ERKKI ILVESKOSKI

Association of Apolipoprotein E Genotype with Early and Advanced Atherosclerotic Lesions

Autopsy and Clinical Studies

■

ACADEMIC DISSERTATION

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the Faculty of Medicine of the University of Tampere,
for public discussion in the small auditorium of Building B,
Medical School of the University of Tampere,
Medisiinarinkatu 3, Tampere, on April 27th, 2001, at 13 o'clock.

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To my wife Reetta

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, which are referred to in the text by their Roman numerals I-V. In addition, some unpublished data are presented.

I Ilveskoski E, Perola M, Lehtimäki T, Laippala P, Savolainen V, Pajarinen J, Penttilä A, Lalu KH, Männikkö A, Liesto KK, Koivula T and Karhunen PJ (1999): Age-dependent association of apolipoprotein E genotype with coronary and aortic atherosclerosis in middle-aged men: an autopsy study. Circulation 100:608-613.

II Ilveskoski E, Järvinen O, Sisto T, Karhunen PJ, Laippala P and Lehtimäki T (2000): Apolipoprotein E polymorphism and atherosclerosis: association of the ε4 allele with defects in the internal elastic lamina. Atherosclerosis 153:155-160.

III Ilveskoski E, Loimaala A, Mercuri MF, Lehtimäki T, Pasanen M, Nenonen A, Oja P, Bond MG, Koivula T, Karhunen PJ and Vuori I (2000): Apolipoprotein E polymorphism and carotid artery intima-media thickness in a random sample of middle-aged men. Atherosclerosis 153:147-153.

IV Ilveskoski E, Lehtimäki T, Laaksonen R, Janatuinen T, Vesalainen R, Nuutila P, Laippala P, Karhunen PJ and Knuuti J: Improvement of coronary artery reactivity by lipid-lowering therapy with pravastatin is modulated by apolipoprotein E genotype: a placebo-controlled PET study in mildly hypercholesterolemic young men. (submitted)

V Ilveskoski E, Lehtimäki T, Erkinjuntti T, Koivula T and Karhunen PJ (1998): Rapid apolipoprotein E genotyping from mailed buccal swabs. J Neurosci Meth 79:5-8.

ABBREVIATIONS

AD	Alzheimer´s disease
AN(C)OVA	analysis of (co)variance
Apo	apolipoprotein
BMI	body mass index
bp	base pair(s)
CAD	coronary artery disease
CFR	coronary flow reserve
CHD	coronary heart disease
CA	coeliac artery
CI	confidence interval
DNA	deoxyribonucleic acid
FH	familial hypercholesterolemia
HDL	high density lipoprotein
HSDS	Helsinkin Sudden Death Study
HSPG	heparan sulfate proteoglycan
IAP	International Atherosclerosis Project
IDL	intermediate density lipoprotein
IEL	internal elastic lamina
IMA	inferior mesenteric artery
IMT	intima-media thickness
kDa	kilo Daltons
LAD	left anterior descending (coronary artery)
LDL	low density lipoprotein
LRP	LDL receptor-related protein
MI	myocardial infarction
Mmax	mean maximum (intima-media thickness)
NO	nitric oxide
PDAY	Pathobiological Determinants of Atherosclerosis in Youth
PET	positron emission tomography
RCA	right coronary artery
SMA	superior mesenteric artery

SMC	smooth muscle cell
VLDL	very low density lipoprotein

INTRODUCTION

Atherosclerosis is the main cause of morbidity and mortality in Finland (Pyörälä et al. 1985) and in other Western countries (Keys 1970, Tunstall-Pedoe et al. 1994). As stated by Russell Ross, "atherosclerosis is not merely a disease in its own right, but a process that is the principal contributor to the pathogenesis of myocardial and cerebral infarction, gangrene and loss of function in the extremities" (Ross 1993). Coronary heart disease (CHD) is the most common cause of death in Finland, although there is a clear decline in mortality rate (Salonen et al. 1983, Tuomilehto et al. 1989**,** Salomaa et al. 1996). Traditional risk factors for CHD include hypercholesterolemia, hypertension, smoking and diabetes (Jousilahti et al. 1998, Wilson et al. 1998). Beside the involvement of these factors, CHD has a strong genetic component. Family studies have estimated heritability of CHD to be 56-63% (Nora et al. 1980) and also twin studies have supported the importance of genetic susceptibility to CHD (Koskenvuo et al. 1992, Marenberg et al. 1994). In Finnish mono- and dizygotic twins, the heritability of CHD is estimated to be 45% in men <60 years and 13% in men >60 years (Koskenvuo et al. 1992). CHD and atherosclerosis are complex traits, which means that they do not exhibit classic Mendelian inheritance attributable to a single gene locus. Rather, multiple genetic factors, incomplete penetrance and high frequency of disease-causing alleles characterize the genetics of atherosclerosis (Lander and Schork 1994).

The four most widely used genetic methods used to identify predisposing genes in complex traits like atherosclerosis are linkage analysis, allele-sharing methods, animal models and association studies (Lander and Schork 1994). Candidate gene approach utilizes genes, which are thought to predispose to the complex trait under investigation. Numerous association studies have implicated apolipoprotein E (apoE), a polymorphic gene with three common alleles (ε2, ε3, ε4) and six subsequent genotypes, to be an important candidate gene for atherosclerosis in several populations (Mahley and Huang 1999). The differences in the biological function of the three apoE isoforms are very well characterized. In addition, apoE is known to have a considerable role in the regulation of serum total and low-density lipoprotein (LDL) cholesterol levels (Davignon et al. 1988). However, there is no knowlegde, which phase of atherosclerosis apoE affects and to which phenotype of atherosclerosis apoE is related.

This thesis is based on four different study series, which all characterized a single phenotype of atherosclerotic disease in different arteries. Examination of two autopsy series consisting of over 800 subjects made it possible to relate apoE genotypes to the area of early and advanced atherosclerotic lesions of the coronary arteries and aorta and also to histological changes of the internal elastic lamina (IEL) of the mesenteric artery wall. One clinical series was utilized to study the role of apoE polymorphism in coronary artery reactivity measured by positron emission tomography (PET). Furthermore, the effect of apoE genotype on pravastatin induced changes in coronary function was examined. Relation of apoE genotypes to carotid artery intima-media thickeness (IMT) measured by ultrasound was examined in another clinical sample. Finally, a rapid DNA sample collection method for apoE genotyping was developed.

REVIEW OF LITERATURE

1. Atherosclerosis

1.1. Structure of normal arteries

Three main layers, intima, media and adventitia, can be distinguished from normal arteries (Geer and Haust 1972, Stary et al. 1992). Elastic and muscular arteries differ in the composition and thickness of these layers. The intima, the innermost layer of the arterial wall, consists of endothelium and a subendothelial part containing sparse connective tissue and only few smooth muscle cells (SMCs) and macrophages. The subendothelial part of the intima is composed of two layers: the inner layer is the proteoglycan layer and the layer underlying it is called the musculoelastic layer. IEL is a wavy structure separating the intima from the media. The media contains contractile type SMCs producing collagen and elastic fibers. External elastic lamina separates the media from the outermost layer of the arterial wall, the adventitia. The adventitia is connective tissue and contains vasa vasorum and also nerves, which enter the media (Geer and Haust 1972, Stary et al. 1992).

The thickness of human intima is not uniform and it varies with location (Stary et al. 1992). These differences are present in everyone from infancy, and are strictly physiological changes due to variation of shear and tensile forces in different segments of the arteries. This so called adaptive (or eccentric) intimal thickening is a self-limited process forming at certain locations of arterial tree, mostly at bifurcations and orifices (Geer and Haust 1972). In microscopy, the cellular composition of the site with adaptive intimal thickening is quite similar to that of arterial intima in general but there are some proportional differences (Stary et al. 1992). Adaptive intimal thickening itself has no clinical consequences because it does not obstruct the lumen. However, the adaptive intimal thickening and atherosclerosis may share a relationship since they both form at same, relatively constant locations (Stary et al. 1992, Stary et al. 1994). These location have been called atherosclerosis-prone or progression-prone areas, at which the same mechanical forces may both stimulate intimal thickening and enhance lipoprotein deposition. Although advanced lesions form earlier at the progression-prone locations, adaptive intimal thickening is not a necessary change for atherosclerosis, it only marks the areas where lesions tend to occur (Stary et al. 1992).

1.2. Definition and classification of atherosclerosis

Definition of atherosclerosis. Arteriosclerosis is defined as pathological thickening of the vessel wall due to accumulation of cellular elements within it. Atherosclerosis, in turn, is a form of arteriosclerosis that affects the intima of muscular and elastic arteries whereas small arteries and arterioles are injured by other mechanisms.

Macroscopic classification of atherosclerosis. The first large-scale autopsy survey on atherosclerosis was the International Atherosclerosis Project (IAP) in 1960s (reviewed by Solberg and Strong 1983), which examined over 23,000 sets of aortas and coronary arteries using a standardized evaluation method (Guzman et al. 1968). This study gave important basic knowledge of the characteristics and risk factors for autopsy-verified atherosclerosis in aorta and coronary arteries (Eggen et al. 1964, McGill 1968, Geer et al. 1968, Eggen and Solberg 1968). To expand the knowledge of risk factors in the young, a group of pathologists in the USA organized a multicenter autopsy study in young trauma victims (Pathobiological Determinants of Atherosclerosis in Youth, PDAY study) in the late 1980s. In both the IAP and the PDAY studies the evaluation of atherosclerosis was based on procedures developed for the IAP, in which the atherosclerotic lesions are stained with Sudan IV and graded visually as fatty streaks, fibrous plaques and complicated lesions by several pathologists (see Table 1) (Guzman et al. 1968).

Histological classification of atherosclerosis. In the beginning of 1990s, a new classification of atherosclerotic lesions was presented by the Committee on Vascular Lesions of the Council on Atherosclerosis, American Heart Association (Stary et al. 1992, Stary et al. 1994, Stary et al. 1995) (Table 1). This classification is based on microscopical examination of histological arterial samples. Early lesions with no disorganization of normal intimal structure are designated as type I, II and III lesions and advanced lesions containing structural disorganization and thickening of the intima are called type IV, V and VI lesions (Table 1) (Stary et al. 1992, Stary et al. 1994, Stary et al. 1995).

Type I lesions are the most initial and usually invisible with naked eye. These lesions develop already in infants and children, and are the most frequent lesion type in their arteries. Type I lesion preferentially forms at a location with adaptive intimal thickening, and histologically, it consists of small, isolated groups of macrophages and macrophage foam cells (Stary et al. 1994).

Type II lesions are fatty streaks, which may or may not be visible on gross inspection as yellow-coloured lesions of variable size and shape. Most of the invisible fatty streaks come visible when stained red with Sudan III or Sudan IV. Type II lesion increases the intimal thickness by less than a millimeter and is present from childhood. The type II lesion contains more macrophages with and without lipid droplets than the type I lesion, and the intimal SMCs also contain lipid, but the most of the lipid is in macrophage foam cells. However, some extracellular lipid already exists, as well as few mast cells and T-lymphocytes (Stary et al. 1994). Type II lesion can be further classified as **type IIa** or progression-prone and as **type IIb** or progression-resistant lesion. Type IIa lesion colocalizes with adaptive intimal thickening and contains more lipid, and is therefore more rapidly proceeded to type III. Type IIb found in locations with thin intima does not progress or progresses much slower to type III (Stary et al. 1994).

Type III lesion is also called preatheroma or intermediate lesion, and it represents a transitional lesion between early and advanced lesions. Type III lesions are present in young adults, do not obstruct the lumen, and colocalize with adaptive intimal thickening. A confluent lipid core has not yet developed, but this lesion is characterized with extracellular lipid pools accumulated below the foam cell layers slightly disrupting the intimal structure. Type III lesions are important because their presence predicts future advanced lesions at the same location (Stary et al. 1994).

Type IV lesion, i.e. atheroma, is the first lesion classified as advanced since intimal structure is severely disorganized. A confluent lipid core is present in the deep part of the intima, which has probably developed from isolated lipid pools of type III lesion. The intima above the lipid core consists of macrophages and SMCs with or without lipid droplets, mast cells and lymphocytes, but this cap contains no collagen layers. Type IV change is usually invisible in coronary angiography, because the lesion does not narrow the lumen. Instead, the clinical significance of the type IV lesion is due to collagen-poor layer above the lipid core that makes the plaque prone to suddenly rupture producing hematoma and thrombus, and an acute coronary syndrome (Stary et al. 1995).

Type V lesion or fibroatheroma increasingly narrows the arterial lumen and the lesion is characterized with increased collagen content. If collagen is present in the fibrous layer or cap above the lipid core the lesion is classified as Va. A fibrotic lesion with calcification as a major component is referred as Vb (or type VII). Type Vc (or type VIII) lesion in turn has only minor lipid deposits and consists mainly of connective tissue. The type Va lesions are also prone to progress to type VI lesion by rupturing and subsequent

formation of mural thrombi. If multiple events occur, a multilayered type V lesion forms, and the lumen is further narrowed (Stary et al. 1995).

Type VI or complicated lesion is formed by disruption of the surface (type VIa), hematoma or hemorrhage (type VIb), or thrombosis (type VIc) of the type IV or V lesion. This episode may lead to acute coronary syndrome or sudden death, or be clinically silent. There may be a return to type V lesion, or another thrombotic complication (Stary et al. 1995).

Table 1. Comparison of the classification of atherosclerotic lesions by Stary and coworkers with the classification of the International Atherosclerosis Project (IAP) and with findings in the carotid ultrasonography (modified from Stary et al. 1995).

Histological classification by Stary et al. (1995)	**Macroscopic classification of IAP (Guzman et al. 1968)**	**Appearance in carotid B-mode ultrasonography***
Intimal thickening	Not visible or may be mistaken as a raised lesion	Intima-media thickening
Early lesions		
Type I lesion	Not visible	Intima-media thickening
Type II lesion	Fatty streak, usually visible and stains with Sudan IV	Intima-media thickening
Intermediate lesion		
Type III lesion	Fatty streak or fibrous plaque	Intima-media thickening or "soft plaque"
Advanced lesions		
Type IV lesion	Fibrous plaque	"Soft plaque" (if no mineralization)
Type Va lesion	Fibrous plaque	"Soft plaque" (if no mineralization)
Type Vb (or VII) lesion	Calcified lesion	"Hard plaque"
Type Vc (or VIII) lesion	Fibrous plaque	"Soft plaque" (if no mineralization)
Type VI lesion	Complicated lesion	"Soft plaque" (if no mineralization)

*According to Salonen and Salonen 1993.

1.3. Main hypotheses for the development of atherosclerosis

During the last decades, increasing knowledge of the pathogenesis of atherosclerosis have given rise to new hypotheses and to continuous modification of old theories of this complex process. There are several different theories, which all emphasize different aspects of the atherosclerotic process.

Monoclonal hypothesis. Benditt and Benditt proposed in 1973 that all SMCs in the lesion are of monoclonal origin. They hypothesized that the mechanism behind the monoclonal proliferation of SMCs is a chemical mutagen or a virus (Benditt and Benditt 1973).

Lipid infiltration hypothesis. This hypothesis states that LDL cholesterol is a sufficient but not necessarily a necessary cause of atherosclerosis. It emphasizes the role of high lipid levels as risk factors but does not explain why LDL is taken up by the macrophages and SMCs and accumulated in the intima (Steinberg 1983).

Response-to-injury hypothesis. According to a response-to-injury hypothesis atherosclerosis is like a wound healing process due to endothelial injury. The theory has been under a continuous modification. When the theory was presented by Ross and Glomset in 1973, denudation of the endothelium was thought to be the first injuring step (Ross and Glomset 1973), and later the role of SMC proliferation was emphasized in the formation of advanced lesions (Ross and Glomset 1976). In 1986, Ross presented the modified response-to-injury hypothesis, which was further updated in 1993 (Ross 1986, Ross 1993). These theories still considered endothelial dysfunction important but they also emphasized the role of oxidation, cellular interactions, growth-factor (like platelet-derived growth factor, PDGF) release of different cells and inflammation in the pathogenesis.

Oxidation hypothesis. With high lipid levels, LDL is accumulated beneath the endothelium, where it is modified by several oxidation pathways (Steinberg et al. 1989, Steinberg 1997). Macrophages have scavenger receptors on their surfaces and they can internalize oxidized LDL, which lead to foam cell formation. Oxidized LDL also injures endothelium and SMCs, is a chemotactic agent for monocytes, and stimulates the replication of monocyte-derived macrophages (Steinberg et al. 1989). A cycle of continuous accumulation of monocytes and foam cells has formed, leading first to formation of fatty streaks (Ross 1999). There is evidence that macrophage foam cell formation is a protective effort to remove lipid from the arterial wall. On sites in arteries

with retracted endothelial cells, underlying foam cells have been observed to struggle towards circulation (Ross 1993).

Response-to-retention hypothesis is kind of an extension of the lipid infiltration theory. It states that the key event in the early atherogenesis is the retention of atherogenic apolipoprotein B (apoB)-rich lipoproteins, mainly LDL, under the endothelium, and that the retention of these lipoproteins is both necessary and sufficient to provoke the lesion formation in artery wall (Williams and Tabas 1998). The hypothesis rests on recent findings in genetically engineered mice: it has been shown that defective binding of apoB-100 on arterial proteoglycans protects from atherosclerotic lesions, even during significant hyperlipidemia (reviewed by Williams and Tabas 1998).

Inflammation hypothesis. In 1999, the role of inflammation was brought into the hypothesis (Ross 1999). All atherosclerotic lesions represent a state of chronic inflammatory process of the intima (Ross 1999). According to the response-to-injury hypothesis, endothelial dysfunction, a result of high or oxidized LDL, smoking, high blood pressure, diabetes mellitus, and other factors, is the key event that initiates the atherosclerotic cascade. Dysfunction of the endothelium leads to increase in its adhesiveness and permeability. Increased expression of selectins and adhesion molecules by the injured endothelium results in increased attachment of blood monocytes and T-lymphocytes to them, and the cells migrate into the intima. In addition, at lesion-prone sites with appropriate shear stress and turbulence, the expression of adhesion molecules is increased. As the injuring process continues the endothelium also produces cytocines and growth factors leading to continuous inflammation provoking more macrophages and lymphocytes (Ross 1999). In addition, the role of infection by micro-organisms like *Chlamydia pneumoniae* has been proposed to be important in evoking the immune system (Saikku et al. 1988).

1.4. Changes of the internal elastic lamina and their role in atherosclerosis

Intimal migration and proliferation of SMCs play an important role in the development of the fibrous tissue in atherosclerotic lesions (Ross and Glomset 1973, Ross and Glomset 1976). SMCs originate from different lineages in different parts of the arterial tree, and therefore they may respond differently to stimuli by cytokines and chemotactic factors in

different arteries (Ross 1999). There are two phenotypes of SMCs: the contractile and the synthetic phenotype. In the atherosclerotic intima, SMCs have changed from contractile to synthetic phenotype capable of responding to growth stimulating factors (Ross 1993). However, there is no consensus about the question on the origin of the SMCs that give rise to the intimal lesion. SMCs have been observed in the intimas of children under the age of 5 (Stary 1987) suggesting that they are a normal component of the intima. In response to injury, these SMC could therefore proliferate and form the lesion (Ross 1986). On the other hand, migration of medial SMCs into the intima is known to occur: SMCs have been observed in gaps or fenestrae of the IEL (Wissler 1967). Although based on limited evidence, it has been proposed by Sims et al. that defects in the IEL play a role in the pathogenesis of intimal thickening and atherosclerosis (Sims 1985, Sims et al. 1989). According to the hypothesis, the defects in the continuity of the IEL and the inability to form a reduplicated elastin membrane would allow SMCs to migrate into the intima. Indeed, an association of structural changes in the IEL with intimal thickening and with atherosclerosis has been reported in humans (Sims 1985, Sims 1989, Sims et al. 1989, Sims et al. 1993, Sisto 1990, Järvinen et al. 1996) and also in experimental animals (Nakatake et al. 1985). There are also lines of evidence from the studies on animal models that hypercholesterolemia induces fragmentation in the IEL (Hayashi et al. 1991, Kwon et al. 1998).

1.5. Plasma lipoproteins as risk factors for atherosclerosis

Plasma lipoproteins. In blood, insoluble lipids are transported in soluble lipoproteins, which all consist of a surface layer of hydrophilic lipids including free cholesterol and phospholipids and of a core containing hydrophopic cholesteryl esters and triglycerides (Mayes 2000). There are five major lipoprotein classes: chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), LDL and high density lipoprotein (HDL) (Shen et al. 1977). From these all but HDL increase the risk of coronary artery disease (CAD), and small dense LDL particles have been shown to be particularly atherogenic (Steinberg and Gotto 1999).

Serum total and LDL cholesterol. The causal relationship between atherosclerosis, CAD and cholesterol is well established. Based on the Oslo and PDAY autopsy studies,

serum total and LDL cholesterol are positively correlated with aortic and coronary atherosclerosis (Solberg and Strong 1983, PDAY Research Group 1990). The Framingham study was the first large prospective study to show that serum total cholesterol level is a risk factor for CAD (Castelli 1984). Since that numerous epidemiologic studies have confirmed the positive relation between CAD and serum total and, particularly, LDL cholesterol (Braunwald 1997, Thompson 1999, Steinberg and Gotto 1999). The relationship between serum cholesterol and CAD mortality is a continuously graded one and independent of other risk factors, as shown in the Multiple Risk Factor Intervention Trial including over 360,000 men (Stamler et al. 1986). In addition, patients with familial hypercholesterolemia (FH) characterized by extremely high levels of serum LDL cholesterol due to defective LDL receptor suffer their first myocardial infarction (MI) in early middle-age (Hill et al. 1991, Miettinen and Gylling 1988).

Cholesterol lowering interventions. The risk of CAD morbidity and mortality can be reduced by reducing serum cholesterol by 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (i.e. statins). This has been shown in secondary prevention studies on patients at very high risk (Scandinavian Simvastatin Survival Study group 1994) and on individuals with mildly elevated serum total and LDL cholesterol levels (The Long-Term Intervention with Pravastatin in Ischaemic Disease study group 1998). Also according to primary prevention trials the incidence of CAD events is reduced in men and women with high cholesterol levels by statin treatment (Shepherd et al. 1995, Downs et al. 1998). In addition, intervention trials in which coronary angiography has been performed, have shown that cholesterol lowering with statins slows lesion progression or even regresses coronary stenoses (MAAS Investigators 1994, Jukema et al. 1995, reviewed by Archbold and Timmis 1999). All this data support the causality of the relationship between serum cholesterol and CAD.

HDL cholesterol. There is evidence provided by epidemiological and autopsy studies that low HDL cholesterol, particularly the low HDL_2 fraction, is correlated with the risk for cardiovascular disease (Solberg and Strong 1983, Miller 1987, Salonen et al. 1991, Barter and Rye 1996, de Backer et al. 1998, Steinberg and Gotto 1999). HDL is itself antiatherogenic, probably due to its involvement in reverse cholesterol transport (Glomset 1968, Spady 1999), but the inverse relationship with CAD may also be explained in part by the fact that low HDL cholesterol is very often associated with elevated levels of atherogenic lipoproteins and other risk factors (Wu 1999).

Triglycerides. The association between serum triglycerides and CAD is not quite clear. In some studies, after controlling for the effects of total cholesterol and HDL, the association disappears (reviewed by Austin 1991). There are, however, several studies in which triglyceride remains a significant predictor for CAD after adjustments for other risk factors (Austin 1991, Manninen et al. 1992, Burchfiel et al. 1995, Assmann et al. 1996).

1.6. Coronary flow reserve (CFR) measured by positron emission tomography

Endothelial dysfunction is, as indicated before, the key event and an important abnormality in early atherogenesis (Ross 1999). The state of endothelial function can be evaluated by measuring endothelium-dependent vasodilatation. For coronary circulation, this can be performed non-invasively by positron emission tomagraphy (PET) (Bergmann et al. 1984, Krivokapich et al. 1989, Hutchins et al. 1990, Araujo et al. 1991). PET is based on the detection of two photons created in an annihilation reaction between a positron (infused into circulation) and an electron from tissue. In PET, the myocardial blood flow is measured both at rest (basal) and during hyperemia created by administration of a vasodilator like dipyridamole or adenosine into the circulation. The ratio of hyperemic and basal blood flow is called coronary flow reserve (CFR) which an integrated measure of endothelial function and smooth muscle relaxation (reviewed by Pitkänen 1998, Knuuti and Nuutila 1999).

PET studies have shown that reduced response of coronary arteries to vasodilatating agents and reduced CFR are already present in asymptomatic middle-aged men with high risk for CAD (Dayanikli et al. 1994), in young men with FH (Pitkänen et al. 1996) or with familial combined hypercholesterolemia type IIB (in type IIB both serum cholesterol and triglyceride concentrations are elevated) (Pitkänen et al. 1999). Further, serum total and LDL cholesterol, and also autoantibody titer against oxidized LDL have been observed to have a significant inverse correlation to CFR (Dayanikli et al. 1994, Pitkänen et al. 1997, Raitakari et al. 1997). Clearly, myocardial blood flow indexes measured by PET associate with all traditional CAD risk factors. Therefore, due to its noninvasive nature PET can be used to detect early vascular abnormalities in healthy volunteers at risk for CAD.

1.7. Intima-media thickness (IMT) measured by B-mode ultrasonography

Carotid artery IMT can be measured noninvasively with B-mode ultrasound imaging which is a validated method used in several epidemiological studies (Mercuri 1994). IMT is a measure of the distance between media-adventitia and intima-lumen interfaces, i.e. it measures the combined thickness of the intima and media. B-mode ultrasonography cannot distinguish fatty streak from intima-media thickening but fibrofatty plaque and complicated lesions give their characteristic echos (Salonen and Salonen 1993). The comparison between findings in B-mode ultrasonography and atherosclerotic lesions determined at autopsy is shown in table 1.

On the basis of several epidemiological studies, the carotid IMT measured by ultrasound can be considered as a well-characterized measure of atherosclerosis. The carotid IMT and its progression are associated with age, male sex, high systolic blood pressure, cigarette smoking, and high serum LDL cholesterol concentration (Tell et al. 1989, Heiss et al. 1991, Prati et al. 1992, Salonen and Salonen 1993, Espeland et al. 1999). In addition, carotid artery atherosclerosis assessed with ultrasound is associated with CAD and CAD severity (Craven et al. 1990, Wofford et al. 1991).

2. Apolipoprotein E (ApoE)

2.1. ApoE protein structure

The history of apolipoprotein E (apoE) begins in the early 1970s, when Shore and Shore (1973) found a new arginine-rich protein component of VLDL. This protein was later isolated and designated as apoE (Utermann 1975). Later, studies on type III hyperlipoproteinemia patients led to the observation that apoE is a genetically polymorphic protein with three common isoforms: E2, E3 and E4 (Utermann et al. 1975, Utermann et al. 1977). The combinations of the isoforms lead to phenotypes E2/2, E3/2, E4/2, E3/3, E4/3 and E4/4. The isoforms result from cysteine-arginine interchanges at positions 112 in E4 ($cys_{112} \rightarrow arg$) and 158 in E2 ($arg_{158} \rightarrow cys$) of the 299 amino acid chain of the mature apoE polypeptide (Figure 1) (Weisgraber et al. 1981, Rall et al. 1982). These amino acid substitutions cause charge differences between the three isoforms

because E2 has two neutral cysteine residues, E3 has one cysteine (site 112) and E4 has no cysteine (relative charges 0, +1 and +2, respectively). The charge differences were utilized in apoE phenotyping by isoelectric focusing (Utermann et al. 1977, Warnick et al. 1979, Utermann et al. 1982, Menzel and Utermann 1986, Lehtimäki et al. 1990) until a genotyping technique was developed (Hixson and Vernier 1990).

ApoE protein contains two domains (Figure 1): a 22-kDa aminoterminal domain (residues 1-191) and a 10-kDa carboxyl-terminal domain (residues 216-299) (Wetterau et al. 1988, Aggerbeck et al. 1988). The aminoterminal domain is responsible for receptor binding. The receptor binding region is located in the vicinity of residues 136-150 (Innerarity et al. 1983, Weisgraber et al. 1983) but the 171-183 region is also essential for receptor binding, which is probably due to its interaction with the region actually binding to the receptor (Lalazar and Mahley 1989). It has been observed by X-ray crystallography that four alpha-helixes of the aminoterminal domain form a four-helix bundle (Rall et al. 1982, Wilson et al. 1991, Segrest et al. 1992). The carboxyl-terminal domain is in turn the major lipid-binding domain (Weisgraber 1990) and it mediates the tetramerization of lipid-free apoE (Aggerbeck et al. 1988). Its structure is predicted to be highly helical and to have a long class A amphipathic helix (Segrest et al. 1992). The residues between 223-230 in the C-terminal domain are detrimental for the binding of apoE to the proteoglycan biglycan (Klezovitch et al. 2000).

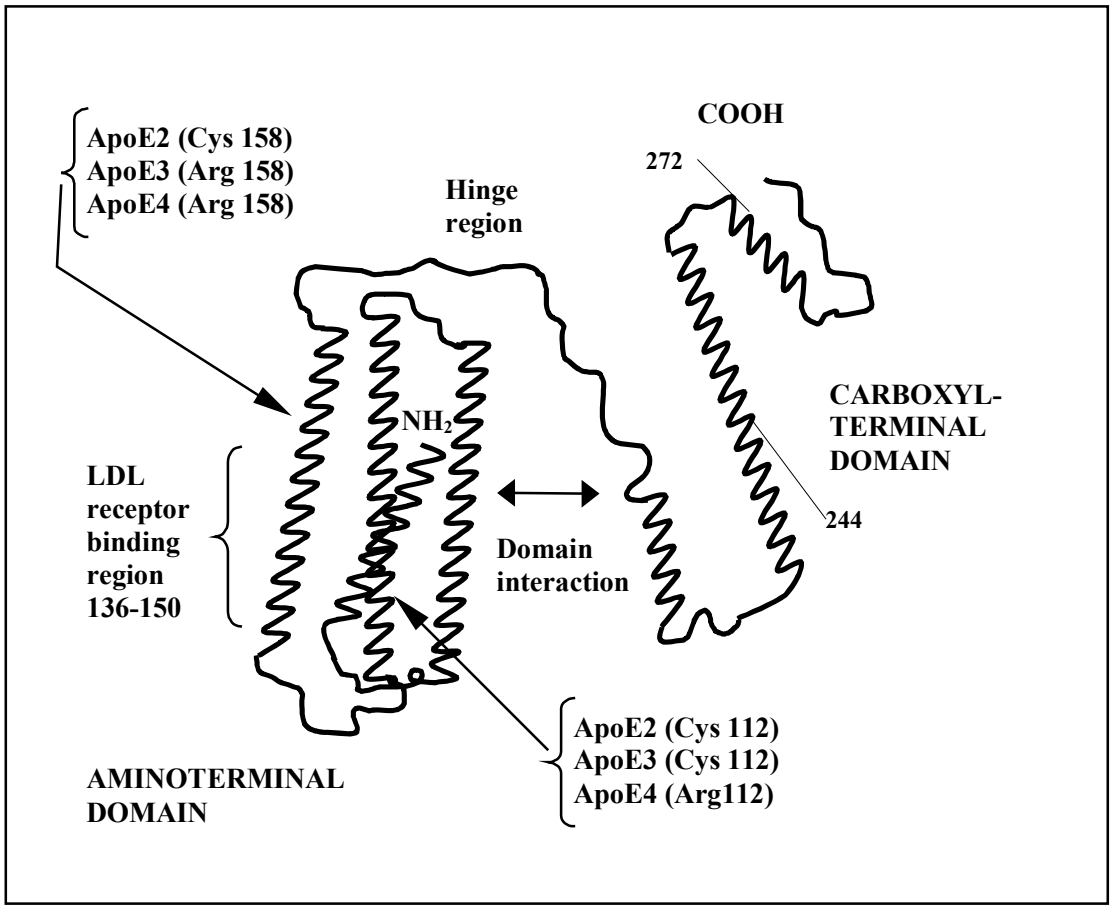


Figure 1. Model of the domain structure of apoE protein (modified from Weisgraber and Mahley 1996). The positions 244 and 272 indicate the region critical for lipoprotein binding (Dong et al. 1994).

In the apoE E4 isoform, but not in the E3, there is an interaction between amino-terminal and carboxyl-terminal domains. The positive charge (arginine) at position 112 of E4 changes the conformation of arginine 61 side chain (Weisgraber 1990, Dong et al. 1994), and the domain interaction is further mediated by interaction of arginine 61 and glutamic acid 255 (Dong and Weisgraber 1996). This results in changes in the lipid-binding properties of carboxyl-terminal domain, and therefore explains an observed preference of the E4 variant for VLDL (Gregg et al. 1986, Steinmetz et al. 1989). The domain interaction is absent in the E3 isoform and its preference is for HDL.

Poor binding of the E2 isoform to the LDL receptor compared to the E3 (Weisgraber et al. 1982) is also explained by changes in the conformation of the apoE protein. The change of arginine at 158 to cysteine causes salt-bridge reorganization, which is mainly responsible for the decreased receptor binding of the E2 (Wilson C et al. 1994,

Dong et al. 1996). However, also the carboxyl-terminal domain plays a critical role: if it is removed, the E2 attains full binding activity to the LDL receptor (Innerarity 1984, Dong et al. 1998).

2.2. ApoE biosynthesis

ApoE is syntesized in several organs and cells. Most of the apoE is synthesized in the liver parenchymal cells (Blue et al. 1983, Elshourbagy et al. 1985, Kraft et al. 1989) and is involved in lipid metabolism while secreted as a component of VLDL (Mahley 1988). In non-human primates, it has been estimated that 60-80% of total body apoE mRNA is synthesized by the liver (Newman et al. 1985). Kraft et al. (1989) studied liver transplantation patients and found <10% of plasma apoE to be derived from extrahepatic tissues. They also noticed that the recipient's apoE phenotype in plasma had changed to the donor's phenotype.

Large amounts of apoE are synthesized in central nervous system by astrocytes (Elshourbagy et al. 1985, Boyles et al. 1985). In the peripheric nerve, apoE, secreted by macrophages, participates in regeneration (Boyles et al. 1989). In addition, apoE is synthesized in various tissues including kidney, adrenals, spleen, testis, ovary, heart, and lung (Blue et al. 1983, Driscoll and Getz 1984, Zannis et al. 1985). Macrophages but not monocytes, are also a significant source of apoE (Basu et al. 1981, Basu et al. 1982, Zannis et al. 1985, Auwerx et al. 1988). The role of apoE secreted by arterial wall macrophages is discussed in more detail later.

The primary translation product of apoE, pre-apoE (317 amino acids in length), has an 18 amino acid signal peptide, which is intracellularly cleaved (Zannis et al. 1984). ApoE is then glycosylated with carbohydrate chains containing sialic acid. The secreted apoE contains 2, 4, or 6 sialic acid residues, but because of extracellular desialiation, plasma apoE sialo content is reduced, and most of the apoE in plasma is in asialo form (Zannis and Breslow 1981, Zannis et al. 1984, Zannis et al. 1986). It was proposed that glycosylation plays a role in cellular processing and secretion, but Wernette-Hammond et al. (1989) showed that apoE secretion does not depend on glycosylation. Although apoE is a secretory protein, a substantial proportion of the synthesized apoE remains in the cells

and is degraded (Dory 1991, Mazzone et al. 1992). The role of this intracellular apoE is unclear.

2.3. ApoE gene

ApoE (MIM 107741) gene is located on chromosome 19q13.2 (Das et al. 1985, Myklebost and Rogne 1986) about 5.5 kb upstream of the gene encoding apolipoprotein C-I (Lauer et al. 1988). It is 3597 nucleotides in length and shares a structural similarity with several other apolipoprotein genes (Lauer et al. 1988). ApoE gene consists of four exons (lengths 44, 66, 193, and 860) separated by three introns (lengths 760, 1092, and 582) (Das et al. 1985, Paik et al. 1985). The TATA box element of apoE gene begins 33 bp upstream from the transcription initiation site. ApoE gene encodes a mRNA of 1163 nucleotides.

ApoE gene has numerous regulatory elements (Paik et al. 1988, Chang et al. 1990), which bind nuclear transcription regulating proteins. Regions between –360 bp and –80 bp and within the first intron are important in the regulation of the apoE gene (Smith et al. 1988). The transcription factor SP-1 plays a major role, and it modifies the upstream regulatory element 1 activity by binding to the –161 to –141 sequence (Chang et al 1990). SP-1 has also other, less important binding sites in apoE promoter region. Further, apoE promoter regions from –48 to –74 and from –107 to –135 bind transcription factor AP-2 (García et al. 1996).

The common apoE variants E2, E3 and E4 are determined at the DNA level by alleles ε2, ε3 and ε4, which differ from one another by single point mutations in the first base of the each codon in exon 4. In comparison to the ε3 variant, in the ε4, there is a change of thymidine to cytosine at codon 112 (TGC → CGC) and in the ε2, cytosine is replaced by thymidine at codon 158 (CGC → TGC) (Utermann et al. 1980, Zannis et al. 1981, Breslow et al. 1982). It has been suggested that the ε4 allele is the ancestral form, from which the ε2 and ε3 alleles are derived (Mahley and Rall 1999, Fullerton et al. 2000). Several other polymorphic sites in the apoE gene have been identified, which determine several rare apoE variants (de Knijff et al. 1994), and recently, apoE gene and its flanking regions were resequenced and the researchers found 22 diallelic sites defining 31 distinct haplotypes of the apoE gene (Fullerton et al. 2000).

There is a great variance between populations in the apoE allele frequencies. The ε3 allele is the most common allele in every population. The ε2 allele frequency is highest in Papua-New Guinea (de Knijff et al. 1994) and lowest in Nigerian Blacks (0.027) (Sepehrnia et al. 1989) and in Japanese (0.037) (Eto et al. 1986). The ε2 allele is totally absent in Amerindians and Australian aboriginals (Kamboh et al. 1991, de Knijff et al. 1994). Further, the ε4 allele is most frequent in Pygmies (0.41) (Zekraoui et al. 1997), in Nigerian Blacks (0.30) (Sephrnia et al. 1989), in Sudan (0.29) (Hallman et al. 1991) and in Australian aboriginals (0.26) (Kamboh et al. 1991), and lowest in Chinese populations (Davignon et al. 1988). There is a clear decreasing north/south gradient for the frequency of the ε4 allele in Europe (Lucotte et al. 1997). In Finland, the ε4 allele frequency is the highest (0.19-0.23) and the ε2 allele frequency is the lowest (0.04) of all Caucasian populations (Ehnholm et al. 1986, Lehtimäki et al. 1990). There are no regional differences in the apoE allele frequencies in Finland (Lehtimäki et al. 1991) with the exception of a very high frequency of the ε4 allele (0.30) in the Saami (Lehtinen et al. 1996).

2.4. ApoE in lipid metabolism

Human lipid metabolism consists of two different transport systems, namely exogenous and endogenous lipid transport systems (Mayes 2000). The **exogenous transport system** is responsible of delivering ingested lipid from the gastrointestinal tract into the circulation and to the liver and peripheral cells. After the dietary fat is taken up by the intestinal mucosal cells, the lipids are secreted as chylomicrons from the enterocytes into the circulation through ductus thoracicus. Chylomicrons, triclyceride-rich lipoproteins carrying the dietary lipids, have mainly apoB-48 and apoCs as their apolipoproteins. In the circulation, lipoprotein lipase hydrolyzes the triclycerides to free fatty acids and glycerol and the chylomicrons acquire apoE from the HDL (Chappell and Medh 1998). These smaller triglyceride-poor lipoproteins are called chylomicron remnants and they are taken up by the liver via LDL receptors (apoB,E receptor) (Brown and Goldstein 1986) or by LDL receptor-related protein (LRP) (apoE or remnant receptor) (Herz et al. 1988). In the LDL receptor binding process of the chylomicron remnants, apoE serves as a ligand because apoB-48 is lacking the receptor binding domain of the apoB-100 (Chappell and

Medh 1998). Heparan sulfate proteoglycans (HSPG) participate the binding of apoE to LRP, and in addition, HSPG can act alone as a remnant receptor (reviewed by Mahley and Ji 1999). Normal function of apoE is therefore essential for the receptor binding and plasma clearance of chylomicron remnants while apoE is involved in all three remnant clearance pathways i.e. (1) LDL receptor, (2) LRP and (3) HSPG pathways.

The **endogenous lipid transport system** delivers cholesterol synthesized in the liver to peripheral cells (Mayes 2000). VLDL, IDL, LDL and HDL are the four lipoprotein classes involved in the system. Liver synthesizes triglyceride-rich apoE and apoB-100 containing VLDL particles and secretes them into circulation, where they are rapidly cleared from plasma by conversion first to VLDL remnant and then to IDL and LDL. In the conversion process of VLDL to LDL, the apoE:apoC and cholesterol:triglyceride ratios are continuously increased. IDL is formed from VLDL when lipoprotein lipase hydrolyses the triglycerides of the VLDL core, and some apoE, apoC and free cholesterol are removed (Chappell and Medh 1998). As VLDL and IDL both contain apoE as one of their apoproteins, they can be removed from the plasma by binding to both LDL receptor and LRP in the liver. Further, IDL remaining in plasma is converted to LDL, in which almost all triglycerides, apoE and apoC are removed. LDL carries cholesterol and cholesteryl esters to peripheral tissues and its only apolipoprotein is apoB-100, which binds LDL to LDL receptors of the liver and peripheral tissues (Brown and Goldstein 1986).

HDL is the lipoprotein responsible for the transport of cholesterol from peripheral cells to the liver; it is the main vehicle of reverse cholesterol transport system (Glomset 1968). HDL is synthesized in the liver and intestine (Schmitz and Williamson 1991) and there are two main subclasses of HDL, namely HDL_2 and HDL_3. ApoE containing HDL is present in the circulation but only in low concentrations (Weisgraber and Mahley 1980), and *in vitro* it can be taken up by the liver via apoB,E receptors (Mahley et al. 1984).

Clearly, apoE has a critical role in lipid metabolism by participating remnant catabolism and by mediating uptake of all apoE-containing lipoproteins, i.e. chylomicron remnants, VLDL, IDL, and HDL.

2.5. Receptors for apoE

LDL receptor. ApoE is recognized by all members of the constantly growing LDL receptor gene family. LDL receptor was the first family member to be characterized. Brown and Goldstein (1986) showed in their classic work that LDL receptor was the main receptor responsible for regulating the plasma cholesterol levels by mediating the uptake of apoB-containing lipoproteins to the liver. Mutations in the LDL receptor gene lead to FH (Brown and Goldstein 1986). LDL receptor binds avidly apoE (Bersot et al. 1976), which has 25-fold affinity for the LDL receptor compared to the LDL (Innerarity and Mahley 1978), probably due to interaction of four apoE molecules with four LDL receptors (reviewed by Weisgraber 1994). The interaction of LDL receptor and apoE is essential for normal lipoprotein metabolism, which is dramatically shown in patients with type III hyperlipoproteinemia. It is a genetic lipoprotein disorder characterized by hypertriglyceridemia and hypercholesterolemia because of accumulation of atherogenic β-migrating VLDL particles due to mutations in apoE gene. These mutations result in defective binding of apoE to LRP and LDL receptor but also defects in the HSPG pathway are necessary (Mahley et al. 1990, Chappell and Medh 1998, Mahley and Ji 1999). For instance, the binding of the E2 isoform to apoE receptors is defective (Schneider et al. 1981, Weisgraber et al. 1982). However, it has been shown that the receptor binding activity of the E2 isoform is modulated by lipid composition of the lipoprotein particles (Weisgraber 1994) and only 1-2% of the E2 homozygotes develop type III hyperlipoproteinemia (autosomal recessive disorder). The disorder is expressed only with additional environmental (for example diet) or genetic factors (Mahley et al. 1990). In addition, there are rare mutated forms of apoE, which always cause defective remnant clearance leading to type III hyperlipoproteinemia (dominant disorder) and increased risk of CAD (Mahley et al. 1990, Chappell and Medh 1998).

LDL receptor-related protein (LRP). LRP belongs to the LDL receptor family and it is expressed in several tissues including liver and arterial wall (Herz et al. 1988, Luoma et al. 1994). It is the same molecule as the α_2-macroglobulin receptor (Kristensen et al. 1990). In atherosclerotic lesions, LRP is expressed by macrophages and SMCs in humans and rabbits (Luoma et al. 1994, Hiltunen et al. 1998). As discussed above, LRP plays an important role in remnant catabolism. ApoE-rich lipoproteins are ligands for LRP (Beisiegel et al. 1989), which acts as a remnant receptor in the liver (reviewed by Herz

1993). In concert to defective binding to LDL receptor, the affinity of apoE E2 isoform for LRP is only 40% of that of E3 or E4 (Kowal et al. 1990).

Other apoE receptors. VLDL receptor is also a member of LDL receptor family but its exact functions in humans are still to be determined (Willnow 1999). VLDL receptor binds VLDL and IDL containing apoE but not LDL (Takahashi et al. 1992) and it is expressed in atherosclerotic lesions where it may mediate the lipid uptake of SMCs (Hiltunen and Ylä-Herttuala 1998). Other apoE receptors include apoE receptor 2, LR11, LR7/8 and gp330 (megalin). They are mainly expressed in the brain (St Clair and Beisiegel 1997, Schneider et al. 1997).

2.6. ApoE phenotypes and serum lipids

In most of the populations studied, the apoE ε4 allele is associated with higher and the ε2 allele with lower total and LDL cholesterol and apoB concentrations, as compared to the ε3 allele (Utermann et al. 1979, Bouthillier et al. 1983, Ehnholm et al. 1986, Ordovas et al. 1987, Davignon et al. 1988, Hallman et al. 1991, Dallongeville et al. 1992, Frikke-Schmidt et al. 2000b). ApoE genotype has been suggested to explain 7% of the variance of total cholesterol (Davignon et al. 1988) and to account for 16% of genetic variance in LDL cholesterol (Sing and Davignon 1985). The cholesterol-lowering effect of the ε2 allele is suggested to be two to three times the cholesterol-raising effect of the ε4 allele (Davignon et al. 1988). However, there are populations like Turkish, where only minor effects of apoE polymorphism on lipids have been observed (Mahley et al. 1995). Diet and gender have been shown to interact with the effect of apoE polymorphism on serum lipids. In other words, the associations are probably context-dependent (Hegele et al. 1994, Reilly et al. 1994, Lussier-Cacan et al. 2000). For an example, the cholesterol raising effect of apoE ε4 allele is increased with an increasing dietary intake of saturated fatty acids and cholesterol (Tikkanen et al. 1990, Lehtimäki et al. 1995a), and by female gender (Schaefer et al. 1994).

The associations of apoE polymorphism with triglycerides and HDL cholesterol are not consistent but according to a meta-analysis by Dallongeville et al. (1992), the carriers of the ε2/2, ε3/2, ε4/3 and ε4/2 genotypes have higher triglycerides than the ε3/3 carriers, and the ε4/3 genotype is associated with the lowest HDL values. Similar

observations were recently done in a large Danish study (Frikke-Schmidt et al. 2000b). In addition, the ε2 allele is associated with postprandial triglyceridemia but this association does not increase the MI risk (Dallongeville et al. 1999). The plasma apoE concentration is highest in carriers of the E2/2 isoform, intermediate in those with the E3/3 and lowest in carriers of the E4/4. The catabolic rates of correlate with the plasma concentration: the rate is highest in individuals with E4/4 and lowest in those with E2/2 (Davignon et al. 1988).

Why is there an association of apoE polymorphism with serum LDL cholesterol concentration? One explanation is the differences in the remnant clearance rates between isoforms (Davignon et al. 1988) It is known that in the case of E2, the remnants are cleared from the circulation at a slower rate than normal (Weintraub et al. 1987) due to defective binding to the LDL receptor. This leads to up-regulation of the LDL receptor and to lowering of the LDL cholesterol (Davignon et al. 1988). In contrast, the remnant clearance in E4 is more efficient than in E3 (Weintraub et al. 1987). This is probably based on the fact that the apoE isoforms have different preferences for lipoprotein particles. The E4 isoform is more associated with VLDL, IDL and LDL, whereas the E2 isoform is more associated with HDL (Gregg et al. 1986, Steinmetz et al. 1989, Dong and Weisgraber 1996). Higher concentration of apoE in remnant particles in individuals with the E4 may then lead to their increased uptake by the liver. In addition, it was recently suggested that homozygosity for the E4 isoform is related to increased binding activity of VLDL to LDL receptor (Bohnet et al. 1996, Mamotte et al. 1999), which may also contribute. The resulting high rate of remnant clearance may cause down-regulation of the LDL receptor and elevation of the LDL cholesterol in the subjects with E4 isoform (Davignon et al. 1988). Other explanations of the association between apoE polymorphism and LDL include influence of the apoE isoforms on cholesterol absorption, on cholesterol synthesis, and on bile acid formation (Kesäniemi et al. 1987, see also Lehtimäki 1992).

Several investigators have reported a greater sensitivity of the ε4 allele carriers to dietary interventions (Miettinen et al. 1988, Tikkanen et al. 1990, Mänttäri et al. 1991, Lehtimäki et al. 1992, Lopez-Miranda et al. 1994) but also opposite findings exist (Boerwinkle et al. 1991, Lefevre et al. 1997). Further, there are numerous studies on the influence of the apoE polymorphism on the response to statins, most of which have found no association between apoE genotypes and LDL cholesterol reduction by lovastatin (O'Malley and Illingworth 1990, Ojala et al. 1991, Sanllehy et al. 1998, Korhonen et al. 1999), pravastatin (Watanabe et al. 1993, Berglund et al. 1993) or simvastatin (de Knijff et

al. 1990). In contrast, some investigators have reported worse response in the ε4 carriers (Carmena et al. 1993, Ordovas et al. 1995, Ballantyne et al. 2000).

3. ApoE and atherosclerosis

3.1. ApoE and atherosclerotic lesions

ApoE is present in normal human arterial wall (Ylä-Herttuala et al. 1988) but the amount of apoE protein and mRNA is highly increased in early and advanced atherosclerotic lesions of humans and rabbits (Murase et al. 1986, Badimon et al. 1986, Crespo et al. 1990, Vollmer et al. 1991, Salomon et al. 1992). In lesions, apoE is expressed and synthesized mainly by non-foam cell macrophages and foam cells (Rosenfeld et al. 1993, O'Brien et al. 1994), and maybe also by SMCs (Vollmer et al. 1991). In vitro, apoE synthesis rate of macrophages is regulated transcriptionally by cholesterol content of the cell (Basu et al. 1981, Mazzone et al. 1987, Mazzone et al. 1989).

Most lines of evidence from the pivotal role of apoE in lipid metabolism and particularly in arterial wall biology come from mice models but also human examples exist. Subjects with a rare familial apoE deficiency, in which apoE production is markedly depressed, develop premature cardiovascular disease and xanthomas due to accumulation of VLDL and IDL particles in the circulation (Schaefer et al. 1986). This is also observed in mice with disrupted apoE gene (apoE knock-out mice), which develop severe atherosclerosis even on a low fat diet (Plump et al. 1992, Zhang et al. 1992). ApoE's protective role from vascular disease is further established by the fact that mice overexpressing apoE are highly resistant to diet-induced hypercholesterolemia (Shimano et al. 1992). The antiatherogenic effect of apoE, however, is not entirely mediated by lipid metabolism. Shimano et al. (1995) created transgenic mice expressing apoE only locally in the vascular wall and found atherogenesis to be inhibited in those mice. Further, apoE knock-out mice with macrophage-spesific apoE expression and with atherogenic lipid profile have less atherosclerosis than mice without macrophage apoE production (Bellosta et al. 1995). This supports the hypothesis that apoE has local effect in the arterial wall which are independent of its effect on lipid clearance. However, apoE secretion by arterial macrophages has a strong regressive impact on foam cell formation in early lesions but it

is not beneficial in later phases of disease (Hasty et al. 1999). Conversely, if only apoE gene of macrophages is knocked out, the mice develop more lesions than controls (Fazio et al. 1997). Mice models have also been used to examine differences between apoE isoforms (Tsukamoto et al. 1999). If human apoE gene is transfected to the liver of apoE-deficient mice, a regression of pre-existing lesions can be observed together with reduction in cholesterol levels but this is specific for gene transfer of human apoE E3 isoform. Transfer of the E4 isoform does not induce lesion regression, despite its efficient reducing effect on cholesterol levels (Tsukamoto et al. 1999).

Why is arterial wall apoE antiatherogenic? HDL is involved in reverse cholesterol transport, and in the artery wall it is responsible of accepting cholesterol from foam cells (reviewed by Rothblat et al. 1999). In the atherosclerotic lesions, apoE produced by macrophages (Mazzone and Reardon 1994), but not exogenous apoE (Granot and Eisenberg 1995), facilitates efflux of cholesterol from foam cells to HDL_3. Cullen and coworkers demonstrated that the macrophages from subjects with different apoE phenotypes differ in their cholesteryl efflux capabilities in order E2/2>E3/3>E4/4 (Cullen et al. 1998). This provides one possible explanation for suggested differences in the function of apoE isoforms in arterial wall. Further, HDL_3 is known to regulate the secretion of apoE from macrophages (Dory 1991) and macrophage apoE production is increased by apoA-I, a constituent of HDL (Rees et al. 1999, Bielicki et al. 1999). However, mice models have shown that in apoE knock-outs, normalization of cholesterol efflux from macrophages is not accompanied by changes in lesion progression (Zhu et al. 1998, van Eck et al. 2000). This suggests that the beneficial effect of apoE is not only a result of promotion of cholesterol efflux from cholesterol-loaded macrophages. Indeed, there is further evidence on the antiatherogenic effects of apoE produced by vessel wall macrophages. ApoE inhibits SMC migration and proliferation by affecting cell signalling events (Ishigami et al. 1998). ApoE increases macrophage NO production (Vitek et al. 1997) and suppresses platelet-derived growth factor–induced SMC proliferation by activating inducible nitric oxide (NO) synthase (Ishigami et al. 2000). The activation of platelet NO synthase by apoE mediates inhibition of platelet aggregation by HDL particles rich in apoE (Desai et al. 1989, Riddell et al. 1997). In addition, apoE has also found to inhibit proliferation of several cell types, including T-lymphocytes, endothelial cells and tumor cells (Hui et al. 1980, Kelly et al. 1994, Vogel et al. 1994, Browning et al. 1994), to have antioxidant activity (Miyata and Smith 1996), and to reduce lipoprotein lipase mediated retention of LDL by extracellular matrix (Saxena et al. 1993). Finally, apoE

associates with extracellular matrix (Lucas and Mazone 1996). In human coronary artery plaques, apoE is localized to regions enriched with a certain proteoglycan, namely biglycan (O'Brien et al. 1998). This observation suggests that in atherosclerotic lesions proteoglycans might bind apoE and also that apoE might act as a bridging molecule between HDL and biglycan leading to trapping of HDL particles in the arterial wall (O'Brien et al. 1998). In fact, apoE was recently found to be an important determinant of HDL binding to biglycan (Olin et al. 2001).

3.2. Association of apoE polymorphism with coronary heart disease

The association of apoE polymorphism with CHD has been intensively studied since the recognition of the relation of phenotypes to serum lipids. In general, the ε4 allele is thought to be a CHD risk factor and the ε2 to protect from it. However, the proof of the association is not quite indisputable and must be carefully reviewed (Table 2).

The first study of the relation of apoE phenotypes to CAD was performed by Menzel et al. (1983), who found the E3/2 phenotype to be less frequent in angiography patients with CAD than in those without significant stenoses. The protective role of the ε2 allele in MI (Cumming and Robertson 1984**,** Luc et al. 1994, Tiret et al. 1994) and in CAD (Miida 1990) has later been observed in other with case-control design. In women, the ε3/2 genotype protects from CHD (Frikke-Schmidt et al. 2000a). The ε2 allele is associated with less severe CAD in angiography (Wang et al. 1995), and according to an autopsy study, the ε3/2 genotype protects from coronary atherosclerosis (Scheer et al. 1995). In contrast, the PDAY study group found no significant relation of apoE genotype to coronary artery atherosclerosis (Hixson 1991). It has also been suggested that the ε3/2 genotype is associated with earlier CAD age of onset in males compared to the ε3/3 genotype (Moore et al. 1997).

A meta-analysis of nine studies published in 1996 concluded that the ε4 allele is significantly associated with clinical CHD and angiographically verified CAD in both genders (Wilson et al. 1996). The ε4 allele was estimated to increase the risk of CHD by 26% (OR=1.26, 95% CI 1.13-1.41) compared to the ε3 allele. In 1984, Utermann and coworkers found the ε4 allele to be related to decreased risk of MI (Utermann et al. 1984), but since that the associations, if found, have been just the opposite. The ε4 allele has been

associated with increased risk for CAD diagnosed by angiography in case-control studies (Kuusi et al. 1989, Miida 1990, Nieminen et al. 1992, van Bockxmeer and Mamotte 1992, Ou et al. 1998) but there are also angiography studies failing to show any significant association with CAD (Lenzen et al. 1986, Stuyt et al. 1991, Marshall et al. 1994, Lehtinen et al. 1995). The frequency of the ε4 allele was reported to increase linearly with the increase in CAD severity in both men and women, and the effect was independent of lipid values (Wang et al. 1995). In contrast, Reardon et al. (1985) examined a small sample of 107 CAD patients and observed an association of CAD severity only with serum lipids but not with apoE phenotype. In addition, The Framingham offspring study investigated the prevalence of CHD in a large community-based sample of both sexes (Wilson PW et al. 1994). The ε4 allele was associated with CHD, and the association persisted adjustment with conventional risk factors including LDL cholesterol. Other investigators have obtained parallel results (Eto et al. 1989, Corbo et al. 1999, Frikke-Schmidt et al. 2000a). The ε4 allele was also found significantly more frequently in cases of than in controls in the Etude Cas-Témoins sur l'Infarctus Du Myocarde (ECTIM) multicenter study, which examined a large population-based sample of MI patients aged 25-64 (Luc et al. 1994). The investigators suggested apoE polymorphism to explain 12% proportion of the MI cases at the population level. In Finland, no significant association of apoE with MI or CHD death was found in a sample of middle-aged men (Mänttäri et al. 1991) but studies in other populations support the finding of the ECTIM study (Cumming and Robertson 1984, Eichner et al. 1993). Further, it has been reported that there is a synergistic effect of apoE ε4 allele and angiotensinogen polymorphism on early onset of MI (Batalla et al. 2000). The apoE ε4 allele may also predispose to silent myocardial ischemia (Katzel et al. 1993), it is related to paternal history of MI (Tiret et al. 1994) and to early age of CAD onset (Nassar et al. 1999). In young MI survivors, the ε4 allele predicts cardiac death, recurrent MI or revascularization procedure (Brscic et al. 2000). A substudy of Scandinavian Simvastatin Survival Study investigated also whether the apoE ε4 allele determines prognosis of MI survivors (Gerdes et al. 2000). The investigators found that in those treated with placebo, the all-cause mortality risk ratio was 1.8 (95% CI 1.1-3.1) in the carriers of ε4 allele compared to patients without the allele. This was mainly due to an increased risk of coronary death (risk ratio 1.7, 95% CI 0.9-3.2) in patients with the ε4 allele. However, this increase was not statistically significant.

The association of apoE with CHD incidence has been investigated in two Finnish prospective studies on elderly. These reports gave conflicting results too. Stengård et al. (1995) followed two samples of men aged 65-84 from Eastern and Southwestern Finland and found, in both samples, a twofold increase in the ε4 allele frequency among the men who died from CHD. Similar results were obtained in another Finnish follow-up study (Räihä et al. 1997). In the 3.5-year follow-up study of Kuusisto and coworkers, apoE phenotype was not associated with CHD incidence in the elderly subjects aged 65 to 74 years (Kuusisto et al. 1995).

In patients with type II diabetes mellitus, the prevalence of CHD is reported to be highest in men with the apoE ε4/4 or ε4/3 genotype (Laakso et al. 1991). ApoE genotype also modulates macro- and microangiopathy risk of type II diabetics (Ukkola et al. 1993).

There are two studies, which have examined the effect of apoE genotype on the risk of restenosis after coronary angioplasty. Van Bockxmeer and coworkers (1995) found the ε4/4 genotype to be more frequent in patients with restenosis than in those without. In addition, they reported an interaction between apoE and angiotensin-converting enzyme genotypes. In contrast to this finding, Samani et al. (1996) found no association between apoE polymorphism and the risk of restenosis.

Table 2. Association apoE polymorphism with CHD.

Study	Design	Number of subjects	Gender	Age, years (range or mean±SD)	CHD phenotype or event*	Association compared to ε3/3
Menzel et al. 1983	Case-control	1000 vs. 469 vs. 439	Both	37±12 vs. 54±7 vs. 50±10	CAD	E3/2 ⇩
Kuusi et al. 1989	Case-control	91	Male	37-60	CAD	ε4⇧
Miida 1990	Case-control	125 vs. 129	Both	Means of groups between 49-67	CAD	ε4⇧ ε2⇩
Nieminen et al. 1992	Case-control	111 vs. 46	Both	36-65	CAD	ε4⇧
van Bockxmeer and Mamotte 1992	Case-control	91 vs. 172	Male	30-50	CAD	ε4⇧
Ou et al. 1998	Case-control	214 vs. 310	Both	33-70 vs. 47-77	CAD (onset <66 yrs of age)	ε4/3⇧
Stuyt et al. 1991	Case-control	145 vs. 153	Both	50±8 vs. 52±8	CAD	No association
Marshall et al. 1994	Case-control	444 vs. 404	Both	30-69	CAD	No association
Wang et al. 1995	Angiography patients	424	Both	<65	CAD severity	ε4⇧, ε2⇩
Reardon et al.1985	Angiography patients	107	Both	52±8	CAD severity	No association
Lehtinen et al. 1995	Case-control	309 vs. 38	Both	58±8 vs. 54±7	CAD severity	No association
Scheer et al. 1995	Autopsy	130	Both	9-85	Lesion area	ε4/3⇧, ε3/2⇩
Ilveskoski et al. 1999 (I)	Autopsy	700	Male	33-70	Lesion area	ε4/3⇧ in men <53 years
Hixson 1991	Autopsy	720	Male	15-34	Lesion area	No association
Eto et al. 1989	Case-control	109 vs. 576	Both	55±2 vs. 49±1	CHD	ε4⇧, ε2⇧
Corbo et al. 1999	Case-control	150 vs. 110	Male	31-93	CHD (severe disease)	ε4⇧
Wilson et al. 1994	Population-based case-control	189 vs. 1761	Both	40-77	CHD	ε4⇧
Stengård et al. 1995	Prospective	Southwest 369 East 297	Male	65-84	CHD death	ε4⇧ in both samples
Kuusisto et al. 1995	Prospective	1067	Both	65-74	CHD incidence	No association
Utermann et al. 1984	Case-control	523 vs. 1031	Both	Not specified	MI (survivors)	ε4 ⇩, E2/2⇧
Cumming and Robertson 1984	Case-control	239 vs. 400	Both	< 65 vs. 45-60	MI (survivors)	E4/3 ⇧, E3/2 ⇩
Luc et al. 1994	Population-based case-control	574 vs. 680	Male	25-64	MI (survivors)	ε4⇧ ε2⇩
Eichner et al. 1993	Nested case-control	206 vs. 412	Male	35-57	Fatal or nonfatal MI	ε4⇧
Tiret et al. 1994	Case-control	635 vs. 1259	Both	18-26	Paternal history of MI	ε4⇧, ε2⇩
Lenzen et al. 1986	Case-control	570 vs. 624	Male	Not specified	MI (survivors), CAD	No association
Mänttäri et al. 1991	Case-control	136 vs. 132	Male	40-55	MI or coronary death	No association
Nassar et al. 1999	Case-control	150 vs. 150	Both	46±4 vs. 74±5	CHD age of onset	ε4 associated with early onset
Brscic et al. 2000	Case-control	21 vs. 85	Male	23-45	Any CAD event after acute MI	ε4⇧
Gerdes et al. 2000	Follow-up	966	Both	35-70	Major coronary event, coronary death or all-cause mortality	ε4⇧ all-cause mortality. No association with any of the coronary events

* CAD indicates angiographycally determined coronary disease, CHD clinically diagnosed disease and MI myocardial infarction.
⇩ indicates protective effect and ⇧ favouring effect.

3.3. Association of apoE polymorphism with atherosclerosis in other arteries

Carotid IMT. In all of the studies on the association of apoE polymorphism with carotid artery atherosclerosis, the early atherosclerotic changes have been measured as IMT by ultrasonography. In general, the results of these association studies have been controversial (Table 3). The ε3/2 genotype was found to be a risk factor for carotid IMT in the Atherosclerosis Risk in Communities (ARIC) study, which is an American prospective multicenter study including both men and women aged 45-64 years (de Andrade et al. 1995). This finding is supported by the results of Hanon and coworkers (2000), who studied carotid IMT in middle-aged men and women without any cardiovascular disease and observed the ε2 allele to be associated with increased IMT. However, there is another study in which the common carotid IMT was less in subjects with the ε2 allele than in those with the ε3 or ε4 allele (Terry et al. 1996). At least two reports have failed to show any association between apoE genotype and carotid IMT (Kogawa et al. 1997, Sass et al. 1998). Terry et al. (1996) found ε4 carriers to have greater overall mean carotid IMT than the carriers of the ε2 or ε3 alleles. Similar results were obtained from a Finnish study (Vauhkonen et al. 1997), in which the ε4 allele was associated with common carotid IMT in randomly selected controls but not in patients with type 2 diabetes mellitus. Italian investigators observed also an association of the ε4 allele with the common carotid IMT in a randomly selected population sample (Cattin et al. 1997). Zannad and coworkers (1998) found just the opposite: the ε3 allele carriers had the highest carotid IMT compared to the ε3 or ε4 carriers.

Table 3. Association of apoE genotype with carotid IMT.

Study	Sample	Number of subjects	Gender	Age, years	Association compared to ε3/3
de Andrade et al. 1995	Population based	145 vs. 224	Both	45-64	ε3/2 ⇧
Terry et al. 1996	Coronary angiography patients	260	Both	>45	ε2 ⇩, ε4 ⇧
Cattin et al. 1997	Randomly selected	260	Both	45-65	ε4 ⇧
Vauhkonen et al. 1997	Type II diabetics and controls	83 vs. 123	Both	45-64	None in diabetics, ε4 ⇧ in controls
Kogawa et al. 1997	Type II diabetics and controls	356 vs. 235	Both	18-82	None
Zannad et al. 1998	Familial sample	315	Both	10-54	ε2 ⇩, ε4 ⇩, apoE explains 1.5% of the variability
Hanon et al. 2000	Healthy cohort	320	Both	49±12	ε2 ⇧
Ilveskoski et al. 2000 (III)	Random sample	189	Male	50-59	E3/2 ⇩

⇩ indicates protective effect and ⇧ favouring effect.

Ischemic cerebrovascular disease. A recent meta-analysis of nine studies investigating the role of apoE polymorphism in transient ischaemic attack and stroke found that the ε4 allele is a risk factor for ischemic cerebrovascular disease but that the ε2 allele provides no protection from it (McCarron et al. 1999).

Aortic atherosclerosis. The association of apoE genotype with atherosclerotic lesions has been studied in two autopsy studies. Hixson and the PDAY study group found apoE genotype ε4/3 to be associated with greatest and ε3/2 genotype with least involvement of atherosclerotic lesions (Hixson 1991). However, the finding was not supported by an autopsy study on Alaska Natives, which observed no significant association of apoE genotype with aortic atherosclerosis (Scheer et al. 1995).

Peripheral vascular disease. ApoE is not clearly associated with peripheral vascular disease. In a Spanish case-control study, apoE polymorphism was associated with serum lipids but the allele frequencies were similar in patients with and without peripheral vascular disease (Sentí et al. 1992). Similar results were obtained for Italian diabetic patients (Boemi et al. 1995). However, a recently published large study took into account the smoking and found that apoE ε4/3 genotype is associated with peripheral arterial disease in non-smoking diabetics (Resnick et al. 2000).

4. ApoE polymorphism in other pathologies and in longevity

4.1. ApoE polymorphism in other pathologies

Alzheimer's disease (AD). In addition to its importance in lipid metabolism, apoE polymorphism plays an important role in central nervous system by modulating neuronal repair, remodelling and protective cascades (reviewed by Weigraber and Mahley 1996, Mahley and Huang 1999). It has been hypothesized that the apoE E2 and E3 isoforms are effective in repairing and remodelling processes after a neuron damage, whereas the E4 isoform prevents or is ineffective in supporting these processes (Weisgraber and Mahley 1996). The first line of evidence of the important role of apoE in AD pathology was published in 1991. ApoE expression of the astrocytes was observed to be increased in AD (Diedrich et al. 1991) and apoE was shown to localize in extracellular amyloid deposits and in neurofibrillary tangles in AD patients (Namba et al. 1991). The same year a linkage of familial AD was described to chromosome 19 (Pericak-Vance et al. 1991), and based on these findings apoE was suggested to function as a pathological chaperon in neurological disorders with amyloid deposits (Wisniewski and Frangione 1992). Two years later apoE ε4 allele frequency was found to be increased in late-onset familial AD (Strittmatter et al. 1993). There is a clear gene dosage effect of the apoE genotype on the risk and age of onset of AD (Corder et al. 1993). These findings have been extended to subjects with late-onset sporadic AD (Saunders et al. 1993), as well as to several populations and ethnic groups including Finnish subjects (Kuusisto et al. 1994, Lehtovirta et al. 1995, Lehtimäki et al. 1995b). In addition, the ε2 allele is suggested to protect from AD (Corder et al. 1995, Lippa et al. 1997). A large meta-analysis reported the following odds ratios for different apoE genotypes in Caucasian subjects, as compared to ε3/3 genotype: OR=0.6 for ε2/2 and ε3/2, OR=3.2 for ε4/3 and OR=14.9 for ε4/4 (Farrer et al. 1997). The association between apoE polymorphism and AD is thought to be independent of the relation of apoE polymorphism to CAD (Romas et al. 1999, Slooter et al. 1999).

Multiple sclerosis. The role of apoE polymorhism has also been studied in multiple sclerosis. ApoE ε4/4 genotype is suggested to be associated with increased risk of developing multiple sclerosis (Hogh et al. 2000) and the ε4 allele may be related to disease progression (Chapman et al. 1999, Hogh et al. 2000). However, there are also studies reporting no association (Pirttilä et al. 2000).

Head injury. There is an association between the amount of amyloid deposits following head injury with the apoE ε4 allele (Nicoll et al. 1995, Macfarlane et al. 1999). In addition, patients with the ε4 allele are twice as likely as those without the allele to have an unfavourable outcome after head injury (Teasdale et al. 1997, Lichtman et al. 2000). In boxers, apoE ε4 allele is suggested to be related to increased severity of chronic neurological deficits (Jordan et al. 1997).

Gallstones. Two studies have reported an association of apoE polymorphism with the occurrence of gallstones. The apoE ε4/3 genotype is suggested to be associated with gallstone disease (Bertomeu et al. 1996) and the ε2 allele to protect from it (Niemi et al. 1999). This association may be related to differences between apoE isoforms in the hepatic lipoprotein uptake.

4.2. ApoE genotype and longevity

If a gene has a major impact in mortality, the frequency of the "bad" allele should decrease with age. Because apoE ε4 allele is associated with both CAD and AD, studies of apoE polymorphism in longevity have been performed by comparing the allele frequencies in younger and older individuals. In Finns, the frequency of the ε4 allele is shown to be lower in nonagenarians and centenarians than in general Finnish population, and on the other hand, the ε2 allele frequency is observed to be almost twice as high (Kervinen et al. 1994, Louhija et al. 1994). Similar findings have been reported in French centenarians (Schachter et al. 1994), in Swedish (Eggertsen et al. 1993) and in women (Cauley et al. 1993). However, reports from UK and Italy found no association of apoE polymorphism with longevity (Galinsky et al. 1997, Bader et al. 1998). The reports suggest that apoE has a major effect on survival but that the effects depend on the population studied.

AIMS OF THE STUDY

The era of genetic studies has introduced several candidate genes for the risk of atherosclerosis. Among these, apoE is one of the most functionally and epidemiologically studied polymorphisms. However, the impact of apoE genotype on exact arterial phenotypes still remains unclear. Using two autopsy and two clinical series, it was possible to elucidate the association of apoE genotype with four different phenotypes of early or advanced atherosclerotic disease. Accordingly, the following targets were set for the present study:

1. To investigate the association of apoE genotype with coronary function and exact coronary artery atherosclerosis phenotype.

2. To examine the relationship between apoE genotype and autopsy confirmed early and advanced atherosclerotic lesions of the aorta.

3. To relate apoE polymorphism to gaps in the internal elastic lamina of the mesenteric arteries.

4. To assess the role of apoE polymorphism in the carotid artery intima-media thickness.

5. To analyze whether apoE genotype modulates pravastatin induced change of myocardial blood flow.

6. To develop a rapid and non-invasive DNA sample collection method for apoE genotyping.

SUBJECTS AND METHODS

For more detailed information on study subjects and methods, please refer to the original articles I-V.

1. Autopsy series

1.1. The Helsinki Sudden Death Study (I)

Helsinki Sudden Death Study (HSDS) comprises of two autopsy series collected at the Department of Forensic Medicine in Helsinki in 10-year intervals. Both series were collected by similar standards. The first series (A-series, n=400) was collected during 1981-1982 and the second series (B-series, n=300) during 1991-1992. The two series comprised 700 consecutive medicolegal autopsies of males aged 33 to 70 (mean 53.07, SD 9.58, median 54). Of the series of 700 men, arterial samples for the planimetric measurements were available from 596 men for the analysis of the LAD and from 440 men for the analysis of the RCA. Planimetric data of the atherosclerosis in the thoracic (n=256) and abdominal (n=259) aorta were available only for the B-series.

A spouse, a relative or a close friend of the diseased was interviewed within 2 weeks postmortem to receive data on CAD risk factors. The interview consisted of more than 50 detailed questions on smoking habits, alcohol consumption and medical history (Karhunen and Penttilä 1990). All interview data was available in 423 cases of the total of 700.

During the collection of the B-series, pieces of cardiac muscle were taken for future DNA analyses. However, in the A-series, paraffin blocks of histological samples had to be used for extraction of DNA because in early 1980s genetic techniques were still to be discovered and DNA samples were not collected. The study protocol was approved by the Ethics Committee of the Department of Forensic Medicine, University of Helsinki.

1.2. A series of 123 consecutive autopsies from Tampere University Hospital (II)

Initially, an autopsy series of 123 consecutive medical (n=42) and forensic (n=81) autopsies was collected in the Tampere University Hospital in 1993 to evaluate atherosclerosis in abdominal arteries (Järvinen 1996). At that time, no specific DNA samples were taken. Later in 1997, arterial tissue samples in paraffin blocks were used as a source of DNA and thus genetic studies on the series became possible. Subjects were both men (n=90, 73%) and women (n=33, 27%) aged 18-93 years (mean 62.0 years, SD ± 17.7). The study was approved by the Ethics Committee of the Tampere University Hospital.

2. Clinical series

2.1. Random sample of Finnish middle-aged men from Tampere (III)

A random selection of 300 men aged 50 to 59 years were done from ten age-cohorts (n=9058) living in the city of Tampere in Southern Finland. A total of 223 men agreed to participate (74%), while 33 refused and 44 did not answer or could not be reached. Finally, data was collected in 219 participants but 26 men were excluded because of missing data. Thereby, 189 men were included in the analysis of the association between apoE polymorphism and carotid IMT. The Ethics Committee of the UKK Institute approved the study, and all participants gave their written informed consent.

2.2. Positron emission tomography (PET) study (IV)

Fifty-one men from Archipelago Sea Naval Command, Archipelago Coast Guard District, Säkylä Garrison, and from Turku Fire Department were invited to participate the study during routine physical examination. The inclusion criteria were: 1) age 25 to 40 years, 2) total cholesterol levels 5.5 – 9.0 mmol/l measured at routine controls provided by employers, 3) otherwise healthy, and 4) no continuous medication or antioxidant vitamin use. The study was randomised, double-blinded and placebo-controlled with treatment

groups of placebo (n = 26) and pravastatin (40 mg/day for 6 months, n = 25). Pravastatin (Pravachol®) tablets and matching placebos were provided by Bristol-Myers Squibb, Finland. To assess the effect of pravastatin on coronary function, PET measurements were done at baseline and after 6 months treatment. Seven men were rejected (3 from placebo and 4 from pravastatin group) because of the technical problems with the PET measurements. Finally, 44 men were included in the analysis. The study protocol was accepted by the Joint Commission on Ethics of the Turku University and the Turku University Central Hospital. Each subject gave written informed consent.

2.3. Collection of buccal swab samples (V)

To test the reliability of collection of buccal swab samples by mail, samples were collected both from healthy volunteers and from AD patients. During the study of Lehtimäki et al. (1992), apoE phenotypes of 36 subjects had been determined from delipidated serum, and the pellet containing cells was stored at -20 °C for 8 years. The stored buffy coat leucocytes were now used for a comparative apoE genotyping, which was done without the knowledge of the phenotypes. In 1996, some of these subjects were contacted again and advised to take buccal swabs themselves using sterile cottonwool-tipped stick (Difco), which was rubbed on buccal mucosa back-and-forth for 10-20 seconds. The swab was then allowed to dry in the air, after which it was closed in the transport tube and sent back to our laboratory in an envelope by surface mail. In addition, buccal cell samples of 18 AD patients from Memory Research Unit of Helsinki University Hospital were taken by nurses as above and sent to our laboratory by mail. The arrival of the mail took one to three days. The buccal swab samples were then stored at -20°C or +4°C until DNA was isolated.

3. Scoring the atherosclerosis at autopsy

3.1. Macroscopic lesions of atherosclerosis (I)

In HSDS, the area of the different types of atherosclerotic lesions was measured in the RCA and LAD coronary arteries and thoracic and abdominal aortas. The definition of atherosclerosis was based on the protocols used in the IAP (Guzman et al 1968). The vessel wall was stained with the Sudan IV fat-staining method. An area stained red with Sudan IV and showing no any other types of changes underlying it, was classified as fatty streak. An elevated plaque that exhibited no ulceration or thrombosis was considered as a fibrotic lesion. Any plaque area with ulceration or thrombosis was classified as complicated lesion. The area involved with fatty streaks, fibrotic plaques, and complicated lesions was measured by computer-assisted planimetry. The area of different types of lesions was expressed in percentages (%). The total atherosclerotic lesion area of the arterial wall was the total areas of fatty streak and fibrotic lesions. Because the complicated lesions in the arterial wall were always incorporated in either the fatty streak or in the fibrotic lesion area, or both, the complicated lesion area was analyzed separately.

3.2. Histological changes in the artery wall (II)

The measurements of the atherosclerosis are described in detail in Järvinen et al. (1996). Briefly, samples from coeliac artery (CA), and superior and inferior mesenteric arteries (SMA, IMA) were prepared for histological examination. The circumference of the IEL and the thickness of the intima were measured in millimetres (MOP 3 image-analysis system, Reichert-Jung, Eching, Germany). The intimal thickness was defined as the measure from the lumen to the IEL at the area of greatest intimal thickness (Kay et al. 1976). The intimal thickness was divided into two categories according to the median (Md) value of the series (in CA and IMA Md=0.1 mm, in SMA Md=0.2 mm, respectively): 0=no intimal thickening, 1=intimal thickening. Finally, the number of the gaps in the IEL was counted, divided by the circumference of the IEL and expressed as gaps per mm.

4. Ultrasonographic measurements of carotid artery IMT (III)

A high-resolution B-mode ultrasound with a 10 MHz transducer was used (Biosound Phase 2, Biodynamics Inc., Indianapolis, USA), to examine left and right carotid arteries. The examinations were recorded on S-VHS videotapes (Panasonic AG 7530A, Panasonic, Japan) and the tapes were then read off-line at the ultrasound reading center, Wake Forest University, North Carolina, USA. All recordings and measurements were performed by the same certified sonographer and reader, respectively.

Quantitative carotid artery ultrasonography was performed using a standardised protocol (Multicenter Israpidine/Diuretic Atherosclerosis Study, MIDAS) (Mercuri 1994). The protocol involved the scanning of the distal 10 mm of the common carotid artery, the bifurcation and the proximal 10 mm of the internal carotid artery. The distance between the media-adventitia interface and the lumen-intima interface represents the IMT. The maximum IMT of the near and far wall was measured at 12 well-defined arterial segments. The single largest IMT was determined by selecting the largest IMT among the individual maximum IMTs in the 12 standard arterial walls, i.e., the near and far walls of the common, bifurcation, and internal carotid artery at both sides. The mean maximum IMT (Mmax, overall mean) was calculated as the mean of 12 maximum IMTs identified at 12 standard sites (Mercuri 1994).

5. PET method to evaluate myocardial blood flow and coronary flow reserve (IV)

All PET studies were performed after 6-h fast. Two catheters were inserted, one in the antecubital vein of the left hand for the injection of $[^{15}O]H_2O$ and for adenosine infusion, the other in the antecubital vein of the right hand for blood sampling. Myocardial perfusion was measured twice, once at rest and once after the administration of adenosine. Heart rate and blood pressure were monitored during the studies to calculate the rate-pressure product. Electrocardiogram was continuously monitored during the PET study.

The patients were positioned supine in a 15-slice ECAT 931/08-12 tomograph (Siemens/CTI Inc., Knoxville, TN, USA). After transmission scan, the subjects' nostrils were closed and they inhaled $[^{15}O]CO$ for 2 min through a three-way inhalation flap-valve

(0.14% CO mixed with room air, mean dose 3250 ± 235 MBq). After the inhalation, carbon monoxide was allowed to combine with hemoglobin in red blood cells for two min before a static scan was started. During the scan period, three blood samples were drawn at 2 min intervals and blood radioactivity concentration was measured. A 10 min period was allowed for [^{15}O]CO radioactive decay before the flow measurements.

Flow was measured at baseline and 60 sec after the beginning of intravenous administration of adenosine (140 μg×min^{-1}×kg $^{-1}$). 1630 ± 115 MBq of [^{15}O]H_2O was injected intravenously for 2 min (1610 ± 115 MBq at baseline and 1640±120 MBq after adenosine) and dynamic scanning was started (6×5s, 6×15s, 8×30s).

Large regions of interest were placed on representative transaxial ventricular slices in each study covering anterior, lateral, septal and whole free wall of the left ventricle. The regions of interests were drawn on the images obtained at rest and copied to the images obtained after adenosine administration. Values of regional myocardial blood flow (expressed as ml×g^{-1}×min^{-1}) were calculated according to the previously published method employing the single compartment model (Iida et al. 1988**,** Iida et al. 1995). Since no regional differences were found in myocardial blood flow, global left ventricular blood flow was used for further analyses. The CFR was defined as the ratio of myocardial blood flow after adenosine to flow at baseline.

6. Determination of serum lipids and apolipoproteins (III, IV)

In the study III, blood was drawn after a 12-h overnight fast for determination of biochemical analysis. Taking alcohol and exercise were not allowed for 24 hours before sampling. Lipoprotein fractions were assessed from fresh samples by ultrasentrifugation (Carlson 1973) and cholesterol was measured from serum and lipoprotein fractions using an enzymatic method (CHOD-PAP, Boehringer Mannheim, Germany). Triglycerides were measured with enzymatic method (GPO-PAP, Boehringer Mannheim, Germany). ApoB was analysed by using immunonephelometric method (Behring, Behringwerke AG, Marburg, Germany) and lipoprotein (a) (Lp(a)) by using a two-site immunoradiometric assay (Pharmacia, Uppsala, Sweden). In the study IV, the subjects also fasted over night before collection of the blood samples. Plasma triglycerides and total and HDL cholesterol concentrations were analysed by a Cobas Integra 700 automatic analyser with reagents and

calibrators recommended by the manufacturer (Hoffmann-La Roche Ltd., Basel, Switzerland). LDL concentration was calculated using Friedewald's formula (Friedewald et al. 1972).

7. DNA extraction

In the HSDS A-series (I) DNA was extracted from paraffin-embedded samples of cardiac muscle using the method of Isola et al. (1994). In the B-series DNA isolation was performed from frozen (-70°C) cardiac samples by the standard phenol-chloroform method at the Department of Forensic Medicine, University of Helsinki. The frozen cardiac tissue block (-70 °C) was cut into thin pieces with a sterile blade in the freezer. After incubation in proteinase K and 20 % SDS, the samples were loaded in Phase Lock Gel II B Light-tubes (5 prime ® 3 prime, USA). Phenol and chloroform/isoamylalcohol was then added and the tubes were shaken well and centrifuged. The aqueous phase was transferred to a sterile tube, 2 M KCl was added and the tube was shaken gently. DNA was precipitated and collected with a closed Pasteur pipette, air-dried and redissolved. The concentration was measured spectrophotometrically. In the studies II and IV DNA was isolated from the paraffin embedded samples of the mesenteric artery wall (II) or from blood leukocytes (IV) by using commercial kit (QIamp Tissue Kit and Blood Kit, QIAGEN Inc., USA). In the study V, DNA was released both from buccal cell samples using a rapid lysis method described by Higuchi (1989). A cotton-tipped stick containing cells from buccal mucosa was stired in 0.7 ml digestion buffer (0.05M Tris-HCl pH 8.8, 0.015M $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, 0.55 % Triton X-100 and 0.45 % Tween 20) for 30 seconds, after which swabs were discharged. Proteinase K (10 µg/µl) was added and the tubes were incubated at 55 °C for one hour and at 100 °C for 10 minutes. 5 µl of the mixture was used as a template in PCR reactions.

8. ApoE genotyping and phenotyping

In studies I, II, IV and V, the extracted DNA was used for apoE genotyping by polymerase chain reaction and *HhaI* restriction enzyme digestion as described by Hixson and Vernier

(1990) with slight modifications. A 244 bp sequence of the apoE gene including the point mutations was amplified by PCR in a programmable heat block (PTC-100, MJ Research, inc.). The primer sequences used were the ones described by Emi and coworkers: the forward primer (F4) was 5'-ACAGAATTCGCCCCGGCCTGGTACAC-3' and the reverse primer (F6) was 5'-TAAGCTTGGCACGGCTGTCCAAGGA-3' (Emi et al. 1988). The 50 µl reaction contained 5 µl of sample, 2.5 µl of each primer (concentration 20 µM), 1.25 µl of each dNTP (concentration 2 mM), 5 µl PCR buffer and 5 µl dimethyl sulfoxide. "Hot start" was done by adding 1.25 units of *Taq* DNA polymerase (Promega) or of Dynazyme (Finzymes) to the PCR tubes on the heat block at 95 °C in order to increase the specificity of the reaction. Blank control with no DNA and a control with apoE genotype ε4/2 was also run with each set of amplification. PCR conditions were: denaturation in 95 °C for five minutes, followed by 32 cycles (40 cycles in study II) of amplification with annealing for one minute at 60 °C (62 °C in study II), extension for two minutes at 70 °C (72 °C in study II) and denaturation for one minute at 95 °C. A 10 minute final extension at 70 °C (72 °C in study II) ended the reaction. *HhaI* was added directly to the amplification tube under mineral oil and incubated for three hours at 37 °C. The digested fragments with molecular weight marker ϕX174 DNA/*HinfI* (Promega) were run on 12% polyacrylamide gel or on 5% MetaPhor (FMC) agarose gel electrophoresis and visualized by silver staining.

In the study III, apoE phenotyping was performed using delipidated plasma, isoelectric focusing, cysteamine treatment and immunoblotting, as described by Menzel and Utermann (1986), with minor modifications described by Lehtimäki et al. (1990). The verification of correct apoE phenotypes in gel was based on comparison to previously known apoE genotype standards. The phenotyping method is described in more detail elsewere (Lehtimäki 1992). ApoE genotyping and phenotyping were always performed without the knowledge of the clinical data.

9. Statistical methods

All computation was carried out with Statistica Version 5.1 (StatSoft Inc., Tulsa, Oklahoma, USA) (I, II and IV) and SPSS Version 8.0 for Windows (III) on a PC. The means of continuous variables in different genotype groups were compared by using

analysis of covariance (ANCOVA) or ANCOVA with repeated measures. In case of a significant main effect or interaction, Scheffé (I, II), Sidak (III) or least significant difference´(LSD) (IV) post-hoc tests were performed to compare the differences between groups. Non-normally distributed data were analysed in square root or logarithmically transformed form but the results were expressed as crude. The significance of the differences between groups in apoE genotype distribution was calculated using Pearson's χ^2 test. The level of statistical significance was set at $p<0.05$.

RESULTS

1. ApoE isoforms, serum lipids, and their response to pravastatin treatment (III, IV)

In the random sample of 189 middle-aged Finnish men, serum total and LDL cholesterol and apoB levels increased significantly in order E3/2 < E3/3 < E4/3 or E4/4. In post-hoc comparisons, the difference between isoform groups was statistically significant only for E3/2 vs. E3/3 and E3/2 vs. E4/3 or E4/4. The values of the carriers of E3/3 and E4/3 or E4/4 phenotypes tended to be at the same level.

In the PET study, parallel effects were observed in a relatively small sample of young men. Like in the study III, the baseline lipid values did not differ between genotypes ε3/3 or ε4/3. After 6 months of pravastatin treatment, serum total and LDL cholesterol levels had decreased significantly ($p<0.0001$) and the change was independent of the apoE genotype ($p=0.99$ for apoE-time interaction). Total cholesterol decreased by 1.20 mmol/l in men with ε3/3 and by 1.21 mmol/l in men with ε4/3 genotype.

2. ApoE genotype, CFR and coronary atherosclerosis

2.1. ApoE genotype, myocardial blood flow and CFR (IV)

One aim of the study IV was to investigate the relation of apoE genotype to the coronary artery function measured as CFR. Association of the apoE genotypes ε3/3 and ε4/3 with basal and adenosine-stimulated myocardial blood flow was examined in 40 mildly hypercholesterolemic but otherwise healthy men using PET (Figure 2). Before pravastatin treatment, there was no significant association of apoE genotype with basal ($p=0.10$) or adenosine-stimulated flow ($p=0.65$) or with CFR ($p=0.50$). The values of adenosine-stimulated flow were similar in men with ε4/3 (3.38±0.97, n=13) and in men with ε3/3 (3.26±0.77, n=27). The CFR values were also comparable in the two genotype groups (4.26±1.50 for ε3/3 and 3.97±1.36 for ε4/3). Adjustments were done for the possible confounding effects of age, BMI and LDL cholesterol.

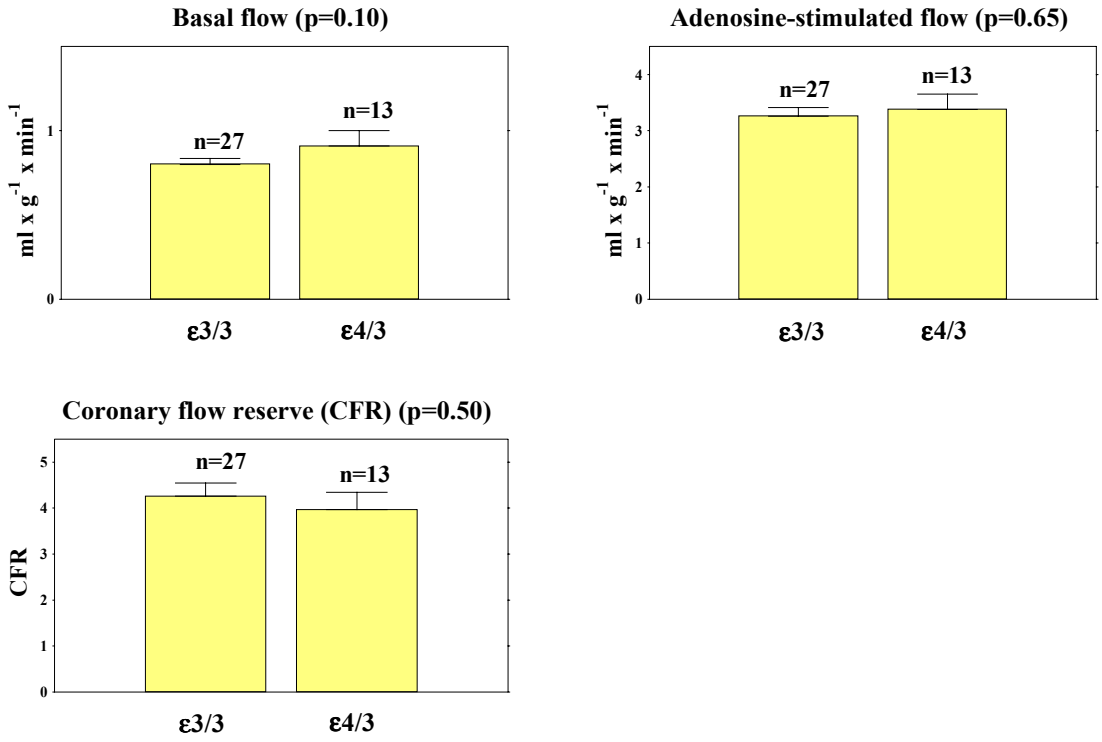


Figure 2. The baseline basal and adenosine-stimulated myocardial blood flow and CFR by apoE genotype (bars indicate mean and whiskers indicate standard error of mean).

2.2. ApoE genotype and atherosclerotic lesions in the coronary arteries (I)

The role of apoE genotype in autopsy verified CAD was studied in two consecutive autopsy series. The association of apoE genotype with coronary artery atherosclerosis was found to be age-dependent; apoE was associated with atherosclerotic lesions on only in men under 53 years old (Table 4).

Fatty streaks. A significant apoE genotype-by-age interaction (p=0.027) was observed on fatty streak area involvement in the RCA, but in the LAD, the interaction reached only borderline significance (p=0.10). In the RCA, apoE genotype was associated with fatty streaks only in men under 53 years. In this subgroup, the carriers of the ε4/3 genotype had on average a 54% increase in the area of fatty streaks of the RCA when compared to the carriers of ε3/3 (p=0.047).

Fibrotic lesions. The association of apoE genotype with fibrotic lesions of the RCA was highly significant (p=0.009) in men under 53, although the interaction of apoE and age was insignificant (p=0.17). The ε4/3 genotype was associated with the higher area

of the fibrotic lesion involvement in the RCA, as compared to the ε3/3 genotype (p=0.0089). In the LAD, no relation between apoE genotype and fibrotic lesions was observed.

Complicated lesions. No significant association of apoE genotype with complicated lesions was observed in either of the coronary arteries.

Total atherosclerotic lesion area. The association of apoE genotype with total atherosclerotic lesion area of the coronary arteries was also age-dependent. In the LAD and RCA, there was a significant interaction of apoE and age on the total atherosclerotic lesion area (p=0.041 and p=0.009, respectively). Again, in men under 53, the ε4/3 carriers had in average a 26% increase in LAD and a 61% increase in RCA total atherosclerotic lesion involvement when compared to the carriers of ε3/3 (p=0.003 and p=0.14, respectively).

Although the carriers of ε3/2 tended to have lower values of atherosclerotic lesion involvement than the carriers of ε3/3 or ε4/3, the differences were insignificant.

Table 4. The association of apoE genotypes with atherosclerotic lesions of RCA and LAD coronary artery in men <53 years (I).

Coronary artery	ε3/2 versus ε3/3	ε4/3 versus ε3/3
RCA (n=183)		
Fatty streak	NS	0.047
Fibrotic lesion	NS	0.009
Complicated lesion	NS	NS
Total	NS	0.003
LAD (n=273)		
Fatty streak	NS	NS
Fibrotic lesion	NS	NS
Complicated lesion	NS	NS
Total	NS	NS

NS indicates not significant, RCA right coronary artery, LAD left anterior descending.

Adjusment for risk factors (unpublished data). The above analyses are adjusted for the effects of age and BMI (I). If the same analyses are carried out in the subgroup with the interview data (n=423) and adjusted for the confounding effects of BMI,

smoking, alcohol consumption, hypertension and diabetes, the results are as follows (Figure 3). In the LAD coronary artery (n=357), there was a significant age-dependent association of apoE genotype with fatty streaks (p=0.023) and total atherosclerotic lesion involvement (p=0.013). In the RCA (n=246), the apoE genotype-by-age interaction on total atherosclerotic lesion area reached borderline statistical significance (p=0.08) whereas other interaction were insignificant (p=0.21 for fatty streaks, p=0.25 for fibrotic lesions, p=0.91 for complicated lesions).

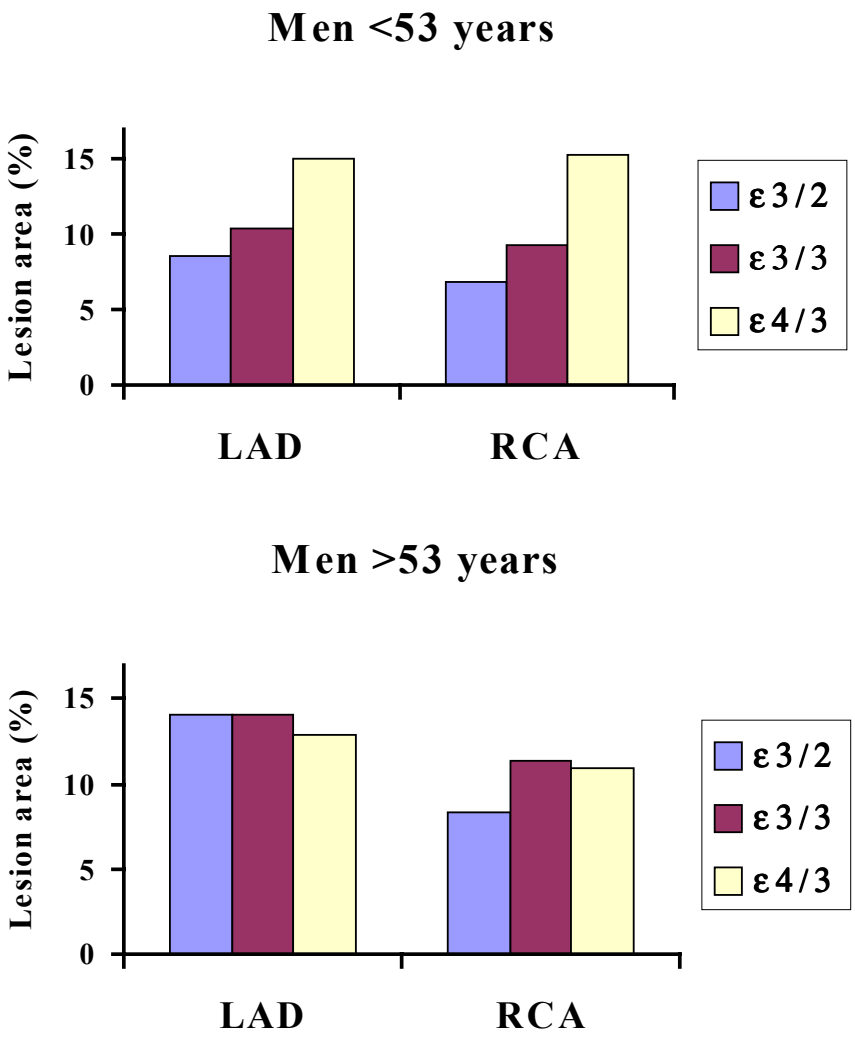


Figure 3. The adjusted mean percentage area of total atherosclerotic lesion involvement in the LAD (left anterior descending coronary artery) (n=357) and RCA (right coronary artery) (n=246) by apoE genotype and age subgroups. P=0.013 and p=0.08 for the apoE genotype-by-age interaction in the LAD and RCA, respectively (ANCOVA adjusted for the effects of BMI, smoking, alcohol consumption, hypertension and diabetes).

3. ApoE genotype and atherosclerotic lesions in the aorta (I)

To examine the relation between apoE genotype and aortic atherosclerosis the B-series (n=300) of the HSDS was analysed. The lesion area was measured separately in thoracic and abdominal aorta.

Unlike in the coronary arteries, the association of apoE genotype with aortic atherosclerosis was not age-dependent (P=NS for apoE genotype-by-age interaction). Therefore, the age was used as a covariate in the analysis. As expected, age had a major effect on atherosclerosis in both parts of the aorta. ApoE genotype had a borderline association with fatty streaks both in thoracic and abdominal aorta (p=0.09 for both), and the carriers of the ε4/3 genotype had the highest area of fatty streak involvement. There was no association of apoE genotype with fibrotic lesions or total atherosclerosis in the thoracic aorta but in the abdominal aorta, apoE genotype was significantly associated with fibrotic lesion (p=0.010) and total atherosclerotic lesion (p=0.014) area. The ε4/3 genotype was associated with greater total atherosclerotic lesion area involvement than ε3/3 (p=0.034) or ε3/2 (p=0.024). In the ε3/2 genotype group, the atherosclerotic lesion area involvement was comparable to the ε3/3 group. ApoE genotype was neither associated with complicated lesions of the aorta.

4. ApoE genotype and defects in the internal elastic lamina of the mesenteric arteries (II)

Autopsy samples of mesenteric arteries were used to examine the association of apoE genotype with histological changes of arterial wall typical of atherosclerosis. Analysis were performed for all subjects including men and women but also subgroup of only males was analysed separately due to low number of females (n=32).

A highly significant association of the intimal thickness with the IEL defects was found in all three mesenteric arteries examined (CA, SMA and IMA). No relation of apoE genotype with intimal thickness was observed in any of the three arteries.

The apoE genotype was associated with the number of IEL defects in CA (p=0.027) when all subjects were included. The carriers of the ε4/3 or ε4/4 genotype expressed higher number of defects than carriers of ε3/3 (p=0.032). In men, an association

was found also with defects in the IMA (p=0.041) in addition to CA (p=0.033). The men with ε4/3 or ε4/4 genotype had higher number of IEL gaps in their CA than the men with ε3/3 (p=0.035). In IMA, the IEL of the men with the ε4 allele was characterised by higher number of gaps than the IEL of the men with the ε3/2 genotype (p=0.041) but the difference between the ε4/3 or ε4/4 and ε3/3 genotypes was insignificant.

5. ApoE genotype and carotid artery IMT (III)

The relation between apoE phenotype and carotid artery IMT was examined in a random sample of 189 Finnish men. The associations were studied separately for the each three IMT values of three individual segments of the carotid artery and for the overall mean IMT (Mmax) (Figure 4).

ApoE E3/2 phenotype had a favourable effect on the carotid IMT. The carriers of E3/2 had 8.9% and 10% lower common and Mmax IMT values than the carriers of the E3/3 (p=0.028 and p=0.07, respectively) when adjusted for the effects of age and BMI. The statistical significance of these associations disappeared with further adjustment for serum lipids.

A surprising association was found between E3/3 phenotype and internal carotid IMT. Compared to E4 allele carriers the men with E3/3 had 18% higher IMT of the internal carotid segment (p<0.01) and this association persisted after the adjustment for serum lipids.

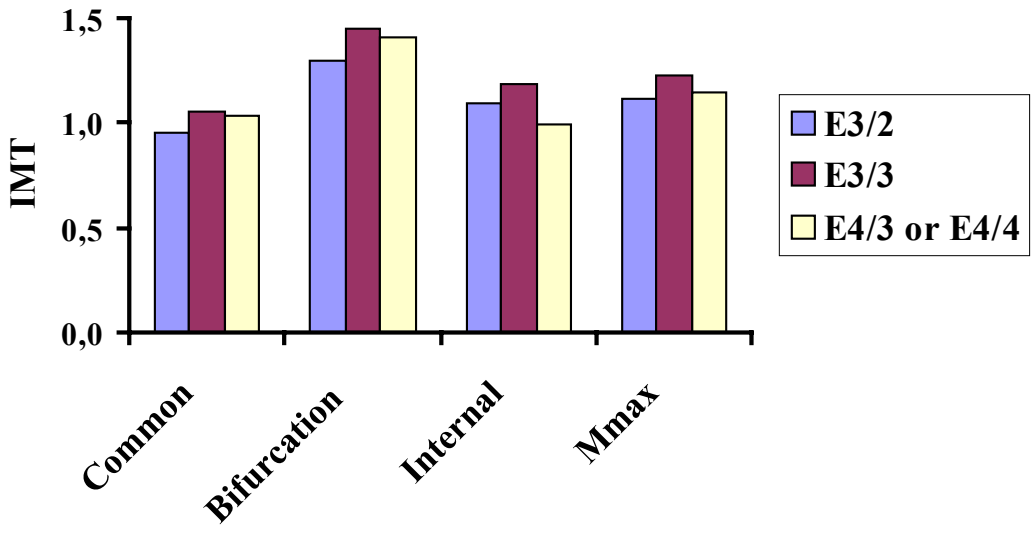


Figure 4. Carotid IMT in different segments by apoE phenotype (unadjusted means). Mmax indicates mean maximum IMT.

6. ApoE genotype and improvement of coronary function by pravastatin (IV)

Pravastatin did not induce significant change in basal myocardial blood flow. However, the change in adenosine-stimulated myocardial blood flow was dependent on the apoE genotype (p=0.016). Hence, in men with the apoE ε3/3 genotype the adenosine-stimulated flow increased by 32.5% after pravastatin treatment, whereas in the men with the ε4/3 genotype the values slightly decreased (p=0.0009 for ε3/3 versus ε4/3). Adenosine-stimulated flow did not change in the subjects with placebo (p=0.39).

The change in CFR from baseline to 6 months was dependent on the apoE genotype (p=0.02), and the difference of the change among the genotypes in the pravastatin group was of borderline statistical significance (p=0.05 for ε3/3 versus ε4/3). Thus, CFR improved by 17.8% in men with the ε3/3 and decreased by 11.9% in men with the ε4/3 genotype. Again, there were no significant responses in the placebo group.

7. ApoE genotyping from mailed buccal swabs (V)

DNA was isolated succesfully from all mailed buccal swabs taken by the subjects themselves or from the buccal cell samples of 18 Alzheimer's dementia patients taken by the nurses. ApoE genotypes determined from blood leucocytes and buccal cell samples of the healthy controls did not differ. Thus, apoE genotypes can be determined both from blood samples and buccal swabs and there are not any significant differences in the quality of amplification or fragment separation in the electrophoresis. ApoE phenotypes of the controls were also compared to their genotypes determined from blood leucocytes. Both methods gave exactly the same results (see study V Figure 1).

DISCUSSION

1. Study subjects and methodological considerations

Study subjects. In the present studies (I-IV), two autopsy and two clinical series were utilized to examine the relation of apoE genotype to different phases and phenotypes of atherosclerosis. All of these four study samples were completely independent and consisted of unrelated Finnish males. The Finns are known to be very homogeneous population due to geographic isolation and are therefore suitable for candidate gene based association studies (Peltonen et al. 1995).

An important question is, can the results of the different studies (I-IV) be generalized to Finnish people. We studied only men and thus the results cannot be extrapolated to women. In the HSDS, the cadavers were victims of sudden death or trauma who were subjected to a medicolegal autopsy, and in the study II, a medical or forensic autopsy was performed to the subjects. The selection of these individuals as study subjects may have led to a selection of individuals with more severe atherosclerosis than in individuals selected randomly (selection bias). The CHD risk factors may also have been different in these subjects. This was the case for alcohol consumption, which was very high in the HSDS subjects (mean 92 grams or 7-8 drinks per day, median 60 g/day). However, the apoE allele frequencies in the studies I and II were comparable to Finnish population and all statistical adjustments for confounding effects of risk factors were done. Therefore, the subjects (I,II) can be considered as representative sample as available for autopsy examinations. Further, the subjects of the study III were a random sample of middle-aged men from ten age-cohorts. In that study, we could not control the possible confounding effect of advanced atherosclerotic diseases because coronary angiography was not performed on our subjects, which could have biased the outcome. The participants of the PET study (IV) were also a random sample consisting of Finnish coast guards and firemen who were hypercholesterolemic but otherwise healthy. All subjects had normal PET measurements and it is highly unlikely that any of them had significant stenosis in their coronary arteries. However, because of their occupation, the subjects may be healthier and in better physical condition than average people.

Genetic method. Atherosclerosis does not show Mendelian inheritance, which indicates a complex pattern of inheritance with a significant influence of environmental factors (Lander and Schork 1994, Cambien 1996, Funke and Assmann 1999). The genetic dissection of atherosclerosis is methodologically difficult due to its polygenic etiology, several phenotypes and slow development before manifestation of clinically significant atherosclerotic changes late in life. In addition, predisposing genes have incomplete penetrance and individuals with no predisposing allele may still get the disease as a result of environmental risk factors (Lander and Schork 1994). Consecutively, family members carrying the disease are not easily identified from non-affected individuals making e.g. linkage analyses very difficult to perform (Lander and Schork 1994). In the present studies (I-IV), candidate gene approach was used to investigate the role of apoE genotype as a determinant of atherosclerosis phenotype and coronary function. Candidate gene approach together with autopsy materials is suitable in studying complex diseases such as atherosclerosis with several phenotypes. In addition, association studies based on candidate genes are sensitive to find genetic effects (Risch and Merikangas 1996). But of course, the limitation of this approach is that the candidate gene have to be identified before studies can be performed.

The candidate gene selection is generally based on knowledge about the pathophysiology of the disease and it is known that the association studies are most meaningful when applied to candidate genes having a clear biological relation to the trait (Lander and Schork 1994). In the case of the present study, apoE was chosen as the candidate gene because of well-characterized differences between the isoforms in biological function. Also data from apoE knock-out mice and all available biochemical, metabolic and genetic studies done for apoE during past thirty years support the importance of apoE in pathology of cardiovascular diseases (Davignon et al. 1988, Mahley and Huang 1999).

In genetic analysis of complex traits, one of the most important preconditions is the proper characterization of the phenotype of the studied trait, e.g. phenotype of the atherosclerotic lesion or change. In each of the four study series (I-IV), the extent of atherosclerosis was measured as continuous variables, i.e. lesion area, IMT, number of gaps in the IEL and myocardial blood flow. Instead of a pure case-control comparison, the statistical means of these variables were compared between apoE genotypes to find an association of an exact atherosclerosis phenotype with the apoE genotype.

Classification of atherosclerotic lesions. In the HSDS (I), atherosclerotic lesions were classified according to the classification protocol of the IAP (Guzman et al. 1968), which is based on fat staining with Sudan IV and classification of the lesions by naked eye as fatty streaks, fibrotic lesions and complicated lesions. In addition, unlike in the IAP, the exact area of lesion involvement was measured in square millimetres by planimetry. The classification of fatty streaks correctly is one problem of this standardized classification method. It is known that if lesions have developed in regions with adaptive intimal thickening, they may be located somewhat deeper under the endothelium, and may not become visible if stained (Stary et al. 1994). However, most of the fatty streaks stain well with Sudan fat-staining method. Another problem rises from the distinguishing between adaptive intimal thickening and raised lesions. After death, the site of arterial wall with adaptive intimal thickening may project into the lumen, and at autopsy, the change may be mistaken for a raised lesion (Stary et al. 1992). This can also have happened in our series. Today, a new standardized histological classification exists (Stary et al. 1992, Stary et al. 1994, Stary et al. 1995), which characterizes all lesion types in more detail. This method was not available at the time of collection of the two series of the HSDS but it should be used in future studies.

PET methodology. An important advantage of the PET technique used in study IV is its non-invasive nature: it can be safely performed to healthy volunteers. We used PET to detect early atherosclerotic changes in young men. PET is not the best possible method for detecting narrowings in the epicardial coronary arteries. Rather, the CFR is more functional parameter of the whole coronary system (Pitkänen 1998), and we wanted particularly information on the functional changes typical for early atherosclerosis. However, there is also a correlation between coronary vasodilator response and the degree of stenosis (Uren et al. 1994). The CFR, when assessed by using adenosine as a vasodilator, measures both endothelium and smooth muscle dependent vasodilatation of the artery wall. Due to the direct stimulation of A_2-adenosine receptors on smooth muscle cells, the vasodilatation induced by adenosine has been considered to occur through endothelium-independent mechanisms. This contention is contradicted by a finding that the coronary flow response to adenosine is related also to endothelium-dependent vasodilatation (Leipert et al. 1992, Mayhan 1992), which is probably due to shear stress associated release of vasodilating substances from endothelial cells with preserved function (Rubanyi et al. 1986). Thus, the coronary flow response to adenosine may

preferably be an integrated measure of endothelial function and vascular smooth muscle relaxation.

The developed DNA collection method. In the study V, we collected successfully DNA samples for apoE genotyping by surface mail. Using the present procedure DNA samples can be taken, collected and transported easily and rapidly, and if instructions are good patient can even take a buccal swab himself without a visit to a laboratory. All these lead to high patient acceptance. The method is also adaptable for genotyping of dementia patients because cooperation needed is minimal. The mailing did not affect the quality of DNA extracted. Problems with sample contamination were not observed either. During genetic studies blood is usually drawn for other laboratory samples and DNA can therefore be isolated from blood samples. However, in our laboratory, this method has been successfully used in studies, in which DNA samples were not originally collected. In the small sample of 36 subjects (V), we also compared apoE feno- and genotyping methods, and like Hansen et al. (1994) we found a good agreement between the results.

2. ApoE genotype and coronary function

The response-to-injury hypothesis states that the endothelial dysfunction due to injuring agents is the key event leading to the development of an atherosclerotic lesion (Ross 1999). In endothelial dysfunction, the ability of the endothelial cells to secrete vasoactive molecules is impaired (Ross 1999). Measuring of coronary function using PET can be used to evaluate endothelial dysfunction in coronary arteries, as discussed above. Hypercholesterolemia is considered to be one of the most important of the insults to the endothelium and it has shown to be associated with impaired coronary function in young hypercholesterolemic men (Pitkänen et al. 1996, Pitkänen et al. 1999). On the basis of these findings and based on the fact that apoE polymorphism is associated with serum total and LDL cholesterol concentrations (Davignon et al. 1988), it can be hypothesized that apoE genotype would be associated with endothelial dysfunction. This hypothesis was tested in the study IV by measuring coronary reactivity by PET. There were no associations of the apoE genotypes ε3/3 and ε4/3 with any of the myocardial blood flow indexes. The subjects were 40 hypercholesterolemic otherwise healthy men aged 26 to 40

years. In general, subjects of this age have already fatty streaks and some raised lesions in their coronary arteries but have not usually developed any clinically significant stenoses (Eggen and Solberg 1968). The subjects can therefore be considered as a representative sample of early atherosclerosis, with which apoE polymorphism does not seem to associate when measured as coronary function. Before the present study, PET has not been used to investigate the role of genetic risk factors like apoE polymorphism in coronary function. Neither has the role of apoE polymorphism been examined in any other form of early coronary atherosclerosis.

3. ApoE genotype and atherosclerotic lesions

In general, the apoE ε4 allele is considered to be a risk factor for CAD (Table 2). This impression is largely based on studies that have investigated the association of apoE polymorphism with CAD phenotype representing an advanced state of atherosclerosis like stenosis detectable by angiography or MI. Before the present study (I), there were only two autopsy studies (Hixson et al. 1991, Scheer et al. 1991) giving any information on the association with lesions, which do not obstruct the lumen and are clinically silent. In addition, the results of these two studies are conflicting. The study of Scheer et al. (1995) found the ε4/3 genotype to be related to coronary but not to aortic atherosclerosis, whereas the PDAY study group found no significant association of apoE genotype with coronary atherosclerosis (Hixson 1991). In contrast, in the PDAY series the aortic atherosclerosis increased significantly in order ε3/2 < ε3/3 < ε4/3 (Hixson 1991). Neither of the studies investigated the age-dependence of the association. Our findings in the HSDS autopsy series show that the apoE polymorphism is strongly associated with fatty streaks and total atherosclerotic lesion area both in the coronary arteries and aorta (I). In the coronary arteries, but not in the aorta, the association was age-dependent, i.e. the association was found only in men aged between 33 and 52 years at the time of their death. In these men, the apoE ε4/3 genotype was associated with greater atherosclerotic lesion involvement than the ε3/3 genotype. There were no significant differences between the ε3/3 and ε3/2 genotypes although the carriers of the ε3/2 tended to have the lowest lesion area. Therefore, the ε3/2 genotype does not seem to provide any significant protection from coronary or aortic atherosclerosis.

Most of the studies on the association of apoE polymorphism with CAD have been presented as if the effects of apoE genotype are independent of traditional CAD risk factors. However, there are reports suggesting that apoE genotype modifies the relationship between lifestyle-related risk factors and CAD (Srinivasan et al. 1994, Srinivasan et al. 1999, Kardia et al. 1999), although opposite findings also exist (Boer et al. 1997). In our study, we examined whether the association of apoE with atherosclerosis is different at different ages and found that the association disappeared in older men (53 to 69 years). Although the ε4 allele frequency is known to decrease by age (Kervinen et al. 1994, Louhija et al. 1994, Schachter et al. 1994), the allele frequencies were identical in both age subgroups in our series. Therefore, this does not explain our finding. Further, the observed age-dependence is in agreement with the fact that the genetic susceptibility of CAD, as well as of other complex traits, decreases with increasing age (Koskenvuo et al. 1992, Marenberg et al. 1994). In addition to the present study (I), there are other studies suggesting such an age-dependence for the effects of apoE genotype. The association of apoE genotype with serum cholesterol weakens at older ages (Jarvik et al. 1997) and apoE polymorphism does not seem to play any role in CHD or stroke incidence of old (65 to 74 years) Finnish subjects (Kuusisto et al. 1995).

4. ApoE genotype and gaps in the IEL

At present, the meaning of the injury of the IEL during the atherosclerotic process is unclear. During embryogenesis, gaps in the IEL allow the SMCs to migrate into the intima when arterial wall develops in fetuses at 30-33 weeks (Stary et al. 1992). In addition, discontinuities of the IEL have been detected already at very early age when no atherosclerosis has developed yet (Levene 1956, Sims et al. 1993). However, several pieces of evidence have been reported supporting the hypothesis that the fragmentation of the IEL plays a role in the intimal thickening and atherosclerosis by allowing migration of SMCs from the media to the intima (Sims 1985, Sims et al. 1989, Sisto 1990, Järvinen et al. 1996). Similarly, the intimal thickness was significantly associated with the IEL gaps in mesenteric arteries also in the present study (II).

What could be the mechanism behind the association between the IEL defects and atherosclerosis? Metalloproteinases have an important role in atherosclerosis and

arterial remodelling (Galis et al. 1994, Nikkari et al. 1995). The finding that macrophages containing 92-kD gelatinase have been detected in regions of IEL disruption in temporal arteritis (Nikkari et al. 1996) supports possible metalloproteinase-induced fragmentation of the IEL during atherosclerosis. Further, it has been shown that in atherosclerotic plaques macrophage-derived foam cells and SMCs express matrix metalloproteinases (Henney et al. 1991, Galis et al. 1994, Galis et al. 1995, Li et al. 1996) which might digest the IEL of the artery wall. However, it is not known for sure, does the number of gaps just represent the severity of atherosclerosis or do the gaps play a role in the pathogenesis of atherosclerosis.

Why is the apoE ε4 allele associated with higher number of gaps in the IEL than the ε2 or ε3 allele? Hypercholesterolemia has been found to be associated with discontinuity of the IEL in the coronary arteries of pigs (Hayashi et al. 1991, Kwon et al. 1998). Based on this finding it can be hypothesized that the high level of serum total and LDL cholesterol in subjects with ε4 allele might lead to defects in the IEL (Figure 5). There are no previous studies on the association of apoE genotype with IEL gaps, and nor has the effect of hypercholesterolemia on the IEL been studied in human subjects. This discussion is, therefore, highly speculative. Further, the causal relationship is unknown. The association of apoE genotype with IEL defects might as well be only a marker of increased atherosclerosis (intimal thickness) related to the ε4 allele, as discussed above. However, there are some reports providing evidence on the interaction of lipid fractions with elastin thus making the described hypothesis possible. It has been suggested that arterial elastin interacts with serum LDL or VLDL in a way which leads to a transfer of lipids to the elastin (Kramsch and Hollander 1973). Fatty acids have also been found to accumulate in elastin in atherosclerosis (Claire et al. 1976), which is an important factor in the fragmentation of elastin, and maybe also in the fragmentation of IEL.

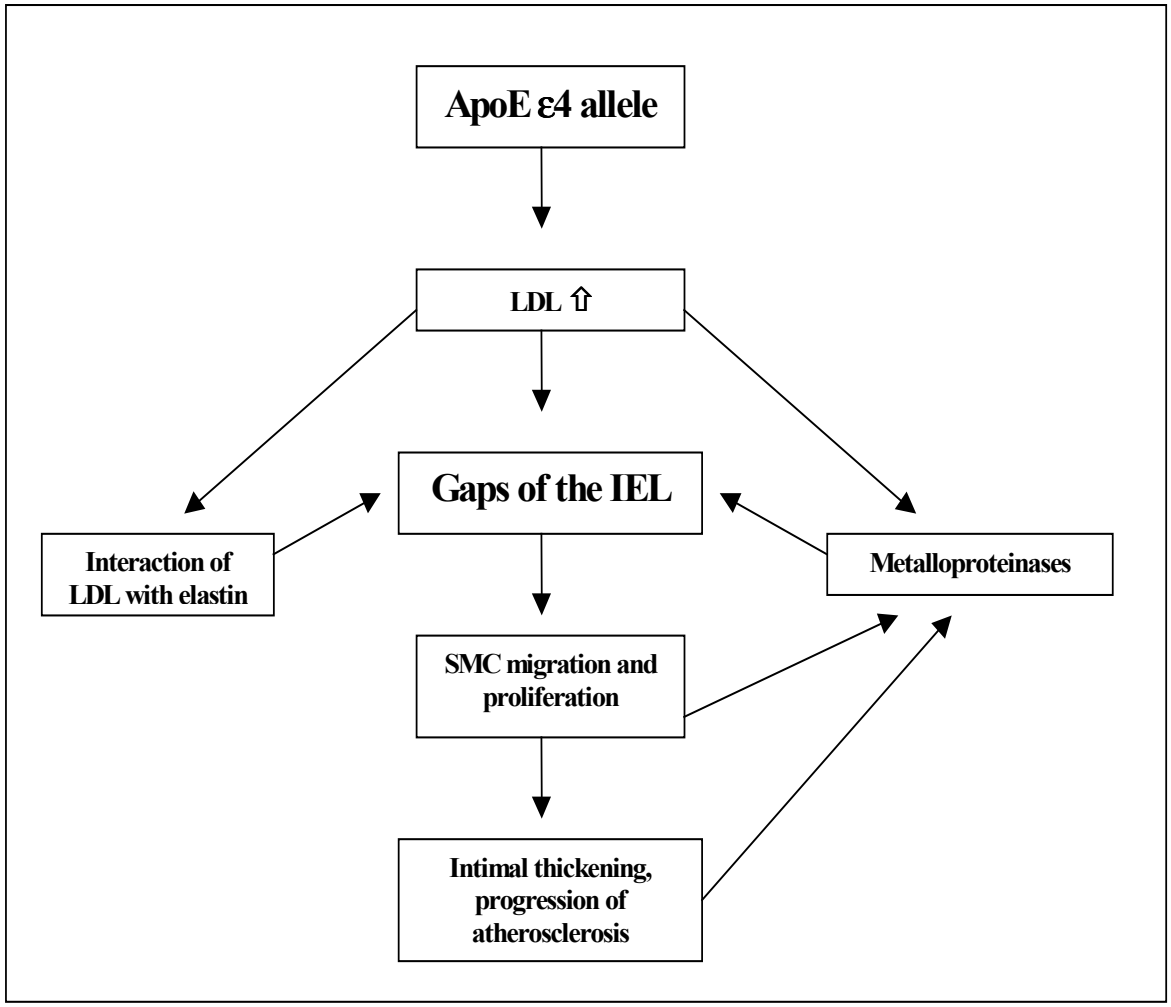


Figure 5. Possible hypothetic mechanisms how the apoE ε4 allele may be involved in the formation of IEL gaps, and its consequences.

5. ApoE genotype and carotid IMT

The aim of the study III was to examine the role of apoE polymorphism in a randomly selected middle-aged (aged 50-59 years) male population from Tampere. Contrary to our hypothesis, we found no relationship between the ε4 allele and increased carotid artery IMT but the carriers of the apoE E3/2 phenotype had lower intima-media thickness (IMT) values in common carotid artery and lower Mmax IMT values than the E3 homozygotes. The statistical significance weakened after adjustments for serum lipids, which suggests that the protective effect of the E3/2 phenotype on carotid atherosclerosis could be mediated at least partly through cholesterol metabolism. The ε2 allele is known to be associated with lower total and LDL cholesterol values (Davignon et al. 1988) which was

also shown in our study (III), in which the association of apoE polymorphism with lipids was particularly due to significant difference between the E3/2 and E3/3 carriers.

As reviewed in table 3, the previous studies on this topic have shown conflicting results. In contrast with our results, the ε4 allele was significantly associated with greater carotid IMT than the ε3 allele in three previous studies (Terry et al. 1996, Cattin et al. 1997, Vauhkonen et al. 1997). There are two reports with similar findings to ours supporting the protective role of the ε2 allele in carotid atherosclerosis (Terry et al. 1996, Zannad et al. 1998). In the contrast, two reports suggest the ε2 allele to be a risk factor for carotid atherosclerosis (de Andrade et al. 1995, Hanon et al. 2000). In addition, two studies with negative results exist (Kogawa et al. 1997, Sass et al.1998).

The differences in subjects and study design could have accounted for controversy between the results. Maybe the most obvious reason is the confounding effect of gender. We studied only men whereas others included both sexes. It has been shown in autopsy studies that men have more plaques in their carotid arteries than women (Solberg and Eggen 1971) and parallel results have been detected also by ultrasound (Tell et al. 1989). Another explanation is selection bias. The subjects of one previous study were selected from patients who had undergone coronary angiography (Terry et al. 1996). This may have lead to selection of patients with more advanced atherosclerosis because coronary angiography is not performed without symptoms. Another study excluded patients with evident cerebrovascular or CHD symptoms (de Andrade et al. 1995), which in turn leads to selection of more healthy study subjects. We avoided selection biases by choosing a random population-based sample from 10 age-cohorts including 9058 men.

6. The role of apoE genotype in improvement of coronary function by pravastatin treatment

Impaired coronary reactivity is related to oxidized LDL, which is believed to cause endothelial dysfunction by decreasing the availability of nitric oxide (John et al. 1998, Laufs et al. 1998). It has been shown that statins have favorable effects on arterial vasomotion by reducing the susceptibility of LDL to oxidation (Aviram 1996). In addition, statins have anti-inflammatory and anticoagulant properties and they stabilize atherosclerotic plaques (reviewed by Davignon and Laaksonen 1999, Bellosta et al. 2000).

In CAD patients, studies using invasive methods have shown the beneficial effect of statin treatment on function of coronary (Anderson et al. 1995, Treasure et al. 1995) and forearm arteries (Stroes et al. 1995, O'Driscoll et al. 1997). However, there are also negative results (Vita et al. 2000). PET studies have demonstrated improvement of CFR after 6 months of statin therapy in patients with CAD symptoms (Gould et al. 1994, Baller et al. 1999, Guethlin et al. 1999, Yokoyama et al. 1999). In the present study (IV) it was shown that lipid-lowering therapy for 6 months with pravastatin (40 mg/day) improves coronary artery reactivity in young subjects with mild hypercholesterolemia but that this effect is apoE genotype-dependent. Adenosine-stimulated flow and CFR were increased in men with the ε3/3 genotype but not in men with the ε4/3 genotype. There are no previous studies on this topic but the modulating effect of the apoE genotype on statins has been examined in advanced CAD in two previous reports. It has been reported that the increased CHD mortality risk associated with the ε4 allele (Stengård et al. 1995) can be reduced by statin treatment (Gerdes at al. 2000). Gerdes and coworkers analyzed a subgroup of MI survivors from the Scandinavian Simvastatin Survival Study and found that simvastatin treatment reduced the mortality risk particularly well in the ε4 allele carriers. In another study, CAD patients were treated for 2.5 years with fluvastatin and the CAD progression was assessed with quantitative angiography (Ballantyne et al. 2000). The investigators observed no differences between apoE genotypes in benefits on CAD progression. Our results suggest that in the carriers of ε4/3 genotype, pravastatin treatment does not improve endothelial function. Although our results seem contradictory to the previous studies, the effects of statins and the possible interaction between apoE and statins can be different in early and advanced phase of atherosclerosis.

The clearest reason for the genotype-dependent improvement in coronary function would have been differences in the reduction of serum cholesterol levels but this was not the case. Both genotypes responded equally to lipid lowering effects of pravastatin, as expected based on previous reports (O'Malley and Illingworth 1990, Ojala et al. 1991, Watanabe et al. 1993). Therefore, the differences must be mediated by other mechanisms. It can be speculated that the apoE genotype can modulate the antioxidant properties of statins. In fact, the apoE ε4 allele is reported to be associated with decreased antioxidant activity (Miyata and Smith 1996) suggesting that in men with the ε4/3 genotype, LDL in the arterial wall might be more susceptible to oxidation than in men with the ε3/3 genotype. Thus it is possible that pravastatin treatment at the used dosage in subjects with

the apoE ε4/3 genotype may be inefficient in affecting LDL oxidation in the coronary artery wall itself and in improving coronary reactivity.

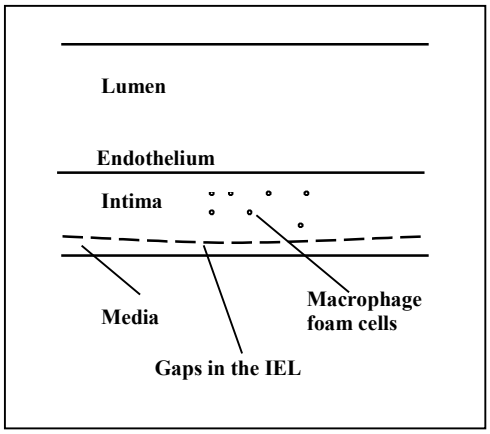
The strength of our PET study is that it used randomized, placebo-controlled and double-blind study design, which makes the findings more reliable. The limitation was clearly the small number of subjects, which made it impossible to compare the associations of apoE genotypes other than the ε3/3 and ε4/3. However, PET measurements are expensive and laborious to perform and the study series of 51 participants can be considered quite large among PET series.

To summarize, we found that 40 mg pravastatin per day does not improve the coronary artery endothelial function in the ε4/3 genotype carriers. In the future, it would be valuable to test whether the men with ε4/3 genotype would benefit from a higher dose of pravastatin. It is possible that with higher dose of lipid lowering therapy, the coronary function would improve equally also in the men carrying this risk gene.

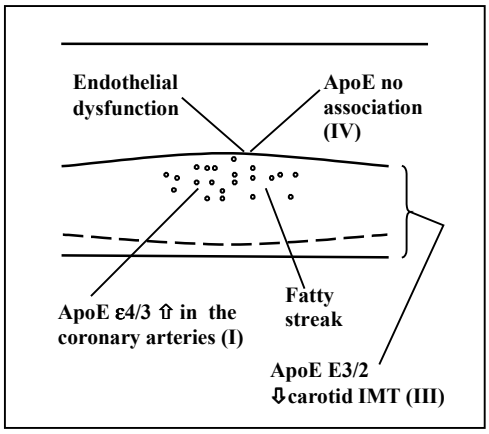
7. ApoE genotypes in early and advanced atherosclerosis

One target of this thesis was to examine the role of apoE genotype in both early and advanced phenotypes of atherosclerosis. The following picture series (Figure 6) summarizes the findings of the studies I-IV.

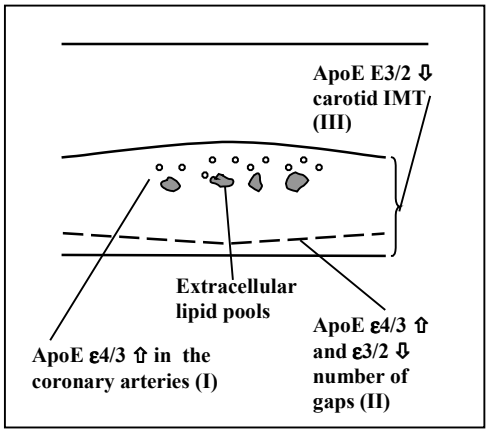
Figure 6. Schematic drawing of the role of apoE genotypes in different phases of atherosclerosis (modified from Stary et al. 1995). ⇩ indicates protective effect and ⇧ favouring effect.



Type I lesion. This lesion is macro-scopically invisible and it occurs already in infants and children (Stary et al 1995). The role of apoE in the development of the type I lesion is unknown.

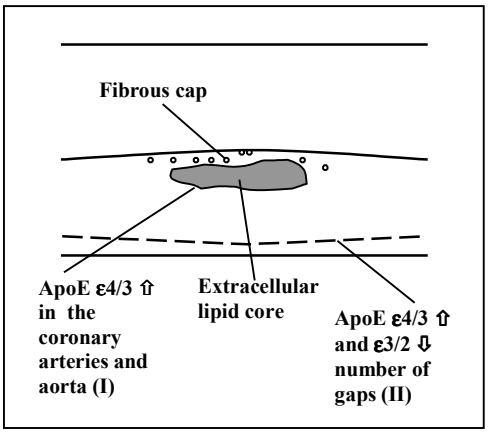


Type II lesion (fatty streak). ApoE genotype seems to affect the formation of fatty streaks and intimal thickening probably by its effects on cholesterol metabolism. However, also local effects of apoE in the arterial wall are involved. ApoE genotype may not have any effect on endothelial function.

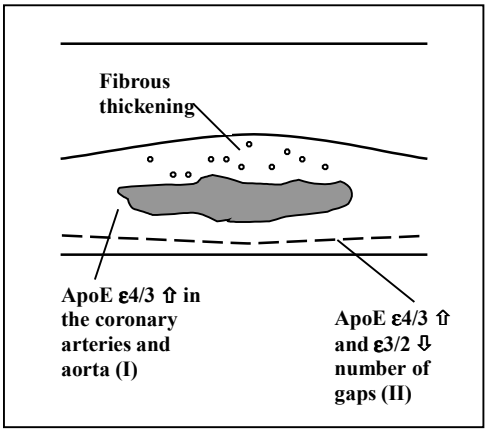


Type III lesion (preatheroma). ApoE genotype is also associated with this phenotype of atherosclerosis. The ε4/3 genotype may increase the number of gaps in the IEL and thereby allow the further growth of the lesion.

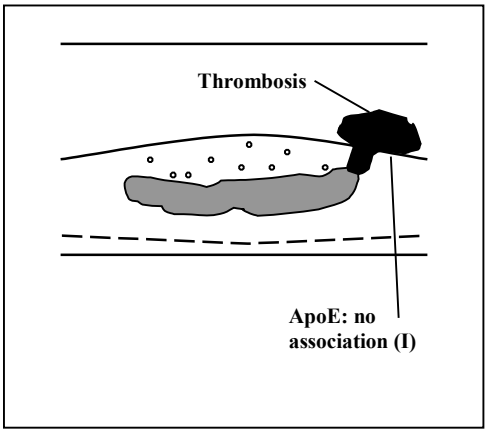
Figure 6 (continued).



Type IV lesion (atheroma). This lesion type has major clinical importance because it is prone to rupture. In the study I, the type IV lesion was probably classified as a fibrotic lesion. ApoE genotype was also associated with this phenotype of atherosclerosis.



Type V lesion (fibroatheroma). Several studies (Table 2) including ours suggest that apoE genotype is associated with advanced atherosclerotic lesions and CAD.



Type VI lesion (complicated lesion). There are no studies on the association of apoE genotype with occurrence of autopsy verified thrombosis. However, several clinical studied have found that apoE genotype is related to MI, and therefore, apoE must also be involved in occurrence of thrombosis.

SUMMARY AND CONCLUSIONS

Apolipoprotein E is an important candidate gene for atherosclerosis. Most previous studies suggest that the apoE ε4 allele is associated with increased risk of atherosclerosis but conflicting results have been published. The present studies examined two autopsy and two clinical series to further characterize the association of apoE genotypes with atherosclerosis in the coronary, carotid and mesenteric arteries and in the aorta. By using candidate gene approach, the target of this thesis was to investigate, whether apoE genotype is involved in occurrence of early or more advanced phenotypes of atherosclerosis. The role of apoE genotype in pravastatin induced improvement of coronary function was also examined. In addition, a rapid DNA sample collection method for apoE genotyping was developed. The findings and conclusions are:

1. In the coronary arteries, apoE genotypes are not related to coronary function in hypercholesterolemic but otherwise healthy young men. However, the apoE ε4/3 genotype is a risk factor for early and advanced atherosclerotic lesions in middle-aged men (<53 years) whereas in older men (>53 years) apoE polymorphism is not associated with atherosclerotic changes of the coronary arteries. ApoE genotype can therefore be considered as an age-dependent risk gene for advanced CAD.

2. In the abdominal aorta, the apoE ε4/3 genotype is associated with increased area of advanced atherosclerosis in all age groups.

3. In the mesenteric arteries, apoE ε4/3 genotype is associated with increased number of gaps (defects) in the IEL. The present study design was unable to determine, whether this association is just a marker of hypercholesterolemia and increased atherosclerosis related with the apoE ε4/3 genotype or a sign of a local effect of apoE. Nevertheless, this association suggests one possible new mechanism for the local effects of apoE polymorphism in the pathogenesis of atherosclerosis.

4. In carotid artery, the apoE E3/2 phenotype is associated with lower values of IMT than other phenotypes (III). The protective effect of the ε2 allele on early carotid atherosclerosis may be mediated by beneficial effects on serum lipids. However, no

significant protective effect for the ε2 allele could be shown in the coronary arteries or aorta (I).

5. In men with the apoE ε3/3 genotype, coronary function can be improved by treatment with pravastatin 40 mg/day for 6 months but those with the apoE ε4/3 genotype do not benefit from the treatment at this dose. Because pravastatin reduced serum cholesterol values in both genotype groups as efficiently, this apoE genotype-dependent improvement of coronary function must be mediated by other mechanisms.

6. Buccal cell samples taken by patients themselves can be easily collected by mail and apoE genotypes can be reliably determined from these DNA samples.

Based on these lines of evidence, it can be concluded that the apoE gene is an important susceptibility locus for atherosclerosis but its effects in different arteries with different anatomical locations and in different phases of atherosclerosis seem to be diversified.

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