Expression profiling of human macrophages and atherosclerotic plaques to identify genes and mechanisms that modulate the development of atherosclerosis

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“The whole problem with the world is that fools and fanatics are so certain of themselves, but wiser people so full of doubts”

-Bertrand Russell

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Abstract

Macrophages play an important role in atherosclerosis, a disease that affects large and medium size arteries and causes clinical manifestations such as myocardial infarction or stroke. The aim of this thesis was to identify genes that are important in the development of atherosclerosis. Genes that have their major site of expression in macrophages or in atherosclerotic plaques, or are differently expressed in macrophages from subjects with atherosclerosis compared with macrophages from control subjects may affect atherogenesis.

By comparing DNA microarray expression profiles of macrophages and atherosclerotic plaques with expression profiles from major tissues and cell types, macrophage and plaque specific genes were identified. The macrophage specific anti-inflammatory cytokine interleukin 1 receptor antagonist (IL1RN) was down regulated by oxidized low-density lipoprotein (LDL), suggesting a novel pro-inflammatory role of oxidized LDL. Immunohistochemistry showed that the plaque specific gene chemokine CC motif ligand 18 (CCL18) co-localized with macrophages in the plaques. In addition, macrophages from subjects with atherosclerosis had more than two-fold higher gene expression of CCL18 than macrophages from subjects without atherosclerosis. CCL18 is chemotactic for leukocytes and may therefore contribute to plaque inflammation. A promoter region polymorphism of the CCL18 gene was associated with increased macrophage CCL18 gene expression, but not with an increased risk of coronary heart disease (CHD).

Comparison of macrophage expression profiles from subjects with atherosclerosis and control subjects identified 27 genes with an altered expression. Among these genes, CD44 and insulin receptor substrate 2 (IRS2) were both expressed at higher levels in macrophages from subjects with atherosclerosis compared with macrophages from control subjects. Immunohistochemistry showed that IRS2, an intracellular signaling molecule important in metabolism, was expressed in macrophages and endothelial cells in human carotid plaques. The C allele of the -765C→T SNP in the promoter region of the IRS2 gene was associated with increased macrophage expression of IRS2, and subjects homozygous for the C allele had 40% increased risk of coronary

heart disease. The receptor CD44 mediates adhesion of monocytes to the vascular wall, a crucial step in atherosclerosis. CD44 expression correlated with secretion of interleukin 6 (IL-6) in macrophages, and IL-6 augmented CD44 expression in macrophages. In addition, CD44 deficient mice had lower circulating IL-6 than wild type mice. This suggests a positive feed-back loop between IL-6 and CD44, and that CD44 may affect atherosclerosis progression by modulating the inflammatory response.

In conclusion, IRS2 might be a new susceptibility gene for atherosclerosis and CHD. CCL18, IL1RN and CD44 may play important roles in the development of atherosclerosis.

List of papers

The thesis is based on the following papers:

I. Svensson P-A, Hägg DA, Jernås M, Englund MC, Hultén LM, Ohlsson BG, Hulthe J, Wiklund O, Carlsson B, Fagerberg B, Carlsson LM. *Identification of genes predominantly expressed in human macrophages.* Atherosclerosis **2004** 177(2):287-90.

II. Hägg DA, Olson FJ, Kjelldahl J, Jernås M, Thelle DS, Carlsson LMS, Fagerberg B, Svensson P-A. *Expression of chemokine (C-C motif) ligand 18 in human macrophages and atherosclerotic plaques.* Atherosclerosis **2008.** Accepted for publication.

III. Hägg DA, Jernås M, Wiklund O, Thelle DS, Fagerberg B, Eriksson P, Hamsten A, Olsson B, Carlsson B, Carlsson LM, Svensson P-A. *Expression profiling of macrophages from subjects with atherosclerosis to identify novel susceptibility genes.* Int J Mol Med. **2008** 21(6):697-704.

IV. Hägg DA, Sjöberg S, Hultén LM, Fagerberg B, Wiklund O, Rosengren A, Carlsson LM, Borén J, Svensson P-A, Krettek A. *Augmented levels of CD44 in macrophages from atherosclerotic subjects: a possible IL-6-CD44 feedback loop?* Atherosclerosis **2007** 190(2):291-7.

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Abbreviations

ABCA1 ATP-binding cassette, subfamily A, member 1

ApoE Apolipoprotein E

CAD Coronary artery disease

CCL2 Chemokine CC motif ligand 2

CCL18 Chemokine CC motif ligand 18

CHD Coronary heart disease

CVD Cardiovascular disease

HA Hyaluronic acid

HDL High density lipoprotein

IFNγ Interferon gamma

IL1RN Interleukin 1 receptor antagonist

IL-1α Interleukin 1 alpha

IL-1β Interleukin 1 beta

IL-4 Interleukin 4

IL-6 Interleukin 6

IRS2 Insulin receptor substrate 2

LDL Low density lipoprotein

LDLR LDL receptor

LXRα Liver X receptor alpha

LXRβ Liver X receptor beta

MI Myocardial infarction

mmLDL Minimally modified LDL

MSR1 Macrophage scavenger receptor 1

oxLDL Oxidized LDL

PLAUR Plasminogen activator, urokinase receptor

QTL Quantitative trait locus

RE Restriction enzyme

RFLP Restriction fragment length polymorphism

ROS Reactive oxygen species

SLC11A1 Solute carrier family 11 member 1

SMC Smooth muscle cell

SNP Single nucleotide polymorphism

TIA Transient ischemic attack

TNFα tumor necrosis factor alpha

1. Introduction

The thrifty genotype hypothesis suggests that mankind have encountered long periods of famine at the course of evolution [1]. During these periods, food has been both scarce and energy-poor, and food-gathering strenuous. The evolutionary pressure should therefore benefit genetic variants that favor energy storage, e.g. fat accumulation, as well as low metabolic turnover. The clash between the Paleolithic genotype and our modern lifestyle with around the clock access to unlimited amounts of energy-dense food, in combination with an increased life span, have contributed to the rise of so called welfare diseases such as obesity, type II diabetes and cardiovascular disease (CVD). In addition, the global spreading of the westernized life-style has made CVD the leading global cause of death. In 2005, WHO estimated that CVD caused 30% (17.5 million people) of all deaths world-wide. According to the Swedish National Board of Health and Welfare, 42% of all Swedish deaths in 2006 were CVD related. For 2008, the indirect and direct cost of CVD in USA is estimated to 448.5 billion dollars, about twice the estimation for cancer [2]. The corresponding sum in Sweden 2002 was 22 billion Swedish crowns, according to the Swedish National Board of Health and Welfare. Thus, effective treatment of CVD is a global concern. To reduce morbidity and mortality as well as medical expenses, it is necessary to increase our understanding of the mechanisms and epidemiology of CVD.

2. Atherosclerosis

Atherosclerosis, the main cause of CVD, is a disease that affects large and medium size arteries in the body. Atherosclerotic plaques are characterized by the accumulation of lipids and fibrous elements in the artery wall, and develop slowly over decades. Atherosclerosis may cause clinical manifestations such as angina pectoris, myocardial infarction (MI), stroke and gangrene. The atherosclerotic plaques are classified according to their morphology, as defined by the American Heart Association [3, 4] (table 1).

Table 1. American Heart Association definitions of atherosclerotic plaques

Plaque Type	Characteristics	
Type I lesion	Microscopic amounts of lipids in the intima, isolated groups of macrophages	No clinical manifestations.
Type II lesion	Fatty streak formation, presence of foam cells	No clinical manifestations.
Type III lesion	Extracellular lipids form pools between SMC layers, causing structural alteration of the vascular wall.	No clinical manifestations.
Type IV lesion	Lipid core, intima disorganization.	Clinical manifestations.
Type V lesion	Formation of fibrous tissue. Type Va has a lipid core surrounded by fibrous tissue, Type Vb is also calcified and Type Vc is fibrous plaque without a lipid core.	Clinical manifestations.
Type VI lesion	Type IV or V lesions with complications. Type VIa has a disrupted surface, Type VIb has hematoma or hemorrhage and Type VIc thrombosis.	Clinical manifestations.

The first type of lesion, initial lesion or Type I lesion, is characterized by microscopic amounts of lipids in the intima, and isolated groups of macrophages containing lipid droplets [5, 6]. This type of lesion can be seen already in infants [5]. Lipoproteins, mainly in the form of low-density lipoproteins (LDL), from the bloodstream diffuse through the vessel wall and are retained in the intima [7, 8] where they may become oxidized [9]. Oxidized LDL (oxLDL) is chemotactic for monocytes [10] and is taken up by macrophages [11] and smooth muscle cells (SMC) [12].

Gradually, the lipid accumulation in the intima leads to the formation of a fatty streak. The fatty streak is visible as yellow colored streaks or spots on the intima surface. Both lipid-loaded macrophages (foam cells) as well as macrophages without lipid

droplets are present in the lesion, which also contains SMCs with lipid droplets, T-cells [13, 14] and mast cells [15]. The fatty streak gradually develops to form a lipid core by an increase in formation and fusion of lipid droplets [6]. As the plaque develops, the lipid core thickens the artery wall. At first, the thickening is directed outwards, by enlarging the external artery boundary. After reaching a critical point, the growth is directed inwards, decreasing the lumen area [16]. The deep intima SMCs and intercellular matrix are gradually dispersed by the lipid core, and these cells have disturbed morphology and are often calcified [4]. As the atherosclerotic plaque formation progresses, it may cause clinical complications, such as myocardial infarction or stroke. The two lesion types that are most prone to plaque surface rupture are Type IV and Type Va [17-20]. Rupture typically occurs in regions with high prevalence of inflammatory cells [21, 22] and high foam cell/SMC ratio [20, 23]; and is facilitated by release of proteases from macrophages [24].

2.1. Clinical manifestations of atherosclerosis

Atherosclerosis may lead to complications such as angina pectoris, MI and stroke, although many atherosclerotic plaques never cause clinical manifestations. These complications often manifests decades after the onset of atherosclerotic lesion formation.

Angina pectoris is caused by partial obstruction of the coronary arteries. The occlusion results in impaired supply of blood and oxygen. Angina pectoris is divided into two subgroups, stable and unstable angina. The *stable angina* is usually free of symptoms at rest, but show symptoms such as chest pain at physical activity. A patient often knows how active he or she can be before experiencing symptoms [25]. Angina is defined as *unstable* if it is severe and of new onset, if it occurs at rest or after minimal physical activity; or if it is more frequent, severe or more prolonged than previously [26].

A *myocardial infarction* is the occlusion of a coronary artery, most often caused by a ruptured local plaque, followed by thrombosis. The occlusion cause ischemia and lack of oxygen, which in turn may cause damage or death of the heart muscle [27].

Stroke is usually caused by thrombosis, emboli or hemorrhage. This leads to ischemia and oxygen deprivation, which may cause necrosis of the surrounding brain tissue. A *transient ischemic attack* (TIA) causes the same symptoms as stroke, but with temporary effects that usually only lasts a few minutes. A TIA is usually a warning signal that the patient is at high risk of suffering from a future stroke [28].

2.2. Risk factors for atherosclerosis

Several risk factors for atherosclerosis and CVD have been identified. The main risk factors are summarized in table 2.

Table 2	**Risk factors for atherosclerosis and CVD**
Age	Although early lesions can be prevalent already in infants [5], the risk of CVD strongly increases with high age [29].
Sex	Middle-aged men, below the age of 60, have 2-5 times higher risk of CHD than women, but the sex dependent difference decreases with age [30, 31].
Smoking	Smoking is a strong environmental risk factor for MI, especially in young women [32]. Two years after smoking cessation, the risk of stroke is significantly reduced, and is equal to that of non-smokers after five years [33].
Hypertension	High blood pressure is a strong risk factor of CVD [34].
Obesity and physical inactivity	Both obesity [35] and physical inactivity [36] are risk factors for CVD, as they influence other risk factors such as blood lipids, blood pressure and insulin resistance [37].
Serum cholesterol	High LDL cholesterol [38], low HDL cholesterol [39] and particularly the LDL/HDL ratio (i.e. the apolipoprotein B /apolipoprotein A-I ratio) [40] are all risk factors of CVD, and reducing serum cholesterol levels with statins clearly reduce CVD risk [41].
Type II diabetes	Patients with type II diabetes have a 2-4 fold increased risk of CVD [42].
Family history	Estimations suggest that the hereditary factors may constitute as much as 40% of the total CVD risk profile [43].

Smoking and physical inactivity are life style choices, whereas age, sex and genetics are strictly biological factors. Hypertension, obesity, serum cholesterol and type II diabetes are affected by both environmental and biological factors [43]. The risk factors may contribute to bigger or lesser extent in certain genetic backgrounds, and the effect varies in different populations [44], making it hard to estimate to what extent the genetic profile affects the risk of atherosclerosis and CVD. The INTERHEART study, in which some 15000 cases from 52 countries around the world

were enrolled, found that about 90 % of the total risk for MI could be attributed to smoking, apolipoprotein B/apolipoprotein A-I ratio, hypertension, abdominal obesity, physical inactivity, low intake of fruit and vegetables, psychosocial factors, and non-moderate alcohol consumption [45]. This study also showed that although family history was a significant risk factor for myocardial infarction, it mainly contributed to the total risk profile by affecting the other risk factors studied.

2.3. Inflammation in atherosclerosis

For a long time, atherosclerosis was regarded mainly as a lipid storage disease. However, accumulating data during the later part of the 20th century have showed that an inflammatory response contributes to the pathogenesis of atherosclerosis [46]. The oxLDL particles have several pro-inflammatory properties, including activation of endothelial cells, causing them to express the adhesion molecules vascular cell adhesion molecule 1 and intercellular cell adhesion molecule 1 [47]. Rolling leukocytes adhere to the activated endothelial cells [48], and enters the intima responding to chemotactic molecules such as chemokine cc motif ligand 2 (CCL2, also known as monocyte chemoattractant protein-1) [49, 50], which is in turn produced by the activated macrophages [51].

The inflammatory process accelerates foam cell formation through down regulation of cholesterol efflux transporters ATP-binding cassette, subfamily A, member 1 (ABCA1) [52, 53] and ATP-binding cassette, subfamily G, member 1 [53]. Interferon gamma (IFNγ) is produced in the atherosclerotic plaque [54] and induces the production of the T-cell chemotactic proteins chemokine cc motif ligand 5, chemokine cxc motif ligand 10 and chemokine cxc motif ligand 11 in the vessel wall [55]. The T-cells further accelerates the inflammatory process by secretion of pro-inflammatory cytokines such as IFNγ and CD40 ligand [56].

2.3.1. The macrophage

The macrophage is part of both the innate and the adaptive immune system of vertebrates. Its main role is to phagocytose pathogens, cellular debris and other compounds harmful to the body; and to activate other immune cells through secretion

of signal molecules and as an antigen presenting cell. Macrophages are differentiated from monocytes that enter damaged or diseased tissue from the blood stream. Other types of macrophages are situated in tissues that are frequently exposed to pathogen invasion, such as alveolar macrophages in the lung and kupffer cells in the liver.

The macrophage is considered a key cell type in atherosclerosis, and the presence of foam cells is considered a hallmark of the early stages of atherosclerosis. Monocytes enter the vessel wall and are differentiated into macrophages, a crucial step in atherogenesis [57]. The differentiation is induced by granulocyte-macrophage colony-stimulating factor, colony-stimulating factor 1 and colony-stimulating factor 3, expressed by the endothelial cells exposed to oxLDL [58]. Macrophage uptake of oxLDL is mediated through scavenger receptors [59], mainly CD36 [60] and macrophage scavenger receptor 1 (MSR1) [61], causing accumulation of large amounts of cholesterol within the macrophage [61]. The oxLDL particles are metabolized and the lipids are stored as droplets in the cytoplasm, resulting in a foamy phenotype, hence the designation foam cells [62, 63].

The macrophages produce platelet-derived growth factor [64] which stimulates SMC proliferation [65]. They also secrete vascular endothelial growth factor [66] that induces neovascularization, which is necessary for plaque growth [67]. The oxLDL particles are cytotoxic [68] and may cause apoptosis [69], contributing to the formation of a necrotic core within the atherosclerotic lesion [70]. The necrotic core consists of a lipid pool and cellular debris. The mechanism behind oxLDL-induced macrophage apoptosis is not fully clarified, but the up regulation of the anti-oxidative glutathione and thioredoxin pathways in macrophages after exposure to oxLDL [71] suggests an increase in oxidative stress burden. Markers of oxidative stress are found in areas of the plaque that are eroded or prone to rupture [72]. The macrophages are particularly numerous in the shoulder regions of the plaque and around the lipid core [73], and dying foam cells are located next to the lipid core [74].

3. Genetics of complex diseases

Although several risk factors for CVD have been identified, they cannot explain all variations in CVD incidence, and why some individuals may be exposed to several risk factors without having any symptoms, whereas others are affected although doing "everything right". For instance, stroke is twice as common in Scotland as in southeast England, which cannot be fully explained by traditional risk factors or socioeconomic factors [75]. An individual's total risk of CVD is comprised both of the genetic background and environmental risk factors (figure 1).

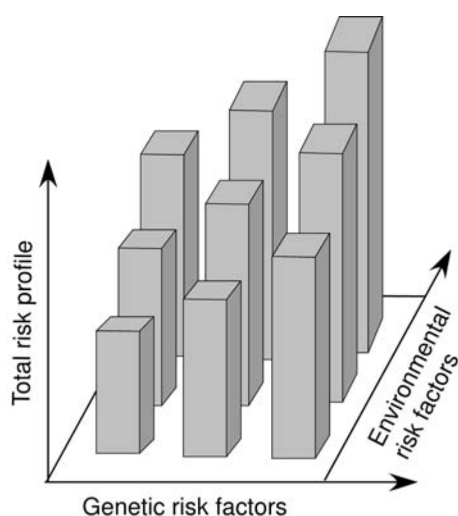

Figure 1. Schematic overview of how the risk of CVD is comprised of genetical and environmental risk factors. The more risk alleles a person has, and the more environmental risk factors that are present, the greater the total risk of CVD.

Although rare mendelian disorders such as Tangier's disease (caused by a mutation in the ABCA1 gene [76]) or hypercholesterolemia (caused by mutations in the LDL receptor (LDLR) gene [77]) cause aggravated atherosclerosis, the etiology of atherosclerosis is in most cases a complex interaction between environmental and genetic factors. Susceptibility genes, or rather certain alleles of these genes, are often frequent within the population but with a rather modest contribution to an individual's total risk of developing a complex disease such as atherosclerosis. For complex diseases, the disease may be caused by several different mechanisms, where many affected subjects are non-carriers of a certain risk allele. If a disease gene has low penetrance, it may be common in unaffected subjects as well. In addition, a risk allele may require a certain genetic background, gene-gene interactions, or the presence of

environmental conditions to contribute to the total risk profile. Copy number variation has recently emerged as a hot topic in genetics. 12% of the genome were found to contain large (>1 kb) DNA sequences with different copy numbers, containing both genes and disease loci [78].

3.1. Genetics of gene expression

The definition of a gene is a sequence of DNA that encodes a protein. Variations in the DNA sequence may influence protein synthesis through several different mechanisms. These DNA variations can be insertion or deletion of single or stretches of nucleotides, or single nucleotide polymorphisms (SNP), where one nucleotide has been replaced by another. Polymorphisms located in the transcribed region of a gene may affect secondary or tertiary structure and stability of the mRNA molecule, thereby affecting protein translation. Polymorphisms that cause amino acid substitutions may affect function or stability of the encoding protein. If a polymorphism is located in the promoter region of a gene, it may affect mRNA transcription efficiency (figure 2). A promoter region polymorphism that affects transcription is called a *Cis*-acting effect. A *trans*-acting effect is when gene expression is affected by another protein or compound. *Cis*-acting effects have been shown to affect tumorigenesis [79] and the response to asthma drug treatment [80]. Several studies have been performed to estimate the extent of *cis*-acting effects on gene expression. Cowles *et al.* showed that 6% of 69 mouse genes contained *Cis*-acting elements [81]. Two studies of monozygotic twins showed that less than 2% of all genes were differently expressed within the twin pairs, and up to 14% differed between unrelated subjects [82, 83]. However, differences in experimental procedures and lack of power make it hard to estimate how frequent these variations really are and to what extent they affect gene expression. In addition, gene expression may be affected by environmental or epigenetic factors. It is plausible to assume that a large number of human genes have *Cis*-acting polymorphisms, but to what degree transcription must be affected to have an impact on phenotype, and how much feedback control or trans-regulatory elements can compensate, remains elusive [84].

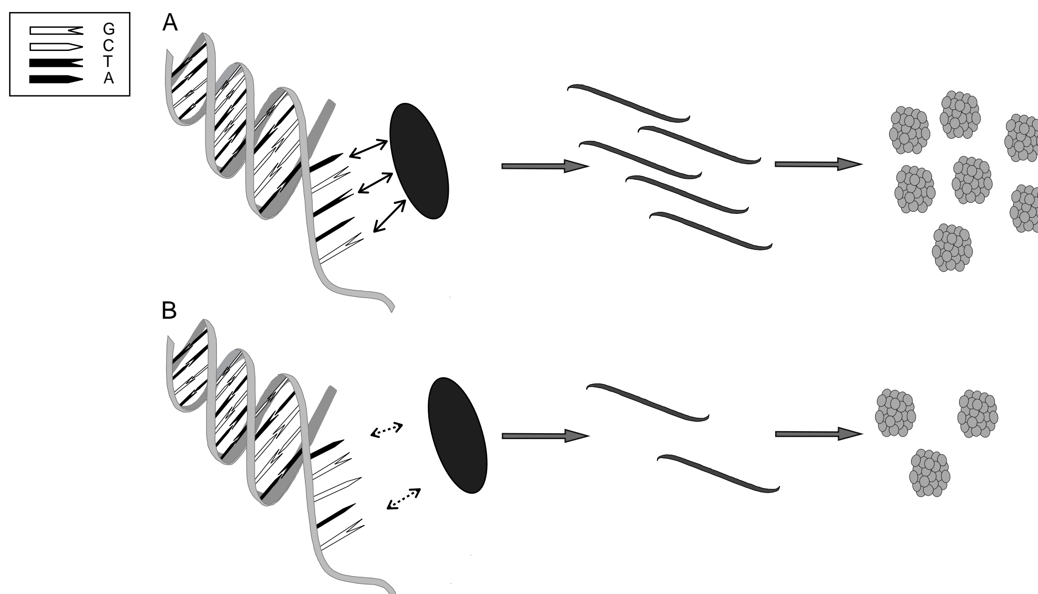

Figure 2. Transcription factors recognize specific DNA sequences and binds to them, which facilitates transcription of downstream genes. A thymine in figure A is substituted for a cytosine in figure B, which alters the recognition site for the transcription factor, causing weaker interaction with the DNA strand. This results in a decrease in mRNA transcription, which may cause a subsequent lower protein yield.

3.2. Genetics of atherosclerosis and CVD

The roughly sixty known mendelian disorders that cause atherosclerosis only account for a small percentage of all cases of atherosclerosis and CVD, although they may provide insights of function of genes and pathways that are important in atherogenesis [43]. There are likely hundreds of genes that to some degree affect the development of atherosclerosis. So far, over a hundred transgenic or genetic knock-out animal models have been shown to modulate atherosclerosis, although it is not certain that such extreme interventions in animals are physiologically relevant in humans. About 40 quantitative trait loci (QTL) for atherosclerosis have been identified in man, and about 30 in mice [85]. The most convincing human QTL so far is 9p21.3, which has been confirmed in 10 different populations [86-88], although the gene or genes causing this association are yet to be discovered. Association studies have found hundreds of potential susceptibility genes for atherosclerosis and CVD, although factors such as weak power and population biases suggest that a large fraction of these genes are in fact false positives. Based on the great variation in penetrance and relative risk, the

susceptibility genes are here roughly classified according to their contribution to the total risk of atherosclerosis and CVD (figure 3). This categorization is for illustrative reasons, and the boundaries between these categories are fluctuant and should not be viewed as a definitive classification.

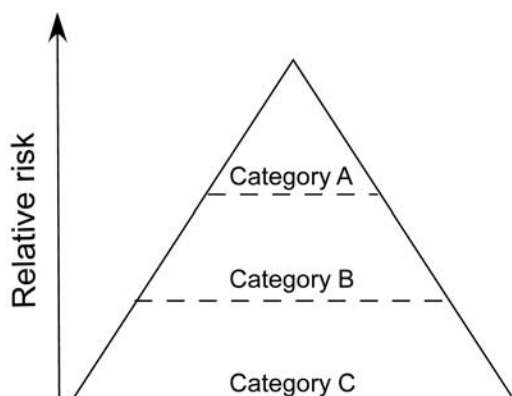

Figure 3. Category A genes have low population prevalence, but high risk of atherosclerosis and CVD. Category B genes have lower risk, but have higher prevalence. Category C genes have low risk and high population prevalence.

Category A consists of genes that have low population prevalence and high penetrance. Example of such genes are classical mendelian disease genes such as LDLR (familial hypercholesterolemia) [89], ABCA1 (Tangier disease) [90], and LDL receptor adaptor protein 1 (autosomal recessive hypercholesterolemia) [91]. Such polymorphisms typically affect function of the gene product through amino-acid substitution, which may have an impact on protein structure and activity.

Category B genes are genes that cause an intermediate increased risk of atherosclerosis and CVD, or genes that cause an increased risk regardless of genetic background or influence of environmental risk factors, that is they have been found to contribute to risk in several studies. Meta-analyses have found association between apolipoprotein E (ApoE) [92], 5,10-@methylenetetrahydrofolate reductase [93], cholesteryl transfer protein [94], plasminogen activator inhibitor 1 and fibrinogen β-chain [95] with CVD.

Category C genes are typically rather frequent within the population, but with either a low contribution to total risk, or requiring specific genetic backgrounds or contribution from environmental factors to contribute to the total risk. These alleles

typically contribute to disease risk by affecting the amount of protein synthesized rather than protein function.

It is difficult to determine if an unsuccessful repeating of a positive association is due to variations in phenotype assessment, differences in genetic background or exposure to other risk factors, lack of power, population stratification or if it is a false positive. Using a cut-off of $p<0.05$ for positive association means that 5% of all findings are Type I errors, false positives, but this figure is likely much higher due to the factors mentioned above.

Population stratification is used to describe the phenomena when a population or cohort consists of several ethnic groups or subpopulations. If the polymorphism is more prevalent in one group, and this group is for some reason overrepresented for the disease, this will cause a false positive association [96]. If the contribution of a polymorphism is weak, it will in most cases not show up in a case-control study of a few hundred subjects. This means that a positive association is likely due to sample variation, and that the strength of the association is over-estimated in a positive result. This makes the finding harder to reproduce, and requires a larger validation population [97]. Variations in genetic background and environmental factors explains some of the difficulties in repeating association studies in man, whereas discoveries in experimental animals are more easily reproduced [98].

In a comparison of association studies with different complex traits over 600 positive hits were found [97]. Of these, 166 had been studied at least three times, and only six were consistently repeated. Of the remaining 160, 97 were repeated at least once. Extrapolation of these data suggests that 3.6 % of all associations are category B genes according to the classification used here. The 58 % that were repeated at least once are likely category C genes. The remaining 38% that failed to replicate are either false positives or category C genes, requiring specific circumstances to contribute to total risk.

4. Aims

The aim of this thesis was to identify mechanisms that are important for atherogenesis, and to increase our understanding of why some people are more susceptible to atherosclerosis and CVD than others. The specific aims were:

To identify genes predominantly expressed in macrophages and atherosclerotic plaques, and to investigate if they affect atherogenesis.

To identify genes with an altered expression in macrophages from subjects with atherosclerosis compared to macrophages from control subjects, and to evaluate them as susceptibility genes for atherosclerosis.

5. Study cohorts

5.1. The Göteborg Atheroma Study Group

The Göteborg Atheroma Study Group was initiated to create a biobank consisting of human endarterectomies, surgically removed carotid plaques, from patients with clinical atherosclerotic disease. The patients were diagnosed with stroke, TIA or amaurosis fugax. In addition, whole blood, serum and plasma from the patients were collected. More information can be found at www.wlab.gu.se/GASG/.

5.2. The INTERGENE study

The INTERGENE study was designed to study the INTERplay between GENEtical susceptibility, environmental factors and psychosocial background for the risk of CVD. This cohort is comprised of randomly sampled individuals from a source population, consisting of all inhabitants that were living in the region of Västra Götaland (western Sweden) at April 1, 2001, aged 25 to 74 at the time of sampling. The sampling continued until the end of December 2004 when 3600 members of the target population sample had been examined. During the same time period slightly more than 800 male and female survivors of coronary heart disease (CHD), myocardial infarction or unstable angina, were identified from the same source population as the sampled cohort. Already known coronary cases with new attacks as well as first time patients were included. 617 cases willing to participate were included. Blood samples were drawn from four weeks to several months after the coronary event in order to avoid the acute phase effect. At this point the majority of the CHD cases were on lipid lowering drugs which are reflected in their lipid profile. A total of 617 control subjects who were matched for sex and age (±2 years) with the CHD cases were recruited from the above mentioned cohort of 3600 subjects. The control subjects had no known clinically CVD at the baseline examination and CVD incidence reported during the study period. The INTERGENE research program and procedures are further described at www.sahlgrenska.gu.se/intergene and by Berg *et. al* [99, 100].

5.3. The Macrophage INTERGENE study

The Macrophage INTERGENE study was designed to identify novel susceptibility genes for atherosclerosis and CHD. The study consists of 15 subjects with sub-clinical atherosclerosis and a family history of CHD, and 15 control subjects. All participants were identified from the INTERGENE study. By choosing subjects with a family history of CHD, the chance of finding susceptibility genes for atherosclerosis are likely to be increased. Additional reasons for not directly studying patients with CHD are that the clinical event or the medication that these patients often receive may contribute to gene expression alteration. The outline of this study is described in figure 4.

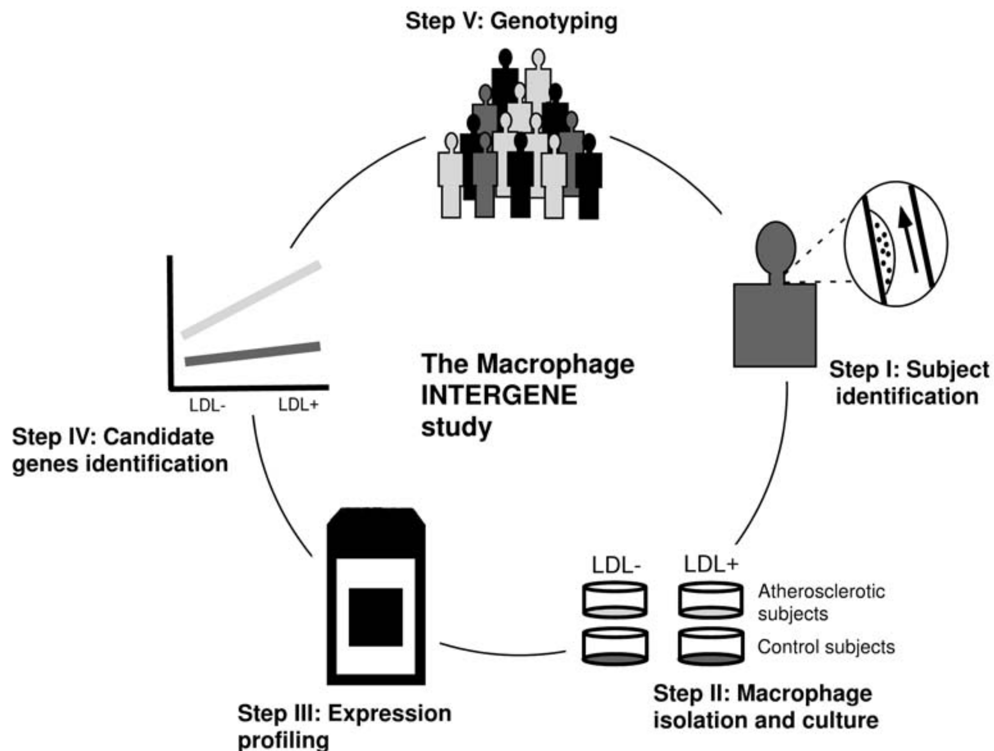

Figure 4. The gene identification strategy used in the Macrophage INTERGENE study. The subjects were identified from the INTERGENE study, containing 800 subjects with a history of CHD (black), 400 first-degree relatives to the cases (dark grey) and 3600 matched control subjects (light grey). 15 subjects with atherosclerosis were identified in the relative group and 15 subjects with no atherosclerosis were selected from the control group, Step I. Macrophages were derived and cultured with or without mmLDL for 24 h, Step II. Macrophage RNA was used to generate DNA microarray expression profiles, Step III. Genes with different expression between atherosclerotic subjects and controls were identified, Step IV. The subjects of the INTERGENE population were genotyped for candidate gene SNPs, Step V.

All participants were screened with ultra sound in the carotid and femoral arteries for atherosclerotic plaques. An intima thickness 50% thicker than its neighboring sites was considered as an atherosclerotic plaque [101]. Inclusion criteria in the case group were at least one plaque in either the carotid or the femoral artery, and a first-degree relative with a history of CHD. Inclusion criteria in the control group were no detectable plaque in the carotid or femoral arteries and no family history of CHD. Exclusion criteria for both groups were age <40 years, clinical cardiovascular disease, type II diabetes, severe hypercholesterolemia, severe chronic disease, current infection (C-reactive protein >5mg/L) and smoking during the last ten years. Addition exclusion criterion for the control group was hypertension. The atherosclerotic subjects and the control subjects were matched for age and sex. However, the atherosclerotic subjects had higher systolic blood pressure and higher total cholesterol levels (table 3).

Table 3	Subjects with atherosclerosis	Control subjects
Age	58.2±2.6	57.0±2.1
Glucose (mM)	5.0±0.2	5.1±0.2
SBP (mmHg)**	144.2±4.2	126.4±3.6
DBP (mmHg)	77.9±2.9	74.2±2.2
Total cholesterol (mM)*	6.0±0.2	5.3±0.2
LDL cholesterol (mM)	3.8±0.2	3.2±0.2
HDL cholesterol (mM)	1.4±0.1	1.6±0.1
Triglycerides (mM)	1.8±0.3	1.4±0.1

Table 3. Characteristics of the subjects in the Macrophage INTERGENE study. The subjects were matched by age and sex. * p=0.034, ** p=0.0052.

From both groups, monocyte-derived macrophages were isolated from buffy coats and cultured with or without minimally modified LDL (mmLDL) to model foam cell formation. Macrophages were chosen since they are regarded as a key cell type in atherogenesis, and alterations of macrophage gene expression may be important in atherosclerotic plaque formation. In addition, by differentiating the monocytes to macrophages in cell culture for seven days, the influence of environmental factors are reduced. RNA was isolated from the macrophages and foam cells and used for DNA microarray analysis. Genes that were differently expressed ($p<0.05$) in both macrophages and foam cells from subjects with atherosclerosis compared with macrophages from control subjects were selected for further analysis. Genes that were

regulated in one direction in macrophages and the other in foam cells were excluded. Further, the genes identified were cross-referenced with public databases such as PubMed, to see if these genes have been studied in the context of atherosclerosis or CVD previously, if there are transgenic mouse models, if they are located in a QTL linked to atherosclerosis or any of its risk factors or if there are any known polymorphisms that might affect gene expression. Candidate genes may be genotyped in the INTERGENE study to test for association with CHD.

6. Methodological considerations

The methodological procedures used in this thesis are described in detail in each paper respectively. Below I will focus on and discuss some of the critical methodological aspects of the thesis.

6.1. Macrophage cell culture

There are several sources of macrophages or macrophage-like cells for *in vitro* studies of atherosclerosis. In this thesis, human monocyte-derived macrophages, isolated from peripheral blood mononuclear cells, were used. Other common sources are bone marrow-derived macrophages, cell lines and peritoneal macrophages isolated from the abdominal cavity in mice. Example of cell lines are murine tumor-derived RAW 264.7 and J774.A1, and human lymphoma-derived U937 and leukemia-derived THP-1, which proliferate as monocyte-like cells and can be *in vitro*-differentiated into macrophage-like cells in culture. Embryonic stem cells from mice have also been used as a source of macrophages to study foam cell formation [102]. These cells were found to resemble mouse peritoneal macrophages more than cell lines RAW 264.7 and J774.A1 [103].

Cell line experiments have the advantage of being highly reproducible, which means experiments can be performed at smaller scale which will reduce cost. High reproducibility is desired for functional and mechanistic studies, which require low variability. Cell lines are more easily transfected than primary cells, they are easy to store and no donors are needed to start up an experiment. However, cell lines are derived from tumor cells and are kept through many passages, which may affect properties of the cells.

Human primary cells are probably a more accurate model to study a disease process. However, humans are genetically diverse, and thereby human cells have the disadvantage of showing large variability. In addition, the cells may be affected by a number of factors from the host, such as variations in diet, exercise, smoking and drinking habits etc.

6.2. DNA microarray analysis

Microarray technology was developed to study the expression of hundreds or thousands of genes simultaneously. By attaching pieces of nucleic acid, probes, complementary with mRNA from genes of interest to a surface, the variation of gene expression in different samples can be estimated by measuring the amount of mRNA hybridization to the probes. Thus, multiple genes can be studied under identical experimental conditions.

Several different platforms or technologies for microarray have been developed, ranging from the first variants with a few hundred genes to microarray chips representing all predicted genes in human genome and transcript variants of these genes. In this thesis, the Affymetrix (Paolo Alto, CA) DNA microarray technology was used. The chip types used in this thesis uses 11 probes for each transcript. The probes are 25 base-pairs long and are synthesized directly onto the chip. These probes are complementary with sequences located near the 3' end of the gene. In addition, for each probe matching a sequence of the transcript, there is a mismatch probe, in which one of the bases have been substituted to estimate the amount of background signal from unspecific hybridization. The microarrays that have been used in this thesis are U133A, with ~15000 genes, and U95 with ~10000 genes. Since these projects were performed, new chips have been developed, including the Affymetrix U133 2.0, which is said to include all known genes.

The introduction of SNP chips have provided finer mapping and have been used to identify QTLs for complex disease [88, 104]. However, many of the identified QTLs for atherosclerosis and CVD still spans large chromosomal regions which may contain many genes. Previous studies have shown expression profiling can be used to identify genes within QTLs for different traits [105, 106].

DNA microarray technology has been used for vastly different purposes such as identification of genes involved in geotaxis in fruit flies [107], to predict clinical outcome of renal cancer [108], to identify subtypes of obesity in mice [109] and

classification of cancer subtypes in man [110, 111], and to identify markers for disease [112, 113]. Thus, DNA microarray analysis shows great promise in identifying genes involved in the pathogenesis of complex diseases.

6.3. Genes predominantly expressed in a tissue or cell type

While all cells in a multicellular organism share the same genotype, they display a vast spectrum of different phenotypes. This is partly because only a fraction of all proteins encoded by the DNA is synthesized in the different cell types. By regulating RNA transcription, the cell controls the amount of proteins produced, thereby affecting its phenotype. If the expression levels of a gene encoding a certain protein is much higher in one cell type than in other cell types, it is plausible that this protein is important in that cell type. Examples of important genes expressed only in specific cell types are insulin produced in the pancreatic β-cells and growth hormone produced in the pituitary gland. Such genes may be potential drug targets or be used as markers in histological staining or as biomarkers in blood, saliva or urine if secreted.

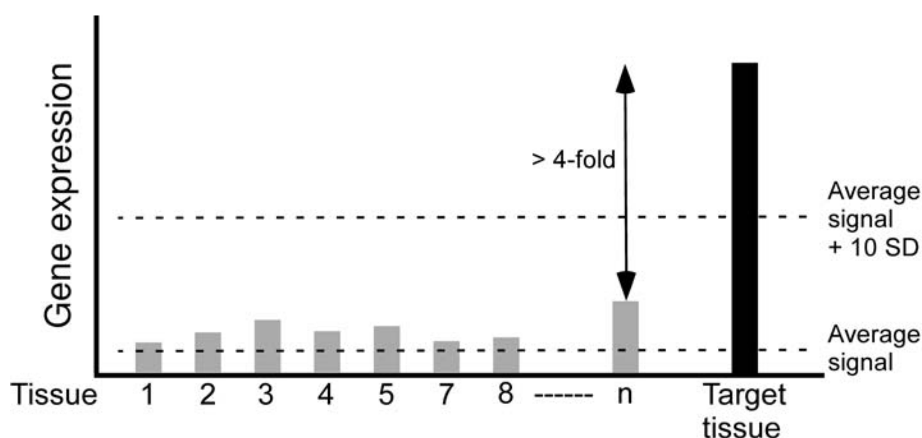

Figure 5. Outline of our identification strategy of genes predominantly expressed in a tissue or cell type. The expression of each gene on the DNA microarray is compared across the tissue panel. Genes that have an expression at least 4-fold higher in the target tissue or cell type than the tissue with the second highest expression, and at least 10 SD above the average expression across the tissue panel are considered as tissue specific.

We have developed a DNA microarray based strategy to identify genes predominantly expressed in a target tissue or cell type (figure 5). This strategy is based on comparing expression profiles from the target tissue with reference profiles from a tissue panel,

consisting of expression profiles of several other tissues and cell types. Ideally, the tissue panel should include all human cell types. In paper I, 56 reference expression profiles were used, and in paper II, 80 reference expression profiles were used. The expression profiles were obtained as duplicates from the GNF data base [114]. The expression of each gene is compared across the tissue panel to identify the tissue or cell type with the highest expression. Genes that have at least four times higher expression in the target tissue than the tissue or cell type with the highest expression of the reference profiles, and at least ten SD above the average expression of the reference profiles are considered as predominantly expressed in the target tissue.

6.4. Genotyping analysis

Repeat elements and SNPs are common in the genome, and may affect gene expression and protein function. Several different methods for detection of these variations exist, which may be more or less suitable depending on the polymorphism and genetic surroundings. Three different methods for genotyping analysis have been used in this thesis.

6.4.1. TaqMan based genotyping analysis

TaqMan based genotyping (Applied biosystems, Santa Clara, CA) is suitable for SNP genotyping. The technology is based on a polymerase enzyme with endonuclease activity. The PCR is performed using two primers, and with two probes, oligonucleotides complementary to the alleles of the SNP. A fluorescent reporter molecule is attached to one end of the probe and a quencher molecule is attached in the other end of the probe. The quencher prevents the reporter from emitting light when the probe is intact. The two probes have different reporter molecules, and during each PCR cycle, the probes are digested by the endonuclease activity of the polymerase. If the subject is homozygous for one allele, there will be fluorescence from only one reporter. Correspondingly, heterozygous subjects will have fluorescence from both probes. This method is reliable and requires very little starting material. However, since it is a standardized method with fixed buffer-solution and PCR cycles, it is not always possible to design primers and probes that works at these

conditions. In addition, if the SNP is located near another SNP, it can be difficult to design primers and probes.

6.4.2. RFLP based genotyping analysis

If it is not possible to design primers or probes for a TaqMan based assay, restriction fragment length polymorphism (RFLP) assay may be used. This method is based on the use of regular PCR and restriction enzymes (RE). REs recognize specific DNA sequences, where they cut the DNA strands. If a polymorphism is altering, or creating, an RE recognition site, RFLP may be used to detect this polymorphism. A PCR is performed and the RE is added to the product. After incubation, electrophoresis is performed to separate DNA fragments based on size. If the restriction site is present, the product is cleaved into two smaller products, and if it is not, one larger band is visible on the gel. Correspondingly, if the subject is heterozygous, there will be three bands. This analysis requires more manual work and more starting material than the TaqMan based analysis. In addition, since the restriction enzyme is added to the PCR product, there is a risk of cross contamination. There is also a risk for incomplete RE digestion, which may cause false heterozygous calls.

6.4.3. Fragment analysis

Repeated elements are common in the genome. If a short sequence is repeated several times, it is quite common that there will be several alleles for this element. A common method to estimate the number of repeats for a short sequence is fragment analysis. A PCR product of the region surrounding the repeat element is generated using a fluorescently labeled primer. The product is then separated using capillary electrophoresis. By using a ladder consisting of marked oligonucleotides of known lengths, and often reference samples of known genotypes, it is possible to estimate the number of repeats of each allele. Like RFLP based analysis, there is a risk of cross contamination since the ladder has to be added to the PCR product. This method is also more labor and time consuming than TaqMan analysis, and is less accurate, since it is sometimes hard to distinguish two alleles. In addition, repeat elements of various lengths typically results in more PCR errors.

7. Results and discussion

7.1. Papers I & II

The aim of papers I & II was to identify genes predominantly expressed in human macrophages and carotid plaques respectively. Such genes are likely to be important for cellular function, and genes predominantly expressed in macrophages or carotid plaques may be important in atherogenesis. Potentially, proteins encoded by such genes could be used as biomarkers for atherosclerosis and CVD. In Paper I, 23 genes were identified as predominantly expressed in human macrophages (table 4) and in Paper II, eight genes were predominantly expressed in human carotid plaques (table 5). These lists should not be viewed as the definite "macrophage"- or "plaque-genes", but rather as the result of a screening process to identify genes that may be important in macrophage and plaque biology. For example, the known macrophage marker CD68 did not meet our criteria as macrophage specific.

Genes such as MSR1, capping protein gelsolin-like and integrin alpha are known to be highly expressed in macrophages, whereas genes such as KIAA0930 and hexokinase 3 were previously not known to be mainly expressed in macrophages. Several of the identified macrophage specific genes were previously known to affect atherosclerosis. For example, inhibition of tumor necrosis factor (ligand) superfamily, member 14 signaling reduces hyperlipidemia in LDLR$^{-/-}$ mice [115]. Another macrophage specific gene, plasminogen activator, urokinase receptor (PLAUR) was further studied by our group and we found that PLAUR was highly expressed in carotid plaques, and that PLAUR protein expression correlated with plaque instability [116]. This shows that our hypothesis that macrophage specific genes may be important for atherosclerosis was adequate.

Studies of gene expression in atherosclerotic plaques are complicated by the difficulty in obtaining an optimal control tissue, as the properties of arteries vary between different regions of the body [117]. In addition, as the plaque develops the cellular composition of artery wall is altered by the infiltration of SMCs, macrophages and other cells. By viewing the plaque as a unique tissue, and comparing it with a panel of

reference expression profiles, the problem of obtaining a relevant control tissue is circumvented.

Table 4. Genes predominantly expressed in human macrophages

Probe set #	Name	Symbol	Fold[1)]	2nd tissue[2)]
31742_at	Tumor necrosis factor (ligand) superfamily, member 14	TNFSF14	32.2	T-cells
34041_at	Chemokine (C-C motif) ligand 22	CCL22	24.5	Pancreas
1057_at	Cellular retinoic acid binding protein 2	CRABP2	15.1	Nasal mucosa
41516_at	SNARE protein Ykt6	YKT6	12.2	-
677_s_at	Acid phosphatase 5, tartrate resistant	ACP5	11.3	Spleen
31859_at	Matrix metalloproteinase 9	MMP9	10.8	Spleen
38391_at	Capping protein (actin filament), gelsolin-like	CAPG	10.2	Lung
37068_at	Phospholipase A2, group VII	PLA2G7	10.0	Thyroid
41783_at	Cellular retinoic acid binding protein 2	CRABP2	9.7	THY+
41169_at	Plasminogen activator, urokinase receptor	PLAUR	9.2	HUVEC
33106_at	Nuclear receptor subfamily 1, group H, member 3 (LXR-alpha)	NR1H3	8.8	Omental Adipocyte
37061_at	Chitinase 1 (chitotriosidase)	CHIT1	8.5	Pancreas
33646_g_at	GM2 ganglioside activator protein	GM2A	8.4	Placenta
38745_at	Lipase A, lysosomal acid, cholesterol esterase	LIPA	7.0	Thymus
512_at	Nuclear receptor subfamily 1, group H, member 3 (LXR-alpha)	NR1H3	6.7	Liver
36753_at	Leukocyte immunoglobulin-like receptor, subfamily B, member 4	LILRB4	6.5	Thymus
35820_at	GM2 ganglioside activator protein	GM2A	6.2	Placenta
36372_at	Hexokinase 3 (white cell)	HK3	6.0	Whole Blood
37980_at	CBF1 interacting corepressor	CIR	5.8	WSU
189_s_at	Plasminogen activator, urokinase receptor	PLAUR	5.7	HUVEC
36758_at	Solute carrier family 38, member 6	SLC38A6	5.1	K-562
36833_at	Galactosidase, alpha	GLA	5.0	HL60
37603_at	Interleukin 1 receptor antagonist	IL1RN	5.0	Fetal liver
39433_at	KIAA0930 protein	KIAA0930	4.8	HL60
38533_s_at	Integrin, alpha M	ITGAM	4.3	Whole Blood
39981_at	Macrophage scavenger receptor 1	MSR1	4.2	S.c. AT
32387_at	Lysophospholipase 3 (lysosomal phospholipase A2)	LYPLA3	4.1	Heart

1) Fold change between macrophages and the tissue or cell type in the reference panel with highest expression
2) The tissue or cell type with highest expression in the reference panel

As for the macrophage specific genes discussed above, several of the genes identified as plaque specific, such as complement component 3a receptor 1 [118] and matrix metalloproteinase 12 [119], have been implicated in atherosclerosis, again suggesting that our approach produces results relevant for the disease.

Table 5. Genes predominantly expressed in human carotid plaques

Probe set #	Name	Symbol	Fold[1)]	2nd tissue[2)]
32128_at	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	CCL18	11.3	Lymph node
209924_at	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	CCL18	9.2	Thymus
214770_at	macrophage scavenger receptor 1	MSR1	7.4	Pons
204580_at	matrix metalloproteinase 12 (macrophage elastase)	MMP12	6.4	Superior cervical ganglion
209071_s_at	regulator of G-protein signaling 5	RGS5	4.7	Thyroid
214265_at	integrin, alpha 8	ITGA8	4.4	Superior cervical ganglion
201792_at	AE binding protein 1	AEBP1	4.3	Uterus corpus
214954_at	KIAA0527 protein	KIAA0527	4.1	Ciliary ganglion
209906_at	complement component 3a receptor 1	C3AR1	4.0	Monocytes

1) Fold change between macrophages and the tissue or cell type in the reference panel with highest expression

2) The tissue or cell type with highest expression in the reference panel

7.1.1. IL1RN

One of the macrophage specific genes in Paper I, interleukin receptor 1 antagonist (IL1RN) is a signal molecule with anti-inflammatory properties, inhibiting signaling of interleukin 1 alpha (IL-1α) and interleukin 1 beta (IL-1β) by interacting antagonistically with the IL-1 receptor. Several animal studies have implicated an important role for IL1RN in atherosclerosis. IL1RN deficient mice have lower cholesterol levels, while over expression of IL1RN in $LDLR^{-/-}$ mice increases plasma cholesterol while reducing lesion size [120]. $ApoE^{-/-}IL1RN^{+/-}$ mice have an increased lesion size compared with $ApoE^{-/-}$ [121], and $ApoE^{-/-}$ mice that receive IL1RN infusion have decreased lesion size [122], whereas $ApoE^{-/-}IL\text{-}1\beta^{-/-}$ mice have reduced plaque formation [123].

We found that IL1RN was down regulated by oxLDL, both on RNA and protein level, suggesting that oxLDL acts pro-inflammatory partly by inhibiting this signal molecule. The high expression of IL1RN in macrophages found in our study suggests that the effects of IL-1α and IL-1β may be attenuated when studying macrophages *in vitro*. Genetic studies in humans have found inconclusive association between a repeat

element in the IL1RN gene with CAD [124-126]. The IL-1α&β/IL1RN ratio is increased in subjects with unstable angina compared to subjects with stable angina [127], and recombinant IL1RN have beneficial effects in patients with rheumatic arthritis, without an increased risk of infection and few side effects [128]. This suggests that IL1RN reduce the inflammatory process of atherosclerosis, however it may have non-beneficial effects on plasma cholesterol. A clinical trial where MI patients are treated with recombinant IL1RN is currently conducted in the UK [129] and may further elucidate the effects of this cytokine in man.

7.1.2. LXRα

The other gene selected for further analysis in Paper I was nuclear receptor subfamily 1, group H, member 3, more commonly referred to as liver X receptor alpha (LXRα). LXRα is a transcription factor, involved in cholesterol homeostasis. Several studies have implicated a role of LXRα in atherosclerosis, such as inhibition of IL-6 [130] and activation of ApoE [131]. $ApoE^{-/-}LXR\alpha^{-/-}$ mice have increased lesion size and more cholesterol accumulation in peripheral tissues compared with $ApoE^{-/-}$ mice, and this effect is reduced by ligand activation of liver X receptor beta (LXRβ) [132]. We found that LXRα expression is down regulated by hypoxia in macrophages. This could contribute to the acceleration of atherosclerosis caused by hypoxia [133], since absence of LXRα and LXRβ cause a substantial increase in macrophage accumulation of cholesterol [134]. However, while over expression of LXRα cause up regulation of cholesterol efflux gene ABCA1 in RAW264.7 macrophages [135], we found no down regulation of ABCA1 in macrophages by hypoxia, data not shown. $ApoE^{-/-}$ mice receiving $LXR\alpha\beta^{-/-}$ bone marrow have increased lipid levels and increased atherosclerotic lesion size than mice receiving $LXR\alpha\beta^{+/+}$ bone marrow [134]. This together with our finding that LXRα is predominantly expressed in human macrophages suggests that macrophage expression of LXRα is important in atherogenesis.

7.1.3. CCL18

In paper II, eight genes were identified as predominantly expressed in human carotid plaques. The gene with the highest fold change between carotid plaque and the tissue

with the second highest expression was chemokine CC motif ligand 18 (CCL18). Chemokines are a large super family of low-weight molecules with chemotactic properties inducing leukocyte migration, growth and activation [136]. CCL18 expression was localized to a subset of macrophages in carotid plaques, and was expressed at higher levels in macrophages from subjects with atherosclerosis compared with macrophages from control subjects. The G allele of the rs2015086 SNP in the CCL18 promoter region was associated with higher macrophage CCL18 gene expression, although this was based on a small number of subjects.

The role of CCL18 in atherosclerosis is poorly investigated. *In situ* hybridization has shown that CCL18 is expressed in macrophages in human atherosclerotic plaques [137]. CCL18 is expressed at higher levels in symptomatic carotid plaques than in asymptomatic carotid plaques from the same individual [138] and in unstable regions of carotid plaques compared with stable regions of the same plaque [139]. A recent study showed that high plasma levels of CCL18 were associated with an increased risk of acute coronary event during an 18 month follow up period [140]. CCL18 is chemotactic for macrophages [141] and naïve T-cells [142-144]. Depletion of several other chemokines in atherogenic mouse models result in smaller plaques with fewer infiltrating macrophages [49, 145-149]. Since many chemokines thus seems pro-atherogenic, and CCL18 has a similar role in attracting macrophages and T-cells, this indicates that CCL18 is pro-atherogenic. There are however factors that makes functional studies of CCL18 in atherogenesis difficult. No agonist receptor for CCL18 has been identified, although CCL18 has been shown to have antagonist effects on chemokine CC motif receptor 3 [150]. Furthermore, rodents lack a CCL18 homologue, although it has been shown that human CCL18 is chemotactic for murine T-cells [151].

High levels of CCL18 have been reported in a number of diseases [152], and it has been suggested as a biomarker for Gaucher's disease [153] and for pulmonary fibrosis in systemic sclerosis [154]. However, we found no differences in CCL18 serum levels between patients with symptomatic cerberovascular disease and control subjects, suggesting that it may not be a suitable marker for cerberovascular disease.

7.2. Papers III & IV

In paper III & IV, the aim was to identify novel susceptibility genes for atherosclerosis and CHD using gene expression analysis. By comparing the expression profiles from the Macrophage INTERGENE study, 27 genes were identified as differently expressed in macrophages from subjects with atherosclerosis compared to macrophages from control subjects. Of these, seven genes were also significantly regulated by mmLDL, and six genes were located within QTLs previously linked to CVD (table 6). Three genes, CD44, insulin receptor substrate 2 (IRS2) and solute carrier family 11 member 1 (SLC11A1) were selected for further analysis.

7.2.1. IRS2

IRS2 was expressed at higher levels in macrophages from subjects with atherosclerosis compared with macrophages from control subjects, and IRS2 expression was also increased by mmLDL stimulation. IRS2 mediate intracellular signaling in response to insulin, insulin-like growth factor 1, growth hormone and various cytokines [155]. Polymorphisms in the IRS2 gene have been linked to both obesity [156] and diabetes [157], and subjects with metabolic syndrome have lower IRS2 expression in white blood cells compared with metabolic healthy subjects [158], which indicates that normal function of this gene is required for a balanced and healthy metabolism.

The implication of IRS2 in the context of atherosclerosis has only been studied to a limited extent, and the underlying mechanisms are poorly understood. IRS2$^{-/-}$ mice develops insulin resistance [159], with significantly higher triglyceride and cholesterol levels, as well as higher systolic blood pressure compared to wild type mice [160]. IRS2$^{+/-}$ApoE$^{-/-}$ murine macrophages have increased uptake of acetylated LDL caused by an increased expression of CD36 and MSR1, as well as an increased expression of CCL2 [158]. IRS2$^{+/-}$ApoE$^{-/-}$ mice have increased atherosclerosis compared to ApoE$^{-/-}$ mice [161], while ApoE$^{-/-}$ mice reconstituted with ApoE$^{-/-}$IRS2$^{-/-}$ bone marrow have smaller lesion than mice reconstituted with ApoE$^{-/-}$IRS2$^{+/+}$ cells

Table 6. Genes with altered expression in macrophages from subjects with atherosclerosis compared with macrophages from control subjects

		Average signal intensity				**Fold change**				**t-test**					
Gene symbol	*Probe set #*	*Ath baseline*	*Ath mmLDL*	*Cont baseline*	*Cont mmLDL*	*Baseline Ath/ Cont*	*mmLDL Ath/ Cont*	*Cont mmLDL/ Baseline*	*Ath mmLDL/ Baseline*	*Baseline Ath/ Cont*	*mmLDL Ath /Cont*	*Cont mmLDL/ Baseline*	*Ath mmLDL /Baseline*	*Genomic region*	*Known QTL*
ABCC3	208161_s_at	139	210	96	126	**1.44**	**1.67**	**1.31**	**1.51**	**0.0232**	**0.0032**	**0.0456**	**0.0000**	17q22	
ANP32A	201051_at	319	334	379	382	**0.84**	**0.88**	1.01	1.05	**0.0108**	**0.0266**	0.8535	0.2200	15q22.3-q23	
ARF6	214182_at	131	120	204	178	**0.64**	**0.67**	0.88	0.91	**0.0139**	**0.0301**	0.1021	0.4399	7q22.1	
CAMK2G	212757_s_at	71	100	87	123	**0.83**	**0.81**	**1.42**	**1.40**	**0.0471**	**0.0191**	**0.0035**	**0.0017**	10q22	
CD44	204489_s_at	631	684	434	466	**1.45**	**1.47**	1.07	1.08	**0.0135**	**0.0248**	0.4127	0.5042	11p13	FCHL [162]
CD86	205685_at	132	142	109	109	**1.20**	**1.30**	1.00	**1.08**	**0.0212**	**0.0125**	0.9905	**0.0190**	3q21	
COPS7A	209029_at	219	209	254	244	**0.86**	**0.86**	0.96	0.96	**0.0410**	**0.0060**	0.4012	0.4743	12p13.31	H.M. [163]
CRAT	209522_s_at	58	73	46	57	**1.26**	**1.28**	1.25	**1.27**	**0.0220**	**0.0416**	0.0627	**0.0008**	9q34.1	
EPB41L1	212339_at	108	104	137	152	**0.79**	**0.69**	1.10	0.97	**0.0341**	**0.0034**	0.0879	0.6667	20q11.2-q12	MI [164]
EPB41L1	212336_at	85	71	118	97	**0.72**	**0.73**	0.82	0.83	**0.0152**	**0.0304**	0.0922	0.1041	20q11.2-q12	MI [164]
FLJ14146	218546_at	34	38	63	58	**0.53**	**0.66**	0.91	1.13	**0.0017**	**0.0151**	0.2125	0.3515	1q42.11	
IMPA2	203126_at	95	235	69	181	**1.38**	**1.30**	**2.62**	**2.47**	**0.0009**	**0.0144**	**0.0000**	**0.0000**	18p11.1	
INSIG2	209566_at	55	54	44	38	**1.25**	**1.42**	0.86	0.98	**0.0327**	**0.0327**	0.1789	0.7131	2q14.2	
IRS2	209184_s_at	127	246	96	206	**1.33**	**1.19**	**2.15**	**1.93**	**0.0079**	**0.0269**	**0.0000**	**0.0000**	13q34	
KIAA1522	212048_s_at	265	314	228	241	**1.16**	**1.30**	1.06	1.19	**0.0288**	**0.0407**	0.5219	0.0970	1p34.3	
LILRB2	207697_x_at	265	192	208	148	**1.28**	**1.30**	**0.71**	**0.73**	**0.0217**	**0.0176**	**0.0001**	**0.0000**	19q13.4	
LYPLA2P1	216606_x_at	130	122	155	145	**0.84**	**0.85**	0.93	0.94	**0.0015**	**0.0371**	0.1546	0.3801	6p21.32	
MMP19	204575_s_at	425	384	551	469	**0.77**	**0.82**	**0.85**	0.90	**0.0170**	**0.0448**	**0.0304**	0.0806	12q14	
MMP2	201069_at	39	55	24	36	**1.63**	**1.54**	1.49	1.40	**0.0153**	**0.0267**	0.0671	0.0847	16q13-q21	
MXRA7	212509_s_at	88	90	180	195	**0.49**	**0.46**	1.08	1.02	**0.0350**	**0.0103**	0.3040	0.8368	17q25-q25.2	
NCOA4	210774_s_at	1227	1453	953	1181	**1.29**	**1.23**	**1.24**	1.18	**0.0176**	**0.0310**	**0.0043**	0.0556	10q11.2	
PTCRA	215492_x_at	73	60	96	86	**0.76**	**0.71**	0.89	0.83	**0.0169**	**0.0308**	0.2364	0.0899	6p21.3	CAD [165]
SLC11A1	210423_s_at	627	549	452	406	**1.39**	**1.35**	0.90	0.88	**0.0301**	**0.0321**	0.1664	0.0709	2q35	CHD [166]
SLC12A7	218066_at	282	305	328	363	**0.86**	**0.84**	1.10	1.08	**0.0316**	**0.0219**	0.0719	0.1199	5p15	
SLC12A8	219874_at	53	88	27	61	**1.94**	**1.45**	**2.21**	**1.65**	**0.0096**	**0.0443**	**0.0001**	**0.0036**	3q21.2	
SLC7A11	209921_at	67	206	36	130	**1.86**	**1.58**	**3.63**	**3.09**	**0.0305**	**0.0430**	**0.0002**	**0.0000**	4q28-q32	
STUB1	217934_x_at	412	417	495	513	**0.83**	**0.81**	1.04	1.01	**0.0215**	**0.0391**	0.4998	0.8438	16p13.3	CHD [167]
ZNF232	219123_at	50	58	63	74	**0.78**	**0.78**	**1.17**	1.17	**0.0128**	**0.0079**	**0.0482**	0.1359	17p13-p12	

Ath = subjects with atherosclerosis
Cont = subjects without atherosclerosis
Baseline = unstimulated macrophages
mmLDL = macrophages stimulated with mmLDL for 24 h

FCHL = familial combined hyperlipidiemia
Homol = homology to mice susceptibility loci Athsq2

[168]. Thus, there are differences in the effects on atherosclerosis between systemic and hematopoietic knock out of IRS2 in the development of atherosclerosis. High IRS2 expression in macrophages may be pro-atherogenic, an effect that might be augmented by the increased IRS2 expression by mmLDL. We found association between high macrophage expression of IRS2 and the C allele of the -765C→T SNP, and subjects homozygous for the C allele for this SNP had an increased risk of CHD.

IRS2 mediates signaling in response to several stimuli, including insulin and interleukin 4 (IL-4) [155]. Insulin signaling up regulates tumor necrosis factor alpha (TNFα) in THP-1 macrophages [169], but down regulates nuclear factor kappaB and CCL2 in human mononuclear cells after infusion [170]. IL-4$^{-/-}$ApoE$^{-/-}$ mice have 27% smaller plaques than ApoE$^{-/-}$ mice [171]. LDLR$^{-/-}$ mice receiving IL-4$^{-/-}$ bone marrow have smaller plaques than those receiving IL-4$^{+/+}$ bone marrow [172], while IL-4 injections decrease plaque formation in mice [173]. IL-4 is an anti-inflammatory cytokine, but may have other properties that make it pro-atherogenic. Endothelial cells incubated with IL-4 have increased production or reactive oxygen species (ROS) and CCL2 production, and decreased NO production. This is abolished by blocking of PI-3K [174], a signaling target of IRS2 [155]. The PI-3K catalytic subunit p110δ is primarily expressed in leukocytes [175]. IL-4 mediates p110δ signaling via IRS2 phosphorylation in B-cells [176], and blocking of p110δ in human neutrophils decrease ROS production [177]. Therefore, one hypothesis is that IRS2 may act pro-atherogenic by contributing to an increased oxidative stress in the vascular wall, although the mechanism behind the link between high macrophages IRS2 expression and atherosclerosis is not known.

7.2.2. SLC11A1

SLC11A1 expression was increased in macrophages from subjects with atherosclerosis compared with macrophages from control subjects. In addition, SLC11A1 is located in a QTL previously linked to CHD [166], supporting the hypothesis that this may be a candidate gene for atherosclerosis.

A repeat element in the SLC11A1 promoter region was first described by Blackwell *et. al,* who identified four different alleles [178] that have an impact on gene expression [179]. Since the SLC11A1 gene expression was higher in macrophages from subjects with atherosclerosis than in macrophages from control subjects, we genotyped these subjects for the promoter repeat element. Subjects homozygous for allele 2, 2/2-subjects, had significantly higher gene expression than non2/2-subjects, however only three of 27 successfully genotyped subjects were 2/2-subjects. This is in contrast to the results reported by Searle *et al* who reported higher expression of allele 3 using a luciferase assay [179]. The high macrophage expression of SLC11A1 seen in our study may suggest that SLC11A1 is pro-atherogenic. Since SLC11A1 is a transporter of Fe^{2+} [180], and Fe^{2+} enhances LDL oxidation [181], high SLC11A1 may contribute to increased formation of oxidized LDL.

7.2.3. CD44

CD44 was selected for further investigation because it had increased expression in macrophages from subjects with atherosclerosis compared with macrophages from control subjects, and was located in a QTL previously linked with hyperlipidemia [162]. CD44 is a cell surface receptor with hyaluronic acid (HA) as its primary ligand [182], and CD44 is required for cell adhesion to HA [183, 184]. Different functions of CD44 include adhesion of lymphocytes to endothelial cells [185], as well as homing [186, 187], migration [188, 189], rolling [190] and activation [191] of leukocytes. Bacterial lipopolysaccaride and various pro-inflammatory cytokines enhance CD44-HA binding in monocytes, while the anti-inflammatory cytokines IL-4 and interleukin 13 inhibit this binding [192]. The activation of CD44 induce the release of IL-1β and TNFα from monocytes [193].

CD44 stimulates IL-6 secretion in B-cells [194], synovial cells [195], and polynuclear blood cells [196], and we found that $CD44^{-/-}$ mice had decreased circulating levels of IL-6. In myeloma cells, IL-6 induces CD44 expression, and also the binding with hyaluronic acid [197]. We found that CD44 macrophage expression correlated with IL-6 levels in cell culture supernatants, and that IL-6 induced CD44 expression in cultured human macrophages. Taken together, these results suggest a possible feed-

back loop between IL-6 and CD44 in human macrophages. IL-6 deficiency causes obesity and decreased glucose tolerance in mice [198], and IL-6$^{-/-}$ApoE$^{-/-}$ mice have increased plaque formation and serum cholesterol [199] but reduced number of macrophages within the plaque [200]. On the other hand, IL-6 injection in ApoE$^{-/-}$ increases plaque formation and plasma levels of IL-6, IL-1β and TNFα [201], but has no effect on plaque formation in LDLR$^{-/-}$ mice [202]. Interestingly, ApoE$^{-/-}$CD44$^{-/-}$ mice have a 50-70% reduction of atherosclerotic lesions compared to ApoE$^{-/-}$ mice, and a striking 90% reduction of infiltrating macrophages [203]. This suggests that the high expression of CD44 in macrophages from subjects with atherosclerosis may be pro-atherogenic by modulating the inflammatory process.

7.3. Summary

IL1RN was predominantly expressed in human macrophages, at very high expression levels. This suggests that under normal conditions, IL1RN acts as a strong inhibitor of inflammation, by blocking signaling from IL-1α and IL-1β. The down regulation of IL1RN by oxLDL may therefore be an important step in the inflammatory response caused by this molecule (figure 6).

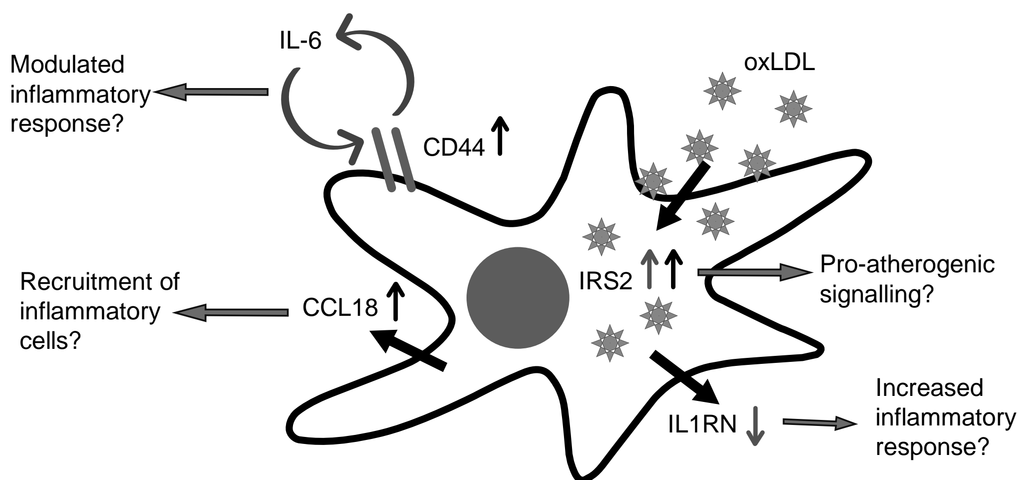

Figure 6. Schematic summary of the major findings of this thesis. Oxidatively modified LDL are taken up by macrophages and induces up regulation of IRS2 and down regulation of IL1RN, grey up or down arrows. IRS2, CD44 and CCL18 where all expressed at higher levels in macrophages from subjects with atherosclerosis compared to macrophages from control subjects, black up arrows.

The high CCL18 expression in carotid plaques, in combination with higher expression in macrophages from subjects with atherosclerosis suggests that high CCL18 expression is pro-atherogenic, possibly by recruiting inflammatory cells (figure 6).

IRS2 macrophage expression was up regulated by oxidatively modified LDL (figure 6), and high macrophage expression of IRS2, caused by the C allele of the -765C→T SNP, was associated with atherosclerosis (figure 6). This allele was also associated with CHD, suggesting that IRS2 is a novel susceptibility gene for atherosclerosis and CHD.

The positive feed-back loop between IL-6 and CD44 may cause aggravated atherosclerosis by modulating the inflammatory response, figure 6.

8. Concluding remarks

The specific aims of this thesis were to identify genes predominantly expressed in macrophages and carotid plaques; and to identify genes with altered expression in macrophages from subjects with atherosclerosis compared with macrophages from control subjects. Twenty three macrophage specific and eight plaque specific genes were identified. Several of these genes have been implicated in atherosclerosis previously, which supports our hypothesis that tissue specific genes may be important in the pathophysiology of atherosclerosis. In addition, the microarray based strategy used to identify genes in this thesis has also been used to identify genes predominantly expressed in adipocytes [204], and could be applied on other tissues and cell types as well to search for novel disease mechanisms.

By comparing the expression profiles from the Macrophage INTERGENE study, we identified 27 genes with an altered expression in macrophages from subjects with atherosclerosis compared to macrophages from control subjects. Given the large heterogeneity of atherosclerosis, where several different mechanisms may be involved in the development of the disease, the relatively small sample size is a therefore a limitation of this study. We tried to compensate for this by cross referencing the identified genes with public databases, to further increase the chance of selecting relevant genes. Another consideration is that even if a gene is differentially expressed between subjects with atherosclerosis and control subjects, it could be an effect of the disease, rather than affecting the disease. Genotyping analysis was performed in the larger INTERGENE case-control cohort. However, not all atherosclerotic plaques cause clinical manifestations, and a gene that affects atherosclerosis may thus not be a susceptibility gene of CVD. When designing a study to identify genes that affect the risk of developing a complex disease, large cohorts are desired. In addition, for heterogeneous diseases, in depth phenotyping for separation of subjects with similar diagnosis but with different underlying causes would most likely increase the power of association studies. In this thesis, microarray analysis was used to identify genes that modulate the risk of developing atherosclerosis and CVD, and the genes were further analyzed separately. For complex diseases, caused by multiple genes, it may

be more efficient to study gene-gene interactions and how expression pattern of multiple genes influence disease susceptibility and the field of system biology is emerging as a hot field for research. In addition, combination of several screening methods could further facilitate the identification of genes that are important in complex diseases. For instance, combination of gene expression arrays with SNP arrays could greatly increase the probability of identifying genes relevant to diseases. In the future, as DNA sequencing is rapidly becoming faster and cheaper, full genome sequencing will most likely be a powerful tool when analyzing complex diseases.

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