GENETIC AND IMMUNOLOGICAL RISK FACTORS AND CAROTID ARTERY ATHEROSCLEROSIS

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Abstract

Atherosclerosis is a multifactorial disease with numerous genes and environmental factors affecting its initiation and progression. During the past years many candidate genes for atherosclerosis have been suggested, and it has also become evident that the immune system plays a part in atherogenesis. Early atherosclerotic changes can be effectively detected by measuring carotid artery intima-media thickness (IMT). In the present study the associations between IMT and polymorphisms of three candidate genes for atherosclerosis were studied: endothelial nitric oxide synthase (eNOS), apolipoprotein E (apoE) and paraoxonase-1 (PON1). To assess the role of immunological factors determining carotid atherosclerosis, CRP and circulating autoantibodies to oxidised LDL were studied in relation to IMT. The study population consisted of 519 hypertensive and 526 control subjects from a middle-aged population in Oulu, Finland. The results showed that the investigated polymorphisms of eNOS and PON1 genes were not associated with IMT, suggesting that these polymorphisms are not major risk factors for atherosclerosis in the general Caucasian population. A significant interaction between the apoE polymorphism and smoking in relation to IMT was observed among men, indicating that carriers of the ε4 allele may be particularly prone to the atherogenic effects of smoking. This interaction was especially clear in hypertensive subjects. CRP was strongly associated with IMT before adjusting for confounding factors. After the adjustment, this association disappeared. The finding suggests that instead of early atherosclerosis CRP may be related to the later phases of the disease. This may partly explain the strong correlation between CRP and future cardiovascular events. IgM type of autoantibodies binding to oxidised LDL were inversely associated with IMT, and this finding remained after adjusting for previously known risk factors for atherosclerosis, implying a possible protective role for these antibodies in atherogenesis.

Keywords: apolipoproteins E, arteriosclerosis, autoantibodies, C-reactive protein, carotid arteries, endothelial nitric oxide synthase, oxidized LDL, paraoxonase-1, polymorphism
Acknowledgements

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Oulu, December 2003

Jarkko Karvonen
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<tr>
<td>AMI</td>
<td>acute myocardial infarction</td>
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<tr>
<td>ANCOVA</td>
<td>analysis of covariance</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>apo</td>
<td>apolipoprotein</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>BIF</td>
<td>carotid artery bifurcation</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
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<td>CAAD</td>
<td>carotid artery atherosclerotic disease</td>
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<tr>
<td>CAD</td>
<td>coronary artery disease</td>
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<tr>
<td>CCA</td>
<td>common carotid artery</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>CuOx</td>
<td>copper-oxidised</td>
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<td>EBCT</td>
<td>electron beam computer tomography</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>HDL</td>
<td>high-density lipoprotein</td>
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<td>ICA</td>
<td>internal carotid artery</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<td>IMT</td>
<td>intima-media thickness</td>
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<td>IVUS</td>
<td>intravascular ultrasound</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>lipoprotein(a)</td>
</tr>
<tr>
<td>LVM</td>
<td>left ventricular mass</td>
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<tr>
<td>MDA</td>
<td>malondialdehyde</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
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<tr>
<td>NS</td>
<td>not significant</td>
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<tr>
<td>OxLDL</td>
<td>oxidised LDL</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PON-1</td>
<td>paraoxonase-1</td>
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<tr>
<td>RIA</td>
<td>radio immuno assay</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<td>SE</td>
<td>standard error</td>
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<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<td>VLDL</td>
<td>very-low-density lipoprotein</td>
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References
1 Introduction

Atherosclerosis is a major cause of death in the Western countries. It is manifested in various forms including coronary artery disease and stroke. Traditional risk factors for atherosclerosis, such as elevated plasma LDL-cholesterol, smoking, hypertension and male gender, predict only a part of the future cardiovascular events. In fact, half of all patients with coronary artery disease do not have any established risk factors (Hennekens 1998). Atherosclerosis is now generally recognised as a multifactorial condition with numerous genes and environmental factors affecting the risk. Although the clinical complications of atherosclerosis occur at older age, the disease process begins decades earlier. Therefore identifying the individuals with the highest risk would allow for more efficient primary prevention of cardiovascular diseases. Measuring carotid artery intima-media thickness (IMT) with ultrasonography is a valid method to investigate early atherosclerotic changes, and there is strong epidemiological evidence showing that carotid artery IMT correlates well with future cardiovascular events (Smith, Jr. et al. 2000).

Even though it is clear that genetic factors strongly affect the risk of atherosclerosis, there are currently few genes that have consistently been shown to associate with the increased risk. Genetic polymorphism is a common feature in the human genome, and it means that a gene exists in a population in two or more different alleles, the frequencies of which are at least 1% in the studied population (Schafer & Hawkins 1998). Many candidate genes associated with atherosclerotic diseases are polymorphic. Most polymorphisms have no effect on the function of the protein the gene is coding. However, several polymorphisms are functional and have been described to be associated with atherosclerosis risk (Zannad & Benetos 2003). Nitric oxide has many functions that could prevent atherosclerosis. Therefore, the endothelial nitric oxide synthase (eNOS) gene has been of interest in several association studies (Wang & Wang 2000). Apolipoprotein E (apoE) has a central role in lipid metabolism, and the genetic polymorphism of the apoE gene has been linked to atherosclerosis in numerous studies. Recently, evidence for a gene-environment interaction between apoE gene polymorphism and smoking has been published (Humphries et al. 2001). The paraoxonase-1 (PON1) gene is one of the latest strong candidate genes for atherosclerosis (Mackness et al. 2002a). PON1 is able to protect LDL cholesterol from oxidation, which is a key event in atherogenesis. The
possible role of PON1 as a candidate gene of atherosclerosis is further highlighted by the fact that the activity of PON1 is largely determined by genetic polymorphisms.

There is increasing evidence suggesting that atherosclerosis is an inflammatory disease affected by innate and acquired immunity (Ross 1999). C-reactive protein (CRP) is a part of the innate immune system and in many studies the risk for cardiovascular events has been associated with elevated serum concentrations of CRP. However, there is less information available on the relationship of CRP and early subclinical atherosclerotic changes, such as increased carotid IMT. The role of adaptive immunity in atherosclerosis is complex. It is known that autoantibodies against oxidised LDL exist in vivo, but the role of these antibodies in atherogenesis is still unclear.

The OPERA (Oulu Project Elucidating Risk of Atherosclerosis) provides a good opportunity to study the associations between carotid artery IMT and various genetic and other risk factors, since the study sample was drawn from a geographically limited area with an ethnically and genetically homogenous population. In the present work, the relationship of carotid IMT and the polymorphisms of the three candidate genes mentioned (eNOS, apoE and PON1) were studied. In addition, the association between carotid IMT and CRP was investigated. Finally, the circulating autoantibodies to oxidised LDL were studied in relation to carotid artery IMT.
2 Review of the literature

2.1 Definition of atherosclerosis
Atherosclerosis refers to the accumulation of lipids and fibrous elements in large or medium-sized arteries leading to thickening and hardening of the vessel wall (Ross 1999). The arterial wall consists of three anatomically distinct layers: intima, media and adventitia, which are separated by two thin elastic layers, the inner and outer layer. Atherosclerosis is a disease of intima. The most common clinical complications of atherosclerosis are coronary artery disease (angina pectoris, myocardial infarction, sudden cardiac death), cerebrovascular disease (transient ischaemic attack, stroke), aortic disease (aneurysms) or insufficient circulation in the lower extremities (claudication or gangrene).

2.2 Pathogenesis of atherosclerosis
The theories on the pathogenesis of atherosclerosis remain somewhat hypothetical, because atherosclerosis is a multifactorial disease with a very large number of factors affecting it. Currently the leading hypothesis is the updated response-to-injury theory (Ross 1999). This theory is based on the idea that atherosclerosis is an immune, inflammatory or healing response to injury in the intima.

Atherosclerosis develops in the so-called lesion-prone sites in which the mechanical force of blood flow (shear stress) is low or turbulent (Stary et al. 1992). Atherosclerosis is thought to be initiated by endothelial dysfunction characterised by abnormal constriction and loss of nitric oxide-dependent dilatation of the affected vessels. In normal conditions endothelial cells secrete nitric oxide and cytokines that inhibit smooth muscle cell migration and proliferation (Consigny 1995). Dysfunctional endothelium produces adhesion and vasoactive molecules, cytokines and growth factors and is more permeable to lipoproteins and other plasma constituents. Compared to normal endothelial cells, dysfunctional endothelium also has impaired anticoagulant function (Ross 1999).
Histologically atherosclerosis can be classified into types I to VI (Stary et al. 1995) as shown in Figure 1. Type I and II lesions are often referred to as early lesions, type III lesion as intermediate or transitional lesion and type IV–VI lesions as advanced lesions.

Fig. 1. Classification of atherosclerotic lesions. Modified from Stary et al. (1995).

Type I lesions (initial lesions) are found in regions of intima where intimal thickening has occurred. The lesion contains small, isolated groups of lipid-rich macrophages (foam cells). The number of foam cells is, however, so small that type I lesions are normally not visible to the eye (Stary et al. 1994).

Type II lesions (fatty streaks) are characterised by large numbers of foam cells and can therefore often be seen as yellow streaks on gross inspection (Stary et al. 1994). Fatty streaks frequently develop at sites where endothelial injury has increased the permeability of endothelium to allow LDL cholesterol to penetrate into the intima. LDL becomes trapped in the intima and is oxidised by free radicals and other oxidising agents produced by endothelial cells, macrophages and smooth muscle cells. Also, other modifications of the structure of LDL particles occur. As a result of the modifications, the macrophages recognise the modified LDL and take up these particles via scavenger receptors. Since the scavenger receptors are under no feedback control, large amounts of modified LDL can be incorporated into macrophages, leading to the formation of foam cells (Witztum & Steinberg 1991). T lymphocytes and isolated mast cells have also been identified in type II lesions. In addition, smooth muscle cells migrate from the media into the intima. Foam
cell formation, T-cell activation and smooth muscle cell migration are mediated by a large number of cytokines (Ross 1999).

Type III lesions (preatheromas, intermediate lesions, transitional lesions) form a bridge between fatty streaks and advanced lesions. Whereas in type II lesions most of the lipid is in cells (mainly macrophages but also smooth muscle cells), in type III lesions small separate pools of extracellular lipid are found. However, the lipid core has not yet developed as in the case of advanced lesions (Stary et al. 1994).

Type IV lesions (atheromas) are the first class of advanced lesions. The small isolated pools of extracellular lipids in type III lesions are conflated to form the lipid core. The region between the lipid core and the endothelial surface contains macrophages, isolated smooth muscle cells, T-cells and mast cells and only minimal amounts of collagen. Therefore type IV lesions may be vulnerable to fissures and ruptures, even though they often fail to narrow the vascular lumen (Stary et al. 1995). In fact, the thickening of the vascular wall may lead to compensatory enlargement of the arteries until approximately 40% stenosis is present (Glagov et al. 1987). This may explain why approximately 70% of patients with coronary disease present with acute myocardial infarction or sudden death, not angina as the first symptom (Nissen 2001).

Type V lesions (fibroatheromas) are characterised by a new fibrous connective tissue layer (fibrous cap) that walls off the lesion from the lumen. The fibrous cap contains substantial amounts of collagen and smooth muscle cells. In the deeper layers of the lesion a mixture of leukocytes, lipids and debris is found (Ross 1999). In some type V lesions the lipid core and other parts of the lesion are calcified (Stary et al. 1995). Type V lesions generally narrow the lumen of the vessel more than type IV lesions.

Type VI lesion (complicated lesion) is defined as type IV or V lesion with disruption of the lesion surface, haematoma, intrallesional haemorrhage or thrombotic deposit. The rupture of the lesion often occurs at the shoulders of the fibrous cap where macrophages enter, accumulate and are activated and where apoptosis may occur. Plaque rupture leads to release of cholesterol crystals from the lesion and an exposure of intimal collagen and matrix to blood flow, which accelerates platelet adhesion and aggregation leading to thrombosis. Finally thrombosis may lead to e.g. myocardial infarction, stroke or gangrene (Lee & Libby 1997).

2.3 Detection of atherosclerosis

Probably the most valid method currently available for the assessment of atherosclerosis is the intravascular ultrasound (IVUS) (Nissen 2001). It provides accurate quantitative images of atherosclerotic plaques with excellent sensitivity. IVUS has been thought to be especially useful in clinical decision-making since it also detects plaques that do not narrow the arterial lumen that appear commonly (Glagov et al. 1987, Nissen 2001) but cannot be detected by conventional angiography. However, IVUS is an invasive method with limited availability, and thus it has not been widely used in clinical studies.

There are also various non-invasive methods available for determination of atherosclerosis. It has been demonstrated that endothelial dysfunction, one of the key events in early atherosclerosis, may be present years before structural changes appear in
the arteries (Celermajer et al. 1992). The original method for studying endothelial function included cardiac catheterisation (Ludmer et al. 1986) and was therefore inconvenient for large studies. Since then several non-invasive methods have been developed, including the measurement of endothelium-dependent dilatation in peripheral arteries using ultrasound (Celermajer et al. 1992), transthoracic doppler/echocardiography (Hozumi et al. 1998), cardiac magnetic resonance imaging (Davis et al. 1997) and positron emission tomography (Vallabhajosula & Fuster 1997).

Measuring coronary artery calcification provides another method to study early arterial wall alterations. Electron beam computer tomography (EBCT) presents high resolution and enables quantitative assessment of coronary artery calcification (Agatston et al. 1990, Fallavollita et al. 1994). However, EBCT has low specificity for clinically relevant obstructive coronary artery disease (CAD) (Achenbach et al. 1998), and some plaques may not contain calcium, which also diminishes the predictive value of the method. In addition, in a study by Achenbach and co-workers (1998) it was found that technical problems impairing the quality of the images occurred in approximately 25% of the coronary arteries studied.

Atherosclerosis is a disease of the vascular wall characterised by increased wall thickness. Therefore, the measurement of the IMT of the large arteries close to the skin (i.e. carotid and femoral arteries) provides yet another tool to study the early atherosclerotic changes. Measuring IMT of the carotid arteries by B-mode ultrasound is a fairly simple, safe, inexpensive, precise and reproducible method (Aminbakhsh & Mancini 1999). An increase in the IMT of the carotid arteries is considered to reflect early atherosclerosis, although it might also reflect an adaptation to environmental factors, such as hypertension. However, several lines of evidence support the view that IMT is a reliable marker for atherosclerosis. First, there is a close relationship between IMT and a number of traditional cardiovascular risk factors such as male sex, age, overweight, hypertension, hypercholesterolaemia and smoking in the general populations (Salonen & Salonen 1990, Gariepy et al. 1998, Ferrieres et al. 1999, Mannami et al. 2000). Also, a cumulative effect of several risk factors on IMT has been shown as Persson and co-workers (1994) have reported an association between the Framingham risk score and IMT. Second, there is a strong association between angiographically detected CAD and IMT (Crouse et al. 1995) as well as between CAD and the progression of IMT (Crouse et al. 2002).

The third line of data suggesting that IMT represents atherosclerotic changes comes from the prospective clinical studies. The KIHD study (Salonen & Salonen 1991) was carried out in a large (n = 1,228) healthy middle-aged Finnish male population. The results of this study showed that intima-media thickening (at or above 1 mm) of the common carotid artery (CCA) was associated with an over two-fold greater risk for acute myocardial infarction (AMI) during 3 years. Another large (7,289 women and 5,552 men) cohort study, the ARIC study, showed that in a middle-aged population of four US communities intima-media thickening (at or above 1 mm) of the CCA resulted in a 2- to 5-fold greater risk for a coronary event over 4–7 years’ time (Chambless et al. 1997). In the ARIC study, also the risk of stroke was increased when IMT of the CCA was at or above 1 mm (Chambless et al. 2000). CHS study is the other large (5,858 subjects, aged 65 years or older) study carried out in the USA investigating the relationship between IMT and cardiovascular events. The results of the CHS study showed that IMT of the
CCA at or above 1.18 mm was associated with an approximately four-fold greater risk for AMI or stroke during the 6 years’ follow-up (O’Leary et al. 1999). The Rotterdam study was carried out in a Dutch population (7,983 subjects, aged 55 years or older) and the results showed that the risk of AMI and stroke increased by 40% per each standard deviation (0.16 mm) of IMT of the CCA over a period of 6 years (Bots et al. 1997). Also, the CLAS study, where the study subjects were CAD patients, showed that the increased IMT of the CCA and the progression in IMT were associated with coronary events (relative risk of 3.1 for every 0.03 mm increase in IMT per year) during the 9 years of follow-up (Hodis et al. 1998). All these studies demonstrate that IMT predicts future coronary events and stroke efficiently.

The fourth aspect of evidence are the numerous therapeutic trials on IMT. Many studies have shown that compared to placebo, statins significantly decrease the progression of IMT, e.g. the KAPS (Salonen et al. 1995) and CAIUS (Mercuri et al. 1996) studies among asymptomatic high-risk subjects and the REGRESS study (de Groot et al. 1998) among coronary patients. Similar results have also been reported in studies comparing a calcium antagonist with placebo (Pitt et al. 2000) and an angiotensin-converting enzyme inhibitor with placebo (Lonn et al. 2001). There is also some evidence that a high dietary intake of vitamin E (McQuillan et al. 2001), vitamin E supplementation (Azen et al. 1996) or combined vitamin E and C supplementation (Salonen et al. 2003) reduces the progression of IMT, even though some other studies report no benefit from supplemental antioxidant vitamin use in relation to IMT (McQuillan et al. 2001, Hodis et al. 2002). Finally, ultrasonographically detected IMT is closely related to other markers of subclinical atherosclerosis, i.e. to EBCT (Davis et al. 1999) and endothelial dysfunction (Hashimoto et al. 1999) and also shows a close correlation to microscopically measured IMT (Pignoli et al. 1986, Persson et al. 1994).

Since measuring IMT is a relatively new method, no international standards are available on how the measurements should be carried out. The two main approaches used so far are the manual measurements at multiple carotid sites in both near and far walls on the basis of a video image (Howard et al. 1993) and the automated computerised measurement of only the far wall of the CCA (Gariepy et al. 1998). The precision of the measurements has been studied using wedge phantoms and the results have shown that changes as small as 0.03–0.05 mm can be detected (Salonen et al. 1993). The reproducibility of the measurements has been proven excellent in both manual (Tang et al. 2000) and computerised measurements (Graf et al. 1999). However, the computerised method is currently available only for the CCA, whereas the manual method also covers the carotid bifurcation (BIF) and the internal carotid artery (ICA), thus giving more information on the whole carotid tree. This is important as atherosclerotic plaques are often found in the ICA. As of yet there are no threshold values for normal and abnormal IMT, even though some studies have defined the distribution of IMT in the general healthy populations (Howard et al. 1993, Denarie et al. 2000). Nevertheless, epidemiological data strongly indicate that the increased risk of future myocardial infarction or stroke is associated with IMT at or above 1 mm.
2.4 Risk factors for atherosclerosis

Atherosclerosis is a multifactorial disease and the risk of atherosclerosis is affected by many environmental and genetic factors as well as by interactions between these risk factors (Sing et al. 1992). The concept of risk factor was introduced by the Framingham Heart study in the 1970s (Kannel et al. 1976). The major risk factors for atherosclerosis are well-known. Grundy and co-workers (1999) have divided them into three categories: independent, predisposing and conditional risk factors. The independent risk factors include cigarette smoking, hypertension, elevated serum total and LDL cholesterol, low serum HDL cholesterol, diabetes mellitus and advancing age. The predisposing risk factors include overweight, abdominal obesity, physical inactivity, family history of premature atherosclerotic diseases, ethnic characteristics and psychosocial factors. The conditional risk factors include elevated serum triglycerides, small LDL particles, elevated serum homocysteine, elevated serum lipoprotein(a), prothrombotic factors (e.g. fibrinogen) and inflammatory markers (e.g. CRP). Studies have shown that the traditional risk factors (e.g. age, smoking, blood pressure and cholesterol concentration) predict only part of the future cardiovascular events (Heller et al. 1984, Vartiainen et al. 1994). Novel risk factors, such as genetic and immunological factors, may thus have a substantial influence on the age of onset and the frequency and severity of clinical symptoms, as well as response to therapy in atherosclerotic diseases. Indeed, in a Swedish twin study (Marenberg et al. 1994), the relative hazard of death from CAD when one's twin died of CAD before the age of 55 years, as compared with the hazard when one's twin did not die before 55, was 8.1 for monozygotic twins and 3.8 for dizygotic twins among men. Among women, when the co-twin died of CAD before the age of 65 years, the relative hazard was 15.0 for monozygotic twins and 2.6 for dizygotic twins. The genes affecting the risk for atherosclerosis are mostly unknown, even though some rare genetic mutations leading to premature CAD have been found (Funke & Assmann 1999).

2.5 Association studies in atherosclerosis

Basically, there are two approaches to achieve genetic information on the pathophysiology of complex diseases, such as atherosclerosis (Lander & Schork 1994). First, in linkage analysis the whole genome is searched for loci (i.e. regions of the genome) linked to the disease and then the responsible focus is narrowed down until at least one gene is identified. This is a time-consuming and expensive method and with regard to complex diseases success has been limited. For example, many gene loci linked to high blood pressure have been pointed out, but so far no single gene has been identified at the molecular level by linkage alone (Lifton et al. 2001). The second possibility, which is also used in the present work, is association analysis. A typical association study has a case-control or a population-based cohort design in which the allele frequencies of subjects with and without a certain phenotype are compared when studying qualitative traits (Lander & Schork 1994). Quantitative traits, such as the intima-media thickness, can be studied either by comparing the genotype and allele frequencies between different classes of the trait or by comparing the values of the
quantitative trait between different genotype groups. There are three possible explanations, in addition to pure chance, for a positive association observed in a genetic association study (Lander & Schork 1994). First, the gene actually has a causative role in the development of the disease. In that case, the same positive association is expected to occur also in other populations. Second, there is linkage disequilibrium between the disease allele and a marker allele, meaning that the disease allele and the marker allele are situated in close proximity on the same chromosome in such a way that many generations are required for the alleles to be separated by recombination. In that case, the same association is not necessarily seen in other populations because the associating marker allele may be different in different populations e.g. due to the so-called founder effect. Third, a selection or another bias has led to a false positive association.

Because whole-genome studies have proven arduous with very limited success in complex diseases, the so-called candidate gene approach has become popular in the field of atherosclerosis research. In this method the relationship between one candidate gene and the disease is studied and usually the remainder of the genome is ignored. The choice of candidate genes arises either from the positional identification by linkage analysis or more frequently because the gene product can be plausibly argued to contribute to the disease process. In animal models, the transgenic techniques have been a powerful tool in studying the functional role of the candidate genes. In humans, however, association studies are the mainstay of the candidate gene approach. The heritability of carotid artery atherosclerosis has been well documented. Duggirala et al. (1996) reported that genetic factors account for up to 66% of the variation in carotid IMT. In another study, the genes were estimated to explain up to 30% of the variation in carotid IMT (Zannad et al. 1998). Also, the two most recent studies (Xiang et al. 2002, North et al. 2002) found a significant heritability of carotid IMT. A number of candidate genes for increased carotid artery IMT have been studied, including the genes of the renin-angiotensin aldosterone system, genes affecting endothelial mechanisms, inflammation and haemostasis, genes of cytokines, extracellular matrix, the adrenergic system and neuropeptide Y and genes related to the metabolism of lipids and homocysteine (Zannad & Benetos 2003).

2.6 Endothelial nitric oxide synthase and atherosclerosis

Nitric oxide is a versatile molecule, its actions ranging from haemodynamic regulation to anti-proliferative effects on vascular smooth muscle cells. Nitric oxide (NO) is produced from L-arginine by the nitric oxide synthases, endothelial NOS (eNOS), neural NOS (nNOS), and inducible NOS (iNOS) (Marletta 1994). The neural NOS is expressed in neurons and skeletal muscles and is responsible for the physiological production of NO in these tissues. The inducible NOS is generally not expressed, but may be induced in most cells by abnormal processes such as inflammation. Constitutively expressed eNOS is present mainly in the vascular endothelium and produces low concentrations of NO, which is necessary for good endothelial function and integrity (Palmer et al. 1987, Marletta 1994). NO also regulates the antithrombotic properties of the endothelium by inhibiting platelet aggregation (Mellion et al. 1981) and adhesion (Radomski et al. 1987). In addition, NO inhibits leukocyte chemotaxis and adhesion to endothelium (Bath et al.
and the proliferation (Garg & Hassid 1989) and migration (Sarkar et al. 1996) of vascular smooth muscle cells. Finally, NO has been shown to inhibit LDL oxidation (Hogg et al. 1993). Because of these multiple actions, endothelial NO may have an important role in the protection against atherogenesis. Indeed, it has been reported that reduced basal NO release may predispose humans to atherosclerosis (Oemar et al. 1998). Furthermore, knockout mice (eNOS -/-) have been examined for the development of atherosclerosis in an apoE-deficient mouse model (Knowles et al. 2000, Kuhlencordt et al. 2001). The results indicate a protective role for eNOS in atherosclerosis and this antiatherosclerotic effect cannot be fully explained by the influence on blood pressure.

Several polymorphisms in the promoter region, exons and introns of the eNOS gene have been identified (Wang & Wang 2000). Three single nucleotide polymorphisms (at positions –1468 bp, –922 bp and –786 bp) have been detected in the promoter region of the eNOS gene. However, it has been suggested that sequences extending only to –144 bp are essential for the promoter activity (Karantzoulis-Fegaras et al. 1999). The substitution of T to C at –786 bp has been associated with coronary spasm in Japanese populations (Nakayama et al. 1999, Yoshimura et al. 2000) and with CAD in an Italian population (Colombo et al. 2003). The other polymorphisms in the promoter region have not been reported to associate with cardiovascular pathology. Single nucleotide polymorphisms have also been found in introns 2, 11, 12, 18, 22 and 23. There is no evidence for an association between cardiovascular diseases and the sequence variation in these parts of the gene. The eNOS gene also harbours tandem repeats in introns 2, 4, 8 and 13. In intron 4 the smaller allele, eNOS4a, contains four 27-bp tandem repeats and the larger allele, eNOS4b, contains five repeats. There is evidence that eNOS4a is associated with hypertension (Uwabo et al. 1998), CAD (Wang et al. 1996, Fowkes et al. 2000) and AMI (Ichihara et al. 1998, Hooper et al. 1999). The high number of CA repeats in intron 13 has also been reported to associate with hypertension (Nakayama et al. 1997) and CAD (Stangl et al. 2000), but there is no evidence for an association between cardiovascular diseases and the tandem repeats in introns 2 and 8.

Only two polymorphisms in the exons of the eNOS gene have been found. The polymorphism in exon 6 does not lead to amino acid change. In exon 7, however, there is a polymorphism which leads to the substitution of glutamate (Glu) to aspartate (Asp) at position 298. It has been reported that this polymorphism is likely to alter the function of the protein (Tesauro et al. 2000), even though it has also been demonstrated that this altered function does not affect endothelium-dependent vasodilatation (Schneider et al. 2000). There are studies showing that the Asp298 allele has a positive relation to hypertension (Miyamoto et al. 1998, Shoji et al. 2000a), coronary spasm (Yoshimura et al. 1998), CAD (Hingorani et al. 1999, Colombo et al. 2002) and AMI (Shimasaki et al. 1998, Hibi et al. 1998). Previously, only one study has been published where the Glu298Asp polymorphism with regard to IMT has been investigated. Lembo and associates (2001) studied 375 consecutive subjects who attended a hypertension centre for the first time. The subjects were divided into two categories according to the presence of carotid plaques (IMT ≥ 1.5 mm). It was found that the risk of having carotid plaques was approximately three times higher in subjects who were homozygotic for the Asp298 variant compared with subjects who were homozygotic for the Glu298 variant, and the risk was independent of the other common risk factors (age, blood pressure and smoking).
2.7 Apolipoprotein E and atherosclerosis

Apolipoprotein E is found in chylomicrons, VLDL and HDL. It is mainly synthesised in the liver but also in many other organs including brain, spleen, and kidney (Siest et al. 1995). ApoE plays an essential role in lipoprotein metabolism by acting as a ligand for two receptors, the LDL receptor (also known as the apoB/E receptor) found in the liver and other tissues and an apo E-specific chylomicron remnant receptor found in the liver. The coordinate interaction of these lipoprotein complexes with their receptors forms the basis for the metabolic regulation of cholesterol. ApoE gene contributes more to the normal cholesterol variability than any other gene identified so far in cholesterol metabolism (Sing & Davignon 1985). In addition, apoE may contribute to arterial protection from atherosclerosis via other mechanisms, such as directing cholesterol efflux mechanisms with the aid of apoA1 and the ATP binding cassette transporter 1, preventing platelet aggregation by facilitating the production of nitric oxide and inhibiting the proliferation of T-lymphocytes and endothelial cells (Bocksch et al. 2001). There is also evidence for antioxidant activity of apoE (Miyata & Smith 1996). Furthermore, other functions of apoE unrelated to cholesterol transport and vascular protection have been found (Mahley 1988). For example, markedly high quantities of apoE are found at sites of peripheral nerve injury and regeneration (Ignatius et al. 1986), which may reflect the general tissue response to an increased cholesterol demand for cellular membrane construction (Siest et al. 1995).

Polymorphism at the apoE gene locus results in three common alleles found in most populations: ε2, ε3 and ε4. These determine six apoE phenotypes: E2/2, E2/3, E2/4, E3/3, E4/3 and E4/4. The isoforms differ from each other by a single amino acid at positions 112 and 158 (Weisgraber et al. 1981). Apo E3 contains cysteine at 112 and arginine at 158. Apo E2 has cysteine at both positions and E4 has arginine on both sites. The frequencies of the ε2, ε3 and ε4 alleles in the Caucasian population are approximately 8, 77 and 15%, respectively (Davignon et al. 1988). However, the allele frequencies vary markedly between different populations. Northern Europeans have higher frequencies (0.17–0.23) of the ε4 allele than subjects in most populations studied (Ehnholm et al. 1986, Lehtimäki et al. 1990, Gerdes et al. 1992) and in addition, a gradient for the ε4 allele frequency decreasing from Finland to the south of Europe has been reported (Tiret et al. 1994). Finns also have a relatively low frequency (0.04) of the ε2 allele (Ehnholm et al. 1986, Lehtimäki et al. 1990).

The functional effects of apoE polymorphism on lipoprotein metabolism are mediated through the hepatic binding, uptake, and catabolism of chylomicrons, chylomicron remnants, VLDL, and HDL subspecies (Sing & Davignon 1985, Davignon et al. 1988). E4 has been suggested as the ancestral form of apoE (Fullerton et al. 2000), but E3 is currently the most common isotype in all human populations investigated so far (Eichner et al. 2002). The E2 isoform confers defective binding capacity for the LDL receptor (approximately 1% of the capacity of E3 and E4) and up-regulation of the liver LDL receptors resulting in lower concentrations of plasma LDL cholesterol compared to E3. On the contrary, E4 is associated with normal receptor-binding capacity and down-regulation of hepatic LDL receptors and thus with elevated concentrations of plasma LDL cholesterol when comparing to the most common isotype, E3. In addition, apo E2 is associated with low, and apo E4 with abundant cholesterol absorption...
(Kesäniemi *et al.* 1987). Altogether, it is estimated that the apoE polymorphism explains 16–17% of the genetic variance and 8–9% of the total variance in the plasma cholesterol concentration in different populations (Sing & Davignon 1985, Boerwinkle *et al.* 1987). The apoE polymorphism also determines the plasma concentration of apoE: the ε2 allele is associated with a higher concentration and the ε4 allele with a lower concentration (Boerwinkle *et al.* 1987).

Several studies have assessed the relationship between apoE polymorphism and atherosclerotic diseases. A meta-analysis of 14 observational studies showed that the ε4 allele is associated with coronary disease among both men and women (Wilson *et al.* 1996). Most of these studies were cross-sectional studies, but the association has also been shown in a prospective study in a Finnish population (Stengård *et al.* 1995). Further, another Finnish study (Ilveskoski *et al.* 1999) has confirmed the association between the ε4 allele and atherosclerosis using autopsy series, even though in this study the association was only seen in early middle aged subjects, whereas at older age the allele was not related to atherosclerosis. Also, prognosis after myocardial infarction seems to be determined by the apoE polymorphism (Gerdes *et al.* 2000), and the ε4 allele associates negatively with longevity (Kervinen *et al.* 1994, Louhija *et al.* 1994). Finally, the ε4 allele has been shown to be associated with carotid artery atherosclerosis (Terry *et al.* 1996, Cattin *et al.* 1997, Vauhkonen *et al.* 1997, Haraki *et al.* 2002), although not in every study (de Andrade *et al.* 1995, Kogawa *et al.* 1997, Zannad *et al.* 1998, Sass *et al.* 1998, Ilveskoski *et al.* 2000, Hanon *et al.* 2000, Herejsi *et al.* 2000, Güz *et al.* 2000, Slooter *et al.* 2001) (Table 1).

In addition to the ε2/ε3/ε4 polymorphism several other polymorphisms of the apoE gene have recently been identified in various populations (Nickerson *et al.* 2000, Fullerton *et al.* 2000, Stengård *et al.* 2002). These polymorphisms also seem to have a substantial role in explaining the interindividual variation in lipid concentrations, and the role of the different combinations of these additional polymorphisms varies between different populations (Stengård *et al.* 2002).

There are numerous studies of interactions between the apoE ε2/ε3/ε4 polymorphism and different environmental factors. Many studies have suggested a gene-diet interaction between the apoE polymorphism and dietary fats. A meta-analysis of these studies (Ordovas *et al.* 1995) indicates that subjects carrying the ε4 allele are more responsive with regard to LDL cholesterol lowering in response to dietary fat and cholesterol restriction than subjects carrying the ε3 or ε2 allele. It has also been reported that ε2 carriers have greater responsiveness to the lipid-lowering drugs than ε3 and the ε4 carriers (Nestel *et al.* 1997). Moreover, the apoE genotype may influence the blood pressure increasing effect of alcohol consumption (Kauma *et al.* 1998). A gene-gene interaction has been suggested with the angiotensin-converting enzyme gene (van Boekxmeer *et al.* 1995) as well as an interaction between apoE and physical activity (Taimela *et al.* 1996). Finally, an interaction between smoking and apoE polymorphism has been reported by Humphries and co-workers (2001). In this prospective study, apoE genotype and smoking habits were obtained for 2,303 healthy middle-aged men and the subjects were followed up for eight years on average. The results showed that among non-smokers, the risk for CAD was much the same irrespective of genotype. Among smokers, however, subjects who were carriers of the ε4 allele had a significantly increased risk of CAD compared to subjects not carrying the ε4 allele.
Table 1. Studies analysing associations between apolipoprotein E polymorphism and IMT.

<table>
<thead>
<tr>
<th>Author</th>
<th>Study population</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terry et al. (1996)</td>
<td>130 subjects with CAD, 130 controls (men and women)</td>
<td>The ε4 carriers had the highest IMT in CCA and ICA. In BIF there was no difference between genotypes.</td>
</tr>
<tr>
<td>Cattin et al. (1997)</td>
<td>260 randomly selected subjects (men and women)</td>
<td>The ε4 carriers had greater IMT in CCA than the ε3 carriers. In BIF, ICA and maximal mean IMT there was no difference between genotypes.</td>
</tr>
<tr>
<td>Vauhkonen et al. (1997)</td>
<td>83 subjects with NIDDM, 123 controls (men and women)</td>
<td>In the control subjects the ε4 carriers had greater IMT in CCA than the ε3 carriers. In the NIDDM subjects there was no difference between genotypes.</td>
</tr>
<tr>
<td>Haraki et al. (2002)</td>
<td>96 asymptomatic men</td>
<td>The ε4 allele was positively associated with IMT of CCA.</td>
</tr>
<tr>
<td>de Andrade et al. (1995)</td>
<td>145 subjects with carotid atherosclerosis, 224 controls (men and women)</td>
<td>The ε2/3 genotype was associated with CAAD (IMT &gt; 2.5 mm at any site, IMT of ICA &gt;1.7 mm, IMT of BIF &gt; 1.8 mm or IMT of CCA &gt; 0.6 mm)</td>
</tr>
<tr>
<td>Kogawa et al. (1997)</td>
<td>356 subjects with NIDDM, 235 controls (men and women)</td>
<td>No difference was found in IMT between genotypes either in NIDDM patients or in the controls.</td>
</tr>
<tr>
<td>Zannad et al. (1998)</td>
<td>76 families from the general population</td>
<td>The ε2 and ε4 alleles were associated with lower IMT in CCA than the ε3 allele.</td>
</tr>
<tr>
<td>Sass et al. (1998)</td>
<td>150 subjects without cardiovascular diseases (men and women)</td>
<td>No difference was found in IMT of CCA between genotypes.</td>
</tr>
<tr>
<td>Ilveskoski et al. (2000)</td>
<td>189 randomly selected men</td>
<td>IMT of ICA was higher in the ε3 carriers than in the ε4 carriers.</td>
</tr>
<tr>
<td>Hanon et al. (2000)</td>
<td>320 asymptomatic subjects with cardiovascular risk factors (men and women)</td>
<td>The ε2 allele was associated with CCA hypertrophy (IMT &gt; 0.66 mm).</td>
</tr>
<tr>
<td>Güz et al. (2000)</td>
<td>269 subjects undergoing haemodialysis (men and women)</td>
<td>No difference was found in IMT of CCA between genotypes.</td>
</tr>
<tr>
<td>Horejsi et al. (2000)</td>
<td>114 hyperlipidemic subjects (men and women)</td>
<td>No difference was found in IMT of CCA between genotypes.</td>
</tr>
<tr>
<td>Slooter et al. (2001)</td>
<td>5,401 subjects from a population-based cohort (men and women)</td>
<td>The ε2/3 carriers had lower IMT of CCA than the ε3/3 carriers. The ε4 allele was not statistically significantly associated with IMT.</td>
</tr>
</tbody>
</table>

BIF, carotid bifurcation; CAAD, carotid artery atherosclerotic disease; CAD, coronary artery disease; CCA, common carotid artery; ICA, internal carotid artery; IMT, intima-media thickness; NIDDM, non insulin-dependent diabetes mellitus.

2.8 Paraoxonase-1 and atherosclerosis

Paraoxonase-1 is a member of a multigene family with at least three PON genes, designated PON1, PON2 and PON3. PON1 was found first and most research has focussed on it. PON1 is expressed mainly in the liver but also in kidneys, heart, brain, small intestine and lungs (Primo-Parmo et al. 1996). PON1 is involved in the metabolism of xenobiotics, such as paraoxon (Mackness et al. 1996), but despite intensive studies its
physiological substrate is still unknown. However, the highly conserved structure of the gene among mammalian species (81–91% identity in nucleotide sequence) is thought to suggest an important role for the PON1 catalysed reactions (Primo-Parmo et al. 1996). Within a given species the three PON genes share approximately 70% similarity in the nucleotide sequence.

The PON1 enzyme is located on HDL particles and the antioxidant activity of HDL is largely due to the activity of PON1. PON1 has been shown to protect LDL against oxidative modifications by hydrolysing LDL-associated oxidised phospholipids and cholesteryl-ester hydroperoxides and destroying the pro-inflammatory molecules involved in the initiation and progression of atherosclerotic lesions (Mackness et al. 1993, Watson et al. 1995, Navab et al. 1996, Hedrick et al. 2000). HDL from species lacking PON1, such as birds, (Mackness et al. 1998a) and HDL in the presence of PON1 inhibitors (Aviram et al. 1998) loses its capacity to prevent LDL from oxidation. In addition, experiments with PON1 knockout mice strongly indicate the potential of PON1 to protect against LDL peroxidation and atherogenesis (Shih et al. 1998, Rozenberg et al. 2003). It has also been demonstrated that PON1 accumulates in the atherosclerotic lesions as they advance, but it is not clear whether the cells of the arterial wall secrete PON1 or whether PON1 is taken up from circulation (Mackness et al. 1997a).

PON1 has two common coding region amino acid polymorphisms (Humbert et al. 1993). The first polymorphism leads to the substitution of leucine (Leu) to methionine (Met) at position 55 and the second to the substitution of glutamine (Gln) to arginine (Arg) at position 192. These polymorphisms are in linkage disequilibrium with Leu at position 55 associated with Arg at position 192. The 10- to 40-fold variability in the activity of PON1 among individuals is largely due to these polymorphisms (Davies et al. 1996). In addition, five polymorphisms in the promoter region have been recently identified (Leviev & James 2000, Brophy et al. 2001). They also strongly influence the serum concentration and activity of PON1.

When using paraoxon as a substrate, the Met55 allele is associated with lower and the Leu55 allele with higher activity of PON1 (Mackness et al. 1998b). Higher serum PON1 concentration is also assigned to the Leu55 variant (Garin et al. 1997). Similarly, the Gln192 allele exhibits lower and the Arg192 allele higher activity towards paraoxon (Davies et al. 1996). Despite the high PON1 activity several studies have shown a positive association between the Arg192 allele and CAD (Ruiz et al. 1995, Serrato & Marian 1995, Sanghera et al. 1997, Zama et al. 1997, Pati & Pati 1998, Sanghera et al. 1998a, Pfohl et al. 1999), while in several other studies a lack of association has been reported (Antikainen et al. 1996, Suehiro et al. 1996, Herrmann et al. 1996, Rice et al. 1997, Ombres et al. 1998, Ko et al. 1998). Also, there are some studies reporting a positive association between the Leu55 allele and atherosclerotic diseases (Garin et al. 1997, Schmidt et al. 1998) as well as some studies reporting no association (Zama et al. 1997, Sanghera et al. 1998b, Gardemann et al. 2000). Finally, there is one prospective study in a Finnish population reporting that increased risk for myocardial infarction is associated with the Met/Met55 genotype (Salonen et al. 1999). There are also some association studies with regard to PON1 polymorphisms and carotid atherosclerosis (Cao et al. 1998, Dessi et al. 1999, Leus et al. 2000, Malin et al. 2001, Markus et al. 2001, Fortunato et al. 2003). The results of these studies (Table 2) have been contradictory.
2.9 C-reactive protein and atherosclerosis

Acute phase proteins are produced by the liver in response to different clinical conditions including infection, inflammation or trauma (Gabay & Kushner 1999). The production of these agents is induced by cytokines (mainly interleukin-6) released from the jeopardised tissue. CRP is one of the best-known acute phase reactants. It is part of the pentraxin protein family. The structure of these proteins is well conserved among different species and they have been thought to precede the development of the adaptive immune response (Du Clos 2000). Also, the fact that no deficiencies of CRP have ever been found suggests a crucial role of the protein.

During acute-phase reactions CRP can increase up to 100-fold (Gabay & Kushner 1999). The physiological role of CRP is yet unknown. It is not even clear whether CRP is a proinflammatory or an anti-inflammatory molecule overall. A number of ligands to which CRP binds have been identified (Gabay & Kushner 1999). These include C-polysaccharide of the pneumococcus (from which the name CRP is derived) and the phospholipids of the damaged cell walls. It has been demonstrated that CRP has the ability to mediate phagocytosis of various pathogenic microorganisms functioning as an opsonin and to activate the classical complement pathway (Du Clos 2000). Experimental studies have also shown that CRP protects mice from pneumococcal infection (Mold et al. 1981, Szalai et al. 1995) and could also be protective against the development of autoimmunity (Du Clos et al. 1994).

There is accumulating evidence that CRP is a risk factor for cardiovascular disease in humans. Recently Ridker and associates (2002) published a study where 28,000 initially healthy women were followed for a mean of eight years for the occurrence of AMI, stroke, coronary revascularisation, or death from cardiovascular causes. The results

It has been speculated that circulating CRP only reflects the general inflammation occurring in the atherosclerotic process and is not an active component in the pathogenesis of the disease. However, several lines of evidence also support the view that CRP has a role in atherogenesis. First, chronic infections giving rise to CRP are also associated with increased risk for cardiovascular diseases (Leinonen & Saikku 2002). Second, CRP is found in atherosclerotic lesions (Reynolds & Vance 1987, Hatanaka et al. 1995) and infarcted myocardium (Lagrand et al. 1997). Finally, CRP has been shown to have proatherogenic properties in vitro: CRP may activate endothelial cells to produce adhesion molecules (Pasceri et al. 2000). It may also decrease the production of eNOS in endothelial cells (Venugopal et al. 2002) and enhance the uptake of LDL by macrophages (Zwaka et al. 2001).

Table 3. Studies analysing associations between CRP and IMT.

<table>
<thead>
<tr>
<th>Author</th>
<th>Study population</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blackburn et al. (1999)</td>
<td>1,051 dyslipidaemic subjects (men and women)</td>
<td>A positive relationship was found between CRP and the severity of carotid stenosis. In multivariate analysis the association remained significant in male subjects only.</td>
</tr>
<tr>
<td>Hashimoto et al. (2001)</td>
<td>179 subjects with cardiovascular risk factors (men and women)</td>
<td>The number of plaques developing during 35 months’ follow-up was associated with the baseline CRP.</td>
</tr>
<tr>
<td>Wang et al. (2002)</td>
<td>3,173 subjects from a population-based cohort (men and women)</td>
<td>After adjustments CRP was associated with carotid stenosis (≥ 25%) in women but not in men. Similarly, women in the fourth CRP quartile had a significantly higher IMT of ICA than those in the lowest CRP quartile.</td>
</tr>
<tr>
<td>Zoccali et al. (2002)</td>
<td>90 subjects undergoing haemodialysis (men and women)</td>
<td>In the follow-up study (15 months) IMT changes in CCA were significantly related to CRP in subjects with initially normal IMT (≤ 0.95 mm).</td>
</tr>
<tr>
<td>Winbeck et al. (2002)</td>
<td>411 consecutive neurological inpatients (men and women)</td>
<td>Subjects with elevated (≥ 5 mg/l) CRP had significantly larger IMT of CCA than subjects with normal (&lt; 5 mg/l) CRP.</td>
</tr>
<tr>
<td>Hak et al. (1999)</td>
<td>186 women selected from the general population</td>
<td>The association of CRP with IMT of CCA was weak and limited to ever-smokers.</td>
</tr>
<tr>
<td>Manzi et al. (1999)</td>
<td>175 women with systemic lupus erythematosus</td>
<td>CRP was not an independent determinant of plaques.</td>
</tr>
<tr>
<td>Hulthe et al. (2001b)</td>
<td>391 randomly selected healthy men</td>
<td>There was no significant association between tertiles of CRP and IMT or plaque occurrence in CCA and BIF.</td>
</tr>
</tbody>
</table>

BIF, carotid bifurcation; CCA, common carotid artery; ICA, internal carotid artery; IMT, intima-media thickness.
2.10 Lipoprotein oxidation and atherosclerosis

The transformation of the fatty streaks to intermediate and advanced lesions has many characteristics of a chronic inflammatory process (Ross 1999). Indeed, there is now much experimental evidence in animal models showing that the immune system plays an important role and may even substantially alter the course of the atherogenesis (reviewed in Hansson 2001).

Lipoprotein oxidation is thought to enhance atherogenesis by a number of different mechanisms (Berliner et al. 1995, Ylä-Herttuala 1998, Hansson et al. 2002). First, oxidised LDL (OxLDL) is chemotactic for monocytes and T cells and is readily taken up by macrophages via scavenger receptors, thus enhancing the accumulation of cholesterol into the macrophages and the formation of foam cells. Second, oxidation of LDL in the artery wall affects gene regulation of vascular cells and increases the expression of adhesion molecules and chemotactic proteins by endothelial cells of the intima, which may contribute to the atherogenic process. Third, OxLDL may regulate the expression of pro-inflammatory genes in macrophages by activating the peroxisome proliferator-activated receptor γ (PPARγ) (Ricote et al. 1998). Finally, the oxidation of LDL results in structural modifications, which make it highly immunogenic and induce humoral immune responses.

During LDL oxidation a variety of highly reactive breakdown products, such as malondialdehyde (MDA) are generated (Esterbauer et al. 1991) which further modify closely associated lipids and proteins into immunogenic epitopes. Interestingly, when MDA-modified LDL was prepared with guinea pig and murine LDL and monoclonal antibodies were generated, the antibodies recognised their respective oxidation-derived epitopes not only on OxLDL, but also on a variety of other similarly derived peptides and proteins (Palinski et al. 1989, Palinski et al. 1990). These epitopes were therefore termed oxidation-specific epitopes. The antibodies generated have been used in establishing the presence of such epitopes in atherosclerotic lesions of animal models and humans (Palinski et al. 1989, Rosenfeld et al. 1990, Palinski et al. 1994).

OxLDL induces a humoral immune response also in vivo. There are numerous reports of autoantibodies to the oxidation-specific epitopes of OxLDL in plasma both in humans and in animal models (Palinski et al. 1989, Orekhov et al. 1991, Palinski et al. 1994). In animal models, autoantibody titres to epitopes of OxLDL show a strong correlation with the measures of atherosclerosis. For example, it has been reported that autoantibodies to OxLDL rise progressively over time in cholesterol-fed LDL-receptor-deficient mice and at time of sacrifice the antibody titre correlates with the extent of atherosclerosis present (Palinski et al. 1995b). Such antibody titres also rise in chow-fed C57BL/6 mice as they age, though the levels of antibody titres are lower (Reaven et al. 1999). Extremely high titres of autoantibodies to OxLDL are observed in cholesterol-fed apoE-deficient mice (Palinski et al. 1994). In addition, it has been demonstrated that the autoantibodies are present in atherosclerotic lesions, in part as immune complexes with OxLDL (Ylä-Herttuala et al. 1994). Autoantibodies to OxLDL are also found in human populations, both in patients with clinically evident cardiovascular disease as well as in apparently normal populations. Extensive data have been accumulated suggesting that the titres of these antibodies may be an indicator of the severity of atherosclerosis or its rate of progression. It was initially reported that the baseline titre of autoantibodies to
MDA-LDL was an excellent predictor of the progression of carotid IMT over a 2-year period in a group of healthy Finnish males (Salonen et al. 1992). Since that initial report studies have shown a relationship between elevated autoantibody titres to epitopes of OxLDL and risk factors for atherosclerosis (Maggi et al. 1993) or various cardiovascular diseases such as carotid atherosclerosis (Maggi et al. 1994), CAD (Lehtimäki et al. 1999), AMI (Puurunen et al. 1994) and peripheral vascular disease (Bergmark et al. 1995). In contrast, several other studies have failed to observe a positive correlation between the antibodies and atherosclerosis (Uusitupa et al. 1996, Wu et al. 1999, Fukumoto et al. 2000).

In light of the conflicting results from studies in human populations the potential role of the autoantibodies to OxLDL in the atherogenic process is complex and still remains unsolved. There are, however, data to suggest that the immune responses to OxLDL not only reflect but also actively modulate the atherogenic process. It has been shown in animal models that immunisation with OxLDL induces high levels of antibodies to OxLDL and reduces atherosclerosis (Palinski et al. 1995a, Ameli et al. 1996, Freigang et al. 1998, Zhou et al. 2001). However, other evidence indicates that the immune responses may advance atherogenesis. For example, if the CD40 signalling is inhibited in mice prone to atherosclerosis the cell-mediated immune responses are decreased and the lesion formation is reduced by 60% (Mach et al. 1998). Moreover, a cross between mice developing severe atherosclerosis (apoE knockout) and immune-deficient mice showed a significant reduction in atherosclerosis when they were fed a regular diet, but no reduction when fed a high-fat diet (Daugherty et al. 1997). Finally, immunological mechanisms appear to play an important role in transplant-associated atherosclerosis (Shi et al. 1996).
3 Aims of the study

The aim of this study was to investigate genetic and immunological risk factors for early atherosclerotic changes in a population-based cohort. The main questions were:

1. Is endothelial nitric oxide synthase Glu298Asp polymorphism associated with blood pressure, cardiac left ventricular mass and carotid artery intima-media thickness?
2. Is there an interaction between apolipoprotein E polymorphism and smoking with regard to carotid artery intima-media thickness?
3. Are paraoxonase-1 Leu55Met and Gln192Arg polymorphisms associated with carotid artery intima-media thickness?
4. What is the relation of C-reactive protein to carotid artery intima-media thickness?
5. Are antibodies to oxidised LDL associated with carotid artery intima-media thickness?
4 Subjects and methods

4.1 Subjects

This study is a part of the OPERA (Oulu Project Elucidating Risk of Atherosclerosis) project, which is a population-based, epidemiological study addressing the risk factors and disease end-points of atherosclerotic cardiovascular diseases. The study population consisted of 600 hypertensive (300 men and 300 women) subjects and 600 controls (300 men and 300 women) living in the City of Oulu. Both hypertensive and control subjects were 40–59 years old at the time of selection (September 1, 1990). The hypertensive subjects were randomly selected by age stratification (15 men and 15 women per year) from the Social Insurance Institute register for reimbursement of antihypertensive medication. According to the register they were entitled to a special refund (higher reimbursement class) of antihypertensive medication endorsed later than August 1980. The criteria of the special refund in 1990 were as follows: diastolic blood pressure 105 mmHg or more during a few months’ follow-up unless the patient shows signs of target organ damage (left ventricular hypertrophy, heart failure, CAD, cerebro-vascular disease, renal insufficiency or hypertensive retinopathy), in which case the diastolic blood pressure limit was 100 mmHg. If the patient was young (men below 50 and women below 40 years), had a family history of cardiovascular disease or sudden death at an early age, had diabetes or severe dyslipidaemia or had a systolic blood pressure above 180 mmHg (above 200 mmHg in subjects older than 50 years), he or she was eligible for the higher reimbursement even when the diastolic blood pressure level during the follow-up was 100–104 mmHg. In the case of nephropathy, diastolic blood pressure of 95 mmHg was sufficient for entitlement to the special refund. For each hypertensive subject, an age- and sex-matched control was randomly selected from the national health register (including all inhabitants), excluding subjects with the right to reimbursement for hypertension medication. Only the male population was included in study II. In study III, hypertensive males and females were pooled and control males and females were pooled respectively. In study IV, the whole population was pooled into one group.

The participants visited the research laboratory of the Department of Internal Medicine for laboratory tests, a physical examination and a detailed interview covering past
medical history, current and former medication use and physical activity. Alcohol consumption was determined by the method of Khavari and Farber (Khavari & Farber 1978) and calculated as grams of absolute alcohol per week. Smoking history was obtained by a questionnaire used in the WHO MONICA study (Rose et al. 1982). The lifetime smoking burden was calculated as pack-years (1 pack-year = 20 cigarettes smoked/day in one year). In the analyses, smokers included both current and ex-smokers. Echocardiography and carotid ultrasonography were performed on separate visits 6–12 months later.

All subjects volunteered for the study, which was approved by the Ethical Committee of the Faculty of Medicine, University of Oulu. The study was carried out according to the instructions of the Declaration of Helsinki and an informed consent was obtained from each participant.

4.2 Clinical methods

Body mass index (BMI) was calculated as weight (kg) divided by height squared (m²). Waist circumference was measured to the nearest 0.5 cm with a tape measure midway between the lower rib margin and the iliac crest in light expiration. Hip circumference was measured at the point yielding the maximum circumference over the buttocks. Blood pressure was measured according to the recommendations of the American Society of Hypertension (American Society of Hypertension 1992) in a sitting position from the right arm with an oscillometric device (Dinamap® model 18465X, Criticon Ltd., Ascot, UK) after an overnight fast and after a 10–15-minute rest. Three measurements were made at 1-minute intervals. The means of the last two measurements were used for the analyses.

4.3 Carotid ultrasonography

Throughout the study a duplex ultrasound system with a 7.5 MHz scanning frequency in B-mode, pulsed Doppler mode and colour mode (Toshiba SSA-270A, Toshiba Corp., Tokyo, Japan) was used according to the same protocol by a single trained radiologist without knowledge of the clinical data. The ultrasonographic assessment of carotid arteries was performed while the subject was in a supine position with his head turned away from the sonographer at an angle of 45°. Each carotid system was imaged in anterior oblique and lateral planes, transversally and longitudinally. The examiner consistently aimed for the clearest image of the near and far wall of the carotid arteries. The scan head was kept perpendicular to the arterial walls, and the transducer laterally angled to give optimal visualisation. The Doppler mode was used to identify the vessels and to evaluate flow disturbances. Each scan of the common carotid artery began just above the clavicle and moved past the bifurcation and along both the internal and external branches as far distally as possible. The whole scanning procedure was recorded on a Super-VHS videocassette recorder (Panasonic AG-7330, Matsuhita Electric Industrial
Co., Ltd., Osaka, Japan). All measurements were performed about one year later from the video image on the monitor of the ultrasound device using its electronic calipers.

The structures measured were IMT and the number of atheromatous plaques. IMT was defined as the distance between the media-adventitia interface and the lumen-intima interface (Fig. 2). IMT was measured at five locations on each side, both on the near and the far wall, i.e. at a total of 20 sites: the internal carotid artery about 1 cm distal from the flow divider, the bifurcation enlargement and three locations of the common carotid artery: proximal, middle and distal at about 1–1.5 cm intervals, depending on the length of the vessel, the most cranial measuring point being about 1 cm proximal from the bifurcation. The examiner searched for the thickest point of IMT for measurement at each site, avoiding, however, sites with atheromatous plaque. Because near-wall measurements may be difficult to perform accurately (Wendelhag et al. 1991), data from the far walls were used in this study. The mean IMT was defined as the mean of ICA, BIF and the highest three of CCA measurements. Arterial plaque was defined as an echogenic structure encroaching into the vessel lumen with a distinct area, resulting in IMT more than 50% greater compared to the neighbouring sites (Giral et al. 1991).

Fig. 2. Longitudinal echo image of right common carotid artery. The arrows indicate the lumen-intima interface and the media-adventitia interface of the far wall of the vessel. Intima-media thickness is measured from the distance between these interfaces.

The intra- and interobserver reproducibility of the IMT measurement was assessed in 31 randomly selected subjects. The repeat measurements were performed from the videotapes 1.5 years after the examination without knowledge of the original results. The variability was estimated using the mean absolute difference between paired measurements. The intrareader variability and the correlation coefficient (Pearson) were 3% and 0.97 for the mean IMT and 9.9% and 0.94 for the maximal IMT, respectively. The
respective interreader variability and correlation were 7.2% and 0.93 (mean IMT) and 12.8% and 0.92 (maximal IMT).

### 4.4 Echocardiographic methods

All echocardiographic examinations were performed with Hewlett-Packard Sonos 500 (Hewlett-Packard Company, Massachusetts, USA) by a single trained cardiologist without knowledge of the clinical data. The left ventricular internal diameters and wall thickness were measured from M-mode recordings under 2-D guidance according to the recommendations of the American Society of Echocardiography (Sahn et al. 1978). The left ventricular mass (LVM) values were calculated from the measurements according to the corrected equation (Devereux et al. 1986):

\[
LVM (g) = 0.8 \times \{1.04 \times [(IVS + LVID + PWT)^3 - LVID^3]\} + 0.6,
\]

where IVS is the end-diastolic interventricular septum thickness, LVID is the end-diastolic left ventricular internal dimension and PWT is the end-diastolic posterior wall thickness. The LVM index (LVMI) was calculated by dividing LVM (g) by body surface area (m²). Body surface area was determined by the Dubois equation (Du Bois & Du Bois 1916):

\[
\text{Body surface area (m²)} = 0.007184 \times [\text{weight (kg)}]^{0.425} \times [\text{height (cm)}]^{0.725}
\]

The cut-off points for left ventricular hypertrophy (LVH) were >134 g/m² among men and >110 g/m² among women (Devereux et al. 1984). These points are widely used and based on values of the general population and represent the 97th percentile of LVMI.

### 4.5 Laboratory analyses

All the laboratory test samples were obtained after an overnight fast. Venous blood was drawn into EDTA tubes. Plasma was separated by centrifugation at 2000 rpm for 10 min and kept at 4°C until further analyses. The routine clinical laboratory tests were carried out in the Central Laboratory of the Oulu University Hospital and the lipid and lipoprotein analyses in the Research Laboratory of the Department of Internal Medicine. In the oral glucose tolerance test 75 g of glucose was given to the subjects, and the plasma glucose and insulin levels were determined at 0, 60 and 120 minutes. The venous blood glucose concentration was determined with the glucose dehydrogenase method and the plasma insulin concentration with the double RIA method (AIA-PACK IRI, Tosoh Corp., Tokyo, Japan). The VLDL fraction (d < 1.006 g/ml) was separated from plasma by ultracentrifugation in a Kontron TFT 45.6 rotor at 105 000 g and 15°C for 18 hours. The VLDL fraction was removed from the ultracentrifuged preparation by tube slicing. The plasma HDL cholesterol concentration was determined by mixing 1 ml of the VLDL-free fraction with 25 µl of 2.8% (w/v) heparin and 25 µl of 2 M manganese chloride and by measuring the cholesterol concentration in the supernatant after centrifugation at 1000 g
and 4°C for 30 minutes. The plasma LDL cholesterol concentration was calculated by subtracting the cholesterol concentration in HDL from that in the VLDL-free fraction. The concentrations of total cholesterol and triglycerides in the plasma and lipoprotein fractions were determined by enzymatic colorimetric methods (kits of Boehringer Diagnostica, Mannheim GmbH, Germany, catalogue nos. 236691 and 701912, respectively) using a Kone Specific analyser (Kone Specific, Selective Chemistry Analyser, Kone Instruments, Espoo, Finland). The coefficients of variation for the determination of plasma total cholesterol, HDL cholesterol and triglycerides were 2.1%, 5.5% and 5.3%, respectively. C-reactive protein was measured using commercially available ELISA kits with a detection limit of 0.31 ng/ml (Diagnostic Systems Laboratories, Texas, USA).

4.6 Apolipoprotein E phenotyping

The apolipoprotein E phenotype was determined from delipidated plasma with isoelectric focussing and immunoblotting techniques (Menzel & Utermann 1986), using commercial antibodies (Daiichi Pure Chemical, Tokyo, Japan; Bio-Makor, Rehovot, Israel). In order to evaluate specifically the effect of phenotype E4, all the phenotypes with E4 (E2/4, E4/3 and E4/4) were pooled into one group and the phenotypes without it (E2/2, E2/3, E3/3) into another in the analyses of the current work.

4.7 DNA analyses

DNA was extracted from white blood cells using the Triton X-100 lysis method (Miller et al. 1988). Polymerase chain reaction (PCR) and restriction fragment-length polymorphism (RFLP) analysis were used to determine eNOS and PON1 polymorphisms. Denaturation, annealing and extension was carried out using an automatic thermal cycler (MJ Research, Waltham, USA).

For the eNOS Glu298Asp polymorphism primers were selected to amplify a 248-base pair fragment containing the Glu298Asp polymorphism in exon 7 of the eNOS gene. The oligonucleotides used as primers in the study were 5’-AAGGCAG-GAGAGACAGTG-GATGGA-3’ (sense) and 5’-CCCAGTCAATCCCTTTGGTGCTCA-3’ (antisense) (Shimasaki et al. 1998). DNA amplification was carried out in a final volume of 25 µl using 2.0 mM MgCl₂, 0.4 mM dNTPs, 1.0 µM of each of the primers, 0.5 units of Taq polymerase (Amplitaq Gold, Perkin-Elmer, CT, USA) and 175 ng DNA template. The PCR reaction was carried out with initial denaturation at 95°C for 12 minutes, followed by 35 cycles, each consisting of three steps: denaturation at 95°C (1 minute), annealing at 62°C (1 minute) and extension at 72°C (2 minutes). The reaction was completed with a final extension at 72°C for 10 minutes. The PCR fragments were incubated at 37°C for 1 hour with the restriction enzyme BanII (New England Biolabs Inc., Beverley, USA). Digestion with BanII resulted in 163 bp and 85 bp fragments for the Glu298 allele and a non-digested 248 bp fragment for the Asp298 allele. The digested fragments were
separated and visualised on an ultraviolet transilluminator after electrophoresis on a 3% agarose gel (3:1 NuSieve; Biowhittaker, Rockland, USA) and GelStar® staining (Cambrex, East Rutherford, USA).

For the PON1 Leu55Met and Gln192Arg polymorphisms two sets of primers were designed to flank the polymorphic sites. The primers used for the amplification of the 169 bp DNA fragment for PON155 polymorphism were 5’-GAAGAGTGATGTTATAGC-CCCAG-3’ and 5’-ACTCAGAGCTAATGAAAGCCA-3’. The 99 bp DNA fragment encompassing the PON1192 polymorphism was obtained using the primers 5’-TATTGT-TGCTTGGGACCTGAG-3’ and 5’-CACGCTAAACCCAAATACATCTC-3’. Twenty-five µl of PCR mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.05 mM dNTPs for the PON155 polymorphism and 0.4 mM dNTPs for the PON1192 polymorphism, 0.25 µM of each of the primers, 1 unit of DynaZyme II DNA polymerase (Finnzymes, Espoo, Finland) and 125 ng of DNA template. The PCR reaction for the amplification of both polymorphic regions was carried out with initial denaturation at 95 °C for 5 minutes, followed by 35 cycles, each consisting of three one-minute steps: denaturation at 95 °C, annealing at 61 °C and extension at 72 °C. The reaction was completed with a final extension at 72 °C for 10 minutes. The PON155 (169 bp) PCR product was digested with 5 units of NlaIII (New England Biolabs Inc., Beverley, USA) at 37 °C for three hours. Digestion resulted in 127 bp and 42 bp fragments for the Met55 allele and a non-digested 169 bp fragment for the Leu55 allele. The PON1192 (99 bp) PCR product was digested with 2 units of AlwI (New England Biolabs Inc., Beverley, USA) at 37 °C for five hours. Digestion with AlwI resulted in 63 bp and 36 bp fragments for the Arg192 allele and a non-digested 99 bp fragment for the Gln192 allele. The digested fragments were separated and visualised on an ultraviolet transilluminator after electrophoresis on a 5% agarose gel (4:1 NuSieve; Biowhittaker, Rockland, USA) containing nucleic acid gel stain (GelStar®; Cambrex, East Rutherford, USA).

4.8 Autoantibody measurements to OxLDL

The levels of IgM, IgG and IgG2 autoantibodies binding to MDA-LDL and CuOx-LDL were determined by chemiluminescence based ELISA (Hörkkö et al. 1999). MDA-LDL and CuOx-LDL were generated as previously described (Palinski et al. 1996, Freigang et al. 1998) and the degree of the modification was verified by the TNBS method (Habeeb 1966) and testing monoclonal anti-OxLDL antibody binding (EO6 binding, (Hörkkö et al. 1999)). The same modified LDL preparation was used throughout the study. Antigens at 10 µg/ml in PBS-EDTA (PBS with 0.27 mmol/l EDTA) were incubated overnight at 4°C in white MicroFluor plates (Dynatech Laboratories). The plates were washed three times with PBS-EDTA with an automated plate washer and blocked with PBS-EDTA containing 1% BSA for 30 min. Plasma samples were diluted 1:1000 for IgM, 1:500 for total IgG and 1:50 for IgG2 and incubated 1 hour at room temperature. Plates for IgM and IgG were incubated with an alkaline phosphatase–labelled goat anti-human IgM or IgG (Sigma, Cat# A-3187 or A-9794) for 1 hour at room temperature. Finally, 25µl of a 50% solution of Lumi-Phos530 (Lumigen, Cat no P-501) was added and the luminescence was determined after 90 minutes with Victor2 Luminometer (Wallac, Perkin-Elmer, CT,
USA). Plates for IgG2 were first incubated with biotin-labelled mouse anti-human IgG2 (Pharmingen, Cat no 555874), followed by alkaline phosphatase-labelled NeutrAvidin (Pierce, Cat no 31002) and LumiPhos. Triplicate determinations were performed for each plasma sample. A standard curve of human IgM or IgG and a control plasma sample was added to each plate to correct potential variations between the assays. The inter-assay coefficients of variation were IgM: 13.6% for CuOx-LDL and 10.0% for MDA-LDL; IgG: 11.5% for CuOx-LDL and 9.14% for MDA-LDL; IgG2: 11.9% for CuOx-LDL and 10.3% for MDA-LDL.

4.9 Statistical methods

The SPSS 10.0 for Windows software (SPSS Inc., Chicago, USA) was used in all statistical analyses, except in the determination of the allele effect, where Excel 2000 (Microsoft Corporation, USA) was used.

Allele frequencies were estimated with the gene counting method. The chi-squared test was used to assess the fit of the observed allele frequencies to the Hardy-Weinberg equilibrium and the difference in genotype distributions between the control and hypertensive groups or between the particular subgroups of the study subjects.

Power calculation was used in Study I to assess the power (β-error) of the observed difference in allele frequencies between the hypertensive subjects and controls. The equation used for the calculations was (Pocock 1988):

\[ n = \left\lfloor \frac{\{p_1 (100- p_1) + p_2 (100- p_2)\} \times f(\alpha, \beta)}{(p_2 - p_1)} \right\rfloor, \]

where \( n \) is the number of subjects in each group, \( p_1 \) and \( p_2 \) are the allele frequencies in the groups and \( f(\alpha, \beta) \) is a function of \( \alpha \) (type I error) and \( \beta \) (type II error). The numerical values of \( f(\alpha, \beta) \) were obtained from a table of standardised normal deviation \([f(\alpha, \beta) = (z_\alpha + z_\beta)]\). At a two-tailed \( \alpha \) value of 0.05 and \( \beta \) value of 0.20, \( f(\alpha, \beta) \) has a value of 7.9.

Continuous variables are expressed as means with standard deviation (SD) or standard error (SE) of the mean or with the 95% confidence interval. Triglycerides, lipoprotein(a) [Lp(a)], insulin, the amount of current smoking and lifetime smoking (pack-years) and alcohol consumption were particularly skewed, and logarithmic transformation was therefore used when appropriate.

Unpaired t-test was used to assess the differences of the means between two groups and analysis of variance (ANOVA) to assess the differences between more than two groups. Bonferroni-type multiple comparison correction was not used in this study because it is highly conservative for a large number of comparisons (Altman 1991). When the number of subjects in one or more classes was small, non-parametric tests (Mann-Whitney or Kruskal-Wallis) were used. The frequencies between groups (two or more) were compared using the chi-squared test.

To control the effect of confounding factors on dependent variables, the analysis of covariance (ANCOVA) was used. Further, in Studies I and II, multiple linear regression analysis was used. Adjusted \( R^2 \) values were used to describe the proportion of variance of the dependent variable explained by the model. The beta values (standardised regression
coefficients) were used to compare the effect of independent variables on the dependent variable.

The average effect of the $\varepsilon 4$ allele on the mean carotid artery IMT in hypertensive smoking subjects (Study II) was estimated using the method described previously (Sing & Davignon 1985). The formula used was:

$$\alpha_4 = (f_{44}E_{44} + \frac{1}{2} f_{24}E_{24} + \frac{1}{2} f_{34}E_{34}) / f_{\varepsilon 4} - \mu,$$

where $f$ is the frequency expected on the basis of the Hardy-Weinberg equilibrium, $E$ is the mean value of the mean carotid artery IMT for subjects with the given phenotype and $\mu$ is the mean value of the whole hypertensive smoking group. For statistical testing of the average effect, a sampling-resampling approach was used with 1000 permutations of the data (Kaprio et al. 1991) ($H_0$: allele effect = 0).
5 Results

5.1 Subjects and their basic characteristics

The overall participation rate of the hypertensive subjects was 86.5% (261 men, 258 women) and that of controls 87.7% (259 men, 267 women). Table 4 shows the basic characteristics in the hypertensive and control cohorts. The women were about one year older, because they were examined about one year later than the men. The prevalence of diabetes and CAD was higher among the hypertensive subjects than among the controls. Also, in both genders hypertensive subjects had significantly higher BMI, blood pressure and blood glucose than the control subjects. The mean IMT did not differ significantly between the hypertensive and control subjects.

Table 4. Main characteristics of the hypertensive and control subjects by gender.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hypertensives</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men (n = 261)</td>
<td>Women (n = 258)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>50.5 (5.9)</td>
<td>51.8 (5.9)</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>5 (11.9)</td>
<td>19 (7.4)</td>
</tr>
<tr>
<td>Coronary artery disease, n (%) #</td>
<td>25 (9.6)</td>
<td>30 (11.6)</td>
</tr>
<tr>
<td>Current smokers, n (%)</td>
<td>74 (28.4)</td>
<td>58 (22.5)</td>
</tr>
<tr>
<td>Smoking (pack-years)</td>
<td>15.4 (13.9)</td>
<td>7.7 (12.4)</td>
</tr>
<tr>
<td>Alcohol consumption (g/week)</td>
<td>106 (126)</td>
<td>31 (48)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.4 (4.4)</td>
<td>28.7 (5.3)</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>158 (21)</td>
<td>152 (21)</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>97 (10)</td>
<td>90 (11)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.77 (1.02)</td>
<td>5.73 (1.07)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.60 (0.92)</td>
<td>3.47 (0.92)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.18 (0.31)</td>
<td>1.43 (0.38)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.98 (1.26)</td>
<td>1.60 (1.09)</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>5.1 (1.9)</td>
<td>4.9 (1.8)</td>
</tr>
<tr>
<td>Mean IMT (mm)</td>
<td>0.92 (0.22)</td>
<td>0.83 (0.14)</td>
</tr>
</tbody>
</table>

Values are expressed as means (SD) or number of subjects (%), when appropriate. BMI, body mass index; BP, blood pressure; HDL, high-density lipoprotein; IMT, intima-media thickness; LDL, low-density lipoprotein.

*p<0.05 between hypertensives and controls; # based on disease history (interview).
Table 5 shows the use of antihypertensive and other medication among the study subjects. Twenty-six men and 29 women among the control subjects were on chronic medication affecting blood pressure, since blood pressure-lowering medication is also commonly used for other indications apart from hypertension (e.g. chest pain, ankle oedema, essential tremor, palpitations, etc.). Furthermore, in a smaller portion (7 men and 14 women) of these 55 subjects, these drugs were used to treat hypertension, although the subjects were not listed as special refund recipients at the time of recruiting. These subjects had been entitled to the refund later or for some reason had not applied for the right to refund.

Table 5. Use of selected medications among the hypertensive and control subjects by gender.

<table>
<thead>
<tr>
<th>Medication</th>
<th>Hypertensives</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men (n = 261)</td>
<td>Women (n = 258)</td>
</tr>
<tr>
<td>Antihypertensive medication, n (%)</td>
<td>239 (91.6)</td>
<td>248 (96.1)</td>
</tr>
<tr>
<td>Diuretics, n (%)</td>
<td>61 (23.4)</td>
<td>100 (38.8)</td>
</tr>
<tr>
<td>Beta-blockers, n (%)</td>
<td>132 (50.6)</td>
<td>124 (48.1)</td>
</tr>
<tr>
<td>Calcium-channel blockers, n (%)</td>
<td>53 (20.3)</td>
<td>58 (22.5)</td>
</tr>
<tr>
<td>ACE-inhibitors, n (%)</td>
<td>106 (40.6)</td>
<td>87 (33.7)</td>
</tr>
<tr>
<td>Others, n (%)</td>
<td>13 (5.0)</td>
<td>18 (7.0)</td>
</tr>
<tr>
<td>Lipid-lowering medication, n (%)</td>
<td>13 (5.0)</td>
<td>7 (2.7)</td>
</tr>
<tr>
<td>Acetylsalicylic acid, n (%)</td>
<td>25 (9.6)</td>
<td>15 (5.8)</td>
</tr>
<tr>
<td>Oral antidiabetic medication, n (%)</td>
<td>10 (3.8)</td>
<td>9 (3.5)</td>
</tr>
<tr>
<td>Insulin, n (%)</td>
<td>6 (2.3)</td>
<td>5 (1.9)</td>
</tr>
<tr>
<td>Hormone replacement therapy</td>
<td>–</td>
<td>48 (18.6)</td>
</tr>
</tbody>
</table>

All genotype and phenotype distributions were in agreement with the Hardy-Weinberg equilibrium, and no significant differences were observed in the genotype or phenotype distributions between the hypertensive and control subjects.

5.2 Relation of the eNOS polymorphism to blood pressure, LVM and IMT (Study I)

In both the hypertensive and control group, no significant associations were found between the eNOS genotypes and blood pressure, LVM or mean IMT in either men or women. The lack of association between eNOS genotypes and IMT remained after IMT was adjusted for the covariates (age, BMI, LDL cholesterol, systolic blood pressure and smoking). Furthermore, when the Glu/Asp and Asp/Asp genotypes were combined and compared to the Glu/Glu genotype, no significant associations between the genotypes (Glu/Glu vs. Glu/Asp + Asp/Asp) and the clinical measurements were found in either hypertensive or control subjects. Also, when the analysis was conducted separately for smokers (ex- or current) and non-smokers, there was no association between the eNOS...
Glu298Asp polymorphism and the clinical variables in either hypertensive men or women or control men or women.

Finally, multiple linear regression analysis for systolic blood pressure was performed in the control group, and the eNOS polymorphism was not a significant predictor of systolic blood pressure among either men or women.

5.3 Relation of the apoE variants and smoking to IMT (Study II)

The raw data showed that the hypertensive subjects with the ε4 allele had greater carotid IMT and a higher mean number of plaques than those without ε4. The control subjects showed no significant differences in IMT between the subjects carrying or not carrying the ε4 allele. The existence of an interaction between smoking and the ε4 allele was assessed using ANCOVA. Because the interaction term was a significant determinant of IMT among the hypertensive subjects but not among the controls, the hypertensive subjects were analysed further by smoking status (smoking/non-smoking) and the ε4 allele (present/absent). Among the hypertensive smokers, the mean carotid IMT was significantly higher in the ε4 carriers than in the subjects without ε4 (1.01 mm vs. 0.90 mm, p=0.003). This finding remained even after adjusting for LDL cholesterol and age (Fig. 3). Also, the mean number of plaques was significantly higher in the ε4 carriers than in the non-carriers (2.5 vs. 1.8, p=0.03) among the hypertensive smokers. In the control group, no differences were seen in either the smokers or the non-smokers (Fig. 3).

Fig. 3. Mean intima-media thickness of the internal carotid artery, the carotid bifurcation and the common carotid artery (±SE) in hypertensive and control subjects by smoking status and the absence or presence of the apoE ε4 allele. ‘ε4 absent’ stands for the apoE genotypes ε2/2, ε2/3, ε3/3 and ‘ε4 present’ for the apoE genotypes ε2/4, ε4/3 and ε4/4. The IMT values are adjusted for LDL cholesterol and age and in the control group also for systolic blood pressure. p-values were obtained by comparing the IMTs using analysis of covariance. NS = non-significant p-value.
Finally, a multiple linear regression analysis was performed, which indicated that the $\varepsilon^4$ allele was an independent determinant of IMT in the hypertensive smokers but not in the non-smokers. The estimated average effect of the apoE $\varepsilon^4$ allele on the mean IMT in the smoking hypertensive subjects was 0.088 mm (p<0.001). In the non-smoking hypertensive subjects the allele effect was non-significant. The regression model was able to explain 20.6% ($R^2=0.206$, p<0.001) of the total variance in the mean IMT among the hypertensive smokers and 3.3% (statistically not significant) among the non-smokers.

Figure 4 shows the mean carotid artery IMTs adjusted for LDL cholesterol (and in the control group also for systolic blood pressure) by age groups. The additive effect of age on IMT was particularly obvious in the smoking hypertensive subjects with the $\varepsilon^4$ allele. This effect was weaker in the smoking control subjects with the $\varepsilon^4$ allele. In the other groups, the effect of age was less marked.

![Fig. 4. Mean intima-media thickness of the internal carotid artery, the carotid bifurcation and the common carotid artery in hypertensive and control subjects by age groups, smoking status and the absence or presence of the apoE $\varepsilon^4$ allele. ‘$\varepsilon^4$ absent’ stands for the apoE genotypes $\varepsilon^2/2$, $\varepsilon^2/3$, $\varepsilon^3/3$ and ‘$\varepsilon^4$ present’ for the apoE genotypes $\varepsilon^2/4$, $\varepsilon^4/3$ and $\varepsilon^4/4$. The IMT values are adjusted for LDL cholesterol and in the control group also for systolic blood pressure. p-values were obtained by comparing the four groups in each age range using analysis of covariance. NS = non-significant p-value.](image)

### 5.4 Relation of the PON1 polymorphisms to IMT (Study III)

No significant differences for carotid IMT or the number of plaques were found between the PON1$_{55}$ or the PON1$_{192}$ genotype groups either among hypertensive or control subjects. Adjusting for confounding effects (age, smoking, LDL cholesterol, systolic blood pressure) did not have an impact on the results. Combining the relatively rare Met55 homozygotes with the Leu55/Met55 heterozygotes did not change the results, nor did the combining of the Arg192 homozygotes with the Gln192/Arg192 heterozygotes. Since there are a couple of studies indicating interactions between smoking and the PON1 polymorphisms (Sen-Banerjee et al. 2000, Malin et al. 2001), smokers and non-smokers
were also analysed separately. The results among smokers and non-smokers were similar
and no evidence was found for any interactions between PON1 genotypes and smoking.
Furthermore, when considering both polymorphisms together we could not find an
interaction between the PON155 and the PON1192 polymorphisms. Finally, when testing
the effect of PON1 polymorphisms on IMT no differences were found in the results of
male and female subpopulations.

5.5 Relation of CRP to IMT (Study IV)

Before the adjustment the present data indicated that there was a positive correlation
between CRP and IMT. This correlation was seen in all segments of the vessel, but after
adjusting for the previously known risk factors the statistically significant correlation
vanished in all parts of the carotid artery. Also, the linear trend towards a greater number
of plaques in higher tertiles of CRP disappeared after the adjustment. Also, no correlation
between CRP and the levels of antibodies to OxLDL was found.

5.6 Relationship between autoantibodies
to OxLDL and IMT (Study IV)

The different antibody isotype titres to OxLDL were analysed and the data showed
statistically significant differences in the IMT between the tertiles of the following
subclasses of autoantibodies: IgM to MDA-LDL and to CuOx-LDL and IgG to
CuOx-LDL. These subclasses of autoantibodies were analysed further.

Figure 5 shows the IMT of all the study subjects in various tertiles of IgM
autoantibodies to MDA-LDL in the different parts of the carotid artery. The IMT was
lowest in the highest antibody tertile in all parts of the vessel. This association remained
significant after adjusting for the known risk factors of atherosclerosis (age, gender, LDL
cholesterol, systolic blood pressure, CRP and smoking) (Fig. 5), although the statistical
significance was reached in only one section of the artery. In addition, the calculated
mean IMT was lowest in the highest antibody titre and this difference remained
statistically significant after adjusting for covariates (Fig. 6). Similar results were seen in
the measurements of maximal IMT and number of plaques, even though these were
statistically significant only before adjusting for covariates (Fig. 6).

Before the adjustment also the IgM and IgG autoantibody titres to CuOx-LDL were
negatively associated with IMT and the number of plaques (IgM only). However, after
adjusting for previously known risk factors, the statistically significant differences
disappeared.
Fig. 5. IMT by tertiles of IgM antibodies to malondialdehyde-modified LDL in different parts of the carotid artery. Adjustment was made for age, gender, systolic blood pressure, LDL cholesterol, CRP and pack-years.
Fig. 6. Mean and maximal IMT and the number of plaques by tertiles of IgM antibodies to malondialdehyde-modified LDL. Adjustment was made for age, gender, systolic blood pressure, LDL cholesterol, CRP and pack-years.
6 Discussion

6.1 Study population

In 1990, 7,539 subjects (3,132 men and 4,407 women) were entitled to the special refund of antihypertensive medication in the City of Oulu. Of these, 443 subjects (268 men and 175 women) were aged 40–44 years, 601 (333 men and 268 women) 45–49 years, 757 (400 men and 357 women) 50–54 years and 952 (476 men and 476 women) 55–59 years, accounting for a total of 2,753 subjects. At the same time, 403,000 subjects in the whole country had this benefit. The main characteristics of the control subjects are comparable to those previously reported from the general Finnish population (Vartiainen et al. 1994). The control cohort, however, does not represent the normotensive part of the population, mostly due to the reimbursement criteria of the Social Insurance Institution. Subjects suffering from mild or moderate (diastolic blood pressure < 105 mmHg and systolic < 180 mmHg) uncomplicated hypertension have no right to the special refund (but to a basic refund instead) of antihypertensive medication. Therefore these subjects are included in the control population in this study. Twenty-six men and 29 women (approximately 10% of the control cohort) were on antihypertensive medication, although only 7 of the men and 14 of the women were taking these drugs primarily because of hypertension. Throughout the study these subjects were considered part of the control cohort. Finally, approximately 6% of the subjects in the hypertension cohort were not using any antihypertensive medication at the time of the study, despite the right for the special refund (Table 5). It is possible that at least a part of these subjects were no longer hypertensive (e.g. because of reduction in weight). All these factors may dilute the potential differences between the hypertensive and control groups and make it more difficult to draw conclusions between hypertensive and normotensive subjects.
6.2 Limitations of association studies

There are two crucial points in the candidate gene approach used in the present work. First, the candidate gene must be likely to contribute to the phenotype under study. In other words, there has to be a hypothesis on how the gene affects the phenotype and the candidate gene approach is then used to test this hypothesis. Similarly, information on the effect of the gene polymorphism on the function of the gene product is important but often difficult to find. Second, the population studied must be well controlled for confounding factors, such as gender, age and ethnic origin to avoid biased conclusions. In the current population-based study the subjects were recruited from a geographically limited and ethnically homogenous population, which is an ideal approach to carry out genetic epidemiological studies (Cooper & Clayton 1988).

In the present work polymorphisms of three candidate genes for atherosclerosis were studied: the endothelial nitric oxide synthase, apolipoprotein E and paraoxonase-1. The biological actions of the products of these genes are well-known based on previous studies. Also the effects of the polymorphisms on the gene products have been intensively studied as to apoE and PON1 (Davignon et al. 1988, Mackness et al. 2002b). As for the biological effects of the Glu298Asp polymorphism in the eNOS gene, less information is available but some recent studies on the subject have been published (Tesauro et al. 2000, Schneider et al. 2000, Golser et al. 2003). Another problem of association studies in general is that the remainder of the genome is discarded even though other genes in close vicinity are often in linkage disequilibrium. Therefore even if the polymorphism under investigation is irrelevant for a given phenotype, a neighbouring gene variant with a marked effect on the same phenotype could produce an apparent association (Hilgers & Schmieder 2002). From a purely statistical point of view this consideration is needless because there would still be a valid association between a certain phenotype and the genomic marker. However, no information on the pathological mechanisms of the disease would be gained. Taking these matters into account, a sufficient sample size is especially important because of the increased number of recombination events and therefore a smaller risk for biased results.

Several polymorphisms of the candidate genes for atherosclerosis (including those evaluated in this work) have been studied in different populations with contradictory results. The reproducibility of association studies based on a single nucleotide polymorphism (SNP) has also proved poor in general (Hirschhorn et al. 2002). It is generally supposed that if a true causal association exists between an allele and a disease the same association should be found in different ethnic populations. However, it is treacherous to expect that the same association has to be found in every population since it is possible that gene-gene interaction is required to produce a phenotype. As a consequence, a certain polymorphism could be associated with a phenotype in one ethnic population but not in another. It is not plausible to assume that atherosclerosis is one disease to which the same genes contribute in every population. On the contrary, different genes may predispose to atherosclerosis in different populations. It should also kept in mind that if one allele of a gene is not associated with the disease, it does not rule out the possible role of the whole gene in the disease, but rather suggests that the particular allele does not have role in the pathogenesis of the disease (Gambaro et al. 2000). Therefore, the eNOS and PON1 genes cannot be fully ignored as candidate genes for atherosclerosis,
even though in this study no association was found between the certain polymorphisms of 
these genes and carotid IMT.

Because of the growing number of association studies between complex diseases and 
genetic polymorphisms with often unknown functional significance and the 
irreproducibility of the results, some serious criticism has been raised towards the entire 
proposed that association studies between gene polymorphisms and complex diseases can 
only be used to generate hypothesis but are not suitable for testing them. It is true that 
there are weaknesses in the genetic association studies. One or two SNPs is clearly not 
sufficient to reflect the amount of information that a certain gene contains. For instance, 
the eNOS gene contains several SNPs with numerous possible interactions between them 
(Poirier et al. 1999). The problems of genetic association studies also include the often 
unsatisfactory designation of cases and controls, aetiologic and genetic heterogeneity 
underlying the affected status of cases or the quantitative trait of interest thus increasing 
the probability of false negative results, associations resulting by chance when large 
numbers of markers are tested one at a time, thus increasing the probability of false 
positive results and finally, publication bias favouring positive results (Hegele 2002). 
However, it has also been suggested that if the association studies are carefully done (e.g. 
adequate definition of phenotypes in large enough populations, they can provide 
information both about disease and gene function (Burgner & Hull 2000). The demands 
for a sufficiently large study population and careful determination of the phenotype (IMT) 
are met in this study. It is, of course, clear that if association studies are completed by 
hyphothesis-driven prospective functional investigations of the gene product, more 
information of the possible role of the gene in the disease process will be gained. The 
cross-sectional study design in the present work does not, however, allow these kinds of 
investigations.

6.3 Carotid artery IMT as a measure of atherosclerosis

Various noninvasive techniques are available for identification and monitoring of 
preclinical atherosclerosis. These techniques have the potential to substantially improve 
our ability to identify individuals at risk for cardiovascular disease. Because carotid artery 
IMT has been shown to be an independent predictor of cardiovascular diseases after 
adjustment for traditional risk factors, for example in the ARIC (Chambless et al. 1997) 
and CHS (O'Leary et al. 1999) studies, it is the only noninvasive imaging technique 
currently recommended by the American Heart Association for inclusion in the 
evaluation of risk (Smith, Jr. et al. 2000). It is now generally recognised that IMT serves 
as a surrogate measurement of atherosclerosis (O'Leary & Polak 2002).

There are no international standardised protocols for measuring IMT, and there is 
therefore notable methodological variability in IMT measurements between different 
laboratories. In many studies IMT has been measured in the common carotid artery only 
(Table 1–3), which may dilute the power of the study to observe early atherosclerotic 
changes and partially explain the differences in the result of these studies. The definition 
of a plaque also varies markedly (Hashimoto et al. 2001, Lembo et al. 2001, Fortunato et
(al. 2003) making the comparison between studies more difficult. It is reasonable to assume that several measurements of IMT in the whole carotid tree illustrate the atherosclerotic burden better than measurements in the common carotid artery only (Wikstrand & Wendelhag 1994). Further, in the large CHS study (O'Leary et al. 1999) there was a stronger association between myocardial infarction incident and the IMT of internal carotid artery than the IMT of common carotid artery. This clearly indicates that there is a need for measurements in all sections of the carotid artery when using IMT in the evaluation of cardiovascular risk. Also, the variability of the IMT measurements has been shown to be lowest when IMT is determined using several projections (Kanters et al. 1997). Another problem in the IMT studies is that near-wall measurements are still used even in recent studies (e.g. Cattin et al. 1997, Lembo et al. 2001), even though the near-wall measurements have been considered unreliable because of technical difficulties (Wendelhag et al. 1991). In the present study, the IMT measurements were performed by one trained radiologist at five distinct points in both right and left carotid arteries including the near and far walls, i.e. altogether 20 sites. To avoid inaccuracy in the measurements, only the data from far walls were used in this work. The repeatability of the IMT measurements was good and in line with previous reports (Riley et al. 1992, Kanters et al. 1997).

6.4 Endothelial nitric oxide synthase gene polymorphism and IMT

The eNOS 298Asp variant has previously been reported to associate with cardiovascular pathology including hypertension, coronary spasm, CAD, and stroke (Wang & Wang 2000). In the present population-based cohort study no significant association was found between the eNOS Glu298Asp polymorphism and IMT in either hypertensive or control subjects. Also, the relations between the polymorphism and blood pressure level and echocardiographic left ventricular mass were studied. No differences between genotypes were observed in these measurements, either.

The eNOS gene is in many ways a suitable candidate gene for atherosclerosis. The gene product (endothelial nitric oxide synthase) is well-known and the functions of the ultimate product (i.e. nitric oxide) have been studied widely and are known to be antiatherogenic. Until recently there was only a very limited amount of information available on the functional significance of the Glu298Asp polymorphism. Tesauro et al. (2000) demonstrated on cultured human endothelial and heart cells that the 298Asp variant leads to the generation of two fragments sized 35 kDa and 100 kDa instead of a single 135 kDa product which is seen when the eNOS protein has glutamate at position 298. They concluded that the 298Asp variant is susceptible to cleavage and is therefore likely to alter the function of the protein. In the most recent study on the subject (Golser et al. 2003) the function of the purified recombinant eNOS expressed in the yeast was studied. Golser and co-workers reported that the function of eNOS with aspartate at position 298 is identical with the wild type protein, suggesting that the polymorphism has no functional meaning. This finding is supported by a study in a human population (Schneider et al. 2000) where endothelium-dependent vasodilatation was investigated with regard to eNOS genotype. The forearm blood flow and the responses to different
vasoactive drugs (acetylcholine and nitroprusside) were assessed by plethysmography. Also, L-arginine was infused to test the basal production of nitric oxide. The results showed that the forearm blood flow across eNOS genotypes did not differ after infusion of acetylcholine (endothelium-dependent vasodilatation), sodium nitroprusside (endothelium-independent vascular relaxation) or L-arginine. Thus, Schneider and associates also concluded that the Glu298Asp polymorphism does not have any biological effect. In light of these studies it seems likely that the functional effect of the Glu298Asp polymorphism is small or does not exist at all, which is also in line with the results of the present work.

The present observations are supported by the findings of Cai and associates (1999), who found no association between the eNOS Glu298Asp polymorphism and CAD in white Australians. Similar results were reported by Liyou et al. (1998) in a white population. Poirier and co-workers (1999) found no association between 10 different polymorphisms of the eNOS gene and AMI in European subjects. Further, lack of association between the eNOS Glu298Asp polymorphism and diabetic macroangiopathy has been reported in Finnish subjects (Ukkola et al. 2001). However, as discussed above, the allele could still be associated with cardiovascular diseases in some populations because of linkage disequilibrium. Indeed, there are many studies in Japanese populations showing an association between the Glu298Asp polymorphism and cardiovascular pathology (Miyamoto et al. 1998, Yoshimura et al. 1998, Shimasaki et al. 1998, Hibi et al. 1998) but only one study in a Caucasian population where an association between the 298Asp variant and CAD has been reported (Hingorani et al. 1999). It seems likely that this polymorphism has a greater effect on Japanese than on Caucasian, e.g. Finnish, subjects. However, it is also possible that some selection bias may have occurred because in most of the studies in Japanese populations the study population has consisted of selected clinic patients and their controls, not a population-based sample as in this work.

Interestingly, in terms of the genotype distributions and allele frequencies of eNOS, the Finnish population differs markedly from the Japanese population, the Asp variant being less frequent (<0.001, χ² test) among the Japanese subjects: the frequencies of the Glu and Asp alleles were 90.7% and 9.3% for AMI patients and 91.3% and 8.7% for controls, respectively (Hibi et al. 1998). In contrast, the prevalence of the Asp allele was higher among East Anglian CAD patients (47.8%) and AMI patients (39.6%) (Hingorani et al. 1999) than in the hypertensive (29.9%) or control (28.8%) groups of the present study. The prevalence of the Asp allele was also higher among French AMI patients (34.5%) and their controls (38.8%) and Irish AMI patients (42.9%) and their controls (39.4%) (Poirier et al. 1999) than the in hypertensive and control groups of the current work.

The present results clearly do not support the view that the eNOS Glu298Asp polymorphism is associated with cardiovascular risk as determined by carotid artery IMT. Apparently, the relationship between the eNOS Glu298Asp genotype and carotid atherosclerosis has only been studied in one previous study (Lembo et al. 2001). Lembo and associates studied 375 consecutive subjects who attended a hypertension centre in Italy. They measured the IMT of common carotid artery, bifurcation and internal carotid artery both in the near and far wall and used the maximum IMT detected for the analysis. The authors defined an arterial plaque as maximum IMT at any site of the carotid artery ≥ 1.5 mm. Based on this definition, they found that the Asp allele was more frequent among
the subjects with carotid plaques than among those without them. This study can be criticised for many reasons. First, the use of near-wall measurements may have caused inaccuracy. Second, the absolute IMT values and the number of plaques with regard to eNOS genotypes were not given. Third, the definition of plaques was somewhat artificial because the thickness of the arterial wall varies in different parts of the vessel even without plaques. In the present study an arterial plaque was defined as an echogenic structure encroaching into the vessel lumen with a distinct area, resulting in IMT more than 50% greater compared to the neighbouring sites (Giral et al. 1991) and based on this definition, no association between plaques and the eNOS Glu298Asp genotype was found.

Although the present results do not offer evidence for an association between the eNOS Glu298Asp polymorphism and carotid atherosclerosis, it is possible that the other polymorphisms in the eNOS gene have an effect on atherogenesis. There is already some evidence that the tandem repeat polymorphism in intron 4 of the eNOS gene is associated with CAD in African American (Hooper et al. 1999), Japanese (Ichihara et al. 1998) and Caucasian (Wang et al. 1996) populations. A recent study in a Finnish population showed significant differences among the different eNOS 4a/b genotypes in the improvement of coronary blood flow by statin treatment (Kunnas et al. 2002). So far, no studies on associations between this polymorphism and carotid artery atherosclerosis have been published. Also, no studies on the functional effect of the 4a/b polymorphism are currently available. These studies would be needed to elucidate the possible effect of the polymorphism on the eNOS protein function and to further clarify the position of the eNOS gene as a candidate gene for atherosclerosis.

6.5 Apolipoprotein E gene polymorphisms and IMT

Apolipoprotein E gene has for long been a candidate gene for atherosclerosis. The molecular biology and protein chemistry of apoE and its effects on lipid metabolism are well-known (Davignon et al. 1988). The ε2 allele is associated with lower levels of LDL cholesterol and it could therefore play a protective role in atherogenesis. However, in a meta-analysis (Wilson et al. 1996) the relative odds for CAD among persons with the ε2 allele were 0.98 (95% CI: 0.85–1.14) for both sexes combined when subjects with the ε3/ε3 genotype were used as a reference group. Similar results for the ε2 allele were obtained when the data for each sex were analysed separately. Because the relative odds are close to 1.00 and the 95% confidence interval of the estimate includes 1.00, the data suggest that the relative odds for CAD are neither higher nor lower among persons with the ε2 allele. The ε4 allele is associated with higher levels of LDL cholesterol and could therefore favour the development of atherosclerosis. Indeed, the meta-analysis mentioned above showed that the ε4 allele is associated with higher relative odds for CAD among men, women, and both sexes combined. The relative odds of 1.38 (1.22–1.57) for men indicated that the odds associated with the ε4 allele are 38% higher than in apoE 3/3 men. For women, the odds ratio was 1.82 (1.30–2.54). However, it should be noted that significant heterogeneity exists between the studies. For example, for the association of the ε4 allele with CAD for both sexes combined, one study (Utermann et al. 1984)

The previous reports on the apoE polymorphism and carotid IMT have not been conclusive. Some studies (Terry et al. 1996, Cattin et al. 1997, Vauhkonen et al. 1997, Haraki et al. 2002) have indicated that ε4 allele carriers have greater carotid IMT than ε2 and ε3 carriers. In a recent study in Finnish population, significantly smaller carotid IMT was reported among the carriers of the ε2/3 genotype than among the ε3/3 carriers, but no association between the ε4 allele and carotid artery IMT was found (Ilveskoski et al. 2000). The same observation was made in the Rotterdam study (Sluiter et al. 2001) which is by far the largest study (n = 5,401) on apoE polymorphism and IMT. On the other hand, Hanon and associates (2000) found an association between the ε2 allele and carotid hypertrophy, and de Andrade et al. (1995) reported an association between the ε2/3 genotype and carotid atherosclerosis. One previous study reported greater carotid artery IMT in ε3 allele carriers than in ε2 or ε4 carriers (Zannad et al. 1998). Also, a lack of association between the apoE genotype and carotid artery IMT has been reported (Kogawa et al. 1997, Sass et al. 1998, Horejsi et al. 2000, Güz et al. 2000).

The discrepancy between the results of the previous studies may be due to differences in the study design and the methods used to select the study population. For example, some selection bias may have occurred when patients who had undergone coronary angiography were selected (Terry et al. 1996), as these patients were likely to have more severe atherosclerosis than the population at large. Further, diabetic patients (Vauhkonen et al. 1997), subjects with increased cardiovascular risk (Hanon et al. 2000), patients undergoing haemodialysis (Güz et al. 2000), and subjects with hyperlipidaemia (Horejsi et al. 2000) have been used as study subjects, and these subjects clearly do not represent the general population. It should also be noted that the study population in the present work includes only male subjects, which may partially explain the differences compared to some of the other studies in which both male and female subjects have been included.

In the present study, a significant interaction between the presence of the ε4 allele and smoking in relation to mean carotid IMT was observed, although only in hypertensive subjects. This finding remained even after controlling for the effect of the well-known risk factors of atherosclerosis. In addition, IMT clearly increased with age in hypertensive smokers carrying the ε4 allele, but less so in hypertensive non-carriers of the ε4 allele, hypertensive non-smokers and the control subjects overall. However, the interaction was also seen in the oldest age group of control subjects, suggesting that the interaction may also occur in normotensive subjects but is only seen earlier in the hypertensive subjects because of the damaging effect of hypertension on the vessel wall. The results of the present work are supported by a follow-up study published recently (Humphries et al. 2001). That study showed that while overall the multivariate hazard ratio of CAD for smokers versus nonsmokers was 1.73, the hazard ratio was higher for the smokers carrying the ε4 allele (2.79) and not increased at all for the smokers carrying the ε2 allele (0.85). However, this result could not be reproduced in an even larger (4,484 AMI cases and 5,757 controls) ISIS study (Keavney et al. 2003).
It is possible that smoking and hypertension induce endothelial dysfunction and predispose the vascular wall to atherogenesis with differences in lipid metabolism explaining the association between the ε4 allele and IMT. However, it should be emphasised that, in the regression model, the ε4 allele remained an independent determinant of carotid IMT and there were also no significant differences in triglyceride, total cholesterol, LDL or HDL levels between the ε4 carriers and the subjects without the ε4 allele among those subjects who smoked. The fact that the association was present even after adjustment for LDL and HDL cholesterol suggests that some other mechanism apart from the lipid metabolism may be behind the observed interaction. Potentially, the interaction of the ε4 allele and smoking may be due to increased oxidation of LDL. Fickl and associates (1996) have shown in a group of asymptomatic male subjects that the autoantibodies against MDA-LDL are higher in smoking than in nonsmoking men. This difference was especially clear in subjects older than 30 years. Also, in the current study sample the IgG type of autoantibodies against MDA-LDL were higher among the smokers than among nonsmokers (data not published). Furthermore, the antioxidant activity of the apoE is lower in the E4 phenotype than in the E3 or E2 phenotype (Miyata & Smith 1996). Smith and co-workers (1998) recently reported that among subjects with apoE 4/3 phenotype, there was a significant association between serum apoE and lipid peroxide levels, which was not apparent among subjects with E3/3 or E3/2 phenotypes. In multivariate analysis, apolipoprotein E phenotype was a significant independent contributor to variation in serum lipid peroxide levels, suggesting that the ε4 carriers have lower protection against oxidation than the ε2 or ε3 carriers. The fact that the LDL particle size tends to be smaller among the ε4 carriers compared to the ε2 and ε3 carriers (Haffner et al. 1996) further provides a possible explanation for the observed interaction, since it has been shown that small LDL particles are particularly susceptible to oxidation (Chait et al. 1993) and supposedly even more so in the presence of the harmful effect of smoking. Finally, it has been reported that apoE may inhibit the migration of vascular smooth muscle cells (Ishigami et al. 1998), and possibly the low plasma concentrations of apoE among the ε4 carriers may lead to faster smooth muscle migration, thus accelerating the atherogenic process.

The results of the current study are in line with the existing evidence on the role of apoE in atherogenesis. However, there are some limitations in the present study. First, only men were included in the study population and therefore conclusions cannot be drawn with regard to women based on these results. The pack-years among women were significantly fewer compared to men both in hypertensive and control subjects (Table 4) and assumably this would have diluted the power of the study to observe an interaction between smoking and the ε4 allele among women if they had been included in the study. Second, it is possible although not probable (due to the fairly young age of the study subjects) that selective survival due to increased mortality of the ε4 carriers may have occurred. Third, the serum levels of apoE were not measured in the present study. Measuring the serum levels could have given more information as to whether the observed interaction between the ε4 allele and smoking is really due to the different function of the isoforms or to differences in serum levels of apoE. Finally, only the ε2/ε3/ε4 polymorphism of the apoE gene was studied even though several other polymorphisms also exist (Nickerson et al. 2000).
6.6 Paraoxonase-1 gene polymorphisms and IMT

PON1 polymorphisms have previously been reported to associate with atherosclerotic diseases in many studies (Laplaud et al. 1998). In the present population-based cohort study, however, no association was found between the PON1 Gln192Arg and Leu55Met polymorphisms and carotid artery IMT. The natural substrate of PON1 is still unknown. It is known that PON1 protects LDL from oxidation thus impeding atherosclerosis, but it is highly unlikely that this is the physiological function of the protein, since atherosclerosis has been a prevalent disease only for some decades. It is also known that PON1 is involved in the metabolism of xenobiotics and therefore it has been hypothesised that the natural organophosphate toxins and other esters and cyclic carbons are the physiological substrates for PON1. Nevertheless, the antioxidant mechanisms of PON1 continue to be a subject of growing interest. The effects of the polymorphisms of PON1 have been studied thoroughly and the results have shown consistently that the Leu55 and the Arg192 alleles are associated with higher activity towards paraoxon. Contrary to assumptions, the Arg192 allele has not been reported to associate with increased capability to protect LDL from oxidation, but conversely PON1 with glutamine at position 192 has been shown to protect LDL against copper-induced oxidative modification more efficiently than PON1 with arginine at position 192 (Mackness et al. 1997b, Mackness et al. 1998b). Similarly, HDL isolated from subjects with the Met/Met55 genotype maintained twice as much of the ability to protect LDL after the incubation with LDL and copper-ions as did HDL from subjects with the Met/Leu55 or the Leu/Leu55 genotypes. In light of these and other studies, it has been suggested that PON1 could have two active sites, one for paraoxonase activity and another required for the protection against oxidation of LDL (Aviram et al. 1998). It has become evident that measuring PON1 activity with paraoxon is not a quantitative measurement of the ability of PON1 to protect LDL against oxidative modifications. In addition, dietary modifications have recently been shown to alter serum PON1 activity and the response to dietary modification varies between the PON1 genotypes (Rantala et al. 2002), which may further complicate the interpretation of the measurements of PON1 activity.

The results of the previous association studies investigating the relationship between PON1 polymorphisms and atherosclerotic diseases have been contradictory as reviewed by Laplaud et al. (1998). The discrepancy between the results of the previous studies may be partly due to the fact that most reports on the effect of the PON1 polymorphisms on cardiovascular pathogenesis are based on case–control studies in which selection bias may have occurred. Also, different genetic background, small sample sizes and publication bias may partly explain the differences as discussed earlier. So far not many studies on carotid IMT and PON1 polymorphisms have been published (Table 2). In fact, there are only two previous studies where both the Gln192Arg and the Leu55Met polymorphism have been investigated in relation to IMT (Leus et al. 2000, Fortunato et al. 2003). Leus and associates studied 187 subjects with familiar hypercholesterolaemia and found that when both polymorphisms were analysed separately, no differences in IMT were found between the genotypes. In further analysis, they found that subjects with the Leu/Leu55-Gln/Gln192 genotype had the highest mean IMT of common carotid artery, bifurcation and internal carotid artery when compared to other genotypes.
Fortunato and co-workers studied 310 randomly selected middle-aged women. They defined an arterial plaque as IMT > 1.2 mm at any site of the carotid artery and found that the Leu55 allele was an independent contributor to the number of plaques, whereas the Gln192Arg polymorphism was not a significant determinant of the plaque score. They also investigated the effect of interaction of both polymorphisms on the number of plaques but did not identify any significant risk carrier genotype.

The present work is the first study where both polymorphisms have been studied in a population-based sample of both men and women. Our observation of no association between IMT and either of these polymorphisms alone or both of them together is supported by three studies where the Gln192Arg polymorphism and IMT of common carotid artery were studied and no association was found (Cao et al. 1998, Dessi et al. 1999, Markus et al. 2001). One previous study on PON1 polymorphism and IMT in a Finnish population has been published (Malin et al. 2001). Malin and associates studied the relationship between the Leu55Met polymorphism and carotid artery atherosclerotic disease (CAAD, defined as IMT > 1.7 mm at any site of the carotid artery) in a sample of 199 randomly selected men. They reported that the Leu55 allele is a risk factor for CAAD among nonsmokers, whereas among smokers the Met55 allele increases the risk. The present results do not support this finding as no difference was observed between smokers and nonsmokers in relation to IMT differences between the genotypes. Also, there is some evidence suggesting that the Gln192Arg polymorphism is associated with plasma lipoprotein concentrations. The Gln/Gln192 homozygotes have been reported to have significantly lower levels of plasma apoB-related biochemical variables and lower ratios of total cholesterol/HDL cholesterol, LDL cholesterol/HDL cholesterol and apoB/apoA-I than the heterozygotes and the Arg/Arg192 homozygotes (Hegele et al. 1995). Also, the Arg192 allele has been reported to associate with higher Lp(a) concentrations than the Gln192 allele (Cao et al. 1998). In the present study the PON1 genotypes did not seem to have an effect on the lipoprotein profile.

Although in the present study and also in some other studies no association was detected between the PON1 genotypes and early atherosclerotic changes as assessed with IMT, it is possible that the polymorphisms of the PON1 gene are involved in the later phases of the atherothrombotic process, especially since it has been shown that paraoxonase immunoreactivity is present in atherosclerotic lesions (Mackness et al. 1997a). In addition to the Leu55Met and the Gln192Arg polymorphisms, five promoter region polymorphisms of PON1 have been described (Levie & James 2000, Brophy et al. 2001). It is known that they strongly influence the serum concentration and the activity of PON1 and may also have an association with atherosclerotic diseases (James et al. 2000, Levie et al. 2001, Levie et al. 2002). Further, the other two members of the PON multigene family, namely PON2 and PON3, also harbour polymorphisms, and some studies have reported associations between these polymorphisms and CAD (Sanghera et al. 1998a, Leus et al. 2001, Chen et al. 2003). Therefore it is possible that the promoter polymorphisms of PON1 or the polymorphisms of PON2 and PON3 are associated with early atherosclerotic changes and more studies are needed in the future concerning whether the genetic variations in the genes of PON1, PON2 or PON3 are to some extent responsible for atherosclerosis.
6.7 C-reactive protein and IMT

Atherosclerosis is widely accepted as a chronic inflammatory disease. When comparing atherosclerosis to rheumatoid arthritis, a well-known autoimmune disease, a remarkable pattern of similarities emerges: both disorders show evidence of activation of macrophages, B cells, T cells, and endothelial cells, alteration in the Th1/Th2 ratio and elevation of inflammatory cytokines (Pasceri & Yeh 1999). CRP is an acute phase protein that increases during systemic inflammation. In a direct comparison of inflammatory and lipid markers, CRP was a stronger predictor of future cardiovascular events than all other biomarkers, including LDL cholesterol (Ridker et al. 2000). It can be speculated that the strong predictive value of CRP is due to its stability during storage, its long half-life, its lack of diurnal variation and independence of age and sex (Meier-Ewert et al. 2001).

Convincing evidence has been accumulated suggesting that CRP is strongly associated with future cardiovascular events (Ridker 2003). However, there is only limited amount of evidence that CRP is associated with early atherosclerotic changes, such as increased IMT. Wang and co-workers (2002) studied the association of CRP with IMT in the largest study sample (n=3,173) published so far. Subjects were participants in the offspring cohort of the Framingham Heart study. It was found that before adjustment for traditional risk factors the IMT values of the internal carotid artery were strongly associated with CRP. However, after adjusting for possible confounding factors (age, sex, smoking, systolic blood pressure, diabetes, total to HDL cholesterol ratio and body mass index, among others) the association disappeared totally among men. Among women the difference in IMT of the internal carotid artery between the first and the fourth quartile of CRP remained statistically significant, but there was no clear trend towards higher IMT values in higher CRP quartiles (IMT was 0.86 mm in the first, 0.83 mm in the second, 0.90 mm in the third and 0.99 mm in the fourth quartile). Interestingly, common carotid artery IMT was not associated with CRP even before the adjustment. These results are in line with the present results since it was found in the current work that before the adjustment for previously known risk factors there was a positive correlation between CRP and mean IMT, but after adjusting for the confounding factors the association vanished. Some other studies have also reported no association between CRP and carotid IMT (Hak et al. 1999, Manzi et al. 1999, Hulthe et al. 2001b). There are two cross-sectional studies suggesting an association between CRP and IMT (Blackburn et al. 2001, Winbeck et al. 2002). Blackburn et al. reported that in men, but not in women, the presence of advanced plaques (≥ 40% stenosis) was associated with elevated CRP levels. Winbeck et al. showed that subjects with elevated CRP (≥ 5 mg/l) had significantly higher IMT of common carotid artery than those with CRP < 5 mg/l. The subjects of these studies did not, however, represent the general population as Blackburn and associates recruited dyslipidaemic outpatients and Winbeck and co-workers studied neurological inpatients.

One study has investigated the association between CRP and changes in IMT during a follow-up period (Zoccali et al. 2002). Common carotid artery IMT of 90 subjects undergoing haemodialysis was measured at the start of the study and after 15 months. At the beginning of the study CRP was not an independent determinant of IMT, but after the follow-up CRP was found to be the strongest predictor of the increase in carotid IMT, suggesting that CRP may be a marker of atherosclerotic activity rather than of the extent
of atherosclerosis. This finding is further highlighted by the fact that there is also a strong association between the baseline CRP and the number of plaques developing over time (Hashimoto et al. 2001).

CRP is strongly associated with the traditional risk factors of atherosclerosis and especially those of metabolic syndrome (Fröhlich et al. 2000). This was also the case in the present study (data not published). It has been shown that weight loss is associated with a marked decrease in CRP and other inflammatory markers (Esposito et al. 2003). These findings lead to the speculation that CRP may after all be a marker of increased risk for atherosclerotic diseases rather than their cause. At least, this could be true for early atherosclerotic changes, since the evidence for a positive association between IMT and CRP is weak and lack of association between CRP and another measure of subclinical atherosclerosis, EBCT, has also been reported (Hunt et al. 2001). In addition, it has recently been shown that CRP is a risk factor for stroke independent of carotid IMT (Cao et al. 2003) which provides further evidence for the role of CRP in the later phases of atherogenesis. It has been suggested that CRP may predict the risk of cardiovascular events by indicating inflammation that leads to the atherothrombotic events or by directly interacting with the atherosclerotic vessels promoting thrombosis (Lagrand et al. 1999). This suggestion is in line with the recent finding that elevated CRP is associated with plaque rupture (Sano et al. 2003).

6.8 Autoantibodies against oxidised LDL and IMT

It has become evident that lipoprotein oxidation is one of the early events in the atherosclerotic process. Polyunsaturated fatty acids in LDL particles undergo lipid peroxidation forming hundreds of different kinds of oxidation products, many of which are highly reactive and further modify closely associated proteins and lipids (Hörkkö et al. 2000). These newly formed modified structures are no longer recognised as "self" structures, and therefore, an immune response rises against them. However, the role of the autoantibodies against OxLDL in the atherosclerotic diseases is not clear.

In animal models of atherosclerosis, such as apoE-deficient mice, autoantibodies against OxLDL have been shown to correlate strongly with the measures of atherosclerosis (Palinski et al. 1994). These antibodies have also been intensively studied in human populations, both in patients with cardiovascular disease as well as in normal populations. So far, in most of the studies the relationship between the autoantibodies to OxLDL and measures of atherosclerosis has been positive, suggesting that these antibodies could be markers of the progression of the disease or that they may even play an active role and enhance the progression of the disease. However, the results of the present study clearly demonstrate that those subjects having the highest titres of IgM type of autoantibodies binding to a classical model of OxLDL, MDA-LDL, have the lowest IMT in their carotid arteries. This finding remained after adjusting for traditional risk factors for atherosclerosis suggesting a possible protective role of these autoantibodies in atherogenesis.

There are several lines of earlier data supporting the hypothesis that there are also protective type of antibodies to OxLDL in vivo, and these data also provide important
mechanistic insights. The natural IgM type of monoclonal autoantibodies have been cloned from apoE-deficient mice having extremely high titres of autoantibodies to a wide variety of oxidation-specific epitopes without any exogenous immunisation (Palinski et al. 1996). Most of these antibodies have been shown to be able to block the binding and degradation of OxLDL by macrophages (Hörkkö et al. 1999). Since the uptake of OxLDL by macrophages and formation of foam cells is one of the key events in atherosclerosis, this finding suggests that these antibodies could play a protective role in atherosclerosis. In addition, because oxidised epitopes exist on circulating LDL \textit{in vivo}, these antibodies could enhance the removal of oxidatively modified lipoproteins from plasma and prevent their entrance into the arterial wall (Wiklund et al. 1987). In the present study, after the adjustment for age, gender, systolic blood pressure, LDL cholesterol, CRP and pack-years only IgM type of antibodies were inversely related to carotid artery IMT at statistically significant level. It can be speculated that the IgG type of antibodies do not have similar blocking properties as the IgM. Instead, the IgG molecules have an Fc-domain which may even enhance the uptake of OxLDL through the Fc-receptors on macrophages.

Usually the first exposure to an antigen leads to production of mainly IgM type of antibodies, and the production of IgG antibodies begins a few days later with less intensity. During the second exposure to the same antigen B-cells rapidly generate IgG type of antibodies, and IgM antibodies are produced in a markedly smaller quantity (Abbas et al. 2000). As to autoantibodies against OxLDL, the isotype switching from IgM to IgG does not seem to occur as the IgM subclass of autoantibodies can be continuously measured in substantial quantities in animal models and in humans. In the present study, IgM type of autoantibodies both to MDA-LDL and CuOx-LDL were inversely correlated with IMT before adjusting for the traditional risk factors of atherosclerosis. However, after the adjustments, IgM autoantibodies to CuOx-LDL were no longer associated with IMT. It can be speculated that IgM type of autoantibodies to MDA, but not to other oxidation specific epitopes could be protective. MDA-LDL contains large amounts of MDA whereas CuOx-LDL contains only a small number of MDA molecules among many other epitopes, which might explain the difference in results between the two models of OxLDL.

The other line of data suggesting that there exist protective type of antibodies \textit{in vivo} are the immunisation studies with OxLDL in various animal models. A recent study by Zhou and co-workers (2001) demonstrated that immunisation with MDA-LDL not only protected against atherosclerosis, but the increased titres of antibodies were negatively related to the degree of lesion formation. Immunisation with MDA-LDL results in a strong increase of antibodies, not only to MDA-LDL, but also to a variety of other oxidative neo-epitopes that may be present on OxLDL, such as oxidised phospholipids, oxidised cholesterol, oxidised cholesteryl linoleate and oxidised cardiolipin (Freigang et al. 1998). These results also lead to the speculation that high titres of antibodies to the oxidation-specific epitopes enhance formation of immune complexes with minimally modified LDL, which leads to its rapid removal from the circulation before it enters the vascular wall.

Finally, in addition to the present work there are a few other studies suggesting that high antibody titres to OxLDL could be protective in atherosclerosis. Hulthe and associates (2001a) studied 102 patients with hypercholesterolaemia and 102 control subjects. In the control group there was a significant inverse correlation between IMT of
the carotid bifurcation and IgM titres against OxLDL. In the patient group, only a weak and statistically nonsignificant association was seen. Similarly, Fukumoto and co-workers (2000) reported an independent inverse correlation between the antibodies to OxLDL and maximal IMT in a healthy population.

Thus, although the general view so far has been that the antibodies to OxLDL are related to increased atherosclerosis, there is evidence to suggest the opposite. Atherosclerosis is a chronic inflammatory process that involves a complex interplay of circulating cellular and blood elements (Ross 1999, Hansson et al. 2002). A high amount of OxLDL in circulation does not necessarily lead to high antibody titres to OxLDL. In fact, it has been shown that the amount of circulating OxLDL is inversely related to the antibody titres to OxLDL (Shoji et al. 2000b). The role of the humoral immune response may vary between different patient groups as well as at different stages of the disease.

The present data suggest that increased antibody titres to OxLDL may be an independent protective factor in subclinical atherosclerosis in humans, and may also support the recent views that induction of humoral immune response to oxidised neo-epitopes could be beneficial as a treatment of atherosclerosis in humans and studies pursuing a vaccine against atherosclerosis should be launched (Hansson 2002, Binder et al. 2003).
7 Conclusions

1. The endothelial nitric oxide synthase Glu298Asp polymorphism is not associated with carotid artery intima-media thickness, blood pressure or cardiac left ventricular mass in the current study population. These results imply that the Glu298Asp polymorphism of the eNOS gene is not a significant risk factor for atherosclerosis or other cardiovascular pathology in the general Caucasian population.

2. There is a significant interaction between the apolipoprotein E polymorphism and smoking in relation to carotid artery intima-media thickness among men. The results of the present work suggest that ε4 carriers are especially susceptible to the atherogenic effects of smoking. This interaction is particularly clear in hypertensive subjects.

3. The Leu55Met and Gln192Arg polymorphisms in the PON1 gene do not contribute to the variation in carotid artery intima-media thickness in the present study sample, indicating that these genetic variations are not a major risk factor for cardiovascular diseases in the general Caucasian population.

4. C-reactive protein is not independently associated with carotid artery intima-media thickness, implying that the risk of cardiovascular events linked with elevated CRP could be due to the role of CRP in the later phases of the atherosclerotic process.

5. The IgM type of autoantibodies against oxidised LDL are inversely correlated with carotid artery intima-media thickness, suggesting that they may have a protective role in atherogenesis.
References


