulting in a participation rate of eligible patients of 76%.
Exclusion criteria were: insulin-dependent diabetes mellitus, renal insufficiency, chronic inflammatory disease, malignancy and unwillingness to participate.
Of the 433 patients entering the study, 46 patients did not complete the program. Thus 387 patients completed the program and for each patient included, a sex- and age-matched healthy control person was recruited from the general population of the same county.

Follow- up

In study I, the patients were followed by clinical controls for 24-60 months (40±16 months).

Table 6. Clinical and metabolic characteristics of the study subjects in study IV.

	Patients (n=364)	**Controls (n=364)**	**p-value**
Age	54 (49-57)	54 (49-57)	
Male, %	82	82	
Smokers, %	50	25	< 0.0001
Family history of CHD, %	42	21	< 0.0001
Diabetes, %	11	0	< 0.0001
Hypertension, %	34	6	< 0.0001
Hyperlipidemia, %	70	16	< 0.0001
BMI (kg/m^2)	26.8 (24.7-29.7)	25.6 (23.8-27.8)	< 0.0001
SBP (mmHg)	130 (118-140)	128 (118-140)	ns
DBP (mmHg)	80 (75-88)	80 (78-88)	ns
Glucose (mmol/L)	5.3 (5.0-5.9)	4.8 (4.6-5.2)	< 0.0001
Total cholesterol (mmol/L)	5.0 (4.3-5.7)	5.4 (4.7-6.1)	< 0.0001
Triglycerides (mmol/L)	1.6 (1.2-2.2)	1.2 (0.8-1.6)	< 0.0001
LDL-cholesterol (mmol/L)	3.2 (2.5-3.9)	3.4 (2.9-4.2)	< 0.0001
HDL-cholesterol (mmol/L)	1.1 (0.9-1.3)	1.4 (1.1-1.6)	< 0.0001
CRP (mg/L)	1.5 (0.7-3.4)	1.0 (0.5-1.8)	< 0.0001
IL-6 (pg/mL)	0.8 (0.6-1.4)	0.6 (0.5-1.0)	< 0.0001

Values are median (interquartile range) or percentage. CHD = Coronary heart disease, BMI = Body mass index, SBP = Systolic blood pressure, DBP = Diastolic blood pressure, LDL= low density lipoprotein, HDL= High density lipoprotein, CRP = C-reactive protein, IL-6 = Interleukin-6.

Blood sampling

Study I

Blood samples were drawn at admission to hospital, before initiation of thrombolysis and at every 4 hrs thereafter up to 36 hrs after admission. Further blood samples were taken 42 and 48 hrs after admission for analysis of biochemical markers.
Samples for inflammatory markers were taken at admission and after 48 hrs.

Study II and III

Venous blood samples were taken in vacuum tubes from an intravenous line before and at 2, 4, 6, 8, 10 and 24 hrs after vaccination.

Study IV

Three months after the index cardiac event, venous blood samples were collected in vacuum tubes. The venous blood samples were centrifuged immediately at 1750 g for 20 min at +1°C (EDTA plasma) or after 30 minutes at 2000 g for 20 min at room temperature (citrate plasma and serum). Following centrifugation, aliquots of the samples were analyzed immediately or were stored at -80°C until assayed .

Biochemical analyses

Cardiac markers

CK-MB isoenzyme mass concentration was measured in serum with the Imx CK-MB Microparticle Enzyme Immunoassay (Abbott Diagnostics).

Hemostasis markers

FVIIa concentration was determined in citrated plasma with a clotting assay using soluble recombinant truncated[110].

TF procoagulant activity was quantified with a chromogenic assay using the Actichrome TF kit (American Diagnostica Inc).

Inflammatory markers

High sensitive CRP concentration was analyzed in EDTA plasma by particle-enhanced immunonephelometry (Dade Behring). The lower detection limit was 0.16 mg/l with a coefficient of variation (CV) of 1.4% at 1 and 5 mg/l.
In study I, IL-6 concentration was analyzed in EDTA-plasma by an ELISA with a lower detection limit of 0.104 pg/ml (Biosource). In study II and IV another ELISA was use (R&D-system), with a interassay CV of 7.8% and intraassay CV of 5.8% with a lower detection limit of 0.039 pg/ml.
IL-1β and TNF-α concentrations were determined in serum by an ELISA (Biosource).
Plasma glucose, low and high density lipoprotein cholesterol and plasma triglyceride concentrations were determined with automated by clinical routine analyses.
White blood cells, neutrophils and platelets counts were assessed by clinical routine instrumental analyses.

Antibody analyses

Commercially available ELISA kits were used to determine IgG antibodies against different pathogens with the exception for cytomegalovirus, where an in-house ELISA was used[111]. For Chlamydia pneumoniae, IgA antibodies were also determined.

Results were interpreted as positive or negative according to the manufacturers' instruction. All equivocal results were regarded as negative results.
The following commercial ELISA kits were used: Chlamydia pneumoniae IgA EIA and Chlamydia pneumoniae IgG EIA (AniLabsystems Ltd. Oy, Helsinki, Finland), anti-EBV VCA IgG ELISA (Biotest AG, Dreieich, Germany), Helicobacter pylori Pyloriset^{R} EIA-G III (Orion Corporation Orion Diagnostica, Espoo, Finland) and HerpeSelect^{R}1 ELISA IgG and HerpeSelect^{R} 2 ELISA IgG (FOCUS Technologies, California, USA).

Genetic analysis

DNA was extracted from whole blood using the Qiagen Blood Cell & Culture Midi Kit. Amplification of the region of interest in the FVII gene and IL-6 promoter was performed by polymerase chain reaction (PCR).
The *-174 G>C* genotype was determined by digestion of a 639 bp PCR product (5'-GGGCTGCGATGGAGTCAGAG-3',5'-TCCCTCACACAGGGCTCGAC'-3) using the restriction enzyme NlaIII (New England Bio Labs).
The *–572 G>C* genotype was determined by digestion of a 161 bp PCR product (5'-GGAGACGCCTTGAAGTAACTGC-3', 5'-GGGCTGACTCCATCGCAG-3') with the restriction enzyme BsrBI (New England Bio Labs).
The *Arg353Gln* polymorphism was determined using a PCR fragment amplified by primers (5'-GGGAGACTCCCCAAATATCAC-3', 5'-ACGCAGCCTTGGCTTTCTCTC-3'), digested with the restriction enzyme MspI (New England Biolabs).
Digested PCR products were visualized by electrophoresis on ethidium bromide-stained agarose gels.
Two independent observers blinded to the clinical data determined the FVII and IL-6 genotypes.
Genotyping of the *AnTn tract* at position -373 were performed after sequencing in both forward and reverse directions of PCR products generated by primers flanking the polymorphic sites (5´GGGCTGCGATGGAGTCAGAG-3', 5'TCCCTCACACAGGGCTCGAC'-3).

Statistics

Values are presented as number, mean ± (SD), median (95% confidence interval, interquartile range) or area under the curve (AUC).
Allele frequencies were estimated by gene counting.
A chi-square test was used to compare the observed numbers of each FVII and IL-6 genotype with those expected for a population in Hardy-Weinberg equilibrium.
Differences between basic characteristics of

the groups were tested by chi-square analysis or by unpaired t-test. Associations between different parameters were expressed by calculation of Spearman rank correlation coefficients. Prognostic information was analyzed by Cox regression multivariate analysis in study II. Differences in the response between genotypes in study II and III were tested by repeated measures ANOVA.

In study IV, one-way ANOVA was performed in analyzing concentrations of IL-6 in relation to genotype and factorial ANOVA when relating inflammation markers to categorical parameters.

Correlations between inflammatory markers and pathogen burden were determined by calculation of Spearman rank correlation coefficient. Multivariate analysis was performed by multiple stepwise regression analysis. Since CRP and IL-6 values had a skewed distribution, logarithmic transformation was applied before hypothesis testing. In all statistical analyses, a p-value <0.05 was considered statistically significant.

Ethical considerations

All studies were approved by the Ethics Committee of the Karolinska University Hospital.

All patients and controls gave informed consent for participation in the studies.

Results and Discussion

Study I

This study was performed to evaluate the prognostic value of circulating IL-6 concentrations and two SNPs in the promoter region of the IL-6 gene, the more common -174 G>C and the rare -572 G>C, in patients whose inflammatory system had been stimulated by Q-wave myocardial infarction.

All of the patients were treated with thrombolysis. In all, 222 patients were included but DNA was only available from 208 patients.

The patients were investigated acutely and followed for 24-60 months (40±16 months). Forty-three percent of the patients showed signs of reperfusion determined by vectorcardiography.

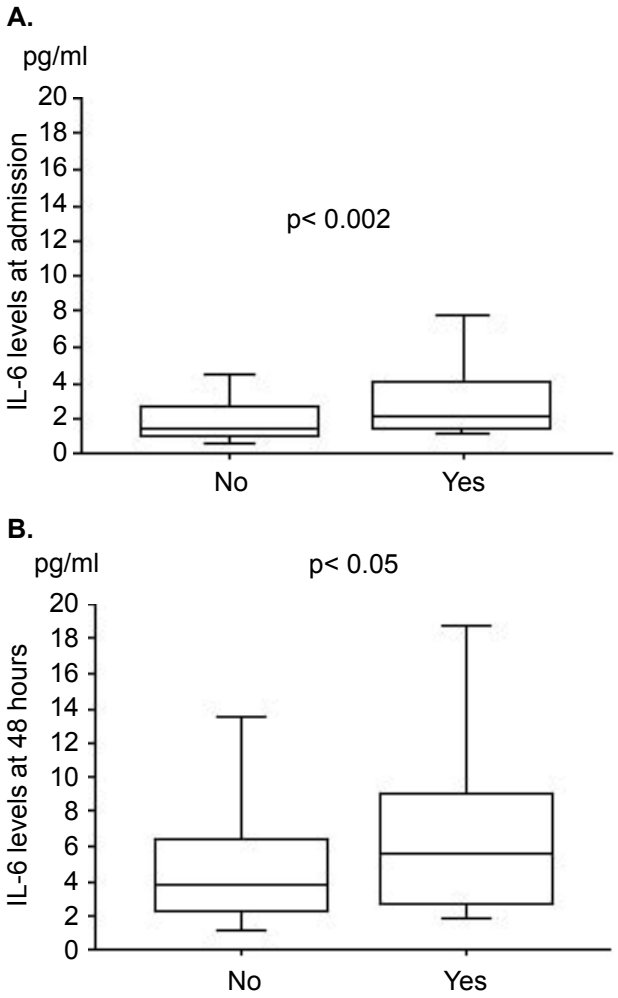

Figure 5. Plasma IL-6 concentrations according to the incidence of cardiovascular death or a new myocardial infarction during follow-up. **A**. IL-6 levels at admission p<0.002. **B.** IL-6 levels 48 hours after admission p<0.05. The box includes observations from the 25th to the 75th percentile, whereas the horizontal lines inside the box represent the median value. Vertical lines outside the box represent the 10th to the 90th percentiles.

During follow-up, 19 patients (8.6%) died of cardiovascular causes and 26 patients (10.3%) suffered a myocardial infarction as a first new cardiovascular event.

The median plasma IL-6 concentration at admission was 1.48 (1.07-2.89) pg/ml and increased as an inflammatory response to myocardial infarction to 4.58 (2.24-7.52) pg/ml at 48 hrs after admission (p<0.0001).

IL-6 levels at admission and at 48 hrs were highly correlated (r=0.237, p<0.001).

Patients who died, or experienced a new myocardial infarction during follow-up had increased plasma concentrations of IL-6 compared to those who surviveed and did not suffer a new myocardial infarction, both at admission (2.06 [1.43-4.08] vs 1.38 [0.97-2.70] pg/ml, p<0.002) and at 48 hrs after admission (5.67 [2.75-9.03] vs 3.78 [2.20-6.37] pg/ml, p<0.05) (figure 5).

Of the traditional cardiovascular risk factors such as BMI, family history of CHD, previous smoking, a medical history of diabetes mellitus and hypertension, none were associated with plasma IL-6 concentrations.

IL-6 genotype distributions were in Hardy-Weinberg equilibrium for both polymorphisms. The frequency of the rare C allele of the -174 G>C polymorphism was 0.48, which is higher than previously reported, but similar to that no-

Table 7. Interleukin-6 (IL-6) concentrations of the -174 G>C and -572 G>C polymorphisms.

	IL-6 (pg/ml)	
	At admission	**At 48 hrs**
-174 G>C		
GG (n=53)	1.36 (1.00-2.36)	4.29 (2.42-6.85)
GC (n=109)	1.63 (1.01-2.89)	3.69 (2.23-7.82)
CC (n=46)	1.44 (1.14-2.96)	4.10 (2.20-6.29)
-572 G>C		
GG (n=192)	1.53 (1.11-2.90)	3.91 (2.23-7.63)
GC (n=16)	1.20 (0.84-1.83)	4.00 (2.56-6.10)
CC (n=0)		

Values are median (interquartile range).

ted in patients and controls in study IV.
The frequency of the rare allele of the -572 G>C polymorphism was 0.04, which is lower than has been reported previously, but again similar to what we observed in patients and controls in study IV.
No associations were found between the -174 G>C and the -572 G>C polymorphisms, and circulating IL-6 concentration neither at admission nor at 48 hrs after admission (table 7).
The -174 G>C polymorphism was not associated with cardiovascular death or a new subsequent myocardial infarction during follow-up.

In multivariate analysis, including the previously described prognostic markers (i.e. diabetes mellitus, left ventricular function and plasma CRP concentrations at 48 hrs[112]); plasma IL-6 concentrations above the median at admission were significantly associated with subsequent cardiovascular death or a new myocardial infarction ($p<0.05$).
A history of diabetes mellitus ($p<0.001$) and a left ventricular function < 60% ($p<0.05$), but not age above 55 years, were associated with a poor prognosis.
In contrast, neither of the IL-6 genotypes nor IL-6 levels determined at 48 hrs contributed significantly to the prediction of risk in the multivariate analysis.

The major finding of this study was that increased plasma IL-6 concentration, measured early during the acute stage of a Q-wave myocardial infarction treated with thrombolysis, were independently associated with an increased risk of future cardiovascular death or a new myocardial infarction.
Previous studies in patients with non-Q-wave myocardial infarction or unstable angina pectoris have shown that increased IL-6 concentrations are independent markers for increased risk of cardiovascular death or myocardial infarction[74,113,75].

The results of this study add to this knowledge by investigating patients with Q-wave myocardial infarction.
The reason for the increased risk attributable to IL-6 in the present study cannot easily be discerned, but the risk was independent of symptom-to-hospital delay, myocardial injury and a history of diabetes mellitus. We have in a previous study[112] shown that CRP levels do not add any prognostic value to age over 55 years, diabetes mellitus or impaired left ventricular

function in patients with Q-wave myocardial infarction. The prognostic advantage of IL-6 over CRP is not easy to explain, but may be related to non-Q-wave myocardial infarction or unstable angina pectoris as Q-wave myocardial infarction is a strong stimulus of inflammation. Yet another explanation might be that IL-6 is a more sensitive marker than CRP.
IL-6 is a fast and strong inducer of the inflammatory response and it is produced by many cell types and a primary determinant of hepatic production of CRP.
The results might have been different if we had chosen another time point for the blood sampling.

Studies investigating the association between IL-6 genotype and circulating IL-6 concentration have come to conflicting results.
Initial studies by Fishman and co-workers showed that the -174 G allele was associated with high unstimulated plasma concentrations of IL-6[114], but in three subsequent studies the -174 C allele has been associated with high unstimulated plasma concentrations of IL-6[115-116] or IL-6 related proteins, such as CRP. However, this was statistically significant only in newborns after birth trauma[116] and in subjects with abdominal aortic aneurysm[115].
Two studies investigating plasma IL-6 concentrations stimulated by coronary artery by pass grafting (CABG) and the impact of the -174 G>C polymorphism came to somewhat contradictory results.
In the study by Brull and coworkers, preoperative IL-6 concentrations did not differ. However, six hrs after the surgical trauma, IL-6 levels were significantly higher in homozygotes for the C allele[117]. These data are in contrast to those from the second study of plasma IL-6 concentrations stimulated by CABG, which showed that the -174 G allele was associated with higher IL-6 levels 24 hrs after the surgical trauma[118]. However, it cannot be excluded that the results of the first study would have been concordant with those of the latter study if the blood samples had been taken at a later time after the surgical trauma, since subjects carrying the G allele appeared to reach peak IL-6 levels more slowly than the C allele carriers in the first study. This possibility is further discussed under study II.

Our findings, which do not show genotype-dependent differences in plasma IL-6 concentration, suggest that the -174 G>C promoter polymorphism might not be a strong modulator of the IL-6 response during myocardial infarction. Further support for this concept is that plasma CRP, being a robust marker of IL-6 activation and less influenced by time, did not differ between the different -174 G>C genotypes.
In our study, the first blood sample was taken approximately six hrs after symptom onset, which is comparable to the study by Brull and coworkers[117]. The second blood sample was taken 48 hrs after admission which is a time-point that did not show associations between genotype and IL-6 levels in the two CABG studies.
There are many potential confounders that might have influenced the results in the three studies, such as differences in medication, different clinical settings i.e. acute myocardial infarction vs. CABG and various time points for blood sampling. Hemodilution is another potential confounder that may have influenced the IL-6 response in the CABG studies.
Studies regarding genetic influence of IL-6 response need to be done in more standardized settings.

Studies investigating the -174 G>C promoter polymorphism as a CHD risk marker have come to divergent results. It has been reported that subjects carrying one or two copies of the -174 C allele have an increased risk of CHD compared with GG subjects.
In the ECTIM (Etude Cas-Temoins sur l'Infarctus du Myocarde) case-control study[78], the risk estimates in the GC and CC genotype groups were similar, but the increase was significant only for the GC group.
The Northwick Park Heart Study (NPHS), a prospective study of healthy men, also reported an increased risk of CHD for subjects who were heterozygotes for the C allele[79]. The same study reported that the -174 C allele was associated with a significantly higher systolic blood pressure. The CVD risk associated with the -174 C allele has been confirmed in a study of patients

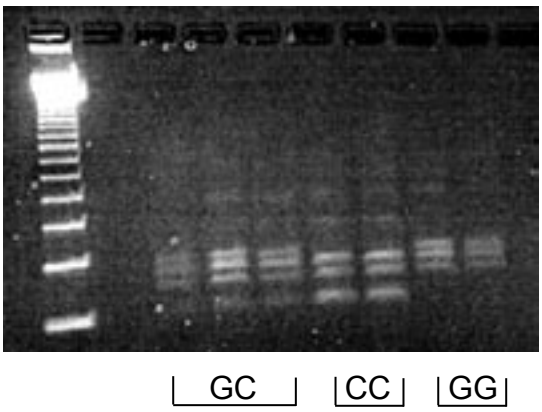

Figure 6. The figure shows genotypes of the -174G>C polymorphism of the IL-6 gene.

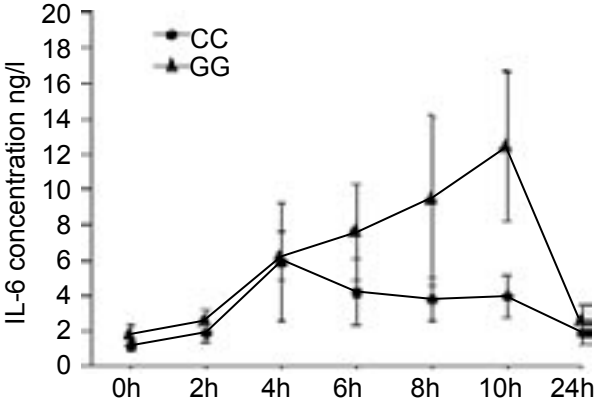

Figure 7. Plasma levels of interleukin-6 (IL-6) 0-24 hrs after vaccination according to genotype.

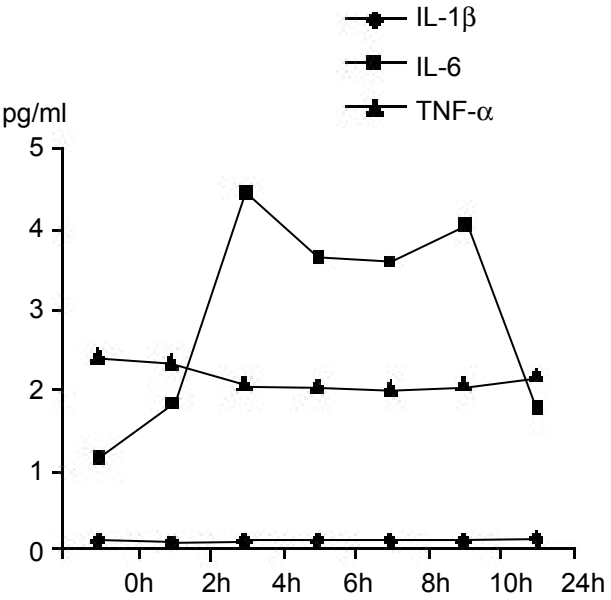

Figure 8. Median concentrations of IL-1β, IL-6 and TNF-α over the day.

with abdominal aortic aneurysm[115].

In contrast to these studies, we failed to show any association between the -174 G>C polymorphism and the risk of suffering a subsequent myocardial infarction or cardiovascular death during follow-up.

This is in line with what we found in study IV, where we also failed to show any associations between -174 G>C IL-6 genotype and patients suffering a first myocardial infarction before the age of 60. Nauck and coworkers have found similar results in a case-control study regarding CHD and healthy controls[80].

The reason for the discrepancy between these studies and others with conflicting results cannot be discerned, but is possibly related to the differences in sample size and to the heterogeneity of the study population.

Study II

This study was conducted to investigate the role of the -174 G>C promoter polymorphism for the induction of circulating IL-6 in vivo in humans. Forty healthy subjects were vaccinated with 1 ml Salmonella typhii vaccine.

Twenty of the subjects were homozygous for the C allele and 18 were homozygous for the G allele.

Vaccination with Salmonella typhii vaccine was chosen as a stimulus because a previous study has shown that it increased plasma IL-6 levels and had a detrimental effect on endothelial function of this vaccine[119].

Plasma concentrations of IL-6 increased from 2 hrs after vaccination and returned to baseline after 24 hrs ($p<0.001$). Overall, individuals with the -174 G genotype had significantly higher plasma concentrations of IL-6 those with the C genotype ($p<0.005$) (figure 7).

Subjects with the G genotype reached a peak in IL-6 levels at 10 hrs whereas the C genotype had no peak but a plateau between 4 and 10 hrs. The differences in plasma concentrations of IL-6 between the two groups were significant at 6 ($p<0.01$), 8 ($p<0.005$) and 10 hrs ($p<0.0005$).

Serum concentrations of TNF-α decreased significantly in both the G and the C group

Table 8. Plasma interleukin-6 (IL-6) concentrations, area under curve (AUC) for IL-6 promoter haplotypes.

	G[A8T12]C	G[A9T11]G	G[A10T10]G	G[A10T11]G
N=	19	12	8	6
AUC IL-6	128 ± 66	259 ± 126	307 ± 124	152 ± 83
p-value		P<0.001	P<0.001	n.s.

Values are mean ± S.D. n.s.; non significant.

($p<0.05$), whereas serum concentrations of IL-1β were unchanged after vaccination.
There were no differences between the two genotypes regarding serum concentrations of IL-1β and TNF-α (figure 8).

Haplotype analysis of the polymorphisms -572[AnTn-373]-174): Four common haplotypes were revealed: G[A8T12]C, G[A9T11]G, G[A10T10]G and G[A10T11]G. All subjects with the -174 C genotype, except one G[A8T12]C/G[A10T10]C, were homozygous for the G[A8T12]C haplotype, which indicate strong linkage disequilibrium between the -174 C and AnTn -373 polymorphism.
Individuals with the -174 G exhibited three haplotypes.
Four were homozygous for the G[A9T11]G, four were homozygous for the G[A10T10]G and two were homozygous for G[A10T11]G haplotypes, respectively. The remaining subjects were heterozygous for the G[A9T11]G/G[A10T10]G (n=4) and G[A9T11]G/G[A10T11]G (n=4) haplotypes, respectively.
Individuals with the G[A9T11]G and G[A10T10]G haplotypes, respectively, had higher IL-6 AUC concentrations than subjects with the G[A8T12]C haplotype (Table 8).

The results of this study showed that healthy subjects, homozygous for the G allele of the -174 SNP, had a stronger inflammatory IL-6 response, induced by vaccination against Salmonella typhii, than individuals homozygous for the C allele. No significant increase in IL-1β and TNF-α concentration was seen. These results indicate that the -174 G>C SNP of the IL-6 gene is functional in vivo with an increased inflammatory response associated with the G allele.

Previous studies have come to conflicting results, particularly if one compares in vitro and in vivo studies. Two in vitro studies, the first by Fishman and coworkers[114] and the second by Terry and coworkers[73] have shown that the G allele of the -174 SNP was associated with an increased transcriptional response to stimuli such as endotoxin or IL-1β.
An ex vivo study of whole blood from healthy subjects stimulated with lipopolysaccharide showed that haplotypes of the IL-6 promoter including the G allele were associated with the highest IL-6 concentration[120]. However, studies investigating the role of the -174 G>C promoter polymorphism for circulating IL-6 concentrations in vivo have come to different results. Unstimulated IL-6 levels were associated with the G allele in healthy subjects, in the first report by Fishman and coworkers[114]. Three studies have investigated stimulated plasma IL-6 levels in vivo. Brull and coworkers showed, in contrast to the in vitro and ex vivo studies, that patients homozygous for the C allele had increased plasma IL-6 concentrations six hrs after CABG[117], whereas in the study by Burzetta and coworkers reported that CABG patients with the G allele had higher plasma IL-6 levels 24 and 48 hours after the procedure[118].
The third in vivo study did not find any differences in plasma IL-6 concentration between genotypes after intravenous endotoxin administration[121].
The conflicting results from these studies indicate that a variated influence of the different stimuli may influence the IL-6 promoter in different ways. It may also be speculated that the effects on the IL-6 promoter are mediated by different transcription factors.
The lack of association between plasma IL-6 levels and the strong inflammatory stimulus

of endotoxin administration indicate a lack of functional importance of the -174 G>C SNP in the response to this stimulus. In contrast, a study investigating the association between the -174 G>C SNP and survival in sepsis have shown improved survival in subjects homozygous for the G allele[122].

The relatively infrequent number and the timing of blood sampling in the CABG[117- 118] and endotoxin[121] studies, may have influenced the results.

Our study indicates that the peak response to a stimulus in subjects homozygous for G allele is later than six hrs, which was the time chosen by Brull and coworkers[117] and this may potentially have affected their findings.

Surprisingly, no TNF-α or IL-1β response was seen. The reason for this is not clear but can involve the nature of the stimulus. Previous studies with LPS-infusions in healthy individuals have shown that low dosage only increased the IL-6 concentration, but high dosage also increased even IL-1β and TNF-α[123].

The Salmonella typhii vaccination used in our study is a mild localized stimulus for cytokine production and this might explain our results. Other possible explanations include short-term peaks that may have been missed due to the interval of blood sampling.

It appears that it is not only the -174 G>C SNP that influence IL-6 expression and production in response to a stimulus. In vitro and in vivo studies have indicated that haplotypes including the four promoter polymorphisms of the IL-6 gene: -174 G>C, -373 AnTn -572 G>C and -597 G>A influence circulating IL-6 levels[73].

Haplotype analysis of the present study sample showed that at least two out of the three common haplotypes, including -174 G, were associated with increased IL-6 levels compared with the only common haplotype including -174 C. Stimulation of the G[A10T11]G haplotype resulted in comparatively low plasma IL-6 concentration which is in agreement with the results of the study by Kelberman and co-workers[124]. The results indicate that the AnTn tract might modulate the influence of IL-6 response of the -174 G>C SNP.

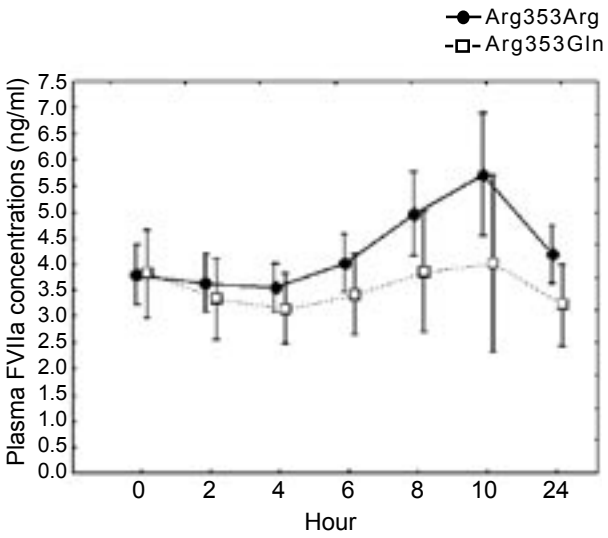

Figure 9. Plasma concentrations of activated coagulation factor VII (FVIIa) before and up to 24 hrs after vaccination in subjects grouped according to genotype.

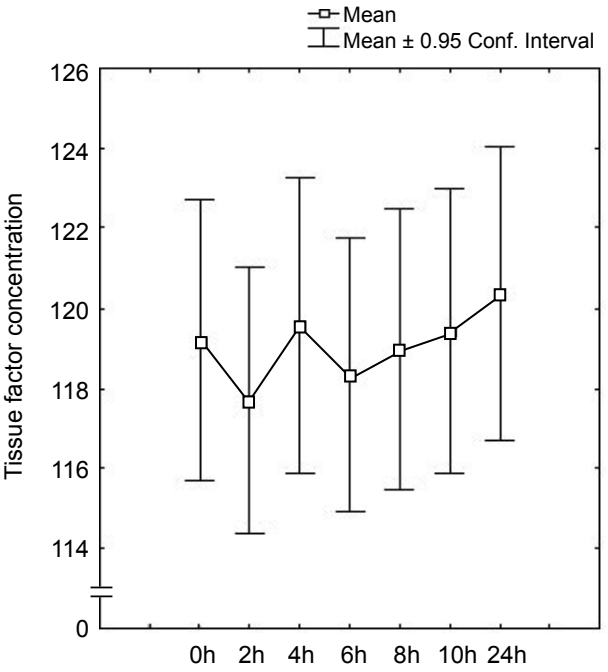

Figure 10. Plasma concentrations of Tissue Factor before and up to 24 hours after vaccination.

Genotyping of the -597G>A was not performed, which is a limitation of the present study. However, previous studies have shown that the -597 A allele is in a strong linkage disequilibrium with the -174 C allele[73].

Considering the central role of IL-6 in a variety of major diseases such as Alzheimer disease, atherosclerosis and cardiovascular disease, cancer, diabetes mellitus type 2, osteoporosis, sep-

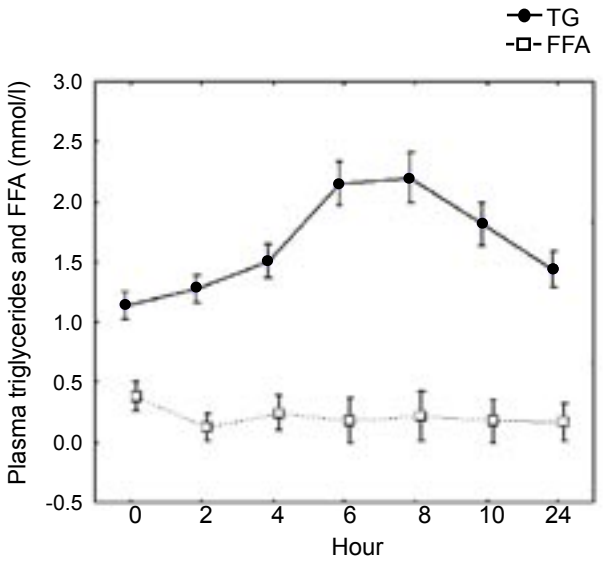

Figure 11. Plasma concentrations of triglyceride and free fatty acids (FFAs) before and up to 24 hrs after vaccination.

sis and systemic-onset juvenile chronic arthritis, the present findings may have major clinical relevance.

Study III

The aim of the study was to investigate if experimental inflammation induces a prothrombotic state in healthy individuals.

Blood from healthy subjects in study II was analyzed, including genotyping for the FVII Arg353Gln SNP.
Twenty-seven subjects were homozygous for the R-allele (ArgArg), whereas 13 individuals were heterozygous (ArgGln). None was homozygous for the rare Q-allele (GlnGln).
Plasma concentrations of FVIIa started to increase 6 hrs after vaccination, and reached a maximum at 10 hrs after vaccination. FVIIa levels returned to baseline after 24 hrs ($p<0.001$).
Subjects, homozygous for the Arg genotype, had higher plasma concentrations of FVIIa compared with heterozygous subjects ($p<0.05$) (figure 9).
The differences in plasma concentrations of FVIIa between the two groups were significant at 6 ($p<0.05$), 8 ($p<0.05$), 10 ($p< 0.05$) and 24 hrs ($p<0.05$).
Tissue Factor (TF) activity was unchanged after vaccination (figure 10).
Plasma levels of triglycerides increased significantly over the day ($p<0.01$). Plasma concentrations of FFAs decreased significantly between 0 and 2 hrs with no change thereafter ($p<0.01$) (figure 11).
We could not find any correlations between the AUCs for IL-6, triglycerides and FVIIa. The increase in plasma FVIIa concentrations was not different between -174 G>C IL-6 genotype.
This study demonstrates that experimental inflammation is associated with an increase in plasma FVIIa concentration. This is of interest since the mechanisms behind the connection between infection, inflammation and thrombosis are unclear. It is also of interest that subjects, homozygous for the common Arg of codon 353 of the FVII gene, had a stronger FVIIa response compared with subjects who were heterozygous, indicating that thrombous formation might be under genetic control to a certain extent.

Studies on FVII and the risk of CHD or CVD death have come to conflicting results [23,105,125-127]. The explanations for this discrepancy are not clear. One reason might be that the study subjects have been at different states of FVII activation. FVII levels are stable in subjects who are unaffected by an acute phase reaction or infection.
In contrast, infection or an inflammatory reaction might result in activation of FVII, particularly in individuals with a certain genetic predisposition.
Girelli and coworkers[108] found that patients with CHD more often hade a previous diagnosis of myocardial infarction if they were homozygous for the Arg-allele. Similarly, when undergoing percutaneous coronary intervention, a strong stimulus of inflammation, carriers of the Arg-allele had an increased risk of periprocedural thrombotic complications[128].
Surprisingly, we could not find any increase in TF activity in our study. Previous studies have shown that IL-6 may induce TF, particularly in combination with impaired endothelial dysfunction. However, although vaccination against Salmonella typhii has been shown to cause endothelial dysfunction, we saw no change in TF levels, challenging this association. The inflammatory stimulus is possibly not strong enough to evoke a response in form of TF expression.

Table 9. Plasma C-reactive protein concentrations and cardiovascular risk factors in patients and controls.

	Patients (n=373)	p value	Controls (n=373)	p value
Sex				
Female	2.09 (0.88-6.36)		0.98 (0.40-1.78)	
Male	1.37 (0.71-3.05)	<0.01	0.96 (0.53-1.83)	n.s
Smoking status				
Never	0.84 (0.42-1.62)		0.78 (0.50-1.53)	
Former	1.57 (0.82-3.67)		0.96 (0.51-1.67)	
Current	2.17 (1.25-5.62)	<0.001*	1.34 (0.61-2.91)	n.s
Diabetes				
No	1.39 (0.68-3.25)			
Yes	2.12 (1.15-5.62)	<0.01		
Hypertension				
No	1.33 (0.88-2.89)		0.95 (0.51-1.75)	
Yes	1.98 (0.94-3.86)	0.04	1.52 (0.78-1.93)	0.05
Hyperlipidemia				
No	1.54 (0.66-3.15)		0.93 (0.50-1.93)	
Yes	1.44 (0.78-3.50)	n.s	1.06 (0.66-1.67)	n.s
Lipid lowering drugs				
No	1.45 (0.71-3.74)			
Yes	1.45 (0.77-3.20)	n.s		

Values are median (interquartile range). * Compared with never smokers.

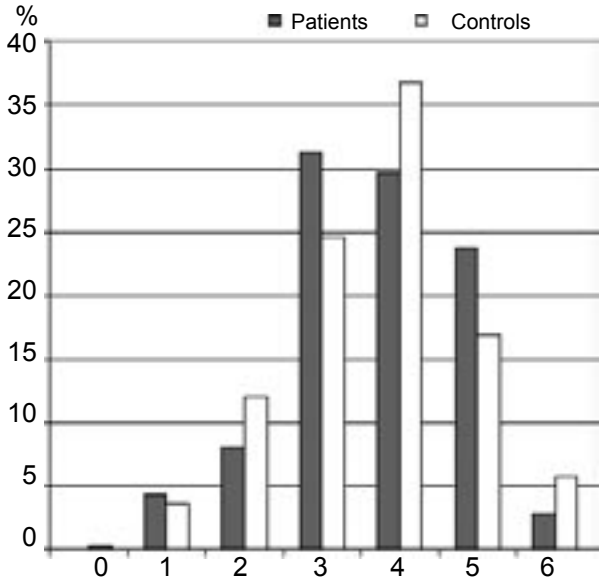

Figure 12. Frequency distribution of patients and controls exposed to infectious pathogens expressed as number of seropositives.

TF is still considered the most important cofactor for activation of FVII.

Another important factor is triglycerides[129-130]. In the absence of TF activation, it is most likely that triglycerides may have triggered the FVII response.

Several studies have shown an increase in FVIIa after intake of a fat-rich meal[131]. However, the participants in this study only received a light meal during the day.

The most probable mechanism for the rise in plasma triglyceride concentration is the inflammation caused by vaccination, as indicated by the increased plasma IL-6 levels. This interpretation is supported by previous studies showing that plasma triglycerides increase after lipopolysaccharide administration in mice and that subjects with bacterial or viral infections have increased serum triglycerides levels[82,132].

The association between inflammation and triglycerides is not clear, but IL-6 has been shown to inhibit adipocyte lipoprotein lipase activity and induce a secretion of hepatic triglycerides in rats[83].

Table 10. Plasma Interleukin-6 levels and cardiovascular risk factors in patients and controls.

	Patients (n=373)	p value	Controls (n=373)	p value
Sex				
Female	0.78 (0.52-1.61)		0.58 (0.43-0.95)	
Male	0.80 (0.59-1.36)	n.s	0.64 (0.47-0.97)	n.s
Smoking status				
Never	0.60 (0.49-0.91)		0.57 (0.43-0.80)	
Former	0.85 (0.60-1.33)		0.64 (0.47-0.91)	
Current	1.22 (0.69-2.00)	<0.001*	0.79 (0.58-1.40)	<0.05
Diabetes				
No	0.77 (0.58-1.34)			
Yes	1.06 (0.72-1.60)	<0.01		
Hypertension				
No	0.74 (0.55-1.27)		0.63 (0.45-0.95)	
Yes	0.95 (0.61-1.61)	<0.05	0.64 (0.47-0.97)	<0.05
Hyperlipidemia				
No	0.73 (0.57-1.26)		0.63 (0.47-0.94)	
Yes	0.88 (0.58-1.48)	n.s	0.63 (0.44-1.01)	n.s
Lipid lowering drugs				
No	0.80 (0.59-1.53)			
Yes	0.81 (0.58-1.34)	n.s		

Values are median (interquartile range). * Compared with never smokers.

In humans, infusion of IL-6 results in increased fasting triglycerides[133].

The study suggests that FVIIa may be a link between inflammation and thrombosis and that IL-6 activates FVII through triglycerides.
This is of clinical interest since one mechanism for the link between inflammation and acute myocardial infarction might be activation of coagulation.

Study IV

This study was conducted to investigate the gene-environmental effects on circulating IL-6 concentrations in patients with a recent myocardial infarction.
Three-hundred-eighty-seven patients with a first myocardial infarction before the age of 60 and 387 healthy controls, matched for age and sex, were investigated. DNA and blood samples for analysis of antibodies and inflammatory markers were obtained from 364 patients and controls. Blood sampling was performed three months after the acute event.
The risk factor profile differed between patients and controls. Total and LDL cholesterol levels were lower in patients, probably as a result of statin therapy in patients.
CRP and IL-6 concentrations were higher among patients compared to controls, CRP 1.5 vs 1.0 mg/l ($p<0.0001$) respectively and IL-6 0.8 vs 0.6 pg/ml ($p<0.0001$) respectively.
CRP and IL-6 both correlated with both age and BMI in both patients and controls.
The relationship between CRP and IL-6 concentrations and other traditional risk factors is shown in table 9 and 10. Regarding the rare allele of the -174 G>C polymorphism, the frequencies were 0.49 in the patient group and 0.48 among control subjects, which is higher than previous reports but similar to those obtained in study I.
There was no difference in genotype distribution between patients and control subjects. No association between CRP concentrations and -

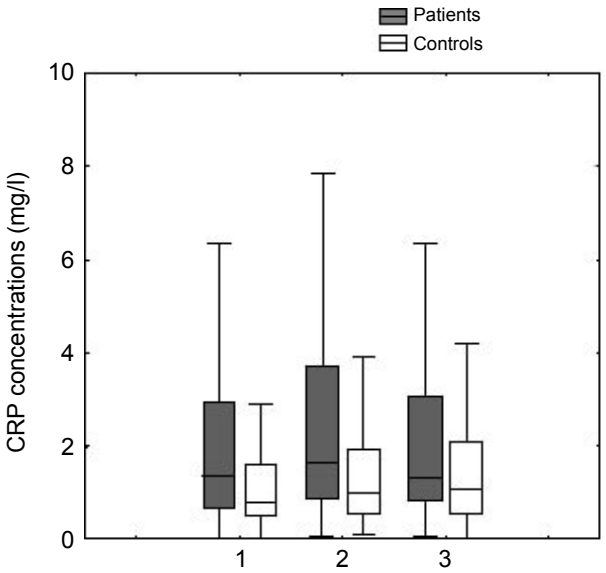

Figure 13. Plasma C-reactive protein concentrations in relation to the number of seropositives in patients and controls clustered into 3 groups, (1=0-3, 2=4, 3=5-6) p < 0-05 for both comparisons.

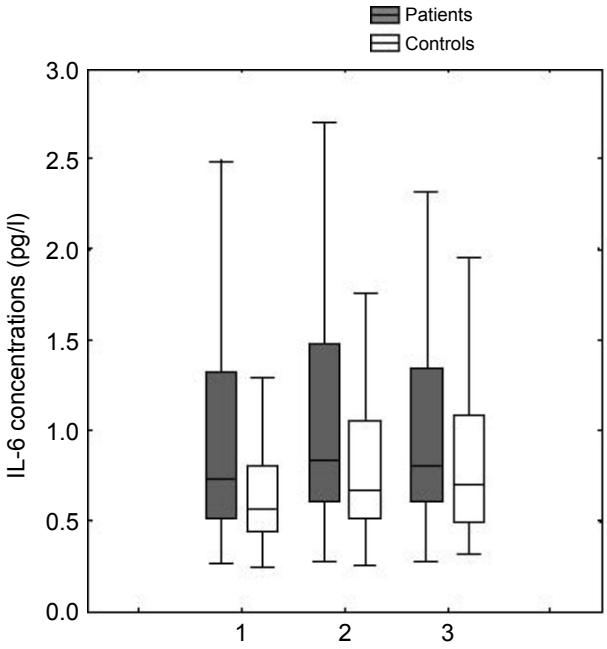

Figure 14. Interleukin-6 (IL-6) concentrations in relation to the number of seropositives in patients and controls clustered into 3 groups, (1=0-3, 2=4, 3=5-6) p < 0-05 for both comparisons.

174 G>C IL-6 genotype could be found.

Patients homozygous for the G allele had higher IL-6 concentrations compared with subjects homozygous for the C allele (p=0.02).

To further explore possible factors which may influence the circulating IL-6 concentration, we investigated the numbers of seropositive antibodies.

Recently, it has been suggested that the number of pathogens rather than individual infectious pathogens to which an individual has been exposed might be a more relevant marker of risk. Pathogen burden expressed as percent of number of seropositives is shown in figure 12.

No differences between patients and controls were seen regarding the number of individual seropositives or total pathogen burden.

Individual pathogens were not associated with CRP or IL-6 concentrations. CRP and IL-6 concentrations increased gradually as the number of seropositive titers increased, but there were no differences between the patients and controls (figure 13 and 14). These results remained unchanged when IgA antibodies were substituted for IgG antibodies against Chlamydia pneumoniae.

Multivariate analysis including age, BMI, gender, presence of hyperlipidemia, or hypertension, number of positive antibodies determinations, -174 G>C genotype, patient-control status, smoking history and statin treatment, showed that up to 35% of the CRP concentration could be explained by including IL-6 concentration (31%), BMI (3%), gender (0.5%) and patient-control status (0.5%).

The variation in plasma IL-6 concentration could be explained up to 15% when including BMI (8%), patient-control status (3%), age (1.5%), IL-6 genotype (1.5%) and smoking status (1%).

In line with previous studies, we found that patients had higher circulating IL-6 concentrations than healthy subjects.

The stimuli responsible for triggering and sustaining circulating levels of IL-6 in patients with CHD are unknown but one explanation might be low grade inflammation. Although associations between inflammation and traditional risk factors such as BMI, diabetes mellitus, hyperlipidemia, hypertension and smoking have been suggested, factors that determine unstimulated circulating IL-6 levels are largely unknown. One determining factor may be functional polymorphisms in the promoter region. The -174 G>C SNP has been reported as functionally important since it influences the transcription rate of the IL-6 gene and expression of IL-6.

Another explanation might be that chronic infections trigger and/or sustain inflammation. During the last decade, studies on the association between infection and pathogen burden have been performed with conflicting results.
Both Zhu and coworkers and Georges and coworkers showed an association between CRP, pathogens and coronary atherosclerosis verified by angiography [134-135]. In contrast, De Backer and coworkers failed to show any association in a case-control study of middle-aged men with or without CHD[136].
In two prospective studies of men with documented coronary atherosclerosis, it was shown that the future risk of myocardial infarction or cardiovascular death was related to pathogen burden independently of CRP. In contrast, in a prospective nested case-control study on healthy postmenopausal women[137].
Ridker and coworkers found no association between multiple antibodies against infectious pathogens on one hand and the subsequent risk of CHD or to CRP concentration on the other.

Few studies on the association between IL-6, CHD and pathogen burden are available. Blankenberg and coworkers found that infection with cytomegalovirus is independently associated with cardiac mortality in patients with CHD, documented by coronary angiography and increased IL-6 levels[138]. In contrast, Georges and coworkers could not find any association between IL-6 and multiple antibodies in a case-control study[135].
Furthermore, Choussat and coworkers did not find any association between the IL-6 response and pathogen burden in patients with ACS[139].
One may speculate that the quality and/or quantity of pathogens are of importance in order to explain the contradictory results. The pathophysiologic mechanisms may differ depending on the pathogens.
We have evaluated a majority of the various pathogens thought to be of importance. Our study did not confirm any association between circulating CRP or IL-6 concentrations to any specific pathogen. However, total pathogen burden was correlated to circulating CRP and IL-6 concentrations in univariate analysis. No differences between patients and controls were seen. In contrast to Georges and coworkers, we did not find any association between IL-6 genotype and pathogen burden. A geographical difference in allele frequencies between the two cohorts may be a possible explanation. Another explanation might be the selection of cases and controls in the study by Georges, where they recruited patients with stable angina pectoris and ACS, and healthy controls through advertisement or through general practitioners in contrast to our study where all patients had a previous acute myocardial infarction and control subjects were selected randomly from a registry kept by the Stockholm County Council.
Regarding other factors known to be associated with increased IL-6 concentrations, BMI, age and smoking showed such association. Furthermore and in agreement with other studies, patients with diabetes mellitus type 2 had higher IL-6 concentration compared to patients without known type 2 diabetes. Another explanation for the variation in circulating IL-6 concentrations might be a genetic influence.
In the present study, we could demonstrate that patients homozygous for the G allele have increased IL-6 concentration compared to healthy subjects homozygous for the G allele as well as patients and healthy controls that were heterozygous or homozygous for the C allele.
We failed to show any difference in allele frequency between patients and controls.

The studies regarding IL-6 concentration and genotype have come to contradictory results which are discussed in study II.
In this study the reason for the discrepancy between genotype-phenotype associations might be that the patients are stimulated by a low-grade of inflammation originating from atherosclerosis.
This study also addressed whether IL-6 genotype distribution between patients with a previous myocardial infarction before the age of 60 and healthy controls differed. As mentioned above, we did not find any difference regarding genotype distribution between CHD patients and healthy controls.
As discussed in the Introduction, previous studies have reported that subjects carrying one or two copies of the -174 C allele have an in-

creased risk of CHD whereas other studies have shown the opposite.
The results in our study is in line with Nauck and coworkers in that they could not demonstrate any association between the -174 G>C SNP and CHD[80]. The contradictory results between these studies and the studies that show associations between CHD risk and genotype cannot easily be explained, but possible reasons might be related to study sizes, ethnic differences between the various study samples and heterogeneity of study groups.

In summary, we could not demonstrate any differences between patients and controls in terms of IL-6 genotype, total number or number of varieties of antibodies to pathogens, indicating that genotype and pathogen burden are not predictors of myocardial infarction.
However, patients, homozygous for the G allele had higher IL-6 concentration which suggests that genetic influence is of importance for circulating IL-6 concentration.

General Discussion

In the studies included in this thesis, circulating IL-6 concentration was assessed and correlated to IL-6 genotype of the -174 G>C SNP both unstimulated and after different stimuli in vivo in health and CHD.
The results show that circulating IL-6 levels are influenced by the G allele, indicating that this polymorhism is functional in vivo. Furthermore, patients who have a concentration of IL-6 above the median in the acute stage of myocardial infarction are at increased risk for a new myocardial infarction or cardiovascular death. Factor VIIa increased as a response to experimental inflammation and this, in turn, could be an important link between inflammation and coagulation and risk of subsequent myocardial infarction.

There is increasing evidence that inflammation plays an important role in atherosclerosis and atherothrombotic complications[3].
IL-6 is a key proinflammatory cytokine produced by many different cells, including adipocytes, B- and T-cells, and endothelial cells. IL-6 regulates expression of adhesion molecules and induces secretion of MCP-1, an important mediator of release of other cytokines such as TNF-α and IL-1β that subsequently amplify the inflammatory reaction[53].
Prospective studies on healthy individuals have shown that plasma levels of IL-6 in the upper quartile of the normal range are predictive of myocardial infarction and of death of CVD[140]. Similarly, studies on patients with unstable angina pectoris or myocardial infarction have shown that patients with high IL-6 concentration run an increased risk of future CVD or myocardial infarction[74-75,113].
The stimuli responsible for triggering and sustaining raised circulating levels of IL-6 in patients with CHD are, however, unknown. Although associations between inflammation and traditional risk factors such as BMI, diabetes mellitus, hyperlipidemia, hypertension and smoking have been suggested, factors that determine the magnitude of unstimulated and stimulated circulating IL-6 levels remain largely unknown.
One explanation for the inter-individual variation of circulating IL-6 might be functional polymorphisms in the regulatory promoter region of the IL-6 gene which influence plasma IL-6 concentration. The -174 G>C SNP has been reported as functionally important since it influences the transcription rate of IL-6 in vitro[114]. However, previous in vivo studies have come to conflicting results regarding which of the alleles that are of importance regarding the association with circulating IL-6 levels and future risk of CHD.
A problem in clinical studies investigating the inflammatory response is confounding factors that influences the results. In study I and study IV on patients with known CHD, there were several potential factors that could potentially influence the IL-6 concentrations, such as medication (e.g. acetylsalicylic acid and lipid lowering drugs) and/or other risk factors.
Patients are not homogeneous in the sense that some of them have a higher burden of atherosclerosis compared to others and this could also have an influence on the circulating IL-6 levels. All these different factors, alone or in combination, might be explanations for the contradictory results obtained in previous studies regarding association between genotype, IL-6 concentration and CVD risk. These confounders make it difficult to come to conclusive results regarding the complex interaction between genotype and phenotype.
In study I, on patients with acute myocardial infarction, we showed that patients with IL-6 concentration above median at admission had an increased risk for cardiovascular death or a new myocardial infarction independently of other cardiovascular risk factors during follow-

up. In multivariate analysis including traditional cardiovascular risk factors and CRP, IL-6 concentration were independently predictive of future CHD. An explanation for this may be that IL-6 is a more sensitive marker of the inflammatory response than CRP.
Previous studies have suggested that CRP is a good marker of the IL-6 response to inflammation and several clinical studies have preferred CRP to IL-6 as a marker of inflammation.
An argument for this is the diurnal variation[141] and the short half-time of IL-6, which makes the timing of the blood sampling critical. Another problem with IL-6 is that the method for analyzing IL-6 is not a simple routine analysis and has to be analyzed with costly and complicated ELISAs.
To further investigate genetic influence on the inflammatory response in vivo with a minimum of confounding factors disturbing the IL-6 response, we examined the effects of a standardized inflammatory stimulus on healthy subjects in study II. The reason we chose vaccination as a stimulus for inflammation was that it has been shown to impair endothelial function and increase plasma IL-6 concentrations in a previous study[119].
The results in our study show that patients, homozygous for the G-allele of the -174 G>C polymorphism in the IL-6 promoter, had an increased response compared with subjects homozygous for the C-allele. Of special interest is that we found the response of circulating IL-6 to occur later in subjects homozygous for the G-allele, indicating that the timing of blood sampling is of importance.
This might be a reason for the opposing result of previous studies regarding IL-6 genotype and IL-6 response to a variety of stimuli such as CABG and CHD.

In study I we failed to confirm the possible association between -174 G>C genotype and the risk of CVD death and a new myocardial infarction. This is in line with the results in study IV, where no association between -174 G>C genotype and risk of myocardial infarction before the age of 60 was found. It is possible that sample sizes, ethnicity and heterogeneity of study groups affected these results.
In order to reach conclusive results regarding the influence of genes encoding inflammatory mediators in a complex disease such as CHD, it is necessary to perform studies on large samples representative of the population.
The multiple of genes involved makes it difficult to single out a specific SNP gene as a cause of CHD.
It has been proposed that infection and/or inflammation might trigger myocardial infarction, but the underlying mechanisms remain unclear.
Thrombosis is a key event in the pathophysiology of the acute manifestations of CHD and a possible explanation for this is that inflammation is of importance for the activation of the coagulation system.
In our experimental inflammation model, we could demonstrate that FVIIa concentration increases as a response to the inflammation and that the response is influenced by genotype.

There are several factors known to activate FVII to FVIIa, of which the most important is TF. Previous studies have shown that infusion of IL-6 can induce TF expression. Much to our surprise, we could not demonstrate any increase in TF activity in healthy subjects exposed to experimental inflammation. One possible explanation might be that the stimulus was not strong enough to trigger TF expression.
The most probable mechanism for the activation of FVII is an increase in plasma triglycerides due to inflammation, as has previously been shown, both in animal and human studies, after lipopolysacharide administration[82]. Another explanation might be that IL-6 induces activation of FVII by itself.
A mechanism for the pathophysiological effect of inflammation in CHD may be that chronic infections trigger and/or sustain inflammation. Several studies have shown associations between CHD, circulating levels of CRP and/or IL-6 and elevated antibody titres against chronic infections such as Chlamydia pneumoniae, cytomegalovirus, Epstein-Barr virus, Helicobacter pylori and herpes simplex type 1 and type 2[136-137,142].
Recently, it has been suggested that the number of pathogens, pathogen burden rather than in-

dividual pathogens to which an individual has been exposed to, might be a more relevant marker of risk for CHD[143].
In study IV, we found no differences in the number of seropositive antibodies between patients and healthy controls which speaks against this theory less likely.

Future directions

The importance of inflammation as a trigger for atherothrombosis is not yet fully determined and must be further evaluated in future studies regarding a multitude of coagulation factors. Furthermore, although IL-6 is a strong risk marker, the influence of genetic variation of IL-6 on the coagulation cascade will have to be elucidated.
The mechanisms that trigger, and sustain inflammation in patients with CHD need to be identified and further evaluated and explored. There is also a need for larger prospective studies in order to show the importance of pathogen burden as part of the mechanism for inflammation and CHD.
Future studies in all these fields should substantially contribute to the knowledge of the complex associations between genotype and phenotype with regard to inflammation, coagulation and CHD.

CONCLUSION

- Circulating interleukin-6 concentrations are influenced by genetic variation in vivo.
- The plasma interleukin-6 concentration at admission due to acute myocardial infarction might be of importance regarding the subsequent cardiovascular risk.
- -174 G>C interleukin-6 genotype did not have an impact on the risk of suffering a myocardial infarction or the risk of a new myocardial infarction or death from cardiovascular causes.
- Inflammation resulting in elevated circulating levels of IL-6 might be an important activator of the coagulation system.
- Environmental factors, such as body mass index but not pathogen burden, influence unstimulated circulating interleukin-6 concentrations.

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