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Insulin resistance, genetic variation and cytokines; associations to myocardial infarction risk

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To Maria & Per
ABSTRACT

Insulin resistance, inflammation and associated genes are considered factors of potential importance for the pathogenesis of cardiovascular diseases, but their relationship to myocardial infarction (MI) and associated risk factors remains unclear.

In the present thesis, insulin resistance and serum levels as well as genetic variants of interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α), were investigated in the population-based study “the Stockholm heart epidemiological program” (SHEEP) (n=5452). The present analyses were restricted to 1643 men and women, who had suffered a first-time, non-fatal MI event, and 2339 healthy controls. Serum, DNA, anthropometric measurements and questionnaire-based information was available for the present analyses.

In paper I, several genes of potential interest for insulin resistance and MI were identified by searching the public genome-databases. The selected genes were also scanned for the presence of previously described, and novel, genetic variants. Two genes, IL-6 and TNF-α, which were thought to be of particular interest, were then selected for further analysis in SHEEP.

In paper II, the association between insulin resistance, measured as fasting-insulin and HOMA, and MI risk was investigated. Insulin resistance, which is closely related to other metabolic risk factors, infers an increased risk for MI. Insulin resistant individuals, particularly women, also appear to be particularly sensitive to the hazards of smoking.

In papers III and IV, polymorphisms in the IL-6 and TNF-α genes were analysed in relation to their intermediate phenotype (i.e. IL-6 or TNF-α protein levels in serum) and to MI risk. Increased serum levels of both IL-6 and TNF-α are associated with an increased risk for MI. The contribution of these inflammatory markers as risk factors for MI seem to be dependent on gender, as the risk estimates were persistently higher for men. Men with elevated levels of IL-6 or TNF-α, who also display symptoms of the metabolic syndrome are at particularly high risk. Increased levels of these cytokines are most probably mainly regulated by non-genetic influences since the genetic variants of IL-6 and TNF-α, with one exception, do not significantly contribute to the determination of cytokine levels. It is possible that genetic variants in the TNF-α gene may be of some relevance for MI development. Also, IL-6 genotypes should be further investigated in relation to insulin resistance.

The work presented in this thesis has led to the following conclusions: (i) insulin resistance is a risk factor for MI particularly through synergistic interaction with smoking and elevated serum levels of IL-6, (ii) there is a clear gender related discordance in gene-phenotype associations as elevated IL-6 and TNF-α serum levels are stronger markers for MI risk among men compared to women, (iii)healthy male IL-6 -174C carriers have increased insulin levels, and (iv) female TNF-α -238A carriers have a reduced risk for MI.
This thesis is based on the following original articles, which will be referred to in the text by their Roman numerals.


II  Anna M Bennet, Kerstin Brismar, Johan Hallqvist, Christina Reuterwall, Ulf de Faire. The risk of myocardial infraction is enhanced by a synergistic interaction between serum insulin and smoking. European Journal of Endocrinology (2002), 147: 641-647

III  Anna M Bennet, Jonathan A Prince, Guo-Zhong Fei, Louise Lyrenäs, YiHui Huang, Björn Wiman, Johan Frostegård, Ulf de Faire. Interleukin-6 serum levels and genotypes influence the risk for myocardial infarction. Atherosclerosis (2003), In press

IV  Anna M Bennet, Merel C van Maarle, Johan Hallqvist, Ralf Morgenstern, Johan Frostegård, Björn Wiman, Jonathan A Prince, Ulf de Faire. TNF-α serum levels and promotor polymorphisms influence the risk for myocardial infarction. Manuscript (2003)
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<tr>
<td>BLAST</td>
<td>Basic local alignment tool</td>
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<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>DASH</td>
<td>Dynamic allele-specific hybridization</td>
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<td>EDNRA</td>
<td>Endothelin receptor A</td>
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<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
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<tr>
<td>HGMD</td>
<td>Human gene mutation database</td>
</tr>
<tr>
<td>HOMA</td>
<td>Homeostasis model assessment</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>Insulin-like growth-factor binding protein-1</td>
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<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin resistance syndrome</td>
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<td>LT-α</td>
<td>Lymphotoxin-alpha</td>
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<tr>
<td>MI</td>
<td>Myocardial infarction</td>
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<tr>
<td>OMIM</td>
<td>Online inheritance in man</td>
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<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
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<tr>
<td>S</td>
<td>Synergy index score</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
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AIMS

The general aim of this thesis was to study genetic and environmental factors, with particular emphasis on insulin resistance, as risk markers for myocardial infarction.

More specifically, the aims were:

- To identify genetic variants of potential importance for the development of the insulin resistance syndrome and MI.
- To study insulin resistance as a risk factor for MI.
- To study the relationship between genetic variation in the IL-6 gene, serum levels of IL-6 and the risk for MI.
- To study the relationship between genetic variation in the TNF-α gene, serum levels of TNF-α and the risk for MI.

Figure 1 Insulin resistance, cytokines, and genetic variation, and potential mechanisms through which they may act as risk factors for MI.
INTRODUCTION

Over the years, great efforts have been made to identify agents that can cause or contribute to the development of MI. Many such factors are life-style related (so called environmental risk factors), but individuals may also be born with a predisposition for the disease carried by variations in their genetic make-up (so called genetic risk factors). MI development is the result of exposure to a cocktail of environmental and genetic risk factors. These may act individually or in combination with other risk factors to influence the development of the disease. A person’s genetic make-up is determined at conception, and his/her environmental experiences continues throughout life. The genetic predisposition may be favourable, or unfavourable, with regard to the risk of developing heart disease. Likewise, environmental exposures may also be beneficial or detrimental. However few, if any, individual genetic or environmental agents are sufficient to predict disease development, and it is the interactions between the genetic and environmental exposures that in the end determine an individual’s phenotype (Figure 2).

Myocardial infarction and Insulin resistance

Myocardial infarction (MI) arises when a coronary artery is obstructed, leading to damage to an area of the myocardial tissue. The underlying cause of MI, atherosclerosis, can start to develop as early as in childhood and progress undetected throughout life. In a large atherosclerotic plaque the outermost
shell, the fibrous cap, can burst. This causes a slight bleeding that can lead to the formation of a thrombus, which can block the vessel leading to inadequate blood flow, giving rise to an MI\(^1\). Most often, MI incidents occur after the age of 50, but it is not too uncommon among younger individuals. Common risk factors for MI include smoking, dyslipidaemia, familial predisposition and high blood pressure. Life-style related factors such as diet, obesity, stress and lack of physical activity also contribute to the disease.

One of the more recently established risk markers for MI is insulin resistance. Insulin resistance is reduced sensitivity of the peripheral tissues to the effects of insulin. In other words, insulin resistance is present when a normal concentration of insulin fails to produce a normal response. Insulin resistant states are characterised by a decreased ability of insulin to stimulate glucose uptake by muscle and fat and also to inhibit hepatic glucose production\(^2\). There is a strong correlation between obesity and insulin resistance\(^3\), and smokers are relatively more insulin resistant compared to non-smokers\(^4\). Many large epidemiological studies have evaluated hyperinsulinaemia, or other markers of insulin resistance, in relation to cardiovascular disease (CVD) and death. Although these studies show some variation with respect to clinical endpoints and subject selection criteria, they are consistently showing a strong relationship between insulin resistance and coronary heart disease (CHD) development\(^5\)-\(^10\). Mostly, the obtained risk estimates vary between 1.5 and 2.5\(^11\)-\(^15\). However, adjustment for multiple confounding factors often result in a significant reduction of these risks. Insulin resistance is a central component of the insulin resistance syndrome (IRS). The syndrome was first described as “syndrome X” by Reaven in 1988\(^16\) but is often also referred to as the “metabolic syndrome”. IRS consists of the close co-existence of metabolic factors associated with risk for CVD and type 2 diabetes, including obesity, dyslipidaemia, hypertension and insulin resistance\(^16\). The syndrome is more common among older people and the prevalence is likely to increase as populations are becoming older and more obese (reviewed in \(^17\)). Evidence supporting the role of IRS as a risk factor for CHD comes from three Scandinavian studies\(^18\)-\(^20\). Prospective data from Finland and Sweden showed that the risk for CHD was increased threefold in subjects with IRS\(^18\). Slightly lower risk estimates were seen among Finish men\(^19\),\(^20\). One of these studies also reported that IRS did not predict CHD events among Finish women\(^19\).

According to a recent review by Wallace and Matthews\(^21\), there is no single quantitative measurement of insulin resistance that is appropriate under all conditions. The most common measurements of insulin resistance, fasting-insulin, the homeostasis model assessment (HOMA) or the euglycaemic clamp, are however closely correlated\(^22\),\(^23\). The euglycaemic
clamp, which is often used as the ‘gold-standard’, is time consuming and relatively complicated and it is therefore not practical to use in epidemiological studies. Instead, fasting serum insulin levels and HOMA are both commonly used in larger studies. Insulin resistance leads to increased serum-insulin levels as the body attempts to compensate for the fact that more insulin is required to maintain normal glucose homeostasis. Therefore, insulin resistant subjects have higher fasting-insulin levels than healthy individuals and fasting-insulin levels can subsequently be used to estimate insulin resistance. HOMA is calculated using an established formula (insulin resistance = [fasting glucose x fasting insulin] 22.5).

Insulin-like growth factor binding protein-1 (IGFBP-1) is a circulating protein that binds to insulin-like growth factor-1 (IGF-1). IGF-1 displays high affinity for the insulin receptor and by binding free IGF-1, IGFBP-1 can prevent IGF-1 from activating the insulin signalling cascade and the stimulation of glucose uptake. IGFBP-1 levels are negatively correlated to fasting insulin levels, LDL cholesterol, triglycerides, blood pressure, body mass index and positively correlated to HDL cholesterol. This suggests that IGFBP-1 may be a marker of insulin resistance, play a part in the metabolic syndrome network and thus also potentially contribute to MI risk.

**IL-6, CRP and TNF-α**

Atherosclerosis can be regarded as an inflammatory disease considering the many inflammatory processes that are active in developing plaque. The growth of an atherosclerotic plaque begins at sites where the arterial endothelium is dysfunctional or injured in some way. Cytokines expressed by cells in the lesion and adhesion molecules on the vascular endothelium act to attract leukocytes (monocytes and macrophages) to the area. These cross and enter into the underlying tissues where they start to engulf modified low density lipoproteins (LDL) and develop into lipid-laden foam cells. If the foam cells die, their lipid contents become part of the necrotic core of the atherosclerotic plaque. Furthermore, the inflammatory response stimulates the migration of smooth muscle cells into the area of inflammation, cells which then contribute to the occlusion of the arterial lumen (reviewed in 26,27).

Apart from the direct role in atherosclerotic plaque growth, several other cardiovascular risk factors are also adversely influenced by acute inflammation and an increasing body of evidence from population-based studies show that circulating inflammatory markers have a role as risk factors for CVD. In this thesis, the circulating inflammatory markers, IL-6,
TNF-α and CRP are evaluated. Apart from the link to CVD, studies also show associations between elevated levels of TNF-α, IL-6 and CRP, abnormal lipid and carbohydrate metabolism, and obesity\textsuperscript{29,30}, as well as for insulin resistance\textsuperscript{31-33}. These markers, which influence carbohydrate and lipid metabolism by impairing insulin signalling and inhibiting LPL activity, are synthesised by adipocytes\textsuperscript{34,35} and are elevated in insulin resistant individuals\textsuperscript{36,37}. The diverse effects of inflammatory markers may help to explain their influence on the development of CVD.

IL-6 is a pleiotropic cytokine that plays a central role in inflammation and tissue injury. IL-6 is produced in response to several factors, including TNF-α, and is an important determinant of CRP production. IL-6 levels increase with age, particularly after the age of 50, possibly as a result of diminished levels of sex-hormones which can suppress IL-6 levels\textsuperscript{38}. Elevated levels of IL-6 has been shown to be associated with an increased risk for CVD. Ridker et al showed a strong association between elevated IL-6 levels and risk for future MI’s in a large cohort of men\textsuperscript{39}. Furthermore, elevated IL-6 was associated with increased risk of CVD mortality both in a mixed-sex group\textsuperscript{40}, and among elderly women\textsuperscript{41}. It is also believed that IL-6 could play some role in the development of insulin resistance. The cytokine can inhibit the insulin receptor, thereby preventing insulin signalling\textsuperscript{42}, and increase insulin secretion by enhancing insulin gene expression\textsuperscript{43}. Smaller studies in humans support this hypothesis\textsuperscript{44,45} but conclusive epidemiological evidence regarding the role of IL-6 in insulin resistance is still warranted.

TNF-α is synthesised by macrophages and smooth muscle cells in the atherosclerotic plaque, but also by adipocytes. One of the properties of TNF-α is that it induces the expression of adhesion molecules on endothelial cells\textsuperscript{46}, thus contributing to the recruitment of leukocytes to developing atherosclerotic plaques. Elevated TNF-α levels are associated with increased risk for recurrent coronary events among men who have suffered an MI\textsuperscript{47}. Elevated TNF-α was also found to predict mortality in patients with advanced heart failure\textsuperscript{48}. Furthermore, TNF-α levels correlate with early carotid atherosclerosis\textsuperscript{49} and results from the BECAIT trial suggest that TNF-α is related to several metabolic disturbances commonly associated with CVD\textsuperscript{50}. TNF-α is involved in several aspects of adipocyte metabolism\textsuperscript{35} and is thought to be involved in the pathogenesis of obesity-associated insulin resistance and type 2 diabetes\textsuperscript{51,52}. A strong correlation between elevated TNF-α levels and insulin resistance, seen among native Canadians, provides further support for this hypothesis\textsuperscript{57}.
C-reactive protein (CRP) is an acute phase protein with a short half-life. The detection of CRP levels in serum is widely used as an indicator of the presence of inflammation or tissue injury. It is synthesised primarily in the liver where the expression is stimulated by cytokines, in particular IL-6. It has been shown that CRP is present in atherosclerotic lesions, but not in normal arterial tissue, and CRP gene expression in atherosclerotic plaques suggests local production. Furthermore, CRP stimulates the production of endothelial adhesion molecules and inhibits nitric oxide (NO) synthesis (reviewed in). Other evidence supporting a role for CRP in atherosclerosis comes from numerous epidemiological studies. Results from prospective studies show that elevated levels is associated with increased risk for CVD incidents or death, an association which may be further enhanced by interaction with the IRS.

The genetics of MI and insulin resistance

A family history of CVD is one of the strongest risk markers for MI, with relative risks being as high as 3.4 for men with 2 or more affected parents or siblings. Also, results from a cohort of more than 20 000 Swedish twins show that the relative contribution to death from CHD due to genetic effects is stronger at younger ages, but the risk of CHD attributable to genetic factors remains highly significant up to the age of 75. The genetic contribution is further confirmed by twin studies which show that the heritability of CHD death is 50-60% among males and 35-55% among females. The mechanisms by which genetic variants contribute to the strong familiarity of CHD are not fully understood but it is likely that the disease can be influenced by a large number of genetic variants. Some may be potent enough to exert a direct impact on the phenotype or give risk to disease. However, CHD is more likely to be caused by genetic variants that contribute to the disease when they occur in combination with other genetic variants, or with particular environmental, behavioural or metabolic factors.

The reason for the clustering of metabolic factors seen in the IRS is not clear. It is possible that changed levels of one component may lead to changes of another component, but there may also be underlying common causes. Twin studies which show that the principal components of the IRS are influenced by a set of common genetic factors, support the latter theory. Data which show that the heritability estimates for insulin are around 50% provides further support for a genetic background to insulin resistance and IRS. Since most of the components of the IRS are by themselves individual risk factors for CVD, it is possible that CVD share
genetic causes with IRS. However, this hypothesis needs to be further evaluated.

There are two approaches to identify candidate genes. Either a genome-wide search is used to identify a chromosomal region that contains the disease causing gene. The other approach, the candidate gene approach, is often used in studies on complex diseases where one expects to identify several candidate genes. In such studies, the association between a particular genetic variant and its relation to a disease or a phenotype is estimated. The selection of candidate genes is usually based on the relevance of the gene, i.e. its corresponding protein, in biological processes that are relevant to development of the studied disease.

**Single nucleotide polymorphisms**

Every individual, with the exception of identical twins, is genetically unique. Even though any two humans are approximately 99.9% identical genetically, millions of differences are still present among the 3.2 billion base pairs of the genome. The most common form, and the most often studied form of genetic variation, are nucleotide changes at one single base pair. These variants are, rather descriptively, called single nucleotide polymorphisms (SNPs). It is estimated that the human genome contains roughly 11 million SNPs. This corresponds to about one SNP every 300 base pairs\(^6\). If the pathogenic effect of a polymorphism occurs late in life, or if the severity of the effect is relatively mild, the genetic variant can be passed down from generation to generation and spread in the population.

If the consequences of a genetic variant are too grave, however, the polymorphism is more likely to be removed from the population by selection.

SNPs can be made up of an insertion or deletion of a single base, or by the substitution of one base for another. For the purpose of this thesis, I shall focus on the latter type. Many SNPs are located in places of the genome where they have no apparent effect on gene function (so called silent SNPs). They sit quietly unnoticed without having an impact on the phenotype of the organism. To avoid doing genotypings that fails to identify disease-causing polymorphisms when conducting association studies, it is common to actively select for SNPs that are likely to have a direct impact on the disease, or other phenotype of interest. Examples of polymorphisms that are commonly analysed are SNPs located in exons which can result in an amino acid exchange and altered protein properties, and SNPs in promoter regions, which might effect gene regulation.
INTRODUCTION

Genetic variation in the IL-6 and TNF-α genes

IL-6 polymorphisms
There are three polymorphisms in the IL-6 promoter region that have received particular attention by the research community. These have also been analysed in the work presented in this thesis. These variants are C/G exchanges at positions -174 and -573, and an A to G exchange at position -598, numbered relative to the transcription start site. There are also several other variants registered in the databases, but I am not aware of any studies where they have been investigated.

The -174C/G polymorphism is the most extensively studied variant. It was first described in 1998 and the less common C allele occurs at an allele frequency of approximately 40%70,71. Initially, it was reported that the C allele was associated with lower expression of IL-6 compared to the G allele70, indicating that the G-allele was disadvantageous. Carriers of the G allele showed higher plasma triglycerides and higher fasting insulin72 suggesting involvement in IRS. A small study of 32 subjects has confirmed an association between the -174G allele and insulin resistance73, but further studies are warranted. In contrast, other reports suggest that the -174C allele is the “bad” variant. Carriers of the -174C allele have higher IL-6 levels six hours after coronary artery bypass surgery74 and has been associated with elevated IL-6 levels in neonates, but not in adults75. Also, Humphries et al76 and Georges et al77 have found the C-allele to be associated with an increased risk for CHD.

Less is known about the -573C/G and the -598A/G polymorphisms. The -174G and the -598G alleles are in strong linkage disequilibrium and most researchers have focused on the former variant only74,77. Carriers of the -573 C allele showed higher IL-6 levels after coronary artery bypass surgery74 but other studies where its relevance for CVD or insulin resistance has been studied are not available. The -573C allele is rare with an allele frequency of about 4-5%,78,79. Large data-sets are thus required to evaluate its function in man and this limitation can perhaps help to explain the lack of epidemiological data evaluating the role of this SNP.

TNF-α polymorphisms
Like the IL-6 gene, there are certain genetic variants of the TNF-α gene that are more widely studied than others. The far most commonly studied polymorphism is the G to A exchange at position -308. Second to that is the G/A SNP at position -238. Also studied in the papers presented here are C to A exchanges at positions -857 and -863 and a T to C exchange at position -1031. Another SNP, a G to A exchange at position -376 has been
identified and is associated with increased susceptibility to malaria. In a previous study on Swedish men this variant was found to be extremely rare and it has not been included in other comprehensive studies where many TNF-α genetic variants were genotyped suggesting that is very rare also in other ethnic groups.

Transcription studies have suggested that the -238A, the -308G and the -863A alleles result in lower rates of transcription of the TNF-α gene. The results regarding the function of the -308G allele must however be regarded as inconclusive since lack of association between this allele and transcriptional regulation has also been documented. The -308A allele was associated with increased MI risk among obese subjects, and a weak association between this allele and a history of MI has also been reported. On the other hand, in a large study by Koch et al where the -308A/G and -863C/A polymorphisms were analysed in relation to MI, these variants were not associated to the disease. Similar conclusions regarding the -308A/G polymorphism, were drawn by Allen et al, who studied coronary artery disease. TNF-α polymorphisms -238A/G and -308G/A have also been implicated to be associated to the development of insulin resistance. The data are however far from conclusive as, although several studies are in support of such a connection, many are also against it. Only few studies are available on the -857C/A and -1031T/C variants. The former is associated with TNF-α secretion from adipose tissue but the latter is not, but their impact on CVD risk or insulin resistance is unknown.

**SNP databases**

During the late 90’s and onwards, there has been tremendous development in our knowledge about the genome and how to study it. Large efforts from the industry and academic institutions have resulted in exponential growth of the number of SNPs available in public databases. There are also company owned databases containing even more entries which have not yet been released to the public.

Many factors have played a part in the rapid growth of the SNP research field. Parallel with the progress of the human genome project, of which a first draft was presented in June 2000, was a rapid development in genotyping technology and accessibility of the public databases. The development of genotyping technology was driven by a wish to reduce labour time, amount of DNA required and, of course, to reduce the overall cost of the individual assay without reducing the reliability of the genotype scoring. The limiting factor for many labs today are not the technology to assay SNPs, but the availability of DNA. We will, no doubt, in the future
see increased use of, and further development of methods for accurate reproduction of DNA samples so that epidemiological materials can be saved even after the samples originally donated by the study participants have been used up.

The SNP databases available today can be crudely divided into two groups. The comprehensive public database dbSNP contains a large number of genetic variants but the entries may not always have been verified experimentally in human populations. The second type of database is less comprehensive but only contain SNPs that have been validated in epidemiological materials and have a documented effect on gene transcription or association to disease. Examples of such databases which are discussed in this thesis are HGMD and OMIM. Links to the SNP databases discussed in the text are given in Table 1.

**Table 1** Useful Links to SNP databases.

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<tr>
<th>Database</th>
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<tr>
<td>dbEST</td>
<td><a href="http://www.ncbi.nlm.nih.gov/dbEST/">http://www.ncbi.nlm.nih.gov/dbEST/</a></td>
</tr>
<tr>
<td>HGMD</td>
<td><a href="http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html">http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html</a></td>
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</table>

**dbSNP**

The largest database of SNPs available today (dbSNP) was established in September 1998 and now contains around 6 million human genetic variants. The data can be submitted from any laboratory but major contributors to the database are laboratories associated with large SNP identification efforts such as the Human Genome Variation Database (HGVbase) database. The database has no requirement or assumption about minimum allele frequency, and even if researchers submitting polymorphisms are encouraged to include validation data, this is not required.

**HGMD, OMIM**

Despite the rapid development of the genome based SNP databases, OMIM and HGMD remain useful guides to the most prominent variants of different genes. In April 2003, the Human Gene Mutation Database (HGMD) contained 33252 disease-associated genetic variants in 1338 genes. The database comprises various types of polymorphisms (SNPs, insertions, deletions, nucleotide repeats etc) within coding, regulatory or splicing regions of human nuclear genes that cause inherited diseases. To be included in HGMD, the editors of the database state that there must be a “convincing association of the polymorphism with the phenotype”.
Currently, the Online Mendelian Inheritance in Man (OMIM) database contains information on 10645 entries. It is a database of human genes and genetic disorders, and it includes references to literature, nucleotide sequences and other related information as well as a text describing findings regarding the particular gene\(^{100}\). OMIM also contains descriptions of published allelic variants with described impact on a disease or phenotype.

**dbEST**
The EST database (dbEST) contains sequence data and other information on “single-pass” cDNA sequences, or Expressed Sequence Tags (ESTs), from a number of organisms\(^{100}\). An EST is a partial sequence of a cDNA clone that corresponds to an mRNA\(^{102}\). The sequences were initially aimed to be used in the sequencing of the human genome\(^{103}\). However, ESTs have been widely used for different purposes: to identify new members of gene families\(^{104}\), for exon identification\(^{105}\) and SNP discovery\(^{106,107}\). The EST database currently contains over 5 million human EST sequences.

\(^{1}\) [http://archive.uwcm.ac.uk/uwcm/mg/docs/new_back.html](http://archive.uwcm.ac.uk/uwcm/mg/docs/new_back.html)
METHODS

The following section is a brief discussion on the studied populations and some of the methods used in this thesis. Details regarding specific laboratory assays are presented in the individual papers.

The Borderline hypertension study

The DNA used to verify a subset of the SNPs identified in paper I, was obtained from 30 participants randomly selected from the borderline hypertension study. The material consists of men living in Åkersberga (a small community 35 km north of Stockholm) who were asked to visit their primary health care centre for blood pressure measurements. 81 men with slightly elevated, but not pharmacologically treated, blood pressures (diastolic blood pressure [DBP] of 85-94 mmHg) and 80 age matched, normotensive controls (DBP< 80 mm Hg) were enrolled in the borderline hypertension study. All subjects were Swedish citizens. The reason for using this study and not the SHEEP material for the SNP verifications of paper I, was that the latter study has a more limited amount of collected DNA. However, as both materials consist of Swedes of approximately the same age and from the same geographical region, variations in SNP frequency due to ethnic variation is likely to be negligible or absent.

The SHEEP material

The Stockholm heart epidemiology program (SHEEP) has been described in detail previously. The full SHEEP study consists of 2246 cases, aged 45-70, who had suffered either a fatal or a non-fatal MI event and their controls (n=3206). Male cases were identified during 1992-1993 (two years) and female cases during 1992-1994 (three years). During the period January – October 1992, the upper age limit was 65 years and from 1st November 1992 and onwards it was set to 70 years.

Case and Control Identification

The cases were recruited from (i) coronary and intensive care units at the internal medicine departments at all the emergency hospitals within the
Stockholm County, (ii) the computerised hospital discharge registry for the Stockholm County, and (iii) death certificates from the National Register of Death Causes at Statistics Sweden. Criteria for MI included (i) certain symptoms according to case history information, (ii) specified changes in blood levels of creatine kinase (CK) and lactate dehydrogenase (LD), (iii) specified ECG-changes, and (iv) autopsy findings. These criteria were those accepted by the Swedish Association of Cardiologists in 1991\textsuperscript{110}, and all hospitalised cases were diagnosed according to these criteria. To ensure that only subjects with first-time MI events were included in the study, each case was checked for earlier MI’s from 1975 and onward in the hospital discharge registry.

One control per case was randomly selected after stratification for age, sex and residential area. The controls were selected from the computerised register of the Stockholm County population within two days of the case event. Each referent candidate was checked for previous MI events since 1975 using the computerised hospital discharge register. Five control candidates per case were sampled at the same time so that another candidate who belonged to the study-base at the case incident could replace non-respondent controls. Occasionally, both the initial and a substitute control were included due to a late reply of the initial control. With this procedure more controls than cases were included.

**Subjects, papers II-IV**
The present papers are restricted to non-fatal MI events only as no blood samples could be obtained from the fatal cases. Cases who had suffered a reinfarction between the initial event and the blood sampling were also excluded. The selection criteria were somewhat different in paper II from papers III & IV as all subjects with diagnosed diabetes were excluded from the former. The subjects were asked in the questionnaire if they were receiving treatment for diabetes. As it was not possible to control if they were diabetic otherwise, all subjects who had not completed the questionnaire had to be excluded from the study population of paper II.

The cases were called to the clinic approximately three months after the MI event. The examination date for the controls was set as close as possible to that of his/her corresponding case to avoid bias due to seasonal variation in the blood parameters. The subjects were asked to arrive to the medical centre after an overnight fast for blood sampling, blood pressure measurements and anthropometrical tests. The blood samples were handled according to prevailing clinical practice for analyses of blood glucose, serum lipids and plasma haemostatic factors. Blood chemical analyses were performed at the Department of Clinical Chemistry at the
Karolinska Hospital. Total cholesterol and triglycerides were analysed in fresh serum samples with a colorimetric method. The samples were frozen immediately after sampling and stored at -70 °C until they were collected and transported to the central SHEEP biobank. The samples were analysed by trained laboratory technicians in a random, blinded manner to reduce any bias. DNA was extracted from blood cells at the Department of Clinical Chemistry at the Karolinska Hospital. The DNA was stored at -70° C until standardised dilutions (2.5 ng/µl), were prepared from these samples for the purpose of papers III and IV. Each sample was placed in an individual well of a 96-well microtiter plate. In total, each plate contained 3 blank samples (buffer only) and DNA samples from 93 individuals. The plates were sealed and stored at -20°C.

**Exposure Parameters**

The selection criteria for the SHEEP sub-studies described in papers II-IV are described in Figure 3.

**Paper II**

In total, the population analysed in paper II consisted of 2762 subjects. However, insulin resistance could only be measured in 749 MI cases (510 men and 239 women) and 1101 controls (705 men and 396 women) as serum was not available from all subjects. Also, some subjects who had failed to comply to the requirement of an overnight fast prior to the blood sampling were excluded from the statistical analyses. Exposure to high levels of insulin or HOMA values defined as above the 90th percentile boundary of the control group, and a second exposure group was defined as those with insulin or HOMA levels between the 75th and 90th percentile limits. Those with levels below the 75th percentile limit of the control group were classified as the unexposed reference group. Exposure to low levels of IGFBP-1 was defined as below the 10th percentile limit, and a second exposure group was defined as those with IGFBP-1 levels between the 10th and the 25th percentile limits. The reference group for IGFBP-1 were all subjects with IGFBP-1 levels above the 25th percentile cut-off limit.

**Papers III & IV**

Papers III & IV were based on all participants of the SHEEP study who had left a blood sample and who had not had a reinfarction between the initial MI and the blood sampling. In total, 1213 cases (852 men and 361 women) and 1561 controls (1054 men and 507 women) fulfilled these criteria, most of whom all had available DNA. However, the analysis of serum levels of IL-6, CRP and TNF-α was restricted to smaller number of samples since the serum from some subjects had been used up previously.
Exposure to IL-6, CRP and TNF-α was classified as between the 25th and 75th percentiles or as above the 75th percentile limit of the control group. Subjects with IL-6, CRP or TNF-α levels below the 25th percentile limit were classified as the unexposed reference category. As the lower detection limit for the IL-6 assay was 0.5 ng/L and more than 25% of the men had IL-6 levels at this level, IL-6 levels <0.5 ng/L was used to define the unexposed reference category for IL-6 rather than using the 25th percentile limit.

**Genotyping**

**Paper I**

Genes with a possible role for the development of IRS were scanned for novel SNPs by comparing their mRNA sequence to the human dbEST using a basic local alignment tool (BLAST). This software aligns the candidate sequence to EST sequences with identical, or similar, nucleotide sequences. Distinct differences between the candidate sequence and the matching ESTs identified potential SNPs, some of which were verified in DNA from 30 subjects from the borderline hypertension material. Thirty-two SNPs in 13 genes were analysed in this manner. Primers were designed to yield a PCR product of 400-500 bp, with the potential SNP located equidistant from the primers. The PCR product was then sequenced, initially using the forward primer only, but some sequences were confirmed using the

**Figure 3** Selection criteria for the SHEEP sub-studies of papers II, III and IV.
reverse primer (i.e. sequencing in the opposite direction) in case the first sequence was unreliable, or in order to confirm the presence of an SNP. The SNPs were identified after multiple alignment of the sequences and localisation of the polymorphic sites.

**Paper III & IV**

For the SNP analyses of papers III and IV, genotyping was done using Dynamic Allele Specific Hybridization (DASH). The reason for selecting this method rather than any other genotyping technology was that it is comparatively fast, inexpensive and requires small amounts of DNA. Another important aspect was that the method was developed by researchers at the Karolinska Institutet and DASH help was close at hand. A diagram illustrating the principle of DASH genotyping is shown in Figure 4. First, PCR primers are designed to yield a short (approximately 50 bp) PCR product with the polymorphic site at the centre. It is necessary that the primers are designed to avoid the formation of secondary structures of the PCR product, which would otherwise prevent the allele specific probe from hybridising. One of the primers is biotinylated so that the product can be immobilised to a streptavidin coated well in a microtiter plate. The non-biotinylated strand is rinsed away with a weak alkali, leaving only single-stranded DNA in the well. An allele-specific oligonucleotide probe (15-17 bp) is then hybridised to the strand, right across the SNP site, and an intercalating dye (SyBR Green) that emits fluorescence (proportional to the amount of double stranded DNA present) upon excitation is added. The allele-specific probe is designed to be perfectly complimentary to the nucleotide sequence surrounding

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**Figure 4** The principle for allele discrimination using DASH technology.

1. **Immobilise PCR product**
2. **Slow heating of the sample whilst monitoring fluorescence**
3. **Plot negative derivative of fluorescence against temperature to determine genotype**
4. **Add allele-specific probe & fluorescent dye**
5. **Remove non-biotinylated strand**

---
the SNP. Thus, a DNA sequence corresponding to the allele-specific probe, will yield a perfect match between the probe-target duplex. Presence of another allele will lead to a one base-pair mismatch. The sample is then slowly heated and fluorescence monitored. Denaturing (melting) of the probe-target duplex is detected as a sharp drop in fluorescence. The temperature at which the probe dissociates is different depending on if there is complete matching between the probe and the target, or if there is a one base mismatch. The single base mismatch in the probe-target duplex will result in a lower melting point, which is then used to distinguish between the alleles present in the sample. The accuracy of the assay (as with most genotyping methods) is dependent on a good quality PCR reaction and a reasonable discrimination between melting points.

**Statistics**

Throughout the statistical analyses of the SHEEP material, men and women were analysed separately. Differences in baseline characteristics were compared using the Wilcoxon rank-sum test for continuous variables or $\chi^2$ test for categorical variables. Odds-ratios (OR) with 95% confidence intervals (95% CI) were calculated using logistic regression. The crude ORs were adjusted for the matching variables age and residential area (sjukhusupptagningsområde). Adjusted ORs were obtained after the confounding variables were included in the regression model (see section on confounding exposures below). Correlations between two continuous variables were obtained using the Pearson correlation coefficient. The association between the geometric mean of a continuous variable and a genetic variant, were assessed using analysis of covariance (ANCOVA) that allowed for adjustment for confounding factors, including the matching variables age and residential area.

**Assessment of biological interaction**

Many factors that can influence the risk of developing CHD have been identified. Millions of people worldwide take drugs to reduce their blood pressure or cholesterol levels, or are in physical training or on diet programmes in order to prevent the onset of CHD. It seems logical that a person with high blood pressure, and who also has high cholesterol levels, is at a higher risk of developing the disease compared to individuals who only have high blood pressure or high cholesterol levels. In order to analyse how different risk factors are inter-related, it is important to be able to assess the presence of such biological interactions. Hypothetically, consider that the effect of exposure A yields an odds ratio of two and that exposure B also results in an odds ratio of two. However, the combination of exposure
A and exposure B yields an odds ratio of six. In other words, the effect of the combined exposure of the risk factors A and B is higher than the sum of the risk inferred by the two separate agents. This concept of biological interaction, as illustrated in Figure 5, takes into account the possibility of synergistic or antagonistic effects. It was first described by Rothman and Greenland\cite{113}, and by their definition, synergistic interaction is departure from the additive effects of each of the risk factors. Two components must thus share in the causal responsibility of the outcome, in this case MI risk.

The presence of interaction can be estimated using the Synergy Index score (S) with 95% CI (see Figure 6). An S-score ≠1 indicates departure from an additive effect between two variables, i.e. S >1 indicates synergistic interaction and S <1 indicates antagonistic effects. The analysis requires large sample sizes to obtain acceptable statistical power and reliable estimates, but apart from this restriction the stringency of the method should be considered advantageous as it reduces the risk of over-interpretation of data. The combination of a too small study population and a rare exposure will result in extremely wide confidence intervals or,
in extreme cases, no estimates at all. An example of this latter situation was seen in paper IV, where several interaction estimates could not be obtained due to too few exposed women. It is also noteworthy that in paper II, the abbreviation “SI”, rather than “S” (which was used papers III & IV) was used to describe the S-score. The abbreviation was changed to avoid confusion as SI is also short for synergistic interaction, and as “S” corresponds better to other publications in the field.

**Other exposures**

The various risk factors were defined in accordance with previous reports on the SHEEP material\(^6\),\(^{10}\).

**Hypertension:** In paper II, subjects receiving anti-hypertensive drug therapy or with a systolic blood pressure (BP) $\geq 160$ mm Hg or diastolic BP $\geq 90$ mm Hg were classified as exposed. The limit for systolic BP was changed to 140 mmHg for papers III & IV to accommodate to changed clinical guidelines for classification of hypertension.

**Hypercholesterolaemia:** Subjects with total cholesterol levels $\geq 6.5$ mmol/L or those receiving lipid lowering medication were classified as exposed.

**Hypertriglyceridaemia:** Hypertriglyceridaemia was defined as having fasting serum triglyceride levels $\geq 2.3$ mmol/L.

**High LDL/HDL quotient:** Subjects with LDL/HDL quotient $>4.0$ were classified as exposed.

**Obesity:** Body mass index (BMI) (kg/m\(^2\)) was analysed as a continuous variable, or a cut-off limit of 28 kg/m\(^2\) (corresponding to the 75\(^{th}\) percentile of the control group) was used for the categorised variable.

**Diabetes:** Subjects who reported that they were controlling diabetes with diet, insulin, or other drug treatment were classified as exposed.

**HRT:** Women who reported that they were receiving hormone replacement therapy (HRT) in the form of oestrogen only, or oestrogen + progesterone, at the time of answering the questionnaire were classified as exposed.

**Jobstrain:** Jobstrain was determined as having low decision latitude but high psychosocial demands as determined by the Karasek-Theorell questionnaire\(^{11}\).

**Smoking:** Former smoking was defined as having smoked daily but having stopped more than two years prior to examination and current smokers were defined as persons who smoked or had stopped smoking within the last two years.

**Physical inactivity:** Subjects who reported inactive leisure time were defined as exposed.
RESULTS

Paper I- SNP searches

In paper I, genes with a possible role for the development of IRS were scanned for previously known SNPs using public databases. Also, novel SNPs were identified by comparing the mRNA sequence of the candidate gene with the human EST database. Mismatches between the known mRNA sequence and the EST sequences indicated potential SNPs. The existence of 32 of the potential SNPs in 13 genes were then verified by sequencing DNA from 30 participants of the borderline hypertension study.

Two novel SNPs were identified after sequencing DNA from subjects of the borderline hypertension study. One was a G/C exchange at position 1471 of endothelin receptor A (EDNRA) and the other was a T/C exchange leading to a Trp to Arg amino acid change at position 514 of the plasminogen activator inhibitor-1 (PAI-1) gene. It is worth noting that the EDNRA and PAI-1 polymorphisms were registered into HGBASE by others (genbank accession numbers and date of identification; rs5335 [1999-06-29] and rs11178 [1999-08-23] respectively). The EDNRA SNP was first reported after it had been identified using a chip assay\textsuperscript{115} and PAI-1 was predicted from alignment of EST sequences\textsuperscript{116}. Both have been validated in human populations. At the time when the genotypings for the purpose of this

<table>
<thead>
<tr>
<th>Gene</th>
<th>Number of polymorphisms in public databases: Before 01.01.01</th>
<th>01.01.01 or later</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OMIM</td>
<td>HGMD</td>
</tr>
<tr>
<td>Insulin</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CRP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>
thesis were made, these SNPs were not considered top-priority and the IL-6 and TNF-α genotypes were analysed instead.

A search of 146 genes of potential relevance for development of insulin resistance, revealed that they contained 614 polymorphisms with a reported influence on phenotype or gene regulation. As the databases have developed markedly since paper I was written, I wanted to get an overview of recent developments of the existing SNP databases. I therefore searched of OMIM, HGMD and dbSNP for polymorphisms in the genes for insulin, IGFBP-1, IL-6, CRP and TNF-α. These genes were chosen since they code for proteins that have been studied in the work presented in this thesis. The results from this search, given in Table 2, show that HGMD and OMIM are not much different now compared to before 1st January 2001, the only difference is one new entry in OMIM for an SNP in the TNF-α gene. However, the number of entries in dbSNP has increased several-fold, the database now contains 61 SNPs in the IL-6 gene compared to five entries registered before 1st January 2001.

**Paper II- Insulin resistance and MI**

In paper II, the association between levels of serum insulin, HOMA and IGFBP-1 as factors related to MI risk and their interaction with life-style related risk factors was evaluated in the SHEEP material. The cases were more insulin resistant compared to the controls but did not differ significantly with regard to IGFBP-1 levels. IGFBP-1 was negatively correlated to insulin and HOMA as expected. The correlation coefficients were similar for cases and controls for both gender.

<table>
<thead>
<tr>
<th></th>
<th>Exposed category</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Crude</td>
<td>Adjusted</td>
</tr>
<tr>
<td><strong>Insulin</strong></td>
<td>&gt;75th percentile</td>
<td>1.6 (1.3-2.1)</td>
<td>1.3 (1.0-1.7)</td>
</tr>
<tr>
<td><strong>HOMA</strong></td>
<td>&gt;75th percentile</td>
<td>1.5 (1.1-1.9)</td>
<td>1.2 (0.9-1.7)</td>
</tr>
<tr>
<td><strong>IGFBP-1</strong></td>
<td>&lt;25th percentile</td>
<td>1.0 (0.8-1.4)</td>
<td>1.1 (0.8-1.4)</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>&gt;75th percentile</td>
<td>2.6 (1.8-3.8)</td>
<td>2.7 (1.7-4.4)</td>
</tr>
<tr>
<td><strong>CRP</strong></td>
<td>&gt;75th percentile</td>
<td>1.9 (1.5-2.5)</td>
<td>1.6 (1.0-2.5)</td>
</tr>
<tr>
<td><strong>TNF-α</strong></td>
<td>&gt;75th percentile</td>
<td>1.6 (1.2-2.1)</td>
<td>1.7 (1.1-2.7)</td>
</tr>
</tbody>
</table>

Table 3 Odds ratios (95% CI) for MI risk among men and women according to exposure to high levels of different biological parameters.

Exposure to elevated levels of insulin or HOMA values were associated with increased risk for MI (Table 3). The estimates were somewhat higher among women compared to men for all three exposure variables. Elevated insulin and HOMA resulted in very similar risk estimates. The cut-off limit to define exposure was set at two levels, above the 90th and above the 75th
percentile limits for insulin and HOMA, and as below the 25th and 10th percentile limits for IGFBP-1. The risk estimates for all three variables were similar for both exposure categories and slightly higher among women compared to men.

A strong synergistic interaction between insulin resistance and smoking was found. Women exposed to both smoking and elevated insulin levels had particularly high risk estimates (co-exposure to insulin and smoking; OR [95% CI], women: 8.1 [4.5-14.8], men: 4.7 [3.0-7.2]) The corresponding S-scores suggested that strong synergism exists between these two risk factors (S-score [95% CI]: women; 3.0 [1.3-6.9], men: 2.6 [1.2-5.6]). There was no synergistic effect between elevated insulin and former smoking, i.e. among men who had stopped smoking at least two years prior to the SHEEP data collection. It was not possible to obtain reliable S-scores for former smoking and insulin resistance as the material lacked a sufficiently large number of women who were former smokers.

**Papers III & IV; Inflammatory markers and MI risk**

**IL-6, CRP, and TNF-α serum levels in relation to MI**

In papers III and IV the SHEEP material was used to analyse serum levels of IL-6, CRP and TNF-α as well as three SNPs in the IL-6, and six SNPs of the TNF-α promoter regions. IL-6 and CRP was analysed in paper III, and TNF-α in paper IV.

Serum levels of IL-6 and TNF-α were higher among male, but not female, cases than controls, and CRP levels were higher among cases of both gender. Elevated levels of IL-6, CRP and TNF-α were associated with approximately two- to three-fold increases in MI risk for men (Table 3). There was no association between elevated IL-6 levels and MI risk among women. CRP was a weaker marker for MI risk than IL-6 among men. No significant associations were found between elevated CRP and MI risk among women after adjustments for confounding variables. The OR for women exposed to elevated TNF-α levels indicated only slightly elevated MI risks with the 95% confidence intervals overlapping one for both the crude and adjusted estimates. These data indicate that there is a clear gender-related difference in the importance of inflammatory markers in CVD, in that MI events appear to be less influenced by elevated levels of inflammatory markers in women compared to men.

Interactions were seen between elevated IL-6 and high levels of fasting insulin, hypercholesterolaemia and to some extent also with smoking...
The combined exposure to elevated IL-6 levels and elevated insulin, hypercholesterolaemia or smoking resulted in OR [95% CI] of 4.6 [2.7-7.7], 4.7 [2.8-7.9], and 8.3 [4.5-15.4] respectively. Unfortunately, there were too few women in the material to draw any conclusions regarding the presence of synergistic interaction between various risk factors and elevated IL-6 levels. Significant synergistic interactions were seen between elevated TNF-α and BMI > 28 kg/m² for men (OR [95% CI] for co-exposure: 3.6 [1.4-21.9], S-score [95% CI]: 5.6 [1.4-21.9]) but not for women (S-score [95% CI]: 0.8 [0.1-5.3]). No significant interactions could be seen between elevated CRP levels and environmental or genetic variables.

**IL-6 and TNF-α polymorphisms in relation to MI**

The studied alleles all occurred at similar frequency from that reported in previous studies²⁴,²⁷,⁸¹. In the IL-6 gene, the −598A/G and −174C/G SNPs were in perfect linkage disequilibrium. Further statistical analyses of the −598A/G polymorphism were therefore not made. No subjects carrying the A allele of the −376A/G TNF-α SNP could be identified when 100 random individuals from the SHEEP material were genotyped, and no further genotyping of this SNP was therefore done.

Some association between the analysed genotypes and risk for MI was observed. The A allele at position −238 of the TNF-α gene appeared to have some protective effects among women (OR [95% CI]: 0.4 [0.1-1.0]). The OR for male carriers of the same allele were also below one, but with a 95% CI overlapping one (OR 95% CI: 0.7 [0.4-1.3]). Men carrying the −174C allele of the IL-6 gene had a somewhat higher MI risk (OR [95% CI]: 1.3 [0.9-2.0]) after adjustments for potential confounding factors.

IL-6 levels were not markedly influenced by the analysed genotypes. However, there was an association between the −174G/C SNP and insulin levels. Male controls homozygous for the −174G allele had significantly higher insulin levels than CC individuals. Heterozygous subjects had higher insulin levels than the CC group but lower levels than the GG group, suggesting an additive mode of inheritance¹¹⁷. Among male controls, the TNF-α −857T allele was associated with higher TNF-α serum levels compared to CC subjects (TNF-α levels [95% CI]: TT + TC: 2.0 [1.7-2.2], CC: 1.6 [1.6-1.7]). There was a trend for higher TNF-α levels for −857T carriers throughout the material but the associations among the male and female cases and the female controls were less clear and not statistically significant.
RESULTS

In paper IV, a rare TNF-α haplotype was found to occur more frequently among cases than controls for both men and women. The haplotype contains the −863A and −1031 T alleles, a combination which is not seen in the more common haplotypes. Only four controls were found to carry the rare haplotype whereas 32 cases had it. There are no previous records of this haplotype despite at least two other publications where TNF-α haplotypes have been investigated, using more or less the same markers as those studied in paper IV. It is possible that the previous studies were too small to pick up this haplotype but it is also possible that it occurred by chance.

IL-6, CRP and TNF-α in relation to insulin resistance

Table 4 shows the results of new analyses that are not presented in the individual papers. Here, the association between high levels of inflammatory markers and insulin resistance was estimated by calculating odds ratios with 95% CI’s. In one set of analyses, insulin resistant subjects were classified as those who had insulin levels >75th percentile of the control group, and in another set of analyses, the same cut off-limit was used but for HOMA values. Those with IL-6, TNF-α or CRP levels >75th percentile were classified as exposed for that variable. The cut-off limits are the same as those described in the individual papers. Both cases and control subjects from the SHEEP subjects analysed in papers III & IV were used for the analyses (n= 2774) as subdivision by case/control status did not seem to influence the point estimates. All diabetic subjects were excluded from the analyses.

<table>
<thead>
<tr>
<th></th>
<th>Men Crude*</th>
<th>Adjusted**</th>
<th>Women Crude*</th>
<th>Adjusted**</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insulin resistance measured as: Insulin levels &gt;75th percentile</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6 &gt;75th percentile</td>
<td>1.2 (0.9-1.6)</td>
<td>1.2 (0.8-1.8)</td>
<td>0.8 (0.5-1.3)</td>
<td>0.6 (0.3-1.2)</td>
</tr>
<tr>
<td>CRP &gt;75th percentile</td>
<td>2.3 (1.7-3.0)</td>
<td>1.1 (0.7-1.8)</td>
<td>2.8 (1.9-4.1)</td>
<td>1.3 (0.7-2.2)</td>
</tr>
<tr>
<td>TNF-α &gt;75th percentile</td>
<td>1.4 (1.0-1.9)</td>
<td>1.2 (0.8-1.8)</td>
<td>1.4 (0.9-2.0)</td>
<td>1.1 (0.6-1.9)</td>
</tr>
<tr>
<td><strong>Insulin resistance measured as: HOMA values &gt;75th percentile</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6 &gt;75th percentile</td>
<td>1.0 (0.8-1.4)</td>
<td>1.1 (0.7-1.6)</td>
<td>0.8 (0.5-1.4)</td>
<td>1.0 (0.5-2.0)</td>
</tr>
<tr>
<td>CRP &gt;75th percentile</td>
<td>2.4 (1.8-3.2)</td>
<td>1.3 (0.9-2.0)</td>
<td>2.5 (1.7-3.7)</td>
<td>1.3 (0.8-2.3)</td>
</tr>
<tr>
<td>TNF-α &gt;75th percentile</td>
<td>1.4 (1.0-2.0)</td>
<td>1.3 (0.8-1.9)</td>
<td>1.3 (0.9-2.0)</td>
<td>1.1 (0.6-1.9)</td>
</tr>
</tbody>
</table>

*Adjusted for age and residential area. **Adjusted for age, residential area, high BMI, hypertension, job strain, physical inactivity, hypercholesterolaemia, current and former smoking, PAI-1, fibrinogen and the use of beta-blockers.
The crude risk estimates show that elevated serum levels of CRP is associated with a two-fold risk for insulin resistance but the ORs were reduced to nearly one after adjustment for potential confounding variables (insulin resistance measured as insulin >75th percentile, OR [95% CI]: Men, crude: 2.3 [1.7-3.0], adjusted: 1.1 [0.7-1.8], Women, 2.8 [1.9-4.1], adjusted: 1.3 [0.7-2.2]). Elevated TNF-α was a somewhat stronger marker for insulin resistance compared to elevated IL-6, with the crude OR for TNF-α being 1.4 for both men and women and the OR for IL-6 being 1.2 for men and 0.8 for women. Similar analyses, but using the IL-6 and TNF-α polymorphisms rather than cytokine levels as the exposure variable, were also conducted. These SNPs did not markedly influence the risk for insulin resistance.
DISCUSSION

General Discussion

Insulin resistance, inflammatory markers in relation to MI
A large number of genes may be of relevance for the development of insulin resistance and MI. Several genes of potential interest for these diseases were identified by searching the public genome-databases. For the purpose of this thesis, two of these genes, were then selected for further analysis in the SHEEP material (IL-6 and TNF-α). Polymorphisms in the IL-6 and TNF-α genes were analysed in relation to their intermediate phenotype (i.e. IL-6 or TNF-α protein levels in serum) and to MI risk. Furthermore, using the SHEEP material the association between insulin resistance, measured as fasting-insulin and HOMA) and MI risk has been analysed. Insulin resistant individuals appear to be particularly sensitive to the hazards of smoking. Increased serum levels of both IL-6 (for men) and TNF-α (both gender) are associated with an increased risk for MI. Increased levels of these cytokines are most probably mainly modulated by non-genetic influences since the genetic variants of IL-6 and TNF-α, with one exception, do not significantly contribute to the determination of cytokine levels.

Our data show that insulin resistance increases the risk for MI among men and women. This is in accordance with prospective epidemiological studies. The OR's remained significant and above one, after adjustments for life style related confounding variables, indicating that insulin resistance is a risk marker, independent from such influences. Further adjustments for components of the IRS resulted in the disappearance of the significant risks, indicating a close relationship between insulin resistance and such factors. Furthermore, elevated levels of IL-6 and TNF-α were associated with increased risks for MI among men but not women. The existing large epidemiological studies on these two cytokines were not stratified by gender, and the finding that the effects are different between men and women therefore represent an important addition to the debate regarding their importance as risk factors for CVD.
Synergistic interaction was found to exist between smoking and insulin resistance among men and women, and also, to some extent, between elevated IL-6 and insulin resistance among men. It must however be noted that even larger study materials are required to obtain reliable statistics regarding the interaction analyses. Smoking is associated with elevated insulin levels\textsuperscript{4,119} and acutely impairs insulin action\textsuperscript{120} and could thus worsen the already harmful effects of insulin resistance. It has been suggested that it is substances other than nicotine in the tobacco smoke which are of importance for insulin resistance development\textsuperscript{128}. This does however require further analyses since contradictory evidence suggests that long-term use of nicotine supplements also elicit insulin resistance\textsuperscript{121}. In a recent publication by Reaven and Tsao\textsuperscript{122}, the authors suggest that it is the adverse consequences of insulin resistance and related disorders that are responsible for the increased risk for CVD among smokers. This would lead to higher prevalence of these abnormalities among smokers, resulting in the observed synergistic interaction. Glucocorticoid dysregulation has been suggested as an underlying mechanism for the increased risk for insulin resistance among smokers\textsuperscript{123,124}. According to the “glucocorticoid theory”, smoking would cause increased cortisol levels that in turn could cause obesity and insulin resistance. In one study, cortisol levels were only 5\% higher among smokers\textsuperscript{125} but in another study cortisol levels were not at all affected\textsuperscript{126}, suggesting that the association is, at best, small. Furthermore, increased cortisol levels would result in immune suppression and inhibited IL-6 synthesis\textsuperscript{127}. We would then be able to observe a negative correlation between smoking and IL-6 levels, or even see that the combined exposure to smoking and IL-6 would have an antagonistic effect. On the contrary, the synergistic interaction between smoking and IL-6, is more likely due to immune response activation by smoking. Smokers have significantly increased levels of fibrinogen\textsuperscript{128}, which is another acute-phase protein that is an important risk factor for CHD. The synergism between smoking and increased IL-6 levels could thus possibly be attributed to increased fibrinogen levels.

There were clear gender-related differences with regard to inflammation as men appeared to be more sensitive to high levels of IL-6 and TNF-\alpha. Elevated levels of these cytokines were associated with MI risk in men whereas female cases and controls did not differ with respect to serum levels of these cytokines. More female cases, compared to controls, reported exposure to jobstrain, whereas male cases and controls were more similarly distributed regarding this variable. Jobstrain, a measurement of perceived exposure to stress at work, appeared to have slight confounding effect as the presence of jobstrain in the regression model influenced the risk estimates somewhat. The hormone cortisol is released in response to stress...
and one of its effects is that it inhibits IL-6 synthesis and action. Subjects who are exposed to jobstrain have documented higher levels of cortisol as well as lower IL-6 levels\textsuperscript{139} raising the possibility that jobstrain protects against elevated IL-6 levels even though it, in itself, is a risk factor for MI\textsuperscript{130}. The difference in response to inflammatory markers between men and women could also be sex-hormone related. Oestrogen and progesterone can inhibit TNF-\(\alpha\) secretion\textsuperscript{131,132}, women receiving HRT display lower IL-6 levels\textsuperscript{133}, and post-menopausal women have fewer active cytokine secreting cells\textsuperscript{134}. HRT-status was not associated with any differences in cytokine levels among the women in the SHEEP material, but it is possible that our estimate of HRT is too crude to reflect oestrogen levels. The previous population-based studies indicate that sex-hormones only have a small, but significant, impact on cytokine levels\textsuperscript{133}. It is however questionable whether or not a small HRT-related reduction in cytokine levels is significant enough to have a pathophysiological meaning.

Paper II is, to our knowledge, the first study where IGFBP-1 has been evaluated in relation to insulin resistance and MI in a larger epidemiological material. There was a weak correlation between IGFBP-1 levels and fasting-insulin. Women with low IGFBP-1 levels showed a tendency towards increased risk for MI but there was no association between low IGFBP-1 levels and MI risk among men. This was also seen by Ruotolo et al, in a study on progress of coronary artery disease among young male survivors of MI\textsuperscript{135}. Low activity of the IGF-1 receptor and low IGF-1 levels are associated with longevity in mice. As IGF-1 and IGFBP-1 are negatively correlated, high levels of IGFBP-1 could, theoretically, therefore also be associated with a longer life-span\textsuperscript{136}. This could have implications for the study and treatment of age-related diseases such as CVD.

Several large epidemiological studies show that elevated baseline levels of CRP is a strong predictor of future cardiovascular risk among both men and women\textsuperscript{60,137}. Furthermore, CRP has been shown to be a stronger marker for CVD than elevated levels of IL-6 or TNF-\(\alpha\)\textsuperscript{41,57,59,138}, and in a large study by Ridker et al, elevated CRP levels was found to be a better predictor of CVD risk than several markers of inflammation and plasma lipids\textsuperscript{96}. In contrast to this large body of convincing evidence, our data showed that elevated IL-6 and TNF-\(\alpha\) were better markers of MI risk compared to elevated CRP levels. The reason for this discrepancy is not clear, but elevated CRP levels seem to predict cardiac deaths to a greater extent than non-fatal MI's\textsuperscript{57} and patients with very high CRP levels have poorer prognosis after cardiovascular events\textsuperscript{139,140}. The exclusion of fatal cases from the present analyses may thus explain why there are associations between elevated IL-6 and TNF-\(\alpha\), but not CRP, and MI risk. Elevated CRP levels were associated to increased insulin
resistance risk, whereas increased IL-6 and TNF-α levels were not. Supporting results was found in a cohort of women where the association between elevated levels of CRP, IL-6, and development of type 2 diabetes was investigated\(^{32}\). In that study, both CRP and IL-6 were found to predict disease development, although the risk estimates for IL-6 were lower and of borderline significance after adjustments for confounding factors. Significant correlations between levels of inflammatory markers and degree of insulin resistance have been reported\(^{36,37}\), but the possibility that the small deviations in TNF-α and IL-6 levels, as those present in the SHEEP material, are not sufficient to induce insulin resistance remains.

**Genetic variation in relation to MI**

Two other publications have reported similar, borderline significant, risks for CHD among carriers of the IL-6 -174C allele, suggesting that this allele may play some part in the pathogenesis of MI. These studies, a case-control study by Georges et al\(^{77}\), and a prospective study by Humphries et al\(^{76}\), showed point estimates of around 1.5, with borderline significance. These risks are similar to the results presented here but our findings were not significant, suggesting that the IL-6 -174C allele may play some part in MI pathogenesis, but it is not likely to be of major significance. Since the TNF-α -238A allele does not appear to influence TNF-α levels, the biological mechanisms underlying the finding that it leads to reduced risk for MI among women must be evaluated in more detail. Previous data suggests that the -238A allele results in lower transcription rate of the TNF-α gene\(^{84}\), but the effect may to too small to be visible as changed protein serum levels. The TNF-α -238A allele has previously been shown to be protective with regard to insulin resistance\(^{85}\), a finding which is in agreement with our results which show that it infers a lowered risk for MI. However, two other larger studies have failed to identify this relationship\(^{89,94}\), suggesting that if this SNP influences the pathogenesis of insulin resistance, the effect is likely to be rather weak.

These results suggest that TNF-α polymorphisms are unlikely to play a major role in insulin resistance development but that the IL-6 -174C/G polymorphism should be further investigated as it is associated with variations in serum insulin levels. The latter finding is supported by data from Fernández-Real et al\(^{73}\), who also report this association from a small sample of men and women. The TNF-α -308A/G SNP was not associated with TNF-α serum levels, or with MI or insulin resistance risk. A weak association between this SNP and MI has previously been shown\(^{88}\), but a larger, more carefully evaluated study does not support the existence of such a relationship\(^{142}\). It is therefore somewhat surprising that Ozaki et al recently reported an association between the lymphotoxin-α (LT-α) 252A/
G and MI risk\textsuperscript{142} with odds ratios between 1.6 and 1.8 with 95% confidence intervals clearly above one. As the TNF-\textit{\textalpha} -308A and LT-\textit{\textalpha} 252G alleles are in almost perfect LD\textsuperscript{143}, similar risk estimates would therefore have been expected to be seen with the TNF-\textit{\textalpha} -308 SNP and MI risk in the SHEEP material. However, it is possible that these discrepancies are attributable to differences in genetic and environmental exposures between the Japanese and Swedish populations.

It is difficult to obtain conclusive evidence regarding the role of genetic variants in the development of complex disease. One polymorphism may result in a positive association in one study which later can not be replicated in another study population. Differences in the outcome of similarly conducted association studies can be due to a number of things, the choice of end-point, the selection of subjects and stringency of the statistics chosen to test the association. Standardised statistical methodology to test for gene-phenotype associations are warranted. Therefore, the possibility still exists that the association between the IL-6 -174G allele and increased insulin levels is a chance finding. It must be confirmed in additional studies and possible mechanisms behind the association should be investigated. However, the association was tested using both parametric and non-parametric statistics, and remained significant after adjustment for potential confounders.

Much work has been conducted with the aim of identifying the individual genetic factors which contribute to the development of IRS and insulin resistance. Twin studies on components of IRS have shown that the heritability estimates for insulin was 48\%\textsuperscript{68} although numbers may vary from 20\% to 60\%\textsuperscript{144,145}. Large efforts have been placed in trying to identify the individual genetic variants that cause the disease, work that has been much helped by the increased user-friendliness and number of entries in the public SNP databases. The use of utility databases such as OMIM and HGMD, that focus on describing functional polymorphisms only, has perhaps decreased during the last few years as the more comprehensive databases now often contain information on allele frequencies and links to publications if such are available. At the time, when paper I was written, the large SNP database dbSNP, which is a central lab tool today, contained only a few entries and was not very user-friendly, a situation that has changed substantially. Today, dbSNP is the most comprehensive public SNP database, and we can here show that its entries outnumber those of HGMD and OMIM by far. Nevertheless the fact still remains that a substantial part of dbSNP contain genetic variants that have never been validated in human populations after their discovery. In fact, it has been
suggested that as much as 68% of the entries of coding SNPs are erroneous.146

Methodological Considerations

The major benefit with case-control studies are the large number of cases that can be included without the extra cost and waiting-time associated with prospective data collection. In fact, the analyses presented in this thesis are based on more cases than most of the leading publications in the field (particularly regarding the genotype data). The large number of cases has enabled stratification by gender and allowed for analyses of synergistic interactions which requires large populations. It is unfortunate that although these analyses have proved to be highly relevant, they are rarely seen in genetic/ molecular epidemiology. Another advantage of the SHEEP-study is that information regarding most common risk factors for CVD as well as DNA and blood is available. More studies where a genotype can be directly related to an intermediate phenotype, as well as to the disease, are warranted.

There are, however, also several sources of error that can arise due to the retrospective data-collection. After an MI event, a case may reflect over what in his, or her, lifestyle could have caused the disease and this could influence the subject’s ability to objectively recall past experiences in the questionnaire. Furthermore, samples taken after the MI event may to some extent be influenced by the MI event itself. MI cases are commonly advised on lifestyle changes that will reasonably have a beneficial effect on important risk factors such as smoking-habits, blood pressure, body-weight or diet, potentially leading to underestimated risks. Hypertension was more common among the controls than among the cases. Blood pressure commonly falls after an MI event and may still be affected three months afterwards due to damage to the myocardium.

In the SHEEP study, the samples were taken three months post-infarction at which time metabolic stability should have been regained according to experiences from young post-MI patients.147,148 The serum samples that were used to obtain insulin, IGFBP-1, IL-6, CRP and TNF-α values had been stored at −70°C for more than five years when they were thawed and analysed. This could potentially have an on the samples and influence assay results but, as described in the methods section, the samples were handled in a way which would minimise the time they were kept at room temperature. Even though no formal studies have been conducted to evaluate this, it can probably be assumed that biological markers measured in samples kept at -70°C should be minimally affected by the storage.
There is no reason to believe that the long storage time has affected the samples markedly as the observed levels of insulin, IGFBP-1, IL-6, CRP and TNF-α closely corresponds to those reported in earlier studies. The DNA was stored at -70°C for several years before the samples were diluted and placed in -20°C, a temperature at which the samples should be very stable.

At the time when the SHEEP study was collected, MI patients were commonly given beta-blockers but, fortunately for these studies, not statins as is common practice today. Beta-blockers can lead to induction of insulin resistance, and statin therapy is an efficient blocker of inflammatory responses and results in reduced cytokine levels. However, only very few subjects in the SHEEP material were being treated with statins since the results from the first large-scale cholesterol lowering trial using statins was only published in 1994. High levels of inflammatory markers are also seen in conjunction with acute inflammation. However, as the acute effects of the MI event would have dissipated by the time of sampling, there is no reason to believe that such exposure differed systematically between cases and controls.

The DNA and serum samples were analysed in a blinded manner to reduce bias and any sample that could not be clearly assayed was either re-analysed or excluded from the subsequent statistical analyses. As explained in the methods section, successful genotyping using DASH is dependent on a well-designed assay that clearly distinguishes between the assay melting points of the alleles. Overall, assays for the SNPs presented here were very reliable and left very few question-marks during the genotype scoring. DNA, or PCR-product contamination of the samples is a common problem when large number of genotypings are performed in a short stretch of time. Contamination of PCR-products from other samples will appear as a small peak in the DASH-output. These peaks are clearly distinguishable from the true peaks which are substantially taller and should therefore not influence the genotyping results.

Conclusions

MI is a complex disease and its development is a consequence of a person's genetic predisposition and his/her exposures to environmental factors throughout life. Unlike in animal-studies, it is not possible to keep the environment of a population of humans constant when assessing the relationship between a genotype and a phenotype. Likewise it is not possible to study a population of genetically identical persons, except for monozygotic twin-pairs. Individuals with unfavourable genotypes may
remain healthy if they are exposed to favourable environment and those with a low-risk genotype may fall ill because of adverse experiences in life. Considering that CHD is a multifactorial disease, it is very unlikely to find susceptibility genes that alone will result in increased MI risks. Instead the goal must be to identify all the ingredients in the cocktail of risk factors that shape our predisposition for MI as this will give important information on disease aetiology. The work presented in this thesis has led to the following conclusions regarding agents in the gene-environment network that underlie MI development: (i) insulin resistance is a risk factor for MI particularly through its synergistic interaction with smoking and elevated levels of IL-6, (ii) there is a clear gender related discordance in gene-phenotype associations as elevated IL-6 and TNF-α levels are stronger risk markers for MI among men compared to women, (iii) healthy male IL-6 -174C carriers have increased insulin levels, and (iv) female TNF-α -238A carriers have a reduced risk for MI.
Hjärtinfarkt kan orskas av många genetiska och omgivningsmässiga faktorer. Ibland kan förekomst av en enda riskfaktor vara tillräckligt för sjukdomens uppkomst. Troligen uppkommer dock de flesta fallen då en individ är exponerad för flertalet riskfaktorer. Riskfaktorernas ”interagerar” och bidrar alla tillsammans till sjukdomens utveckling. Målet med detta doktorandprojekt var att studera betydelsen av genetiska och omgivningsmässiga riskfaktorer, med speciell betoning på insulinresistens (försämrad insulinkänslighet i bl.a. muskler och fett), för uppkomst av hjärtinfarkt.

**Material och metod**

I delarbete I gjordes en kartläggning av gener av potentiellt intresse för uppkomst av insulinresistens och hjärtinfarkt och sedan användes webbaserade metoder för att söka efter genetiska varianter (s.k. polymorfier) i dessa gener. Andra databaser och experimentella metoder användes sedan för att söka efter ytterligare, dittills okända, polymorfier som skulle kunna påverka den individuella genens funktion. För delarbeten II-IV har SHEEP-materialet (Stockholm Heart Epidemiology Programme) använts. Detta är en fall-kontroll studie bestående av 5452 män och kvinnor som fått en hjärtinfarkt samt friska kontrollpersoner. Studiedeltagarna har lämnat blod, svarat på en enkät och genomgått vissa enklare kliniska undersökningar. Från blodproverna har DNA extraherats och blodvärden av insulin, insulin-like growth factor binding protein-1 (IGFBP-1), interleukin-6 (IL-6), C-reaktivt protein (CRP) och tumour necrosis factor-alfa (TNF-α) uppmätts. Män och kvinnor har analyserats separat i samtliga delarbeten för att kunna studera könsskillnader. För att undersöka hur genetiska faktorer, biologiska sambandskedjor (t.ex. insulinresistens, hypertension, lipidförändringar, övervikt) och riskbeteenden (t.ex. rökning och stillasittande) samverkar, gjordes även beräkningar för att uppskatta förekomst av biologisk interaktion.

**Resultat**

Efter att ha studerat internetbaserade databaser presenterade vi i delarbete I en lista över 146 gener och 614 ”single nucleotide polymorphisms” (SNPs) som är potentiellt gemensamma nämnare för uppkomst av både
insulinresistens och hjärtinfarkt. Från de 146 generna valdes två stycken av speciellt intresse (IL-6 och TNF-α) ut för vidare analys i SHEEP-studien (delarbeten III och IV).

Under senare år har man insett att insulinresistens är en viktig riskfaktor för hjärtinfarkt. I delarbete II har insulinresistensens betydelse för uppkomst av hjärtinfarkt studerats i SHEEP-materialet. Som mått på insulinresistens användes faste-insulin och HOMA (homeostasis model assessment) och en jämförelse visade att dessa mått är mer eller mindre likvärdiga i avseende på deras relation till hjärtinfarktrisk. Studien visade att insulinresistenta individer är speciellt känsliga för rökningens skadliga effekter, t.ex. kan en kvinna med insulinresistens minska risken att få hjärtinfarkt från en oddskvot på 8.1 till en oddskvot på 1.7 genom att sluta röka. Ateroskleros (en underliggande orsak till hjärtinfarkt) är en inflammatorisk process och höga nivåer av de inflammatoriska komponenterna IL-6 och TNF-α, tros vara betydande riskfaktorer för sjukdomen. Tidigare studier tyder på att genetiska varianter i generna för IL-6 och TNF-α ger förhöjda nivåer av IL-6 och TNF-α och därmed en ökad hjärtinfarktrisk. I delarbete III mättes IL-6 nivåerna och tre polymorfier i IL-6 genen analyserades i SHEEP materialet. Resultaten visar att risken för hjärtinfarkt ökade med IL-6 nivåerna hos män men inte hos kvinnor. Interaktion mellan IL-6 och flera andra viktiga riskfaktorer (inklusive insulinresistens) ökade risken att insjukna ytterligare. Ingen av polymorfierna påverkade IL-6 nivåerna eller hade någon betydande vikt för uppkomst av hjärtinfarkt som man tidigare misstänkt, men bör studeras ytterligare i relation till insulinresistens. I delarbete IV studerades fem funktionella polymorfier i TNF-α genen och TNF-α serum-nivåer analyserades i SHEEP för delarbete IV. Resultaten visade att förhöjda nivåer av TNF-α ger en ökad hjärtinfarktrisk hos både kvinnor och män. Effekten var speciellt markant hos överviktiga män. Studien visade även på att en polymorfi i TNF-α genen påverkar MI risk och att en annan polymorfi påverkade TNF-α proteinnivåerna något.

**Slutsatser**

Avhandlingsarbetet har lett till följande slutsatser: (i) Insulinresistenta individer förefaller vara speciellt känsliga för rökningens skadliga effekter. (ii) Förhöjda cytokinnivåer av såväl IL-6 (hos män) som TNF-α (hos båda könen) ökar risken för hjärtinfarkt. (iii) En polymorfi i IL-6 genen är associerad med förhöjda insulininnivåer hos män och, (iv) En polymorfi i TNF-α genen minskar risken för hjärtinfarkt hos kvinnor.
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