LEENA VIIRI

Association and Functional Studies on Promoter Polymorphisms within the Apolipoprotein E gene, Regarding Lipid Metabolism and Atherosclerosis

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ACADEMIC DISSERTATION
To be presented, with the permission of
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 June 14th, 2008, at 12 o'clock.

UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION
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Tampere Graduate School in Biomedicine and Biotechnology (TGSBB)
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www.uta.fi/taju
http://granum.uta.fi

Cover design by
Juha Siro

Acta Universitatis Tamperensis 1324
ISBN 978-951-44-7353-1 (print)
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 735
ISBN 978-951-44-7354-8 (pdf)
ISSN 1456-954X
http://acta.uta.fi

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2008

To my beloved family

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List of original communications

This dissertation is based on the following original communications, which are referred to in the text by their Roman numerals (I-IV).

I **Viiri LE**, Loimaala A, Nenonen A, Islam S, Vuori I, Karhunen PJ & Lehtimäki T (2005): The association of the apolipoprotein E gene promoter polymorphisms and haplotypes with serum lipid and lipoprotein concentrations. Atherosclerosis 179: 161-167.

II **Viiri LE**, Raitakari OT, Huhtala H, Kähönen M, Rontu R, Juonala M, Hutri-Kähönen N, Marniemi J, Viikari JSA, Karhunen PJ & Lehtimäki T (2006): Relations of APOE promoter polymorphisms to LDL cholesterol and markers of subclinical atherosclerosis in young adults. J Lipid Res 47: 1298-1306.

III Abboud S, **Viiri LE**, Lütjohann D, Goebeler S, Luoto T, Friedrichs S, Desfontaines P, Gazagnes MD, Laloux P, Peeters A, Seeldrayers P, Lehtimäki T, Karhunen PJ, Pandolfo M & Laaksonen R (2008): Associations of apolipoprotein E gene with ischemic stroke and intracranial atherosclerosis. Eur J Hum Genet.

IV **Viiri LE**, Viiri KM, Ilveskoski E, Huhtala H, Mäki M, Tienari PJ, Perola M, Lehtimäki T & Karhunen PJ. Interactions of functional APOE promoter polymorphisms with smoking on aortic atherosclerosis. (submitted for publication).

In addition, this thesis contains unpublished data.

Abbreviations

AA	abdominal aorta
ABCA1	ATP binding cassette 1
ACAT	acyl-coenzyme A cholesterol acyltransferase
AD	Alzheimer's disease
AHA	American Heart Association
AN(CO)VA	analysis of (co)variance
apo	apolipoprotein
apoE	apolipoprotein E (protein)
APOE	apolipoprotein E (gene)
BMI	body mass index
bp	base pair
BSS	Belgian Stroke Study
CAC	carotid artery compliance
CE(s)	cholesterol ester(s)
CHD	coronary heart disease
CM	chylomicron
CR	chylomicron remnant
CRP	C-reactive protein
CV	coefficient of variation
CVD	cerebrovascular disease
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EMSA	electrophoretic mobility shift assay
FMD	flow-mediated dilatation
GWA	genome-wide association
HDL(-C)	high-density lipoprotein (cholesterol)
HepG2	human hepatocellular liver carcinoma cell line
HL	hepatic lipase
HSDS	Helsinki Sudden Death Study
IAP	International Atherosclerosis Project
ICAM-1	intercellular adhesion molecule 1
IDL	intermediate-density lipoprotein
IEL	internal elastic lamina
IMT	intima-media thickness
IRE	intron regulatory element
IS	ischemic stroke
LAD	left anterior descending coronary artery
LDL(-C)	low-density lipoprotein (cholesterol)

LDLR	low-density lipoprotein receptor
LRP	low-density lipoprotein receptor-related protein
LVA	large-vessel atherosclerosis
MC	mast cell
MCP-1	monocyte chemotactic protein 1
M-CSF	macrophage colony stimulating factor
MI	myocardial infarction
NO	nitric oxide
ox-LDL	oxidized LDL
OR	odds ratio
PAI-1	plasminogen activator inhibitor 1
PDAY	Pathobiological Determinants of Atherosclerosis in Youth
RANOVA	repeated measures analysis of variance
RCA	right coronary artery
RCT	reverse cholesterol transport
SCD	sudden cardiac death
SNP	single nucleotide polymorphism
SMC	smooth muscle cell
SR-BI	scavenger receptor class B type I
SVO	small-vessel occlusion
TASTY	Tampere Autopsy Study
TC	total cholesterol
TG(s)	triglyceride(s)
TOAST	Trial of ORG 10172 in Acute Stroke Treatment
URE	upstream regulatory element
VCAM-1	vascular cell adhesion molecule 1
VLDL(-C)	very low-density lipoprotein (cholesterol)

Abstract

Background. Atherosclerosis is a complex disease in which lipid laden foam cells, fibrotic matrix and calcification gradually accumulate in the arterial wall. The disease begins already in childhood and progresses throughout life. Atherosclerosis can be asymptomatic for decades and develop clinical symptoms, such as heart attack or stroke, later in life. Many risk factors for atherosclerosis have been identified, including advanced age, male sex, high blood pressure, smoking, dyslipidemia and the ε4-allele of the apolipoprotein E gene (APOE).

Objectives. This study is an attempt to clarify the role of the APOE promoter polymorphisms -219G/T and +113G/C and APOE haplotypes in explaining the variation of serum lipid, apolipoprotein and lipoprotein concentrations, as well as studying the possible association of these polymorphisms with the early markers of atherosclerosis, intracranial atherosclerosis as well as ischemic stroke, especially within the most common APOE ε3/ε3 genotype group. Furthermore, we wanted to test whether the alleles of the APOE +113G/C polymorphism have differential effects on APOE transcription.

Subjects and methods. The study was based on five study series: three clinical and two autopsy studies. The first two clinical series altogether comprised 824 subjects (UKK and Cardiovascular in Young Finns Study). They were used to study the associations of the APOE promoter polymorphisms -219G/T and +113G/C and their haplotypes, with several variables of lipoprotein metabolism (I), as well as with longitudinal changes in cholesterol values (II) in apparently healthy Finns. The third clinical series consisting of 237 stroke cases and 326 controls, of Belgian origin, studied the association of the APOE polymorphisms/haplotypes with the risk of ischemic stroke (III). The Tampere Autopsy Study (TASTY, n = 604) was utilized to study the intracranial atherosclerosis within different APOE genotype groups (III). In the Helsinki Sudden Death Study (n = 700), the association of APOE promoter polymorphisms/haplotypes with fatty streak areas in two coronary arteries and the abdominal aorta were explored (IV). Finally, the luciferase assay was used to study the effect of the APOE +113G/C polymorphism on transcriptional efficiency in a human hepatoma cell line. Furthermore, the electrophoretic mobility shift assay (EMSA) was used to study nuclear protein binding to the APOE +113G/C region (IV).

Results. Our results show that both APOE promoter polymorphisms -219G/T and +113G/C, as well as their haplotypes seem to affect various serum lipid/apolipoprotein concentrations, independent of the APOE ε2/ε3/ε4 genotype. The -219T/T and +113C/C-genotype carriers had lower very low-density (VLDL) cholesterol, apolipoprotein B (apoB) and triglyceride (TG) concentrations when compared to G/G carriers (I). The -219T/+113C/ε3 haplotype carriers showed higher low-density lipoprotein (LDL) cholesterol and total cholesterol values throughout the 21 year follow-up compared to non-carriers (II). We did not detect statistically significant differences in any of the studied subclinical markers of atherosclerosis (such as intima-media thickness), between the APOE promoter genotypes or haplotypes (II). Within the APOE ε3/ε3 carriers, there was a statistically significant promoter genotype-smoking interaction on the fatty streak area of

the abdominal aorta (IV). Within non-smokers, -219T- and +113C-allele carriers had larger fatty streak areas in abdominal aorta compared to G/G genotype carriers. Moreover, carriers of haplotype -219T/+113C/ε3 had larger fatty streak areas in abdominal aorta compared to homozygous carriers of haplotype -219G/+113G/ε3. In smokers the situation was opposite. The -219G- or +113G-allele carriers were at increased risk of ischemic stroke, and the -219T-allele carrier men had more severe intracranial atherosclerosis (III). Our functional studies revealed differences in the transcriptional activity of different APOE +113G/C alleles. The C-allele had a higher transcriptional activity compared to the G-allele. The EMSAs indicated that there is a quantitative difference in protein(s) from hepatic nuclear lysate binding to the G- and C-alleles: the G-allele bound nuclear protein(s) with higher affinity compared to the C-allele. A transcription factor RBP-Jκ was shown to bind with higher affinity to the C-allele compared to the G-allele (IV).

Conclusions. The APOE promoter polymorphisms -219G/T and +113G/C associated with variations in serum lipid/apolipoprotein concentration and to some extent also predicted the risk of ischemic stroke. Additionally, our studies showed that the studied APOE promoter polymorphisms interact with smoking, an environmental risk factor, in defining the lesion areas in the abdominal aorta. Moreover, the +113G/C polymorphism was shown to be functional, the C-allele associating with a higher transcriptional activity when compared to the G-allele. The studied APOE promoter polymorphisms are important and functional regulators of lipid metabolism and can, together with smoking, affect the development of atherosclerosis.

Tiivistelmä

Taustaa. Ateroskleroosi eli valtimonkovettumatauti alkaa jo nuoruudessa ja kehittyy oireettomasti vuosikymmenten aikana. Ateroskleroosissa valtimon seinämään kertyy vähitellen rasvatäytteisiä vaahtosoluja, fibroottista soluväliainetta ja kalkkeutumia, jotka vähitellen muodostavat verisuonta tukkivan aterooman. Osa muodostuneista ateroomista on hauraita ja ne voivat revetessään aiheuttaa sydän- tai aivoinfarktin tai sydänäkkikuoleman. Aterosklerooosin riskiä lisäävät monet eri tekijät kuten korkea ikä, miessukupuoli, korkea verenpaine, tupakointi, korkea LDL-kolesteroli ja apolipoproteiini E geenin (APOE) ε4-alleeli.

Tavoitteet. Tämän tutkimuksen tarkoituksena on selvittää kahden APOE geenin säätelyalueen polymorfian, -219G/T ja +113G/C, ja niiden muodostaman haplotyypin yhteyttä veren lipidi- ja apolipoproteiinitasoihin sekä varhaisiin sepelvaltimotautimuutoksiin. Tätä tarkoitusta varten säätelyalueen polymorfioiden merkitystä tutkitaan pääasiassa kaikista yleisimmän APOE ε3/ε3 genotyypin kantajissa. Etsimme myös APOE geeniin polymorfioiden yhteyttä aivoinfarktin ja aivoateroskleroosin riskiin. Lisäksi selvitämme onko +113G/C polymorfialla vaikutusta APOE-geenin luentaan eli transkriptioon.

Aineisto ja menetelmät. Tutkimus perustui viiteen tutkimusaineistoon: kolmeen kliiniseen (UKK, LASERI ja BSS) ja kahteen ruumiinavausaineistoon (HSDS ja TASTY), joista tutkittiin yhteensä 2691 henkilöä. Kahdessa ensimmäisessä aineistossa, jotka sisälsivät terveitä miehiä (UKK-aineisto, n = 299) tai miehiä ja naisia (LASERI, n = 525), tutkittiin APOE geenin polymorfioiden yhteyttä plasman lipidi- ja apolipoproteiinitasoihin (I) sekä niiden pitkittäismuutoksiin ja ateroskleroosin varhaismuutoksiin (II). Kolmas kliininen aineisto (BSS) sisälsi 237 aivoinfarktitapausta ja 326 verrokkia, ja siinä tutkittiin APOE geenin polymorfioiden yhteyttä aivoinfarktiriskiin (III). Lisäksi samojen polymorfioiden yhteyttä aivovaltimoateroskleroosiin (III) tutkittiin kahdessa avausaineistossa, jotka sisälsivät yhteensä 1004 tapausta (HSDS ja TASTY). Neljännessä osatyössä (IV) tutkittiin APOE geenin polymorfioiden sekä geeni-tupakointi interaktion yhteyttä valtimoiden seinämän rasvajuosteiden kokoon (HSDS, n = 700). Lisäksi käytettiin lusiferaasi-analyysiä geenitranskription tutkimiseen sekä EMSA-menetelmää (electrophoretic mobility shift assay) trankriptiotekijöiden sitoutumisen tutkimiseen APOE +113G/C alueelle (IV).

Tulokset. Tuloksemme osoittivat, että tutkitut APOE geenin säätelyalueen polymorfiat ja niiden muodostamat haplotyypit yhdistyivät seerumin lipidi- ja apolipoproteiinitasoihin. Verrattuna -219G/G ja +113G/G genotyyppien kantajiin, -219T/T ja +113C/C genotyyppien kantajilla oli esim. matalammat VLDL-kolesteroli, apolipoproteiini B ja triglyseridipitoisuudet (I). Toisaalta -219T/+113C/ε3 haplotyypin kantajilla oli korkeammat LDL-kolesteroli ja kokonaiskolesterolipitoisuudet läpi 21-vuotisen seurantatutkimuksen verrattuna niihin, jotka eivät kantaneet kyseistä haplotyyppiä (II).

Varhaisissa valtimotautimuutoksissa (kuten kaulavaltimon intima-media paksuudessa) ei ollut tilastollisesti merkitseviä eroja APOE geno- tai haplotyyppien välillä (II). -219G- ja +113G-alleelien kantajilla oli kohonnut aivoinfarktiriski verrattuna -219T/T ja +113C/C genotyyppien kantajiin. Toisaalta -219T ja +113C-alleelien kantajilla oli vaikeampi aivoateroskleroosi verrattuna -219G/G tai +113G/G genotyyppien kantajiin (III). APOE ε3/ε3-genotyypin kantajissa löytyi tilastollisesti merkitsevä geeni-tupakointi-interaktio vatsa-aortan rasvajuosteiden suhteen. Ei-tupakoivien ryhmässä -219T ja +113C alleelien kantajilla oli suuremmat vatsa-aortan rasvajuosteet verrattuna -219G/G tai +113G/G genotyyppien kantajiin. Lisäksi -219T/+113C/ε3 haplotyypin kantajilla oli suuremmat rasvajuosteet vatsa-aortassa kuin -219G/+113G/ε3 haplotyypin suhteen homotsygooteilla miehillä. Tupakoivien ryhmässä tilanne oli päinvastainen (IV). Funktionaaliset tutkimukset osoittivat, että +113G ja C-alleelit eroavat transkriptioaktiivisuudeltaan siten, että C-alleelin aktiivisuus on korkeampi. Toisaalta tutkimus osoitti, että +113G-alleeli sitoo voimakkaammin jotakin vielä tuntematonta transkriptiofaktoria/faktoreita kuin C-alleeli (IV).

Johtopäätökset. Tutkittujen APOE geenin säätelyalueen genotyyppien/haplotyyppien ja seerumin lipidi/apolipoproteiinitasojen väliltä löytyi yhteys niin poikkileikkaus- kuin pitkittäistutkimuksessakin mutta ne eivät näyttäneet yhdistyvän varhaisiin ateroskleroosimuutoksiin. Tutkimuksessa löytyi myös mielenkiintoinen APOE geeni-tupakointi interaktio. Funktionaaliset tutkimukset osoittivat, että APOE geenin säätelyalueen +113G/C polymorfialla on vaikutusta geenin transkriptioon ja että se sitoo transkriptiotekijöitä, joista yhdeksi mahdolliseksi tunnistettiin RBP-Jκ. APOE on tärkeä ja funktionaalinen rasva-aineenvaihdunnan säätelijä ja voi yhdessä tupakoinnin kanssa vaikuttaa ateroskleroosin kehittymiseen.

Introduction

Atherosclerosis is an enormous public health problem being the leading cause of morbidity and mortality in most Western countries. Autopsy studies have shown that young adults have pre-atherosclerotic changes in their vascular tree (Enos et al. 1953, Newman et al. 1986, McGill and McMahan 1998). This indicates that atherosclerosis already starts to develop early in childhood, but develops clinical manifestations such as coronary heart disease (CHD) and stroke only later in life. Atherosclerosis is a complex disease having many risk factors such as hyperlipidemia, hypertension, diabetes, male gender and smoking, as well as multiple genes and moreover, interactions between these that contribute to disease pathology (Ross 1999, Lusis 2000).

One of the most vigorously studied genes in relation to atherosclerosis is the gene encoding apolipoprotein E (apoE = protein, APOE = gene) located at 19q13.2. ApoE is a protein constituent of several lipoproteins and a ligand for lipoprotein receptors, hence playing an important role in lipoprotein metabolism. The major source of apoE is the liver, but it is also synthesized by many other organs and tissues such as astrocytic cells in the brain (Elshourbagy et al. 1985), intestinal cells (Wu and Windmueller 1979), human kidney and adrenal tissue (Blue et al. 1983) as well as macrophages in the arterial wall (Rosenfeld et al. 1993). The APOE gene has many polymorphisms, the best known of which are the ε2/ε3/ε4 variations (Zannis et al. 1981, Mahley 1988). The ε4-allele has been associated with higher cholesterol levels than the ε2 or ε3 (Boerwinkle and Utermann 1988, Lehtimaki et al. 1990), and with higher risk of various diseases such as CHD (Cumming and Robertson 1984, Kuusi et al. 1989), autopsy verified atherosclerosis (Hixson 1991, Ilveskoski et al. 1999), Alzheimer's disease (AD) (Corder et al. 1993, Saunders et al. 1993) and stroke (Pedro-Botet et al. 1992, McCarron et al. 1999). The ε3 allele is the most frequent allele in all human populations (Corbo and Scacchi 1999). The most common genotype group ε3/ε3 is an interesting target for genetic studies because the effects of the ε2/ε3/ε4 polymorphism can be excluded, and the effects of other polymorphisms can be addressed more explicitly. The first polymorphism identified within the APOE promoter region was +113G/C (Mui et al. 1996) and subsequently three other biallelic polymorphisms, -491A/T, -427T/C and -219G/T were described (Artiga et al. 1998b). All of these polymorphisms might potentially affect binding of transcription factors to the APOE promoter region, and thus influence APOE transcription and regulate apoE expression levels. The +113G/C has not previously been studied from a functional perspective but the other three polymorphisms have been shown to affect transcriptional efficiency (Artiga et al. 1998a, Artiga et al. 1998b).

This thesis is based on results from different clinical (3), and autopsy series (2), which represent several subclinical and clinical end-points of atherosclerosis, as well as their risk factors. The first two clinical series were used to study the associations of the APOE promoter polymorphisms -219G/T and +113G/C and their haplotypes, with several

variables of lipoprotein metabolism as well as with longitudinal changes in cholesterol values in apparently healthy Finns. In the third clinical series consisting mainly of Belgian people, the association of the same polymorphisms/haplotypes with risk of ischemic stroke was studied. The fourth series, the Tampere Autopsy Study (TASTY) was utilized to study intracranial atherosclerosis in different APOE genotype groups. In the other autopsy series, the Helsinki Sudden Death Study (HSDS), the association of APOE promoter polymorphisms/haplotypes with fatty streak lesion areas, in two coronary arteries and the abdominal aorta, were explored. Finally, the effect of the APOE +113G/C polymorphism on transcriptional efficiency was studied in a human hepatoma cell line.

Review of the literature

1. Overview of lipoprotein metabolism

Lipids are a diverse group of molecules, which serve many important biological functions in living organisms. The major lipids in the human body are cholesterol, triglycerides (TG) and phospholipids. Cholesterol is mainly synthesized by the body, but some is of dietary origin and absorbed from the intestine together with the cholesterol secreted by the liver and transported into the gut via the biliary duct. Cholesterol is an essential component of serum lipoproteins and most cell membranes in the body and the major precursor for synthesis of adrenal steroids (cortisol and aldosterone), sex hormones (progesterone, estrogens and androgens), vitamin D and hepatic bile acids, which are crucial for the absorption of dietary fat from the small intestine. TGs play an important role in metabolism as energy sources and storage. Along with cholesterol, phospholipids are a major component of all biological membranes (Grundy 1990, Ginsberg 1998).

Since lipids are insoluble in aqueous solutions, they cannot freely circulate in plasma but are carried in particles called lipoproteins, which contain both lipids and proteins (apoproteins or apolipoproteins). Apolipoproteins are important constituents of the lipoproteins and they also mediate the binding of lipoproteins to cell-surface receptors. All lipoproteins contain a hydrophobic core with neutral lipids such as cholesterol esters (CEs) and TG, and a hydrophilic surface coat composed of more polar lipids such as cholesterol, phospholipids and apolipoproteins. There are five major classes of lipoproteins, which are traditionally named according to their density (d): chylomicrons (CMs), very low-density lipoproteins (VLDLs), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs). The higher the ratio of protein to lipid content, the higher the density. Furthermore, the higher the density of a lipoprotein particle, the smaller its particle weight and size is. CMs are the largest and least dense ($d < 0.95$g/ml) of the lipoproteins and are assembled in intestinal mucosa for the purpose of transporting dietary TGs and cholesterol absorbed by the intestinal epithelium. The main apolipoprotein in CMs is apoB-48, but they also contain several other types of apolipoproteins including apoA (I, II & IV), apoC (I, II & III) and apoE. VLDLs ($d = 0.95$-1.006 g/ml) are produced in the circulation by the liver, and are TG rich, like CMs. The major apolipoprotein in VLDL is apo-B100, but it also contains apo-C (I, II and III) and apoE. IDLs ($d = 1.006$ and 1.019 g/ml) are produced by the catabolism of VLDL, and contain TGs and CEs in their core as well as apoB-100 and apoE on the surface. LDLs are derived by the catabolism of IDL in the circulation, are smaller and more dense ($d = 1.019$-1.063) than IDL, and are the main transporter of CEs in plasma. LDL contains one apoB-100 molecule on its surface coat. HDLs are the smallest and most dense ($d = 1.063$-1.21g/ml) of the lipoproteins, and their lipid core is mainly composed of CEs. The surface

coating of HDL contains mainly apoA-I and A-II, but also apoC (I, II & III) and apoE (Grundy 1990, Ginsberg 1998).

1.1. Lipid transport pathways

Lipoprotein metabolism is usually subdivided into three main pathways, which are responsible for the generation and transport of lipids within the body: the exogenous (dietary) and the endogenous pathway (Brown et al. 1981), and the pathway of reverse cholesterol transport (RCT) (Glomset 1968).

Exogenous pathway. The exogenous lipid transport system is responsible for delivering ingested lipids from the gastrointestinal tract into the circulatory system and to the liver and peripheral cells. ApoE has been implicated in affecting the efficiency of cholesterol absorption from the intestine, decreasing it in the order E4>E3>E2 (Kesaniemi et al. 1987, Miettinen et al. 1992). Following digestion and absorption of dietary fat by the intestinal epithelium, TG and CEs are packaged to form CMs (containing mainly apoB-48) in the intestinal mucosa, and secreted via the lymphatic system into circulation. As CMs enter the plasma they acquire apoE (from HDL) and apoC which are needed for their catabolism. In the peripheral circulation, CMs come into contact with the lipoprotein lipase (LPL) located on the surface of capillary endothelial cells. LPL hydrolyzes the TGs of the CMs to glycerol and free fatty acids, which are released into the circulation along with apoA and apoC. The free fatty acids are either oxidized by muscle cells to generate energy, stored in adipose tissue, oxidized in the liver, or used in hepatic VLDL synthesis (Kingsbury and Bondy 2003). The remaining CE-enriched CM remnants (CRs) are rapidly removed from the circulation by the liver through LDLR (Brown and Goldstein 1986) or LDLR-related protein (LRP) (Herz et al. 1988, Cooper 1997). Both receptors have a high affinity for apoE, which therefore has an important role in CR metabolism (Brown and Goldstein 1983, Beisiegel et al. 1989, Kowal et al. 1989). Most of the TG carried by the CMs is used in extrahepatic tissues in contrast to cholesterol, almost all of which is carried to the liver as CEs in the CRs.

Endogenous pathway. The endogenous lipid transport system involves lipoproteins synthesized in the liver, and is responsible for delivering cholesterol to peripheral cells. Nascent VLDL particles are formed in the liver when apoB-100 joins with CEs and TG, and the VLDL particles are then released into circulation. ApoE ε2 carriers have higher hepatic cholesterol synthesis compared to ε3 and ε4 (Kesaniemi et al. 1987, Miettinen et al. 1992), which can affect the cholesterol to apoB ratio in VLDL-particles. Mature VLDL-particles are formed when nascent VLDL-particles acquire CEs, apoC and apoE from HDL. Mature VLDL-particles interact with LPL, releasing fatty acids and glycerol into the circulation along with phospholipids, most apoC and some apoE, which are transferred to HDL. The remaining VLDL-remnants are smaller and TG depleted, and they can either be taken up by the liver via LDLR or transform into IDL. Because IDL also contains apoE, it can be removed from the plasma by binding to LDLR or LRP in the liver. The remaining IDL is modified by hepatic TG lipase, which hydrolyzes TGs. Furthermore, IDL loses its apoE and apoC, and is eventually converted to LDL. In the lipid core, LDL contains almost entirely CEs and is the major cholesterol-carrying lipoprotein in the plasma. LDL contains only one apolipoprotein, apoB-100, which acts as a ligand for

LDLR in the liver and peripheral tissues (Brown and Goldstein 1986). On average, 75% of plasma LDL is taken up by the liver and the rest by extrahepatic tissues, and uptake occurs mostly by receptor mediated pathways, i.e. through LDLR (Brown et al. 1981). After LDL binds to LDLR, the receptor-ligand complex is internalized and carried to lysosomes, where CEs are hydrolyzed to free cholesterol. Then LDLR is recycled back to the cell surface, and free cholesterol is released from the lysosomes and down-regulates the synthesis of 3-hydroxyl-3-methylglutaryl coenzyme A reductase, resulting in decreased cholesterol synthesis. Furthermore, the synthesis of new LDLR is down-regulated. On the other hand, any excess of free cholesterol in the cellular cytoplasm activates acyl-coenzyme A cholesterol acyltransferase (ACAT), which esterifies free cholesterol into CE, which can then be stored as cytoplasmic CE droplets within the cells (Grundy 1990, Kingsbury and Bondy 2003).

Reverse cholesterol transport (RCT). RCT refers to a process where excess cholesterol is removed from peripheral tissues (including the cells in the arterial wall) and transported to the liver for excretion into bile. The most important lipoprotein acting in this process is HDL. Firstly, liver hepatocytes and intestinal enterocytes secrete pre-ß-HDL, which is a disc-shaped lipid-poor particle, containing mostly apoA-I. The ATP binding cassette A1 (ABCA1) promotes efflux of phospholipids and unesterified cholesterol from peripheral cells to pre-ß-HDL. Then the enzyme lecithin-cholesterol acyltransferase (LCAT) esterifies cholesterol to CE, which forms a hydrophobic core and transforms the disc-shaped particles into spherical mature HDL_3. When HDL_3 continues to accept cholesterol, the core expands and HDL_2 is formed. HDL can directly transfer CE to the liver via scavenger receptor class B type I (SR-BI) (Acton et al. 1996, Krieger 1999). SR-BI mediates selective lipid uptake from HDL to cells, which means that lipids, but not apoproteins are transferred from HDL to cells (Glass et al. 1983). Alternatively, CEs can be transferred by CE transfer protein (CETP) to apoB-containing lipoproteins LDL or VLDL, which deliver cholesterol to the liver through interaction with LDLR. In hepatocytes CE is hydrolyzed to free cholesterol and excreted into bile either directly or through conversion to bile acid (Rader 2006). In addition to the major apolipoprotein component of HDL, apoA-I, HDL_2 also contains significant amounts of apoE (Weisgraber and Mahley 1980). HDL with apoE can bind to the same surface receptors as apoB. As a component of HDL apoE can influence cholesterol efflux from cells as part of the RCT system by increasing the size and CE content of the HDL (Mahley et al. 2006, Matsuura et al. 2006). However, concentrations of HDL with apoE are low in humans (Weisgraber and Mahley 1980) and therefore thought to represent only a minor element of RCT.

Regarding atherosclerosis, RCT from macrophages is considered important, although it only represents a minor part of the overall RCT. Monocytes enter the artery wall, where they are converted into macrophages which accumulate cholesterol, transform into foam cells, eventually leading to the formation of an atherosclerotic lesion. Therefore, to avoid the accumulation of free cholesterol and to prevent further atherosclerotic complications, it is important that the cholesterol-loaded macrophages (or foam cells) possess mechanisms to efflux excess cholesterol. Macrophages have several ways of excreting cholesterol into the extracellular environment. The efflux can be mediated by specific molecules such ABCA1 or ABCG1 (Wang et al. 2000, Wang et al. 2004). Cholesterol can also leave macrophages via passive diffusion or SR-BI can efflux it to mature HDL. The ABCA1 has a higher activity for apoA-I and pre-ß-HDL (Wang et al. 2000), whereas ABCG1 and SR-

BI interact primarily with more the mature HDL (such as HDL_2 and HDL_3) (Wang et al. 2004) [reviewed by (Linsel-Nitschke and Tall 2005, Rader 2006)]. ApoE can promote cholesterol efflux from the arterial wall as described in apoE null-mice, which expressed human-apoE in macrophages (Bellosta et al. 1995). Recently, Gafencu and colleagues showed that stress-associated factors (e.g. inflammatory cytokines) reduce macrophage apoE production in atherosclerotic plaques (Gafencu et al. 2007). Consequently, reduction or absence of apoE synthesis by macrophages promotes the development of atheromas. On the other hand, it has been proposed that apoA-I could enhance apoE secretion and cholesterol efflux from macrophage foam cells (Rees et al. 1999). Inflammatory mast cells (MCs) that are present in the arterial intima have been found *in vitro* to inhibit cholesterol efflux from macrophage foam cells through chymase-induced proteolysis of apoproteins of HDL_3 (Lee et al. 1992), which normally acts as one of the initial cholesterol acceptors in RCT.

2. Atherosclerosis

Atherosclerosis is a complex disease of large and medium-sized arteries, characterized by accumulation of lipid laden foam cells, fibrotic matrices and calcification in the innermost layer of the arterial wall. This leads to impaired or loss of blood supply to the heart and brain, eventually leading to coronary heart disease (CHD) or ischemic stroke, which are the most important complications of atherosclerosis. Risk factors for atherosclerosis include various environmental and genetic factors such as smoking, physical inactivity, stress, elevated LDL and VLDL concentrations, or reduced HDL concentrations, elevated blood pressure, diabetes, obesity, male gender and inflammation. Furthermore, interactions between the various risk factors add to the complexity of the disease. Separately, each factor might only have a minor effect, but when combined and their interactions taken into account, they can strongly increase the risk for atherosclerosis. The pathology of atherosclerosis includes a complex series of events, the key steps being the loss of normal endothelial function, lipid entry into the arterial wall [high LDL-cholesterol (LDL-C), low HDL-cholesterol (HDL-C)], leukocyte (monocyte and lymphocyte) recruitment to artery wall, smooth muscle cell (SMC) proliferation and inflammation [for reviews see (Ross 1995, Ross 1999, Lusis 2000, Libby 2002)].

2.1. Artery Structure

Arteries have three main structural layers that are called intima, media and adventitia (Figure 1). Intima is the innermost layer of the arterial wall and is separated from the arterial lumen by a monolayer of endothelial cells. In addition to the endothelial surface, the intima consists of a subendothelial part, which can be divided into two layers. The inner layer is a proteoglycan-rich layer containing nonfibrous connective tissue (proteoglycan ground substance), some SMCs of synthesizing phenotype and isolated macrophages. The outer intimal layer is called the musculoelastic layer and it contains more synthesizing SMCs, elastic fibres and collagen, than the inner layer. The internal

elastic lamina separates the intima from the media, the middle layer of the arterial wall (Stary et al. 1992). The media is comprised of SMCs of contractile phenotype. Blood pressure and blood flow within arteries is controlled by the medial SMCs capable of contracting and relaxing. The media also contains an extracellular matrix consisting of elastic fibers and collagens (Ross and Glomset 1976). The external elastic lamina separates the media from the outermost layer, adventitia, which is composed mainly of loose connective tissue. The adventitia also contains nerves, lymph vessels and a network of small blood vessels (vasa vasorum), which supply blood to the media.

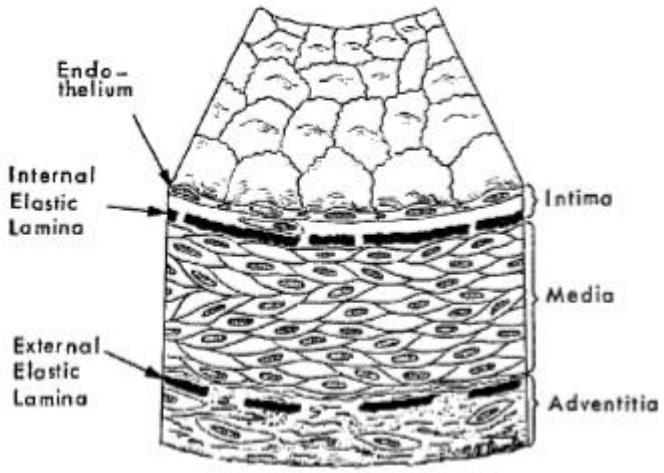

Figure 1. *Structure of normal artery. Reproduced with permission from* (Ross and Glomset 1976). *Copyright © 1976 Massachusetts Medical Society. All rights reserved.*

2.2. Atherosclerotic lesions

In atherosclerosis, the intima of the arteries is affected, most commonly in coronary arteries, aorta, iliac, femoral and cerebral arteries (Ross and Glomset 1973). Macroscopical examination of the degree of atherosclerotic lesions began in the 1960's, when the International Atherosclerosis Project (IAP) examined the degree of atherosclerosis in the first large-scale autopsy survey on atherosclerosis using standardized evaluation methods. In 1985, a multicenter autopsy study called Pathobiological Determinants of Atherosclerosis in Youth (PDAY) was organized to further investigate atherosclerotic risk factors in young men (Wissler 1991). In both of these studies (IAP and PDAY), lesions were stained with Sudan IV and visually graded as fatty streaks, fibrous plaques, complicated and calcified lesions, by procedures developed for IAP (Guzman et al. 1968). This grading was used in the Helsinki Sudden Death Study (HSDS) series which was used in study IV of this thesis.

In the beginning of the 1990's, the American Heart Association (AHA) Committee on Vascular Lesions of the Council on Atherosclerosis presented a new classification of atherosclerotic lesions, which was based on microscopic examination of arterial samples. The first report defines the normal arterial intima and atherosclerosis-prone regions (Stary et al. 1992), the second defines the clinically silent precursors of symptom-producing

atherosclerosis (lesion types I, II and III) (Stary et al. 1994), and the third report gives definitions for advanced types of lesions (type IV, V and VI) (Stary et al. 1995). A few years later, Virmani and colleagues proposed changes to the AHA classification, focusing primarily on lesion types IV, V and VI (Virmani et al. 2000). They composed a simplified scheme for classifying atherosclerotic lesions modified from the AHA recommendations that was based on morphological description. In response to this, Stary published a short clarification to the AHA numerical classification (Stary 2000). The IAP and AHA classifications of atherosclerotic lesions are presented in Table 1.

Table 1. *Classification of atherosclerotic lesions.*

Macroscopic classification by the IAP (Guzman et al. 1968)	Histological classification by AHA (Stary et al. 1994 & 1995)	Histological characteristics of lesion types I to VI (Stary 2000)
Usually not visible, fatty dots	Type I: initial lesion	Isolated macrophage foam cells
Fatty streak, usually visible with Sudan stain	Type IIa: progression-prone type II lesion	Multiple foam cell layers, mainly intracellular lipid accumulation
	Type IIb: progression-resistant type II lesion	
Fatty streak or fibrous plaque	Type III: intermediate lesion (preatheroma)	Isolated extracellular lipid pools added
Fibrous plaque	Type IV: atheroma	Confluent extracellular lipid core formed
Fibrous plaque	Type Va: fibroatheroma	Fibromuscular tissue layers produced
Calcified lesion	Type Vb (Type VII): calcified lesion	Calcification predominates
Fibrous plaque	Type Vc (Type VIII): fibrotic lesion	Fibrous tissue changes predominate
Complicated lesion	Type VI: complicated lesion	Surface defect, hematoma, thrombosis

2.2.1. Development of atherosclerotic lesions

Atherosclerotic lesions consist of endothelial cells, SMCs, and inflammatory cells such as T lymphocytes, macrophages and mast cells (MCs), which can produce inflammatory cytokines as well as proteases, prothrombotic and procoagulant factors (Hansson 2005). Atherosclerotic lesions typically develop in the vicinity of branching points and areas of major curvature, which associate with low shear stress or turbulent flow. The low shear stress might result in prolonged resident times for large, atherogenic lipid particles (e.g., LDL) or even blood cells (e.g. platelets and leukocytes), thus favoring their attachment and infiltration into the arterial wall (Ross 1999). Moreover, changes in blood flow may alter the expression of certain genes that have shear stress response elements in their

promoter region. For example, the intercellular adhesion molecule 1 (ICAM-1) gene expressed in endothelial cells has shear stress elements, and its expression is increased by reduced shear stress (Nagel et al. 1994).

In a normal healthy artery, endothelial cells form a continuous uninterrupted surface, which acts as a selective barrier between the blood elements and the artery wall. Healthy endothelium also maintains the balance between vasodilatation and vasoconstriction, inhibits leukocyte adhesion and SMC proliferation and migration. Furthermore, it inhibits platelet adhesion and aggregation (Bonetti et al. 2003). Damage to the endothelium, causing endothelial dysfunction, is one of the earliest events in the formation of an atherosclerotic lesion (Ross 1999). Endothelial damage or dysfunction can be caused by several cardiovascular risk factors such as elevated or modified LDL, hypertension and smoking, most of which are associated with increased oxidative stress (Cai and Harrison 2000). Endothelial dysfunction increases the permeability of endothelial cells and allows LDL and other particles to penetrate and accumulate in the sub-endothelial matrix. The retention of LDL in the vessel wall involves interactions between apolipoproteinB-100 (apoB-100) of LDL and matrix proteoglycans (Boren et al. 1998). Native LDL is mainly taken up by the LDL receptor (LDLR; apoeB,E receptor), which is strictly regulated by cholesterol and thus does not lead to intracellular accumulation of cholesterol. The LDL that is "trapped" in matrix proteoglycans can undergo modification including oxidation, producing minimally modified LDL, which can stimulate the endothelial cells to produce endothelial cell adhesion molecules, chemotactic proteins such as monocyte chemotactic protein 1 (MCP-1) (Cushing et al. 1990), growth factors such as macrophage colony-stimulating factor (M-CSF) (Rajavashisth et al. 1990) and recruit monocytes into the vessel wall. When the oxidation of LDL continues, it leads to the formation of highly oxidized LDL (ox-LDL), which can rapidly be taken up by macrophages. Macrophages have a limited number of LDLR, which can only bind native LDL, so the uptake of ox-LDL by macrophages is mediated by scavenger receptors (Steinberg et al. 1989), originally described by Goldstein and coworkers (Goldstein et al. 1979), and expression of which is not down-regulated by cholesterol. Therefore, macrophages can internalize large quantities of CE (Witztum and Steinberg 1991) resulting in the formation of foam cells. Ox-LDL inhibits the motility of macrophages from within the artery back to the plasma, thus leading to their trapping in the intima (Steinberg et al. 1989).

The aggregation of macrophages, or foam cells, and leukocytes within the arterial intima produces a fatty streak, an early atherosclerotic lesion present already in childhood as shown, for example, in the PDAY study (Zieske et al. 2002). Besides macrophages, MCs are present in arterial intima (Pollak 1957, Stary 1990). In normal coronary intima, only a few MCs are present, whereas in fatty streaks the amount of MCs is nine fold larger (Kaartinen et al. 1994a). In the aorta, even normal intima contains a significant amount of subendothelial MCs and interestingly, the number seems to decline with progressing atherosclerosis (Kaartinen et al. 1994b). It has been suggested that MCs contribute to the formation of foam cells by promoting intra- and extracellular lipid accumulation in the intima (Kovanen 1996). Due to the accumulation of clusters of lipid-filled macrophages under the endothelium, a fatty streak appears as a yellow streak on the luminal surface of the artery. Fatty streaks increase the thickness of the intima only little (<1mm) and do not obscure blood flow. Although a fatty streak is silent itself, it is crucial to the development

of clinical atherosclerosis because it can develop into a more advanced lesion, the atheroma (McGill 1968, Stary et al. 1994).

When extracellular lipid accumulates, a lipid core is formed and the intima thickens. The transition of a fatty streak to more advanced fibrotic lesion involves the accumulation of foam cells, extracellular lipids, SMCs and SMC-derived extracellular matrices, which gives rise to a fibrous cap (Lusis 2000). In contrast to flat fatty streaks, fibrous lesions are elevated and protrude into the arterial lumen (Ross and Glomset 1973), and may impede blood flow of the artery. Mortality from atherosclerosis is mainly due to complicated lesions, which develop disruptions of the lesion surface such as hemorrhages, hematomas or thrombotic deposits (Stary et al. 1995, Virmani et al. 2000). The development of an atherosclerotic plaque is illustrated in Figure 2.

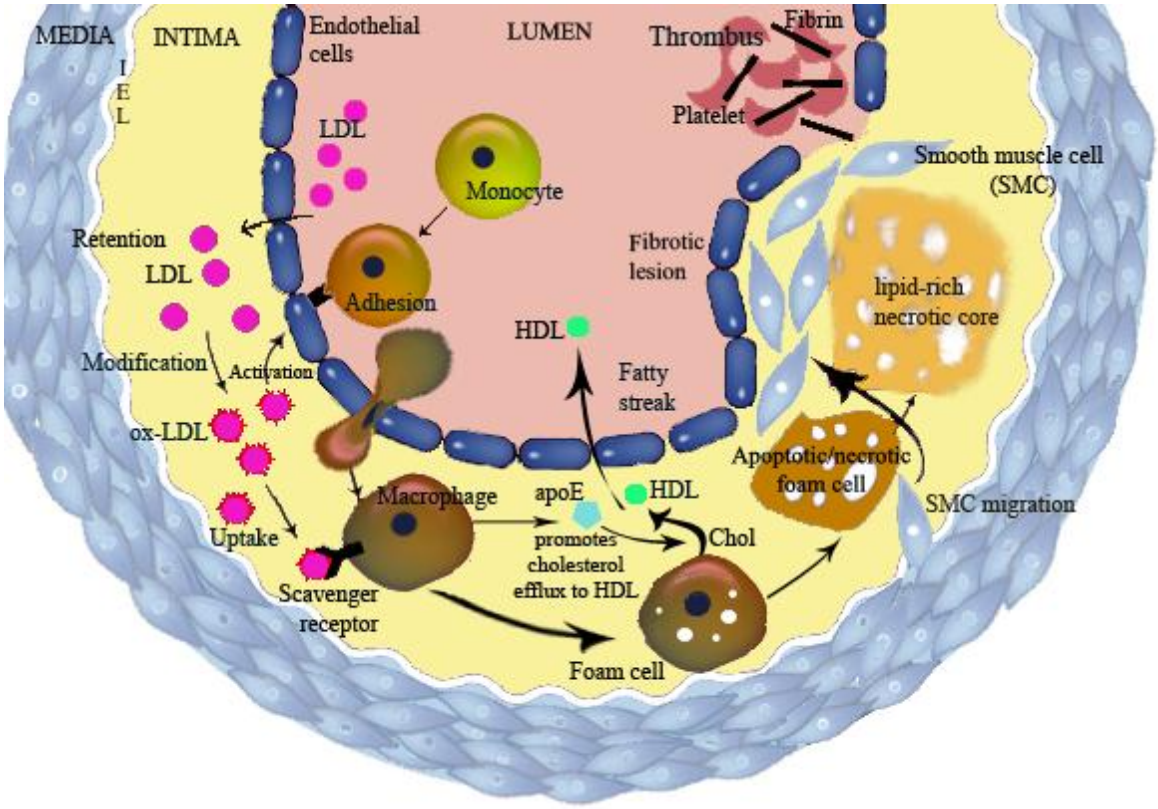

Figure 2. *A simplified illustration of the development of an atherosclerotic plaque. IEL = internal elastic lamina*

2.3. Early markers of atherosclerosis

Although the main complications of atherosclerosis usually occur later in life, the atherosclerotic process itself already begins in childhood. Changes that precede the clinical manifestations of atherosclerosis can be studied using noninvasive ultrasound methods. For example, measurements of the carotid artery wall intima-media thickness (IMT), brachial artery flow-mediated dilatation (FMD), and carotid artery compliance (CAC) have been used as markers of vascular changes related to subclinical atherosclerosis

(Raitakari 1999). IMT represents a structural marker of atherosclerosis, whereas FMD is a functional marker of endothelial health. Independently, they both predict cardiovascular events in populations (O'Leary et al. 1999, Bonetti et al. 2003). Increased carotid IMT correlates with vascular risk factors (Poli et al. 1988, O'Leary et al. 1992) and relates to the severity and extent of CHD (Burke et al. 1995). Several prospective studies have also shown that an increase in carotid IMT predicts the likelihood of cardiovascular events in population groups, independent of traditional risk factors (Salonen and Salonen 1991, Hodis et al. 1998, O'Leary et al. 1999). A recent Finnish study in patients with clinically suspected CHD showed that the maximum carotid IMT was higher in ε4 carriers compared to the ε3/ε3 group (Graner et al. 2007). Furthermore, the ε4 carriers tended to have more severe CHD compared to the ε3/ε3 group, which might partly be explained by differences in lipid variables between the groups. Impaired brachial endothelial function also independently predicts cardiovascular events in patient groups (Gokce et al. 2002, Chan et al. 2003). CAC measures the elasticity of large arteries, which decreases with age and as a result of atherosclerosis. A decrease in CAC is considered to be a risk factor for cardiovascular disease, and decreased arterial elasticity has been implicated as an independent predictor for cardiovascular events and mortality in high-risk individuals (Blacher et al. 1998).

2.4. Complications of atherosclerosis

The most important clinical manifestations of atherosclerosis include coronary heart disease (CHD), cerebrovascular disease (CVD) and peripheral arterial disease. CHD and stroke, a complication of CVD, are briefly reviewed in the next two sections.

2.4.1. CHD

CHD which is also known as coronary artery disease is due to atherosclerosis in the coronary arteries, which deliver blood to the heart. The most important risk factors for CHD include smoking, hypertension, diabetes and hyperlipidemia, and 80-90% of CHD patients have these risk factors (Khot et al. 2003). The main complications of CHD are myocardial infarction (MI), angina pectoris and sudden cardiac death (SCD). MI is defined as the necrosis of cardiac tissue due to ischemia, resulting in the replacement of myocardium with a fibrotic scar. Another common symptom of CHD is angina pectoris, which is defined as chest pain associated with myocardial ischemia (insufficient blood supply to the heart) in the absence of myocardial necrosis. The most severe symptom of CHD is SCD, which is an unexpected death from a cardiac cause. SCD is the most common manifestation of CHD in early middle age and occurs often in subjects without a history of cardiac disease (Zipes and Wellens 1998). Although CHD mortality has been declining since the 1960's in Finland, it still kills over 12 000 people each year. The WHO MONICA project showed that in eastern Finland there is a higher CHD mortality compared to the western part of the country (Tunstall-Pedoe et al. 1999). The incidence of MI is also higher in the eastern Finland compared to the western part, especially in men (Salomaa et al. 2003). The difference between eastern and western Finland in CHD

mortality has been sustained, despite the overall decline in CHD incidence since the 1960's. In addition to regional differences, there are also socioeconomic factors that affect CHD incidence and mortality in Finland. The FINMONICA project showed that CHD mortality due to MI is significantly higher in lower socioeconomic classes compared to higher classes (Salomaa et al. 2001).

2.4.2. Stroke

Cerebrovascular disease results from atherosclerosis in carotid and cerebral arteries, which supply blood to the brain and can cause stroke. In Finland, yearly there is about 18 000 new cases of cerebrovascular disease and it killed about 4500 people in 2006 (Statistics Finland 2006; http://pxweb2.stat.fi/database/StatFin/ter/ksyyt/ksyyt_en.asp; accessed Feb 20th 2008). The two main phenotypes of stroke are ischemic stroke and hemorrhagic stroke. Ischemic stroke is characterized by insufficient blood to supply oxygen and nutrients to part of the brain, and it is most commonly caused by narrowing of the arteries in the neck or head due to atherosclerosis. Ischemic stroke can be further divided to thrombotic and embolic. A thrombotic stroke occurs when cerebral arteries become blocked by the formation of a blood clot within the brain. An embolic stroke is caused by a clot (or embolus) that was formed somewhere other than in the brain itself, most often in the heart (due to e.g. atrial fibrillation) or in the carotid arteries. Hemorrhagic stroke is characterized by subarachnoid bleeding, leading to the accumulation of a blood clot within the closed cranial cavity, resulting from the rupture of a cerebral artery aneurysm or intracerebral hemorrhage from small intracerebral vessels. According to the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification, the classification of stroke includes five categories: 1) large-artery atherosclerosis (or large-vessel atherosclerosis, LVA), 2) cardioembolism, 3) small-artery occlusion (or small-vessel occlusion, SVO), 4) stroke of another determined origin and 5) stroke of an undetermined origin (Adams et al. 1993a). The etiology of stroke involves many risk factors common with atherosclerosis and CHD, such as hyperlipidemia (Pedro-Botet et al. 1992), hypertension, smoking and diabetes (Shaper et al. 1991, Tuomilehto et al. 1996). The clinical phenotype of stroke is complex, but many family and twin studies have shown that stroke has a strong genetic component (Flossmann et al. 2004). A meta-analysis of 120 case-control studies however, did not identify a single gene with a major effect, but rather several genes with common variants each having a modest effect (Casas et al. 2004). Stroke seems to be etiologically extremely heterogeneous and most likely involves multiple genes and their interaction with environmental factors as well (Tournier-Lasserve 2002). The genetic component has been found the strongest in ischemic stroke, due to SVO or LVA, the influence being especially strong in younger (< 65 yrs) patients (Jerrard-Dunne et al. 2003). Specifically, the ε4 allele is associated with a higher risk of stroke, due to LVA (Kessler et al. 1997). The genome-wide studies on stroke are discussed in section 2.5.

2.5. Candidate gene vs. genome-wide association studies

For many years, cardiovascular disease research has tried to unravel the molecular basis of atherosclerosis, and identify predisposing genetic factors. It is thought that many genes are involved in susceptibility to a complex disease like atherosclerosis. Traditionally, candidate gene approach has been widely used in studies trying to determine the underlying causes of cardiovascular diseases. The studied genes have been chosen based on their biologically possible roles in the pathogenesis of the disease, in the development of an intermediate phenotype or in defining risk factors. That is why, for example, genes affecting lipoprotein or lipid metabolism, such as apolipoprotein E gene (APOE), have been studied as possible candidate genes of atherosclerosis. Candidate gene association studies are based on single polymorphisms or haplotypes, and compare allele/haplotype frequencies between cases and controls. Statistically significant differences in the frequencies between the two groups suggest an association with the studied trait. Genome-wide association (GWA) studies, on the other hand, are hypothesis-free and allow the identification of genes involved in biological processes previously unknown to be involved in atherosclerosis, or in other words novel genetic factors contributing to disease risk. This is an advantage specifically when studying complex diseases such as atherosclerosis in which many genes are involved. Secondly, the GWA studies can be used to re-identify well-known disease associated genes and therefore strengthen earlier findings of genes increasing the risk of atherosclerosis. One has to bear in mind however, that genetic association is not equivalent to genetic causation. A genetic variant associated with a trait may be causal or just associated with the causal variant, which lies in the same gene or another gene.

Recently, several genome-wide studies have been published regarding cardiovascular disease (disease of the heart and/or blood vessels), its risk factors or manifestations such as MI and stroke (Gretarsdottir et al. 2002, Helgadottir et al. 2007, Larson et al. 2007, McPherson et al. 2007, Samani et al. 2007). McPherson and colleagues performed a genome-wide association scan using six independent Caucasian populations and showed a 58kb region on chromosome 9p21 (adjacent to genes CDKN2A, ARF and CDKN2B) associated with CHD (McPherson et al. 2007). Similarly, an Icelandic genome-wide study identified an linkage disequilibrium block (containing genes CDKN2A and CDKN2B) on chromosome 9p21 associated with MI (Helgadottir et al. 2007). A large joint genome-wide association study of seven common diseases, one of which was CHD, found a strong association with chromosome 19p21.3, but failed to show any association of APOE with CHD, most probably due to poor coverage of APOE in the Affymetrix 500K chip used in the study (Wellcome Trust Case Control Consortium 2007). Samani and colleagues performed a detailed analysis of the Wellcome Trust Case Control Consortium (WTCCC) study and identified loci strongly associated with CHD, then looked for replication in the German MI Family Study (Samani et al. 2007). Locus 9p21.3 had the strongest association with CHD in both studies. Similarly the clinical manifestations of cardiovascular disease, and also the markers of subclinical atherosclerosis such as IMT, brachial dysfunction and FMD, have been studied using genome-wide approaches. Two studies identified several candidate single nucleotide polymorphisms (SNPs) for subclinical atherosclerosis, but none achieved genome-wide significance (O'Donnell et al. 2007, Vasan et al. 2007).

Gretarsdottir and colleagues were the first to conduct a genome-wide search to identify genes behind the common stroke, including hemorrhagic stroke, ischemic stroke, and transient ischemic attacks (Gretarsdottir et al. 2002). In Icelandic patients they detected linkage to 5q12 and named it STRK1. Later the group reported the candidate gene in 5q12 as being phosphodiesterase 4D (PDE4D), having both a risk-raising and protective haplotype (Gretarsdottir et al. 2003). Helgadottir and colleagues performed a genome-wide scan and identified a four-SNP haplotype of the ALOX5AP gene (on Chromosome 13q) associated with a higher risk of MI and stroke (both ischemic and hemorrhagic) in the Icelandic cohort (Helgadottir et al. 2004). The same haplotype was later shown to associate with ischemic stroke in a Scottish cohort (Helgadottir et al. 2005).

Since blood lipid levels are a major contributor to atherosclerotic risk, GWA studies have attempted to reveal the genetic factors behind these traits. Very recently, several GWA studies have been published in the field, regarding lipid and lipoprotein concentrations (Kathiresan et al. 2007, Kathiresan et al. 2008, Sandhu et al. 2008, Willer et al. 2008). Kathiresan and colleagues used genome-wide association data from three studies and replication association analyses to study the genetic loci behind LDL-C, HDL-C and TGs. They confirmed eight previously identified loci with evidence for association with lipid or lipoprotein concentrations, and also identified six new loci associated with the traits studied. One of the confirmed loci regarding LDL-C was 19q13, of which the nearest genes are APOE-APOC1-APOC4-APOC2 (Kathiresan et al. 2008). Willer and colleagues published a companion manuscript also studying LDL-C, HDL-C and TGs (Willer et al. 2008). In addition to identifying loci involved in the regulation of lipid concentrations, they show that all of the loci associated with increased LDL-C concentrations (including APOE-C1-C4-C2) were also associated with an increased risk of CHD. Another very recent genome-wide association study identified a locus on 1p13.3 as a novel locus for LDL-C (Sandhu et al. 2008).

Thus, both candidate gene approaches as well as GWA studies have pinpointed APOE belonging to the group of important genes associated with the development of atherosclerosis.

3. Apolipoprotein E (apoE)

3.1. Discovery & Structure

In 1973, Virgie Shore and Bernard Shore described a new arginine-rich protein component of VLDL, which was referred to as “arginine-rich protein” (ARP) due to its high arginine content compared to other known apoproteins (Shore and Shore 1973). A couple of years later in 1975, Utermann isolated the same protein and published a partial characterization of it suggesting the designation “apoE” for this protein (Utermann 1975). However it took several years before the term was universally adopted and therefore during the late 1970s, both ARP and apoE were used.

The mature apoE polypeptide contains 299 amino acids (34 kDa), the complete sequence of which was determined in 1982 (Rall et al. 1982). ApoE is a polymorphic

protein with three major isoforms E2, E3 and E4 (Utermann 1975, Utermann et al. 1977), which result from cysteine-arginine interchanges at residues 112 and 158. The most common isoform E3 contains a cysteine at 112 and an arginine at 158, E2 contains cysteine and E4 arginine in both positions (Weisgraber et al. 1981, Rall et al. 1982). These amino acid changes result in charge differences between the three isoforms (relative charges +1, 0 and +2 for isoforms E3, E2 and E4, respectively) and were used in apoE phenotyping by isoelectric focusing (Utermann et al. 1977) before genotyping techniques were developed (Emi et al. 1988, Smeets et al. 1988, Hixson and Vernier 1990).

The apoE protein contains two independently folded structural domains: a 22-kDa N-terminal domain (residues 1-191) and a 10-kDa C-terminal domain (residues 216-299) (Aggerbeck et al. 1988b, Wetterau et al. 1988), which are separated by a flexible linker region (residues 192-215) (Figure 3). The N-terminal domain contains the LDLR binding region (residues 136–150 in helix 4) (Wilson et al. 1991) and the C-terminal domain is responsible for lipoprotein binding (Rall et al. 1982) and apoE self-association (Aggerbeck et al. 1988a). X-ray crystallography studies have revealed that the 22-kDa N-terminal fragment of apoE forms an elongated bundle of four α-helices. The hydrophobic side chains of the 22-kDa bundle are orientated towards the interior of the bundle (Wilson et al. 1991). The region involved in LDLR binding (residues 136–158) is rich in basic amino acids (lysine and arginine) (Innerarity et al. 1983, Weisgraber et al. 1983, Lalazar et al. 1988) and is located on the polar surface of the fourth α-helix (Wilson et al. 1991).

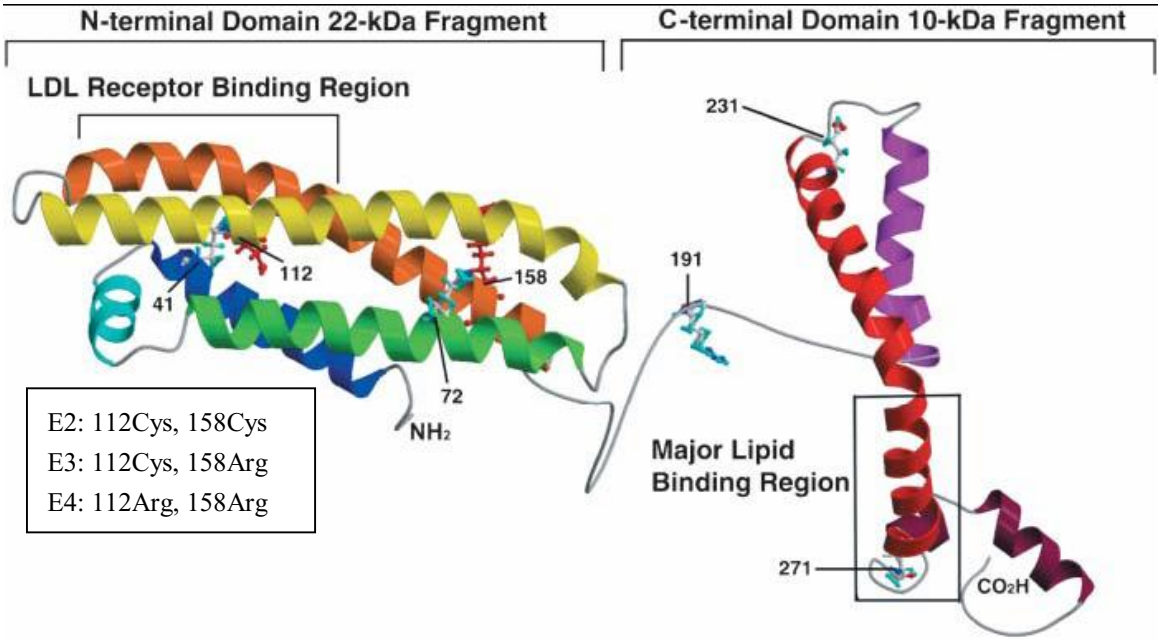

Figure 3. *Structure of the apolipoprotein E protein [modified with permission from* (Chou et al. 2005)*].*

Structure of the C-terminal domain is not as well characterized as that of the N-terminal domain, but it is predicted to contain a long amphipathic α-helix composed of a class A and a class G* helix (Segrest et al. 1992). It has been shown that residues within the segment 267-299 are critical for apoE tetramerization and facilitate lipoprotein association, and residues within the segment 245-266 also contribute to lipoprotein

association (Westerlund and Weisgraber 1993). Choy and colleagues identified an intermolecular coiled-coil helix in the C-terminal domain (residues 218-266), which coincides with the putative lipoprotein-binding surface (Choy et al. 2003).

3.2. ApoE biosynthesis

Most plasma apoE is synthesized in the liver (Blue et al. 1983). In the brain apoE is synthesized mainly by astrocytic cells (Boyles et al. 1985, Elshourbagy et al. 1985) and in the central nervous system by neurons (Xu et al. 1998, Xu et al. 1999), particularly in response to injury. Other types of central nervous system cells including SMCs in larger blood vessels, cells surrounding small vessels, and cells of the choroid plexus, are able to synthesize apoE (Xu et al. 2006). Boschert and colleagues suggested that apoE production in suffering brain neurons would be an endogenous defense mechanism, which could participate in rescuing the neuronal cells (Boschert et al. 1999). ApoE is also produced in other organs such as kidney, adrenal gland, ovary, testis, heart and lung (Blue et al. 1983, Driscoll and Getz 1984). The extrahepatic contribution to serum apoE has consistently been shown to be <10% of the total serum apoE (Kraft et al. 1989, Linton et al. 1991).

The monocyte-derived macrophages are the major cell type and consequently are likely to be the major source of apoE in atherosclerotic lesions. Cholesterol enrichment of macrophages can increase their apoE mRNA expression. Cytokines can also have an effect on apoE production, e.g. interferon-γ decreases, and tumor necrosis factor-α increases apoE production [reviewed by (Larkin et al. 2000)]. Studies done in apoE null mice expressing human apoE only in macrophages have shown that at the level of atheromas, macrophages are the primary providers of apoE, and the expression of human apoE in macrophages clearly reduces atherosclerosis, despite continuing hypercholesterolemia (Bellosta et al. 1995, Zhu et al. 1998). A human hepatocellular liver carcinoma cell line (HepG2) has also been shown to synthesize apoE (Rash et al. 1981, Thrift et al. 1986) and this cell line was used to study the effects of the APOE +113G/C polymorphism on transcriptional activity in study IV of this thesis.

ApoE mRNA is 1163 bp long and results in a primary translation product of 317 amino acids. This pre-apoE contains an 18-amino acid signal peptide at the amino terminal end, which directs the pre-apoE towards the endoplasmic reticulum. The signal peptide is cleaved intracellularly, after which the apoE protein is glycosylated in the Golgi complex with carbohydrate chains containing sialic acid (Zannis and Breslow 1981, Zannis et al. 1984). The modified apoE containing 2, 4 or 6 sialic acid residues is then secreted, and afterwards extracellular desialylation reduces the apoE sialo content. In plasma, apoE is mostly in the asialo, mono or disialo form (Zannis et al. 1986).

3.3. APOE gene

The APOE gene (MIM 107741) is mapped to the long arm of chromosome 19, more precisely to 19q13.2 (Figure 4). APOE is in a cluster with APOC1, APOC4 and APOC2, which are all part of the apolipoprotein gene family. APOE is 3597 nucleotides in length and contains four exons separated by three introns. The lengths of the exons are 44, 66,

193 and 860 nucleotides, and of the introns 760, 1092 and 582 nucleotides (Das et al. 1985, Paik et al. 1985). Paik and colleagues identified the APOE 5' flanking region, as well as the fact that the first intron has numerous positive and negative regulatory elements. This proximal promoter region contains an upstream regulatory element 2 (URE2) between -366 and -246, an URE1 between -193 and -124, a GC box transcriptional control element at -59 to -45, and an intron regulatory element 1 (IRE1) between residues +44 to +262 relative to the transcription initiation site (+1) (Paik et al. 1988). URE1 was later characterized and reported to contain a 30bp enhancer sequence (-169 to -140) named PET (positive element for transcription) and acts as a strong regulator of APOE by binding the transcription factor Sp1 (Chang et al. 1990). URE3, spanning nucleotides -101 to -89 and a specific URE3-binding protein (URE3BP) binding to this region were characterized in 1995 (Jo et al. 1995). Furthermore, a region between -94 and -84 within the URE3 has been shown to contain a binding site for the transcriptional repressor BEF-1, which negatively regulates APOE gene expression in the HepG2 cell line (Berg et al. 1995). Two binding sites for astrocyte- and neuron-associated transcription factor AP-2 have been identified in the promoter region at locations -74 to -48 and -135 to -107 (Garcia et al. 1996). Salero and colleagues isolated two APOE transcription activators Zic1 and Zic2, and identified three binding sites for them within the APOE promoter region at positions -185 to -174, -136 to -125 and -65 to -54 (Salero et al. 2001).

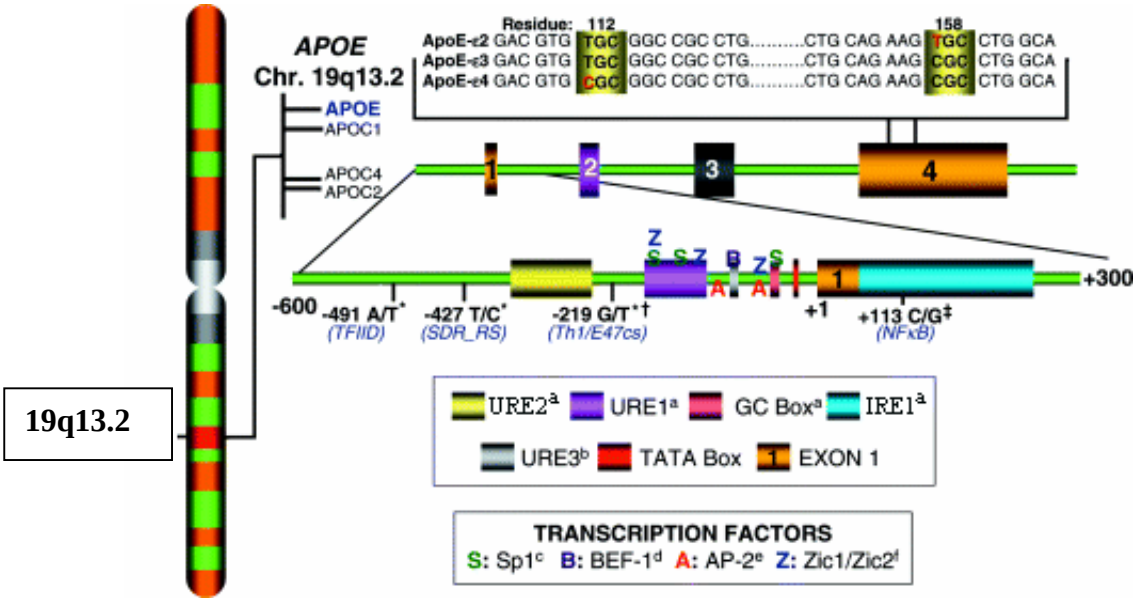

Figure 4. *Human apolipoprotein E gene, its promoter polymorphisms (*Artiga et al. 1998b, †Lambert et al. 1998b, ‡Mui et al. 1996) transcriptional regulatory elements ([a]Paik et al.1988, [b]Jo et al. 1995) and related transcription factors ([c]Chang et al. 1990, [d]Berg et al. 1995, [e]Garcia et al. 1996, [f]Salero et al. 2001). In parenthesis are the predicted transcription factor sites for promoter polymorphisms. Figure modified from* (Laws et al. 2003) *with permission from John Wiley & Sons Ltd.*

3.3.1. APOE ε2/ε3/ε4 and E2/E3/E4

The three major apoE isoforms E2, E3 and E4, discussed earlier, are coded by three alleles of the APOE gene: ε2, ε3 and ε4. These alleles determine six genotypes: ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4 (Zannis et al. 1981, Zannis et al. 1982). The frequencies of the alleles and corresponding genotypes vary in different populations and ethnic groups, but in almost all studied groups, the ε3 allele is the most common and ε2 the least common (Davignon et al. 1988, Hallman et al. 1991, Schiele et al. 2000). The ε4 allele frequency is higher in the northern parts of Europe, compared to the southern parts. For example, in Finland the ε4 frequency is around 20% (17.8 - 22.7%), compared to 8.5% in Greece or 8% in Tunisia (Ehnholm et al. 1986, Lehtimaki et al. 1990, Schiele et al. 2000, Jemaa et al. 2006). There are no regional differences in APOE allele frequencies within Finland.

The three apoE isoforms E2, E3 and E4 have different affinities for the apoE-binding receptors of cell surfaces. E2 is defective in binding to LDLRs due to its' cysteine at amino acid position 158 (E3 and E4 have arginine at this position) (Weisgraber et al. 1982, Hui et al. 1984). This leads to a delayed clearance of apoE2 bearing particles from the circulation, which in turn results in up-regulation of LDLRs and a lowering of serum LDL-C (Boerwinkle and Utermann 1988). E4 on the other hand, has a faster clearance from plasma compared to E3 (Gregg et al. 1986), leading to down-regulation of LDLRs and an increase in plasma LDL-C concentration (Boerwinkle and Utermann 1988).

3.3.2. Other polymorphisms within the APOE gene

In addition to the best known APOE ε2/ε3/ε4 polymorphism discussed earlier, there are several other polymorphisms in the APOE gene region. In 2000, Nickerson and colleagues scanned 5500bp of the APOE gene region for variations. The studied region included the entire coding region (4 exons and 3 introns) containing 3586bp, as well as 1059bp of 5' and 846bp of 3'-flanking regions. They found 22 varying sites, of which 21 were diallelic SNPs and one was an insertion/deletion type variant. Four SNPs located in the coding region (cSNPs), two of which were in exon 4 and are the SNPs defining the common APOE ε2/ε3/ε4 variation. The other two cSNPs were identified in exons 3 and 4 and variation in all four cSNPs resulted in amino acid changes. Seven out of the 21 SNPs were found in the 5' region of the APOE gene (Nickerson et al. 2000). One of these, the -219G/T polymorphism, is situated in a consensus sequence of a potential transcription factor (Th1/E47) binding site and thus is also referred to as the Th1/E47 polymorphism (Lambert et al. 1998b) (Figure 4). -219G/T and two other 5' SNPs in the APOE promoter region were identified in 1998 by Artiga and colleagues, the other two being -491A/T and -427T/C (Artiga et al. 1998b). Both -219G/T and -491A/T were shown to be involved in the regulation of APOE transcription. The -219G allele as well as the -491A allele, have higher transcriptional activity in HepG2 cells when compared to the -219T and -491T alleles, respectively (Artiga et al. 1998b, Bullido et al. 1998). Furthermore, -219T decreased apoE concentrations in human plasma in a dose-dependent manner (Lambert et al. 2000). In U87 human astrocytoma cells -491A and -427C were associated with increased levels of APOE promoter activity (Artiga et al. 1998a). Laws and colleagues have reported that people with the -491A/A genotype have elevated apoE levels in plasma

and in the brain, compared to -491T allele carriers (Laws et al. 1999, Laws et al. 2002). The first described polymorphism within the APOE regulatory region is located in the enhancer region of the first intron (IRE1) at position +113, but until now no functional data was available on the +113G/C polymorphism. Mui and colleagues determined the +113G/C allele frequencies in an Iowa population, and also showed that the +113G allele is strongly associated with the ε4 allele (Mui et al. 1996).

According to the NCBI SNP data base, in total there are 54 SNPs in the human APOE gene region including the 5' and 3' untranslated regions (http://www.ncbi.nlm.nih.gov/sites/entrez, accessed February 27th, 2008). These SNPs include the ones discussed in Nickerson's paper (Nickerson et al. 2000) and thirty additional ones. Of several of the additional SNPs however, the minor allele frequency is very low (≤ 0.02) or allele frequencies are still undefined.

3.4. ApoE-recognizing receptors

ApoE is one apolipoprotein component in several lipoproteins including CMs, VLDL, IDL and HDL, and mediates their uptake by members of the LDLR gene family.

LDLR. ApoE is recognized by all receptors of the LDLR gene family, the best known of which is LDLR, which was characterized by Brown and Goldstein (Brown and Goldstein 1986). The gene encoding LDLR is located on chromosome 19p13.3 (Francke et al. 1984, Lindgren et al. 1985), the same chromosome as the gene for apoE. LDLR is mainly expressed in the liver and to lesser extent in peripheral tissues such adrenal cortex and ovarian corpus luteum (Kovanen et al. 1979, Goldstein and Brown 1987). LDLR is synthesized in the rough endoplasmic reticulum as a precursor, processed in the Golgi apparatus and transported to coated pits on the cell surface (Brown and Goldstein 1986). LDLR binds to apoB-100 and with even higher affinity to apoE (Innerarity and Mahley 1978, Pitas et al. 1979), and is therefore capable of binding LDL (containing only apoB-100), IDL (containing apoB-100, apoE and apoC) and CRs (containing, for example, apoE and apoB-48). Of these, LDL is mainly responsible for delivering cholesterol to liver and extrahepatic cells since the apoE containing lipoproteins are either converted to LDL or rapidly catabolized in the liver (Brown et al. 1981). LDLR is primarily responsible for the catabolism of LDL. Cholesterol esters carried by LDL are delivered into cells through receptor-mediated endocytosis, at the end of which LDLRs are delivered back to the cell surface. Increased intracellular cholesterol leads to down-regulation of LDLR synthesis thus preventing overaccumulation of cholesterol through the receptor-mediated pathway. Functional defects in LDLRs disrupt the normal control of cholesterol metabolism and, by affecting uptake of LDL particles by the liver, can lead to familial hypercholesterolemia, an autosomal dominant condition in which the blood LDL-C concentration is elevated twice above normal (Brown and Goldstein 1986).

LRP. The complete amino acid sequence of LRP was first published by Herz and colleagues (Herz et al. 1988). Later, it was shown that LRP is the same molecule as α2-macroglobulin receptor (Moestrup and Gliemann 1989, Kristensen et al. 1990, Strickland et al. 1990), and therefore in the literature LRP is also called LRP/α2-macroglobulin receptor. LRP recognizes apoE (Beisiegel et al. 1989) and LPL is known to stimulate the binding of apoE to LRP (Beisiegel et al. 1991). Furthermore, hepatic lipase is known to

interact with LRP and mediate lipoprotein uptake (Nykjaer et al. 1994). LRP is mainly expressed in the liver but also in neurons and macrophages (Herz et al. 1988, Moestrup et al. 1992). Moreover, it has been shown that in addition to arterial wall macrophages, also SMCs express LRP in the normal aorta and in early and advanced lesions (Luoma et al. 1994, Lupu et al. 1994). Thus, LRP takes part in lipoprotein uptake by the cells in the arterial wall and contributes to the development of atherosclerotic lesion (Beisiegel 1995).

VLDL receptor. VLDL receptor was first identified in rabbits (Takahashi et al. 1992). Later on the human gene for VLDL receptor was cloned and characterized (Sakai et al. 1994), and localized to 9q24 (Oka et al. 1994). VLDL receptor is highly identical to LDLR but it only binds lipoproteins containing apoE (Takahashi et al. 1992) whereas LDLR binds lipoproteins containing apoE and/or apoB-100. The tissue distribution of LDLR and VLDL receptors is somewhat different: LDLR is mainly expressed in the liver while VLDL receptor mRNA is highly abundant in the heart, brain, muscle and adipose tissue but barely detectable in the liver (Takahashi et al. 1992). Moreover, VLDL receptor mRNA and protein have been shown *in vivo* to be expressed in normal aorta and coronary arteries, as well as in human atherosclerotic lesions by macrophages and SMCs (Multhaupt et al. 1996). It has also been shown that apoE and LPL produced by macrophages enhance the uptake of TG-rich lipoproteins via VLDL receptor (Takahashi et al. 1995). The endothelial expression of VLDL receptor may promote a limited uptake of TG-rich lipoproteins into endothelial cells, where they might undergo intracellular hydrolysis or be directly transported across the endothelium for subsequent uptake by e.g. SMCs (Multhaupt et al. 1996).

3.5. APOE, lipids and atherosclerosis

The APOE ε2/ε3/ε4 polymorphism has been shown to have an impact on plasma cholesterol and TG levels (Utermann et al. 1979). Regarding atherosclerosis, apoE is considered to act mostly as a protective element due to its role in remnant removal and RCT. Benefits of this protectiveness depend on the apoE isoform and its plasma levels, as well as on the cell type secreting it. As stated earlier, apoE plays an important role in lipid and lipoprotein metabolism by binding to members of LDLR family and mediating the uptake of lipoprotein remnant particles by the liver: CRs, VLDL or IDL remnants (Mahley 1988, Beisiegel et al. 1989, Kowal et al. 1989). Many studies have demonstrated that the APOE ε4 allele associates with higher, whilst ε2 with lower concentrations of plasma TC, LDL-C and apoB, when compared to the ε3 allele (Ehnholm et al. 1986, Lehtimaki et al. 1990, Lehtimaki et al. 1991, Dallongeville et al. 1992, Ilveskoski et al. 2000). Lehtimäki and colleagues also showed that the differences between apoE phenotypes in TC and LDL-C remained fairly constant during two follow-ups (six and 21-year) (Lehtimaki et al. 1990, Lehtimaki et al. 1991, Gronroos et al. 2007). Additionally, it is known that APOE ε2 carriers have higher and ε4 carriers lower levels of serum or cerebrospinal fluid apoE compared to ε3/ε3 (Lehtimaki et al. 1995, Schiele et al. 2000, van Vliet et al. 2007). Furthermore, apoE can activate cholesteryl ester transfer protein (Kinoshita et al. 1993) or enzymes involved in the lipoprotein metabolism such as hepatic lipase (Thuren et al. 1991).

ApoE has been shown to be secreted locally in healthy arterial wall (Yla-Herttuala et al. 1988), be present in atherosclerotic lesions (Murase et al. 1986, Babaev et al. 1990, Crespo et al. 1990) and expressed by macrophages within lesion (Werb et al. 1986, O'Brien et al. 1994). This macrophage-produced apoE can facilitate cholesterol efflux from foam cells to HDL_3 (Mazzone and Reardon 1994), which helps to maintain the cholesterol balance in the arterial wall. Cholesterol efflux from macrophages is most efficient in E2 carriers and least efficient in E4 carriers (Cullen et al. 1998). Macrophage-specific apoE production can reduce atherosclerosis even with an atherogenic lipid profile, as shown in apoE knock-out mice (Bellosta et al. 1995). Conversely, the lack of macrophage-specific apoE can promote foam cell formation independently of lipid/lipoprotein levels (Fazio et al. 1997).

ApoE also has several other anti-atherogenic properties, independent of lipid metabolism and transport. ApoE can inhibit platelet aggregation by stimulating platelet nitric oxide (NO) synthase through an interaction with apoE receptor 2 on platelet surfaces (Riddell et al. 1997). NO belongs to the group of endothelium-derived relaxing factors that promote endothelium-dependent relaxation in large arteries (Vanhoutte 2003). Furthermore, NO can decrease production of vascular cell adhesion molecule 1 (VCAM-1), which binds monocytes and lymphocytes to the endothelial wall (Khan et al. 1996). ApoE can inhibit the expression of VCAM-1 on endothelial cells through the stimulation of NO production. ApoE isoforms also stimulate NO release differentially; E3 causes the greatest increase, whilst E4 does not stimulate a significant increase in NO release (Sacre et al. 2003).

In atherosclerosis, SMCs migrate from the media to the intima, where they proliferate and the disease process occurs. These processes are regulated by many factors such as platelet-derived growth factor (PDGF) and ox-LDL, produced within the lesion or derived from circulating cells in the plasma. ApoE can inhibit PDGF-stimulated SMC proliferation and migration (Ishigami et al. 1998), the former being mediated through activation NO production (Ishigami et al. 2000). Anti-migratory functions are mediated by apoE binding to LRP-1 (Swertfeger et al. 2002), leading to cAMP-dependent protein kinase A activation (Zhu and Hui 2003).

C-reactive protein (CRP) is a marker of inflammation, known to be deposited in the arterial intima and plaques during atherogenesis (Zhang et al. 1999), and the concentration of which can be used as a predictor of coronary events (Ridker et al. 1997, Ridker et al. 1998). Elevated CRP also predicts cardiovascular death, MI or stroke in patients with stable CHD (Sabatine et al. 2007). APOE affects CRP plasma levels and ε4 allele carriers have been shown to have lower CRP levels than others in several study populations: in middle-aged Finnish men (Mänttäri et al. 2001), in German patients with and without angiographically documented CHD (Marz et al. 2004), among Japanese Americans (Austin et al. 2004), in Finnish nonagenarians (Rontu et al. 2006), as well as in Finnish children and young adults (Grönroos et al. 2008). The mechanism explaining lower CRP levels in ε4 carriers is still unclear. It has however, been suggested that CRP metabolism could relate to hepatic sterol metabolism and the mevalonate pathway in the liver (Marz et al. 2004). Since ε4 carriers absorb cholesterol more efficiently than other APOE allele carriers (Kesaniemi et al. 1987), their hepatic cholesterol and LDLR synthesis are down-regulated, resulting in increased serum LDL-C concentrations (Miettinen et al. 1992). As a

consequence, the cholesterol producing mevalonate pathway is down-regulated, and could thus explain the reduced CRP levels in ε4 carriers.

3.6. APOE and CHD

Under certain circumstances APOE can increase the risk of atherosclerosis, but this is highly dependent on APOE ε2/ε3/ε4 allele status. Usually the APOE ε4 allele is considered to increase the risk of CHD, whilst ε2 is considered to be protective (Siest et al. 1995). The detrimental role of the APOE ε4 allele is mainly thought to be due to its association with a poor lipid profile (e.g. high LDL-C and low HDL-C). In 1996, a meta-analysis of 14 observational studies, including over 2000 cases, concluded that carriers of the ε4 allele were at greater risk for CHD compared to ε3/ε3 carriers (OR = 1.26; 95% CI 1.13-1.40, both sexes combined). APOE ε2 on the other hand, was not found to have any effect on CHD risk (OR = 0.98; 95% CI 0.85-1.14, both sexes combined) (Wilson et al. 1996). In 2004, a meta-analysis reviewing 48 studies came to the same conclusion as the previous meta-analysis and stated that compared with ε3/ε3 carriers, the APOE ε4 carriers had a 42% higher risk of CHD, whereas the ε2 allele did not statistically significantly associate with CHD risk (OR = 0.98, 95% CI 0.66-1.46) (Song et al. 2004). A more recent meta-analysis on APOE and CHD risk including 121 studies demonstrated that ε2 carriers have a 20% lower risk of CHD whereas ε4 carriers have slightly higher risk compared to ε3/ε3 homozygotes (Bennet et al. 2007). The authors also point out that in smaller studies, the protective effect of ε2 is underestimated and the higher risk of CHD in ε4 carriers compared to ε3/ε3 genotype is overestimated.

There are also studies that have not found any association between ε4 and increased CHD risk. In a prospective population study of Finnish men and women aged 65 to 74 years, the APOE ε4 was not associated with CHD incidence (Kuusisto et al. 1995). The authors suggested that ε4 very likely increases the CHD risk in middle-age, but that its importance as a cardiovascular risk factor decreases with age. Ilveskoski and colleagues also suggested that the APOE ε4 allele could act as a significant risk factor in early middle-age, based on their finding that the atherosclerotic lesion areas in coronary arteries are larger in ε3/ε4 carriers compared to ε3/ε3 carriers, only in men <53 years (Ilveskoski et al. 1999). These two studies and a previous twin study (Marenberg et al. 1994) concluded that the effect of genetic factors on CHD risk decreases with age, whilst other (environmental) factors accumulate.

3.7. APOE, IMT and stroke

IMT. IMT is used to measure atherosclerosis of the carotid arteries and is a strong predictor of future vascular events such as ischemic stroke and MI (Lorenz et al. 2007). Thus, IMT can be used as a surrogate marker for MI and stroke. An increasing IMT is directly associated with an increased risk of MI and ischemic stroke in adults ≥65 years, without a history of cardiovascular disease (O'Leary et al. 1999). IMT has a genetic component and APOE has been proposed to be one of the genes involved. APOE ε4 has been associated with higher IMT in many studies (Terry et al. 1996, Cattin et al. 1997,

Elosua et al. 2004), but negative results have also been published (Beilby et al. 2003, Fernandez-Miranda et al. 2004). A relatively small study involving a random sample of middle-aged Finnish men showed that the ε2/ε3 genotype associates with lower IMT compared to ε3/ε3 homozygotes, but the association weakened after adjusting for CHD risk factors including lipids (Ilveskoski et al. 2000). In the same study, no association was found between the ε4 allele and increased carotid IMT. A very recent meta-analysis showed an association between APOE and carotid IMT, with ε4 associating with the highest and ε2 with the lowest IMT (Paternoster et al. 2008). On the other hand, a recent study on young Finnish adults (aged 24-39 years) showed no significant association between APOE phenotypes and IMT, despite the association of APOE with lipids (LDL-C, HDL-C) in the same population (Gronroos et al. 2008).

Stroke. APOE has been a candidate gene for stroke mainly due to its role in neuronal membrane maintenance and repair (Ignatius et al. 1986, Horsburgh et al. 2000). Studies regarding the association between the ε4-allele and the risk of stroke have given conflicting results. A meta-analysis of nine case-control studies showed that ε4 carriers are associated with ischemic stroke risk (McCarron et al. 1999). A subsequent meta-analysis including data from 120 case-control studies found no significant linkage between ischemic stroke and APOE (Casas et al. 2004). Moreover, a recent meta-analysis found no clear link between ε4 and ischemic stroke, but results from a few studies with information about the ischemic stroke subtypes suggested that ε4 may increase the risk of LVA stroke, with no effect on other subtypes (Sudlow et al. 2006). A meta-analysis conducted in persons of non-European descent showed that ε4 associated with an increased risk of ischemic stroke in people of Asian descent (Chinese and Japanese combined) (Ariyaratnam et al. 2007). Van Vliet and colleagues showed that among APOE ε3/ε3 (and ε3/ε4) carriers in old age, an increase in apoE plasma concentration associates with an increased prevalence and incidence of stroke, independently of other cardiovascular risk factors (van Vliet et al. 2007). The authors speculated that higher plasma apoE levels might reflect higher plasma levels of proatherogenic lipoproteins such as VLDL, LDL and CRs. On the other hand, Couderc and colleagues showed that low apoE levels associated with the presence of stroke (Couderc et al. 1993).

3.8. APOE gene-smoking interactions

Atherosclerosis is a complex disease, the development of which is modified by many factors, both genetic and environmental, as well as interactions between the two. If there are gene-environment interactions, the same genotype can produce different phenotypes under different environmental exposures, or vice versa. For example, a certain genotype might be harmful only in smokers, or the other way around, smoking might be more harmful in carriers of a certain genotype. In 1999, Stengård and colleagues proposed that individuals with different APOE genotypes respond differently to environmental exposures such as smoking (Stengard et al. 1999). Currently, gene-environment interactions are considered to have a crucial impact on the risk of CHD (Talmud 2007). In fact, several studies have explored the APOE-smoking interaction for CHD risk, showing statistically significant results (Humphries et al. 2001, Talmud et al. 2005, Humphries et al. 2007). Humphries and colleagues confirmed the earlier finding of Stengård and

colleagues (Stengard et al. 1999) about the APOE-smoking interaction, extending it to a younger age. They stated that smoking is a risk factor for CHD in all APOE genotypes, but particularly in ε4 carrier men (Humphries et al. 2001). A few years later, a reanalysis of data from the Framingham Offspring Study revealed a statistically significant interaction between smoking and APOE genotype on the risk of CHD (Talmud et al. 2005). In smokers, the ε4-allele increased CHD risk almost 4 fold compared to ε3/ε3 men. Furthermore, a second study was conducted to clarify whether measures of oxidative stress (e.g. ox-LDL) could explain the APOE-smoking interaction. In fact, ε4 carriers were found to have almost 27% higher plasma ox-LDL than other genotypes (Talmud et al. 2005). This could at least partly be explained by the higher amount of reactive oxygen species in ε4 carriers, since ε4 is a poorer antioxidant than ε3 or ε2 (Miyata and Smith 1996). A recent study showed that acrolein (present in cigarette smoke) causes tertiary conformational alterations and oxidative damage to the N-terminal domain of apoE3, which impairs the ability of apoE to interact with LDLR, and also decreases its ability to bind phospholipids vesicles (Tamamizu-Kato et al. 2007). Thus, the role of apoE as a regulator of plasma cholesterol metabolism is disturbed.

There are also studies reporting negative results concerning APOE-smoking interactions regarding implications of atherosclerosis (Keavney et al. 2003, Liu et al. 2003, Loew et al. 2005). Liu and colleagues performed similar analyses to Humphries (Humphries et al. 2001) in their study consisting of MI cases and controls but did not observe APOE-smoking interactions (Liu et al. 2003). Collaborators in the International Study of Infarct Survival had contradictory results on the effect of APOE-smoking interactions on CHD risk, based on analyses using case-control data in MI cases and controls (Keavney et al. 2003). However, their data was later reanalyzed and in fact, smoker ε4 carriers were shown to have a significantly greater CHD risk compared to non-smokers (Humphries et al. 2003).

The effect of the APOE-smoking interaction on IMT and ischemic stroke has also been investigated by several study groups. Djoussé and colleagues were the first to find a synergistic effect between smoking and ε4 allele on the risk of carotid atherosclerosis (Djousse et al. 2002). They suggested that subjects with the ε4 allele could be more susceptible to the detrimental effects of smoking on carotid atherosclerosis and thus at a higher risk of ischemic stroke. A Finnish study observed the APOE-smoking interaction affecting carotid IMT, but the interaction was statistically significant only within hypertensive men (Karvonen et al. 2002). Among hypertensive men, the mean carotid IMT was greater in ε4 carriers compared to ε4 non-carriers. The authors proposed that smoking and hypertension might induce endothelial dysfunction and predispose the vessel to plaque formation. They also suggested that the effect of the ε4-smoking interaction on IMT could be explained by differences in lipid metabolism, increased oxidation of LDL and also by the smaller, more oxidation-prone, LDL particle size in ε4 carriers. A Hungarian study group found the presence of the APOE ε4 allele increased the unfavorable effects of smoking as well as hypertension, diabetes and drinking, on the incidence of ischemic stroke (Szolnoki et al. 2003). A subsequent study in young adults detected a significant APOE-smoking interaction on ischemic stroke, the influence of smoking being greater in the group of ε3/ε4 carriers than in subjects with the ε3/ε3 genotype (Pezzini et al. 2004).

3.9. APOE -219G/T and +113G/C polymorphisms

As was discussed in section 3.3.2., 54 SNPs are currently identified in the APOE gene region. In this study, we concentrated mainly on two of the promoter SNPs -219G/T and +113G/C because they both possess the ability to affect APOE transcription. Of these two SNPs up till now, the -219G/T SNP (also known as Th1/E47cs) has been studied more intensively and been associated at least with MI (Lambert et al. 2000, Ye et al. 2003), AD (Lambert et al. 1998a) and premature CHD (Viitanen et al. 2001). It has also been shown to have a functional activity, the -219G-allele associating with higher promoter activity than the T-allele (Artiga et al. 1998b, Ramos et al. 2005). To date, no functional data has been available regarding the +113G/C SNP and transcriptional activity of the APOE gene. The -219G/T polymorphism has an effect on APOE mRNA expression in human AD brain tissue (Lambert et al. 1998a) and the -219T-allele has been associated with an increased risk of AD (Lambert et al. 1998a, Lambert et al. 1998b). Higher beta-amyloid loads have also been associated with -219T/T carriers in patients >70 years (Lambert et al. 2001). On the contrary, in a Finnish Study consisting of subjects >85 years, the APOE -219T/ε3 haplotype associated with lower beta-amyloid deposition in the brain and its frequency was decreased among AD patients compared to the -219G/ε3 haplotype (Myllykangas et al. 2002). Moreover, the +113G-allele and the +113G/ε3 haplotype associated with increased neuropathologically verified AD. Mui and colleagues found the +113G-allele to be statistically significantly associated with AD, but the significance of the result disappeared when the ε4-allele was used as a covariate in the analysis (Mui et al. 1996). The APOE -219T/T genotype associated with lower plasma apoE concentration compared with -219G-allele carriers in a large multicentre case-control study of MI, also within ε3/ε3 carriers (Lambert et al. 2000). Furthermore, the -219T-allele was associated with an increased risk of MI, independent of the APOE ε2/ε3/ε4 polymorphism. However, no association was found between the -219G/T polymorphism and plasma lipid or lipoprotein levels. In a Finnish study the -219T-allele, as well as the ε4-allele, associated with early-onset CHD (Viitanen et al. 2001). The alleles were also shown to associate with characteristics of insulin resistance syndrome, both separately and together. Another study done in the UK investigated the effects of APOE ε2/ε3/ε4 and -219G/T polymorphisms on the severity of CHD (Ye et al. 2003). Their results show that the -219T/T genotype associates with an increased severity of CHD, independent of APOE ε2/ε3/ε4, but there were no statistically significant differences in plasma cholesterol or TG values between the different APOE -219G/T genotype groups. One study investigated the possible association of the -219G/T polymorphism with carotid plaques or IMT, but found no statistically significant effects (Debette et al. 2006). To our knowledge neither the -219G/T nor the +113G/C polymorphism have been studied relating to early markers of atherosclerosis, atherosclerotic lesion areas or the risk of ischemic stroke.

Aims of the study

ApoE plays an important and functional role in plasma lipoprotein metabolism and therefore, contributes to the development of atherosclerosis e.g. in coronary arteries, in the abdominal aorta, as well as in cerebral arteries. The APOE ε4 allele of the APOE ε2/ε3/ε4 polymorphism is associated with increased plasma lipid concentrations and has been shown to associate with an increased risk of atherosclerosis; however its role in ischemic stroke has remained controversial. The most common genotype group, APOE ε3/ε3 carriers, has been overlooked in many studies investigating the role of the APOE gene in atherosclerosis or ischemic stroke. Furthermore, the roles of two other existing polymorphisms, -219G/T and +113G/C, in the APOE gene promoter region, which affect or are thought to affect APOE transcription, are less studied. Moreover, functional studies on +113G/C are still missing. These APOE polymorphisms might also be associated with the risk of atherosclerosis or stroke, and were therefore selected for this study.

The specific aims of the present study were:

1. To study the role of APOE gene promoter polymorphisms and haplotypes in lipoprotein metabolism, especially within APOE ε3/ε3 carriers.

2. To study APOE promoter polymorphisms in lipoprotein metabolism in a longitudinal setting and to search for associations between the APOE promoter polymorphisms and markers of subclinical atherosclerosis.

3. To examine the relationship between APOE promoter genotypes and early atherosclerotic lesion areas and to search for genotype-environment interactions.

4. To explore the role of APOE promoter polymorphisms in the development of intracranial atherosclerosis and as predictors of ischemic stroke.

5. To study the role of the APOE promoter polymorphism +113G/C in regulating APOE transcription.

Study subjects

1. Clinical series

1.1. A random sample of Finnish middle-aged men (I)

Originally, 300 men aged 50-59 years were randomly selected from ten age-cohorts (n = 9058), living in the city of Tampere in Finland. From those 300 men who were invited to participate, 223 (74%) agreed, 33 refused and 44 did not answer or could not be reached. Detailed medical histories were collected, with particular emphasis on cardiovascular and metabolic diseases, as well as chronic medications. From 217 participants all the required data including APOE genotypes was obtained, and these were included in the statistical analyses. The study was approved by the Ethics Committee of the UKK Institute and all participants gave their written informed consent.

1.2. Cardiovascular Risk in Young Finns Study (II)

The Cardiovascular Risk in Young Finns Study was launched in 1980, in five university hospital cities in Finland. 3596 randomly selected boys and girls aged 3, 6, 9, 12, 15 and 18 years entered at the start of the study. All subjects participating in 1980 were invited to follow-up studies in 1983 and 1986 and the 21 year follow-up in 2001. Cardiovascular risk factors, including body mass index (BMI), blood pressure, smoking, alcohol consumption and serum lipids were measured in 2001. Additionally, carotid artery IMT, FMD and CAC were measured by ultrasonography. This study consisted of a sub-sample of 928 subjects from the young Finns Study. The study was approved by all local Ethics Committees and all participants gave their written informed consent in 2001 and their parents gave it in 1980.

1.3. The Belgian Stroke Study (III)

The Belgian Stroke Study (BSS) was a case-control study including 237 cases and 326 ethnic- and gender-matched controls. The patients were selected from 7 stroke units databases in Belgium and had SVO or LVA stroke [according to the TOAST classification (Adams et al. 1993b)] occurring between 45 and 60 years of age. The control group consisted of healthy volunteers, without stroke and living in the same area as the cases. All study subjects were of Central European origin (>90% Belgians). The study protocol was

approved by the Ethics Committees of all participating hospitals. Written informed consent was obtained from all patients before study entry.

2. Autopsy series

2.1. Tampere Autopsy Study (III)

The TASTY series collected in Tampere comprised 388 men (mean age 59.7 years) and 216 women (mean age 68.2 years) aged 0-97 years, who were subjected to medicolegal autopsy at the Department of Forensic Medicine, University of Tampere during 2002-2004. The cause of death was disease (most of which was of cardiac origin) in 56.6%, accident in 26.8%, suicide in 11.8%, suspected medical error in 2.5%, homicide in 0.5% and unknown in 1.5% of the cases. The study was approved by the Board of Medicolegal Affairs of Finland.

2.2. Helsinki Sudden Death Study (III and IV)

The HSDS comprised two autopsy series collected at the Department of Forensic Medicine, University of Helsinki during 1980-1981 (A-series, n = 400) and 1991-1992 (B-series, n = 300), to study the background and risk factors behind sudden pre-hospital death. Medico-legal autopsies were performed if there was a sudden out-of-hospital death. The HSDS contained 700 Finnish men in total, aged 33 to 70 (mean age 53 years, median 54 years) and whose cause of death were cardiac in 41%, other disease in 20%, and suicides or accidents in 39% of the cases. Atherosclerotic lesion areas were measured in coronary arteries and the abdominal aorta. A spouse, relative or close friend of the deceased was interviewed within two weeks post-mortem, in order to collect data on possible risk factors for atherosclerosis. This was possible in 500 cases. The questionnaire included questions concerning hypertension, diabetes, as well as smoking and drinking habits. The Ethics Committee of the Department of Forensic Medicine, University of Helsinki, approved the study.

Methods

1. DNA extraction and APOE genotyping

In study I, DNA was extracted from lymphocytes using a commercial kit (Qiagen inc. Valencia CA, USA). In study II DNA was extracted from whole blood using a DNA extraction robot (Biorobot M48, Qiagen GmbH, Hilden, Germany) with the MagAttract blood Mini M48 kit (Qiagen GmbH, Hilden, Germany). In study III DNA was extracted from whole blood using a commercial kit (Qiagen inc. Valencia CA, USA) (BSS). In the TASTY series DNA was isolated from ~5ml of frozen post-mortem blood, using the standard salt precipitation method. APOE ε2/ε3/ε4 genotyping was performed as described by Hixson & Vernier (Hixson and Vernier 1990). In the A-series of HSDS, DNA was extracted from paraffin-embedded cardiac muscle samples using the salt precipitation method. In the B-series of HSDS, DNA was isolated from frozen (-70°C) cardiac muscle with the standard phenol-chloroform method.

The polymorphisms genotyped in these studies were APOE ε2/ε3/ε4 (SNPs rs429358 and rs7412), -219G/T (rs405509) and +113G/C (rs440446). APOE promoter genotyping was done using the 5' nuclease assay (Holland et al. 1991) and fluorogenic allele-specific TaqMan MGB probes in the ABI Prism® 7900 HT sequence detection system (BSS and TASTY), or with the ABI Prism® 7000 sequence detection system (HSDS) (Applied Biosystems, California, USA). Some of the studies (I and III:HSDS) used assays we designed and for identification of the promoter polymorphism -219G/T; the probe sequences were 5'-VIC-CCCAGTAATcCAGACAC-3' and 5'-6-FAM-CGCCCAGTAA TaCAGA-3'. The probe sequences for the identification of the intron polymorphism +113G/C were 5'-VIC-ACCCTGGGAAgCC-3' and 5'-6-FAM-CCCTGGGAAcCCCT-3'. The rest of the studies (study II, study III: BSS and TASTY) used pre-designed Applied Biosystems assays.

Universal PCR conditions were used: first 2 min at 50°C and 10min at 95°C, and then 40 cycles of 95°C for 15sec and 60°C for 1min. The PCR reaction was done in 50µl, 25µl (96-well plate) or 5µl (386-well plate) reactions, containing 1x Master mix with a Rox passive reference (MedProbe, Oslo, Norway) or TaqMan® Universal PCR Master Mix with AmpErase® UNG (Applied Biosystems, CA, USA), 900nM of each primer, 200nM of each probe and the template DNA. The genotyping was performed manually (ABI Prism 7000) or called automatically by SDS software (ABI Prism 7900HT).

2. Biochemical analyses

In study I, blood samples were taken after a 12-hour fasting period, and sera for all determinations, but lipoproteins were stored immediately at -70°C until analyzed. Lipoprotein fractions were assessed from fresh blood samples by ultracentrifugation. Cholesterol was measured from serum and lipoprotein fractions using an enzymatic method (CHOD-PAP, Boehringer Mannheim, Germany). TGs were also measured by an enzymatic method (GPO-PAP, Boehringer Mannheim, Germany). Apolipoprotein B (apoB) was analyzed using an immunonephelometric method (Behring, Behringwerke AG, Marburg, Germany) and lipoprotein(a) using a two-site immunoradiometric assay (Pharmacia, Uppsala, Sweden).

In study II, blood samples were taken after a 12-hour fasting period in 2001. Serum cholesterol and TG concentrations were determined enzymatically (Olympus System Reagent; Olympus Diagnostica GmbH, Hamburg, Germany) in a clinical chemistry analyzer (AU400, Olympus Optical, Ltd., Mishima, Japan). HDL-C was analyzed after precipitation of VLDL and LDL using dextran sulfate and $MgCl_2$. LDL-C was calculated using the Friedewald formula: LDL-C = total cholesterol (TC) – HDL-C – TG / 2.2 (Friedewald et al. 1972). Subjects having a TG concentration greater than 4mmol/l (within the APOE ε3/ε3 carriers there were 6) were excluded from the statistical analyses. Serum apolipoproteins apoA-I and apoB were analyzed immunoturbidometrically (Orion Diagnostica, Espoo, Finland). Sensitive CRP was measured using a latex turbidometric immunoassay (Wako Chemicals GmbH, Neuss, Germany). Homocysteine concentrations were analyzed with a microparticle enzyme immunoassay kit (Imx assay, Abbott Laboratories, Tokyo, Japan).

3. Ultrasound measurements

In the Young Finns Study (II), ultrasound measurements were performed between September 2001 and January 2002 using Sequoia 512 ultrasound mainframes (Acuson, CA, USA) with a 13.0 MHz linear array transducer. Ultrasound technicians followed a standardized protocol to scan the left common carotid artery. The image was focused on the posterior (far) wall of the left common carotid artery. From an angle showing the greatest distance between the lumen-intima interface and the media-adventitia interface, a magnified image was recorded. The distance between the media-adventitia interface and the lumen-intima interface defined the carotid artery IMT. A 5s moving scan was recorded and stored in digital format and one reader blinded to participants' details analyzed the digitally stored scans using ultrasonic calipers. From the 5-second clip image, the best quality end-diastolic frame was selected and to derive maximal carotid IMT, at least four measurements of the common carotid far wall were taken approximately 10mm proximally to the bifurcation. The reproducibility of IMT measurements was assessed by re-examining a random sample of 60 participants 3 months after the initial visit. Furthermore,

the reproducibility of IMT image analysis was assessed by reanalyzing 113 scans by a second observer.

To asses carotid artery elasticity indices, the best-quality cardiac cycle was selected from the 5s clip images and the common carotid diameter, 10mm from the carotid bifurcation was measured at least twice at both the end diastole and end systole. The means of these measurements were used as the end-diastolic and end-systolic diameters. Using these diameters and simultaneous brachial blood pressure measurements, CAC was then calculated as follows: ([Ds-Dd]/Dd)/(Ps-Pd), where Ds is the systolic diameter; Dd, diastolic diameter; Ps, systolic blood pressure and Pd, diastolic blood pressure. The between-visit coefficient of variation (CV) was also estimated.

To evaluate brachial artery FMD, the left brachial artery diameter was measured at rest and during reactive hyperemia. Increased flow was induced by inflation of a pneumatic tourniquet placed around the forearm to a pressure of 250 mm Hg for 4.5 min, followed by release. Three arterial diameter measurements were performed at end-diastole at rest and 40, 60 and 80 seconds after cuff release. The vessel diameter in scans after reactive hyperemia was expressed as a percentage relative to the resting scan value (100%). The average of three measurements at each time point was used to derive the maximum FMD. The 3-month between-visit CV was assessed for brachial artery diameter and for FMD measurements.

4. Defining ischemic stroke and assessing the intracranial atherosclerosis

In BSS series (III), ischemic stroke due to SVO or LVA stroke was defined by the TOAST classification (Adams et al. 1993b). In the Finnish autopsy series (TASTY & HSDS), the atherosclerosis of each of the nine branches of the circle of Willis was scored semi-quantitatively as follows: 0 = normal, 1 = slight (streaks with or without elevated fibrous lesions), 2 = moderate (fibrous lesions that cause <50% stenosis), 3 = severe (>50% stenosis with extensive atherosclerosis; fatty, fibrous, calcified lesions), giving a range of scores from 0 to 27. This circle of Willis atherosclerosis score, reflecting large vessel pathology, was used in statistical analyses.

5. Measuring the fatty streak area

At autopsy, the proximal parts of the left anterior descending coronary artery (LAD), right coronary artery (RCA) and the abdominal aorta were collected for analysis. To measure the area of different types of atherosclerotic lesions, the vessels were dissected free, opened and attached to a card, and then fixed in 10% formalin. The arteries were stained for fat with Sudan IV. The measurements were based on the protocols of two international studies: The IAP, Standard Operating protocol 1962 (Guzman et al. 1968) and the WHO study group in Europe (Uemura et al. 1964). A flat lesion that stained red with Sudan IV without showing any other types of changes was regarded as a fatty streak. The area

involved with fatty streaks and the total areas of the coronary segments were assessed by computer-assisted planimetry, which measures the area in square millimeters. The lesion areas were expressed in percentages (%), by dividing the lesion area by the total area of the arterial wall and multiplying by 100%.

6. Haplotype reconstruction

APOE haplotypes were calculated using the Stata (8.0) software (I) or the PHASE program (version 2.0.2) (Stephens et al. 2001, Stephens and Donnelly 2003) (II & IV). In studies I and II, the haplotypes were calculated only within ε3/ε3 carriers. In study II, the study subjects were categorized into carriers and non-carriers of each haplotype: -219G/+113G/ε3, -219T/+113G/ε3, -219T/+113C/ε3 and -219G/+113C/ε3, the last of which was excluded from statistical analyses because there were only three carriers of this haplotype. In study IV, haplotypes were calculated for the whole study population but only haplotypes with frequency >5% were included into the statistical analyses. Within ε3/ε3 carriers, the study subjects were categorized into three haplotype groups as follows: homozygotes of haplotype -219G/+113G/ε3 (i.e. GG/GG), heterozygotes of haplotype -219G/+113G/ε3 and -219T/+113C/ε3 (i.e. GG/TC) and homozygotes of haplotype -219T/+113C/ε3 (i.e. TC/TC).

7. Functional studies

7.1. Cell culture

The HepG2 cell line was used in study IV. Cells were grown at 37°C in a 5% CO_2 atmosphere in 1 x MEM (Gibco) containing 10% fetal bovine serum, L-glutamine, penicillin, streptomycin, 1 x non-essential amino acids and 1% sodium pyruvate.

7.2. Cloning

To study the possible effects of the APOE +113G/C polymorphism on APOE transcription, a 50bp fragment of the first intron of the APOE gene was cloned into a pGL2-promoter (SV40 promoter) vector (Promega, Madison, USA). The oligonucleotide sequence used for cloning was 5'-GATCGGATCCTAGAAAGAGCTGGGACCCTGGG AA**G/C**CCCTGGCCTCCAGGTAGTCTCAGGAGGATCCGATC-3', where the +113G/C locus is indicated in bold and the restriction site and non-template sequences are underlined. Both +113G- and C-alleles were cloned in two positions, upstream (BglII site) and downstream (BamHI site) relative to the luciferase gene, as well as in both orientations (sense: 5' à 3' and antisense: 3' à 5'). The orientation and correct insert sequences of the reporter constructs were all verified by sequencing prior to use in the

reporter assay. We used the sequencing primers GL1 and RV4 from Promega (Promega, Madison, USA). In total, we used eight different promoter constructs in the luciferase assays.

7.3. Transfections and luciferase assays

The Luciferase Assay system (Promega Corporation, Madison, USA) was used to study the differences in gene expression between the APOE +113G- and C- alleles. Triplicate experiments were performed using the eight different promoter constructs (described in section 7.2.).

Transfections were performed in 12-well tissue-culture plates using the FuGENE® HD transfection reagent (Roche, Indianapolis, USA) at a ratio of 5:2 (DNA:FuGENE). We cotransfected 300ng of the pcDNA3.1-LacZ vector (producing ß-galactosidase; Invitrogen) with 700ng of DNA from each luciferase construct. An empty pGL2-promoter luciferase vector was used as a control. After 24-hours the cells were harvested and lysed in 150µl of reporter lysis buffer (Promega Corporation, Madison, USA). Luciferase activity in cell lysates was assayed using the Luminoskan Ascent (Thermo Labsystems) by incubating 20µl of cell extract with 100µl of Luciferase assay reagent. For ß-galactosidase activity assays, 20µl of cell extract was mixed with 66µl of 1x ONPG (2-nitrophenyl ß-D-galactopyranoside), 201µl of 0.1M sodium phosphate and 3µl of 100X Mg^{2+} solution (0.1 M $MgCl_2$ and 4.5M ß-mercaptoethanol) and incubated for 10-30 minutes at 37°C. Finally the optical density of the solutions was measured at a wavelength of 420nm.

Luciferase activities were normalized for transfection efficiency by dividing by the ß-galactosidase activity in the same extract. The results were displayed relative to the empty luciferase vector.

7.4. Preparation of nuclear extract

Nuclear extracts were obtained from HepG2 cells with a variation in the method of Dignam *et al*. (Dignam et al. 1983). Cells were washed with ice-cold 1 x PBS and resuspended in buffer A [20mM HEPES, pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.1mM EDTA, 0.5mM phenylmethylsulfonyl fluoride, 0.5mM dithiothreitol (DTT), protease inhibitor cocktail (Roche)] and kept on ice for 15 min. The cell lysates were then passed ten times through a 22-gauge needle. After one minute of centrifugation at 12 000g, the nuclei were resuspended in buffer C [20mM HEPES, pH 7.9, 1.5mM $MgCl_2$, 420mM NaCl, 0.2mM EDTA, 1mM DTT, 20% glycerol, with freshly added complete protease inhibitor coctail tablets (Roche)]. Then the nuclei were sonicated for 4x15s with 40% amplitude and 3mm stepped microtip using Sonics Vibra Cell TM 505 sonicator (the solution was incubated on ice in between sonications). Finally, the reaction mix was centrifuged for 15 min at 12 000g, and the supernatant collected and stored at -80°C until use.

7.5. Promoter analysis

We used the web-based tool ConSite (Sandelin et al. 2004) to analyze orthologous pairs of human and mouse APOE sequences, spanning the +113 region using 60% cut-off values for sequence conservation. We also used Genomatix MatInspector software (Cartharius et al. 2005) to predict which transcription factors would bind specifically to either allele of the +113G/C polymorphism.

7.6. Fusion protein production

The GST-RBP-Jκ fusion protein was produced in the Escherichia coli BL21-strain [the plasmid pGST-RBPJ (Liang et al. 2002) was generously provided by Dr. Liang from Emory University, Atlanta]. The fusion protein was purified using Glutathione Sepharose 4B beads (Amersham Biosciences) according to the manufacturer's instructions and eluted using 10mM reduced glutathione. The purity of the protein was checked by running the sample on SDS-PAGE and visualizing the proteins with Coomassie stain. The amount of GST-RBP-Jk protein was quantitated (Kodak Molecular imaging software v.4.0.4) against BSA protein standards. The purified GST-RBP-Jκ was used in an electrophoretic mobility shift assay (EMSA), to study its binding to APOE +113G- and C-probes (see section 7.7.).

7.7. EMSA

EMSAs were carried out using the following oligonucleotide sequences, where the +113G/C polymorphic site is underlined and in bold: 5'-TAGAAAGAGCTGGGACCCT GGGAA**G**CCCTGGCCTCCAGGTAGTCTCAGGA-3' and 5'-TAGAAAGAGCTGGGA CCCTGGGAA**C**CCCTGGCCTCCAGGTAGTCTCAGGA-3'. These were annealed to their complementary sequences to form double stranded DNA oligonucleotide probes. The probes were then end-labeled with γ-^{32}P-ATP (Perkin Elmer, USA) using 10U of T4 polynucleotide kinase (Fermentas) and purified using ProbeQuant™ G-50 Micro Columns (Amersham Biosciences, England).

Pre-binding of nuclear extracts to the non-specific competitor DNA poly(dI-dC) (Sigma-Aldrich) was carried out on ice for 30 min in bandshift buffer containing 50mM Tris-HCl, pH 7.5, 125mM NaCl, 2.5mM DTT, 0.5mM EDTA, 1mM $MgCl_2$ and 4% glycerol. For competition studies, a 10-, 100- or 200- fold excess of unlabeled (cold) +113G/C oligonucleotide was added to the preincubation mixture. For supershift experiments, 2 μg of the antibodies p50 (sc-7178 X), p65 (sc-109 X) or RBP-Jκ (sc-28713X) from Santa Cruz Biotechnology Inc. (U.S.A.) were added to the preincubation mixture. After preincubation, purified labeled double stranded probes were added to the reaction mixture and incubated at +4°C for 30 min. The reaction products were analyzed on 4.5-7.5% non-denaturing polyacrylamide gels, pre-run at 4°C in 1x TBE at 180 V for a minimum of 30 min, then run at 180 V for 3 h. Gels were dried and autoradiographed using KODAK BioMax™ MS film.

The binding of GST-RBPJκ to the ^{32}P-labeled APOE +113G/C probes was studied as follows. The reaction mixture included bandshift buffer, probes (+113G or +113C) and

either 360ng of GST-protein or an increasing amount (20, 50, 100 or 200ng) of GST-RBPJκ fusion protein. Gel electrophoresis and visualization were performed as described above.

8. Statistical analyses

Analysis of variance (ANOVA) or analysis of covariance (ANCOVA) was used to compare the means of continuous variables, and the Chi-square test to compare categorical variables in different APOE genotype/haplotype groups. Non-normally distributed values were logarithmically or square root transformed before AN(CO)VA analyses, but all results are presented as crude values. Longitudinal lipid data were analyzed by repeated measures ANOVA (RANOVA) using APOE promoter genotypes/haplotypes as categorical factors (one at a time) and LDL-C or TC concentrations at different years of follow-up (1980, 1983, 1986, and 2001) as dependent repeated variables (II). In the case of a statistically significant main effect, posthoc tests (with Bonferroni corrections) were used to compare the differences between the genotype/haplotype groups.

Interactions between the APOE genotype and other known atherosclerotic risk factors (age, BMI, hypertension, diabetes, smoking, alcohol use) were tested using an ANCOVA custom model. Genotype and one risk factor at a time, as well as their interaction term, were introduced into the custom model (continuous variables were introduced as covariates and categorized variables as factors). In study III binary logistic regression analysis was used to study associations between APOE genotypes and ischemic stroke. In study IV the transcriptional efficiency in different promoter constructs was compared using Kruskal-Wallis test and pairwise tests were performed using Mann-Whitney U test.

In study I, haplotype analyses were only performed if the individual polymorphisms associated statistically significantly with the same variable. The VLDL-C and TG values were categorized into two groups using the median (Md) as a cut-off value.

Statistical calculations were done using SPSS (versions 12.0 and 14.0) on a personal computer and a two-tailed p-value < 0.05 was considered to be statistically significant.

Results

1. The APOE allele frequencies and haplotypes

The distribution of APOE genotypes and allele frequencies in the different study series are provided in Table 2. All genotypic distributions of alleles were in Hardy-Weinberg equilibrium.

Table 2. *The APOE allele frequencies in the five study populations.*

		-219		+113		ε2/ε3/ε4		
		G	T	G	C	ε2	ε3	ε4
UKK (I)	ALL	0.58	0.42	0.75	0.25	0.06	0.75	0.19
	ε3/ε3	0.68	0.32	0.67	0.33	-	-	-
Young Finns Study (II)	ALL	0.54	0.46	0.72	0.28	0.04	0.76	0.20
	ε3/ε3	0.62	0.38	0.63	0.37	-	-	-
BSS (III)	ALL	0.53	0.47	0.65	0.36	0.08	0.77	0.15
	ε3/ε3	0.55	0.45	0.56	0.44	-	-	-
TASTY (III)	ALL	0.55	0.45	NS	NS	0.06	0.78	0.17
	ε3/ε3	0.62	0.38	NS	NS	-	-	-
HSDS (III & IV)	ALL	0.56	0.44	0.74	0.26	0.06	0.75	0.19
	ε3/ε3	0.63	0.37	0.65	0.35	-	-	-

NS = not studied

The APOE haplotypes were determined for studies I, II and IV. Within the APOE ε3/ε3 carriers, in total four different haplotypes were observed: -219G/+113G/ε3, -219T/+113G/ε3, -219T/+113C/ε3 and -219G/+113C/ε3. For study I, all haplotypes are presented, but in study II the three carriers of the -219G/+113C/ε3 haplotype were excluded from the statistical analyses. In study IV, the haplotypes regarding the whole study population with a frequency >5% were -219G/+113G/ε3, -219G/+113G/ε2, -219T/+113G/ε4 and -219T/+113C/ε3. Within the ε3/ε3 carriers (IV), haplotypes were grouped as follows: homozygotes of haplotype -219G/+113G/ε3 (i.e. GG/GG), heterozygotes of haplotype -219G/+113G/ε3 and -219T/+113C/ε3 (i.e. GG/TC) and homozygotes of haplotype -219T/+113C/ε3 (i.e. TC/TC). Haplotype -219G/+113C/ε3 was not detected in study IV.

2. Association of APOE promoter genotypes and haplotypes with lipoprotein concentrations (I & II)

As it was previously known that APOE ε2/ε3/ε4 polymorphism associates with serum cholesterol levels, we wanted to study whether the APOE promoter polymorphisms -219G/T and +113G/C could further explain the variation in serum lipid, apolipoprotein and lipoprotein concentrations, especially within the most common genotype group - APOE ε3/ε3 carriers.

When the APOE promoter genotype groups were compared within the APOE ε3/ε3 carriers, statistically significant differences in lipid and apolipoprotein concentrations were detected. The -219G/T polymorphism associated statistically significantly with VLDL-C, HDL-C, HDL_2-C, apoB and TG concentrations (Table 3). Compared to -219G/G genotype, the carriers of -219T/T genotype had lower concentrations of VLDL-C (0.43 vs. 0.75 mmol/l, p-value 0.068), apoB (1.13 vs. 1.37 g/l, p-value 0.013) and TG (1.06 vs. 1.68 mmol/l, p-value 0.018). On the contrary, HDL-C concentration was higher in -219T/T carriers compared to +219G/G genotype (1.41 vs. 1.22 mmol/l, p-value 0.033), as was the HDL_2-C (1.02 vs. 0.84 mmol/l, p-value 0.040).

Table 3. *Plasma lipid, apolipoprotein and lipoprotein concentrations in APOE -219G/T genotype groups within APOE ε3/ε3 carriers. Adapted from* (Viiri et al. 2005)*, copyright (2005) with permission from Elsevier.*

	-219			
	G/G (n = 55)	G/T (n = 55)	T/T (n = 12)	p-value
Total cholesterol, mmol/	5.60 (0.95)	5.47 (0.87)	5.14 (0.96)	0.190
VLDL-cholesterol, mmol/l	0.75 (0.42)	0.62 (0.38)	0.43 (0.26)	**0.008**
LDL-cholesterol, mmol/l	3.63 (0.91)	3.62 (0.70)	3.30 (0.91)	0.343
HDL-cholesterol, mmol/l	1.22 (0.22)	1.23 (0.26)	1.41 (0.27)	**0.034**
HDL_2 –cholesterol, mmol/l	0.84 (0.22)	0.83 (0.25)	1.02 (0.26)	**0.033**
HDL_3 –cholesterol, mmol/l	0.38 (0.08)	0.40 (0.07)	0.40 (0.13)	0.665
ApoB, g/l	1.37 (0.30)	1.31 (0.30)	1.13 (0.27)	**0.016**
ApoA-I, g/l	1.47 (0.19)	1.48 (0.19)	1.58 (0.22)	0.218
Lp(a), mg/l	359 (466)	185 (253)	273 (290)	0.078
Triglycerides, mmol/l	1.68 (0.87)	1.44 (0.71)	1.06 (0.50)	**0.016**

Values are mean (SD). Differences between genotype groups were tested by analysis of covariance, smoking status as a covariate.

The +113G/C genotypes showed highly similar associations as the -219G/T genotypes within ε3/ε3 carriers (Table 4). Compared to the +113G/G genotype, the +113C/C genotype carriers had statistically significantly lower concentrations of VLDL-C (0.43 vs. 0.75 mmol/l, p-value 0.012), apoB (1.13 vs. 1.37 g/l, p-value 0.012) and TG (1.06 vs. 1.67 mmol/l, p-value = 0.021). The HDL-C concentration, on the other hand, was higher in the +113C/C carriers compared to the +113G/G (1.41 vs. 1.22, p-value 0.032). Compared to

the +113G/G genotype, Lp(a) concentration was lowest in the heterozygous +113G/C genotype group (171 vs. 376 mg/l, p-value 0.042).

Table 4. *Plasma lipid, apolipoprotein and lipoprotein concentrations in APOE +113G/C genotype groups within APOE ε3/ε3 carriers. Adapted from* (Viiri et al. 2005)*, copyright (2005) with permission from Elsevier.*

	+113			
	G/G (n = 54)	G/C (n = 56)	C/C (n = 12)	p-value
Total cholesterol, mmol/l	5.62 (0.93)	5.46 (0.90)	5.14 (0.96)	0.154
VLDL-cholesterol, mmol/l	0.75 (0.42)	0.62 (0.38)	0.43 (0.26)	**0.010**
LDL-cholesterol, mmol/l	3.65 (0.90)	3.60 (0.71)	3.30 (0.91)	0.308
HDL-cholesterol, mmol/l	1.22 (0.23)	1.23 (0.26)	1.41 (0.27)	**0.034**
HDL_2 –cholesterol, mmol/l	0.84 (0.22)	0.83 (0.25)	1.02 (0.26)	**0.033**
HDL_3 –cholesterol, mmol/l	0.38 (0.08)	0.40 (0.07)	0.40 (0.13)	0.595
ApoB, g/l	1.37 (0.30)	1.32 (0.30)	1.13 (0.27)	**0.014**
ApoA-I, g/l	1.47 (0.19)	1.48 (0.19)	1.58 (0.22)	0.224
Lp(a), mg/l	376 (481)	171 (214)	273 (290)	**0.043**
Triglycerides, mmol/l	1.67 (0.87)	1.46 (0.71)	1.06 (0.50)	**0.022**

Values are mean (SD). Differences between genotype groups were tested by analysis of covariance, smoking status as a covariate.

Haplotype analyses suggested that the second most common haplotype APOE -219T/+113C/ε3 was statistically significantly less often found in the above median VLDL-C and TG groups when compared to the most common -219G/+113G/ε3 haplotype (Table 5).

Table 5. *Estimated haplotype frequencies in groups below and above the median (Md) VLDL-cholesterol and triglyceride (TG) concentrations (total frequency is 246). Adapted from* (Viiri et al. 2005)*, copyright (2005) with permission from Elsevier.*

haplotype			VLDL				TG			
-219	+113	ε2/ε3/ε4	<Md	>Md	OR (95%CI)	p-value	<Md	>Md	OR (95%CI)	p-value
G	G	ε3	76	89	1	ref.	76	89	1	ref.
G	C	ε3	1	1	2.3 (0.1, 37.5)	0.57	1	1	0.9 (0.1, 13.9)	0.912
T	G	ε3	1	0	-	-	1	0	-	-
T	C	ε3	54	24	0.4 (0.2, 0.7)	0.001	50	28	0.5 (0.3, 0.8)	0.009

OR = odds ratio, CI = confidence interval, ref. = reference haplotype

2.1. *Longitudinal changes in cholesterol concentrations (II)*

Our first study (I) showed that the APOE promoter polymorphisms -219G/T and +113G/C associated with various serum lipid and apolipoprotein concentrations. Therefore, we wanted to test the associations in a longitudinal setting and for this purpose used a 21 year follow-up study series. In both sexes, LDL-C and TC concentrations changed statistically significantly over time ($p < 0.001$) and the difference between genotypes remained constant throughout the follow-up period (from 1980 to 2001). In males, there was a clear difference in LDL-C and TC values between the APOE promoter genotypes: the heterozygous -219G/T and +113G/C carriers had approximately 0.3 mmol/l higher LDL-C and TC concentrations when compared to the G/G carriers (Figure 5).

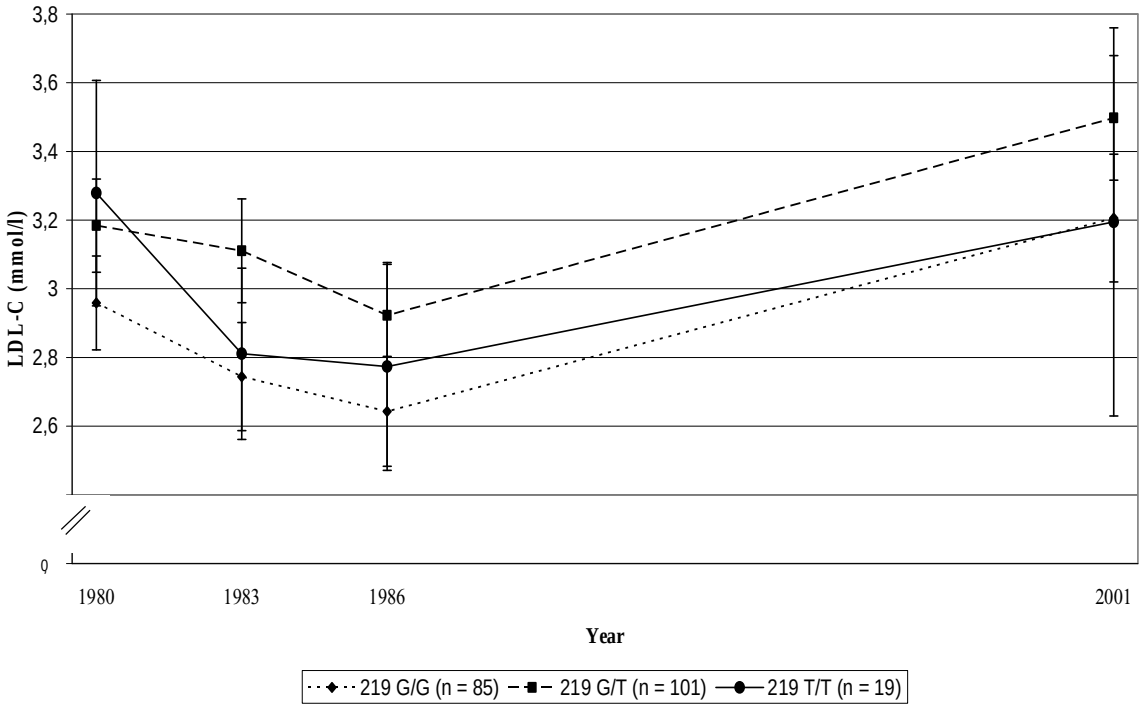

Figure 5. *Longitudinal changes (from 1980 to 2001) in LDL-cholesterol concentrations in the male APOE -219G/T genotype groups (G/G, G/T and T/T). RANOVA main effect for genotype p = 0.012. Error bars represent 95% confidence intervals of the mean. The figure for the +113G/C genotype groups is almost identical to this one. Total cholesterol concentrations followed the same pattern, but with higher values.*

Haplotype analyses showed that male carriers of the -219T/+113C/ε3 haplotype had about 0.3 mmol/l higher LDL-C concentrations throughout the follow-up period, compared to the non-carriers of this haplotype ($p = 0.007$). Moreover, the -219T/+113C/ε3 carriers had 0.2 mmol/l higher TC concentrations over time when compared to haplotype non-carriers ($p = 0.012$) (Figure 6). In females, LDL-C and TC concentrations changed over time, but cholesterol concentrations were not statistically significantly different between the APOE promoter genotypes or haplotypes.

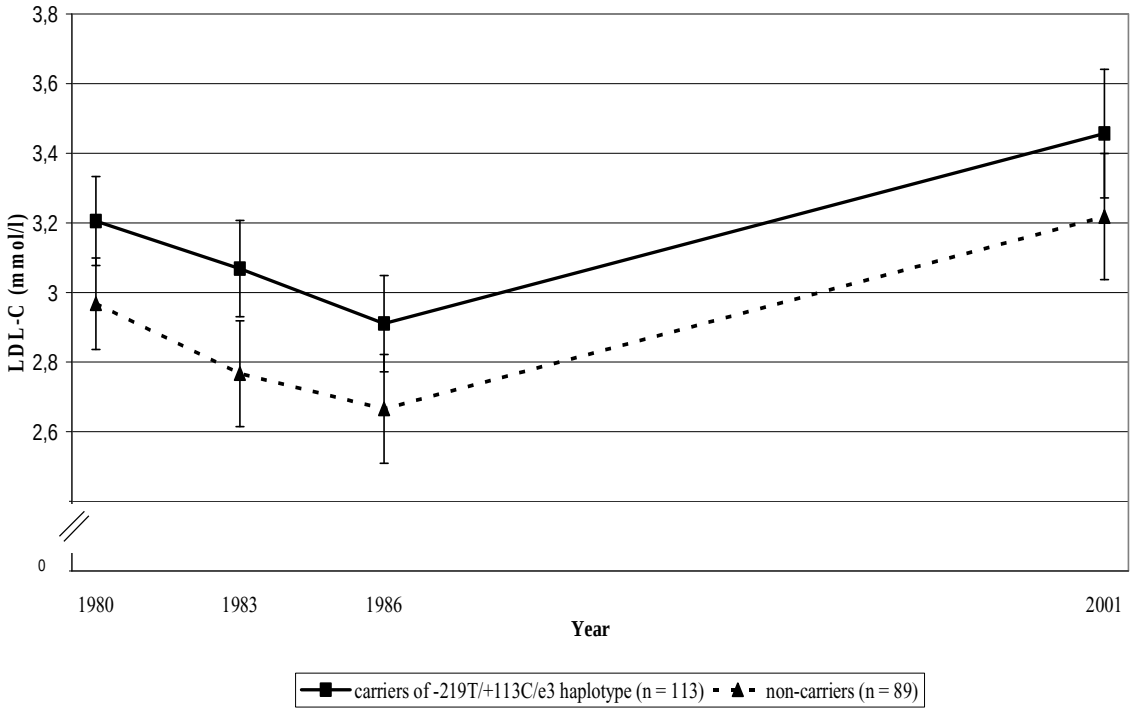

Figure 6. *The longitudinal changes (between the years 1980 to 2001) of LDL- cholesterol concentrations in the APOE –219T/+113C/ε3 haplotype carrier and non-carrier males (RANOVA, main effect for haplotype p = 0.012). Time-haplotype interaction was statistically non-significant.*

3. APOE promoter polymorphisms and early atherosclerotic changes (II & IV)

3.1. Ultrasound measurements (II)

Ultrasound methods can be used to study early atherosclerotic changes and it was previously unknown whether the APOE promoter polymorphisms -219G/T and +113G/C associate with these. We tested possible associations of the APOE promoter polymorphisms -219G/T and +113G/C with a structural marker of atherosclerosis (carotid artery IMT), a functional marker of endothelial health (FMD) and with a marker of the elasticity of large arteries (CAC). We did not detect any statistically significant differences in these markers between the studied APOE promoter genotypes in either sex. The between-visit CVs for IMT, CAC and FMD were 6.4%, 16.3% and 26.0%, respectively; CVs for diastolic carotid diameter and brachial artery diameter measurements were 2.7% and 3.2%, respectively.

3.2. Morphologic measurements (IV)

Using the HSDS autopsy data, we studied the association of the APOE promoter polymorphisms -219G/T and +113G/C and the APOE haplotypes with fatty streak areas measured in two coronary arteries (LAD and RCA) and the abdominal aorta. We also wanted to address the interesting issue of gene-environment interactions in determining atherosclerosis risk; hence we tested for APOE genotype-environment interactions regarding the fatty streak areas. We focused our analyses mainly on the APOE ε3/ε3 carriers, to control for the previously known effects of the APOE ε2/ε3/ε4 polymorphism on lesion areas (Ilveskoski et al. 1999) and to concentrate on detecting the possible effects of the two promoter polymorphisms.

Within the whole study population, including all the APOE ε2/ε3/ε4 genotypes, the APOE ε2/ε3/ε4 polymorphism did not associate statistically significantly with fatty streak areas in neither coronary arteries nor abdominal aorta and no statistically significant interactions with environmental factors were found. Within the APOE ε3/ε3 carriers, the promoter polymorphisms -219G/T and +113G/C were not independently associated with fatty streak areas, but there was a strong genotype-smoking interaction (-219G/T, interaction p = 0.009; +113G/C, interaction p = 0.004) on fat area in abdominal aorta (Table 6).

Table 6. *Mean (SD) fatty streak areas (%) within APOE ε3/ε3 carriers by -219G/T and +113G/C genotype groups in the Helsinki Sudden Death Study.*
Table modified from study IV.

	-219				
	G/G	G/T	T/T	p-value*	genotype-smoking interaction p-value †
LAD	3.7 (1.0)	4.4 (1.3)	4.7 (1.9)	0.261	0.993
RCA	3.7 (1.8)	4.0 (2.0)	4.4 (2.4)	0.783	0.467
AA	11.8 (1.7)	10.9 (1.9)	10.5 (1.2)	0.556	**0.009**
	+113				
	G/G	G/C	C/C		
LAD	4.0 (1.4)	4.5 (1.2)	3.9 (0.8)	0.551	0.629
RCA	3.7 (1.9)	4.0 (2.0)	4.5 (2.0)	0.752	0.910
AA	12.3 (1.9)	10.7 (1.7)	10.1 (1.2)	0.182	**0.004**

ε2+ = ε2/ε2 + ε2/ε3, ε4+ = ε3/ε4 + ε4/ε4; LAD = left anterior descending coronary artery, RCA = right coronary artery, AA = abdominal aorta, *p-value is from the analysis of variance, genotype as a factor. †Interaction p-value is from the analysis of covariance with genotype and smoking (smokers + ex-smokers vs. non-smokers) as factors. Data was square root-transformed before the analyses.

We then performed ANOVA analyses comparing the fatty streak areas of the abdominal aorta between different promoter genotype groups in groups of non-smokers and smokers separately. These analyses showed that when comparing to the -219G/G homozygotes within non-smokers, the -219T-allele carriers associated with statistically significantly larger abdominal aorta fatty streak areas (-219T+ vs. G/G: 12.7% vs. 5.9%, p = 0.007; Figure 7).

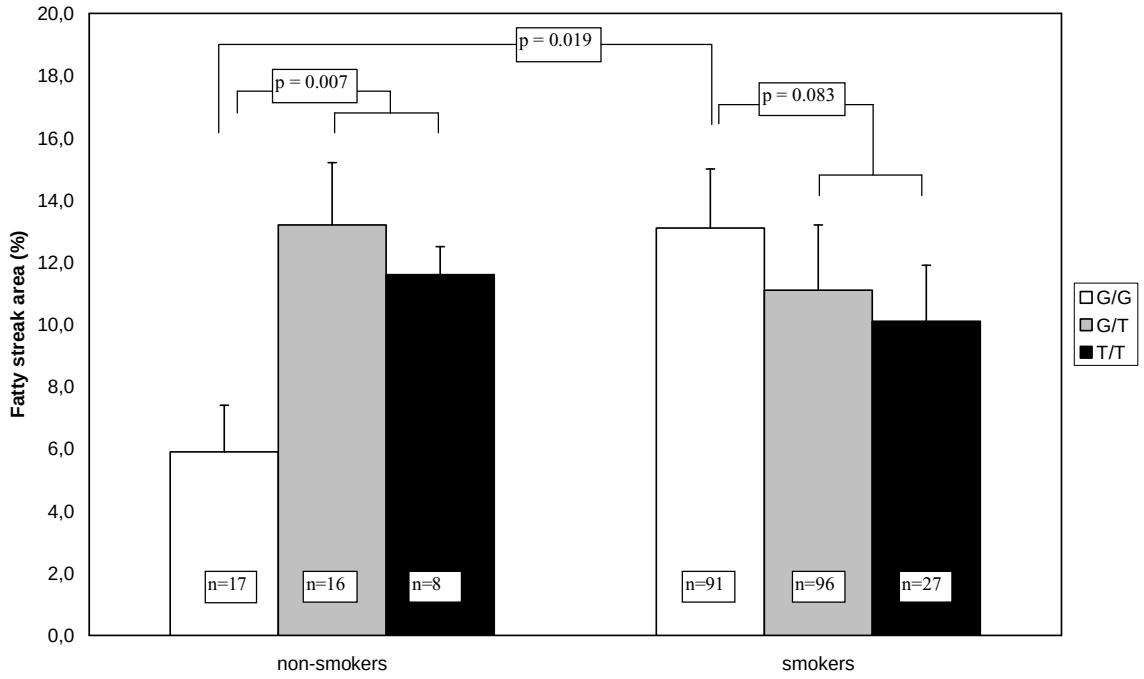

Figure 7. *Mean (+SD) fatty streak area (%) in the abdominal aorta in non-smokers and smokers by APOE -219G/T genotype groups within ε3/ε3 carriers. Figure modified from study IV.*

Additionally, the -219G/G genotype carriers had larger abdominal aorta fatty streak areas in smokers compared to non-smokers (-219G/G smokers 13.1% vs. -219G/G non-smokers 5.9%, p-value = 0.019; Figure 7, Table 7).

Table 7. *Abdominal aorta fatty streak area (%) by -219G/T genotype and smoking within APOE ε3/ε3 carriers.*

-219 genotype	smoking*	n	Fatty streak area, mean (SD)	p-value
G/G	no	17	5.9 (1.5)	ref.
G/T	no	16	13.2 (2.0)	0.203
T/T	no	8	11.6 (0.9)	1.000
G/G	yes	91	13.1 (1.9)	**0.019**
G/T	yes	96	11.0 (2.1)	0.234
T/T	yes	27	10.1 (1.8)	1.000

*smoking: no = never smoker, yes = current or ex-smoker
p-values are pairwise comparisons from analysis of variance

When comparing to the +113G/G homozygotes, the +113C-allele carriers associated with larger abdominal aorta fatty streak areas within non-smokers (+113C+ vs. G/G: 12.9% vs. 6.3%, p = 0.010; Figure 8). In smokers, on the other hand, the +113C-allele carriers

associated with smaller lesion area compared to the +113G/G genotype (+113C+ vs. G/G: 10.5% vs. 13.9%, p = 0.010).

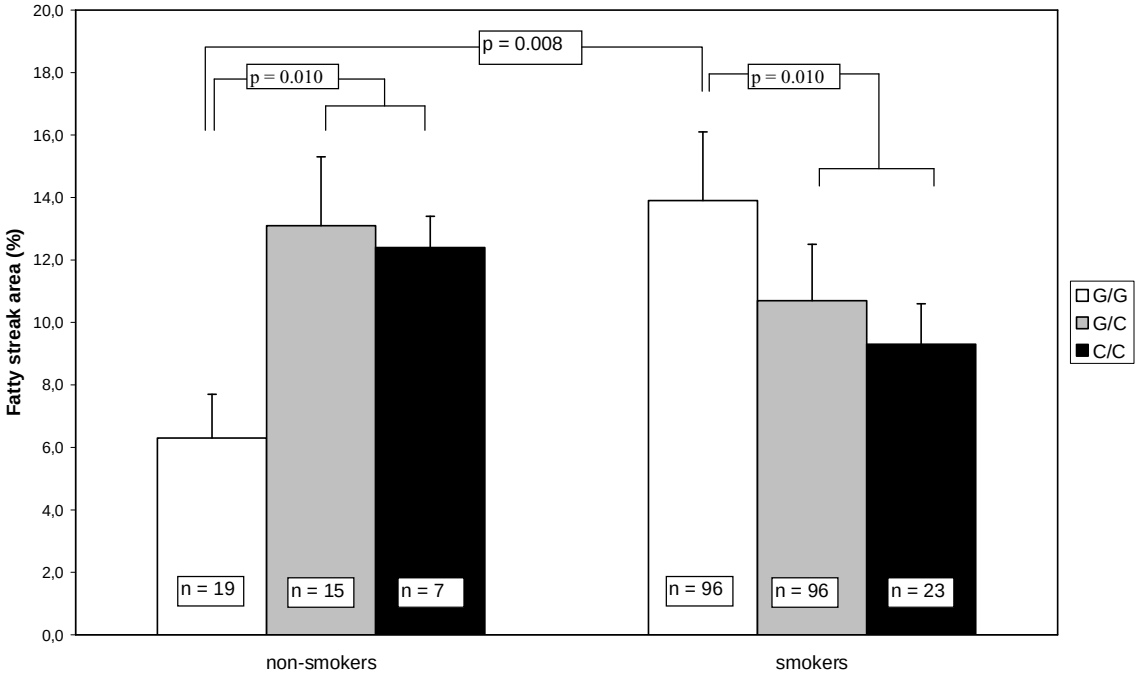

Figure 8. *Mean (+SD) fatty streak area (%) in the abdominal aorta in non-smokers and smokers by the APOE +113G/C genotype groups within ε3/ε3 carriers. Figure modified from study IV.*

Moreover, there was a statistically significant difference in abdominal aorta fatty streak area between the +113G/G non-smokers and smokers (+113G/G smokers 13.9% vs. +113G/G non-smokers 6.3%, p-value = 0.008; Figure 8 and Table 8).

Table 8. *Abdominal aorta fatty streak area (%) by APOE +113G/C genotype and smoking within APOE ε3/ε3 carriers.*

+113 genotype	smoking*	n	Fatty streak area, mean (SD)	p-value
G/G	no	19	6.3 (1.4)	ref.
G/C	no	15	13.1 (2.2)	0.329
C/C	no	7	12.4 (1.0)	1.000
G/G	yes	96	13.9 (2.2)	**0.008**
G/C	yes	96	10.7 (1.8)	0.438
C/C	yes	23	9.3 (1.3)	1.000

*smoking: no = never smoker, yes = current or ex-smoker
p-values are pairwise comparisons from analysis of variance

The APOE haplotypes did not differ regarding fatty streak areas of the studied arteries within the whole HSDS study population. When comparing other haplotypes to the -219G/+113G/ε3 non-smokers, the -219G/+113G/ε3 smokers and -219T/+113G/ε4 smokers had statistically significantly larger abdominal aorta fatty streak areas (12.6% vs. 8.2%, p-value 0.005 and 12.4% vs. 8.2%, p-value 0.041, respectively; Table 9).

Table 9. *Abdominal aorta fatty streak areas (%) in different APOE haplotype and smoking groups in the Helsinki Sudden Death Study population.*

-219/+113/ε2ε3ε4 haplotype	smoking*	n	Fatty streak area, mean (SD)	p-value
GG3	no	70	8.2 (1.7)	ref.
GG2	no	10	8.2 (0.5)	1.000
TG4	no	28	10.1 (2.0)	1.000
TC3	no	40	10.5 (2.0)	1.000
GG3	yes	366	12.6 (2.1)	**0.005**
GG2	yes	39	13.8 (2.5)	0.075
TG4	yes	131	12.4 (1.9)	**0.041**
TC3	yes	203	11.1 (1.8)	0.434

*smoking: no = never smoker, yes = current or ex-smoker, p-values are from analysis of variance

Within APOE ε3/ε3 carriers, there was a statistically significant APOE haplotype-smoking interaction on fatty streak area in the abdominal aorta (interaction p = 0.003). Further analyses showed that within non-smokers, compared to men carrying two copies of the -219G/+113G/ε3 haplotype (i.e. GG/GG) the haplotype -219T/+113C/ε3 carriers (i.e. GG/TC and TC/TC groups) had larger abdominal aorta fatty streak areas (12.9% vs. 5.9%, p = 0.008; Figure 9). Within smokers, the -219T/+113C/ε3 haplotype carriers associated with smaller abdominal aorta fatty streak areas compared to -219G/+113G/ε3 homozygotes (10.3% vs. 13.3%, p-value 0.024; Figure 9).

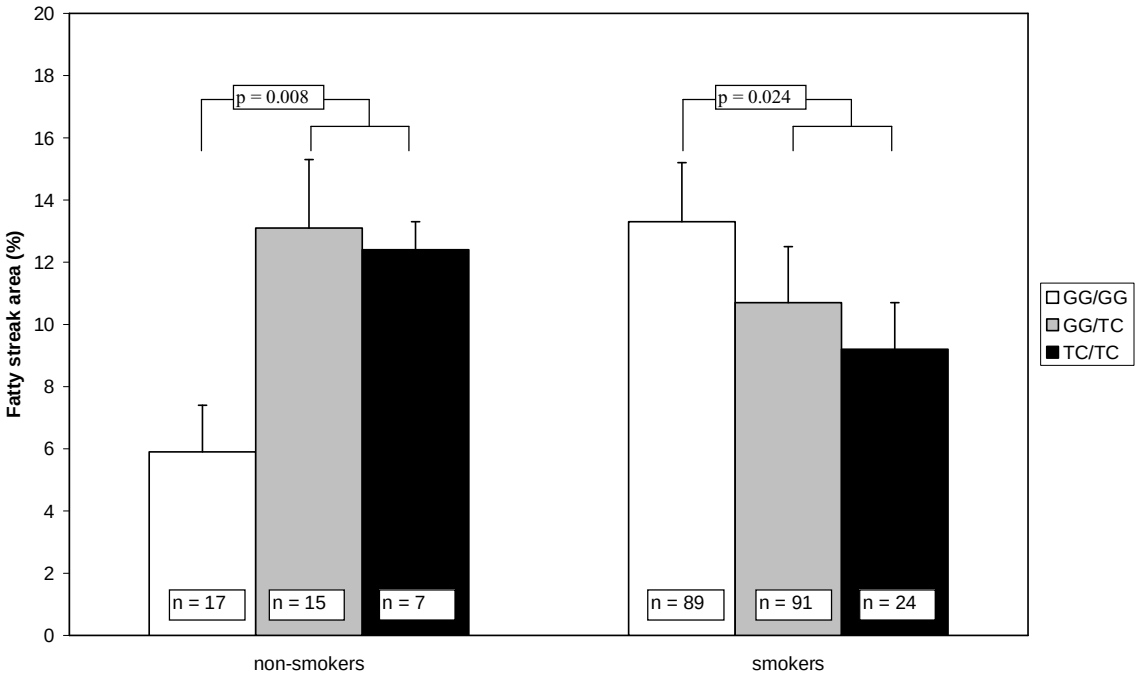

Figure 9. *Mean (+SD) fatty streak area (%) in the abdominal aorta of non-smokers and smokers in APOE haplotype groups within APOE ε3/ε3 carriers (GG/GG = homozygous for -219G/+113G haplotype, GG/TC = heterozygous for haplotypes -219G/+113G and for -219T/+113C and TC/TC = homozygous for -219T/+113C haplotype). Figure modified from study IV.*

4. APOE polymorphisms and ischemic stroke & intracranial atherosclerosis (III)

The association of the APOE promoter polymorphisms -219G/T and +113G/C with ischemic stroke or intracranial atherosclerosis had not previously been studied and there were controversial studies concerning the association of the APOE ε4 allele with ischemic stroke. We used the BSS series to study whether the APOE ε2/ε3/ε4 polymorphism or the promoter polymorphisms -219G/T and +113G/C associate with ischemic stroke.

We did not find statistically significant differences in the APOE ε4 allele frequencies between the ischemic stroke cases and control subjects. However, there were differences in the APOE promoter genotype frequencies between cases and controls (Table 10). Specifically, both the -219G- and +113G-allele carriers were at a higher risk of ischemic stroke compared to -219T/T or +113C/C genotype carriers (OR 6.2, 95%CI 1.6-24.3, p = 0.009 and OR 7.1, 95%CI 1.7-29.9, p = 0.007, respectively). However, if compared to homozygotes of the more common allele i.e. to the -219G/G or +113G/G carriers, the -219T- or +113C-allele carriers were not at statistically significantly higher risk of ischemic stroke (Table 10).

Table 10. *Genotype frequencies (%) for ischemic stroke cases (IS) and controls in the Belgian Stroke Study population. Adapted by permission from Macmillan Publisher Ltd: European journal of Human genetics* (Abboud et al. 2008)*, copyright (2008).*

Genotype	IS (n = 237)	Controls (n = 326)	OR (95% CI)	p-value
APOE				
ε 4+	25.2	25.2	0.9 (0.5-1.5)	0.715
ε 4-	74.8	74.8	1.0	ref.
APOE -219 (ε3/ε3)				
G +	84.1	76.4	**6.2** (1.6-24.3)	**0.009**
T/T	15.9	23.6	1.0	ref.
G/G	29.2	31.9	1.0	ref.
T+	70.8	68.1	1.2 (0.4-3.4)	0.744
APOE +113 (ε3/ε3)				
G +	85.0	76.4	**7.1** (1.7-29.9)	**0.007**
C/C	15.0	23.6	1.0	ref.
G/G	31.3	31.4	1.0	ref.
C+	68.7	68.6	1.4 (0.5-4.1)	0.561

IS = ischemic stroke, OR = odds ratio, CI = confidence interval, ref. = reference group.
The p-values are from binary logistic regression analyses with age, hypertension, hyperlipidemia, diabetes, obesity, smoking and alcohol consumption as covariates.
ε4+= carrier of the ε4 allele, ε4- = non-carrier of the ε4 allele, G+ = carrier of the G-allele

We used two Finnish autopsy studies (TASTY and HDSD) to explore whether the APOE polymorphisms associated with intracranial atherosclerosis. Our results showed that there was a tendency towards a higher atherosclerosis score in ε4 allele carriers when compared to non-carriers (5.4 vs. 4.8, p = 0.051). In men, ε4 allele carriers had statistically significantly higher atherosclerosis score compared to non-carriers (5.4 vs. 4.6, p = 0.044; Table 11).

The APOE -219G/T polymorphism was studied within the APOE ε3/ε3 genotype group. There were no statistically significant differences in the circle of Willis atherosclerosis score when comparing the -219G-allele carriers to the T/T homozygotes (Table 11). However, when the -219T-allele carriers were compared to the -219G/G homozygotes, there was a statistically significant difference in the atherosclerosis score in men (5.1 vs. 4.0, p-value 0.014).

Table 11. *Mean atherosclerosis score in the Helsinki Sudden Death Study and Tampere Autopsy Study series. Adapted by permission from Macmillan Publisher Ltd: European Journal of Human Genetics* (Abboud et al. 2008), *copyright (2008).*

	All			Men			Women		
	n	score	p-value	n	score	p-value*	n	score	p-value*
ε4+	289	5.4 (6.0)	**0.051**	222	5.4 (5.9)	**0.044**	67	5.6 (6.4)	0.596
ε4-	626	4.8 (5.6)		492	4.6 (5.4)		134	5.7 (6.5)	
Within ε3/ε3									
-219G+	458	4.8 (5.6)	0.960	370	4.6 (5.3)	0.591	88	5.9 (6.5)	0.399
-219T/T	75	4.9 (5.7)		57	4.7 (5.4)		18	5.4 (6.7)	
-219G/G	208	4.5 (5.3)	0.145	173	4.0 (4.9)	**0.014**	35	7.2 (6.6)	0.168
-219T+	325	5.0 (5.7)		254	5.1 (5.5)		71	5.1 (6.4)	

Values are presented as mean (standard deviation). Score = the circle of Willis atherosclerosis score. p-values are from analysis of covariance with age, gender and BMI, or *with age and BMI as covariates. ε4+ = carrier of the ε4 allele, ε4- = non-carrier of the ε4 allele, G+ = -219G/G and -219G/T, T+ = -219G/T and T/T.

5. The APOE +113G/C polymorphism and transcriptional activity (IV)

It has previously been shown that the APOE -219G/T polymorphism affects APOE transcription, the G-allele having higher transcriptional activity than the T-allele (Artiga et al. 1998b). It was also known that the APOE +113G/C polymorphism, identified in the late 1990's (Mui et al. 1996), is situated within the IRE1 region known to posses enhancer activity (Paik et al. 1988). However, the effect of the APOE +113G/C polymorphism on APOE gene transcription was unknown. We decided to address this issue, by first cloning a 50bp region (from +89 to +138) of the first intron of the APOE gene into a transcription vector. We used eight different promoter constructs and compared their effects on the transcriptional activity in HepG2 cells. Our results showed that most of the constructs carrying the C-allele possessed a higher transcriptional activity than the corresponding constructs with a G-allele. This was most prominent when we compared the C- and G-alleles cloned in a sense orientation upstream of the luc-gene, or cloned in an antisense orientation downstream of the luc-gene (Figure 10). The +113C-allele cloned in a sense orientation in front of the luciferase gene enhanced transcription 1.37 fold, whereas the +113G-allele repressed it 0.98 fold compared to the empty vector. The difference in promoter activity between these two constructs was statistically significant ($p = 0.002$, Table 12). The transcriptional activity for the antisense C-allele construct downstream of the luciferase gene was about 1.6-fold higher compared to the antisense G-allele downstream of the luciferase gene ($p = 0.002$; Figure 10).

Table 12. *Transcriptional activity of eight promoter constructs in HepG2 cells relative to the empty vector. Results are shown as mean (SD).*

+113 allele	orientation	location	transcriptional efficiency		p-value
empty vector	-	-	0	1	-
G	sense	5'	-0.024	0.98 (0.12)	**0.002**
C	sense	5'	0.372	1.37 (0.14)	
G	antisense	5'	-0.089	0.91 (0.12)	**0.002**
C	antisense	5'	0.133	1.13 (0.06)	
G	sense	3'	-0.208	0.79 (0.14)	0.818
C	sense	3'	-0.169	0.83 (0.16)	
G	antisense	3'	-0.044	0.96 (0.21)	**0.002**
C	antisense	3'	0.545	1.55 (0.14)	

Pairwise p-values are calculated using Mann-Whitney U test.

Transcriptional studies clearly showed a difference in the transcriptional activity between the APOE +113G- and C-alleles in HepG2 cells. Therefore, in order to study protein-binding to this intron region *in vitro*, we performed EMSA assays. These assays reveal whether the differential promoter activities were related to differences in protein binding to the APOE intron region. Our results showed distinct shifts in the +113G and +113C EMSA assays, suggesting a difference in the composition or amount of nuclear protein binding to the G- and C-alleles. Specifically, the G-allele band was constantly more intense compared to the C-allele band.

In earlier studies, NFκB was proposed to bind to the +113G/C region (Bullido and Valdivieso 2000). This was confirmed by our promoter analyses with ConSite (Sandelin et al. 2004), which showed that the p50 subunit of transcription factor NFκB binds with different affinities for the +113G- and C-alleles. The affinity score for the +113G allele was 7.0 and the +113C allele 8.7 units. Supershift EMSAs, with antibodies for p50 and p65 (tested to be functional in a Western blot), did not give any positive results (data not shown).

The Matinspector (Genomatix software package) analyses predicted that +113C would recruit the two additional transcription factors RBP-Jκ and RFX1. Core similarity and matrix similarity for RBP-Jκ were 1.0, and 0.843, and for RFX1 0.881 and 0.920, respectively. With RBP-Jκ we conducted an experiment to verify this prediction. RBP-Jκ recognizes the DNA sequence ag/c**CGTGGGAA**<u>c</u>ta/t (core recognition sequence written in bold, the underlined c corresponds to the +113C-allele) (Tun et al. 1994). Firstly, we tried to study the binding of RBP-Jκ to the +113 region using supershift EMSA, but the antibody for RBP-Jκ proved to be non-functional in a Western blot with HepG2 nuclear lysate (data not shown). Secondly, we decided to conduct EMSA using prokaryotically produced transcription factor RBP-Jκ. This experiment showed a four fold affinity of RBP-Jκ to the +113C-allele when compared to the G-allele and the intensity of the band in EMSA grew with increasing input of RBPJκ-protein.

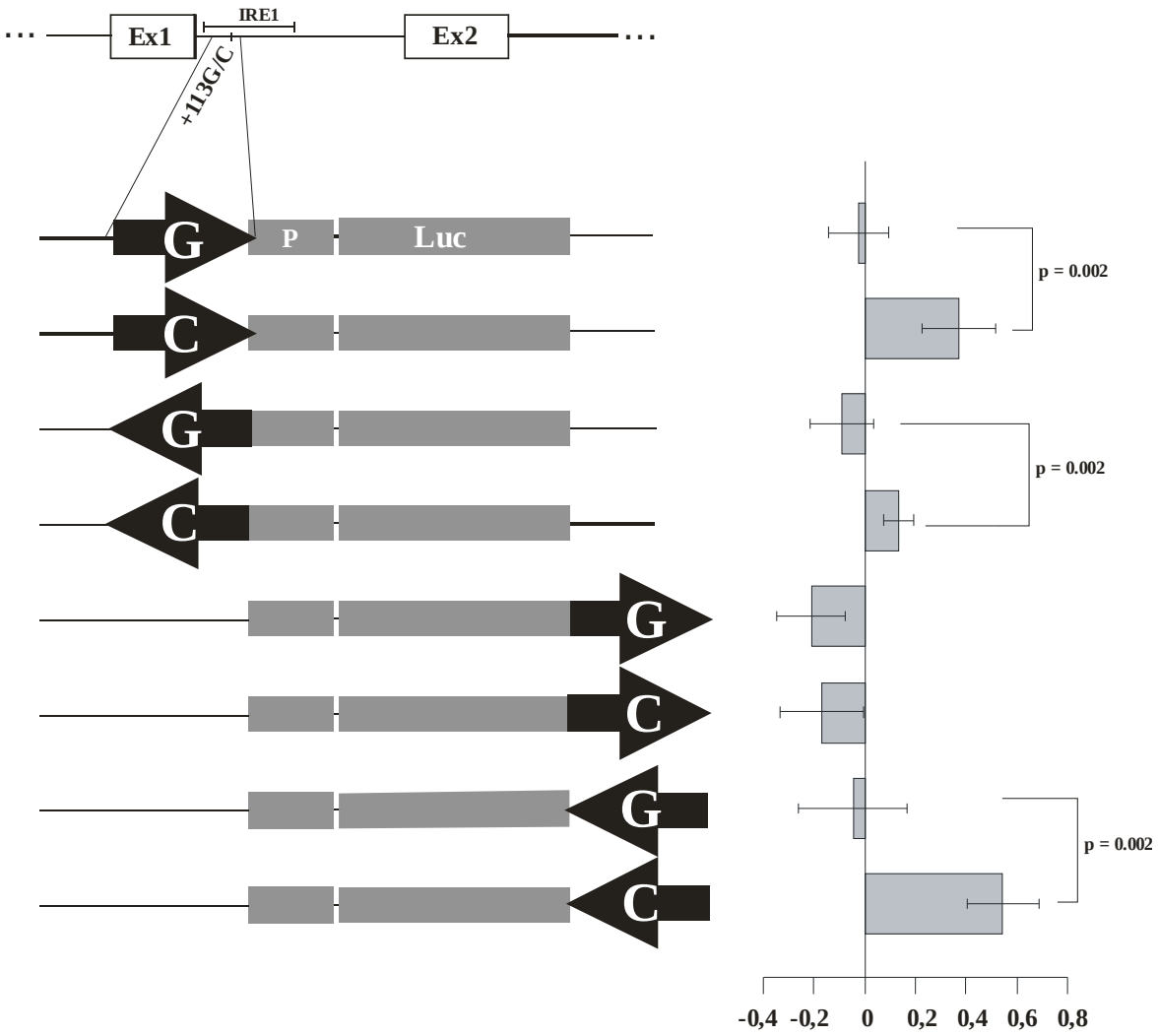

Figure 10. *To study the effects of APOE +113G/C promoter polymorphism on transcriptional activity in HepG2 cells, eight promoter constructs (shown on the left) were used in the luciferase assay system. Arrows indicate whether the insert was cloned in a sense or antisense orientation and the letter inside the arrows indicate the +113G/C allele. Transcription efficiency measurements, which were done in duplicate from three independent experiments, are presented on the right (results are shown as mean with the bars representing standard deviation). Figure adapted from study IV.*

Discussion

1. Study subjects

In the present studies (I-IV), three different clinical and two autopsy series were used to examine the association of two APOE promoter polymorphisms with several lipid, apolipoprotein and lipoprotein variables, early atherosclerotic changes, atherosclerotic lesions, intracranial atherosclerosis and the risk of stroke. Two of the clinical series composed of Finns, the third one mostly of Belgian people. Both autopsy series included only Finnish people. It is important to consider whether the results of the different studies apply to Finnish people in general.

Subjects in the clinical series. Subjects in study I were Finnish men aged 50-59 years. The series did not include women and the age distribution was fairly limited. However, subjects were randomly selected and therefore represented the middle-aged population of Finnish men quite well. The initial study population consisted of 219 men, which was cut down to 122 when the analyses were limited to ε3/ε3 carriers. This can be considered rather a small final study group for a genetic epidemiological study, and it is thus possible that the results are not completely unfailing. Study II consisted of a sub sample of a large multi-center study, the Cardiovascular Risk in Young Finns Study. This study series was randomly selected from different parts of the country, equally from genders, from eastern and western parts of Finland and from rural and urban areas. The subjects belonged to six different age groups, which have been followed over 20 years so the longitudinal changes of various cardiovascular risk factors and manifestations can be studied. Results in the study can probably be generalized for populations consisting of white, apparently healthy individuals. The third clinical series (III) consisted mostly of Belgians and represented stroke patients of a fairly young age (<60 years), however it included gender-matched (about 66% male and 34% women), but older controls (mean age 70 years). The control group was deliberately selected to be older in order to decrease the likelihood of them later suffering from ischemic stroke. The subjects represented both Caucasian men and women.

Subjects in the autopsy series. The subjects included in the two Finnish autopsy series were victims of sudden death or trauma and had been subjected to medicolegal autopsy. The TASTY series comprised both men and women and with a broad age range (range 0-97 years, mean age 62.7 years). The HSDS series only included men and results therefore are not applicable to women. The CHD risk factor distribution may also be different in these subjects; for example, the alcohol consumption was fairly high in the HSDS series. The studied APOE promoter genotype frequencies however, were comparable to other Finnish study series, and HSDS subjects can therefore be thought to represent Finnish middle-aged men. The autopsy subjects of both series were not selected randomly and may have more severe atherosclerosis so selection bias cannot be fully

excluded. One additional limitation of these autopsy series was the lack of blood sample data. Nevertheless, autopsy studies are currently the best way of studying, at the vessel-wall level, the very early steps of the pathogenesis of atherosclerosis, such as accumulation of lipids into arterial wall.

2. Methodological considerations

Candidate gene approach. Atherosclerosis and CHD are complex traits whose mode of inheritance does not follow Mendelian laws. During the past two years, a large number of genome-wide scans and association studies have been performed in relation to CHD, its risk factors and manifestations. These genome-wide studies can be used to identify new disease associated genes or re-identify old disease genes and therefore they serve an important function in trying to unravel the complex genetics behind, for example, CHD. The candidate gene selection is based on these genome-wide studies or on knowledge about the pathophysiology of the disease. The gene can then be investigated using population based case-control studies, which compare the genotype frequencies in unrelated individuals with and without the studied trait as was done, for example, in study III, regarding the ischemic stroke cases and controls. The APOE gene was chosen as the candidate gene in the present study, owing to the wide knowledge about its biological functions concerning lipid metabolism and atherosclerosis. We specifically wanted to examine the most common genotype group APOE ε3/ε3 and study the possible roles of the two promoter polymorphisms (-219G/T and +113G/C) in defining the risk of atherosclerosis within this group. This approach undeniably reduced the study sample sizes. However, since the effects of the common APOE ε2/ε3/ε4 variation could be excluded, it allowed us to study the effects of the promoter polymorphisms on a neutral background.

Classification of atherosclerotic lesions. Methods such as coronary angiography or ultrasound imaging used to evaluate atherosclerosis in living humans only provide information about the extent of arterial lesions, which significantly narrow the lumen. These methods have limited ability to visualize lesions at the vessel-wall level and therefore, autopsy studies are needed to study early atherosclerotic lesions. In the HSDS series (IV), the atherosclerotic lesions were classified according to the IAP protocol (Guzman et al. 1968), by visual inspection after staining of the arterial samples with Sudan IV. Some fatty streaks that develop in regions with adaptive intimal thickening may be deeper under the endothelium and may not become visible when stained (Stary et al. 1994), which can make the correct classification of fatty streaks somewhat problematic. Most fatty streaks, however, stain well with the Sudan staining method. At the time of collection of the HSDS series, the IAP protocol was in use, but currently a new histological classification is available (Stary 2000).

3. APOE promoter polymorphisms and lipoproteins (I & II)

Apolipoprotein E is a multifunctional protein that has many roles in lipoprotein metabolism: it is involved in cholesterol absorption in the intestine, hepatic lipoprotein secretion, lipoprotein metabolism in the circulation, as well as serving as a ligand for cellular lipoprotein uptake. Therefore, it is highly important to study the many variations of the APOE gene and their possible associations with various aspects of lipid and lipoprotein metabolism. The APOE ε2/ε3/ε4 polymorphism has already been studied intensively, with regards to e.g. lipid levels and CHD risk [reviewed by (Bennet et al. 2007)]. However, the polymorphisms -219G/T and +113G/C within the APOE regulatory region have previously not gained much attention and were thus the focus of our interest.

Cross-sectional studies (I, II). Our cross-sectional study on a group of Finnish middle-aged men (I) showed that within the APOE ε3/ε3 carriers, the -219T/T and +113C/C genotypes associated with lower VLDL-C, apoB and TG concentrations and with a higher HDL-C concentration, compared to -219G/G or +113G/G genotypes, respectively. The -219T/+113C/ε3 haplotype was shown to associate with lower VLDL-C and TG values compared to the -219G/+113G/ε3 haplotype. Our study was the first to analyze the +113G/C polymorphism and -219/+113/ε haplotype in relation to serum lipid and lipoprotein levels. There were however, previous studies on the association of the -219G/T polymorphism with fasting lipid or lipoprotein levels, but the results were mostly negative (Lambert et al. 2000, Moreno JA et al. 2003, Ye et al. 2003). Lambert and colleagues found no differences in serum lipid levels between -219G/T genotype groups (Lambert et al. 2000). Their study subjects were from various regions within France and Ireland, and from a wider age group compared to our more genetically homogenous study subjects of 50 to 59 year old men from the Tampere region of Finland. Furthermore, they studied -219G/T and lipids without stratifying the APOE ε2/ε3/ε4 genotypes, as Ye and colleagues (Ye et al. 2003), whereas we concentrated on the ε3/ε3 subjects. The study by Moreno and colleagues, which did not find differences between -219G/T genotypes in basal state plasma lipid concentrations, involved only ε3/ε3 carriers as did our study, but was relatively small and included only 51 Spanish study subjects (Moreno JA et al. 2003). Viitanen and colleagues studied families from eastern Finland and the -219T-allele tended to associate with higher TC, LDL-C and TG and lower HDL-C concentrations than -219T non-carriers, but not with statistical significance (Viitanen et al. 2001). This was also in combination with ε3/ε4 or ε4/ε4 genotypes and therefore, not directly related to our results. Furthermore, their study included both men and women, whereas ours only included men. Our second cross-sectional analyses (II) involved both men and women and was composed of a younger (24-39 years) study population of Finns, but no statistically significant differences in any of the measured lipid or lipoprotein variables between the studied APOE promoter genotype groups were found.

The 21 year follow-up study (II). The 21 year follow-up (from 1980 to 2001) study on young Finns, showed that within the APOE ε3/ε3 group the promoter polymorphisms -219G/T and +113G/C and haplotype -219T/+113C associated with longitudinal changes of LDL-C and TC in males. Throughout the follow-up period, -219T- and +113C-allele carriers, as well as the carriers of haplotype -219T/+113C, had higher LDL-C and TC concentrations compared to -219G/G, +113G/G, or non-carriers of the haplotype

-219T/+113C. In females such differences were not found. There were no previous studies on the association of these promoter polymorphisms and APOE haplotypes with lipid/lipoprotein/apolipoprotein variables. However, an earlier study using the Cardiovascular risk in Young Finns Study population showed that the APOE ε2/ε3/ε4 polymorphism associated with LDL-C and TC in a six year follow-up, and the differences in cholesterol values between APOE phenotypes remained fairly constant throughout the follow-up from 1980 to 1986 (Lehtimaki et al. 1990). A later 21-year follow up of the same Cardiovascular risk in Young Finns Study population showed that the differences in LDL-C and TC values were maintained throughout the follow up with the ε4 carriers having the highest and ε2 carriers the lowest values (Gronroos et al. 2007). It is not surprising therefore, that the APOE promoter polymorphisms have similar effects within the most common APOE genotype group ε3/ε3.

The results of studies I and II are somewhat contradictory since in study I the -219T- and +113C-alleles and -219T/+113C/ε3 haplotype associated with lower VLDL-C, apoB and TG concentrations while in study II the same alleles and haplotypes associated with higher TC and LDL-C concentrations. These results could suggest a lower production of VLDL and apoB in the liver of -219T- and +113C-allele carriers and on the other hand a higher rate of VLDL catabolism to LDL, which was not seen in the smaller cross-sectional study (I) but was evident in the larger longitudinal study (II). The rather a small study sample size in study I can be considered as a limitation, but in study II there were more ε3/ε3 carriers and the constant difference in cholesterol values between the genotypes and haplotypes throughout the long follow-up do make those results trustworthy. The lack of apoE concentration measurements in both of these studies (I, II) makes it impossible to deduce whether the associations of the APOE promoter polymorphisms with cross-sectional or longitudinal lipid/apolipoprotein values could be mediated through differential APOE transcription. Association analyses do not give any possibility to make conclusions about the mechanisms behind the discovered associations. Still, it seems that both the promoter polymorphisms, and their haplotype, are somehow involved in regulating serum lipid concentrations in Finns, but not necessarily in all other populations or in all age groups.

4. APOE promoter polymorphisms and early atherosclerotic changes (II & IV)

Since atherosclerosis develops clinical symptoms only later in life, many different markers for subclinical atherosclerosis, such as IMT, CAC and FMD, have been used to estimate the risk of future coronary events. IMT has been studied extensively in relation to APOE ε2/ε3/ε4 polymorphisms but, to our knowledge, the role of the APOE promoter polymorphisms -219G/T and +113G/C in determining IMT, CAC or FMD had not been earlier investigated.

Preclinical indicators of atherosclerosis (II). Our study was the first to examine the possible associations of the APOE promoter polymorphisms -219G/T and +113G/C with preclinical indicators of atherosclerosis. We did not find any statistically significant differences in the IMT, CAC or FMD values between the APOE promoter genotypes

within ε3/ε3 carriers. Our study is in agreement with a study by Debette and colleagues, who did not find any association of -219G/T with IMT in an elderly (≥65 years) French population (Debette et al. 2006). Grönroos and colleagues studied the same Finnish study population as we did, and found no statistically significant differences in IMT, CAC and FMD between the APOE ε2/ε3/ε4 groups (Gronroos et al. 2008). There was a fairly large variation in the CAC and FMD measurements in our study population but only a small variation in IMT measurements and in diameter measurements of brachial and carotid arteries, suggesting that much of the variation in CAC and FMD relates to physiological fluctuations in vascular function and not to measurement error. It is possible, however, that weak relationships between APOE promoter polymorphisms and CAC or FMD may have gone undetected due to rather large variations in the data.

Fatty streaks (IV). We found a statistically significant APOE promoter genotype-smoking interaction on the fatty streak area of the abdominal aorta (IV). In non-smokers, the -219T- and +113C-allele or haplotype -219T/+113C/ε3 carriers had statistically significantly larger mean percent area of fatty streaks compared to -219G/G or +113G/G genotypes or homozygous carriers of haplotype -219G/+113G/ε3. In smokers the -219T- and +113C-allele and -219T/+113C/ε3 haplotype carriers associated with lower lesion areas than the -219G/G or +113G/G genotype carriers or homozygous carriers of haplotype -219G/+113G/ε3. There were no previous studies exploring the APOE -219G/T or +113G/C polymorphisms and fatty streak areas or their interactions with smoking. Smoking is known to impact many phases of atherosclerosis and predispose individuals to different clinical syndromes such as angina, sudden death and stroke (Ambrose and Barua 2004). Smoking is known to be a risk factor particularly for atherosclerosis of the abdominal aorta (McGill et al. 1997) and arteries of the lower limb (Price et al. 1999), being one of the most important predictors of the obstructive peripheral atherosclerosis leading to gangrene or amputation of lower limbs (McGill 1988/1). Our finding on the interaction of smoking with abdominal aorta atherosclerosis is therefore in line with this clinical evidence. Furthermore, the interactions of APOE ε2/ε3/ε4 genotypes with smoking has been shown to have an impact on CHD risk (Talmud 2007). This has been explained through an effect on LDL oxidation since the different apoE isoforms have different vulnerability to oxidation, E4 being most susceptible and E2 the least susceptible (Jolivalt et al. 2000). Since the promoter polymorphisms were studied primarily within the ε3/ε3 group, the oxidation susceptibility difference between E2, E3 and E4 can not explain the differences we saw in fatty streak areas in the abdominal aorta between promoter genotype and haplotype groups within smokers and non-smokers. Given that both studied promoter polymorphisms affect APOE transcription, we hypothesize that smoking somehow disrupts or interferes with transcription factor binding to these areas. This could lead to differential APOE gene expression and possibly through regulating serum lipid values could eventually cause differential lipid accumulation in aortic walls. Unfortunately, we did not have lipid, apoE protein or mRNA concentrations measured in this study series, so this is highly speculative. We also cannot exclude the possibility that smoking might have an effect on the translational rather than transcriptional level. Our association analyses provide new information considering APOE promoter genotype-dependent and smoking-specific regulation of fatty streaks in the abdominal aorta; nevertheless, the molecular mechanisms behind these specific associations remain unclear.

5. APOE polymorphisms and stroke & intracranial atherosclerosis (III)

Strokes cluster in families, with twin and family studies showing that genetic factors influence the incidence of ischemic stroke (Flossmann et al. 2004). Since the first report of a positive association of the APOE ε4 allele with ischemic stroke (Pedro-Botet et al. 1992), there have been numerous studies on the subject, some confirming (McCarron et al. 1999, Saidi et al. 2007), others contradicting (Coria et al. 1995, Basun et al. 1996, Duzenli et al. 2004, Slooter et al. 2004, Sturgeon et al. 2005, Parfenov et al. 2007) the association between ε4 and ischemic stroke. Our analyses on the Belgian study population found no evidence of ε4 being associated with the risk of ischemic stroke (III). This is in line with the majority of previous studies and additionally, with two large meta-analyses, which found no clear association between ε4 and ischemic stroke (Casas et al. 2004, Sudlow et al. 2006). It has been suggested that accumulation of other risk factors with age would dilute the genetic effect of APOE (Ilveskoski et al. 1999). This does not explain our negative results because the ischemic stroke cases in our study were relatively young (<60 years) and therefore, the genetic effect should have been rather strong. Frikke-Schmidt and colleagues studied ischemic cerebrovascular disease cases in three groups with mean ages of 43, 63 and 70 years, but found no association with ε4 in any of these groups (Frikke-Schmidt et al. 2001), which is in agreement with our results. Interestingly, we discovered that ε4 carriers tended to have a higher circle of Willis atherosclerosis score compared to non-carriers, although the association was statistically significant only in male subjects. In contrast to our study, an earlier Finnish study found no association between the APOE ε2/ε3/ε4 polymorphism and cerebral atherosclerosis (Kosunen et al. 1995). Their study population was a specifically selected group of AD patients, excluding those patients with AD and major cerebrovascular disease, which might partly explain their negative result regarding ε4 and cerebral atherosclerosis.

To our knowledge, this is the first study to investigate the role of the APOE promoter polymorphisms -219G/T and +113G/C in defining a risk of ischemic stroke. We showed that within the ε3/ε3 group, the G-allele carriers of either of the studied promoter polymorphisms had an increased risk of ischemic stroke, which could not be explained with increased intracranial atherosclerosis. There are most probably also other mechanisms than those leading to increased intracranial atherosclerosis which are involved in the development of ischemic stroke. These other mechanisms might be related to plasma levels of apoE, since both studied promoter polymorphisms are involved in the regulation of APOE transcription, and since increased (van Vliet et al. 2007) *and* decreased (Couderc et al. 1993) plasma apoE levels have both been suggested to be associated with increased stroke risk. The -219G-allele is previously known to associate with higher levels, whereas the +113G-allele associates with lower levels of gene transcription, as we showed in study IV. Regardless of these opposing effects on APOE transcription, both -219G and +113G-alleles associated with an increased risk of ischemic stroke in our study. However, when carriers of the -219T- or +113C-allele were compared to common allele homozygotes (i.e. G/G genotype), there were no statistically significant differences in stroke risk; but in men the -219T-allele carriers had higher Circle of Willis atherosclerosis score. This is in

agreement with the results of our longitudinal study that showed higher cholesterol values in -219T- and +113C-allele carriers.

Despite the still lacking explanation for the increased risk of stroke with APOE promoter G-allele carriers, this study gives new possibilities for stroke risk assessment within the group of APOE ε3/ε3 individuals, although the results have to be confirmed in other studies to gain verification.

6. APOE +113G/C functional studies (IV)

It was previously known that the APOE +113G/C polymorphism is located within the IRE1 region spanning the nucleotides +44 to +262, and known to possess promoter-enhancing activity (Paik et al. 1988). Our promoter analyses also showed that the +113G/C region is evolutionary conserved (>60%) between human and mouse, suggesting that it is important in the regulation of APOE transcription. There were however, no previous reports on the possible effects of this polymorphism on transcriptional activity. Our functional studies showed that the promoter constructs containing the +113C-allele possessed higher transcriptional activity compared to the G-allele, suggesting that the enhancer activity of this region is specifically dependent on the +113C-allele. Our *in vitro* protein binding assays showed that both alleles were able to bind proteins from the hepatic nuclear lysate, suggesting that this region can recruit transcription factors in hepatic cells. Thus, it is likely that the +113G/C region can regulate APOE gene transcription also *in vivo*. Furthermore, our studies suggested an affinity difference in protein(s) binding to the different alleles: the G-allele seemed to bind protein(s) with a somewhat higher affinity. However, since the DNA-protein complexes reciprocally competed in the competition experiments, we hypothesize that they most likely contain mostly the same protein(s), but possibly in different amounts.

It has been suggested that transcription factor NFκB binds to the +113G/C region (Bullido and Valdivieso 2000). Our promoter analyses with Consite (Sandelin et al. 2004) augmented this by showing that the p50 subunit of NFκB binds with different affinities to the +113G- and C-alleles. However, we were unable to confirm the binding in EMSA assays. Another promoter analysis with Matinspector (Genomatix software package) predicted that +113C would recruit two additional transcription factors RBP-Jκ (also known as RBP or CBF1) and RFX1. Indeed, our EMSA assay with prokaryotically produced transcription factor RBP-Jκ showed that this protein binds with a higher affinity to the C-allele when compared to the G-allele. RBP-Jκ is a known transcriptional repressor in mammalian cells, and human RBP-Jκ recognizes the DNA sequence ag/c**CGTGGGAA**<u>c</u>ta/t (core recognition sequence written in bold) (Tun et al. 1994). The underlined c corresponds to +113C, which explains in part why the RBP-Jκ bound with a higher affinity distinctly to the C-probe in our EMSA experiment. Normally RBP-Jκ acts to repress transcription and is the principal target of the Notch signaling pathway. In the absence of a Notch signal, RBP-Jκ binds to DNA and recruits corepressor complexes containing histone deacetylases to induce transcriptional silencing. However, upon ligand binding, Notch signaling converts the RBP-Jκ from a transcriptional repressor to a transcriptional activator [reviewed by (Mumm and Kopan 2000, Ehebauer et al. 2006)]. In

conclusion, our *in vitro* experiments propose that RBP-Jκ would be a good candidate for +113 region-mediated regulation of APOE gene transcription, but this admittedly warrants for more *in vivo* studies. There are most probably also other - currently unidentified - transcription factors, which bind to the +113G/C region and take part in regulating APOE transcription.

Summary and Conclusions

In this thesis, a candidate-gene approach and association analyses were used to study various aspects of atherosclerosis in coronary arteries, aorta, and intracranial large vessels. Furthermore, we wanted to clarify the role of two APOE promoter polymorphisms -219G/T and +113G/C, in defining stroke risk and in the initial step of atherosclerosis - fatty streak formation. We were also interested in the gene-environment interactions, which are nowadays considered to have an essential impact on the risk of CHD. Moreover, we aimed to explore the previously unknown effect of APOE +113G/C on transcriptional efficiency. The main findings and conclusions are as follows:

1. Within APOE ε3/ε3 carriers in a random sample of Finnish middle-aged men, the -219T/T and +113C/C carriers had lower concentrations of VLDL-C, apoB, TG and higher concentrations of HDL-C, compared to G/G genotype carriers. The -219T/+113C/ε3 haplotype also associated with lower VLDL-C and TG concentrations compared to carriers of the -219G/+113G/ε3 haplotype (I). These results suggest that the studied APOE promoter polymorphisms take part in defining serum lipid and apolipoprotein levels, independently of APOE ε2/ε3/ε4 polymorphism.

2. Longitudinal analyses revealed that male carriers of the -219T/+113C/ε3 haplotype had higher concentrations of LDL-C and TC throughout a 21-year follow up, compared with non-carriers of the same haplotype. However, there were no statistically significant differences in subclinical atherosclerotic markers (IMT, FMD or CAC) measured by ultrasound methods (II). These results further strengthen the notion that the studied APOE promoter polymorphisms act as modulators of lipoprotein metabolism, but they do not seem to be the major determinants of IMT, FMD or CAC.

3. In the abdominal aorta, a statistically significant APOE promoter genotype-smoking interaction was detected regarding the fatty streak area. Within non-smokers, the -219T- and +113C- allele as well as the -219T/+113C/ε3 haplotype carriers had larger fatty streak areas compared to the G/G genotype or homozygous -219G/+113G/ε3 haplotype carriers. In smokers, the associations were opposite (IV). These findings suggest that the -219G/T and +113G/C polymorphisms and the APOE haplotype, together with smoking, affect the formation of early atherosclerotic lesions in middle-aged men.

4. The APOE ε4 allele did not associate with ischemic stroke. The -219G- and +113G-allele carriers were at increased risk of ischemic stroke compared to -219T/T or +113C/C carriers, and the risk was independent of APOE ε2/ε3/ε4 polymorphism. The intracranial atherosclerosis score did not differ statistically significantly between

-219G-allele carriers and -219T/T genotype carriers. However, in men the -219T-allele carriers had higher Circle of Willis atherosclerosis score when compared to the -219G/G homozygotes.

5. Functional studies showed that the APOE +113C-allele has higher transcriptional activity compared to the +113G-allele in HepG2 cells. *In vitro* protein binding studies showed that both the +113G- and +113C-alleles can bind proteins from hepatic nuclear lysate. These results suggest that +113G/C is a functional polymorphism and can take part in regulating APOE transcription.

Based on these results, it can be concluded that the APOE promoter polymorphisms -219G/T and +113G/C and their haplotypes, take part in many phases of the atherosclerosis process and also act as potential new factors in defining the risk of ischemic stroke, independent of APOE ε2/ε3/ε4. It is important to note that the +113G/C polymorphism was shown to be functional and therefore presents itself as a highly interesting target for future studies in the field of atherosclerosis.

Acknowledgements

This study was carried out at the Departments of Forensic Medicine and Clinical Chemistry, Medical School, University of Tampere during years 2003-2008. This research was made possible by financial support from the Pirkanmaa Regional Fund of the Finnish Cultural Foundation, Kalle Kaihari Heart research Fund, Tampere University Hospital Medical Fund, Emil Aaltonen Foundation, the Erasme Funds, the Finnish Foundation for Cardiovascular Research, the Yrjö Jahnsson Foundation, and travel grants from the Tampere Graduate School in Biomedicine and Biotechnology.

Firstly, I want to thank my supervisors Pekka J. Karhunen and Terho Lehtimäki for their guidance and support through the thesis project. They have both kept an optimistic attitude even when everything seemed to go wrong. Pekka has given me the possibility to work independently but has patiently been there to listen and encourage, when needed. Terho has always shown interest in my work and has had time for discussion.

The reviewers of my thesis, Philippa J. Talmud and Petri Kovanen are warmly thanked for their constructive criticism and helpful comments, which truly helped me to improve this thesis. Markus Perola and Timo Koivula are thanked for participating in my thesis committee. Markus is also thanked for the fact that I found my way to Tampere and to the world of cardiovascular research.

I wish to express my gratitude to all my coauthors and collaborators for all their contributions regarding the original communications of this thesis. I am grateful to Heini Huhtala for the help with the statistics and for offering a shoulder to cry on. Markku Mäki is gratefully acknowledged for giving me the opportunity to carry out the functional studies in the lab of Coeliac Disease Study Group. I also want to thank the whole Coeliac Disease Study Group for all the help and guidance while I was working there.

I also want to thank previous and present members of the Forensic Medicine Department: Sirkka Goebeler, Erkki Ilveskoski, Titta Frantsila, Anni Oksala, Seija Kivimäki, Jussi Mikkelsson, Elina Ollikainen, Outi Lumme, Mervi Seppänen, Sari Tuomisto and all others. Eloise Kok is thanked for all the discussions and fun moments during the last year and also for correcting the English and help with creating figures.

Carita Eklund, Jaana Renko and Kati Huuskonen are thanked for all the discussions and delightful company during meeting and congress trips in Finland and around the world. Riina Sarkanen and Ulla Jalonen, as well as Erja Kerkelä are thanked for friendship and supportive conversations throughout the years.

I also want to acknowledge all my friends and family. My sister Sirpa and our gang from Pori: Sanna, Annu, Iitu and Jonna who have all been there to listen, encourage and offer non-scientific viewpoint on life. Many thanks also to Aleksi, Katri, Tommi, Leena and Nuppu as well as our beautiful goddaughter Hilma for all the fun moments during these years. During my maternity leave in the middle of the thesis project, I got to know many wonderful people, and I want to thank “maalismammat” and especially Johanna,

Sanna and Titta for sharing the amazing moments with (and without J) our firstborns. Ulla, Tommi and Henna Viiri are thanked for all the support and lovely times in Lapland, which were nice breaks from the scientific work. The most loving and warm thanks go to my parents, Ritva and Tauno, without whom this thesis project would not have been possible. They have helped in many ways, most importantly by taking care of Pyry during my long hours in the lab and writing this thesis. Kiitos äiti ja isä! Pyry is the light of my life, so full of joy and laughter. He reminds me of what is most important in life ♡

Finally, I want to thank my husband Keijo, the love of my life, for always believing in me, supporting me and being there through the good and the bad. This would mean nothing without you by my side.

Tampere, May 8th 2008

Leena Viiri

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Original Communications

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ELSEVIER

Atherosclerosis 179 (2005) 161–167

ATHEROSCLEROSIS

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The association of the apolipoprotein E gene promoter polymorphisms and haplotypes with serum lipid and lipoprotein concentrations

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Received 19 May 2004; received in revised form 27 August 2004; accepted 1 October 2004
Available online 10 December 2004

Abstract

Background: Apolipoprotein E (ApoE) is known to modulate lipoprotein transport and metabolism. The common APOE ε2/ε3/ε4 polymorphism explains part of the variation in plasma cholesterol levels. Polymorphisms of the APOE gene regulatory region are suggested to be involved in explaining variation of lipoprotein levels within the APOE ε2/ε3/ε4 genotypes.
Objectives: To study the associations of the APOE gene promoter polymorphisms −219G/T and +113G/C and their haplotypes with serum lipid and lipoprotein concentrations, especially within the most common APOE ε3/ε3 genotype group.
Subjects and methods: From 219 middle-aged Finnish men, APOE genotypes were determined and haplotypes estimated. Plasma lipoproteins were isolated by ultracentrifugation and their lipids were measured.
Results: The studied APOE promoter polymorphisms and haplotypes associated with certain lipid variables independently of the APOE ε2/ε3/ε4 genotype. Within the APOE ε3/ε3 group, both −219G/G and +113G/G genotypes associated statistically significantly with higher levels of very low-density lipoprotein (VLDL) cholesterol, apoB and triglycerides, and tended to associate with lower HDL-cholesterol concentrations than the other genotypes. Compared with the −219T/+113C/ε3 haplotype, the more common −219G/+113G/ε3 haplotype was found more frequently among the group having high (over median) VLDL-cholesterol and triglyceride concentrations (OR 2.6, $p < 0.001$ and OR=2.1, $p = 0.009$, respectively).
Conclusions: In addition to the APOE ε2/ε3/ε4 polymorphism, the promoter polymorphisms −219G/T and +113G/C as well as their haplotype modulate lipid and lipoprotein concentrations in middle-aged Finnish men.

Keywords: Apolipoprotein E (apoE); Apolipoprotein E gene (APOE); Polymorphism; Promoter; APOE promoter polymorphisms; Haplotype; Lipids; Lipoproteins

1. Introduction

Apolipoprotein E (apoE), a major component of circulating lipoproteins, is mainly synthesized in liver and is found in serum on chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL) and high-density lipoproteins (HDL). ApoE plays an important role in the lipid metabolism by acting as a receptor-binding ligand

Abbreviations: ANOVA, analysis of variance; ANCOVA, analysis of covariance; ApoE, apolipoprotein E; APOE, apoE gene; apoB, apolipoprotein B; BMI, body mass index; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; Lp(a), lipoprotein (a); VLDL, very low density lipoprotein

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doi:10.1016/j.atherosclerosis.2004.10.004

for the low-density lipoprotein (LDL)-receptor to mediate the binding and uptake of lipoproteins [1].

Apolipoprotein E gene (APOE) located at 19q13.2 [2] encodes polypeptide of 299 amino acids [3]. Three alleles (ε2, ε3 and ε4) of the gene encode three major apoE isoforms E2, E3 and E4 [4,5] that differ in the extent to which they bind to apoE and LDL-receptors. E2 is defective in binding to the LDL-receptors [6] leading to delayed clearance of the apoE2-bearing lipoprotein particles from the circulation. This results in up-regulation of the LDL-receptors and lowering of the serum LDL-cholesterol [7].

Several polymorphisms have been identified also in the regulatory region of the APOE gene [8,9]. The often-studied −219G/T polymorphism has been associated with Alzheimer's disease (AD) [10], myocardial infarction [11] and premature coronary heart disease [12]. Myllykangas et al. found that in elderly subjects carrying the most common APOE ε3/ε3 genotype, the −219G/T genotype modulates the deposition of beta-amyloid in the brain and the AD risk [13]. Recently, it was reported that the −219G/T polymorphism affects the severity of coronary artery disease independently of the APOE ε2/ε3/ε4 polymorphisms [14]. Moreno et al. demonstrated that the −219G/T polymorphisms could participate in the regulation of the metabolism of triglyceride-rich lipoproteins during the postprandial period [15]. Another polymorphism has been identified in the intron 1 enhancer region (IE-1) at site +113 [16], but to our knowledge, there are no studies on its association with lipid or lipoprotein concentrations. There are no previous data available about the APOE haplotypes formed according to these previously found gene loci and their association with fasting lipid levels of ultracentrifugally isolated serum lipoprotein fractions.

We studied the associations of the APOE promoter region polymorphisms −219G/T and +113G/C and the APOE haplotype with serum lipid and lipoprotein concentrations in a random sample of 219 middle-aged Finnish men. We were especially interested in studying associations within the most common APOE ε3/ε3 genotype group.

2. Materials and methods

2.1. Study subjects

Originally 300 men aged 50–59 years were randomly selected from 10 age-cohorts (n = 9058) living in the city of Tampere in Finland. From these 300 men who were invited by letter to participate, 223 (74%) agreed to, 33 refused and 44 did not answer or could not be reached. Finally, all required data was obtained for 219 participants, who constitute the final study population. The study was approved by the Ethics Committee of the UKK institute, and all participants gave their written informed consent.

Detailed medical histories were collected, with particular emphasis on cardiovascular and metabolic diseases and chronic medications. Weight and height were recorded and the body mass index (BMI) was calculated (kg/m^2). Blood pressure was recorded from the dominant arm with a mercury sphygmomanometer after 15 min of supine rest. Three measurements in supine, two in standing and one in sitting position, were performed and the average of six measurements was recorded as resting systolic and diastolic blood pressure values. The systolic and diastolic blood pressure was reported in mm of Hg.

The mean age of the study subjects was 54.1 ± 2.9 years and BMI 27.3 ± 3.8 kg/m^2. The mean systolic and diastolic blood pressure and their standard deviations were 132 ± 17 and 84 ± 10 mmHg, respectively. There were 53 current smokers, 72 ex-smokers and 92 non-smokers (2 participants had missing data on smoking) in the study population. None of the subjects had had a symptomatic cerebrovascular event. However, a total of 65 study subjects had continuous medication; 45 were treated for hypertension, 7 for diabetes mellitus, 9 for ischemic heart disease and 4 for hyperlipidemia but none of them received statin treatment. There were no statistically significant differences in the fore mentioned characteristics between the different APOE ε2/ε3/ε4, –219G/T and +113G/C genotype groups when compared with analysis of variance (ANOVA) or the Chi-squared test (data not shown).

2.2. Lipoprotein and serum lipid determinations

Lipoprotein and serum lipid determinations were done as described by Ilveskoski et al. [17].

2.3. ApoE phenotyping and APOE genotyping

ApoE phenotyping was performed using delipidated plasma, isoelectric focusing, cysteamine treatment and immunoblotting, as described by Menzel and Utermann [18], with minor modifications described by Lehtimäki et al. [19]. The verification of correct apoE phenotypes in gel was based on comparison to previously known APOE genotype standards.

DNA was isolated from lymphocytes with a commercial kit (Qiagen Inc. Valencia, CA). APOE gene promoter and intron polymorphisms were genotyped using 5′nuclease assay [20] and fluorogenic TaqMan probes (Roche Molecular Systems) in ABI Prism® 7000 Sequence Detection System (Applied Biosystems, California, USA). The probe sequences for the identification of the promoter polymorphism −219G/T were as follows: 5′-VIC-CCCAGTAATcCAGACAC-3′ and 5′-6-FAM-CGCCCAGTAATaCAGA-3′. The probe sequences for the identification of the intron polymorphism +113G/C were: 5′-VIC-ACCCTGGGAAgCC-3′ and 5′-6-FAM-CCCTGGGAAcCCCT-3′.

2.4. Statistical analyses and haplotype construction

Statistical calculations were done using SPSS (version 11.5) software on a PC. Smoking (current smokers/non- and

former smokers) did not differ between the genotype groups when tested with the Chi-squared test (results not shown). Interactions between the APOE promoter genotypes and smoking were tested with analysis of covariance (ANCOVA). Due to statistically non-significant results in these tests, subgroup analyses were not performed but smoking status was used as a covariate in all comparisons. Non-normally distributed data, i.e. VLDL-cholesterol, triglyceride and lipoprotein (a) [Lp(a)] values, were analyzed in logarithmically transformed form but are expressed as crude values. ANCOVA was used to compare the means of serum lipoprotein and lipid values in different −219G/T and +113G/C genotype groups, and *p*-values less than 0.05 were considered statistically significant. The differences between the genotype groups were analyzed with pairwise comparisons using Bonferroni correction for multiple comparisons. To study the associations of the promoter polymorphisms with serum lipid variables excluding the effects of the APOE ε2/ε3/ε4 alleles, we focused the analyses on the most common (56.2%) APOE ε3/ε3 genotype group.

Haplotypes were calculated using Stata (8.0) software, if individual polymorphisms associated statistically significantly with the same variable. The haplotype analyses were performed only within the APOE ε3/ε3 genotype group in order to standardize the effects of the APOE ε2/ε3/ε4 polymorphisms on the studied traits, and to focus on studying variation within this most common genotype group. Before performing haplotype analyses, VLDL-cholesterol and triglyceride variables were divided into two groups using the median (Md) as a cut-off value (0 = below Md, 1 = above Md). For haplotype analyses, the phase was first resolved by applying E-M algorithm, after which the frequencies of different haplotype combinations were estimated from the saturated model (i.e. in which polymorphisms of the two loci were found to be in linkage disequilibrium). Haplotype-specific odds ratios (OR) and their confidence intervals (CI) were calculated comparing the haplotypes comprising of the least common alleles with the other haplotypes. We also checked the two loci for conditional independence with Stata 8.0 using the module for haplotype analysis [21].

3. Results

3.1. The APOE allele frequencies of the study population

The frequencies of the ε2, ε3 and ε4 alleles of the APOE gene were 0.06, 0.75 and 0.19, respectively, corresponding the frequencies in other Finnish studies [12,19]. The frequencies of the G and T alleles of the −219G/T polymorphism were 0.58 and 0.42 in the whole study population, and 0.68 and 0.32 within the APOE ε3/ε3 genotype group ($n = 123$). Frequencies of the G and C alleles of the +113G/C polymorphism were 0.75 and 0.25 for the whole study population, and 0.67 and 0.33 within the APOE ε3/ε3 genotype group. Genotypic distributions of alleles were in Hardy–Weinberg equilibrium for all the above polymorphisms, and the previously described linkage disequilibrium (LD) for the −219G/T, +113G/C and APOE ε2/ε3/ε4 polymorphisms [22] was verified in this study (data not shown).

3.2. The APOE promoter genotypes, serum lipids and lipoproteins

We have earlier shown, using this same data, that the apolipoprotein B (apoB), serum total and LDL-cholesterol concentrations differ statistically significantly between the APOE ε2/ε3/ε4 genotype groups [17]. Within the whole study population in the present study, the −219G/T polymorphism did not associate with the above lipid variables, but it associated with the Lp(a) concentration ($p = 0.030$). When testing within the most common APOE ε3/ε3 genotype group the −219G/T polymorphism showed statistically significant association with VLDL-, HDL- and HDL_2-cholesterol concentrations ($p = 0.008$, 0.034 and 0.033, respectively), as well as with apoB ($p = 0.016$) and triglyceride ($p = 0.016$) concentrations (Table 1). Compared to the more rare −219T/T carriers, the −219G/G carriers had higher VLDL-cholesterol (0.75 versus 0.43, $p = 0.011$), apoB (1.37 versus 1.13. $p = 0.013$) and triglyceride concentrations (1.68 versus 1.06, $p = 0.018$). Furthermore, the −219G/G group had lower HDL- and HDL_2-cholesterol concentrations compared to the -219T/T carriers (1.22 versus 1.41, $p = 0.033$ and 0.84 versus 1.02. $p = 0.040$, respectively).

In the whole study population, comprising all the APOE ε2/ε3/ε4 genotypes, the +113G/C polymorphisms associated statistically significantly with VLDL-cholesterol ($p = 0.002$), HDL-cholesterol ($p = 0.036$), HDL_2-cholesterol ($p = 0.043$), apoB ($p = 0.039$) and triglycerides ($p = 0.007$). Within the ε3/ε3 carriers, the +113G/C polymorphisms associated with VLDL-cholesterol ($p = 0.010$), HDL-cholesterol ($p = 0.034$), HDL_2-cholesterol ($p = 0.033$), apoB ($p = 0.014$), Lp(a) ($p = 0.043$) and triglycerides ($p = 0.022$; Table 2). Compared to the C/C carriers, the G/G carriers had higher concentrations of VLDL-cholesterol (0.75 versus 0.43 mmol/l, $p = 0.012$), apoB (1.37 versus 1.13 g/l, $p = 0.012$), and triglycerides (1.67 versus 1.06 mmol/l, $p = 0.021$). Additionally, the G/G carriers had lower HDL-concentration (1.22 versus 1.41 mmol/l, $p = 0.032$) and HDL_2-concentration (0.84 versus1.02, $p = 0.040$) than the C/C carriers.

3.3. The APOE haplotypes and serum lipids and lipoproteins

To exclude the effects of the different APOE isoforms (ε2/ε3/ε4), we performed the haplotype analyses within the most common (56.2%) homozygous APOE ε3/ε3 genotype group. The four identified haplotype groups, generated by the alleles in the −219 and +113 loci in addition to the ε3 allele, differed in regard to the serum VLDL-cholesterol and triglyceride concentrations. The haplotype analyses suggested that the most common haplotype (67%) -219G/+113G/ε3 asso-

Table 1
Plasma lipid and lipoprotein concentrations in the APOE -219G/T genotype groups for the whole study population and for the APOE ε3/ε3 genotype group

Variable	−219G/T genotype			*p*-value
	G/G n_{all} = 74, $n_{\varepsilon3/\varepsilon3}$ = 55	G/T n_{all} = 101, $n_{\varepsilon3/\varepsilon3}$ = 55	T/T n_{all} = 42, $n_{\varepsilon3/\varepsilon3}$ = 12	
Total cholesterol (mmol/l)				
All	5.50 (0.92)	5.46 (0.86)	5.55 (0.98)	0.931
ε3/ε3	5.60 (0.95)	5.47 (0.87)	5.14 (0.96)	0.190
VLDL-cholesterol (mmol/l)				
All	0.76 (0.45)	0.69 (0.46)	0.59 (0.37)	0.078
ε3/ε3	0.75 (0.42)	0.62 (0.38)	0.43 (0.26)	0.008
LDL-cholesterol (mmol/l)				
All	3.52 (0.87)	3.54 (0.74)	3.72 (0.95)	0.569
ε3/ε3	3.63 (0.91)	3.62 (0.70)	3.30 (0.91)	0.343
HDL-cholesterol (mmol/l)				
All	1.22 (0.24)	1.23 (0.30)	1.24 (0.25)	0.869
ε3/ε3	1.22 (0.22)	1.23 (0.26)	1.41 (0.27)	0.034
HDL_2-cholesterol (mmol/l)				
All	0.85 (0.24)	0.84 (0.28)	0.85 (0.26)	0.925
ε3/ε3	0.84 (0.22)	0.83 (0.25)	1.02 (0.26)	0.033
HDL_3-cholesterol (mmol/l)				
All	0.38 (0.08)	0.39 (0.08)	0.39 (0.09)	0.683
ε3/ε3	0.38 (0.08)	0.40 (0.07)	0.40 (0.13)	0.665
ApoB (g/l)				
All	1.33 (0.30)	1.31 (0.30)	1.30 (0.27)	0.650
ε3/ε3	1.37 (0.30)	1.31 (0.30)	1.13 (0.27)	0.016
ApoA-1 (g/l)				
All	1.47 (0.20)	1.48 (0.22)	1.48 (0.19)	0.997
ε3/ε3	1.47 (0.19)	1.48 (0.19)	1.58 (0.22)	0.218
Lp(a) (mg/l)				
All	338 (429)	205 (270)	265 (284)	0.030
ε3/ε3	359 (466)	185 (253)	273 (290)	0.078
Triglycerides (mmol/l)				
All	1.65 (0.87)	1.56 (0.92)	1.36 (0.70)	0.138
ε3/ε3	1.68 (0.87)	1.44 (0.71)	1.06 (0.50)	0.016

Values are mean (S.D.). Differences between means were tested by ANCOVA, smoking status as a covariate. Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; ApoB, apolipoprotein B; ApoA-1, apolipoprotein A-1; Lp(a), lipoprotein (a).

ciates statistically significantly more often with higher than median VLDL-cholesterol (Md = 0.57 mmol/l) and triglyceride concentrations (Md = 1.32 mmol/l) than the haplotype −219T/+113C/ε3 (OR = 2.6, $p = 0.0007$ and OR = 2.1, $p = 0.0086$, respectively; Table 3). However, when checked for conditional independence of the two promoter loci, it appeared that the polymorphisms could affect the traits also independently.

4. Discussion

The genetic impact of the APOE ε2/ε3/ε4 polymorphism on serum lipid and lipoprotein concentrations, mainly total cholesterol, LDL-cholesterol and apoB, has been shown in numerous studies [17,19,23–25]. Yet the possible role of the APOE promoter polymorphisms −219G/T and +113G/C in regulating the serum lipids and lipoproteins is somewhat unclear. We studied the association of these two polymorphisms and the APOE haplotype with various lipid and lipoprotein variables in a randomly selected group of middle-aged Finnish men.

Our results showed that within the most common APOE ε3/ε3 genotype group the −219G/G and +113G/G genotypes associated with higher apoB, triglyceride and VLDL-cholesterol concentrations and lower HDL-cholesterol concentrations than the more rare −219T/T and +113C/C genotypes. Additionally, the −219G/+113G/ε3 haplotype associated statistically significantly more often with higher (over median) VLDL-cholesterol and triglyceride concentrations than the −219T/+113C/ε3 haplotype.

To our knowledge, there are no previous studies relating the +113G/C polymorphisms or APOE −219/+113/ε3 haplotypes with fasting lipid levels of ultracentrifugally isolated serum lipoprotein fractions. The ultracentrifugation method for assessing lipoprotein fractions is quite laborious and time-consuming and is seldom used in large epidemiological studies.

Table 2
Plasma lipid and lipoprotein concentrations in the APOE +113G/C genotype groups for the whole study population and for the APOE ε3/ε3 genotype group

Variable	+113G/C genotype			*p*-value
	G/G n_{all} = 118, $n_{\varepsilon3/\varepsilon3}$ = 54	G/C n_{all} = 87, $n_{\varepsilon3/\varepsilon3}$ = 56	C/C n_{all} = 12, $n_{\varepsilon3/\varepsilon3}$ = 12	
Total cholesterol (mmol/l)				
All	5.50 (0.86)	5.53 (0.95)	5.14 (0.96)	0.296
ε3/ε3	5.62 (0.93)	5.46 (0.90)	5.14 (0.96)	0.154
VLDL-cholesterol (mmol/l)				
All	0.78 (0.50)	0.62 (0.35)	0.43 (0.26)	0.002
ε3/ε3	0.75 (0.42)	0.62 (0.38)	0.43 (0.26)	0.010
LDL-cholesterol (mmol/l)				
All	3.51 (0.81)	3.68 (0.83)	3.30 (0.91)	0.188
ε3/ε3	3.65 (0.90)	3.60 (0.71)	3.30 (0.91)	0.308
HDL-cholesterol (mmol/l)				
All	1.21 (0.28)	1.22 (0.25)	1.41 (0.27)	0.036
ε3/ε3	1.22 (0.23)	1.23 (0.26)	1.41 (0.27)	0.034
HDL_2-cholesterol (mmol/l)				
All	0.83 (0.27)	0.83 (0.25)	1.02 (0.26)	0.043
ε3/ε3	0.84 (0.22)	0.83 (0.25)	1.02 (0.26)	0.033
HDL_3-cholesterol (mmol/l)				
All	0.38 (0.08)	0.39 (0.07)	0.40 (0.13)	0.555
ε3/ε3	0.38 (0.08)	0.40 (0.07)	0.40 (0.13)	0.595
ApoB (g/l)				
All	1.33 (0.29)	1.32 (0.30)	1.13 (0.27)	0.039
ε3/ε3	1.37 (0.30)	1.32 (0.30)	1.13 (0.27)	0.014
ApoA-1 (g/l)				
All	1.47 (0.22)	1.47 (0.19)	1.58 (0.22)	0.215
ε3/ε3	1.47 (0.19)	1.48 (0.19)	1.58 (0.22)	0.224
Lp(a) (mg/l)				
All	292 (381)	221 (280)	273 (291)	0.423
ε3/ε3	376 (481)	171 (214)	273 (290)	0.043
Triglycerides (mmol/l)				
All	1.69 (0.98)	1.42 (0.69)	1.06 (0.50)	0.007
ε3/ε3	1.67 (0.87)	1.46 (0.71)	1.06 (0.50)	0.022

Values are mean (S.D.). Differences between means were tested by ANCOVA, smoking status as a covariate. Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; ApoB, apolipoprotein B; ApoA-1, apolipoprotein A-1; Lp(a), lipoprotein (a).

The previous studies on association of the −219G/T polymorphisms with fasting lipid or lipoprotein levels have been negative, [11,14,15] contrary to the results of this study. Moreno et al. [15] suggested that the −219T-allele would predispose to lower levels of postprandial apoE and prolonged postprandial lipemia. The basal state plasma lipids, however, did not differ between the −219G/T genotype groups in their study of 51 healthy APOE ε3/ε3 men [15]. Our results in the whole study population are similar concerning the basal state lipids. However, within the most common APOE ε3/ε3 genotype group the plasma triglyceride, apoB and VLDL- and HDL-cholesterol concentrations in the fasting state differed between the −219G/T genotype groups; the −219G/G genotype associated with higher lipid and apoB levels, and lower HDL-cholesterol levels than the −219C/C genotype group. Lambert et al. observed no major effects on lipids by the −219G/T polymorphism despite its effect on the plasma apoE concentration [11]. In our study, the lipid profile, i.e.

Table 3
Estimated haplotype frequencies for groups below and over median lipid concentrations for the APOE ε3/ε3 genotype group (total frequency is 246)

Haplotype			VLDL-cholesterol		OR (95% CI)	*p*-value	Triglyceride		OR (95% CI)	*p*-value
−219G/T	+113G/C	ε2/ε3/ε4	<Md	>Md			<Md	>Md		
G	G	ε3	76	89	2.6 (1.6, 4.9)	0.0007	76	89	2.1 (1.3, 3.9)	0.0086
G	C	ε3	1	1	2.2 (0.6, 57.7)	0.56	1	1	1.8 (0.9, 45.6)	0.681
T	G	ε3	1	0	–	–	1	0	–	–
T	C	ε3	54	24	1	Ref.	50	28	1	Ref.

Md: median, OR: odds ratio, CI: confidence interval.

increased plasma apoB, triglyceride and VLDL-cholesterol concentrations, in subjects with the G-ε3/G-ε3 genotype may indicate that in these subjects the liver VLDL synthesis is increased and/or that their VLDL catabolism (conversion of VLDL to IDL and LDL) is impaired and/or that their clearance from the plasma is altered compared to subject with other genotype combinations. However, there were no differences in the levels of plasma LDL-cholesterol between the different APOE promoter genotype groups which might refer that the found genotype differences are mainly resulted from increased liver VLDL (apoB) synthesis or inadequate clearance of these particles in subject with the G-ε3/G-ε3 genotype. It has been shown in both animal and human studies that large amounts of apoE increase the production of VLDL in the liver and at the same time impair the VLDL lipolysis resulting in increased serum VLDL-cholesterol and triglyceride concentrations [26,27]. Due to the fact that promoter polymorphisms affect the apoE production, we hypothesize that the increased VLDL-cholesterol in G/G genotype carriers in our study could partly be explained by the abovementioned effect of apoE on VLDL production and lipolysis [26,27]. However, kinetic studies of these lipoproteins in subjects with different APOE (promoter) genotype combinations are needed to solve this question. Interestingly, Tomiyasu et al. have suggested that the apoE content of human VLDL affects its metabolism so that, for example, the dense VLDL particle containing apoE has lower fractional catabolic rate than the dense VLDL particle without the apoE [28].

The aforementioned studies by Lambert et al. [11] and Moreno et al. [15] include either *younger* or *wider* age cohort of people compared to our study population; we had a randomly selected sample of middle-aged men from 50 to 59 years, an age group in which dyslipidemia is more often found. Since we had no exclusion criteria, our study population quite faithfully represents – from healthy to diseased – the population of middle-aged men in Finland. Moreover, our Finnish study population is genetically more homogenous compared to the ECTIM Study [11] or Spanish population [15] partly due to the long isolation and a fairly recent population bottleneck. Therefore, our results might not be applicable for larger, more diverse populations containing women, children and very old people. Besides the differences in the study populations and design, the somewhat discrepant results between our study and the aforementioned studies [11,15] might be due to additional loci affecting the APOE gene transcription and serum lipid and lipoprotein metabolism [29]. Furthermore, in different populations there might be different combinations of SNPs explaining the variation in a particular trait such as serum lipid concentrations [29]. In accordance with this suggestion we observed that the APOE-219/+113/ε3 *haplotype* could modify the serum triglyceride and VLDL-cholesterol concentrations. Thus the variation in certain lipid variables could, at least partly, be explained by the studied APOE haplotype. Interestingly, our preliminary data suggest that some of the genotypes, which in the present study associated with higher lipid levels, tend to associate with larger atherosclerotic lesion areas in coronary arteries in an autopsy series (unpublished data).

In summary, in addition to the common polymorphic sites 3937 and 4075 resulting in the APOE ε2/ε3/ε4 alleles, loci in the regulatory region of the APOE gene explain variation in different components of the lipid metabolism [29]. According to our results, the −219G/T and +113G/C polymorphisms of the APOE gene could explain part of the variation in serum triglyceride, apoB, VLDL- and HDL-cholesterol concentrations, especially within the APOE ε3/ε3 genotype group. These polymorphisms also form a haplotype, which associated with elevated triglyceride and VLDL-cholesterol concentrations.

Acknowledgements

This study has received financial support from the grants of Medical Research Fund of Tampere University Hospital, Finnish Foundation for Cardiovascular Research, The Academy of Finland (grant number 104821), Kalle Kaihari Heart Research Fund, The Emil Aaltonen Foundation, The Yrjö Jahnsson Foundation, The Elli and Elvi Oksanen Fund of the Pirkanmaa Fund under the auspices of the Finnish Cultural Foundation and the Finnish Medical Foundation.

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Relations of APOE promoter polymorphisms to LDL cholesterol and markers of subclinical atherosclerosis in young adults

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Abstract The common apolipoprotein E (apoE) gene (APOE) ε2/ε3/ε4 polymorphism explains part of serum lipid variation, and polymorphisms in the APOE promoter region have been proposed to participate in the regulation of serum lipid levels within the most common APOE ε3/ε3 genotype group. We determined APOE −219G/T and +113G/C promoter genotypes and estimated APOE haplotypes in 525 participants of the Cardiovascular Risk in Young Finns Study. We studied the associations of the APOE promoter polymorphisms and their haplotypes with cross-sectional and longitudinal serum lipid and apolipoprotein concentrations as well as with flow-mediated dilatation (FMD), carotid artery compliance (CAC), and intima-media thickness (IMT) within the APOE ε3/ε3 carriers. We found no significant association between the APOE promoter genotypes and serum lipids [low density lipoprotein-cholesterol (LDL-C), HDL-C, and triglycerides], apolipoproteins (apoA-I and apoB), or brachial artery FMD, CAC, or carotid IMT in either men or women. In longitudinal analyses in males, the carriers of heterozygous genotypes (−219G/T or +113G/C) and, furthermore, carriers of the −219T/+113C/ε3 haplotype had significantly higher LDL-C and total cholesterol concentrations throughout the 21 year follow-up period compared with homozygous G allele carriers or noncarriers of the −219T/+113C/ε3 haplotype. Such associations were not found in females. In summary, the APOE promoter polymorphisms −219G/T and +113G/C as well as their haplotype are associated with longitudinal changes in LDL-C and total cholesterol concentrations in young Finnish males but do not seem to be major determinants for FMD, CAC, or carotid IMT in males or females.—Viiri, L. E., O. T. Raitakari, H. Huhtala, M. Kähönen, R. Rontu, M. Juonala, N. Hutri-Kähönen, J. Marniemi, J. S. A. Viikari, P. J. Karhunen, and T. Lehtimäki. **Relations of APOE promoter polymorphisms to LDL cholesterol and markers of subclinical atherosclerosis in young adults. *J. Lipid Res.* 2006.** 47: **1298–1306.**

Supplementary key words lipid • intima-media thickness • flow-mediated dilatation • carotid artery compliance

Apolipoprotein E [apoE (protein); APOE (gene)] plays an important role in lipoprotein metabolism, which contributes to the development and progression of atherosclerosis, a disease starting already in childhood. Therefore, APOE is one of the most vigorously studied genes in relation to this disease. In addition to the effects of the commonly known APOE alleles ε2, ε3, and ε4 on serum lipid levels, APOE promoter region polymorphisms also have been shown to be associated with serum lipid concentrations, especially within APOE ε3/ε3 carriers (1). Srinivasan and colleagues (2) suggested in their 16 year follow-up study that the APOE ε2/ε3/ε4 polymorphism tends to influence the longitudinal change in serum low density lipoprotein-

Manuscript received 20 January 2006 and in revised form 8 March 2006.

Published, JLR Papers in Press, March 9, 2006.
DOI 10.1194/jlr.M600033-JLR200

Abbreviations: apoE, apolipoprotein E protein; APOE, apolipoprotein E gene; BMI, body mass index; CAC, carotid artery compliance; CRP, C-reactive protein; CV, coefficient of variation; FMD, flow-mediated dilatation; LDL-C, low density lipoprotein-cholesterol; IMT, intima-media thickness.

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cholesterol (LDL-C) concentrations. The relations between the APOE promoter polymorphisms and lipid levels, however, have not yet been studied in a longitudinal setting.

Ultrasound methods can be used to study early atherosclerotic changes. For example, measurements of carotid artery wall intima-media thickness (IMT), brachial artery flow-mediated dilatation (FMD), and carotid artery compliance (CAC) have been used as markers of vascular changes related to subclinical atherosclerosis (3). IMT represents a structural marker of atherosclerosis, whereas FMD is a functional marker of endothelial health; independently, they both predict cardiovascular events in populations (4, 5). CAC, on the other hand, measures the elasticity of large arteries, the decrease of which is considered to be a risk factor for cardiovascular disease. A recent meta-analysis by Elosua and coworkers (6) found no association between the APOE genotype and common carotid artery IMT in women, but in men the APOE ε2 allele associated with smaller common carotid artery IMT compared with the ε3 allele. The possible association of the APOE promoter polymorphisms −219G/T and +113G/C with IMT, FMD, or CAC has not been studied previously. We wanted to address this question along with studying whether the serum lipid, apolipoprotein, or C-reactive protein (CRP) concentrations differ between APOE promoter genotype groups in young Finns. The individuals were participants in the prospective multicenter Cardiovascular Risk in Young Finns Study, which was launched in 1980 to obtain data on the risk factors of coronary heart disease (7). We also wanted to investigate whether the possible differences between the genotypes/haplotypes in lipid concentrations remained constant throughout the follow-up period of 21 years from childhood to adulthood. To exclude the previously known effects of the common APOE ε2/ε3/ε4 polymorphism and to test the independent effects of the APOE promoter polymorphisms, we focused our studies on APOE ε3/ε3 carriers.

SUBJECTS AND METHODS

Study subjects

This study consists of a subsample of subjects from the ongoing Cardiovascular Risk in Young Finns Study, which was launched in 1980 in five university cities in Finland with medical schools and their surrounding rural communities. Details of the study design have been presented elsewhere (7). In short, the study included 3,596 randomly selected boys and girls aged 3, 6, 9, 12, 15, and 18 years. All subjects participating in 1980 were invited to follow-up studies in 1983 and 1986 and to the 21 year follow-up study in 2001. Cardiovascular risk factors, including smoking, alcohol use, diabetes, hypertension, body mass index (BMI), blood pressure values, and serum lipids were measured in 2001. Additionally, carotid artery IMT, FMD, and CAC were measured by ultrasonography in 2001. ApoE ε2/ε3/ε4 phenotype analyses were carried out in 1986, and the APOE promoter polymorphisms −219G/T and +113G/C were genotyped in 2005. In total, our random subsample consists of 928 cases, the APOE genotype frequencies of which were as follows: ε2/ε2, 2 (0.2%); ε2/ε3, 47 (5.1%); ε2/ε4, 22 (2.4%); ε3/ε3, 535 (57.5%); ε3/ε4, 289 (31.1%); and ε4/ε4, 33 (3.6%). From the 535 APOE ε3/ε3 carriers, we studied 525 in whom the APOE promoter genotyping was successful.

Subjects gave written informed consent in 2001, and their parents gave it in 1980. The study was approved by local ethics committees.

Clinical characteristics

Height and weight were measured, and BMI was calculated. Blood pressure was measured with a random zero sphygmomanometer (Hawksley and Sons, Ltd., Lancin, UK). The mean of at least three measurements was used in the analysis. Smoking habits, history of diabetes, and alcohol use were ascertained as part of a self-administered questionnaire. Smokers were categorized according to daily smoking into ever or never smokers.

Biochemical analyses

In 2001, serum lipid (apoA-I and apoB) concentrations were determined in the laboratory of the National Public Health Insti-

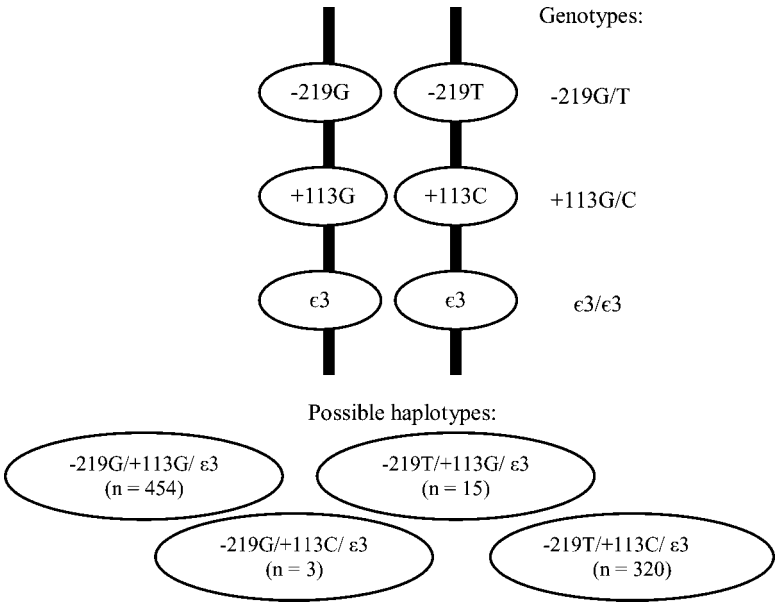

Fig. 1. Schematic representation of two chromosome strands with three single nucleotide polymorphisms [apolipoprotein E gene (APOE) −219G/T, APOE +113G/C, and APOE ε2/ε3/ε4] forming genotypes and haplotypes. The observed frequencies of haplotype carriers in this study are presented in parentheses.

tute in Turku, Finland. Serum total cholesterol and triglycerides were determined enzymatically (Olympus System Reagent; Olympus Diagnostica GmbH, Hamburg, Germany) in a clinical chemistry analyzer (AU400; Olympus Optical, Ltd., Mishima, Japan). HDL-C was measured enzymatically from serum supernatant after precipitation of LDL and VLDL with dextran sulfate and $MgCl_2$ (8). LDL-C was calculated by the Friedewald formula: LDL-C = total cholesterol − HDL-C − triglyceride ÷ 2.2 (9). Subjects having triglyceride concentrations of >4 mmol/l (n = 6 within the APOE ε3/ε3 carriers) were excluded from this analysis. Serum apoA-I and apoB were analyzed immunoturbidometrically (Orion Diagnostica, Espoo, Finland). Sensitive CRP was measured using latex turbidometric immunoassay (Wako Chemicals GmbH, Neuss, Germany). The lower detection limit reported for the assay was 0.06 mg/l. Serum homocysteine concentrations were measured with a microparticle enzyme immunoassay kit (Imx assay; Abbott Laboratories, Tokyo, Japan).

Ultrasound measurements

Ultrasound studies were performed using Sequoia 512 ultrasound mainframes (Acuson, CA) with a 13.0 MHz linear array transducer, as described previously (10). In short, to measure carotid IMT, the image was focused on the posterior (far) wall of the left carotid artery. A minimum of four measurements of the common carotid far wall were taken ~10 mm proximal to the bifurcation to derive mean carotid IMT values. The between-visit (two visits 3 months apart) coefficient of variation (CV) of IMT measurements was 6.4% (10).

To asses CAC indices, several 5 s image clips of the beginning of the common carotid artery were acquired. From these clips, the best-quality cardiac cycle was selected and manually analyzed to measure systolic and diastolic common carotid diameters, as described previously (11). The 3 month between-visit CV was 2.7% for diastolic carotid diameter and 16.3% for CAC.

To evaluate brachial artery FMD, the left brachial artery diameter was measured at rest and during reactive hyperemia, as described previously (12). In short, increased flow was induced by inflation of a pneumatic tourniquet placed around the forearm to a pressure of 250 mm Hg for 4.5 min, followed by release. Three measurements of arterial diameter were performed at end-diastole at a fixed distance from an anatomic marker at rest as well as 40, 60, and 80 s after cuff release. The vessel diameter in scans after reactive hyperemia was expressed as a percentage relative to the resting scan value (100%). The 3 month between-visit CV was 3.2% for brachial artery diameter and 26.0% for FMD measurements (12).

ApoE phenotyping and APOE promoter genotyping

ApoE ε2/ε3/ε4 phenotyping was done as described previously (13). The APOE gene promoter −219G/T and intron +113G/C

TABLE 1. Clinical and biochemical characteristics and preclinical indicators of atherosclerosis in APOE −219 promoter genotype groups within APOE ε3/ε3 carriers

		−219			
Characteristic	Sex	G/G	G/T	T/T	*P*
Number	F	86	151	42	0.024
	M	103	118	26	
Age (years)	F	31.6 (5.1)	31.5 (5.0)	30.9 (4.9)	0.783
	M	32.1 (4.9)	31.2 (5.2)	32.1 (4.3)	0.658
BMI (kg/m^2)	F	24.5 (4.0)	24.4. (4.6)	24.4. (4.8)	0.966
	M	25.7 (3.4)	25.8 (3.4)	25.8 (4.4)	0.996
Systolic blood pressure (mm Hg)	F	116.7 (12.7)	114.8 (10.8)	114.0 (9.7)	0.341
	M	128.4 (13.5)	129.0 (12.4)	130.7 (8.8)	0.693
Diastolic blood pressure (mm Hg)	F	72.7 (8.4)	70.9 (7.7)	69.8 (6.2)	0.099
	M	75.4 (9.0)	74.8 (8.5)	76.3 (7.9)	0.698
Daily smoking (never/ever)	F	54/30	84/66	22/19	0.382
	M	50/52	55/61	15/11	0.638
Total cholesterol (mmol/l)	F	5.03 (0.83)	5.00 (1.02)	5.20 (0.88)	0.477
	M	5.14 (1.08)	5.29 (1.01)	5.02 (1.23)	0.389
LDL-cholesterol (mmol/l)	F	3.11 (0.72)	3.07 (0.80)	3.33 (0.67)	0.146
	M	3.30 (0.91)	3.49 (0.91)	3.24 (1.16)	0.239
HDL-cholesterol (mmol/l)	F	1.40 (0.29)	1.39 (0.31)	1.39 (0.33)	0.951
	M	1.18 (0.27)	1.20 (0.27)	1.16 (0.29)	0.767
Triglycerides (mmol/l)	F	1.15 (0.55)	1.18 (0.62)	1.06 (0.45)	0.522
	M	1.44 (0.90)	1.40 (0.97)	1.45 (1.14)	0.769
Apolipoprotein A-I (g/l)	F	1.60 (0.26)	1.58 (0.28)	1.56 (0.29)	0.689
	M	1.42 (0.21)	1.44 (0.19)	1.39 (0.20)	0.523
Apolipoprotein B (g/l)	F	1.00 (0.23)	1.00 (0.25)	1.03 (0.21)	0.646
	M	1.11 (0.27)	1.13 (0.29)	1.09 (0.35)	0.720
CRP (mg/l)	F	1.00 (0.41;2.42)	1.01 (0.46;2.92)	1.13 (0.26;2.49)	0.555
	M	0.65 (0.24;1.51))	0.63 (0.35;1.52)	0.56 (0.25;3.39)	0.908
Homocysteine (μmol/l)	F	8.60 (7.00;10.23)	8.40 (7.40;10.40)	8.00 (5.95;9.90)	0.164
	M	9.85 (8.50;11.53)	9.80 (8.70;11.40)	10.65 (7.75;11.65)	0.955
IMT (mm)	F	0.57 (0.09)	0.57 (0.08)	0.56 (0.09)	0.718
	M	0.59 (0.09)	0.58 (0.11)	0.59 (0.09)	0.837
CAC (%/10 mm Hg)	F	2.36 (0.75)	2.30 (0.82)	2.31 (0.89)	0.884
	M	2.08 (0.67)	2.05 (0.64)	1.94 (0.56)	0.636
FMD (%)	F	9.42 (3.72)	8.89 (4.52)	7.77 (4.84)	0.148
	M	7.61 (4.37)	7.46 (4.21)	8.03 (4.46)	0.846

APOE, apolipoprotein E gene; BMI, body mass index; CAC, carotid artery compliance; CRP, C-reactive protein; F, female; FMD, flow-mediated dilatation; IMT, intima-media thickness; M, male. Values are expressed as means (SD), n values or median (Q^{25};Q^{75}). Total n values differ slightly regarding BMI and FMD as a result of some missing measurements. *P* values are by ANOVA. Nonnormally distributed variables (triglycerides, CRP, and homocysteine) were log-transformed before analyses.

polymorphisms were genotyped using 5′ nuclease assay (14) and fluorogenic TaqMan probes (Roche Molecular Systems) in the ABI Prism® 7000 Sequence Detection System (Applied Biosystems).

Haplotype reconstruction

Haplotypes were reconstructed using the PHASE program (version 2.0.2) (15, 16). A schematic illustration of the genotypes and haplotypes used in this study is presented in **Fig. 1**. For purposes of statistical analyses, the study subjects were categorized into carriers and noncarriers of distinct haplotypes: −219G/+113G/ε3, −219T/+113G/ε3, and −219T/+113C/ε3. There were only three carriers of the haplotype −219G/+113C/ε3, so this haplotype was excluded from all statistical analyses.

Statistical analyses

The APOE genotype frequencies were first tested for Hardy-Weinberg equilibrium. Then, the genotype frequencies were compared between men and women. Because the genotype frequencies differed significantly between the sexes within the APOE ε3/ε3 carriers, all further analyses were performed separately for men and women. This was true for haplotype frequencies as well. The distributions of cardiovascular risk factors and vascular parameters (measured in 2001) were compared between the APOE genotype groups using ANOVA (continuous variables) and the Chi-square test (categorical variables). Nonnormally distributed triglyceride, CRP, and homocysteine concentrations were log-transformed before the analyses, but the results are expressed as crude.

The longitudinal lipid data were analyzed by repeated-measurement ANOVA using the APOE promoter genotypes or haplotypes as categorical factors (one at a time) and LDL-C or total cholesterol concentrations (one at a time) at different years of follow-up (1980, 1983, 1986, and 2001) as dependent repeated variables. In case of a statistically significant main effect, posthoc tests (with Bonferroni correction) were used to compare the differences between the genotype/haplotype groups. Statistical calculations were done using SPSS (version 12.0) on a personal computer.

RESULTS

Characteristics of the study population

The final study population consisted of 525 APOE ε3/ε3 carriers in whom the APOE promoter genotyping was successful. The frequencies of the −219G and −219T alleles were 0.62 and 0.38, respectively, and those of

TABLE 2. Clinical and biochemical characteristics and preclinical indicators of atherosclerosis in APOE +113 promoter genotype groups within APOE ε3/ε3 carriers

		+113			
Characteristic	Sex	G/G	G/C	C/C	*P*
Number	F	93	147	39	0.054
	M	107	112	27	
Age (years)	F	31.7 (5.1)	31.4 (4.9)	30.8 (4.9)	0.586
	M	31.9 (5.0)	31.2 (5.2)	31.9 (4.3)	0.581
BMI (kg/m^2)	F	25.0 (4.4)	24.2 (4.5)	24.0 (4.5)	0.289
	M	25.7 (3.4)	25.6 (3.3)	26.0 (4.5)	0.855
Systolic blood pressure (mm Hg)	F	116.5 (13.0)	115.0 (10.5)	113.2 (9.6)	0.294
	M	128.3 (13.2)	129.0 (12.6)	130.3 (8.9)	0.760
Diastolic blood pressure (mm Hg)	F	72.5 (8.4)	71.0 (7.7)	69.5 (6.3)	0.112
	M	75.2 (8.9)	74.8 (8.6)	76.0 (7.8)	0.805
Daily smoking (never/ever)	F	57/33	83/64	21/17	0.527
	M	52/54	53/57	15/12	0.787
Total cholesterol (mmol/l)	F	5.00 (0.85)	5.00 (1.01)	5.19 (0.91)	0.552
	M	5.13 (1.05)	5.30 (1.02)	5.02 (1.21)	0.311
LDL-cholesterol (mmol/l)	F	3.11 (0.75)	3.07 (0.78)	3.30 (0.68)	0.246
	M	3.31 (0.90)	3.51 (0.92)	3.26 (1.14)	0.220
HDL-cholesterol (mmol/l)	F	1.38 (0.27)	1.40 (0.32)	1.41 (0.33)	0.879
	M	1.18 (0.27)	1.19 (0.27)	1.16 (0.29)	0.801
Triglycerides (mmol/l)	F	1.14 (0.55)	1.19 (0.62)	1.05 (0.46)	0.425
	M	1.41 (0.84)	1.41 (0.98)	1.43 (1.13)	0.872
Apolipoprotein A-I (g/l)	F	1.58 (0.24)	1.58 (0.30)	1.58 (0.30)	0.997
	M	1.42 (0.21)	1.44 (0.19)	1.38 (0.20)	0.452
Apolipoprotein B (g/l)	F	1.00 (0.24)	1.00 (0.25)	1.03 (0.22)	0.761
	M	1.11 (0.26)	1.14 (0.29)	1.09 (0.35)	0.582
CRP (mg/l)	F	1.03 (0.42;2.45)	0.99 (0.45;2.93)	1.13 (0.24;2.49)	0.676
	M	0.66 (0.24;1.53)	0.60 (0.35;1.42)	0.57 (0.26;3.36)	0.887
Homocysteine (μmol/l)	F	8.60 (7.00;10.50)	8.40 (7.40;10.13)	8.00 (6.10;9.90)	0.211
	M	9.75 (8.50;11.53)	9.85 (8.68;11.48)	10.60 (7.80;11.60)	0.951
IMT (mm)	F	0.57 (0.09)	0.57 (0.08)	0.56 (0.09)	0.710
	M	0.59 (0.09)	0.58 (0.11)	0.58 (0.09)	0.818
CAC (%/10 mm Hg)	F	2.32 (0.78)	2.31 (0.80)	2.36 (0.91)	0.955
	M	2.07 (0.66)	2.07 (0.65)	1.96 (0.56)	0.726
FMD (%)	F	9.25 (3.82)	8.98 (4.48)	7.59 (4.94)	0.139
	M	7.56 (4.36)	7.43 (4.29)	8.15 (4.40)	0.772

Values are expressed as means (SD), n values or median (Q^{25};Q^{75}). Total n values differ slightly regarding BMI and FMD as a result of some missing measurements. *P* values are by ANOVA. Nonnormally distributed variables (triglycerides, CRP, and homocysteine) were log-transformed before analyses.

the +113G and +113C alleles were 0.63 and 0.37. The promoter genotype distributions were in Hardy-Weinberg equilibrium and similar to those seen in previous Finnish studies (1, 17, 18). Age, BMI, systolic or diastolic blood pressure, and smoking did not differ significantly between the APOE promoter genotype groups in the ε3/ε3 carriers (**Tables 1, 2**). Also, alcohol consumption was similar in different genotype groups (data not shown). There were only four diabetic patients within the APOE ε3/ε3 carriers, and there was no significant difference in their distribution into different APOE promoter genotype groups.

Cross-sectional analyses

There were no major differences in serum cholesterol, triglyceride, apolipoprotein, CRP, and homocysteine concentrations between the APOE promoter genotype groups in men or women. In addition, IMT, CAC, and FMD values did not differ between the APOE promoter genotype groups in men or women. (Tables 1, 2).

Longitudinal changes in serum cholesterol values

In both sexes, LDL-C and total cholesterol concentrations changed over time ($P < 0.001$), but there was no interaction between time and APOE promoter genotypes, meaning that the differences between genotypes remained fairly constant through the follow-up period (from 1980 to 2001) (**Fig. 2**). The initial decrease in lipid curves represents the effect of puberty. In males, the APOE promoter genotypes associated significantly with the longitudinal change in LDL-C values (−219, $P = 0.012$; +113, $P =$

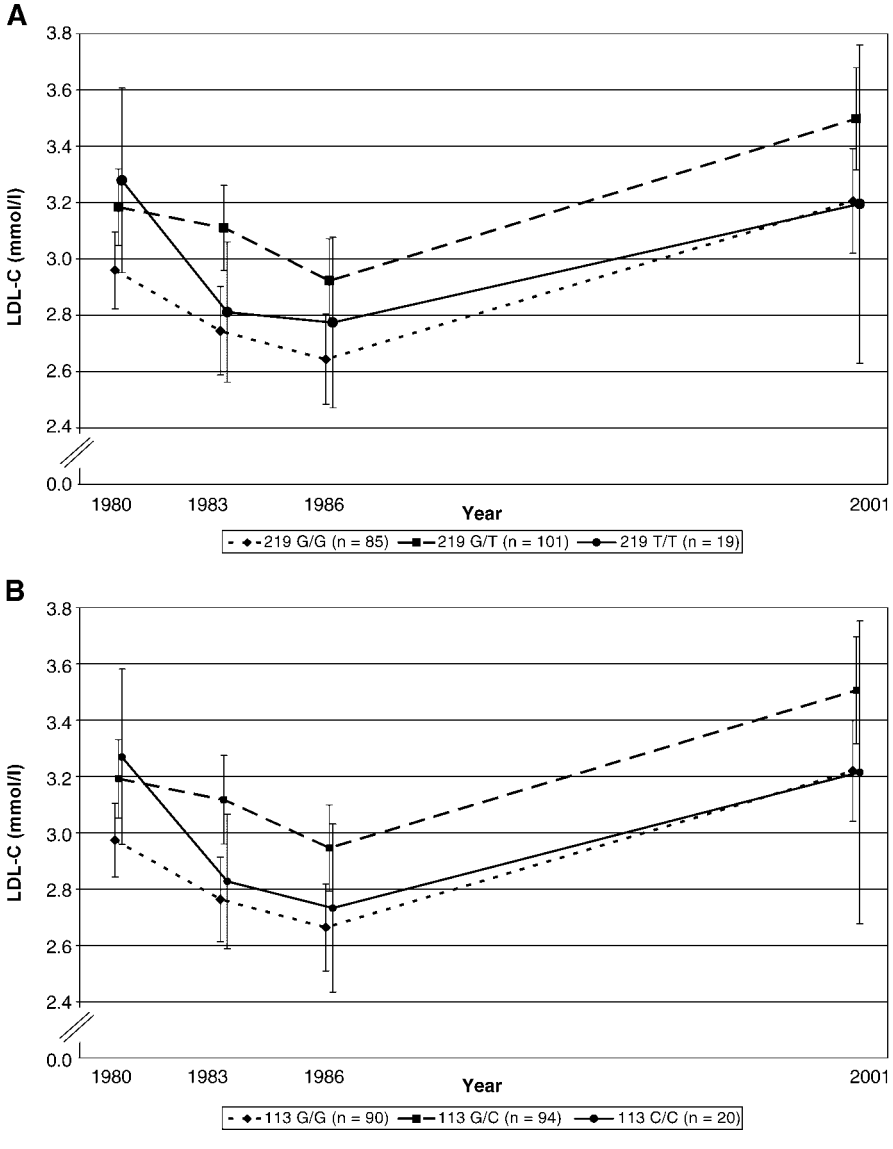

Fig. 2. Longitudinal change (from 1980 to 2001) of low density lipoprotein-cholesterol (LDL-C) values in males in APOE −219 genotype groups (G/G, G/T, and T/T; repeated-measurement ANOVA main effect for genotype, $P = 0.012$) (A) and in APOE +113 genotype groups (G/G, G/C, and C/C; repeated-measurement ANOVA main effect for genotype, $P = 0.013$) (B). Total cholesterol values follow the same patterns, although with higher values. Error bars represent 95% confidence intervals of the mean.

0.013). The APOE −219G/T and +113G/C genotype carriers had significantly higher LDL-C values over time compared with the −219G/G and +113G/G carriers, respectively (Fig. 2). The difference in LDL-C values between the heterozygotes and G/G homozygotes remained fairly constant (∼0.3 mmol/l) over time (−219, $P = 0.009$; +113, $P = 0.010$). Similarly, the APOE promoter genotypes associated significantly with the longitudinal change in total cholesterol values (−219, $P = 0.017$; +113, $P = 0.015$). The APOE −219G/T and +113G/C genotype carriers had significantly higher total cholesterol values over time compared with the G/G carriers: the difference between the heterozygotes and G/G homozygotes remained fairly constant (∼0.3 mmol/l) over time (−219, $P = 0.014$; +113, $P = 0.012$).

The longitudinal analyses also revealed that males carrying the −219T/+113C/ε3 haplotype had significantly higher LDL-C concentrations throughout the follow-up period compared with noncarriers of this haplotype. Furthermore, the difference in LDL-C values between the carriers and noncarriers of the −219T/+113C/ε3 haplotype remained relatively constant (on average, 0.3 mmol/l; $P = 0.007$) over time (**Fig. 3A**). Similarly, the total cholesterol concentration was higher in −219T/+113C/ε3 haplotype carriers compared with noncarriers of this haplotype, and the difference was on average 0.2 mmol/l throughout the follow-up period ($P = 0.012$) (**Fig. 4A**).

In females, LDL-C and total cholesterol concentrations changed over time ($P < 0.001$), but there were no statistically significant differences in longitudinal LDL-C or total

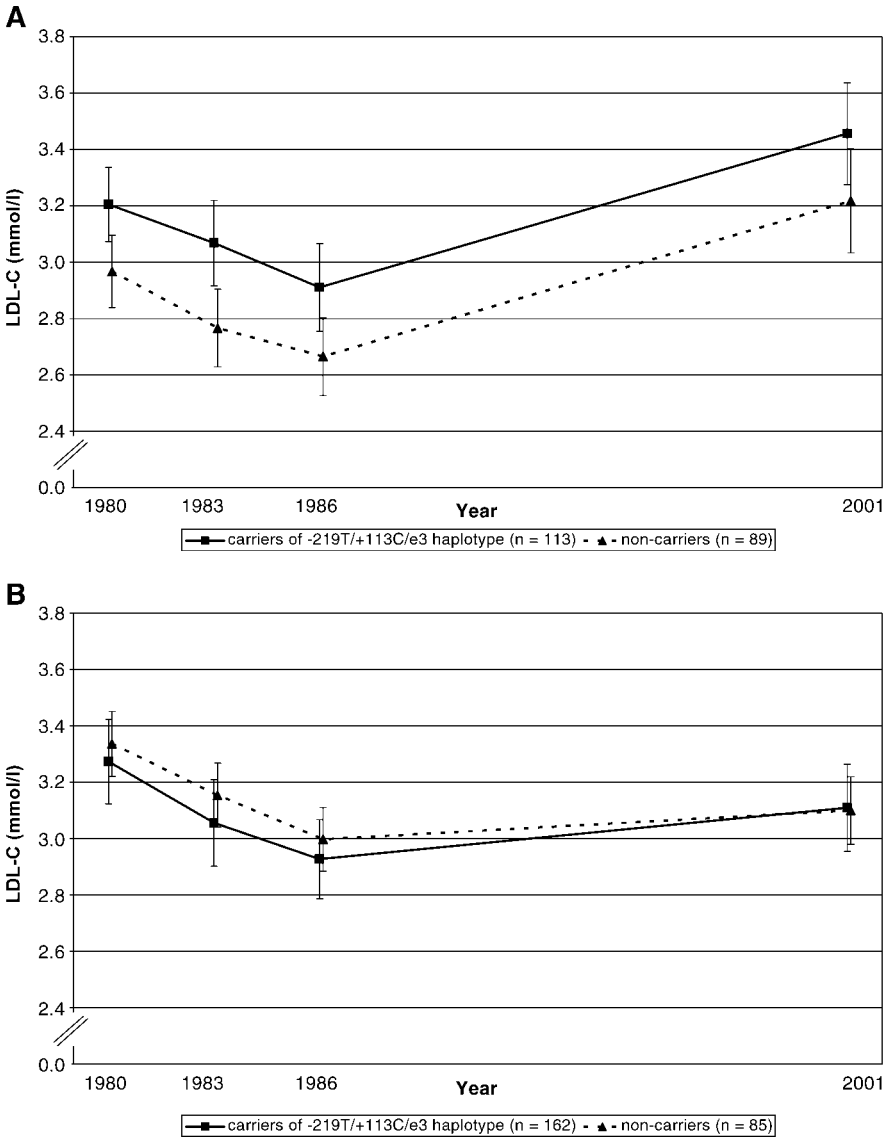

Fig. 3. Longitudinal change (from 1980 to 2001) of LDL-C values in APOE −219T/+113C/ε3 haplotype carriers and noncarriers in males (repeated-measurement ANOVA main effect for haplotype, $P = 0.007$) (A) and in females (repeated-measurement ANOVA main effect for haplotype, $P = 0.492$) (B). The time-haplotype interaction was statistically nonsignificant in both sexes. Error bars represent 95% confidence intervals of the mean.

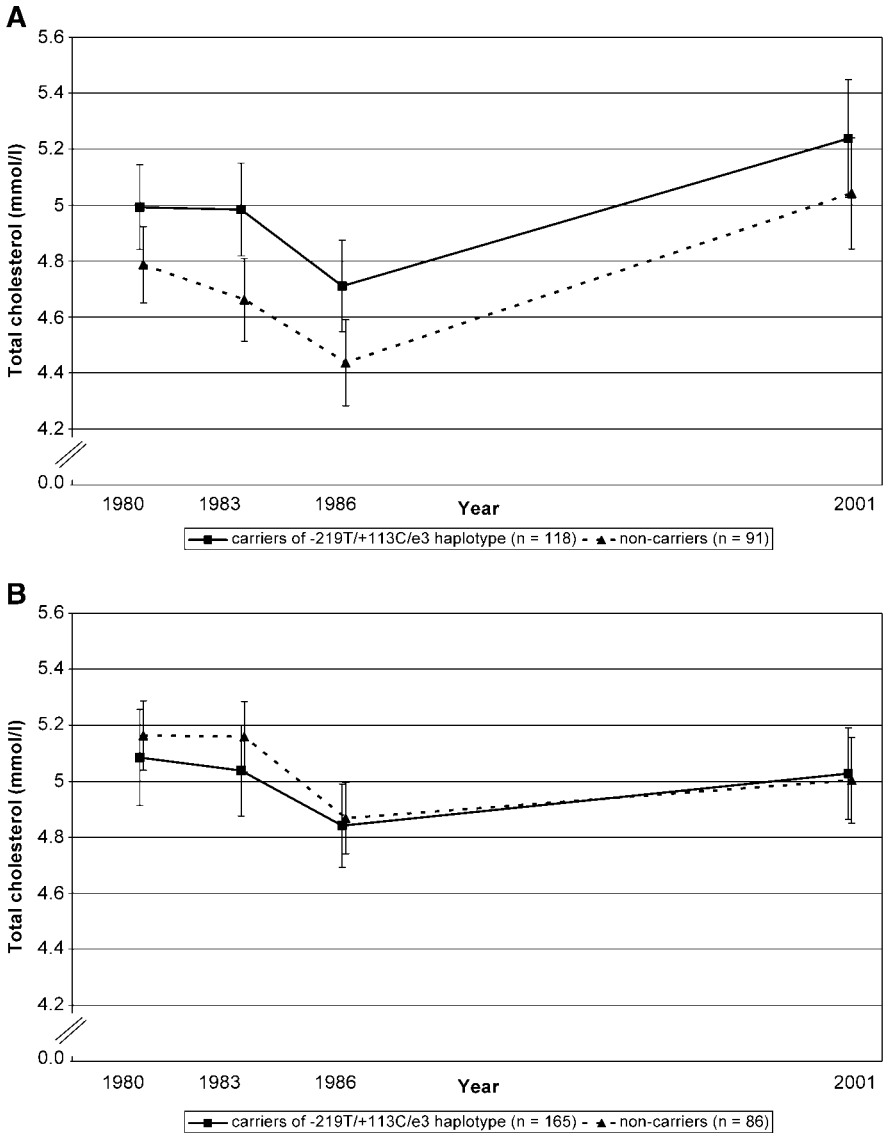

Fig. 4. Longitudinal change (from 1980 to 2001) of total cholesterol values in APOE −219T/+113C/ε3 haplotype carriers and noncarriers in males (repeated-measurement ANOVA main effect for haplotype, $P = 0.012$) (A) and in females (repeated-measurement ANOVA main effect for haplotype, $P = 0.580$) (B). The time-haplotype interaction was statistically nonsignificant in both sexes. Error bars represent 95% confidence intervals of the mean.

cholesterol values between the APOE haplotype groups (Figs. 3B, 4B).

DISCUSSION

In this study, males carrying the heterozygous genotypes (−219G/T or +113G/C) or haplotype −219T/+113C/ε3 had higher LDL-C and total cholesterol values throughout the 21 year follow-up period (from 1980 to 2001) compared with homozygous G allele carriers or noncarriers of the −219T/+113C/ε3 haplotype. In females, such differences in longitudinal cholesterol values between genotypes or haplotypes were not seen. Nor did we find any association of APOE promoter genotypes with the early measures of atherosclerosis in men or women in cross-sectional analyses.

We previously showed that the APOE −219G/+113G/ε3 haplotype associates with higher concentrations of VLDL-C and triglycerides in middle-aged (50–59 years) Finnish men (1). Similar associations were not found in this study of younger (24–39 years) men and women; instead, associations with longitudinal change in LDL-C and total cholesterol concentrations were recognized. We also previously showed the common APOE ε2/ε3/ε4 polymorphism to be associated with LDL-C and total cholesterol values in a 6 year follow-up study (13). The association of APOE promoter genotypes and APOE haplotypes with longitudinal changes in lipid values, however, had not been studied previously. It is also noteworthy that in this

study the follow-up period is rather long (21 years) compared with that in many other studies.

The mechanisms underlying the associations of the studied APOE promoter polymorphisms and haplotypes with serum lipid concentrations are presumably diverse. The APOE −219G/T polymorphism was shown previously to affect APOE transcription, the T allele associating with lower APOE promoter activity (19, 20). Moreover, Lambert and coworkers (21) have shown that the −219T allele associates with lower plasma apoE concentration compared with the −219G allele. Therefore, the APOE −219G/T polymorphism could affect various parts of lipid metabolism, such as cholesterol absorption from the intestine, cholesterol uptake by the liver, and cholesterol synthesis, in which apoE has an important role. Functional studies of the +113G/C polymorphism are still missing, but the locus is known to locate within an enhancer region in intron 1, which suggests that it could participate in the regulation of APOE transcription (22) and hence also play a part in lipid metabolism. Our genetic association study is somewhat limited because we do not have the apoE concentrations measured. Therefore, we cannot make any definite conclusions about the effects of the APOE haplotypes on apoE plasma levels. Neither can we make any explicit conclusions about the exact mechanisms of the found associations of the APOE promoter genotypes and haplotypes with the lipid concentrations.

The relation between the APOE ε2/ε3/ε4 polymorphism and IMT has been studied extensively, some studies suggesting an association between APOE and higher IMT (23–25) and others failing to show such an association (26–28). The possible associations of the APOE −219G/T and +113G/C promoter polymorphisms with IMT, carotid elasticity, or indicators of endothelial function have not, to our knowledge, been studied previously. We found large variation in FMD (CV, 26%) and CAC (CV, 16%) measurements but small variation in IMT as well as brachial and carotid artery diameter measurements. This suggests that much of the variation of FMD and CAC relates to physiologic fluctuation in vascular function and not to measurement error. Therefore, although our study excludes major effects of the APOE promoter polymorphisms on FMD and CAC, it is possible that, as a result of the large variation in the data, weak relations may have been undetected.

In conclusion, our findings indicate that within the group of young Finnish APOE ε3/ε3 carriers, the APOE promoter polymorphisms −219G/T and +113G/C and their haplotype play a part in explaining the longitudinal changes in serum cholesterol concentrations in men, but they do not seem to have an effect on the markers of subclinical atherosclerosis.

The authors thank Marita Koli and Nina Peltonen for their skillful technical assistance and Irina Lisinen and Ville Autio for their skillful statistical assistance. This study was financially supported by the Kalle Kaihari Heart Research Fund (to L.E.V.), the Emil Aaltonen Foundation (to T.L.), the Academy of Finland (Grants 53392 and 34316), the Social Insurance Institution of Finland, the Turku University Foundation, the Juho Vainio Foundation, the Finnish Foundation of Cardiovascular Research, the Finnish Cultural Foundation, and the Tampere and Turku University central hospital medical funds.

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European Journal of Human Genetics (2008), 1–6

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ARTICLE

Associations of apolipoprotein E gene with ischemic stroke and intracranial atherosclerosis

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The apolipoprotein E *(APOE)* ε4 allele is associated with elevated cholesterol and risk of atherosclerosis. However, its role in ischemic stroke (IS) remains controversial. We investigated a possible link between IS or the severity of intracranial atherosclerosis and the *APOE* promoter polymorphisms −219G/T and +113G/C, involved in regulating *APOE* transcription. We genotyped subjects from a multicentric Belgian case–control study, including 237 middle-aged patients with IS due to small- or large-vessel atherosclerotic stroke and 326 ethnicity- and gender-matched controls and a Finnish autopsy series of 1004 non-stroke cases, who had received a quantitative score of atherosclerosis in the circle of Willis. The *APOE* ε4+ genotype did not associate with IS, but was related to more severe intracranial atherosclerosis score in men (5.4 *vs* 4.6, $P=0.044$). Within the most common *APOE* ε3/ε3 genotype group, the risk of IS associated with the G-allele of the tightly linked −219G/T (OR = 6.2; 95% CI: 1.6–24.3, $P=0.009$) and +113G/C (OR = 7.1; 95% CI: 1.7–29.9, $P=0.007$) promoter polymorphisms. There was no difference in the severity of intracranial atherosclerosis between −219G/G genotype carriers and non-carriers. This study suggests a multifaceted role of apoE on the risk of cerebrovascular diseases. The *APOE* ε4+ genotype did not predict the risk of IS but was associated with severity of subclinical intracranial atherosclerosis in men on the autopsy study. In contrast, the promoter variants were significant predictors of IS, suggesting that quantitative rather than qualitative variation of apoE is related to IS.
European Journal of Human Genetics advance online publication, 27 February 2008; doi:10.1038/ejhg.2008.27

Keywords: *APOE* gene; ischemic stroke; intracranial atherosclerosis

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Received 2 October 2007; revised 5 January 2008; accepted 22 January 2008

Introduction

A large number of candidate gene association studies have attempted to identify genes involved in stroke.[1] The etiology of stroke is heterogeneous, and familial predisposition contributes only moderately (OR = 1.3–1.76) to

the risk of all stroke.[2] One way to increase the power of such studies would be to select a predefined phenotype. Genetic influence has been found to be the strongest in ischemic stroke (IS) due to small-vessel occlusion (SVO) or large-vessel atherosclerosis (LVA), as defined by the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification. This influence was especially strong in younger (<60 years) patients.[3–5] Thus, middle-aged SVO and LVA stroke patients seem to be a suitable study population for studies attempting to identify genes involved in the pathogenesis of IS.

Despite the association of the *APOE* ε4 allele with elevated LDL cholesterol levels and slightly elevated cardiovascular risk,[6] a recent meta-analysis found no clear link between the *APOE* ε4 allele and IS.[7] On the other hand, elevated plasma concentration of apoE is a risk factor for stroke.[8,9] Furthermore, it has been shown that apoE concentration is strongly associated with cardiovascular mortality in old age, independently of *APOE* genotype and plasma lipids.[10] Plasma level of apoE is dependent on total protein, albumin level, body mass index (BMI) and alcohol consumption. Moreover, plasma apoE is largely liver-derived and could be regulated by hepatic factors. Besides the *APOE* ε2/ε3/ε4 genotype affecting the structure of apoE protein, the biological activity of apoE can also be influenced by genetic factors that modify its synthesis and quantity. It has been shown that genetic variation in the *APOE* gene promoter, the −219G/T single nucleotide polymorphism (SNP) located in the regulatory region of the *APOE* gene and the closely linked +113G/C SNP located in the intron 1 enhancer region, may affect the transcription of *APOE* gene.[11] Carriers of the −219G/G-genotype had 10–20% higher apoE plasma concentrations compared to −219T/T genotype.[12] Moreover, the −219G/T polymorphism has been reported to associate with atherogenic lipid and lipoprotein profile,[13,14] as well as with coronary atherosclerosis.[15] However, the role of these SNPs in IS and in cerebral atherosclerosis has not been studied before.

In this study, we tested the association between the *APOE* ε4, −219G/T and +113G/C SNPs and IS in a setting of a case–control study of 237 middle-aged patients and 326 ethnicity- and gender-matched controls. To evaluate whether any positive result may be related to predisposition to intracranial atherosclerosis, we also tested whether these genetic variants are associated with atherosclerosis of the circle of Willis in an independent Finnish autopsy series.

Material and methods

The Belgian stroke study (BSS)

BSS is a case–control study. The 237 cases had SVO or LVA stroke (TOAST classification), all occurring between 45 and 60 years of age. The patients were selected from the databases of seven stroke units in Belgium. All patients were of central European origin (>90% were Belgians). Cardiovascular risk factors (hypertension, diabetes mellitus, hyperlipidemia (hypercholesterolemia or hypertriglyceridemia), alcohol consumption (>20 g/day), smoking (former, current and never), obesity (BMI >30)) were recorded.

The control group was composed of 326 gender- and ethnicity-matched healthy volunteers without a history of stroke and living in the same area. As the mean age of stroke occurrence is typically around 70 years of age, and because we selected only younger (<60 years) stroke patients, we explicitly selected older controls than cases to decrease the likelihood that they would later in their life get an IS. Stroke patients had higher prevalence of conventional cardiovascular risk factors than controls. Therefore, to avoid a potential selection bias due to the different cardiovascular risk factors and differences in the mean age, all our genetic results were adjusted for cardiovascular risk factors and age.

Optimal methods to identify and control for population stratification in genetic association studies are not established. A recent study showed that grandparental country of origin provided better control for stratification than the SNP-based approach.[16] In this study, ethnicity was checked up to the four grandparents. The ethical committees of all participating hospitals approved the study protocol. Written informed consent was obtained from all patients before study entry.

The Finnish autopsy series

The Finnish autopsy series comprised of two cross-sectional population-based autopsy studies. In total, the two series included 1004 medico-legal Caucasians autopsy cases that had died suddenly out of hospital, or were found dead. The atherosclerosis of each of the nine branches of the circle of Willis was scored semi-quantitatively as follows: 0 = normal, 1 = slight (streaks with or without elevated fibrous lesions), 2 = moderate (fibrous lesions that cause <50% stenosis), 3 = severe (>50% stenosis with extensive atherosclerosis (fatty, fibrous and calcified lesions)), giving a range of scores from 0 to 27. The cases were from two studies: the Tampere Autopsy Study (TASTY) ($n = 604$) and the Helsinki Sudden Death Study (HSDS)[17] ($n = 400$). The TASTY comprised both men (64.3%, mean age 59.7 years) and women (35.7%, mean age 68.2 years), whereas the HSDS series included only men (mean age 53.7 years). In addition to gender and age, BMI was also recorded. The Finns are particularly suitable for genetic association studies being a homogenous Caucasian population, which results from genetic isolation.[18]

Genotyping

In the BBS, DNA was isolated from samples of whole blood, which had been stored frozen at −20°C, with a

commercially available kit (Qiagen Inc., Valencia, CA). In the autopsy series, DNA isolation was performed either from frozen blood samples (TASTY) with the salt precipitation method or from frozen (−70°C) cardiac muscle samples (HSDS) with the standard phenol-chloroform method. Genotyping was carried out by using the 5′ nuclease assay and fluorogenic allele-specific TaqMan MGB probes in the ABI Prism 7900 HT sequence detection. The DNA and PCR master mix (together 5 μl) were pipeted into the 384-well plates using Tecan Freedom EVO 100 instrument and instrument software V4.8 (Tecan Schweitz AG, Switzerland). To monitor genotyping errors, random duplicates were run in parallel with unknown samples. Allele-specific fluorescence generated from each probe during the PCR amplification was measured with the allelic discrimination analysis module. The nucleotide sequences of primers and probes used in the PCR were deduced from the public databases and synthesized in conjunction with Applied Biosystems. The SNPs evaluated in this study were *APOE* $\varepsilon2/\varepsilon3/\varepsilon4$, −219G/T (rs405509) and +113G/C (rs440446). Because of technical problems in the PCR of the +113G/C SNP and a tight linkage disequilibrium ($D' = 0.86$ $r2 = 0.44$; calculated by the Stata 8.0 program, STATA Corporation, TX, USA) between the –219G/T and +113G/C SNPs, only the −219G/T SNP was analyzed from all of the autopsy cases. The mean genotyping success was >95%.

Statistical analysis

Data were analyzed using the SPSS software (version 12.0, SPSS Inc., Chicago, IL, USA). The clinical data were compared between the IS cases and controls, using a binary logistic regression analysis with age as continuous covariate. To examine the effect of the *APOE* $\varepsilon4$ allele, study populations were divided into $\varepsilon4$ allele carriers ($\varepsilon3/4$ and $\varepsilon4/4$) and non-carriers ($\varepsilon2/2$, $\varepsilon2/3$ and $\varepsilon3/3$). Since the $\varepsilon2/\varepsilon4$ genotype was rare ($n = 18$) and difficult to assign in a group ($\varepsilon2$ and $\varepsilon4$ allele carriers usually have opposite effects), it was excluded from the analyses. A binary logistic regression analysis with smoking, hypertension, alcohol consumption, obesity, diabetes and hyperlipidemia as dichotomous covariates and age as continuous covariate was used to evaluate the association of the genetic variants with IS. To study the association of the promoter polymorphism with IS excluding the confounding effects of the *APOE* $\varepsilon2/\varepsilon3/\varepsilon4$ genetic variation, we performed the analysis within the most common *APOE* $\varepsilon3/\varepsilon3$ genotype group. Furthermore, analysis of covariance (ANCOVA) with age and BMI as continuous covariates was used to compare the mean atherosclerosis scores between the studied genotypes. We analyzed four SNPs that are in linkage disequilibrium and located in the same gene. Therefore, applying a multiple comparison statistics, such as Bonferroni correction, is inappropriate, because the individual SNPs are not independent.

Results

BSS series

The frequencies of the $\varepsilon2$, $\varepsilon3$ and $\varepsilon4$ alleles were 0.07, 0.80 and 0.14, respectively ($\varepsilon2/\varepsilon4$ was left out from the calculations). Within the *APOE* $\varepsilon3/\varepsilon3$ carriers, the frequencies of the −219G and −219T alleles were 0.55 and 0.45, and the frequencies of the +113G and +113C alleles were 0.56 and 0.44, respectively. All genotype frequency distributions were in Hardy–Weinberg equilibrium in cases and in controls. Stroke patients had higher prevalence of conventional cardiovascular risk factors, such as hypertension, hyperlipidemia, current smoking and alcohol consumption than controls (Table 1). The *APOE* $\varepsilon4$ carrier frequency did not differ significantly between the IS patients and controls (Table 2). Within the most common *APOE* $\varepsilon3/\varepsilon3$ genotype group, both the −219G and +113G allele carriers were more common among the IS cases than controls (OR = 6.2; 95% CI 1.6–24.3, $P = 0.009$ and OR = 7.1; 95% CI 1.7–29.9, $P = 0.007$, respectively) after adjustment for all recorded risk factors and age (Table 2). With a frequency of ~50% for the at-risk allele at an α level of 0.05, our sample was evaluated to have 95% power to detect an RR of 1.6 for heterozygote and 3.2 for homozygotes ('genetic power calculator': http://statgen.iop.kcl.ac.uk/gpc/cc2.html).

Finnish autopsy series

The frequencies of the $\varepsilon2$, $\varepsilon3$ and $\varepsilon4$ alleles were 0.05, 0.79 and 0.17, respectively ($\varepsilon2/\varepsilon4$ was left out from the calculations). Within the *APOE* $\varepsilon3/\varepsilon3$ carriers, the frequencies of the −219G and −219T alleles were 0.62 and 0.38, respectively. All APOE allele frequencies were similar to the frequencies in the BSS series. The carriers of the *APOE* $\varepsilon4$ tended to have higher intracranial atherosclerosis score (5.4 *vs* 4.8, $P = 0.051$) compared to non-carriers, but this association was statistically significant only among men (5.4 *vs* 4.6, $P = 0.044$). Within the *APOE* $\varepsilon3/\varepsilon3$ genotype group, there was no significant difference in the circle of Willis atherosclerosis score between carriers and non-carriers of the *APOE* −219G allele. (Table 3)

Discussion

Several studies have addressed the role of *APOE* $\varepsilon4$ in stroke, but the results have been inconsistent.[7] In our selected Belgian stroke population, no association was seen between the *APOE* $\varepsilon4$ allele and IS. However, in the Finnish autopsy study, we found out that men carrying the *APOE* $\varepsilon4$ allele had significantly higher mean intracranial atherosclerosis score compared to the $\varepsilon4$ non-carriers. Previous autopsy studies have revealed a link between the *APOE* $\varepsilon4$ allele and larger coronary and aortic atherosclerotic lesion areas in men.[17,19] Thus, it seems that the *APOE* $\varepsilon4$ allele may have a gender-specific role in the development of atherosclerosis in different vascular beds. The $\varepsilon4$ allele is

Table 1 Clinical characteristics of Belgian stroke study population and odds ratios for ischemic stroke

Characteristic	*IS*	*Controls*	*OR (95% CI)*	*P-value*
Hypertension (%)	636	380	5.4 (2.7–10.5)	0
Diabetes (%)	164	108	1.3 (0.6–3.0)	467
Hyperlipemia (%)	582	365	2.7 (1.4–5.1)	0.002
Smoking status (%)				
Current	557	93	4.7 (2.3–9.7)	0
Former	15.1	21	1.3 (0.5–3.0)	0.571
Never	29.2	69.8	1	Ref.
Alcohol consumption >20 g/day (%)	30.4	21.2	2.2 (1.1–4.3)	0.026
Obesity, BMI > 30 (%)	16.6	13.2	0.6 (0.3–1.4)	0.243
Male (%)	67	66		

BMI = body mass index; IS = ischemic stroke; OR = odds ratio; Ref. = reference.
The *P*-values are from binary logistic regression analysis, adjusted for age.

Table 2 Genotype frequencies (%) for Ischemic stroke cases and controls in the Belgian stroke study population

Genotype	*IS (n = 237)*	*Controls (n = 326)*	*OR (95% CI)*	P-*value*
APOE				
ε 4+	25.2	25.2	0.9 (0.5–1.5)	0.715
ε 4−	74.8	74.8	1	ref.
APOE −219 (ε3/ε3)				
G +	84.1	76.4	**6.2** (1.6–24.3)	**0.009**
T/T	15.9	23.6	1	ref.
APOE +113 (ε3/ε3)				
G +	85	76.4	**7.1** (1.7–29.9)	**0.007**
C/C	15	23.6	1	Ref.

CI = confidence interval; G+ = carrier of the G-allele; IS = ischemic stroke; OR = odds ratio; Ref. = reference; ε4− = non-carrier of the ε4 allele; ε4+ = carrier of the ε4 allele.
The *P*-values are from binary logistic regression analyses with age, hypertension, hyperlipidemia, diabetes, obesity, smoking and alcohol consumption as covariates.

known to be associated with high LDL cholesterol level,[6] which is an important factor in the early development of atherosclerosis. Therefore, it can be hypothesized that the ε4 allele mainly affects the initial stages of cerebral atherosclerosis. In addition, ε4 also seems to have an independent role in cerebral small vessel disease, as suggested by its effect on MRI white matter hyperintensities in a study by Hogh *et al*.[20]. These authors propose that ε4 may interact with other cardiovascular risk factors, such as hypertension, in affecting lipid metabolism and cellular repair mechanism.[20] In fact, a previous study showed that *APOE* ε4 has a decreased antioxidant activity compared to other alleles.[21]

The role of the *APOE* promoter polymorphisms −219G/T and +113G/C in IS or cerebral atherosclerosis had not been studied before. Our results suggest that G-allele carriers of both polymorphisms are at an increased risk of IS. This association was significant within the most common *APOE* ε3/ε3 genotype group, indicating that it is most probably independent of the *APOE* ε2/ε3/ε4 genotype. The *APOE* gene promoter polymorphism −219G/T affects the transcriptional activity of the *APOE* gene, in particular the G-allele is associated with higher transcription than the T-allele.[11] Consequently, G-allele carriers have significantly higher plasma concentrations of apoE.[12] Recent studies suggested that the apoE level is related to stroke.[9,8] Our results provide the evidence that a genetic determinant of higher levels of apoE increases the risk of IS.

ApoE seems to have a multifaceted role on atherosclerosis and vascular events in humans. Our findings suggest that the association of the G-alleles of both promoter SNPs with the risk of IS would involve mechanisms other than those leading to an accelerated development of stable intracranial atherosclerosis. One hypothesis would be that increased apoE level predispose to an unstable atherosclerotic plaque. This is supported by studies on apoE −/− mice. Although these animals develop severe atherosclerosis, they rarely have spontaneous plaque rupture and thrombosis.[22] Furthermore, a recent study showed that increased apoE deposits in early

Table 3 Mean atherosclerosis score in Helsinki Sudden Death Study (HSDS) and Tampere Autopsy Study (TASTY) series

	All			*Men*			*Women*	
	N	*Score*	P-*value*	N	*Score*	P-*value**	N	*Score*	P-*value**
ε4+	289	5.4 (6.0)	0.051	222	5.4 (5.9)	**0.044**	67	5.6 (6.4)	0.596
ε4−	626	4.8 (5.6)		492	4.6 (5.4)		134	5.7 (6.5)	
−219 (within ε3/ε3)									
G+	458	4.8 (5.6)	0.960	370	4.6 (5.3)	0.591	88	5.9 (6.5)	0.399
T/T	75	4.9 (5.7)		57	4.7 (5.4)		18	5.4 (6.7)	

ε4+ = carrier of the ε4 allele; ε4− = non-carrier of the ε4 allele; G+ = carrier of the G-allele (APOE −219G/G and −219G/T).
Values are presented as mean (SD).
The *P*-values are from analysis of covariance (ANCOVA) with age, gender and body mass index as covariates.
*The *P*-values are from analysis of covariance (ANCOVA) with age and body mass index as covariates.

atherosclerotic lesions distinguish symptomatic from asymptomatic patients.[23] A second mechanism may be related to the finding that apoE has proinflammatory properties.[10,24] Elevated apoE level lead to chronic inflammation that may contribute to atherosclerosis. Another possibility is that apoE influences the risk of IS by a mechanism independent of atherosclerosis. ApoE is known to play a role in the coagulation pathway, in particular, by affecting vitamin K1 metabolism. Vitamin K1 is a chylomicron-bound essential cofactor for the synthesis of several blood coagulation factors. Chylomicron uptake and clearance are affected significantly by *APOE* genotype,[25] leading to marked fluctuations in plasma concentrations of vitamin K1.[26] Low vitamin K1 levels have been measured, especially in patients carrying the $\varepsilon 4$ allele[26] and are associated with a poor outcome in hemorrhagic stroke patients.[27] Thus, possible interactions between *APOE* variants and vitamin K1 metabolism might be related to the risk of IS independently of the development of atherosclerosis.

A possible explanation of the observed difference of *APOE* effect on cerebral atherosclerosis in men and women could be related to the gender-dependent effect of apoE isoforms on immune system activation[28,29] or to the influence of sex hormones on apoE protein production.[30]

In summary, this study revealed a multifaceted role of *APOE* gene on IS and subclinical intracranial atherosclerosis. The *APOE* $\varepsilon 4+$ genotype was associated with the severity of subclinical intracranial atherosclerosis in men, but was not a predictor of IS. On the other hand, the promoter variants affecting apoE synthesis were significant predictors of IS, suggesting that quantitative rather than qualitative variation of apoE is related to IS.

Acknowledgements

We thank the laboratory technicians Nina Peltonen and Ana Lopes Cruz for their help. This study was funded by Erasme Funds, FNRS, Emil Aaltonen Foundation (TL), Medical Research Fund of Tampere University Hospital, the Pirkanmaa Regional Fund of the Finnish Cultural Foundation, the Finnish Foundation for Cardiovascular Research and the Yrjö Jahnsson Foundation.

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