Role of the OX40 ligand/receptor pair in coronary artery disease

Massimiliano Ria

Stockholm 2006
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Published and printed by Larsenics Digital Print AB
Sundbyberg, Sweden
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ISBN 91-7140-950-5
"An honest man, armed with all the knowledge available to us now, could only state that in some sense, the origin of life appears at the moment to be almost a miracle, so many are the conditions which would have had to be satisfied to get it going."

“Un uomo onesto, armato soltanto della conoscenza a noi disponibile, potrebbe affermare soltanto che, in un certo senso, l’origine della vita sembra essere al momento piuttosto un miracolo, tante sono le condizioni che devono essere soddisfatte perché si realizzi”

Francis Crick
(premio Nobel, scopritore con Watson della struttura del DNA)

To my family
ABSTRACT

Atherosclerosis is the pathological basis for coronary artery disease (CAD), the leading cause of morbidity and mortality in developed countries. CAD and atherosclerosis have long been known to have a familial component and result from the interaction of several genes with a wide range of environmental and lifestyle factors. Because of this complexity, applying the positional cloning approaches to find new CAD susceptibility genes has resulted disappointing and most of them are still unknown. Evidence from epidemiological studies implies a possibility for CAD susceptibility genes independent of classical risk factors. Identification of such genes might reveal novel intriguing biological pathways, making quests for new susceptibility genes in a hypothesis-independent manner worthwhile. Mapping of quantitative traits in mouse is showing to be particularly useful for such purposes. Despite there is no convincing animal model of CAD in a genetically tractable species, manipulated mouse models and inbred strains have been proved informative for aspects of atherosclerosis, the underlying cause of CAD.

Based on these considerations we investigated the genetic susceptibility to atherosclerosis with the aim to find new possible genetic risk factors implicated in development of its clinical complications, like myocardial infarction and CAD. We applied a combined approach based on identification of loci that have quantitative effects (QTLs) in mouse, and evaluation of the homologous candidate genes in a human context. This strategy applied to strains susceptible to diet-induced atherosclerosis (C57BL/6) identified Os40l as the gene underlying the atherosclerosis-susceptibility locus Ath1 on chromosome 1. Os40l was shown to control lesion formation in female mice and a specific genetic variation in the human counterpart was shown to be associated with MI in women. Our efforts finalized to dissect the mechanism behind the observed association between OX40L and MI revealed a novel promoter polymorphism (-921C>T) indicated by haploChip analysis to regulate OX40L transcriptional activity in vivo. This together with EMSA studies suggests that -921C>T is the functional polymorphism responsible for lower gene expression and the observed increased risk of MI in women. To further evaluate the role of OX40L in relation to CAD we performed transmission-based tests in trio families and observed that a “mirror” of the haplotype previously found was more frequently transmitted to affected offspring, results being statistically significant only in the British subsample. However, in Swedish females the minor rs38506416 G-allele appeared to increase the risk of CAD and MI, in line with our previous findings. Overall, results support the view that genetic variation in OX40L contributes to the development of CAD reinforcing the hypothesis that interactions between the OX40L gene and gender might influence genetic susceptibility. Other evidences from our studies suggested that genetic variation in OX40, encoding the receptor of OX40L, also plays a role in the pathogenesis of MI, thus indicating the OX40L/OX40 pathway as a novel important factor contributing to atherosclerosis and CAD.

In conclusion, we reported that specific genetic variation in the OX40L/OX40 couple appears to promote a pro-inflammatory state destabilizing the atherosclerotic plaque and making it particularly prone to rupture. Since activated immune cells are proposed to initiate plaque rupture, OX40L and OX40, being involved in the recruitment and activation of T-cells, might presumptively play an important role in atherogenesis. In addition, due to its characteristics the OX40 ligand/receptor pair may be an excellent target for therapy.

ISBN 91-7140-950-5
RIASSUNTO

L'aterosclerosi è la base patologica per la malattia coronarica (MC), una delle principali cause di morbilità e mortalità nei paesi sviluppati. È noto da tempo che la MC e l'aterosclerosi hanno una componente familiare, essendo il risultato dell'interazione di più geni con l'ambiente e lo stile di vita. A causa di questa complessità, l'applicazione della strategia del clonaggio per posizione (positional cloning) per trovare nuovi geni coinvolti nella MC si è rivelata deludente, e la maggior parte dei geni responsabili di tale malattia è ancora sconosciuta. I risultati di alcuni studi epidemiologici suggeriscono che potrebbero esistere geni in grado di contribuire alla suscettibilità per la MC indipendenti dai classici fattori di rischio. L'identificazione di tali geni potrebbe rivelare nuovi interessanti meccanismi biologici, giustificando la ricerca di nuovi fattori genetici indipendentemente da ipotesi preformulate. Il mappaggio nel topo dei cosiddetti tratti quantitativi si è finora rivelato particolarmente utile a tale scopo. Malgrado non esista un modello animale adatto allo studio della MC in una specie manipolabile geneticamente, modelli e ceppi ricombinanti di topo possono contribuire allo studio di diversi aspetti dell'aterosclerosi, la causa principale alla base della MC.

Partendo da queste considerazioni abbiamo valutato il ruolo di nuovi fattori di rischio genetici coinvolti nell'aterosclerosi e in particolare nelle sue complicazioni cliniche, quali l'infarto cardiaco e la MC. Abbiamo applicato un approccio basato sull'identificazione di loci per i tratti quantitativi (QTL) nel topo e sulla analisi dei geni corrispondenti nell'uomo. Questa strategia applicata ad animali che sviluppano aterosclerosi in seguito ad alimentazione con dieta ricca di grassi (C57BL/6) ha permesso di identificare Ox40l come il gene presente nella parte del cromosoma 1 (chiamata Ath1) che precedenti studi hanno indicato essere associata allo sviluppo di aterosclerosi. In questo studio abbiamo dimostrato che Ox40l controlla la formazione delle placche aterosclerotiche nelle femmine di topo e che una specifica variante genetica (aplotipo) nell'omologo gene umano (OX40L) è più frequente nelle donne che hanno subito un infarto. I nostri ulteriori sforzi finalizzati a capire il meccanismo dietro la relazione osservata tra il gene OX40L e l'infarto hanno rivelato la presenza di una nuova mutazione (-921C>T) nella regione che regola il funzionamento del gene. Un'analisi funzionale eseguita con l'innovativa tecnica haploChIP ha dimostrato che questa mutazione regola l'attività di trascrizione di OX40L in un contesto come quello in vivo. Questi studi, insieme a quelli eseguiti con la tecnica EMSA, suggeriscono che -921C>T è la mutazione funzionale responsabile sia della minore espressione genica di OX40L, sia dell’aumentato rischio di infarto osservato nelle donne.
Per analizzare ulteriormente il ruolo di OX40L in relazione alla MC abbiamo eseguito dei test di trasmissione familiare di geni e abbiamo trovato che la frequenza dell’aplotipo di OX40L complementare a quello trovato precedentemente era più alta nelle persone che avevano avuto un infarto, facendo pensare che avere questa variante genetica possa aumentare la probabilità di ammalarsi. Nelle donne svedesi la variante allelica più rara della mutazione rs38506416 sembra aumentare il rischio di MC e infarto, in linea con le nostre scoperte precedenti. In generale, questi risultati supportano l’idea che la variabilità genetica di OX40L contribuisce allo sviluppo della MC, rafforzando l’ipotesi che esista un’interazione tra questo gene e altri fattori legati al sesso che potrebbe influenzare la predisposizione genetica per questa malattia. Dai nostri studi emerge che anche la variabilità genetica di OX40, il gene che codifica per il recettore di OX40L, può influenzare la patogenesi dell’infarto, indicando così il sistema OX40L/OX40 come un nuovo importante fattore che contribuisce allo sviluppo dell’aterosclerosi e della MC. Per concludere, i nostri risultati qui presentati evidenziano che la variabilità genetica del sistema OX40L/OX40 sembra promuovere uno stato infiammatorio che destabilizza la placca aterosclerotica, rendendola particolarmente soggetta alla rottura. Poiché OX40L ed OX40 sono coinvolte nel reclutamento e nell’attivazione di linfociti T che si pensa possano favorire la rottura della placca, queste due proteine potrebbero ragionevolmente giocare un ruolo importante nel processo di aterogenesi, oltre a rappresentare un eccellente bersaglio per una futura terapia farmacologica.
LIST OF ORIGINAL ARTICLES

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals (I-IV):


*contributed equally.


### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>apoB</td>
<td>Apolipoprotein B</td>
</tr>
<tr>
<td>apoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CD40</td>
<td>CD40 receptor</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>CRD</td>
<td>Cysteine-rich domain</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalitis</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft-versus-host disease</td>
</tr>
<tr>
<td>haploChIP</td>
<td>Haplotype-specific chromatin immunoprecipitation.</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IBD</td>
<td>Identical by descent</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage disequilibrium</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low density lipoprotein receptor</td>
</tr>
<tr>
<td>LTα</td>
<td>Lymphotoxin alpha</td>
</tr>
<tr>
<td>MDR</td>
<td>Multifactor dimensionality reduction</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T-cells</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidized low density lipoprotein</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
</tbody>
</table>
PCR  Polymerase chain reaction
PROCARDIS Precocious Coronary Artery Disease
QTL  Quantitative trait locus
RA   Rheumatoid arthritis
RI   Recombinant inbred
SAA  Serum amyloid A
SCARF Stockholm Coronary Atherosclerosis Risk Factor
ScR  Scavenger receptor
SHEEP Stockholm Heart Epidemiological Program
SMC  Smooth muscle cell
SNP  Single nucleotide polymorphism
TCR  T-cell receptor
TDT  Transmission disequilibrium test
TF   Tissue factor
Th   T-helper
Tk   T-killer
TNF  Tumor necrosis factor
TNFR Tumor necrosis factor receptor
TNFRSF4 Tumor necrosis factor receptor superfamily member 4
TNFSF4 Tumor necrosis factor superfamily member 4
TLR  Toll-like receptor
VCAM-1 Vascular cell adhesion molecule 1
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1 INTRODUCTION

In this section, firstly the processes and the players involved in development of atherosclerosis, including an overview of the immune system, will be described; secondly a description of the OX40 ligand/receptor system, the focus of this thesis, and of the other members of the tumor necrosis factor ligand/receptor superfamily is provided; in the last part a review of the genetic tools used for dissecting complex diseases is presented.

1.1 ATHEROSCLEROSIS

Atherosclerosis, a disease of the large arteries, is the single most important contributor to cardiovascular disease (CVD). Atherosclerosis is characterized by inflammation and accumulation of lipids in the subendothelial layer\(^1,2\) that progress over decades. The atheroma, as it is called, is preceded by fatty streaks that are prevalent already in young people but may or may not cause symptoms. If it progresses into vulnerable plaques susceptible to rupture and subsequent thrombosis\(^3\), it leads, by occluding the arterial blood flow, to ischemia of the heart, brain, or lower extremities, resulting in infarction. The different manifestations of CVD, mainly myocardial infarction (MI), stroke and peripheral artery disease, are together a leading cause of morbidity and mortality in developed countries and will soon likely become the major health problem worldwide\(^4\). Atherosclerosis represents a heavy socio-economic burden on our societies. Despite the fact that incidence and case fatality of CVD is falling due to changes in lifestyle and the use of new pharmacological approaches to lower plasma cholesterol concentrations\(^5,6\), CVD continues to be the principal cause of death in the United States and Europe\(^7,8\), and it is rising in countries where wealth and food supply are increasing. In Asian countries the metabolic state is deteriorating and an explosion of metabolically related syndromes is expected in the near future.

1.1.1 Clinical complications of atherosclerosis

Atherosclerosis goes unnoticed until complications occur, such as angina or MI. The lesions of atherosclerosis occur principally in large and medium-sized elastic and muscular arteries and can lead to MI, stroke and peripheral artery disease, depending on the part of the body that suffers from ischemia.
1.1.1.1 Acute coronary syndrome

Myocardial ischemia results in a spectrum of clinical presentations called acute coronary syndrome, characterized by a common pathophysiology including clinical manifestations like unstable angina, ST elevation MI and non-ST elevation MI. Acute MI occurs when the blood supply does not meet the myocardial demand, and it usually manifests with chest pain often radiating toward the left arm, the back or the lower jaw. The metabolic changes associated with sudden onset of ischemia caused by occlusion of a major coronary artery include cessation of aerobic metabolism, reduction of creatine phosphate, initiation of anaerobic glycolysis, and accumulation of glycolytic products in the tissue. These changes are associated with contractile failure and electrocardiographic alterations. In addition, systemic elevations of pro-inflammatory agents such as C-reactive protein (CRP), tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6), are observed in relation to myocardial ischemia and subsequent tissue damage.

1.1.1.2 Other atherosclerosis-related diseases

In stable angina pectoris, atherosclerotic plaques are slowly growing inwards, gradually narrowing the lumen and reducing blood flow. Chest pain during exercise is due to myocardial ischemia appearing when the oxygen demand exceeds the oxygen supply.

Another consequence of the ongoing inflammatory processes in atherosclerotic plaques is aneurysm formation. Extracellular matrix (ECM) breakdown in the atherosclerotic wall may lead to dilation and eventually to rupture of the entire vessel wall.

1.1.2 A complex disease

Atherosclerosis is a complex disease where development of the phenotype is triggered by the interaction between genes and a wide range of environmental and lifestyle factors. With a few exceptions, each of the genetic risk factors involves multiple genes. This complexity can be clearly observed in genetic crosses in animals maintained under similar environmental conditions. Another level of complexity involves the interactions between risk factors, often not simply additive. Population migration studies, on the other hand, clearly show that the environment explains much of the variation in disease incidence between populations. Thus, the common forms of coronary
artery disease (CAD) result from a combination of an unhealthy environment, genetic susceptibility and increased lifespan.

**Risk Factors**
The risk factors of atherosclerosis are numerous and can be separated in two groups depending on their ability to be modified in order to reduce the progression of the disease - environmental factors and factors with an important genetic component. In the modifiable group, lifestyle and behavioral factors are accounted for such as smoking, diet, physical exercise, stress and concomitant infectious diseases. The more predetermined group includes gender, age and family history. A third category including for example hypercholesterolemia, hypertension and diabetes has a significant genetic component but is also influenced by lifestyle. Although some of the risk factors are debated, a few such as cholesterol levels, smoking, hypertension and diabetes are relatively undisputed; in particular the relative abundance of different plasma lipoproteins appears to be of primary importance, as raised levels of atherogenic lipoproteins are a prerequisite for most forms of the disease. Nowadays, common preventive approaches combine lifestyle changes, such as diet, smoking cessation, or physical activity, and treatment with lipid or blood pressure lowering drugs. Nevertheless, a substantial proportion of events occur in patients without established risk factors.

**Cholesterol levels:** Elevated levels of low density lipoprotein (LDL) and reduced levels of high density lipoprotein (HDL) predispose to atherosclerosis. The association of total serum cholesterol and LDL cholesterol levels with risk of CAD is direct and continuous. HDL levels are inversely correlated with CAD risk. The main causes of reduced HDL are cigarette smoking, obesity, and physical inactivity. Low HDL is also associated with the use of androgenic and related steroids (including anabolic steroids), β-blockers, hypertriglyceridemia, and genetic factors.

**Hypertension:** High diastolic or systolic blood pressure is a risk factor for stroke, MI, and cardiac and renal failure. Recent data suggest that hypertensive persons are more predisposed to the development of diabetes, a risk factor for atherosclerosis itself (see below), than are normotensive persons.

**Diabetes:** Both insulin-dependent and non-insulin-dependent diabetes mellitus are associated with earlier and more extensive development of atherosclerosis as part of a metabolic unbalance that includes dyslipidemia and glycosylation of connective tissue. Hyperinsulinemia damages the vascular endothelium. Diabetes is a particularly strong risk factor in women and considerably counteracts the protective effect of female hormones.

**Obesity:** Some studies have found that obesity, particularly abdominal obesity in men, is an independent risk factor for CAD. Hypertriglyceridemia, obesity,
diabetes mellitus and insulin resistance appear to be important independent risk factors in persons with lower LDL or HDL levels and in the young.

**Smoking:** Smoking increases the risk of peripheral artery disease, CAD, cerebrovascular disease, and graft occlusion after reconstructive arterial surgery. Smoking is particularly hazardous in persons at increased cardiovascular risk. There is a dose relationship between the risk of CAD and the number of cigarettes smoked daily. Passive smoking may also increase the risk of CAD. Men and women are both susceptible, but the risk for women may be greater. Nicotine and other tobacco-derived chemicals are toxic to the vascular endothelium. Cigarette smoking increases LDL and decreases HDL levels, raises blood carbon monoxide (and could thereby produce endothelial hypoxia), and promotes vasoconstriction of arteries already narrowed by atherosclerosis. It also increases platelet reactivity, which may favour platelet thrombus formation, and increases plasma fibrinogen concentration and hematocrit, resulting in increased blood viscosity.

**Physical exercise:** Several studies have associated a sedentary lifestyle with increased CAD risk, and others have shown that regular exercise may be protective. Concomitant infectious diseases: *Chlamydia pneumoniae* infection or viral infection may play a role in endothelial damage and chronic vascular inflammation that may lead to atherosclerosis.

**Gender and age:** Epidemiological studies have established the greater prevalence and earlier development of cardiovascular diseases (hypertension, atherosclerosis, heart failure) in men compared with premenopausal women.

**Family history:** The importance of genetics and environment in human CAD has been examined in many family and twin studies. Within a population, the heritability of atherosclerosis (the fraction of disease explained by genetics) has been high in most studies, frequently above 50%.

### 1.1.3 Pathogenesis of atherosclerosis: an inflammatory disease

The understanding of atherosclerosis pathophysiology has evolved substantively over time. Decades ago, because high plasma concentrations of cholesterol, in particular LDL cholesterol, are one of the principal risk factors, atherosclerosis was considered by many as a degenerative process largely caused by lipid overloading and accumulation within the artery wall. The emerging knowledge of vascular biology promoted focus on growth factors and proliferation of smooth muscle cells (SMCs) in the 1970s and 1980s. A combination of these views led to the concept of the atheroma as a mass of cellular lipid debris covered by a capsule of proliferated SMCs.
In the past twenty-five years, however, evidence has accumulated that inflammatory processes are crucial for initiation and progression of atherosclerosis and its complications. Whereas atheroma was previously regarded mostly as a mild lesion, the current notion that inflammation and immune responses contribute to atherogenesis has changed that view. Formerly focused on luminal narrowing due to the bulk of atheroma, the current concepts recognize the biological features of the atheroma as critical determinants of its clinical significance. Atherosclerosis is a pathological process that takes place within the intima and involves modified lipids, inflammatory cells, SMCs and endothelial cells (ECs). Nowadays it is widely recognized as a series of highly specific cellular and molecular responses elicited by retention of lipoproteins in the arterial intima, that can best be described, comprehensively, as an inflammatory disease.

1.1.4 Atherogenesis

Atherosclerosis is a slowly progressing disease that starts in early adolescence: Initial steps in the progression, the fatty streaks, have been reported even in infants. The atherosclerotic process is not linear but progresses at different levels simultaneously, however the formation of each atheroma appears to follow an evolution that includes growth and changes in the lipid pool, ECM and inflammatory components (Figure 1).

1.1.4.1 Lesion initiation: LDL accumulation and modification

Atheromas preferentially occur focally and do not affect all portions of the arterial tree in an equal manner; in fact they tend to form at predictable sites characterized by altered turbulent blood flow patterns as branching points. Disturbed flow yields low shear stress on the endothelium at sites of lesion predilection, leading to decreased transcription rate of genes harbouring a shear stress response element, like constitutive endothelial nitric oxide synthase (eNOS), and resulting in a reduced endogenous production of nitric oxide (NO) by ECs. Since NO, in turn, can inhibit transcription of several genes like vascular cell adhesion molecule-1 (VCAM-1), lower levels of NO might contribute to the augmented levels of adhesion molecules and hence to leukocyte recruitment. Fluid shear stress has also an effect on EC morphology. Where blood flow is uniform and laminar, cells are ellipsoid in shape and aligned in the direction of flow, while in regions of arterial branching or curvature where flow is disturbed, cells have polygonal shapes and no particular orientation and show increased permeability to LDL.
Figure 1. Cell infiltration into the intima. Lymphocytes and monocytes interacting with ECs are recruited to the locus of inflammation, promoted by antigens such as oxLDL. Here cells differentiate and proliferate, stimulating the recruitment of other cells.
The atherogenic process is initiated by infiltration of lipids into the arterial intima. LDL diffuses passively through EC junctions in the intima and is retained by proteoglycans in the ECM through interaction with the apolipoprotein B (apoB) component of LDL. Here trapped LDL undergoes modification such as oxidation, lipolysis, proteolysis and aggregation. One of the most significant modifications in early lesion formation is oxidation, as a result of exposure to the oxidative waste and to the action of different self-defense mechanisms of vascular cells. Concerning specific proteins, evidence has been provided for myeloperoxidase, paraoxonase, 15-lipoxygenase and NOS. The product of NOS is NO, a potent oxidant produced in two forms by both ECs and macrophages; while the former (eNOS) has an antiatherogenic vasodilator effect, the latter (iNOS) serves antimicrobial functions that may also promote lipid oxidation and atherogenesis. Oxidized LDL (oxLDL) has been shown to have many proatherogenic properties, among them activation of endothelium to release adhesion and pro-inflammatory molecules, including chemotactic proteins and growth factors (see below), and inhibition of production of the form of NO with vasorelaxant properties.

1.1.4.2 Inflammation: leukocyte recruitment

The normal arterial endothelium resists prolonged contact with blood leukocytes. When ECs undergo inflammatory activation, as in the presence of oxLDL, they express various leukocyte adhesion molecules such as VCAM-1, ICAM-1, P-selectin and E-selectin and pro-inflammatory molecules, including chemotactic proteins. This set of different molecules mediates the entry of specific types of leukocytes into the artery wall. The first step in adhesion, the “rolling” of monocytes and T-cells along the endothelial surface, is mediated by interaction with adhesion molecules as VCAM-1 which bind to carbohydrate on leukocytes and firmly anchor them to the endothelium. Once adherent to the ECs, leukocytes enter the intima by diapedesis at the junctions between ECs. Various chemoattractant cytokines (chemokines) seem to participate in this process; particularly, monocyte chemoattractant protein-1 is capable of recruiting monocytes into the arterial intima. T/lymphocyte recruitment is facilitated by other chemoattractants, including a trio of CXC chemokines induced by interferon-γ (IFN-γ) that bind CXCR3 receptor expressed on T-cell surfaces.
1.1.4.3 The “fatty streak”: leukocyte activation and foam cell formation

Once resident in the arterial intima, monocytes acquire the morphological characteristics of macrophages, undergoing a series of changes that lead ultimately to foam cell formation. The monocytes increase their expression of a group of receptors that recognize a wide array of ligands from modified lipoproteins, such as the scavenger receptor-A, CD36 and CD68. Extensively modified LDL particles are rapidly taken up by macrophages through these receptors, such that cholesteryl esters accumulate in cytoplasmic droplets. The expression of scavenger receptors (ScRs) is regulated by peroxisome proliferator-activated receptor-γ (PPAR-γ), a transcription factor whose ligands include oxidized fatty acids, and by cytokines such as TNFα and IFN-γ. These lipid-laden macrophages, known as foam cells, characterize the early atherosclerotic lesion called “fatty streak”. Macrophages within the atheroma also secrete a number of growth factors and cytokines involved in lesion progression and complication. The cytokine macrophage colony-stimulating factor (M-CSF) stimulates the proliferation and differentiation of macrophages, and influences various macrophage functions such as expression of ScRs. Granulocyte–macrophage colony-stimulating factor may also promote inflammation in the atheroma, aiding the survival of monocytes. Macrophages actively secrete apoliprotein E (apoE), and this may promote cholesterol efflux to HDL, thereby inhibiting the transformation of macrophages to foam cells. In parallel T-cells in the intima may encounter antigens such as oxLDL or heat shock proteins (HSPs) and produce cytokines that influence the activity of other cells.

1.1.4.4 From the fibrous plaque to the advanced lesion

Fatty streaks typically evolve into more complex atheromas through multiplication of SMCs, which, as a consequence of cytokines and growth factors released by T-cells and macrophages, accumulate in the plaque and lay down an abundant ECM that gives rise to a fibrous cap that walls off the lesion. Continuous migration and replication of cells followed by lipid loading leads to programmed cell death (apoptosis). The resulting mixture of cells, lipids and debris forms what is called the necrotic core. Following the interaction between CD40 ligand (CD40L) on macrophages and its receptor CD40 on T-cells, the former secrete several inflammatory mediators as tissue factor (TF) and matrix metalloproteinases (MMPs), while the latter can polarize into T-helper cells (Th) secreting pro-inflammatory cytokines (Th1) or anti-inflammatory cytokines (Th2). Initially wall thickening is compensated by an enlargement of the vessel area in response to increasing plaque burden that
leaves the lumen unaltered, known as “positive remodeling”. This compensation has only a partial effect, and as the lesion becomes thicker the arterial lumen narrows until it obstructs flow and leads to clinical manifestations in the coronary circulation such as unstable angina pectoris or acute MI. Continuing influx and activation of macrophages causes release and accumulation of several classes of proteolytic enzymes such as collagenases, gelatinases,stromolysin and cathepsins, that act towards different components of the ECM and degrade it leading to thinning of the fibrous cap and sometimes hemorrhage from the vasa vasorum, which in turn may result in thrombus formation. As several cycles of lipid deposition, cell accumulation, necrotic and apoptotic processes and proteolytic activity follow one to each other the lesion matures into what is called the advanced complicated lesion.

1.1.4.5 Instability, plaque rupture and thrombosis

The atherosclerotic plaque may grow slowly and over several decades produce a severe stenosis or may progress to total arterial occlusion. Development of CVD was previously believed to be caused by the mere stenosis caused by large plaques. However, several studies have shown that it is not the atherosclerotic narrowing of the lumen that causes the infarction but rather a thrombus on the surface of an activated plaque that leads to a sudden occlusion. Some plaques remain stable for a long period of time, but others may undergo spontaneous fissure or rupture, exposing the plaque contents to flowing blood. The reason why these plaques, deemed to be unstable or vulnerable, are likely to rupture, resides mainly in their composition. The concept of the vulnerable plaque was established almost twenty years ago. The vulnerable plaque is characterized by a large, lipid-rich core\textsuperscript{60, 61}, surrounded by clusters of macrophages\textsuperscript{62, 63} and other inflammatory cells\textsuperscript{64-67}, and an overlying thin fibrous cap\textsuperscript{68}, with a reduced number of SMCs and a decreased amount of collagen\textsuperscript{61, 69, 70}. Maintenance of the fibrous cap reflects matrix production and degradation, which in turn depend on molecules produced by inflammatory cells as described previously. In fact, rupture frequently occurs at the lesion edges, which are rich in foam cells, suggesting that factors contributing to inflammation may also influence thrombosis. The stability of atherosclerotic lesions may also be influenced by calcification\textsuperscript{71} and neovascularization\textsuperscript{72}, common features of advanced lesions.

Three different types of plaque rupture have been observed\textsuperscript{73}.

\textbf{Superficial erosion} is a phenomenon by which, as the plaque enlarges and bulges into the lumen, the subendothelium becomes exposed to blood at sites of endothelial retraction or tear, following desquamation of ECs. This can be
due to both EC death and degradation of ECM components of the subendothelial basal membrane. Platelets can adhere to dysfunctional endothelium, exposed collagen and macrophages, become activated and start aggregating by the effect of von Willebrand factor, possibly leading to mural thrombus formation. The thrombus may embolize and rapidly occlude the lumen to precipitate an acute ischemic syndrome, or gradually become organized and incorporated into the plaque, contributing to its growth. Release of growth factors from the aggregated platelets may increase SMC proliferation in the intima and aid integration of the thrombus in the plaque. Even if common, it is most often asymptomatic and accounts for approximately one-quarter of fatal events.

**Intraplaque hemorrhage.** Pathological neovascularization of the vessel wall is a consistent feature of atherosclerotic plaque development and progression of the disease. The new blood vessels that form in the plaque, due to secretion of angiogenic mediators by inflammatory cells, may be particularly fragile and prone to micro-haemorrhage. Thrombosis *in situ* within plaques leads to thrombin generation, which, in addition to cleaving fibrinogen, triggers platelet release of growth factors such as platelet-derived growth factor stimulating SMC migration and proliferation. Activated platelets also elaborate transforming growth factor beta that stimulates interstitial collagen synthesis by SMCs. Thus, a silent microvascular hemorrhage within the intima could contribute to the observed discontinuous growth of the plaque.

**Fracture of the fibrous cap.** The combination of a lipid core, accumulation of macrophages in the shoulder region and a thin, collagen-poor fibrous cap makes the plaque less resistant to the mechanical forces of the blood stream. The fibrous cap can no longer resist the forces of the flowing blood and eventually it will break. As the fibrous cap ruptures, the extremely thrombogenic lipid-rich core is exposed to the blood stream. The thrombogenicity of the lesion core is likely to depend on the presence of TF, a key protein in the initiation of the coagulation cascade, secreted by ECs and macrophages. When TF is allowed to contact the circulating coagulation proteins, platelets are recruited to the site initiating thrombus formation. The expression of other molecules mediating thrombosis, such as plasminogen activator, may also be important.

Even though ruptured fibrous cap causes about three-quarters of acute MI, most episodes are probably asymptomatic. When the prevailing fibrinolytic mechanisms overbalance the pro-coagulant pathways, a limited mural thrombus, rather than an occlusive blood clot, forms. However, activation of the healing response may lead to reabsorption of the mural thrombus and formation of a more fibrous lesion.
1.1.5 Triggers of inflammation

The concept that inflammation occupies an essential position in the pathophysiology of atherosclerosis is widely recognized, knowledge of the details, however, remains vague. Over the past decades two main hypotheses have been proposed to explain how atherosclerosis is initiated: the response-to-injury hypothesis and the response-to-retention hypothesis. The most likely scenario is the result of an interaction of both these processes.

1.1.5.1 The response-to-injury hypothesis

A hypothesis that an injury to the endothelium might precipitate the atherosclerotic process was proposed in 1973 and has continually been modified since. Initially, endothelial denudation was proposed as the first step in atherosclerosis while recently endothelial dysfunction is emphasized. The response-to-injury hypothesis postulates that endothelial injury by various mechanisms may cause endothelial dysfunction; among possible causes are production of free radicals, lipoprotein (a), homocysteine, infectious agents and combinations of these and other factors. The consequences of the injury lead to loss of endothelium and to compensatory responses that alter its normal homeostatic properties, resulting in increase of adhesiveness and permeability with respect to leukocytes or platelets, acquisition of procoagulant properties and production of vasoactive molecules. Manifestations of the dysfunction of the endothelium caused by injury include also increased trapping of lipoproteins in the artery. If the inflammatory response does not effectively neutralize or remove the offending agents, it can continue indefinitely. In doing so, the inflammatory response stimulates chemotaxis of monocytes and T-lymphocytes that in turn induce migration of SMCs from the media into the intima. If these processes continue, they can thicken the artery wall, which compensates by gradual dilation, so that up to a point, the lumen remains unaltered (positive remodeling). Such a prolonged state results in cycles of accumulation of inflammatory cells and SMCs, which eventually lead to formation of a core of lipid and necrotic tissue covered by a fibrous cap, called advanced lesion.

1.1.5.2 The response-to-retention hypothesis

The key processes of atherosclerosis is not yet fully clear, but there is much evidence pointing towards mechanisms responsible for retaining lipids in the vessel wall (see above). Subendothelial retention of atherogenic lipoproteins is a key event in instigating atherogenesis. By itself, infiltration of LDL would
not cause development of atherosclerosis, but once LDL binds to proteoglycans in the ECM of the intima they appear to exhibit increased susceptibility to oxidative modification, probably because of an increased retention time in the intima where they can be subjected to modifications. The response-to-retention hypothesis postulates that an elevation in plasma LDL levels results in increased infiltration of LDL into the arterial wall, leading to lipid accumulation in SMCs and in macrophages (foam cells). LDL also augments SMC hyperplasia and migration into the subintimal and intimal region in response to growth factors. LDL is modified or oxidized in this environment and is rendered more atherogenic. Small dense LDL cholesterol particles are also more susceptible to modification and oxidation. The modified or oxidized LDL is chemotactic to monocytes, promoting their migration into the intima, their early appearance in the fatty streak, and their transformation and retention in the subintimal compartment as macrophages. ScRs on the surface of macrophages facilitate the entry of oxidized LDL into these cells, transferring them into lipid-laden macrophages and foam cells. Oxidized LDL is also cytotoxic to ECs and may be responsible for their dysfunction or loss from the more advanced lesion.

1.1.5.3 Other candidate antigens

In addition to modified LDL, several other atherosclerosis-associated antigens have been proposed. One of them is the HSP molecule. These proteins are produced by injured cells and act as chaperones to limit the denaturation of other cellular proteins. Interestingly, HSPs are also released by monocytes exposed to LDL. High levels of antibodies against several variants of HSPs have been found at early stage of atherosclerosis in human.

Another candidate antigen is the \( \beta_2 \)-glycoprotein Ib, a phospholipid-binding protein present on platelets and ECs. \( \beta_2 \)-glycoprotein Ib has also been detected in human atherosclerotic plaques, and antibodies against \( \beta_2 \)-glycoprotein Ib are found in lesions.

Finally, certain microorganisms and viruses may be involved in atherogenesis. For example, high titers of antibodies against \textit{Chlamydia pneumoniae} and \textit{Helicobacter Pylori} are found in patients with CVD, while \textit{Herpes simplex} and \textit{Cytomegalovirus} have been found in human atherosclerotic plaques.
1.2 IMMUNITY AND ATHEROSCLEROSIS

Recent experimental, clinical and epidemiological studies have shown that inflammatory and immune mechanisms are involved in atherosclerosis. In addition to the already mentioned features of inflammation, lesions present also characteristics of immunity like dendritic cells (DCs)\(^9\), mast cells\(^7\), a few B-cells\(^6\), probably natural killer T (NKT) -cells, auto-antibodies\(^7\) and complement components\(^8\). DCs are common antigen presenting cells in the plaque\(^5\), and they are said to be the only cell type that can activate naïve T-cells\(^3\). Mast cells are immune effectors and are able to modify lipoproteins and digest matrix components\(^7\), \(^9\). Activated mast cells promote macrophage derived foam cell formation\(^1\). The roles of B-cells and NKT-cells in human atherosclerotic lesions have to be further elucidated; B-cells appear to be scarce in plaques and are found predominantly in the adventitia and the periadventitial connective tissue\(^3\). Local production and accumulation of IgG antibodies is prominent in lesions in humans as well as in animals\(^1\). Available data strongly suggest that both innate and adaptive immuno/inflammatory mechanisms are major determinants of plaque complications. The studies performed to characterize the role of immunity in atherosclerosis are difficult to interpret and often give contradictory indications. However, autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis (RA) and antiphospholipid (Hughes) syndrome supply strong evidence for a link between immunity and atherosclerosis. The incidence of CVD is significantly increased in individuals affected by these disorders\(^1\) and cardiovascular mortality is a major cause of death among these patients\(^1\). Recently, the major-histocompatiblity-complex (MHC) class II transactivator (MHC2TA) gene has been associated with increased susceptibility to RA, multiple sclerosis and MI\(^1\), reinforcing once more the concept that inflammatory and immune components play a role in atherosclerosis. Evidence that inflammation and cholesterol levels are associated and important for development of atherosclerosis has been obtained also from trials, indicating that statins have not only an effect as lipid-lowering drugs but also possess inherent anti-inflammatory properties\(^1\).

1.2.1 The immune system

The main purpose of the immune system is to protect against infectious agents. There are several lines of defense. The first one consists of physical barriers, like skin and surface of mucous membranes, and chemical barriers, like pH and a variety of molecules such as lysozymes. The second line is the
natural or innate immune system where mononuclear phagocytes are pivotal. They express a limited number of highly conserved receptors to recognize and bind foreign antigens. Vertebrates have a third line of defense, which is often referred to as the specific or adaptive immune system. It is constituted of B- and T-cells (or T-lymphocytes) and is characterized by the ability to continuously change and adapt in response to invasion as well as a remarkable property of “memory” allowing a fast secondary response. Thus, adaptive immunity is specific but much slower than innate immunity.

1.2.1.1 Innate immunity

When the physical and chemical barriers are insufficient to prevent foreign pathogens from entering the body, the immune system takes over the responsibility to do it.

The strategy of the innate system is not to recognize every possible pathogenic antigen, but rather to focus on a few, highly conserved structures present in large groups of microorganisms that are often encountered. These structures are termed pathogen-associated molecular patterns (PAMPs) and can be found for example on lipopolysaccharide (LPS), lipoteichoic acid from Gram positive bacteria, peptidoglycan and nucleic acid variants normally associated with viruses, such as double-stranded RNA. The receptors of the innate system, such as Toll-like receptors (TLRs), that evolve to recognize the PAMPs, are referred to as pattern recognition receptors and are present in the body from the beginning since they are genetically determined. Once the PPRs identify the PAMPs, the cells are stimulated to exert their functions very rapidly. The main players of the innate system are the mononuclear phagocytes. They originate from the bone marrow and become monocytes in the blood, to mature subsequently into macrophages once they enter the tissue. Their main role is to clean up the tissue from undesired particles, but they can also behave as antigen-presenting cells.

1.2.1.2 Adaptive immunity

The adaptive system can discriminate slight differences among antigens and can therefore recognize an infinite variety of them. An important feature of adaptive immunity is the capability to remember encounter with an antigen and exert a more efficient and rapid response upon a second exposure to the same antigen. It can be divided into humoral immunity, mediated by B-cells, and cellular immunity, mediated by T-cells.
1.2.1.2.1  B-cells and the humoral response

The humoral response is orchestrated by B-cells. They are produced in the bone marrow as naïve B-cells expressing membrane-bound specific antibodies known as B-cell receptors (BCR), which have the ability to recognize soluble antigens. After binding to the BCR, the antigen is internalized by endocytosis, processed and presented on the cell surface by the class II MHC. Once activated, the naïve B-cells proliferate and give rise to plasma cells and memory B-cells. Plasma cells secrete highly specific immunoglobulins that recognize antigens and tag them in order to facilitate uptake and destruction, directly by macrophages or indirectly via the complement system. Memory B-cells have a long lifespan and are rapidly activated after a second infection by the same pathogen.

1.2.1.2.2  T-cells and the cellular response

The cellular response consists of T-cells, which have highly specific and diverse receptors (T-cell receptor (TCR)) that recognize antigens when processed and presented to them in association with the MHC. This antigen presentation usually requires cell-cell contact between the T-cell and an “antigen-presenting-cell” (APC) such as macrophages, DCs or B-cells. Depending on the surface expression of co-receptors that aid the antigen recognition by TCRs, two main classes of T-cells can be identified: CD8+ and CD4+ T-cells. CD8+ T-cells recognize surface structures and kill by cell-to-cell contact potentially harmful self-cells that have been infected by viruses or otherwise transformed, such as tumor cells. They are also known as T-killer (Tk) or T-cytotoxic (Tc) cells. Activated CD4+ T-cells, or Th-cells, produce large amounts of cytokines to signal, activate or recruit other cells, such as neutrophils, thus initiating a local inflammatory response. The activation and clonal expansion of these cells also provide signals that are essential for differentiation and activation of B-cells. Th-cells can be divided into different subpopulations, based on the production of functionally distinct cytokine profiles. The major subpopulations are denoted Th1, producing predominantly pro-inflammatory cytokines like IL-1, IL-12, TNFα and IFN-γ, and Th2, producing predominantly anti-inflammatory cytokines, like IL-4, IL-5, IL-6 and IL-10. Th1-cells activate CD8+ T-cells, Th2-cells activate B-cells and they also regulate each other. The classification of CD8+ as Tk and CD4+ as Th is not unconditional since some CD4+ can act as T-killers while some CD8+ can exert an effect similar to T-helper cells.
1.2.1.2.3 B- and T-cell activation

MHC class I and II and antigen presentation
The MHC class I and II molecules are antigen-presenting structures. Each class presents antigens to a different subset of T-cells. The MHC I molecule is specialized in presenting small endogenous peptides from proteins synthesized in virus-infected cells or produced in cancerous cells, to CD8+ T-cells. The MHC II molecule is specialized in presenting exogenous antigens that have been taken up and processed by APCs to T-cells expressing the co-receptor CD4.

T-cell receptor and antigen recognition
The TCR is a receptor present on the membrane of T-cells and is responsible for recognizing antigens bound to MHC molecules. It is generated by recombinational events involving a random joining of gene segments that leads to a nearly infinite repertoire of antigen specificity. The TCR associates with co-receptors that are vital for propagating the signal into the cell. These co-receptors, together with the TCR, form what is known as the TCR complex.

Naïve T-cells get activated when they recognize an antigen presented by APCs. In that case the TCR binds the MHC-peptide complex assisted by binding of CD4/CD8 molecule; then ligation of the co-stimulatory molecule CD28 on T-cells by CD80 or CD86 on the APC induces up-regulation of CD40L on T-cells, which in turn will bind CD40 on APCs, contributing to the process \(^{111,112}\). Subsequently, T-cells go on to differentiate into effector cells, which can interact with B-cells in germinal centers and/or migrate out of the lymphoid organs and carry out their effector functions in the peripheral tissues.

B-cell activation and antibody production
Activation of B-cells may require involvement of Th-cells or not. In the first case, interaction between antigen and BCRs induces up-regulation of the MHC II molecules. The MHC II-antigen complex is recognized by Th-cells that become active. Th-cells will then express CD40L and activate B-cells through interaction with CD40, that proliferate and differentiate into plasma and memory cells. Sometimes the antigens are able to bind BCRs and activate B-cells regardless of their specificity.

1.2.2 Adaptive immunity in atherosclerosis
The role of innate immunity in atherosclerosis has already been extensively discussed (see section 1.1.4 describing the atherogenic process). The impact of adaptive immunity in atherogenesis has been studied in various mouse models,
suggesting that lymphocytes play a role mainly in development of early atherosclerosis. Although only few B-cells are detected in the lesions, several studies suggest that they may inhibit the development of vascular pathologies in animal models\textsuperscript{113}. In addition, a subgroup of B-cells produces the natural anti-PC antibody that also recognizes an epitope on oxLDL\textsuperscript{114}. Activated T-cells represent approximately between 10\% and 20\% of the cell content in advanced human lesions\textsuperscript{96, 115}. CD8\(^+\) T-cells are an important subset of all T-cells, and although their role is still poorly understood, it has been suggested that they might be responsible for some of the apoptosis associated with atherosclerosis\textsuperscript{116, 117}. CD4\(^+\) T-cells constitute the vast majority of T-cells found in atheromas and are the ones that recognize LDL-derived peptides within MHC II. OxLDL-reactive CD4\(^+\) T-cells probably recognize apoB-derived oligopeptides carrying adducts formed during oxidation\textsuperscript{118}. They have been found in plaques, lymph nodes, and in the blood of patients with atherosclerosis, and most of them have a Th1-cell phenotype\textsuperscript{118, 119}. Th1-cells have an essential role in regulating the functions of SMCs (collagen formation) and macrophages (collagen degradation) that crucially regulate the integrity of the fibrous cap of the plaque. Specifically, the Th1-cytokine IFN-\(\gamma\) strongly inhibits the production of interstitial collagens by vascular SMCs, which confer stability to the fibrous cap, and can also inhibit the proliferation of SMCs, thereby reducing the stabilizing and collagen-synthesizing cellular component of the plaque\textsuperscript{120}. Also, proteases originating mainly from activated macrophages in plaques can degrade collagen\textsuperscript{121, 122}.

\subsection*{1.3 THE TNF/TNFR SUPERFAMILY}

Presence of the antigen-specific TCR is not enough for full T-cell activation. As already discussed, this is usually realized only in the presence of additional receptor-ligand interactions such as the engagement of CD28 by its ligands CD80 and CD86, altogether referred to as B7 molecules. When these interactions take place at the same time as TCR engagement and allow T-cell survival, they are defined as co-stimulatory signals. Although the majority of T-effector cells are short-lived, some antigen-experienced cells remain as long-lived memory cells\textsuperscript{123}. T-cells receive activation or survival signals at each stage of the response, including naïve, effector, and memory stages. Members of the tumor necrosis factor (TNF) and TNF receptor (TNFR) superfamilies are key mediators of survival signaling in T-cells subsequent to the initial effects of CD28-B7 interaction.
The TNF/TNFR superfamily consists of a set of cytokines and cognate receptors that mediate different physiologic and pathologic activities. They are critically involved in regulation of essential biological functions and maintenance of homeostasis of the immune system. Membrane-bound and/or soluble ligands of the TNF family interact with one or more specific, membrane-bound or soluble receptors which together comprise the corresponding TNFR family. The majority of the members of this TNF/TNFR superfamily is expressed in the immune system, but they have been adapted for apparently dissimilar processes such as host defense and organogenesis. Activation of the TNFR members via their ligands affects cell proliferation, survival, differentiation and apoptosis of responding cells. These biological activities encompass beneficial effects for the host in inflammation and protective immune responses in infectious diseases as well as crucial roles in organogenesis of secondary lymphoid organs and the maintenance of lymphoid structures throughout the body. On the other hand, some members of the TNF/TNFR superfamily can exert host-damaging effects, for instance in sepsis and autoimmune diseases (e.g. RA, psoriasis, inflammatory bowel disease).

Currently more than 40 members of the TNF/TNFR superfamily have been identified, and more knowledge about their central biological role is emerging; members of this group are now being targeted for therapies against common human diseases such as atherosclerosis, autoimmune disorders, osteoporosis, allograft rejection, and cancer.

1.3.1 Structure

The receptors and ligands in the TNF/TNFR superfamily have exclusive structural features that couple them directly to signaling pathways for cell proliferation, survival, and differentiation. Most members of the TNF-like receptors are type I transmembrane proteins characterized by cysteine-rich domains (CRDs) in their ligand-binding extracellular regions, a feature of the TNFR family. There is a significant variation in the number of CRDs among the receptor family members, from only a partial CRD, up to six CRDs (Figure 2). The TNF family ligands are type II transmembrane proteins that are biologically active as self-assembling, noncovalent trimers. The external surfaces of the ligand trimers differ widely within the family. Some ligands can have both membrane integrated and soluble forms, the latter released from the cell membrane after proteolytic
cleavage, mainly by metalloproteinases induced by various stimuli; other ligands are expressed only as soluble molecules, as lymphotoxin-α (LTα).

Figure 2. Protein structure of the TNFR superfamily members.

1.3.2 Signaling

Most members of the ligand family (TNF) interact with more than one receptor of the corresponding cognate family (TNFR). However, each receptor/ligand system appears to have unique and non-redundant functions. Upon receptor stimulation, some of the TNFR family members are cleaved from the cell surface (e.g. TNFR2, 4-1BB) or directly expressed as soluble isoforms lacking the transmembrane domain (e.g. TNFR2, 4-1BB, and FAS) but still being capable of binding its cognate ligand. This represents a cellular mechanism to antagonize ligand-induced receptor stimulation, presumably in pathophysiological conditions.

Based upon their intracellular sequences and signaling properties, TNF receptors can be classified into three major groups. The first group, including FAS and TNFR1 (receptor of LTα) among others, contains a death domain in the cytoplasmic tail. Activation of these DD-containing receptors by their corresponding ligands can lead to recruitment of intracellular adaptors such as FAS-associated death domain (FADD) and TNFR-associated DD (TRADD). These molecules, in turn, activate the caspase cascade and subsequently induce apoptosis. The second group, including TNFR2 (receptor of TNF), CD40, and OX40, accounts for receptors containing one or more
TNFR-associated factor (TRAF)-interacting motifs (TIMs) in their cytoplasmic tails. Activation of TIM-containing TNF receptors leads to recruitment of TRAF family members, and activation of multiple signal transduction pathways such as nuclear factor kappa B (NF-κB), Jun N-terminal kinase (JNK), p38, extracellular signal-related kinase (ERK) and phosphoinositide 3-kinase (PI3K) (Figure 3). The third group of TNF receptor family members does not contain functional intracellular signaling domains or motifs, instead they compete with the other two groups of receptors for their corresponding ligands by hampering the activation of signal transduction pathways by other TNF receptors.

![Figure 3. Regulation of TNFR family members signal transduction. Anti-apoptotic or differentiative signals are indicated with green lines, pro-apoptotic signals with red (Modified from Dempsey 2003).](image)

### 1.3.3 The TNF/TNFR superfamily and immunity

Members of the TNF/TNFR superfamily play a crucial role in establishing a robust immune system by positively regulating T-cell and B-cell viability, survival and differentiation. TNF/TNFR superfamily interactions can influence T-cell responses in a number of ways. They influence inflammation and innate immunity, lymphoid organization, and activation of APCs. They can also provide direct signals to T-cells. OX40L/OX40, 4-
1BBL/4-1BB, CD30L/CD30, LIGHT/HVEM, CD70/CD27, and GITRL/GITR principally support activation, survival, and functionality of T-cells; BAFF/BAFFR has a similar function for B-cells, while RANKL/RANK functions for DCs. Each TNF/TNFR family interaction appears to have certain characteristic or unique functions with regard to the overall immune response. These functions may be derived from differential expression patterns on varying lymphoid cells, variations in the duration of expression of the inducible molecules, and the sites in the body where expression of ligands/receptors is located. The role of some TNF/TNFR superfamily members in atherosclerosis is discussed below.

### 1.3.4 TNF and LTα ligand/receptor systems

TNF and LTα are two important members of the TNF ligand family; they are structurally similar and display 50% amino acid homology\(^{141}\).

TNF, also known as TNFα, is synthesized by activated macrophages\(^{142}\), Th1-cells, mast cells and NK-cells, first as a type II transmembrane protein and subsequently cleaved to a soluble form\(^{143}\). TNF is a cytotoxic and potent pro-inflammatory agent that acts through NFκB. It also inhibits key metabolic enzymes. A recent report has assigned a pro-atherogenic role of TNF due to its ability to initiate expression of adhesion molecules, chemoattractants and other pro-inflammatory factors of the vascular wall, and to induce SrA expression and oxLDL uptake by macrophages\(^{144}\). Gene targeting of TNF leads to reduced atherosclerosis, similar to the effects of IFNγ\(^{145, 146}\).

LTα, also known as tumor necrosis factor β (TNFβ), is one of the first cytokines to be discovered. It is produced in the early stages of vascular inflammatory processes as a soluble molecule, primarily by activated T- and B-lymphocytes\(^{144, 147}\). Several important functions of LTα have recently been identified that demonstrate a significant role for this cytokine in lymphocyte activation and proliferation. LTα promotes inflammatory and chemoattractant responses\(^{148-150}\) and B-cell proliferation\(^{151}\). LTα is also involved in CD8+ T-cell activation\(^{152}\). Recent studies presented evidence that LTα is important for the function of T-cells in their interaction with other T-cells, B-cells and APCs, thus playing a critical role in the development and maintenance of a normal immune response and in the development of autoimmune disease\(^{153, 154}\). A recent report demonstrated that LTα was expressed in atherosclerotic lesions in mice and that loss of LTα reduced their size\(^{155}\), confirming the concept that T- and/or B-cells secrete LTα within the developing lesion to promote atherogenesis.
1.3.5 The CD40L/CD40 system

Beyond the originally described source, the B-lymphocyte, CD40 is present on a variety of immune cells, including those implicated in atherogenesis such as ECs, SMCs, macrophages and APCs. CD40L is largely expressed on CD4+ T-cells and activated platelets\textsuperscript{156-160}. CD40 but not CD40L, is constitutively expressed \textit{in vitro} and shows basal expression in a variety of tissues; expression of the receptor is sensitive to \textit{in vitro} stimulation with inflammatory cytokines\textsuperscript{161} and is probably regulated by NF-\textkappa B and STAT pathways\textsuperscript{162}. The ligand is also present as a soluble, proteolytically cleaved form, which seems to be biologically active and able to interact with the receptor\textsuperscript{163}. High concentrations of sCD40L are assumed to represent abundant CD40L expression on cells\textsuperscript{164, 165}. Interaction between CD40L on T-cells and CD40 on APCs is important for T-cell activation. CD40 may function as a master switch for T-cell costimulation because of its ability to induce B7 family ligands as well as several TNF family ligands\textsuperscript{112}. In the past several years, the CD40L/CD40 interaction has been identified as a key process affecting the stability of atherosclerotic plaques\textsuperscript{166}. Interruption of the CD40L/CD40 pathway in hypercholesterolemic mice by treatment with inhibitory antibodies against CD40L or targeted CD40L gene deletion can decrease the size of coronary plaques as well as the inflammatory cell content\textsuperscript{58, 167-169}. In addition, ligation of CD40 expressed by macrophages increases the production of matrix-degrading proteases\textsuperscript{158}, including the collagenases of the MMP family\textsuperscript{170}, which weaken the fibrous cap by degrading ECM components. Evidence suggests also that an enhanced CD40L/CD40 interaction promotes pro-thrombotic activity by increasing the macrophage expression of TF, the potent procoagulant expressed in the lipid core of the plaque,\textsuperscript{158, 160} and through direct regulation of endothelium procoagulant activity\textsuperscript{171}. Since platelets can also express CD40L\textsuperscript{159} when activated, a positive loop can amplify the local inflammatory response, once a thrombus begins to form. The CD40L/CD40 pathway has a role also in potentiating an inflammatory response of the endothelium, including the release of vascular endothelial growth factor (VEGF). A prominent expression of these molecules has been observed in the vessel intima of advanced lesions\textsuperscript{157}.

1.3.6 The OX40L/OX40 system

OX40 expression is induced on activated T-cells at the peak of the primary immune response\textsuperscript{172} mainly in CD4+ T-cells, but CD8+ T-cells can also express OX40 when properly activated\textsuperscript{173, 174}. Activated T-cells expressing
OX40 have been identified at various sites in a number of inflammatory and autoimmune diseases such as experimental autoimmune encephalitis (EAE)\textsuperscript{175}, inflammatory bowel disease\textsuperscript{176}, Crohn’s disease\textsuperscript{177} and RA\textsuperscript{178}, skin diseases\textsuperscript{179}, graft-versus-host disease (GVHD)\textsuperscript{180} and cancer\textsuperscript{181}. In contrast to OX40, OX40 ligand (OX40L) is expressed on a number of cell types, primarily activated professional APCs such as DCs\textsuperscript{182, 183}, B-cells\textsuperscript{173, 174} and macrophages\textsuperscript{184}. Other types of cells can display OX40L under certain circumstances, including ECs\textsuperscript{185}, SMCs\textsuperscript{186}, mast-cells\textsuperscript{187}, NKT-cells\textsuperscript{188} and activated T-cells\textsuperscript{189}.

![Figure 4. OX40L expressed on APCs stimulates T-cell proliferation and differentiation by interacting with OX40.](image)

OX40L and OX40 are not constitutively expressed on most immune cells but are inducible hours to several days after activation\textsuperscript{183, 190, 191}, suggesting that they are highly regulated. TCR engagement is known to be crucial for OX40 expression, and CD28 signaling synergizes to up-regulate OX40 expression\textsuperscript{190, 192}. The peak level of OX40 on T-cells \textit{in vitro} largely coincides with that of OX40L on APCs\textsuperscript{191, 193}. Signals from TLR4, CD40, and the immunoglobulin receptor, either alone or with cytokine signals, can promote OX40L expression on DCs and B-cells\textsuperscript{183, 193, 194}. Expression of OX40L on these cell types is transient, indicating that the OX40L/OX40 system functions only in presence of certain responses to injury and specific antigens.

\subsection{1.3.6.1 OX40L/OX40 system in inflammation and immunity}

Growing evidence has shown that OX40 regulates the expression and survival of CD4+ and CD8+ T-cells when stimulated by its ligand on different APCs\textsuperscript{195}. Studies with OX40L or OX40 knockout mice have revealed that the
presence of OX40L on DCs is crucial for T-cell expansion\textsuperscript{193, 196, 197}. OX40L/OX40 interaction presumably occurs within 48 hours after antigenic stimulation, when OX40 and OX40L are induced on T-cells and DCs, respectively. Thus, OX40 signals prolong T-cell survival beyond the effector phase of the immune response and increase the number of memory T-cells by stimulating production of anti-apoptotic proteins that inhibit T-cell death\textsuperscript{190, 198}. However, OX40L on B-cells can sustain CD4\textsuperscript{+} T-cells 3-4 days after activation\textsuperscript{199}, suggesting that there may be sequential effects of OX40L expressed by different APC subsets.

Accumulating evidence has also supported the notion that OX40 ligation does not play a role in determining Th1/Th2 polarization but rather contributes to ongoing Th1 or Th2 responses. OX40 ligation has greater effects on Th2 responses because of the higher levels of OX40 on Th2 cells\textsuperscript{200}. OX40/OX40L can influence the number of T-cells accumulating at particular sites in different ways. Evidence that OX40 could directly influence migration comes from studies showing that OX40L expression on DCs induces increased CXCR5 expression by T-cells\textsuperscript{192}. Also, OX40L was reported to be expressed on activated ECs and to mediate the adhesion of OX40 expressing T-cells to vascular ECs resulting in enhanced T-cell recruitment to inflammatory sites\textsuperscript{185}. This interaction can provide T-cells with co-stimulatory signals\textsuperscript{201} and induce ECs to produce Rantes/CCL5, a CC chemokine implicated in T-cell migration\textsuperscript{202}.

1.3.6.2 OX40L/OX40 system in disease

Interactions between OX40 and its ligand are implicated in an increasing number of diseases such as autoimmune diseases, infections, allergies, alloresponses and cancer, supporting the notion of OX40L/OX40 as a potential therapeutic target\textsuperscript{203}. A large body of evidence suggests an important role for OX40L/OX40 in maintenance of inflammatory and autoimmune diseases. In mice, OX40L/OX40 interaction is required for the induction of a number of autoimmune and inflammatory diseases such as EAE\textsuperscript{175}, collagen-induced arthritis\textsuperscript{178}, colitis\textsuperscript{204} contact hypersensitivity reactions\textsuperscript{205}, and diabetes\textsuperscript{206}. In humans, OX40 and its ligand are observed in inflamed tissues in several autoimmune and inflammatory diseases\textsuperscript{207-209}. Evidence suggests a pathogenic role for OX40L in asthma\textsuperscript{186} and in the development of RA\textsuperscript{210}. 

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1.3.6.3 Modulation of OX40L/OX40 system for therapeutic treatment

Therapeutic targeting of TNF and TNFR members appears to hold much promise for manipulating immune responses in positive and negative ways. The ability of several different TNFR family members to transiently control T-cell activation/survival at distinct stages in the immune response may be important in providing multiple points to fine-tune the system. However, ligation of TNFR family members can be protective or harmful, depending on how they are manipulated and the degree of inflammation. Transient stimulation through co-stimulatory TNFR family members can enhance antiviral and anticancer responses \textit{in vivo}, and blockade of these pathways can be beneficial for autoimmunity and transplantation. In contrast, the use of agonistic antibodies that stimulate anticancer or antiviral responses under conditions of strong immune or inflammatory pressure leads to suppression of responses, apparently by induction of inhibitory cytokines. Similarly, constitutive expression of the co-stimulatory ligands tends to have devastating consequences in mice, with autoimmunity or profound immunosuppression as result. Thus, great care must be taken in future clinical applications of co-stimulatory TNF family members to human disease.

With its strong co-stimulatory function and pivotal role in memory T-cell generation, the OX40L/OX40 pair is one of the optimal targets of immune intervention. Evidence from several studies illustrates the feasibility of immune intervention for treating diseases that specifically target OX40+ T-cells \cite{211, 212}. For example, treatment of mice with OX40-Ig blocks the interaction of OX40 with its ligand on APCs and ameliorates pulmonary infections, without preventing virus clearance \cite{211}. Suppression of this late co-stimulatory pathway via OX40 has clear therapeutic potential for the treatment of dysregulated lung immune responses.

1.3.6.4 Animal models

Several effects of TNFR family members observed in gene-targeted mice are not “all or nothing,” but rather appear to affect T-cell survival quantitatively, with quite subtle effects in some models \cite{124} (Table 1). Deletion of the OX40L and OX40 genes leads to no obvious abnormalities in terms of viability or fertility of mice, organization of primary and secondary lymphoid tissues, or early development of T- and B-cells and DCs \cite{193, 196, 213}. However, ablation of OX40L or OX40 resulted in critical defects of CD4+ T-cells survival during active immune response \cite{196, 194, 214}.
Table 1. Abnormal immune responses in OX40-deficient and OX40L-deficient mice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Immune response examined</th>
<th>Results of phenotypic features</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C57BL/6 x 129) hybrid</td>
<td>Influenza virus infection</td>
<td>Impaired CD4+ T-cell proliferation and IFN-γ production; reduction in CD4+ T-cell infiltration</td>
<td>195</td>
</tr>
<tr>
<td>C57BL/6</td>
<td><em>Ex vivo</em> T-cell proliferation after CD3 stimulation</td>
<td>Reduced T-cell proliferation</td>
<td>225</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>T-cell responses to protein antigen</td>
<td>Reduced effector T-cell function and impaired memory T-cell generation</td>
<td>226</td>
</tr>
<tr>
<td>C57BL/6</td>
<td><em>Ex vivo</em> T-cell proliferation and survival after antigen stimulation</td>
<td>Marked reduction in T-cell survival despite almost normal proliferation</td>
<td>189, 197</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Used as donor in experimental GVHD</td>
<td>Reduced GVHD mortality</td>
<td>217</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Airway hyperreactivity using ovalbumin</td>
<td>Diminished inflammation; impaired Th2-cell responses; impaired reactivation of Th2 memory cells</td>
<td>212</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Experimental autoimmune encephalomyelitis</td>
<td>Reduced symptoms; impaired function of infiltrated T-cells</td>
<td>208</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Regulatory T-cell number and function</td>
<td>Reduced numbers of CD4+CD25+ T-cells; less <em>ex vivo</em> suppressive function of OX40-deficient regulatory T-cells</td>
<td>213</td>
</tr>
<tr>
<td>BALB/c</td>
<td>CHS in response to haptens</td>
<td>Reduced CHS responses; impaired APC function of dendritic cells</td>
<td>196, 204</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>CD4+ T-cell responses to protein antigen</td>
<td>Impaired effector CD4+ T-cell responses; reduced memory CD4+ T-cell function</td>
<td>192</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Experimental autoimmune encephalomyelitis</td>
<td>Reduced symptoms; impaired function of antigen-specific T-cells</td>
<td>216</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Airway hyperreactivity using ovalbumin</td>
<td>Suppressed lung inflammation; reduced Th2-cell responses</td>
<td>199, 213</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Infection with <em>Heligmosomoides polygyrus</em></td>
<td>Reduced Th2-cell responses; compromised host defense</td>
<td>217</td>
</tr>
<tr>
<td>NOD</td>
<td>Spontaneous diabetes development</td>
<td>No disease symptoms</td>
<td>205</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>TH2-cell responses in B-cell-deficient mice transferred with exogenous B-cells</td>
<td>Less Th2-cell responses induced in mice transferred with OX40L-deficient B-cells</td>
<td>198</td>
</tr>
<tr>
<td>129/Sv, BALB/c</td>
<td>Used as recipient in experimental GVHD</td>
<td>Reduced GVHD mortality</td>
<td>217</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Experimental inflammatory bowel disease</td>
<td>No disease symptoms in OX40L/Rag2 double-deficient mice transferred with CD4–CD25+ T-cells</td>
<td>215</td>
</tr>
</tbody>
</table>

CHS, contact hypersensitivity; NOD, non-obese diabetic; Rag2, recombinase-activating gene 2.
CD4+ T-cells from OX40 knockout mice did not sustain a proliferative response leading to severe impairment of development of memory cells because of ineffective activation of the signaling pathway inducing antiapoptotic proteins. Similarly, transgenic expression of OX40L on DCs led to an increased number of CD4+ T-cells while blocking the OX40L dramatically reduced the number of T-cells. Nevertheless, a constitutive expression tends to have devastating consequences in mice, with autoimmunity or profound immunosuppression as result. Knowledge of the role of OX40L/OX40 signaling in generation and survival of CD8+ T-cells is poor and results obtained so far are apparently contradictory. The same can be said with regard to development of humoral immune responses. Some studies with OX40 or OX40L gene-targeted mice have demonstrated quantitative impairments in T-dependent antibody responses, while other reports have shown no defects.

1.4 GENETIC SUSCEPTIBILITY TO CORONARY ARTERY DISEASE

CAD and atherosclerosis, the latter a key factor in the development of CAD, have long been known to “run in families”. A small proportion of cases can be attributed to rare, highly penetrant, monogenic effects, but most have a complex etiology, involving a genetic component and important interactions with the environment.

1.4.1 Genetic component

Early lesions can develop from fatty streaks into atherosclerotic plaques, which in turn can remain silent, progressively narrowing the lumen and causing restriction of the flow and ultimately angina, or can suddenly occlude vessels through acute thrombosis, which leads to MI, the main clinical manifestation of CAD. Therefore, the clinical entity of CAD shows a large degree of variability among patients.

The measure of the genetic component for a trait is called heritability and is defined as the percentage of the total variance of the trait that is explained by inheritance. It can be estimated by examining the increased similarity of trait values in related individuals as compared with unrelated individuals. For example, if a trait has a genetic component, monozygotic twins, sharing 100%
of their genome, are likely to resemble each other to a greater extent than are dizygotic twins, who share on average 50% of their genome. Heritability also depends on the age of the individuals, the ethnic groups, and the environment. Another measure of the importance of genetics is the increased risk to a relative of an affected individual as compared with the general population. For example, it has been reported that the relative risk of CAD is 5-fold to 7-fold increased if having a first-degree relative with premature CAD. Animal models of atherosclerosis also provide dramatic evidence for the importance of genetic contributions. For example, inbred strains of mice, each with a unique genetic background, differ widely in the development of atherosclerosis.

The importance of environment, on the other hand, has been clearly demonstrated in population migration studies. For example, Japanese individuals have a much lower incidence of CAD than do American individuals, but Japanese Americans who have adopted a Western lifestyle have about the same incidence as do other Americans. Another example of the effect of environment is the Pima Indians of Arizona, who before adapting a Western lifestyle had a modest incidence of obesity and diabetes, which is now increasing dramatically. From these and other studies, it appears that the difference in CAD incidence between populations is due primarily to environmental factors, albeit populations do differ in genetic susceptibility.

### 1.4.2 Susceptibility genes

Because of the complexity of CAD, applying the positional cloning approaches that have transformed the understanding of Mendelian disorders has proven to be difficult and disappointing, despite the fact that molecular genetic studies of rare Mendelian diseases have identified a few mutations that cause premature CAD, such as low density lipoprotein receptor (LDLR) for familial hypercholesterolemia, ABCG5/8 for sitosterolemia, and ABCA1 for Tangier disease. However, these studies explain only a fraction of the individual inherited risk for common, multifactorial CAD, and there is still a large gap in our knowledge of the genes that are determining susceptibility for most patients with multifactorial CAD.

Currently, there is no convincing animal model of CAD in a genetically tractable species. Genetically manipulated mouse models and inbred strains are informative for certain aspects of atherosclerosis, the underlying cause of CAD, and for lipid intermediate phenotypes, but not for important aspects of human MI. Therefore, acquisition of new knowledge in this area is likely to depend to a large extent on human genetics.
Recent advances in genetic approaches to understanding CAD involve genome-wide linkage mapping and large-scale gene-association studies as core human genetics strategies. These have been complemented by comparative genomics studies and mapping of quantitative traits in the mouse. The genes identified through these combined approaches have diverse functions: they include transcription factors that are implicated in vasculogenesis (MEF2A)\textsuperscript{227}, signaling molecules that are involved in inflammation (LT-A)\textsuperscript{228}, innate (TLRA)\textsuperscript{229} and adaptive (MHC2TA)\textsuperscript{109} immunity, and novel apolipoproteins (APOA5)\textsuperscript{230}, as well as genes for which the physiological roles are as yet unclear. It is noteworthy that only a few of these genes are obvious candidates for CAD, emphasizing the power of genetics to generate new insights into the molecular basis of complex diseases.

1.5 GENETICS OF COMPLEX DISEASES

Genetic characters for which the genotype at one locus is both necessary and sufficient for the character to be expressed, given the normal genetic and environmental background of the organism, are called Mendelian, that is characters which obey Mendel's laws in regard to their hereditary transmission. The term “complex genetic disease” refers to any phenotype that does not exhibit classic Mendelian inheritance.

The gene mutations studied by Mendel, and those more recently discovered by positional cloning, are those with large effects and strong genotype-phenotype correlations. They are in fact the “low-hanging fruits” that are relatively easy to harvest. Now, however, we are left with the great majority of fruits at the top of the tree with no obvious way to reach them. In genetics terms, these are the numerous genes of smaller effect that are likely to underlie most common, familial traits and diseases in humans. Thus, now that most Mendelian diseases have been mapped and most genes at least partially cloned, the new challenge in human genetics is to disentangle the genetic determinants of non-Mendelian diseases, which represent the majority of causes of morbidity and mortality and a

Figure 5. Most characters are the result of the interaction of minor and major genetic determinants with the environment (Adapted from Strachan & Read 1999).
considerable burden for healthcare in the developed world. In fact, most of common disorders such as diabetes mellitus, hypertension, CVDs, autoimmune diseases, cancer and lipid abnormalities are regarded as complex genetics diseases. The underlying background of complex diseases, also called multifactorial diseases, is most likely a combination of several factors, where susceptibility genes interact with each other and with the environment (Figure 5). The genes involved in complex genetic traits are often referred to as susceptibility genes to distinguish them from the causative genes behind the Mendelian monogenic disorders.

1.5.1 Factors contributing to complexity

To date, most of the identified gene variations underlie human Mendelian diseases. For complex genetic diseases, progress has been disappointing due to the small effect of each locus, suggesting the requirement for extremely large sample sizes. While in Mendelian disorders, where disease generally affects families, it is not uncommon to find large pedigrees with many affected members, in complex disorders large affected families are very rarely found. Even if such a situation is encountered, it is not always easy to determine whether the observed phenotype is caused by the complex disease in question or by a rare Mendelian trait. In addition, there are many factors that further contribute to the complexity of complex genetic traits. Penetrance is one of them and is defined as the probability that an individual who has the genotype will manifest the character231. A situation where the correlation between genotype and phenotype is not straightforward, so that carrying a susceptibility allele does not entirely predict the disease status, is described as reduced or non-penetrance depending on the severity of the effect. Conversely, there are also individuals who manifest the disease without carrying the susceptibility allele, a phenomenon called phenocopies. Two other important factors are locus and allelic heterogeneity. In locus heterogeneity, a disease is caused by variation at more than one locus. Allelic heterogeneity, on the other hand, means that there are several alleles at the same locus that are causing the disease. Both forms of heterogeneity can hamper genetic analysis.

However, it is important to note that even if the identified gene is not responsible for the disease in the majority of patients, it can still provide important information about the underlying disease mechanisms.
1.5.2 Genetic recombination and distance

Genetic mapping is the process of locating the positions of specific genes on a chromosome, or in other words, of defining how often two loci are separated by meiotic recombination. Thus, the proportion of offspring who are recombinant, called recombination fraction, is used as a measure of genetic distance. Loci that are on different chromosomes or on the same chromosome but far apart, will segregate independently with a recombination fraction $\theta = 0.5$. On the other hand, if the loci are syntenic and lie closely, the recombination fraction will be as smaller as closer are the loci, with no recombination ($\theta = 0$) when they are too close for a recombination event to separate them, because only a crossover located precisely in the small space between the two loci will create recombinants. Therefore, sets of alleles on the same small fragment tend to be transmitted together as a block. This block, a set of different alleles, is known as haplotype and can be tracked for mapping purposes as alleles at a single polymorphic locus. Genetic distance, which is different from physical distance, is measured in centimorgan (cM) on a genetic map. The genetic distance, being expression of recombination, is also affected by a phenomenon called interference, by which the presence of a point of crossover (chiasma) inhibits the formation of a second chiasma nearby. Another consequence of genetic distance being expression of number of meiosis is that genetic distances differ widely within chromosomal regions and between sexes, for example telomere regions recombine more frequently and have therefore sparser maps compared to the Y chromosome where, outside the pseudoautosomal region, there is no recombination and thus no genetic map.\(^{232}\)

1.5.3 Genetic markers

Since double heterozygotes are required for defining recombination and since people heterozygous for two different diseases are extremely rare, genetic mapping of human diseases depends on markers. Genetic markers reflect natural sequence variations in the genome and any Mendelian character can in principle be used as a genetic marker. Disease-marker mapping requires framework maps of markers spaced at an interval no greater than 20 cM across the genome. Markers should preferably be highly polymorphic to increase the likelihood of informative meioses. The two main markers used for genetic analysis are microsatellites and single nucleotide polymorphisms (SNPs).
1.5.3.1 Microsatellites

Microsatellites are short (2-6 base pairs (bp)), tandemly repeated DNA sequences that are widely spread in the genome. They are highly polymorphic and have been wisely used for genetic analysis, especially in genome-wide screens. They are more frequent in the genome than SNPs.

1.5.3.2 Single nucleotide polymorphisms

Polymorphisms are variations in the genome sequence. The most abundant and commonly used polymorphisms are the SNPs. SNPs are single base pair positions in the genomic DNA at which different alleles exist in normal individuals. The effect of a SNP is dependent on its location in the genome, e.g. whether located in coding sequences, non-synonymous/synonymous etc. SNPs can cause disease but are most commonly part of the normal human variation. They are more common than any other polymorphisms and occur at a frequency of approximately 1 in 1250 bp throughout the genome\(^2\). The bi-allelic nature of SNPs has made them amenable to high-throughput genotyping. There are currently (dbSNP Build 126, May 4 2006) almost 12 millions (5.6 millions validated) human SNPs identified and publicly available at NCBI (http://www.ncbi.nlm.nih.gov/SNP/snp_summary.cgi).

1.5.4 Mapping of complex diseases

Mapping is usually performed by two types of genetic analysis, which assess if the deviation from the normal recombination fraction between two loci, the phenotype (disease) locus and a marker, is significant.

1.5.4.1 Linkage analysis

In multigenerational pedigrees with many affected family-members, classical linkage analysis (parametric, model-dependent) is commonly used to find the gene responsible for the disease in this specific family. Linkage is the tendency for two loci on the same chromosome to be inherited together more often than would occur by chance alone. Linkage analysis can be very powerful, provided that one can specify the correct mode of inheritance (model) in terms of parameters such as penetrance and disease allele frequency. As mentioned above, large multigenerational families with a complex trait are rare; therefore the focus has instead turned towards smaller nuclear families, especially affected sib-pairs. In this case, no mode of inheritance can be determined and
classical linkage analysis is not fully adequate. Instead, nonparametric (model-free) methods have been developed. Nonparametric sib-pair methods are based on the notion that if there is linkage, affected individuals will be more similar in those parts of the genome close to a disease susceptibility gene than would be expected by chance. The sib-pair methods are often referred to as allele-sharing methods. The most common allele-sharing method is the “affected sib-pair test”, which compares the observed number of affected sib-pairs sharing zero, one or two alleles identical by descent (IBD) with that expected under no linkage (¼:½:½). IBD-status can however often not be determined unequivocally, for instance when parent genotypes are missing. Therefore, the most commonly used programs today are based on a likelihood-ratio method, which maximizes the likelihood of the data with respect to the probabilities of pairs sharing 0, 1 and 2 alleles IBD.

1.5.4.2 Association analysis

Association studies test whether a particular allele occurs at higher frequency among affected than unaffected individuals. Basically, there are two main types of association studies. The first is the case:control study, in which a comparison is performed between the allele frequencies in a set of unrelated affected individuals to that in a set of unrelated controls.

The other type of association study is the family-based approach, most commonly the transmission disequilibrium test (TDT) in which trios (ideally an affected individual and both parents) are studied. TDT investigates whether the frequency of alleles transmitted from heterozygous parents to affected offspring is significantly different to the frequency of the non-transmitted alleles. In general, family-based methods are less powerful than case:control studies, but because of the intra-familial comparisons, they are less susceptible to population stratification, which potentially can be a problem in case:control studies.

Association analysis can be performed as a direct test of association, i.e. if the polymorphism(s) in question may have the functional consequences responsible for the observed phenotype. The alternative is to perform an indirect test, which means that the marker tested is in very close proximity to the variant responsible for the functional outcome. Indirect testing relies on so called linkage disequilibrium (LD).
1.5.4.3 Linkage disequilibrium and haplotypes.

Linkage disequilibrium is a non-random association at the population level of alleles at adjacent loci, i.e. two specific alleles at two closely located loci are found together on the same chromosome more often than would expected by chance. The extent of LD is dependent on several factors both on the molecular level such as recombination and mutation rate as well as demographic and evolutionary factors such as migration, population growth and admixture between populations. LD is expected to be higher in populations derived from relatively few founders, such as Sardinians, French Canadians and populations in some parts of Finland.

There are two main ways of measuring the level of LD: the absolute value of $D'$ ($|D'|$) and $r^2$, in both cases a value equal to 1 is called perfect LD. One main drawback of $|D'|$ is that values can be highly inflated, in the case of small sample sizes. It is also sensitive to allele frequencies and can be inflated for SNPs with rare alleles. In addition, intermediate values can be rather difficult to interpret. On the other hand, the term $r^2$ has the advantage that it does not show the same inflation for small sample sizes and intermediate values are more easy to interpret than for $|D'|$, but it is still very sensitive to allele frequencies.

The degree of LD between two alleles is dependent on how old the two polymorphisms are, i.e. when they appeared in the population, and on the degree of recombination between them. Markers that are located close to each other generally have higher LD that those located far apart. It has, however, recently been shown that there is a high degree of variation in the extent of LD. The already mentioned haplotype blocks are regions with blocks of markers with high LD. These blocks are broken by areas with high recombination rates, known as recombination hot spots. As a result of LD, alleles located close to each other tend to be inherited together in a haplotype; the length and number of involved alleles of the haplotypes varies between regions and is decreased by each generation, due to the events of meiosis.

During the last few years there has been an increased interest in the use of haplotypes for analysis of complex genetic traits because it has been shown that common haplotypes can capture most of the genetic variation in a region. Haplotypes can be deduced in family-based analysis by studying the inheritance patterns from parents to offspring. In case:control studies however, where the phase is unknown, the haplotype distribution has to be inferred by statistical methods. The genome-wide extent of LD and the haplotype distribution in the genome has become the focus of intense studies in the hope that it might facilitate whole-genome association studies in complex human diseases. The success of this idea is highly dependent on a comprehensive...
knowledge of the patterns of LD throughout the genome. To facilitate this, The International HapMap Project (http://www.hapmap.org) was founded in 2002. Through the HapMap project over 1 million of common SNPs have been genotyped in 269 individuals from four different populations (European ancestry, Yoruban (Nigeria), Japanese and Han Chinese), followed by additional genotyping in selected regions.

1.5.4.4 Mapping strategies

In principle, linkage and association are totally distinct phenomena. Association is simply a statistical statement about the co-occurrence of alleles or phenotypes and can have many possible causes, not all genetic. Linkage, on the other hand, is a specific genetic relationship between loci (not alleles or phenotypes). It produces by itself an association within families but not in the general population. However, if two supposedly unrelated persons with a disease have actually inherited it from a distant common ancestor, they may well also tend to share particular ancestral alleles at loci closely linked to the disease locus. Where the family and the population merge, linkage and association merge.

In many ways linkage and association provide complementary data. Linkage analysis, whether parametric or nonparametric, operates over a long chromosomal range. Association tests have the opposite characteristics. LD rarely extends over more than a megabase, so a genome screen by association analysis would involve a huge number of tests; on the other hand, a positive result would localize the susceptibility factor rather accurately. A natural study design is therefore to start with a genome-wide screen by linkage, probably in affected sib-pairs, and then, once an initial localization has been achieved, to narrow the candidate region by LD mapping.

1.5.5 Identification of candidate genes in complex diseases

From a methodological point of view, genetic studies can be divided into those that require knowledge of the chromosomal location of the disease locus (positional cloning) and those that do not depend on this knowledge (candidate gene approach). In reality, when trying to identify a disease gene, several parallel approaches are used, depending on the findings and the new possibilities arising from technical advances. Most genes are identified by defining a candidate gene on the basis of both its chromosomal location and its properties (positional candidate approach).
1.5.5.1 Positional cloning

Positional cloning has been successfully used to locate disease-causing genes for monogenic/Mendelian disorders. Although the situation is more complicated in complex genetic diseases, performing a genome-wide screen is usually the first step towards gene identification here as well. Genome-wide screening has the advantage that it is performed in a hypothesis-independent manner and therefore does not require any prior knowledge of the underlying disease mechanisms. Genome-wide screens or scans are performed by typing a set of microsatellite markers evenly spread across the genome. Initially, an accepted average density of markers is of one microsatellite every 10cM, for a total of 300-400 markers. Wherever a region of interest is found, it is usually saturated with a denser set of markers in an extended number of families. In the case of pedigrees with a seemingly Mendelian mode of inheritance, parametric methods are more powerful and should be used for analysis. However, if there is no clear mode of inheritance, nonparametric methods are more commonly used. To further increase the information from the data and to facilitate the localization of the highest peak of recombination, data for more than two loci can be analyzed simultaneously, shifting from two-point to multi-point analysis.

1.5.5.2 Candidate gene approach

Instead of performing an unbiased, non-hypothesis-driven screen of the whole genome in search for susceptibility genes, the candidate gene approach focuses on genes chosen on the basis of existing knowledge of their properties or mechanisms and their expected involvement in the disease process or pathways. This method works much better when applied to predefined candidate, subchromosomal regions, rather than the whole genome.

1.5.5.3 Positional candidate gene approach

A purely position-independent approach will rarely succeed because molecular pathology is too complicated. Predictions of the biochemical function of an unknown disease gene are often proved wrong, once the gene is isolated. On the other hand, a purely positional approach is inefficient because candidate regions identified by positional cloning usually contain dozens of genes. It can be very time-consuming to identify every transcript from the region and excessively laborious to screen them all for mutations. This is why, in reality, most disease genes have been and are currently identified by a combination of the two strategies described above. With this approach, the chromosomal
location of a candidate region is defined as tightly as possible, then genes in this region are prioritized based on e.g. their pattern of expression, likely function, homology with other relevant human genes or their implication in model organisms.

### 1.5.6 Quantitative trait loci

As already discussed, much of the genetic variation that underlies disease susceptibility and morphology is complex and is governed by loci that have quantitative effects on the phenotype. These, called quantitative trait loci (QTLs), are responsible for most of the genetic diversity in human disease susceptibility and severity, but gene-gene and gene-environment interactions make these loci difficult to analyse. However, with the development of new genetic techniques and with more information about the mammalian genome, QTLs are becoming more commonly used to identify the molecular players that underlie complex phenotypes.

A quantitative trait is defined as one that has measurable phenotypic variation owing to genetic and/or environmental influences. This variation can consist of discrete values or can be continuous. Sometimes a threshold must be crossed for the quantitative trait to be expressed; this is common among complex diseases.

A QTL is a genetic locus, the alleles of which affect this variation. Generally, quantitative traits are multifactorial and are influenced by several polymorphic genes and environmental conditions, so one or many QTLs can influence a trait or a phenotype. Sometimes a cluster of closely linked polymorphic genes is responsible for the quantitative variation of a trait. These are difficult to separate by recombination events and therefore might be detected as one QTL. These traits can also be influenced by loci that have large discrete effects, often called Mendelian loci; the same locus might be considered to be both a QTL and a Mendelian locus depending on the alleles that are examined: some alleles might cause quantitative effects whereas others might cause all-or-none effects. The distinction between Mendelian loci and QTLs is artificial, and the classification of genetic (and allelic) effects should be considered as a continuum. At one end of the spectrum is the dichotomous Mendelian trait with only two detectable and distinct phenotypes, which are governed by a single gene. At the other end are traits that are likely to be affected by many genes, each of which contributes a small portion of the overall phenotype. Between these two extremes are traits that are regulated by more than one genetic locus, and possibly also by environmental factors, which show several
intermediate phenotypes. Generally, the more loci that are involved in determining a quantitative trait, the more difficult it is to map and identify all of the causative QTLs and the different effect sizes of each individual QTL. Finally, interactions between QTLs have also to be taken in account.

1.5.6.1 Concordance of human and mouse atherosclerosis QTLs

In recent years, QTL mapping in mammals (humans, mice, rats and pigs) has identified hundreds of chromosomal regions containing genes affecting complex phenotypes like asthma, atherosclerosis, diabetes, hypertension and obesity and their number is increasing. For example, 27 and 21 individual atherosclerosis QTLs have been reported so far in humans and mice, respectively\(^{245}\). The ultimate goal of QTL mapping is to identify the genes underlying these polygenic traits and to gain a better understanding of their physiology and biochemistry. However, only half of the human atherosclerosis QTLs has an obvious candidate gene\(^{245}\).

Recent data suggest that variations underlying certain complex pathologic changes are in large part shared between mouse and humans, so QTLs for the same trait but from different species map to syntenic chromosomal regions. In particular for atherosclerosis, 62% of the 21 mouse QTLs are concordant with human QTLs, and 63% of the 27 human QTLs are concordant with mouse QTLs, suggesting that more than half of the mouse and human atherosclerosis QTLs share the same underlying genes\(^{245}\) (Figure 6). Thus, identifying them in one species should greatly facilitate identifying them in the other.

1.5.6.2 QTL mapping in animal models

One promising approach for dissection of complex traits involves the use of animal models to detect QTLs. Advantages include an order of magnitude greater power to detect linkage, and good mouse and rat animal models for traits relevant to CAD have been identified\(^{223}\).

The overall strategy for this approach involves the use of recombinant inbred (RI) strains. Each RI strain is in fact inbred and has been developed from intercrossing two parental strains for 20 generations, during which time the genomes of the progenitors are broken into homozygous intervals of different length. In recent years, investigators have initiated QTL studies with crosses between inbred strains that differ in their susceptibility to atherosclerosis. QTL studies begin with construction of a cross between two selected inbred mouse strains, typically differing in expression of the primary trait of interest. The offspring of the initial mating (F\(_1\) generation) are either crossed back to one of
Figure 6. Chromosome map of (a) mouse and (b) human atherosclerosis QTLs. Each bar to the right of a chromosome represents a QTL and candidate genes are listed to the left of the chromosomes. Stars indicate QTLs that overlap in mouse and human homologous regions (Adapted from Wang 2005).
the parental strains (backcross) or are themselves mated (intercross) to create an F₁ generation. Although the F₁ mice are genetically identical, having one chromosome from each parent, the backcross, or F₂, mice are each genetically unique. This is caused by independent segregation of chromosomes and crossovers that occur in the F₁ meiotic process, which leads to combinations of genes from the original parental strains. Because these recombined regions are inherited as relatively large segments of chromosomes, the parental origins of each portion of the genome can be determined by using evenly distributed polymorphic markers that distinguish the parental strains. By analyzing whether the measured trait (e.g. atherosclerosis) varies significantly across the population on the basis of the parental genotype at any given location in the genome, QTL analysis can identify chromosomal loci that segregate with the trait of interest. Data are analyzed with specific software and presented as a graph with a curve representing the statistical likelihood, represented by a log-of-the odds (LOD) score, of a genetic effect across the length of the chromosome.

Atherosclerosis QTLs in mice have been identified in two models: (1) a high-fat-diet model, in which a diet containing 15% fat, 1.25% cholesterol, and 0.5% cholic acid is used to induce atherosclerosis, and (2) the sensitized model, in which mice deficient in \textit{Apoe} or \textit{Ldlr} are used\textsuperscript{246, 247}. \textit{Apoe}-deficient mice develop atherosclerosis spontaneously when fed a chow diet, their lesions developing faster when they are fed a Western diet (containing 21% fat and 0.15% cholesterol) and more so when they are fed the high-fat diet described above. \textit{Ldlr}-deficient mice also develop atherosclerotic lesions when fed the chow diet, but the lesions are very small and take considerable time to develop; feeding these mice a Western diet or the high-fat diet described above accelerates their lesion formation. Human-concordant QTLs are found for 71% of the mouse atherosclerosis QTLs in the high-fat model, 67% of the ones in a sensitized model, and 40% of QTLs found in both models\textsuperscript{245}. Altogether, more than half of the QTLs found in each model have concordant human QTLs, so it cannot be said that one model is more relevant to human atherosclerotic disease than the other.
2 AIMS OF THE THESIS

The general objective of this thesis was to shed new light onto genetic susceptibility to atherosclerosis and CAD.

The specific objectives were to examine individuals who had suffered precocious MI in order to:

- Identify new genes predisposing to development of atherosclerosis and related clinical complications.
- Characterize the OX40L/OX40 system and investigate the role of their interaction in relation to atherosclerosis and CAD.
- Identify and evaluate functional variants responsible for genetic susceptibility to atherosclerosis and CAD.
3 MATERIALS AND METHODS

3.1 MATERIALS AND SUBJECTS

In this section, an overview of the methods is presented. Extensive explanations and details are reported in the enclosed original articles.

3.1.1 Mice and diet

B6, C3H and C3H/HeJ Tnfsf6 transgenic mice were obtained from the Jackson Laboratory (Bar Harbour, Maine, USA). Tnfsf6-deficient and transgenic mice were generated as described. Genetic positions of all the markers and genes were retrieved from Mouse Genome Informatics. Mice were initially fed a chow diet and then an atherogenic high-fat diet when they were 8-10 weeks old, to induce atherosclerosis. All animal protocols were approved by the Jackson Laboratory Animal Care and Use Committee.

3.1.2 Human subjects: the SCARF study (papers I-III)

The Stockholm Coronary Atherosclerosis Risk Factor (SCARF) project is a case:control study collection designed for investigation of novel biochemical and molecular genetic risk markers for atherosclerosis, CAD and precocious MI consisting of 387 individuals with MI before the age of 60 and 387 sex- and age-matched controls recruited in the Stockholm area, Sweden. Recruitment procedures, inclusion and exclusion criteria, investigation program, and basic clinical characteristics have been described in detail. Ethnicity was recorded on the basis of self-reported origin as far as three generations back, and more than 99% of participants in the study were considered Caucasians. Three months after the index cardiac event in an individual, both that individual and a control subject underwent a structured interview and blood sampling, and the affected individuals in two of the three hospitals were offered routine coronary angiography. A total of 252 affected individuals (66%) went through routine invasive evaluation with quantitative coronary angiography.

3.1.3 Human subjects: the SHEEP study (paper I)

The Stockholm Heart Epidemiology Program (SHEEP) project is a large population-based case:control study aiming to investigate genetic, biochemical and environmental factors predisposing to MI. The subjects, aged 45-70, were
all Swedish citizens and consisted of 2246 MI cases and 3206 unaffected controls. The work presented in this thesis is restricted to 1213 cases (852 men and 361 women) and 1561 matched controls (1054 men and 507 women). The criteria for MI diagnosis were based on guidelines issued by the Swedish Society of Cardiology in 1991 and included: (1) typical symptoms; (2) marked elevation of the enzymes serum creatine kinase and lactate dehydrogenase and (3) characteristic electrocardiogram changes. The case was diagnosed with MI in the presence of two fulfilled criteria.

3.1.4 Human subjects: the PROCARDIS study (paper IV)

The Precocious Coronary Artery Disease (PROCARDIS) study is a European collaborative project involving the United Kingdom, Italy, Sweden and Germany, using genetic techniques to map susceptibility genes for CAD. Families were recruited to implement two study designs based on linkage and TDT analyses. Ascertainment criteria for probands were MI or symptomatic acute coronary syndrome according to modified WHO diagnostic criteria, occurring before the age of 66 years. For the TDT study design, probands and both parents whenever available or one parent and at least one sibling (affected or unaffected), were recruited, along with any further affected siblings.

All subjects gave informed consent to their participation in the studies, the protocols of which had been approved by the ethics committees of the Karolinska University Hospital in Sweden and the different reference institutions in their respective countries.

3.2 CLINICAL ASSESSMENTS OF ATHEROSCLEROSIS

Diet-induced atherosclerotic lesion size at aortic roots was measured in mice fed an atherogenic diet for 10-13 weeks as described. Briefly, hearts and ascending aortas were removed, fixed in 4% formaldehyde and stained with Oil red O. Five sections of the aortic root of each mouse, separated by 80 μm each, were measured. The number of lesions in each section was counted and size was evaluated with the aid of a grid on the microscope eyepiece. The value for each mouse was independently determined by three different individuals and averaged.

Coronary angiographies were carried out either during the initial admission or three months after the onset of MI. Angiograms were analyzed in 15 coronary segments, according to American Heart Association guidelines, by a computerized method developed in Leiden, the Netherlands (Medis QCA-
CMS system) for quantitative coronary angiography. Reference diameter, minimal lumen diameter, percent diameter stenosis, mean segment diameter, segment length, plaque area, segment area and number of significant (>50%) stenoses were measured in each segment. (paper I).

3.3 BIOCHEMICAL ANALYSES

Blood was collected from 4h-fasting mice. Plasma was separated by centrifugation and plasma total cholesterol, HDL cholesterol and triglyceride levels were measured in a chemical analyzer. Very-low and low-density lipoprotein cholesterol levels were calculated by subtracting HDL cholesterol levels from total cholesterol levels. Human plasma concentrations of cholesterol and triglycerides in different lipoprotein fractions were determined by enzymatic methods (SHEEP, paper I) or a combination of preparative ultracentrifugation, precipitation of apoB-containing lipoproteins and lipid analyses (SCARF, paper I-III)\textsuperscript{256}. Serum CRP and serum amyloid A (SAA) were determined by using particle-enhanced immunonephelometric assay (Dade Behring, Liederbach, Germany) (paper III).

3.4 GENETIC ANALYSES

3.4.1 DNA extraction

Genomic DNA of B6 and C3H strains was obtained from DNA resources at the Jackson Laboratory. Genomic DNA of *Prx6-* and *Tnfsf4*-deficient mice was isolated from mouse tail tips by proteinase digestion, phenol:chloroform extraction and ethanol precipitation. Human nucleated cells from frozen whole blood were prepared according to Sambrook et al.\textsuperscript{257} and DNA was isolated by using the Qiagen Blood and Cell Culture DNA kit (Qiagen Nordic AB, Solna, Sweden).

3.4.2 Sequencing

To sequence the mouse candidate genes for *Ath1*, each exon was amplified by polymerase chain reaction (PCR) and the products were sequenced using Big Dye Terminator Cycle Sequencing Chemistry and the ABI 3700 Sequence Detection System (Applied Biosystems).
Parts of the human *OX40L* (also known as *TNFSF4*) gene were sequenced in the genomic DNA from whole blood of 20 healthy subjects (papers I and III). The human *OX40* gene (also known as *TNFRSF4*) and parts of its flanking regions containing the *TNFRSF18* and *CAB45* genes were sequenced in 20 healthy subjects and also in 20 patients (paper II). Sequencing was performed by PCR-amplification and the PCR products were then used as templates for further amplifications as part of the Taq DyeDeoxy terminator cycle-sequencing system (Perkin-Elmer Corp.). When needed, nested primers were used for analysis of overlapping sections in both directions.

### 3.4.3 Genotyping

Polymorphisms of the genes in the *Ath1* human homologous region were retrieved from the SNP database (build 116) of the National Center for Biotechnology Information. DNA for polymorphisms in the *Ath1* region and rs2298212 in *OX40* were amplified using primer pairs with one 5' end biotinylated, captured on streptavidin-coated magnetic beads (Dynabeads, Dynal A.S., Oslo, Norway), treated and analyzed by Pyrosequencing™ technique (Biotage AB, Uppsala, Sweden) as previously described.

Genotyping for SNP rs17568 in *OX40* (paper II), and for SNPs rs10489266, rs10912564, rs10912558 (paper III), −921C>T (papers III and IV) and rs3850641 (paper IV) in *OX40L* was performed on genomic DNA by using TaqMan® Assay on Demand or on Design (Applied Biosystems) according to the manufacturer’s protocol. Post-PCR allelic discrimination was carried out measuring allele-specific fluorescence on the ABI Prism 7000 Sequence Detection System (Applied Biosystems).

PCR genotyping of *Prdx6*-deficient mice and *Tnfsf4*-deficient mice was performed as detailed. Genotyping of *Apoe in Apoe*-deficient mouse was performed according to the protocol provided by the supplier.

### 3.4.4 HaploChIP

The chromatin immunoprecipitation (ChIP) assay was performed as described with some modifications. Preparation of chromatin and immunoprecipitation were carried out using the ChIP-IT™ kit (Active Motif, Carlsbad, CA) according to manufacturer’s instructions with some modifications. For chromatin immunoprecipitation, specific antibodies against phosphorylated serine residues of the CTD of RNA polymerase II (Pol II) were used, with antibodies against total Pol II as a positive control. Allele-
specific loading of Pol II was evaluated by pyrosequencing as described\textsuperscript{261}. The amplification products were captured on streptavidin coated sepharose beads (Amersham Biosciences AB, Uppsal, Sweden) and analyzed as described\textsuperscript{258}. Quantification of the two alleles was performed by measuring the area under the peak corresponding to each allele.

### 3.4.5 EMSA

In electrophoretic mobility shift assay (EMSA) studies, the labeled probes were incubated with nuclear extracts from human U937 and THP-1 cells as described\textsuperscript{262, 263}. Non-radioactive competitor DNAs, either identical, of the opposite allelic variant or of non-specific origin, were added to the labeled DNA. DNA-protein complexes were separated on polyacrylamide gel electrophoresis and gels were read by autoradiography using a phosphorimager (Fuji Imager Analyzer BAS-2500, Image Reader version 1.4E, Image Gauge version 3.45 software; Fuji, Stockholm, Sweden).

### 3.5 QUANTITATIVE REAL-TIME PCR

Total human RNA was prepared with RNeasy kit (Qiagen Nordic AB, Solna, Sweden) and reverse-transcribed into cDNA. Real-time PCR was performed by using TaqMan® Assay on Demand (Applied Biosystems) according to the manufacturer's instructions and the results were normalized to the house keeping gene RPLP0.

Mouse tissues were preserved in RNAlater (Ambion) before extracting total RNA using RNeasy Mini Kit (Qiagen)\textsuperscript{264}. Quantitative real-time PCR was carried out in triplicate as previously described\textsuperscript{264}. The mRNA expression of each gene was normalized to the copies of \( \beta \)-actin mRNA from the same sample.

### 3.6 CELL CULTURE

Cell lines (THP-1, U937 and Epstein-Barr virus-transformed human B-cells) were maintained according to the protocol provided by the supplier. DNA extraction was performed using Genomic-tip 100/G kit (Qiagen Nordic AB, Solna, Sweden) according to the manufacturer’s protocol; yield and efficiency of extraction were measured by making quantitative spectrophotometric absorbance readings at 260 nm.
3.7 IMMUNOHISTOCHEMISTRY

Anti-mouse OX40L IgGs were generated by immunizing rabbits with a synthetic peptide and their specificity was tested by western blotting. Paraffin-embedded sections were stained with hematoxylin and eosin. Specific OX40L binding was detected using biotinylated secondary antibody and avidin–biotin–horseradish peroxidase system (Santa Cruz). Nuclei were counterstained with hematoxylin.

3.8 BIOINFORMATICS

OX40 sequence conservation between human and mouse alignments was assessed using the UCSC Genome Browser resources. Genetic variants affecting potential regulatory regions in both OX40 and OX40L were identified using RAVEN. Potential transcription factor binding sites altered by variants in OX40 and OX40L genes were identified in silico by using the Genomatix® package and evaluated by browsing the TRANSFAC public database with the web-based softwares TESS and PATCH.

3.9 STATISTICAL ANALYSES

Statistical analyses were carried out using the StatView version 5.0 (SAS, Cary, NC) or SAS version 8.0 softwares (SAS). Continuous data are expressed as means ± s.e.m. Allele frequencies were estimated by the gene counting method, and the presence of Hardy-Weinberg equilibrium was tested using the $\chi^2$ method. The $\chi^2$ test was also used to compare the distribution of genotypes between cases and controls, and for comparing categorical data between groups. Differences in continuous variables between groups were tested by analysis of variance (ANOVA) with the Scheffe F test used as a post-hoc test. To obtain a normal distribution before carrying out statistical computations and significance testing, all skewed variables were logarithmically transformed. Odds ratios with 95% confidence intervals, as estimates of the relative risk, were calculated using logistic regression. Pairwise LD coefficients for polymorphisms within the OX40L and OX40 genes were calculated with the EMLD program (papers I and II) and visualized with the Haploview program (version 3.0) using both data available from the HapMap Project (papers II and III) and our own data (paper III). Haplotype frequencies were estimated using the HAPLOTYPER (paper I) and PHASE programs (version 2.1) (papers II and III). The PedCheck program was used to check for
misinheritance in the PROCARDIS trio families. Excess transmission of the minor alleles to affected offspring (CAD and MI phenotypes) was tested using the TDT analysis\textsuperscript{237} implemented in GENEHUNTER\textsuperscript{269} and TRANSMIT\textsuperscript{237}. Gene-gene and gene-environment interactions were investigated using the multifactor dimensionality reduction (MDR) software (BETA version 0.2.1)\textsuperscript{270}. Student's \textit{t}-test was used to compare the mRNA expression levels, the size of the diet-induced atherosclerotic lesions and plasma lipid levels between the mutant mice and their respective controls. All values are expressed as mean ± s.e.
4 SUMMARY OF RESULTS AND DISCUSSION

In this section, a successive presentation of the main results from each paper is provided together with a brief discussion of the most relevant findings.

4.1 OX40 LIGAND IS A SUSCEPTIBILITY GENE FOR Atherosclerosis (Paper I)

The first study was performed to pinpoint the gene underlying Ath1, a previously identified mouse QTL for atherosclerosis, and to evaluate the role of this gene in relation to atherosclerosis and its complications in a human context.

Bioinformatic evaluation revealed that the mouse Ath1 region contains 11 known genes while its human homologous region contains 10 known genes and three putative genes. Most of the genes have known counterparts in both species (Figure 7).

![Figure 7](image_url)

Figure 7. Genes in the Ath1 region. Genes in (a) the mouse Ath1 region and (b) the human homologous chromosome segment between 1q24.3 to 1q25.1 were retrieved from the Ensembl database and Celera Discovery System. Arrows indicate established and putative (*) genes tested in human SNP association studies; each arrow represents one tested SNP. Tnr and TNR, tenascin R; Tnn and TNN, tenascin N; Mrps14 and MRPS14, mitochondrial ribosomal protein S14; Cacybp, calcyclin binding protein; Serpinc1 and SERPINC1, serine (or cysteine) proteinase inhibitor, clade C, member 1; Gas5, growth arrest specific 5; KHLX, Kelch-like protein X; Prdx6 and PRDX6, peroxiredoxin 6; Tnfsf6 and TNFSF6, tumor necrosis factor ligand superfamily member 6 (Fas ligand); C1orf9, chromosome 1 open reading frame 9; Pigc and PIGC, phosphatidylinositol glycan, class C; Dnm3 and DMN3, dynamin 3.
Among the 11 known genes in the \textit{Ath1} region, three (Peroxiredoxin 6 (\textit{Prdx6}), Fas ligand (\textit{Tnfsf6}, also called \textit{Fasl}) and \textit{OX40} ligand (\textit{Ox40l}, also called \textit{Tnfsf4})) have known functions suggesting that they may predispose to atherosclerosis. PRDX6, an antioxidant enzyme that protects mice against oxidative stress\textsuperscript{259}, was the first one to be tested in previous studies, but was excluded based on the fact that both overexpressing\textsuperscript{248} and knockout\textsuperscript{271} \textit{Prdx6} mice do not show differences in susceptibility to diet-induced atherosclerosis. The other two candidates are both expressed on cells that play a role in atherosclerosis, and they both control the proliferation and survival of lymphocytes\textsuperscript{272, 273}. Whereas FASL regulates the pathway to lymphocyte apoptosis\textsuperscript{272, 274}, \textit{OX40L} is involved in lymphocyte proliferation and survival\textsuperscript{273}. In this paper, the candidacy of \textit{Tnfsf6} and \textit{Ox40l} was evaluated by comparison of diet-induced atherosclerosis in mice that were deficient in either \textit{Tnfsf6} or \textit{Ox40l} with their respective controls.

### 4.1.1 \textit{OX40L} underlies the \textit{Ath1} locus in mice

The gene underlying a QTL should have either a coding sequence difference that changes the function of the protein it encodes or a regulatory sequence difference that causes an expression difference between the two parental strains of a cross in which the QTL is found. Therefore, the two candidate genes in the \textit{Ath1} region (\textit{Tnfsf6} and \textit{Ox40l}) were tested for sequence and expression differences between susceptible (B6) and resistant (C3H) mouse strains. While the coding sequences of \textit{Tnfsf6} and \textit{Ox40l} were identical in B6 and C3H mice, their mRNA expression in hearts and aortas varied between strains. In particular, expression of aortic \textit{Tnfsf6} mRNA was significantly higher in the resistant strain whereas expressions of aortic and heart \textit{Ox40l} mRNA were higher in the susceptible strain. Sequencing of regulatory regions of \textit{Ox40l} from B6 and C3H mice revealed nucleotide differences in either of the two strains that could change promoter activities and mRNA expression levels. All these findings were consistent with proatherogenicity of \textit{Ox40l}.

Since recent reports suggest that FASL is antiatherosclerotic\textsuperscript{275, 276}, if an allelic variant of \textit{Tnfsf6} makes C3H mice resistant to atherosclerosis, \textit{Tnfsf6} deficient C3H mice should lose their resistance and become susceptible to atherosclerosis. However, C3H-\textit{Tnfsf6}\textsuperscript{gld} mice, which have naturally mutant \textit{Tnfsf6} and develop generalized lymphoproliferative disease (“gld”), were as resistant to atherosclerosis as were controls (\textbf{Figure 8a, b}), suggesting that \textit{Tnfsf6} did not underlie \textit{Ath1}. When fed either chow or high fat diets, only female \textit{Ox40l} knockouts (\textit{Ox40l}/\textminus) had significantly smaller atherosclerotic lesions and higher levels of plasma total and HDL cholesterol than controls.
On the other hand, female transgenic mice overexpressing Ox40l had significantly larger atherosclerotic lesions than did controls (Figure 8e). The resistance to atherosclerosis of Ox40l−/− B6 mice was due to the Ox40l knock out and not to residual alleles descending from the parental strain of B6 (129) and located on chromosome 1 because Prdx6−/− mice with 129 alleles in the same region were as susceptible to atherosclerosis as were controls271. Therefore, Ox40l must be the gene that caused the difference in susceptibility to diet-induced atherosclerosis between Ox40l−/− and their controls, as confirmed by Ox40l overexpressing animal models.

Interestingly, the increased plasma HDL levels present in Ox40l−/− mice might suggest that the gene underlying Ath1 controls both atherosclerosis susceptibility and HDL cholesterol levels. However, it is unlikely that mutations in Ox40l led to the increased plasma HDL cholesterol levels. Rather,
the \textit{Ath1} congenic region contains alleles from the 129 strain that might be responsible for it, suggesting that \textit{Ox40l} promotes atherogenesis independently of plasma HDL cholesterol levels.

Immunohistochemical analysis of atherosclerotic lesions from B6 \textit{Apoe}-deficient mice revealed that OX40L was expressed in cells that participate in atherosclerosis, such as ECs, macrophages, SMCs and lymphocytes inside the lesions. In contrast, little OX40L staining was detected in the extracellular areas, including the necrotic atheromatous core. Thus, OX40L can be linked to the development of atherosclerosis.

4.1.2 \textit{OX40L} contributes to the risk of developing CAD and MI in humans

Based on these results, an association study was performed to evaluate polymorphisms in human \textit{OX40L} under the hypothesis that this gene affects atherosclerosis. The characteristics of the study groups are shown in Table 2.

**Table 2. Characteristics of the study groups.**

<table>
<thead>
<tr>
<th></th>
<th>SCARF</th>
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<th>SHEEP</th>
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<tbody>
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<td></td>
<td>Patients</td>
<td>Controls</td>
<td>Patients</td>
<td>Controls</td>
</tr>
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<td>N</td>
<td>401</td>
<td>392</td>
<td>1213</td>
<td>1561</td>
</tr>
<tr>
<td>Gender (female/male)</td>
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<td>69/323</td>
<td>361/852</td>
<td>507/1054</td>
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<td>Age (years)</td>
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<td>53 ± 5</td>
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</tr>
<tr>
<td>% Smokers</td>
<td>18/57</td>
<td>23/37</td>
<td>49/26</td>
<td>29/30</td>
</tr>
<tr>
<td>% Type 2 diabetes</td>
<td>10.7</td>
<td>0*</td>
<td>12.1</td>
<td>4.6*</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>27.4 ± 0.2</td>
<td>26.5 ± 0.2*</td>
<td>26.6±0.1</td>
<td>25.5±0.1*</td>
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<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>3.23 ± 0.05</td>
<td>3.52 ± 0.05*</td>
<td>4.22±0.02</td>
<td>3.96±0.02*</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.10 ± 0.02</td>
<td>1.41 ± 0.02*</td>
<td>1.08±0.01</td>
<td>1.29±0.01*</td>
</tr>
<tr>
<td>Plasma triglycerides (mmol/l)</td>
<td>1.66 ± 0.03</td>
<td>1.21 ± 0.02*</td>
<td>1.76±0.01</td>
<td>1.32±0.01*</td>
</tr>
</tbody>
</table>

Values are Mean ± SEM on the number of subjects in group. *P<10\(^{-4}\), compared with the patients in the same study group.

In the first population (SCARF) where \textit{OX40L} was tested, the affected individuals, who had suffered precocious MI, significantly more often were previous smokers and individuals with type 2 diabetes, had higher body mass index (BMI) and plasma triglyceride concentrations, and had lower plasma concentrations of both HDL cholesterol and LDL cholesterol than did controls.

Out of the five tested SNPs evenly distributed across the \textit{OX40L} gene, the minor G allele of SNP rs3850641 was significantly more common in patients than controls. None of the tested SNPs in each of the genes around \textit{OX40L} (Figure 7b) differed in affected individuals versus controls. The association of the SNP rs3850641 with MI was tested in a second human population (SHEEP), revealing a significant difference for the minor allele between
patients (16.9% of 674) and controls (13.4% of 964) in the female group. In both populations, the genotype of the SNP rs3850641 was associated with an increased risk of MI in women but not in men, indicating a gender-specific effect. In addition, haplotypes including the rs3850641 SNP, together with another SNP in the first intron (rs1234315) and a SNP upstream of exon 1 of \textit{OX40L} (rs1234313) (110NN), were significantly more frequent in patients than in controls. In contrast, a haplotype carrying the other allele for these three SNPs (00100) was significantly more frequent in controls than in patients (\textbf{Table 3}). This result suggests that the first three SNPs, while in LD with each other, determine the distribution frequency of \textit{OX40L} haplotypes. \textit{OX40L} haplotypes were also associated with the severity of angiographically measured CAD. These findings suggest that polymorphisms in potential regulatory regions of \textit{OX40L} affected its functions as observed for the mouse homologue.

\textbf{Table 3.} Distribution of the most frequently occurring \textit{OX40L} haplotypes in SCARF patients and control subjects.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Total (%)</th>
<th>Controls (%)</th>
<th>Patients (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>00000</td>
<td>117 (7.5)</td>
<td>55 (47.0)</td>
<td>62 (53.0)</td>
<td>0.60</td>
</tr>
<tr>
<td>00001</td>
<td>30 (1.9)</td>
<td>15 (50.0)</td>
<td>15 (50.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>00100</td>
<td>453 (29.2)</td>
<td>244 (53.9)</td>
<td>209 (46.1)</td>
<td>0.02</td>
</tr>
<tr>
<td>11000</td>
<td>47 (3.0)</td>
<td>21 (44.7)</td>
<td>26 (55.3)</td>
<td>0.03</td>
</tr>
<tr>
<td>11010</td>
<td>162 (10.5)</td>
<td>71 (43.8)</td>
<td>91 (56.2)</td>
<td>0.03</td>
</tr>
<tr>
<td>110NN</td>
<td>210 (13.5)</td>
<td>92 (43.8)</td>
<td>118 (56.2)</td>
<td>0.01</td>
</tr>
<tr>
<td>10010</td>
<td>78 (5.0)</td>
<td>44 (56.4)</td>
<td>34 (43.6)</td>
<td>0.61</td>
</tr>
<tr>
<td>00010</td>
<td>243 (15.7)</td>
<td>125 (51.4)</td>
<td>118 (48.6)</td>
<td>0.52</td>
</tr>
<tr>
<td>10000</td>
<td>382 (24.6)</td>
<td>194 (50.8)</td>
<td>188 (49.2)</td>
<td>0.66</td>
</tr>
<tr>
<td>Sum*</td>
<td>1512 (97.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P values were calculated using the chi-square test for genotype distribution. N denotes any possible genotype. 0 = major allele, 1 = minor allele. *110NN haplotype not included

In addition, a human QTL for early-onset CAD was mapped to a region homologous to mouse \textit{Ath1}, and \textit{OX40L} lies under the lod score peak\textsuperscript{277}. Also, a mouse atherosclerosis QTL was found in a region where \textit{Ox40} (the gene of the receptor for OX40L) is located\textsuperscript{278}. The same region is homologous to a human region where a QTL for MI was recently mapped\textsuperscript{279}. Thus, although there is no “gold standard” for positively identifying a QTL gene, \textit{Ox40} met the criteria recently proposed by the Complex Trait Consortium\textsuperscript{280}, providing strong evidence that it is the gene underlying \textit{Ath1}, and that polymorphisms of \textit{OX40L} may contribute to the development of CAD in humans.
4.2 OX40, THE RECEPTOR OF OX40L, INFLUENCES SUSCEPTIBILITY TO MI (PAPER II)

Based on our previous findings (paper I), the obvious continuation of the investigation was to assess the candidacy of OX40, the receptor of OX40L, as a factor contributing to atherosclerosis and MI. This was the main objective of the second study.

Many reports support the notion that the OX40L/OX40 pathway has a role in mediating interactions between ECs and activated T-cells\(^{185}\). OX40 on T-cells, by interacting with OX40L on different APCs, enhances T-cell functions\(^{203, 273}\). Many receptor/ligand pairs play a role in atherogenesis\(^{144, 155, 166}\), and several studies suggest a possible role for OX40 as well. OX40 is overexpressed in inflammatory tissues\(^{203, 273}\) and \textit{in vitro} induces ECs to produce a chemoattractant for monocytes and T-cells\(^{202}\). The candidacy is further strengthened by the fact that \textit{OX40} co-localizes with an atherosclerosis QTL in mouse\(^{278}\) and with an MI locus in humans\(^{281}\). In addition, the mouse strain expressing the QTL has a genetic variation causing a 15-amino acid insertion in OX40\(^{282}\). Thus the \textit{OX40} gene, as its ligand (paper I), is a strong candidate for both the mouse and human QTLs, implying a role for the OX40L/OX40 pathway in atherosclerosis in both species.

First, in an attempt to evaluate \textit{OX40} as a candidate gene for atherosclerosis and MI in humans, \textit{OX40} and its flanking regions containing the \textit{TNFRSF18} and \textit{CAB45} genes (Figure 9) were screened for relevant polymorphisms in healthy subjects.

\[\text{Figure 9. The OX40 genomic region. OX40 and flanking genes in the human chromosome segment 1p36.3 were retrieved from the NCBI database. Vertical arrows represent SNPs tested in human subjects. Horizontal arrows represent direction of transcription. CAB45, calcium binding protein Cab45 precursor; TNFRSF18, tumor necrosis factor receptor superfamily member 18, three transcript variants have been described, here variant 1 is depicted.}\]
Five previously reported variants were detected in the OX40 gene, one of which (rs17568) was located in a coding region (Table 4), but the substitution was synonymous, with no effect on the protein product.

Table 4. SNPs detected in the OX40 genomic region in 20 healthy controls.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>RefSNP ID</th>
<th>Observed alleles</th>
<th>Position in gene (bp in NCBI)</th>
<th>Frequency of minor allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFRSF18</td>
<td>rs3753344</td>
<td>A/G</td>
<td>385 bp upstream (1182073)</td>
<td>1/40</td>
</tr>
<tr>
<td></td>
<td>rs4081335</td>
<td>C/T</td>
<td>Intron 2 (1180427)</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>rs1815606</td>
<td>G/T</td>
<td>Intron 2 (1180358)</td>
<td>12/40</td>
</tr>
<tr>
<td></td>
<td>rs946416</td>
<td>A/G</td>
<td>Intron 3 (1179613)</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>rs2298213</td>
<td>A/G</td>
<td>Exon 5 coding (1179125)</td>
<td>1/40</td>
</tr>
<tr>
<td></td>
<td>rs3819001</td>
<td>A/G</td>
<td>Exon 5 UTR (1178836)</td>
<td>Not detected</td>
</tr>
<tr>
<td>OX40</td>
<td>rs11260560</td>
<td>A/C</td>
<td>Intron 4 (1187908)</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>rs11260559</td>
<td>C/T</td>
<td>Intron 4 (1187906)</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>rs9661697</td>
<td>A/G</td>
<td>Intron 4 (1187693)</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>rs17568</td>
<td>G/A</td>
<td>Exon 5 coding (1187345)</td>
<td>9/40</td>
</tr>
<tr>
<td></td>
<td>rs2298212</td>
<td>A/G</td>
<td>Intron 5 (1187220)</td>
<td>3/40</td>
</tr>
<tr>
<td></td>
<td>rs2298211</td>
<td>A/C</td>
<td>Intron 5 (1187166)</td>
<td>3/40</td>
</tr>
<tr>
<td></td>
<td>rs2298210</td>
<td>A/G</td>
<td>3‘-UTR (1186776)</td>
<td>1/40</td>
</tr>
<tr>
<td></td>
<td>rs1055324</td>
<td>C/T</td>
<td>3‘-UTR (1186746)</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>rs1055326</td>
<td>C/T</td>
<td>3‘-UTR (1186736)</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>rs2298209</td>
<td>C/G</td>
<td>3’-UTR (1186708)</td>
<td>1/40</td>
</tr>
<tr>
<td></td>
<td>rs2298208</td>
<td>C/T</td>
<td>307 bp downstream (1186322)</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>*596c</td>
<td>C/T</td>
<td>367 bp downstream (1186261)</td>
<td>2/40</td>
</tr>
<tr>
<td>CAB45</td>
<td>rs3766186</td>
<td>G/T</td>
<td>Intron 2 (1202358)</td>
<td>10/40</td>
</tr>
<tr>
<td></td>
<td>rs3753342</td>
<td>A/G</td>
<td>Intron 2 (1200779)</td>
<td>10/40</td>
</tr>
<tr>
<td></td>
<td>rs3753343</td>
<td>A/G</td>
<td>Intron 2 (1200732)</td>
<td>10/40</td>
</tr>
</tbody>
</table>

*Genes are listed from proximal to distal chromosome 1. CAB45, calcium binding protein Cab45 precursor; TNFRSF18, tumor necrosis factor receptor superfamily member 18, transcript variant 1. NCBI SNP database build 120. *Newly described, detected by sequencing of 20 patients. Position in bp from 3’ of the translation stop codon. *Major/minor allele. *Position in NCBI SNP database, reversed order.

A partial LD was observed between rs17568 in OX40 and rs1815606 in TNFRSF18, while no other variants seemed to be located in the same block. This was in agreement with the haplotype analysis, which showed how OX40 and its two flanking genes resided in different haplotype blocks. A previously unknown C>T substitution was detected outside of the gene at position *596 (from 3’ of the translation stop codon) when sequencing an additional 20 postinfarction patients.

Polymorphisms in the OX40 gene having a minor allele frequency of at least 0.05 (rs17568 and rs2298212) were genotyped in a study of precocious MI (SCARF) in order to evaluate potential case:control differences related to genetic variation in OX40. In general, patients had more cardiovascular risk.
factors (e.g. higher prevalence of smoking and of type 2 diabetes; higher BMI and triglyceride levels; lower HDL cholesterol levels) than controls. SNP rs17568 was located in a coding region and in a section with high degree of evolutionary conservation between human and mouse (Figure 10), but was unassociated with the clinical phenotype of MI.

Figure 10. OX40 gene conservation profile between human and mouse obtained using RAVEN software. X-axis: human sequence, Y-axis: mouse sequence; white area indicates a degree of homology > 70%; arrows represent tested SNPs.

However, carriers of the major G-allele in the control group displayed a significantly lower plasma HDL cholesterol concentration (1.39 mmol/l vs 1.59 mmol/l, P<0.02). It is not clear whether OX40 can influence lipid levels, instead the HDL cholesterol concentration could be determined by a polymorphism located elsewhere and in LD with rs17568G. For example, we have shown that this SNP is partially linked to a variant located in TNFRSF18 (rs1815606, Figure 11), suggesting that HDL cholesterol levels could be related to the activity of this gene.

Figure 11. Linkage disequilibrium (LD) blocks. Exons and UTR regions are indicated. Vertical arrows represent SNPs detected in human subjects. Different LD blocks are defined by different colors.
Another OX40 SNP (rs2298212) located in intron 5 was significantly associated with MI; its minor allele seemed to be protective since it was significantly less frequent in patients than in controls (G/A allele frequencies: 0.92/0.08 vs 0.89/0.11, P = 0.035). This result was substantiated by haplotype analysis where the haplotype carrying the major allele for rs17568 and the minor allele for rs2298212 was significantly less common in patients than in controls (0.07/0.10, P = 0.023). There was no difference in genotype distribution between cases and controls for either the rs17568 SNP (genotype frequency for all subjects: 58.3% GG, 35.3% GA and 6.4% AA) or the rs2298212 SNP (81.7% GG, 17.0% GA and 1.3% AA). A second frequent SNP (rs2298211) present in intron 5 was in complete allelic association with rs2298212 and was therefore not examined in the clinical cohorts. Interestingly, the variant associated with HDL cholesterol concentrations (rs17568G) was not in allelic association with these two intronic SNPs (D' value of -0.56), making it less likely to contribute to an increased risk of MI. The degree of LD between SNPs across the OX40 gene (Figure 11) was in agreement with HapMap data in Caucasians (Project public release #14).

It might be hypothesized that any of these two polymorphisms may constitute the “true” functional variant giving rise to the association with MI presented here. However, in silico examination did not clearly support a functional role for SNPs rs2298211 and rs2298212 since no polymorphism was indicated to significantly alter a potential transcription factor binding site; their location in the 3' end of OX40, in a relatively small intron, makes the presence of regulatory elements less likely. Likewise, neither rs2298211 nor rs2298212 changed splice site junctions, yet rs2298211 appeared to alter a putative Lariat Intermediate Branch site (Py80 N Py80 Py87 Pu75 A100 Py95), changing the third position of the consensus sequence for splicing of intron 5. This event could possibly be responsible for formation of an alternative transcript that might be upregulated, leading to increased recruitment and activation of T-cells, which in turn can affect development of atherosclerosis. Thus, further investigations of rs2298211 are needed to establish whether it constitutes a truly functional variant affecting the phenotype. One could speculate that the observed association between rs2298212 and MI might be due to genes that share haplotype structures with this variant and thus influence the phenotype. In the neighbouring chromosomal regions there are, indeed, several potential candidate genes, like members of the phospholipase A2 family, apolipoprotein E receptor 2 and fatty acid-binding protein 3. However our findings indicate that OX40 lies in a separate block that is not linked to neighbouring chromosomal regions.

Since our findings suggested that genetic variation in both OX40L (paper I) and OX40 (current paper) might contribute to the development of
atherosclerosis and its clinical complications, the two OX40 polymorphisms were included in a multilocus model together with nine polymorphisms in OX40L (papers I and III). The aim was to evaluate whether an interaction between variants in the OX40L/OX40 system was implicated in predicting MI. The nonparametric analysis did not detect any interaction between SNPs in the overall group, but a difference between cases and controls was detected when males and females were analyzed separately. The MDR program selected a model including two SNPs in OX40L (rs3850641 and rs10912564) and one SNP in OX40 (rs17568), which was clearly better in predicting MI status in females (60%) than in males (46.4%). Of note, this model included the same SNP that was previously found to be associated with risk of MI in women (paper I). Since the empirical p value obtained by 1000 permutation samples was 0.178, it cannot be excluded that the findings were due to chance patterns in the data.

In summary, after having shown that genetic variation in OX40L is associated with MI, this second study suggests that also variants in other parts of the OX40 signaling pathway might influence susceptibility to MI, even though the functional relationship with development and rupture of atherosclerotic lesions has yet to be clarified. The relevance of these findings is supported by the vital functions fulfilled by OX40 in mammals, as reflected by the high level of evolutionary conservation.
4.3 FUNCTIONAL GENETIC VARIATION IN OX40L IS ASSOCIATED WITH MI IN A GENDER-SPECIFIC MANNER (PAPER III)

The aim of this study was to identify and characterize the functional variant(s) responsible for the previously observed association between OX40L and risk of MI (paper I). Since the observed association with MI involved a haplotype spanning a section of the gene located both upstream and downstream of the transcription start site, the functional polymorphism(s) could be located anywhere along the part of DNA defined by this haplotype. Previous haplotype analyses did not increase the strength of the association to MI compared with analyses of the single SNP rs3850641 (paper I). However, rs3850641, being located in an intron, can hardly be the functional variant, although interactions with a putative enhancer-binding site situated in close proximity cannot be excluded. Still, a functional variant linked to rs3850641 and located in the promoter or in other regulatory regions is a more likely explanation (Figure 12).

In order to identify functional variants and haplotypes contained in the OX40L gene, a comprehensive screening of the OX40L genomic region was performed. Polymorphisms, in addition to the ones already genotyped (paper I), were selected in silico and validated by sequencing in healthy subjects. Three previously reported variants were found in the region across the transcription start site; also, a novel C-to-T substitution was detected 921 base pairs upstream of the transcription start site (–921C>T). Sequencing of substantial parts of intron 1 confirmed the presence of five SNPs. Three of the verified and previously identified SNPs (rs10489266, rs10912564 and rs10912558), along with the newly identified promoter variant (–921C>T), were genotyped in the SCARF cohort. The remaining SNPs were excluded due to low frequency of the minor allele. Analysis of LD revealed the presence of two blocks across the gene, with a hot spot of recombination located in the large intron 1; the first block is defined by SNPs rs1234315, rs10489266, –921C>T and rs3850641 while the second one includes rs10912564, rs1234313, rs10912558, rs3861950 and rs1234312 (Figure 13).
Interestingly, as it was found for the intron 1 rs3850641 polymorphism (paper I), in the female group the minor T-allele of the –921C>T substitution was significantly more frequent in patients than controls (C/T allele frequencies: 0.89/0.11 vs 0.96/0.04, n = 262, P = 0.02). Also, the haplotype containing the minor T-allele for –921C>T and the minor G-allele for rs3850641 SNP was significantly associated with MI in the female subset of the cohort (P = 0.01) (Table 5), suggesting that the rare allele of one of these polymorphisms is responsible for the observed association. This hypothesis is further supported by the fact that the frequency of the complementary CA haplotype carrying both major alleles was increased (P = 0.02) in control subjects. No significant association was reported between haplotypes and risk of MI in the combined group (data not shown).

Table 5. Distribution of the most frequently occurring OX40L haplotypes generated by SNPs –921C>T rs3850641 in SCARF female patients and control subjects (131 individuals/262 alleles).

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Total (%)</th>
<th>Controls (%)</th>
<th>Patients (%)</th>
<th>P value</th>
<th>Females/Tot (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>00</td>
<td>117 (7.5)</td>
<td>55 (47.0)</td>
<td>62 (53.0)</td>
<td>0.60</td>
<td>218/1302 (17)</td>
</tr>
<tr>
<td>01</td>
<td>30 (1.9)</td>
<td>15 (50.0)</td>
<td>15 (50.0)</td>
<td>1.00</td>
<td>16/120 (13)</td>
</tr>
<tr>
<td>10</td>
<td>453 (29.2)</td>
<td>244 (53.9)</td>
<td>209 (46.1)</td>
<td>0.02</td>
<td>3/16 (19)</td>
</tr>
<tr>
<td>11</td>
<td>47 (3.0)</td>
<td>21 (44.7)</td>
<td>26 (55.3)</td>
<td>0.03</td>
<td>25/90 (28)</td>
</tr>
</tbody>
</table>

P values were calculated using the chi-square test for genotype distribution. 0 = major allele, 1 = minor allele.
Among females, but not in the combined male and female group, carriers of the minor –921 T-allele had significantly higher plasma concentrations of CRP (2.50 ± 1.21 mg/l vs 1.21 ± 1.14 mg/l, P=0.03) and SAA (4.21 ± 1.12 mg/l vs 2.72 ± 1.09 mg/l, P=0.05) compared with non-carriers, both being acute phase reactants used as markers of inflammation. No further associations were found with other intermediate phenotypes (data not shown).

To examine whether interactions between genetic variants in \textit{OX40L} and environmental influences are implicated in predicting MI, all genotyped polymorphisms were included in a multilocus model using the MDR software. The analysis did not detect any interactions between SNPs and environmental attributes, neither in the combined group nor when men and women were analyzed separately. However, it is noteworthy that the best predictor of MI in the female group (n=124) identified by MDR was always the one-attribute model including rs3850641. The model selected by MDR was better in predicting MI status in women (60.1%; selected as best model in 95% of cases) than in men (54.1%; 88%).

In order to dissect the mechanism behind the observed association between \textit{OX40L} haplotypes and MI, and to identify the polymorphism(s) responsible for the perturbation of gene expression/activity, allele-specific gene expression \textit{in vivo} in the presence of a natural chromatin structure was evaluated with the haploChIP technique. The influence of the putative regulatory rs3850641 and –921C>T SNPs on phosphorylated active Pol II loading to the \textit{OX40L} gene was measured by pyrosequencing in heterozygous cells for these markers. Screening of nine different human B-cell lines transformed with EBV showed that one cell line was heterozygous for both the –921C>T and rs3850641 SNPs, and measurements of mRNA confirmed that the \textit{OX40L} gene was expressed in these cells (data not shown), as previously reported\textsuperscript{194}. Pyrosequencing demonstrated that there was a clear allele-specific difference in loading of phosphorylated active Pol II to the \textit{OX40L} gene, with a higher Pol II binding to the –921C-allele (\textbf{Figure 14a-d}). The mean C:T ratio was 1.20 after immunoprecipitation when set to 1 in the starting material (total input chromatin). Likewise, fragments bound to phosphorylated Pol II contained predominantly the rs3850641 A-allele, with an A:G ratio of 1.19 (\textbf{Figure 14e-h}). Thus, these findings indicate \textit{in vivo} that the functional significance resides in the haplotype defined by these polymorphisms, i.e. the haplotype containing the –921T and the rs3850641G alleles was associated with lower transcriptional activity. Of note, since the –921 T-allele was shown to be associated with MI in women and with markers of inflammation such as CRP and SAA, a lowered \textit{OX40L} expression appears to be associated with increased risk of MI due to increased systemic inflammation and susceptibility to plaque rupture, as suggested previously (paper I).
Phylogenetic footprinting analysis using the RAVEN software showed that the –921C>T and rs3850641 polymorphisms are located in regions that are highly conserved between mouse and man, indicating that these regions may be of functional importance.

**Figure 14.** Allele-specific loading of phosphorylated Pol II *in vivo*. a-c/e-g. To quantify relative levels of abundance of allele-specific fragments, Pyrosequencing was used to analyse input chromatin used in chromatin immunoprecipitation (ChIP) reactions (a, e); products of ChIP using specific antibodies to total Pol II as positive control (b, f) or phosphorylated serine residues of Pol II CTD (c, g). Graphs show nucleotide sequence along the x axis and intensity of signal along the y axis. Peaks corresponding to the -921C>T (C/TGTGCT) and rs3850641 (A/GTGTAG) polymorphic sites are shown. d/h, The ratios between the C and T alleles of the SNP at nt -921 (d) and the A and G alleles of SNP rs3850641 (h) of phosphorylated Pol II loading compared with input chromatin used in ChIP reactions are shown. Data are expressed as mean ± s.d. Each immunoprecipitation was analysed in two replicates.

EMSA studies, performed to assess whether the –921C>T and/or rs3850641 SNPs affect the binding of nuclear proteins, demonstrated that the rs3850641 SNP does not influence the formation of any protein-DNA complex when using nuclear extracts derived from U937 cells (Figure 15b). On the contrary, EMSA studies showed an allele-specific protein-DNA interaction when nuclear extracts were incubated with oligonucleotide probes spanning the –921C>T polymorphism (Figure 15a). The –921 T-allele promoted the formation of a complex that was not present with the –921C allele (complex 1, Figure 15a). Also, nuclear protein(s) binding both alleles showed an increased affinity to the T compared with the C allele (complex 2, Figure 15a). A similar allele-specific binding pattern was obtained using nuclear extract derived from human THP-1 cells (data not shown). Taken together, the results of the haploChIP and EMSA studies suggest that -921C>T is the functional polymorphism and that the lower transcriptional activity associated with the –921 T-allele is due to binding of one or more transcriptional repressor(s) to the T-allele.
Figure 15. A representative EMSA of nuclear extract derived from U937 cells bound to (a) the -921C>T region of the promoter and (b) the rs3850641 region of intron 1 in OX40L. a. EMSA of a 25 bp DNA fragment containing either the -921C (lanes 1-4 and 9-12) or the -921T site (lanes 5-8 and 13-16). Lanes 1 and 5, no extract; lanes 2 and 6, 0.5 µg of extract; lanes 3 and 7, 1 µg of extract; lanes 4, 8 and 9-16, 2 µg of extract; lanes 9 and 13, -921C and -921T probe, respectively, without competitors; lanes 10 and 14, 921C and -921T probe, respectively, with a 100-fold excess of -921C probe as competitor; lanes 11 and 15, -921C and -921T probe, respectively, with a 100-fold excess of non-specific (X) competitor. Arrows denote the specific DNA-protein complexes associated with the polymorphic sites, stars indicate the location of the wells. All incubations were performed in 20 µl.

In silico evaluation of transcription factor binding sites suggested few potential candidates responsible for the allele-specific effects on transcriptional activity. Analysis of the TRANSFAC database indicated that the –921 T-allele creates a binding site for a protein affecting granulocyte differentiation and proliferation283 (AML1a) and for a factor involved in development of renal epithelium by induction of tumor suppressor genes284 as well as in cell proliferation and carcinogenesis285 (PAX-2). Nevertheless, EMSAs including antibodies against these transcription factors could not confirm binding of these factors (data not shown).

In conclusion, this third study suggests that lowered OX40L expression is associated with an increased risk of MI in women.
4.4 EVALUATION OF THE ROLE OF OX40L IN TRIO FAMILIES (PAPER IV)

The fourth and last study included in this thesis was undertaken to further analyze the role of OX40L as a genetic risk factor contributing to CAD using transmission-based tests in trio families recruited in the PROCADIS study. This approach offers the advantage of freedom from concerns regarding population stratification, which may produce false results in case-control studies and has already proven to be a useful tool for validating candidate genes for complex diseases like CAD.

Check for families with probable error in family structure or with proband lacking genotype data for both SNPs led to a collection of 974 families (220 with data from both parents); 53 (15) from Germany, 366 (78) from Italy, 148 (45) from Sweden and 407 (82) from the UK. These families included 1065 offspring with CAD, 87% of whom had suffered MI (Germany 87%, Italy 88%, Sweden 96% and UK 84%). Based on previous findings (papers I and III), this sample was analyzed for SNPs rs3850641 and –921C>T.

Unlike what was hypothesized, the TDT analyses of PROCARDIS trio families revealed that the major rs3850641A-allele, and not the minor G-allele, tended to be more frequently transmitted to affected offspring in most parts of the sample. Specifically, analysis of the rs3850641 SNP by TRANSMIT in 967 families (1055 offspring) with at least one typed affected child and acceptable genotyping, revealed that the minor G-allele was generally transmitted less frequently than expected to affected offspring (CAD and MI phenotypes) in all four countries (Table 6), results, however, not being statistically significant.

Table 6. TRANSMIT results for CAD and MI phenotypes: deviation from expected transmission of the minor rs385064 G-allele.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Country</th>
<th>Number of families</th>
<th>Number of transmissions (^1)</th>
<th>Equivalent nr of fully informative transmissions (^2)</th>
<th>Observed/expected transmissions of minor allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>All families</td>
<td>967</td>
<td>2110</td>
<td>482</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td>53</td>
<td>128</td>
<td>27.6</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>364</td>
<td>762</td>
<td>150.4</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>Sweden</td>
<td>148</td>
<td>324</td>
<td>75.5</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>UK</td>
<td>402</td>
<td>896</td>
<td>228.9</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>All families</td>
<td>880</td>
<td>1838</td>
<td>433.8</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td>49</td>
<td>112</td>
<td>20.9</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>MI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>325</td>
<td>666</td>
<td>134.9</td>
<td>1.01</td>
<td></td>
</tr>
<tr>
<td>Sweden</td>
<td>146</td>
<td>310</td>
<td>74.4</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>UK</td>
<td>360</td>
<td>750</td>
<td>204.4</td>
<td>0.96</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) 2 times the number of affected offspring; \(^2\) 4 times the variance of observed – expected number of transmissions.

Considering our previous findings, secondary analyses were performed in female offspring, although the proportion of women was too small (12-19% in
the four countries) for having adequate power for analysis according to gender. However, even though numbers were small and results not statistically significant, the minor rs38506416 G-allele appeared to increase the risk of CAD and MI in Swedish women (Table 7), in accordance with our previous results in the Swedish case:control studies (paper I).

Table 7. TRANSMIT results for females only: deviation from expected transmission of the minor rs3850641 G-allele.

<table>
<thead>
<tr>
<th></th>
<th>Number of families</th>
<th>Number of transmissions</th>
<th>Equivalent nr of fully informative transmissions</th>
<th>Observed/expected transmissions of minor allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>All families CAD</td>
<td>165</td>
<td>344</td>
<td>95.6</td>
<td>0.97</td>
</tr>
<tr>
<td>All families MI</td>
<td>133</td>
<td>270</td>
<td>71.9</td>
<td>0.92</td>
</tr>
<tr>
<td>Germany CAD</td>
<td>7</td>
<td>16</td>
<td>1.1</td>
<td>1.16</td>
</tr>
<tr>
<td>Germany MI</td>
<td>7</td>
<td>14</td>
<td>2.3</td>
<td>0.96</td>
</tr>
<tr>
<td>Italy CAD</td>
<td>53</td>
<td>112</td>
<td>20.5</td>
<td>0.84</td>
</tr>
<tr>
<td>Italy MI</td>
<td>47</td>
<td>96</td>
<td>19.3</td>
<td>0.80</td>
</tr>
<tr>
<td>Sweden CAD</td>
<td>22</td>
<td>44</td>
<td>11.9</td>
<td>1.47</td>
</tr>
<tr>
<td>Sweden MI</td>
<td>19</td>
<td>38</td>
<td>9.3</td>
<td>1.41</td>
</tr>
<tr>
<td>UK CAD</td>
<td>83</td>
<td>172</td>
<td>60.9</td>
<td>0.91</td>
</tr>
<tr>
<td>UK MI</td>
<td>60</td>
<td>122</td>
<td>40.5</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Analysis of the –921C>T SNP by TRANSMIT resulted in a total of 968 families incorporated into the analysis, including 1054 affected offspring, and did not reveal any significant deviations from the expected allelic transmission amongst affected offspring, neither in the entire sample nor in individual countries or in women (data not shown).

In conventional TDT analysis using GENEHUNTER, the major rs3850641A-allele and the haplotype containing the major –921C- and rs3850641A-alleles (haplotype 00 in Table 8) were more frequently transmitted to affected offspring, in agreement with TRANSMIT results, irrespective of affection status (data not shown). A statistically significant deviation from the expected 50% transmission rate was reached in the UK subset (Table 8).

Table 8. GENEHUNTER results for UK only complete trios with informative genotyping: deviations from 50% transmission rate.

<table>
<thead>
<tr>
<th></th>
<th>% transmitted</th>
<th>Numbers of observed transmissions</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD Marker –921C</td>
<td>54</td>
<td>24</td>
<td>0.683</td>
</tr>
<tr>
<td>MI Marker –921C</td>
<td>58</td>
<td>19</td>
<td>0.491</td>
</tr>
<tr>
<td>CAD Marker 3850641A</td>
<td>67</td>
<td>52</td>
<td>0.013</td>
</tr>
<tr>
<td>MI Marker 3850641A</td>
<td>72</td>
<td>46</td>
<td>0.003</td>
</tr>
<tr>
<td>CAD Haplotype 00</td>
<td>68</td>
<td>47</td>
<td>0.013</td>
</tr>
<tr>
<td>MI Haplotype 00</td>
<td>73</td>
<td>41</td>
<td>0.003</td>
</tr>
<tr>
<td>CAD Haplotype 01</td>
<td>27</td>
<td>30</td>
<td>0.011</td>
</tr>
<tr>
<td>MI Haplotype 01</td>
<td>24</td>
<td>29</td>
<td>0.005</td>
</tr>
</tbody>
</table>
Interestingly, the haplotype that appeared to be associated with CAD in the non-Swedish parts of the sample was a “mirror” of the haplotype found in our previous studies (paper I). A similar pattern was seen for haplotypes of ALOX5AP, a gene encoding the 5-lipoxygenase activating protein (FLAP), in relation to MI. It is also worth mentioning that since only 22.6% of the families had two parents and the marker heterozygosities were 14% and 27%, respectively, only 3% and 6% of transmissions could be used for this analysis, rendering use of GENEHUNTER impossible for the female subset.

In summary, despite the low frequency of the minor alleles of the two SNPs investigated rendered the study underpowered, results presented support the notion that genetic variation in OX40L contributes to the development of CAD. Also, the present study reinforces the hypothesis that interactions between the OX40L gene and environmental effects, including sex hormones, might influence genetic susceptibility to CAD.
5 GENERAL DISCUSSION

The work presented in this thesis intended to investigate the genetic susceptibility to atherosclerosis, with the aim to identify new possible genetic risk factors implicated in the development of its clinical complications, like MI. This task is difficult considering the multifactorial nature of the traits examined.

However, there is accumulating evidence from epidemiological studies that the genetic component of CAD risk is only partially explained by classic risk factors that are themselves known to be heritable \(^{287-289}\). Thus, susceptibility genes for CAD, independent of classical risk factors such as hypercholesterolemia, hypertension or diabetes, are likely to exist in accordance with the indicated importance of inflammation and innate immunity in the pathophysiology of atherosclerosis \(^{59}\). Therefore, identification of susceptibility genes might disclose novel intriguing biological pathways involved in atherosclerotic plaque formation and/or rupture, granting new potential for prevention and therapy. Hence, despite limitations and problems, it is worthwhile to perform quests for susceptibility genes for CAD and its underlying cause atherosclerosis, in a hypothesis-independent manner with respect to gene function.

In a substantial part of this program (papers I and III) we have shown that Ox40L contributes to atheroma formation in mice and that its human counterpart is associated with MI. We have also found that strains susceptible to atherosclerosis carried genetic variations in the Ox40L promoter region that influenced gene activity, and that genetic variation affected also the human homologue, resulting in a lower expression of OX40L. The putatively functional polymorphism was associated with allele-specific differences in systemic inflammation. However, plaques found in the atherosclerosis-susceptible mice are similar to human early fatty streaks \(^{290-292}\) and do not rupture. Therefore, it appears that harbouring this specific genetic variation in Ox40L promotes a pro-inflammatory state that destabilizes the atherosclerotic plaque, making it particularly prone to rupture. For as yet unknown reasons, this effect seems to be gender-specific, being confined to women. This gender-specific effect was also found in mice, female mice being more susceptible to atherosclerosis than male mice \(^{293}\). Also, previous findings suggest that gender-related factors affect immune reactions in atherosclerosis \(^{294}\). Gender may affect immune responses through different levels of gonadal steroid hormones in the blood \(^{295, 296}\). In particular, estrogen may alter the production of cytokines through interaction with specific receptors on macrophages and mononuclear
leukocytes. Testosterone, on the other hand, is immunosuppressive, and its immune-modulating properties have been proposed to exert a protective effect on atheroma formation. Thus, allele-specific differences in OX40L expression could be differentially modulated by sex hormones.

The fact that OX40L is expressed by several cell types suggests that it has more functions than the originally reported involvement in T-cell activation. Of cells present in the atherosclerotic lesion, ECs, macrophages, mast cells, and SMCs express OX40L. Therefore, the observed genotype-phenotype associations could reflect the net effect of OX40L actions in different cell types. Expression of OX40L on different types of antigen-presenting cells (macrophages, dendritic cells, B-cells, and SMCs) might influence T-cell recognition of antigens. Moreover, OX40L expressed on mast cells may interact with OX40 on T-cells and stimulate their proliferation.

The cross-talk between the two cell types might exert an effect also in the opposite direction, regulating mast cells and their role in inflammation, as has been observed for other members of the TNF/TNFR superfamily. In fact, mast cells can be activated by T-cell-dependent co-stimulatory signals transduced by ligation of lymphotoxin-β and 4-1BBL. Finally, OX40L expressed on ECs was reported to mediate the adhesion of OX40-expressing T-cells to the vascular endothelium and the subsequent migration to distant inflammatory sites, suggesting an involvement of OX40L in lymphocytes recruitment as well. Unstable plaques are particularly rich in activated lymphocytes; therefore all these events, possibly triggered by OX40L, may favour destabilization and rupture of the plaque. It has been indicated that members of the TNF superfamily may interact with more than one cognate receptor. However, studies in gene-targeted mice have shown that, despite cross-interaction between different members of the TNF/TNFR superfamily, each ligand/receptor pair has different, non-redundant functions, suggesting that the OX40L/OX40 system might play a unique role in atherogenesis.

In the study reported in paper IV, we further evaluated the role of OX40L in a collection of trio families. The trio family approach applying TDT analysis has proven to be a useful tool for validating tentative susceptibility genes for complex diseases such as CAD. By examining whether there is any significant difference between the frequency of the alleles transmitted from heterozygous parents to affected offspring and the frequency of the non-transmitted alleles, it is possible to estimate the heritability of a trait. There has been much discussion recently as to the relative merits of transmission-based tests in trio families versus testing for association in case:control series. Although some reports have indicated that the problem of population stratification in case:control studies may have been overstated, recent data suggest that it may yet remain a significant problem. Thus, despite the fact...
that family-based methods are generally considered less powerful than case-control studies, their intra-familial comparisons make them attractive because of freedom from concerns regarding stratification. It is also relevant to note that collection of true negative controls is difficult in CAD, as presymptomatic disease will be common in apparently healthy control individuals. In contrast, use of trio families does not require any control population, thus avoiding any such distortion of results. Another advantage of trio analyses is that a considerable proportion of genotyping errors can be eliminated if they result in Mendelian segregation inconsistencies; this is an important issue since such errors have a negative impact on the power of gene-association studies. Finally, excess mortality for dominant diseases in the carrier parent, sometimes regarded as a possible source of bias, implies that families carrying recessive alleles will be preferentially sampled in trio cohorts. Consequently, as both parents are informative, the power will be optimal in the TDT design.

Our results from the analyzed trio families support the view that genetic variation in \textit{OX40L} contributes to the development of CAD. However, the low frequency of the minor alleles of the two SNPs investigated rendered this study underpowered. Several reasons might account for the lack of replication of our previous results. First, susceptibility genes for human complex diseases, like \textit{OX40L}, are assumed to confer modest risks. Hence, association to such loci may be difficult to replicate. The small effect of each locus necessitates requirement of extremely large samples that are widely believed very difficult to assemble for late-onset diseases like CAD. In this respect, the collection of trio families examined was quite remarkable. Also, factors like penetrance and phenocopies could further contribute to the complexity of the picture. Furthermore, differences between the studied populations (SCARF and SHEEP in paper I; PROCARDIS trios in paper IV) and/or in the selection criteria used might result in selection for different genetic effects. We found the association between MI and \textit{OX40L} in Swedish cohorts. We then studied it in a collection including subjects from three more European countries. Despite that individuals from these areas are known to be genetically homogeneous, as was confirmed when we checked for haplotype heterogeneity, our results showed a different trend in the Swedish subsample compared to the others and the overall group, suggesting that some differences between populations might have affected our results. In addition, the importance of environment should not be disregarded, as it has been shown that the difference in CAD incidence between populations sometimes can be due to environmental factors. Nonetheless, these considerations notwithstanding, we observed an association in the non-Swedish parts of the sample between CAD and a “mirror”
haplotype compared with our previous data (paper I), further supporting the view that genetic variation in \textit{OX40L} contributes to the development of CAD. With respect to the OX40 receptor, our studies (paper II) have shown that the \textit{OX40} gene is highly conserved between species, indicating that OX40 fulfils vital functions in mammals\textsuperscript{203, 273}. One of the few genetic variants reported in public databases caused a synonymous substitution in exon 5 and was found to be associated with HDL cholesterol levels but not with MI. Recent evidence indicates that even synonymous substitutions are subject to constraint, often because they affect splicing and/or mRNA stability\textsuperscript{314}, resulting, at least in inferior organisms, in a biased usage of synonymous codons intended to maximize the rate of protein synthesis\textsuperscript{315-317}. Therefore, keeping in mind that the HDL cholesterol concentration might be influenced by a linked polymorphism located elsewhere, the role of such silent changes should be carefully considered.

5.1 METHODOLOGICAL CONSIDERATIONS

5.1.1 Of mice and men

To date, despite there is no valid animal model of important aspects of human MI and CAD, genetically manipulated mice and inbred strains have shown to be useful for dissecting complex traits relevant to CAD such as atherosclerosis\textsuperscript{223}. Atherosclerosis is governed by many QTLs, each making a small contribution to the overall phenotype. For this reason and due to interactions with the environment, atherosclerosis shows several intermediate phenotypes which makes it difficult to be analyzed. However, recent QTL mapping in mouse has identified several chromosomal regions containing candidate genes for atherosclerosis. It has been shown that there is a quite extensive synteny between mouse and humans for this trait, and the majority of atherosclerosis QTLs has the same underlying genes in both species\textsuperscript{345}. Against this background, we thought that positional cloning of the gene underlying a mouse QTL could lead to identification of the homologous gene contributing to the disease in humans.

However, this approach is not free from obstacles. For example, when using inbred strains for isolating genes responsible for the quantitative variation of a trait, recombination events occurring through generations cannot separate a cluster of closely linked polymorphic genes that might therefore be detected as one QTL. This is the reason why we carefully evaluated every single candidate in the region when we narrowed down the \textit{Ath1} locus. In this case, we have
successfully used a combination of purely positional and candidate gene approaches. This strategy has often been proved to be optimal because it relies at the same time on both freedom from pre-formulated hypotheses and on existing knowledge of gene properties or mechanisms and their expected involvement in the disease. Specifically, the mouse model enabled us to identify the gene and study its expression and lesion size in mice deficient for candidate genes, while the human population with its different haplotype blocks allowed us to test all genes in the region in a hypothesis-driven association study which confirmed that only $\textit{OX40L}$ was associated with MI. Such an approach is more powerful statistically than the typical association study that tests multiple genes with no \textit{a priori} hypothesis. In fact, this strategy allowed us to test the human population accepting a conventional threshold of significance instead of the much smaller one obtained by correcting for multiple testing (Bonferroni), as we would have had to do if we had tested each of the nine genes in the $\textit{Ath1}$ region with no prior hypothesis. Thus, using the animal model to generate a hypothesis allows for a more efficient study of human populations.

5.1.2 Mouse models of atherosclerosis

Many issues of experimental design, including specific model, strain, gender, atherogenic stimulus and selection of the area for lesion size quantification, may influence the outcome and interpretation of mouse model studies. Therefore, a few aspects of animal models need to be further discussed. First, it is noteworthy that a quantitative trait by definition is characterized by measurable phenotypic variation. This variation can consist of discrete values or can be continuous, and sometimes a threshold must be crossed for the quantitative trait to be expressed, as it is often the case among complex diseases. Therefore, it is relevant for the success of a QTL analysis to both carefully define and measure the phenotypes and set the threshold. Measurement of lesions in the aortic root is the most frequently used mode of quantifying diet-induced atherosclerotic lesions in mice\textsuperscript{318,320}, and it was previously used to find the phenotype of $\textit{Ath1}$\textsuperscript{321,323}. Therefore, we used it also in this study according to established procedures for measurements. Quantification was done in the aortic root because this is the only area where atherosclerosis is consistently present in all models naturally susceptible to diet-induced atherosclerosis\textsuperscript{318}.

Another issue is the choice of the animal model. Models used for studying atherosclerosis and identifying related QTLs are essentially divided in two groups on the basis of their natural propensity to develop the disease when fed
a special diet, or as a consequence of genetic manipulation. The ability of mice to develop diet-induced atherosclerosis in a strain-dependent manner has been known for over two decades\textsuperscript{319,321}, and considerable variation in proneness to atherosclerosis has been demonstrated among inbred strains of mice such as C57BL/6\textsuperscript{319,324}, the strain we used in our experiments.

It is clear that gender could exert a major effect on the outcome of atherosclerosis studies\textsuperscript{325,326}. Female mice are generally considered to develop a greater extent of atherosclerosis\textsuperscript{327}, but this has not been a consistent finding\textsuperscript{326,328}. However, we only studied female mice because the \textit{Ath1} phenotype was found in females\textsuperscript{319,321}.

Finally, there has been some concern about the different diets used to induce and accelerate lesion formation. Despite the absence of extensive systematic studies of the role of different fats and cholesterol contents on the development of atherosclerosis in the most commonly used mouse models, evidence obtained so far is controversial. Some studies have shown that inclusion of saturated fat and cholesterol in the diet promotes the same atherogenic process as occurs during feeding with a normal laboratory diet\textsuperscript{329}. However, Paigen and colleagues have shown that a diet containing cholesterol and sodium cholate is absolutely necessary for developing detectable aortic lesions in mice, including C57BL/6\textsuperscript{249}. Thus, what is known as the “Western diet” (saturated fat 21% wt/wt and cholesterol 1.25%) has to be modified by adding sodium cholate (0.5%). On the other hand, feeding mice such a diet leads to enlarged livers, engorged with fat, and gallstone formation as early as after 3 weeks, due to the presence of cholesterol and cholate\textsuperscript{330}; the role of cholate has been questioned also for its propensity to initiate inflammatory processes\textsuperscript{331}. Therefore, Paigen and colleagues manipulated the atherogenic diet by decreasing the levels of these two dietary components rather than excluding them totally, to produce a diet that minimized lipid accumulation in the liver\textsuperscript{249}.

5.1.3 Linkage disequilibrium

Our results reported in papers I and III showed that the variation in OX40L is organized in two LD blocks across the gene. This consideration relies on the D' measurements. As already mentioned, D' values can be affected by allele frequency and sample size. Concerning the first issue, some analyses have suggested that D' is relatively robust to variation in allele frequency\textsuperscript{332,333}, and even if sometimes a significant relationship between D' and mean marker heterozygosity has been detected, it does not influence the results\textsuperscript{334}. Perhaps of greater concern is the possibility that D' can be skewed when one or both markers contain rare alleles\textsuperscript{335}, but this should not be the case with the allele
frequencies of the variants we analyzed. A further complication when using $D'$ to measure LD is the influence of sample size. For small sample sizes $D'$ is upwardly biased, leading to overestimation of LD. Simulations based on suggest that the SCARF sample size may have led to an overestimation of $D'$ by 0.02. It is notable that several studies of LD in human populations have relied on considerably smaller samples than the ones described here.

5.2 TREATMENT

Our results indicate that the OX40L/OX40 pathway may be an excellent target for atherosclerosis therapies. The expression of OX40L in all the major cell types in atherosclerotic lesions suggests that OX40L is not just another addition to the long list of inflammatory molecules. Rather, it plays a central role in regulating and coordinating inflammatory mediators acting through the various cell types on which it is expressed. In addition, many lines of evidence indicate that both OX40L and OX40 are expressed more avidly in inflammatory than in normal tissues. Therefore, targeting the OX40L/OX40 pathway may inhibit local inflammation and the function of OX40L on nonimmune cells without causing danger to global immune competence. It should noted in this context that the OX40L/OX40 interaction has been targeted for treating many chronic inflammatory diseases and atherosclerosis may be the next one.

5.3 FUTURE PERSPECTIVES

Although the precise functional link between OX40 and OX40L, on the one hand, and development and rupture of atherosclerotic lesions on the other, has yet to be elucidated, our studies encourage further studies to better understand the biochemical mechanisms involved. Further analyses performed in vivo and in vitro are required to specifically determine the role of the OX40L protein in MI and to resolve the issue of gender-specific effects. Adequately powered association studies in women might shed new light onto this issue. Also, large-scale longitudinal cohort studies could be valuable in order to assess the predictive power of the novel functional variant we identified, beyond what is provided by established clinical and biochemical risk factors. Finally clinical and experimental studies also need to determine whether there is synergistic contribution of more than one genetic variant.
6 CONCLUSIONS

In these studies OX40L was found as a new susceptibility gene for atherosclerosis and its related complications MI and CAD. In particular, specific functional genetic variation at OX40L appears to promote a pro-inflammatory state destabilizing the atherosclerotic plaque and making it particularly prone to rupture. Importantly, since this effect seems to be gender-specific, harbouring such genetic variation in OX40L might be a novel risk factor for women.

Further evidences suggested that genetic variation in its receptor OX40 also plays a role in the pathogenesis of MI, thus indicating the T-cell activator OX40L/OX40 pathway as a novel important factor contributing to atherosclerosis and CAD. In addition, due to its characteristics the OX40 ligand/receptor pair may be an excellent target for therapy.

Finally, results of these studies prove that it is possible to use data from a mouse QTL model to positionally identify the homologous gene contributing to disease in a human context.
7 ACKNOWLEDGEMENTS

I have thought about this moment for a long time, when I would have had months of work at my back and only this as the last effort to finish the book: I have to reckon it feels good! Now, after many stressful days and sleepless nights I can thank all those that made possible for me, in different ways, to reach this point. And I have to do it properly because after all, this is the only part that most of you will read, right? Everything in this thesis ended up to be very thorough and acknowledgments follow the trend, so let’s start and enjoy the reading!

The work reported in this thesis has been conducted at the Atherosclerosis Research Unit, King Gustaf V Research Institute (GV), Department of Medicine, Karolinska University Hospital during 2001-2006. Here I wish to express my sincere gratitude to the followings.

I had the privilege, or the challenge, of having four (!) supervisors that I want to acknowledge in a special way:

First of all, Professor Anders Hamsten, Head of the Atherosclerosis Research Unit, Department of Medicine, for welcoming me to Sweden, Karolinska Institute and GV, for supporting my desire to learn science and do research, and especially for considering persons as a whole, with their lives, their problems and their peculiarities, in other words for treating collaborators and colleagues not only as work mates but as human beings. This is probably the reason making GV a nice and friendly working environment, I would say almost like a family. Of course also sharing your great knowledge in atherosclerosis and your competence has been precious to me. Thanks for your interest and for endless corrections to improve my writing, I felt really inspired by reading your beautiful and sophisticated linguistic refinements.

Doctor Jacob Lagercrantz, PhD, for close supervision in the first part of my PhD program. I would have appreciated your support also in the second part of my PhD, however this freedom taught me to be more independent. You are not the typical Swede and I have coped very well with that. I was reassured once again that being late is not necessarily a big deal, like I have always thought! Thanks for our discussions, very simple and direct, for understanding my different point of view on life and Swedish system as a European, and for trying to make me feel comfortable all the time. Thanks also for caring about me when I was sick and about my family, and for fixing my countless requests
I wish to thank you also for helping me with writing the papers and for spending nights up, going through my thesis despite family duties were calling you. I have also appreciated the care you have of your big family and how is difficult to be a scientist and a good father at the same time: I will bring this lesson with me.

Associate professor Per Eriksson, for taking over the supervision in the last part of my studies, for introducing me to the exciting field of inflammation and molecular genetics, and for challenging intellectual discussions. Thanks for your contagious optimism and enthusiasm and your many ideas, for teaching me the scientific method and making me feel there is always hope. I thank you also for your endless patience with my thousands of questions; your door is always opened for everyone and I am not sure I will meet someone else in the future with the same attitude. Finally, thanks for showing me how to be a good scientist and to have a lot of different interests at the same time, how a good balance is important and that everything benefits of that. I have enjoined so much our talks about the Boss and good luck with the tickets to next concerts when I will not be here any longer!

Per G Olsson, PhD, my “external” supervisor at AstraZeneca. Despite you are not supervisor on paper your help has been very important. Thanks for your availability, for introducing me to the world of population genetics and haplotypes and for sharing AZ wonderful facilities in the lab. Thanks for letting me work on such an exciting project and establish collaboration with Jackson Laboratory. As part of AZ R&D I thank you also for not patenting the findings making my publication process much easier, still I think you did a mistake… ☺. Finally many thanks for finding time to meet me and explain me the world of doing research in the industry and for your precious advises.

All co-authors for fruitful collaboration and for sharing knowledge and technical skills, in particular:

Doctor Ann Samnegård, PhD, for invaluable work done with angiographies in the SCARF collection, for being so attentive with all details, for critical revision of the papers, for caring about my health and for being a nice person with a nice hobby.

Xiaosong Wang, PhD, Beverly Paigen, PhD, and all the members of Paigen’s group at Jackson Laboratory for such an exciting joint-venture; doctor Naoto Ishii and Professor Kazuo Sugamura for sharing mice resources and knowledge in the OX40L/OX40 field; Anna Bennet, PhD, for
the statistical assistance, Professor Ulf de Faire and Professor Björn Wiman for access to SHEEP material and revision of the paper; Kristina Forsman-Semb, PhD, for introduction to mouse genetics; associate professor Carl-Göran Ericsson and doctor Susanna Boquist for recruitment of individuals involved in the SCARF study; Professor Hugh Watkins on behalf of the PROCARDIS Consortium for access to a unique collection of samples; Professor Martin Farral and Olof Bengtsson, PhD, for valuable assistance with TDT analysis and critic revision of the paper.

Doctor Jacob Odeberg, PhD, and Malin Andersen for tricks and crucial advises on tackling tough genetic sequences.

Senior researchers at GV for contributing to a good academic environment: Ferdinand van’t Hooft, PhD, for taking care of me during my first months at GV, for patience with my English (I still remember when I have asked you to slow down because I could not follow...), for understanding my decisions and for being such a nice person, always happy and enthusiast (and thanks also for landing me your nice TV); associate professor Angela Silveira, for your kindness and for having always an answer, for being so well organized and arranging meetings in GV; associate professor Rachel Fisher, for being so kind, having always punctual and interesting questions and for bringing some British politeness and style to GV; associate professor Eva Ehrenborg, for your positive attitude and for contributing to nice discussions in GV; Professor Mai-Lis Hellenius for showing how to be a successful scientist with style, for being so friendly, for trusting my Swedish knowledge and for landing me your office; associate professor Johan Bjorkegren for your American style and knowing what you want; associate professor Alejandro Bertorello for bringing new perspectives into GV; Professor Jesper Tegner for opening new horizons in medicine.

Present and former colleagues at GV, several of whom I consider very good friends; the Russian gang: Sergey Krapivner for being such an intelligent and nice companion and friend, for our lunch times where I have learned a lot on communism and Russia, for our endless talks about movies and history and for sharing with me doubts, excitements and plans of our PhD-student status (I really miss them), thanks also for being always available and helpful to everyone and all the best for your future; Ekaterina Chernogubova, PhD, for your very nice moods, caring about the others and being so enthusiast about Italy; thanks for coming down with your family to my wedding, that has been very special to me, and good luck to you too wherever in the world you will end up; Sergej Popov, for never agreeing with me especially about Juventus,
Zlatan and Japan, and for guidance in the downloading world, good luck “over there”. Karl Gertow, PhD, Swedish-Italian friend, for being so close to me during my first staying in Sweden, for buffering the impact and for being the bridge between very far away cultures. Thanks for organizing social events after work and for being “capo delle recchie per eccellenza” (try to translate this in Swedish...☺); good luck in Italia. Josefin Skogsberg, PhD, for being such an alive and happy person, for being so elegantly and typically woman, for the laughing and all the discussions on sport: I am sorry but if you really want to support a winning team you should change nationality!☺ Doctor Maria Nastase Mannila, PhD, for your stubbornness and perseverance, really inspiring to me, for your patience and availability to explain me epistatis and for your precious advises; I am sure you will become an excellent cardiologist. Per Sjögren, PhD, for your cool moods, for being “socially active”, for help with small important details during the dissertation process; Dick Wågsäter, PhD, for fruitful and nice collaboration and for being a nice roommate; Monsur Kazi, PhD, for your teaching life journey and for your ability to focus on what is essential; Kerstin Lundell, PhD, for being so competent and contributing to nice room atmosphere; Karin Stenström for your laugh and incredible energy, a real Scandinavian woman; Alexander Kovacs, for your willingness to understand; Maria Kolak, for being hardworking and friendly; Petra Thulin for your funny laughs; Kristina Eneling, for reminding me that life is not only work; Carl Whatling, PhD, for your impressive knowledge, for very British Fridays (when we left the pub after six hours spent drinking I have decided I could not join you every time…) and for being so kind when I visited you at Astra; Tiina Skoog, PhD, for being such a nice person and for caring about my future; Mattias Sjöström, PhD, for keeping such a nice atmosphere in the lab, really a pity you left; Anna Aminoff, Sara Hägg, Zhongpei Chen, Vincent Fontaine, PhD, Anders Mälartsig, PhD, Fei Chen, PhD, Chaoyong Zhu, PhD, Helena Ledmyr, PhD, doctor Marie Björnstedt-Bennermo, PhD, Hanna Björk, doctor Katja Kannisto, PhD, doctor Christina Ahlbeck Glader, PhD, Björn Glinghammar, PhD, Tobias Cassel, PhD, Sofia Larsson, PhD, doctor Sofia Jormsjö-Pettersson, PhD, doctor Camilla Skoglund-Andersson, PhD, and all members of Computational Medicine Group for contributing thoughts, interesting discussions and creating such a pleasant and interesting atmosphere.

Technical personal in the lab: Barbro Burt, for helping with EMSAs, for patience with all new lab members, for having an answer to everything, for keeping the lab going and making transitions smooth; Karin Danell-Toverud for keeping tracks of databases and fridges and pushing me to teach Italian (I would have never imagined myself doing that!); Anita Larsson, Karin
Husman, Birgitta Söderholm and Peri Noori, for holding GV together and excellent technical assistance.

Present and past people working within the administration of GV and Department of Medicine: Ami Björkholm and Gerd Stridh, for help in solving all my administrative troubles; PROCARDIS gang: Karin Björklund-Jonsson for helping me to keep track of Jacob and all nice chats; Ulla Grundstedt and Karolina Anner for making entering the PROCARDIS room such a nice experience, we miss you. Camilla Berg for your precious assistance with PhD matters and for enthusiasm in learning Italian; Caroline Hamilton, Malin Toverud, Karin Blomberg, Helena Öhgren, Christina Hadders-Medin, Annetty Jansson and all the others for doing your job accurately. Magnus Mossfeldt for indispensable IT-support and availability at the most unusual times of the day, I hope you will finish the roof before snow will come!

During these five and a half years I have spent in Sweden, many things happened beside my PhD studies: most of them are good, some are not. I came as a newly graduated youngster and I will leave as a man, with a wife and soon a baby, many more friends, a third language, maybe a second citizenship, some knowledge and a strong desire to become a scientist. I think all this is wonderful and I want to take now the opportunity to thank all of you who had something to do with it.

I obviously start from the person responsible for my coming to Sweden, when I was totally unaware of what was expecting me, my former boss in Milan, doctor Giacomo Ruotolo, who intrigued me with stories about an exotic country very north called Sweden. You can take most of the merit and the blame for my years in Sweden; overall I am really grateful you gave me this opportunity.

I wish to thank also the small community of friends I met here through Antonella and her nice family, that is Beppe (and Cappy), Sandra (and family), Bessy, Silvano and during the years German, Rodrigo, Carla, Adan, Silvia, Francesco, Rosi, Rita, Manuel and many others who joined along the path: you have been like a family for me and among you I met the girl who became my wife, you have been here the grounds I have left at home and despite things changed with some of you I want you to know I will always take with me the beauty of the truth we encountered and experienced together.
My red-and-black “half brother” Krister, for making me feel always welcome to your house, for sharing your TV to watch Milan on Italian channels, for keeping high Milan flag up in the North and for being a good friend. Lives take different directions sometime but I hope we will be able to keep in touch. Thanks also to all gang of “Milan club G. Nordahl Sweden” (Martin, Marco, Anders, Lena, etc.) for sharing your passion.

My dear “Swedish” friends Alberto, Agnieszka, Dorota, Milan, Gosia, Sam, Raul, for being so caring, unselfish and sympathetic, you have been really important during these years, it is a grace to have friends like you.

The Nockebynians who created a unique atmosphere during that “unforgettable” summer 2001: I don’t think I will ever experience again such an exciting and strong friendship that enabled us to stay in contact across the world during these years: Vasilis, Effie, Pablo, Andreas, Igor, Annalisa, Sabrina, Michailo and Sandra, Michelle and Peary, Barbara, Luis and all the others.

F. Richard for supporting me during my dark and painful times. When I felt lost you helped me to find the way and not to despair. Thanks a lot also for being always there, for accompanying me and Ilaria and taking care of our new family. Many thanks also to all guys at Lärkstaden center, for making me feel always welcome and caring so much about the health of my soul.

F. Klaus, and all Eugenia friends (Monica, Gustavo, Alejandro, Mirta, Barbara, Isabel, Jonathan just to cite a few among the others) I have met through the great meetings you have organized during these years: they have been really a reference point for me.

My Italian connection in Sweden: Marta, Alessandra, Giovanni, Marina, Enrico and Eugenia with little Francesco, Luigi and Marzia with little Matteo, Fausto and Tina, and almost-Italian Pam (good luck with your second PhD dissertation!) for all the nice time we spent together and for contributing to make distance from home not so long.

The Uppsala people: Lucia, Signe, Robert, Zerit, Alessio and all the others, you are a great sign to me with all your different stories.

My Karolinska friends, and among them especially Yuri (good luck in US!), Barbara and Theo for great company and support, Alessandro and Mikke for getting along so well and affecting all the others around you, Guillemin for
the great trips to Norway and Scotland and for hating Real Madrid even more than me! 😊

My many flat mates and specifically **Stefania** (too bad you did not give me another chance) and **Michela**, I had my best time in Sweden when I was sharing the apartment with you; **Louis**, you are really a nice person, actually the ideal flat mate, thanks also for teaching me French cuisine; and **Wondossen**, very short but very nice memory.

**Börje** and the **EHR Motor Vespa Service staff** for fixing promptly all the troubles I had with my Vespa, making me able to drive it in Stockholm as I was used to do at home.

My friends in Italy, who helped me with their support to go through such a demanding commitment, in particular **Marcello**, **Annamaria** and **Riccardo**, **Carlo**, **Laura**, **Silvia**, **Sonia**, **Paolo**, **Stefano F.**, **Magali** and **Giorgio** with little **Chiara**, and all the others for being always there when I come home; I regret I did not manage to convince you to visit me here! I am grateful also to my former university mates **Elena**, **Ema**, **Manu**, **Chiara**, **Totò**, **Stefano S.**, **Marta**, **Pj**, **Antonio**, **Rex**, and all the others for never stopping to remind the meaning of our friendship despite being up here. Thanks to **Danilo** for interesting scientific discussion, making me laugh about my home country and for correcting my not-so-good-anymore (!) written Italian language.

Finally, I would like to thank the most important persons in my life: my beloved family; my mother **Mariagrazia** and my father **Giampietro** for unconditional and endless love, care and support in all decisions I take, for advise and help when I can not make it alone, for always encouraging me on to new conquests and for being a model as parents: I hope I will be able to be as good as you when it will be my turn. To my younger sister **Marianna** and her husband **Aldo**, who have gone very fast and while I was playing around with science you got married and had three kids, my wonderful nephews **Jacopo** and **Alessio** and my niece **Cecilia**, who still doesn’t recognize me (bad thing of leaving home!): thanks for tracking every step I take, for making possible for me to be uncle Massi despite being so far and for having such a beautiful family! Thanks to the rest of my family, especially **Robi** for being with us and my uncles and aunties **Gabriele** and **Marisa**, **Giorgio** and **Antonia**, for caring of what your crazy nephew was doing up in the North. Thanks also to **my wife’s parents and relatives** and in particular **Stefania** and **Sergio** for being so helpful and supporting, for welcoming me and always making me feel a member of your family.
Infine, desidero ringraziare le persone più importanti della mia vita: la mia amata famiglia. **Mamma e Papà** per l'amore, la cura ed il sostegno illimitati e incondizionati in tutte le decisioni che prendo, per i consigli e l'aiuto quando non ce la faccio da solo, per incoraggiarmi sempre a nuovi traguardi e per esser un modello come genitori: spero di essere altrettanto quando sarà il mio turno. Mia sorella **Mari** e suo marito **Aldo**, che pur essendo più piccola di me sei andata molto in fretta e mentre io giocavo a fare lo scienziato ti sei sposata e hai avuto tre bambini, i miei meravigliosi nipoti **Jaco, Ale e Ceci**, che ancora non mi riconosce (ecco cosa succede ad andarsene da casa!): grazie per seguirmi nei passi che intraprendo, per farmi essere “lo zio Massi” nonostante sia così lontano e per essere un esempio per tutti con la splendida famiglia che avete messo su! Grazie al resto della mia famiglia, soprattutto a **Robi** per essere con noi e ai miei zii **Gabriele e Marisa, Giorgio e Antonia**, per non smettere di interessarsi a ciò che vostro nipote combina su al Nord. Grazie anche alla famiglia di mia moglie e in particolare a **Stefania e Sergio** per l’aiuto e il sostegno e per avermi accolto facendomi sentire da subito un membro della vostra famiglia.

My dear wife **Ilaria**, beloved companion of my entire life: you have been given to me in the most extraordinary and unpredictable way and the gratitude for your unconditional love, understanding and support, especially during these last months, cannot be described in words here. I love you.

Thanks to all the ones I have certainly forgot and above all I thank **God** for being the anchor I attach to, the only reasonable source of hope that ultimately saves everything else. Faith is the most beautiful and relieving gift one can receive.

This work was supported by AstraZeneca, Sweden, and grants from the National Institutes of Health, from the European Commission, and from the Swedish Heart-Lung foundation, the Swedish Medical Research Council (8691), the Torsten and Ragnar Söderberg foundation, AFA insurance, the Stockholm County Council, the Wallenberg Consortium North (WCN), the Swedish Society for Medical Research, the Leducq Foundation, the King Gustaf V 80th Birthday foundation and the Karolinska Institute.
One evening some time ago I was at the concert of Lars Winnerbäck in Skansen, and listening to his songs in the wonderful and unique Swedish summer inspired me and provoked strong emotions in me. Now it is time to thank also Stockholm, for being the place where I met the girl that became my wife and for being such a blinding beauty, so incredibly beautiful, the most beautiful city in the world! Wherever I will go I will always bring you in my heart (Stockholm i mitt hjärtal). And finally thanks Sweden because despite our conflicting relationship of hatred and love I have spent here my better years: I may like it or not but in my deep I will always remain a bit Swedish...

Jag trivs bäst i öppna landskap, nära havet vill jag bo några månader om året, så att själen kan få ro
Jag trivs bäst i öppna landskap, där vindarna får fart
Där lärkorna slår högt i skyn, och sjunger underbart

(Öppna Landskap, Ulf Lundell)

Stockholm, November 8, 2006
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