Some Epidemiological Aspects of The Common Genetic Polymorphism of Apolipoprotein E

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In memoriam

Jeg ønsker at dedikere dette arbejde til min lillebror *Karsten Gerdes* som døde den 14. juni 2000, kun 45 år gammel. *Karsten* var chef for Vårdcentralen i Hällefors i mange år, og var en pligtopfyldende og meget dygtig læge. Han var højt respekteret, men ind imellem også frygtet for sin styrke og sit temperament — også i privatlivet. Desværre synes *Karsten* at have miskendt en snigende depression, som langsomt formørkede hans sind og til sidst fik ham til at tage en fatal beslutning. Ære være hans minde!

I wish to dedicate this work to my younger brother *Karsten Gerdes* who died on the 14th of June 2000, only 45 years old. *Karsten* was head of the Health Care Center in Hällefors (Sweden) for many years and was a dutiful and very capable physician. He was highly respected, but sometimes also feared for his strength and temper — also in his private life. Sadly, however, *Karsten* seemingly failed to recognize an insidious depression, which slowly clouded his mind and eventually made him take a fatal decision. All honor to his memory!

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Denne afhandling er i forbindelse med de nedenstående anførte tidligere offentliggjorte artikler af Det Sundheds-videnskabelige Fakultet ved Aarhus Universitet antaget til offentligt at forsvares for den medicinske doktorgrad.

Aarhus Universitet, den 22. april 2002

Søren Mogensen
Dekan

The review is based on the following papers, which are marked with **boldfaces** in the text:

1. Gerdes, L.U., Klausen, I.C., Sihm, I. and Færgeman, O. Apolipoprotein E polymorphism in a Danish population compared to findings in 45 other study populations around the World. *Genet.Epidemiol.* 9:155-167, 1992.

2. Hansen, P.S., Gerdes, L.U., Klausen, I.C., Gregersen, N. and Færgeman, O. Genotyping compared with protein phenotyping of the common apolipoprotein E polymorphism. *Clin.Chim.Acta* 224:131-137, 1994.

3. Gerdes, L.U. Interpopulation differentiation of the impact of polymorphic susceptibility genes. *Excerpta Med.Int.Congr.Series* 1066:614-618, 1995.

4. Gerdes, L.U., Gerdes, C., Hansen, P.S., Klausen, I.C. and Færgeman, O. Are men carrying the apolipoprotein ε4- or ε2 allele less fertile than ε3ε3 genotypes? *Hum.Genet.* 98:239-242, 1996.

5. Gerdes, L.U., Gerdes, C., Hansen, P.S., Klausen, I.C., Færgeman, O. and Dyerberg, J. The apolipoprotein E polymorphism in Greenland Inuit in its global perspective. *Hum.Genet.* 98:546-550, 1996.

6. Klausen, I.C., Gerdes, L.U., Hansen, P.S., Lemming, L., Gerdes, C. and Færgeman, O. Effects of apoE gene polymorphism on Lp(a) concentrations depend on the size of apo(a): a study of 466 white men. *J.Mol.Med.* 74:685-690, 1996.

7. Gerdes, L.U., Gerdes, C., Kervinen, K., Savolainen, M., Klausen, I.C., Hansen, P.S., Y.A. Kesäniemi, Færgeman, O. The apolipoprotein ε4 allele determines prognosis and the effect on prognosis of simvastatin in survivors of myocardial infarction. A substudy of the Scandinavian Simvastatin Survival Study. *Circulation.* 101:1366-1371, 2000.

8. Gerdes, L.U., Jeune, B., Ranberg, K.A., Nybo, H. and Vaupel, J.W. Estimation of apolipoprotein E genotype-specific relative mortality risks from the distribution of genotypes in centenarians and middle-aged men: apolipoprotein E gene is a "frailty gene", not a "longevity gene". *Genet.Epidemiol.* 19:202-210, 2000.

9. Gerdes, L.U., Lindholt, J.S., Vammen, S., Henneberg, E.W. and Fasting, H. Apolipoprotein E genotype is associated with differential expansion rates of small abdominal aortic aneurysms. *Br.J.Surg.* 87:760-765, 2000.

Paper no. 6 was also used in the doctoral thesis of Dr. Ib Christian Klausen: Lipoprotein(a) — a genetic risk marker for atherosclerotic diseases — the ethnic variability, and the influence of genetic and environmental factors on lipoprotein(a) concentrations in blood; Faculty of Health Sciences, University of Aarhus, 2000. However, in the present review, the results of that study are interpreted in a new context that was not considered by Dr. Klausen.

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Acknowledgements

I am much indebted to *Professor Ole Færgeman* who introduced me to lipoprotein metabolism in the early 1980s and asked me to help him establish a lipoprotein research laboratory at Aarhus University Hospital, Aarhus Amtssygehus. It was a daunting task, not only because of technical and financial challenges, but also because the field of lipoprotein research — »the cholesterol business« — had been largely ignored in Denmark, sometimes even opposed.

Fortunately, the winds shifted in the late 1980s, and we had some smooth sailing. The small vessel we had launched to explore local cholesterol coastlines was enlarged and equipped for cruising the oceans also of molecular genetics. *Ole* has had a fine sense of where to go. He is a warmhearted and intelligent man who has attracted and inspired a crew of many dynamic researchers over the years. I have enjoyed spending time with him on the bridge, studying and discussing the ever more complicated charts that we have come across. To strain the metaphor, I also liked working in the engine room and fiddling with our navigation instruments. However, I am particularly indebted to *Ole* for being a good and encouraging friend during those periods of my life when I spent much time otherwise alone in the crow's nest or the dinghy. He always believed in my abilities, and I am deeply grateful to him for his confidence.

It has been a pleasure and very instructive to work closely together with *Ib Christian Klausen* and *Peter Steen Hansen* on several projects. They are exceptionally competent researchers, and they became my good friends over the years. It has also been a delight, and still is, to collaborate with my younger brother and very good friend, *Christian Gerdes.* He is bright and thoughtful, and he is always amusing company. I have been fortunate also to enjoy the company of and the improvements of the laboratory and the department introduced by *Finn Heath, Henrik Kjærulf Jensen, Lone Andersen* and *Lone Lemming*, and more recently by *Jesper Møller Jensen, Bent Raungaard* and *Jens Uffe Brorholt-Petersen*. All of this would have come to naught, however, had it not been for abilities and hard work of *Susan Mackenzie, Gitte Glistrup Nielsen, Anette Stenderup* and *Pia Buchtrup Hornbeck* in the laboratory, *Pia Seeberg Møller* in the office, and also *Edith Clausen* in the library. I am grateful to all of them!

I also thank *Inger Sihm, Professor Niels Gregersen* and *Professor Jørn Dyerberg* for collaboration, and *Kari Kervinen, Markku Savolainen* and *Professor Y. Antero Kesäniemi* in Oulu, Finland, for having had the opportunity to collaborate with them on an important project. Moreover, *Jes S. Lindholt, Søren Vammen, Eskild W. Henneberg* and *Helge Fasting* from Viborg Hospital are thanked for the collaboration on a recent project.

I went to work at the Department of Clinical Biochemistry at Odense University Hospital in 1993-4. *Professor Mogens Hørder* introduced me to *Bernard Jeune, Karen Andersen-Ranberg, Hanne Nybo* and *Professor James W. Vaupel* at the Aging Research Center. These people are attempting to understand the ultimate effects of all those determinants of health that others (including me) are only studying in narrow windows, and I was fortunate to be involved in a study of Danish centenarians. I rank *Bernard* very highly for his outlook and warmth, and *Jim* for his intelligence and enthusiasm. My stay in Odense also brought me in contact with another prominent and inspiring American, *Professor Elizabeth Hedlund Corder.*

The complexities of lipoprotein metabolism and cardiovascular diseases seem to me to be surpassed by those of bone metabolism and osteoporosis. During the past year I have had the pleasure of having my horizon widened while working on a project with *Professor Leif Mosekilde, Peter Vestergaard* and *Pernille Hermann* at the Department of Endocrinology at Aarhus University Hospital, Aarhus Amtssygehus.

Much of the definitive research on apolipoprotein E is due to the creativity and work of *Professor Gerd Utermann, Professor Ulrike Beisiegel, Professor Charles F. Sing* and *Professor Robert W. Mahley.* It was a real privilege for me, many years ago, to visit and learn from *Professor Gerd Utermann* in his laboratory in Marburg. Later, *Ulrike Beisiegel* and her coworker *Wilfred Weber* taught me the apolipoprotein E phenotyping method in Hamburg. *Professor Charles F. Sing* of the University of Michigan has been a source of deep inspiration in thinking about the possibilities and limitations of classical molecular genetics. About a year ago, Ole Færgeman and I were guests of *Professor Mahley* at the Gladstone Institute of Cardiovascular Diseases in San Francisco, and some of the concepts that I shall develop in this thesis reflect the discussions that we had on that particular occasion.

Lastly, I wish to thank my children, *Jonas, Sebastian* and *Josefine*, and their mothers, for their patience with me. I have too often spent more time with my computer than with you. And, not least, I wish to thank *Lisbeth* for her support and loving care.

Financial support

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Taulov, June 2002

Ulrik Gerdes

Abbreviations

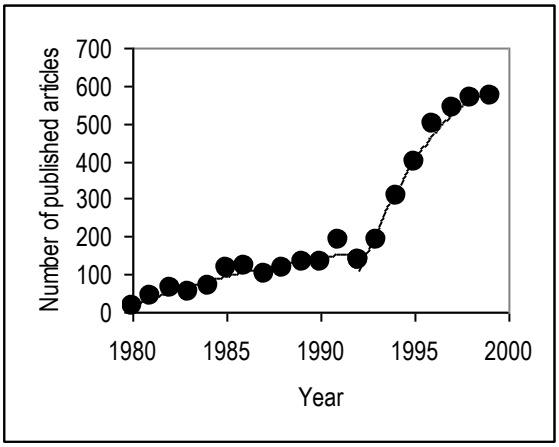
APOE	The apolipoprotein E gene
ApoE	The apolipoprotein E protein
APOE*2, APOE*3 and APOE*4	The three common APOE alleles
ApoE2, apoE3 and apoE4	The three apoE isoforms encoded by APOE*2, APOE*3 and APOE*4
ApoA-I, -II, and –IV	Apolipoproteins A-I, A-II and A-IV
Apo(a)	Apolipoprotein (a)
ApoB	Apolipoprotein B
ApoC-I, -II and III	Apolipoprotein C-I, C-II and C-III
ApoER2	Apolipoprotein E receptor 2 = LRP8
ApoJ	Apolipoprotein J
CETP	Cholesterol ester transfer protein
HDL	High density lipoprotein
HL	Hepatic lipoprotein lipase
HSPG	Heparan sulfate proteoglycans
IDL	Intermediate density lipoproteins
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low density lipoprotein
LDL-R	Low density lipoprotein receptor
Lp(a)	Lipoprotein (a)
LPL	Lipoprotein lipase
LRP	Low density lipoprotein receptor related protein
VLDL	Very low density lipoprotein
VLDLR	Very low density lipoprotein receptor
SNP	Single nucleotide polymorphism
SR-BI	Scavenger receptor class B type I

Contents

Outline of this thesis

Research into the biological functions of apolipoprotein E (apoE) and the effects of the genetic polymorphism of this protein in humans has uncovered an enigmatic little molecule with a surprising variety of different functions, and not less surprising relations to common diseases.

This is reflected in the amount of scientific literature on apoE. Thus, in November 2000 one could retrieve about 4,700 published articles since 1966 on the MEDLINE Internet Grateful Medicine service of the United States National Library of Medicine using the keyword "Apolipoproteins E". Only a score of these papers were published until 1980 and during the following 13 years the publication rate slowly increased to about 200 per year. Most of these papers dealt with the role of apoE in lipoprotein metabolism and the association of the genetic polymorphism with hyperlipidemia and cardiovascular disease.



From 1993 and onwards the publication rate rapidly rose to reach about 600 articles per year. This was due to the findings, first, that the common APOE*4 allele is strongly associated with risk of Alzheimer's disease [Strittmatter et al., 1993; Corder et al., 1993], and secondly, that mice whose apoE gene is disrupted provide excellent animal models for studying atherosclerosis [Plump et al., 1992; Zhang et al., 1992].

The present thesis considers a small fraction of the literature on the subject, and it is focused on some epidemiological aspects of the common genetic polymorphism of apoE that both cross-link and widen the evidence for the importance of this protein for development of disease.

The presentation is structured as follows:

1. Apolipoprotein E (apoE) and its gene (APOE)

Summary of the chapter

ApoE consists of 299 amino acid residues and a variable amount of sialic acid. The amino-terminal domain contains the receptor-binding region and the carboxy-terminal domain contains the lipid-binding region, but the two domains influence each other's biochemical properties. The APOE gene is on chromosome 19q13.2 and has four exons, three exons, and a complex promotor region with many regulatory elements. There are many variant alleles and most have their origin in simple nucleotide polymorphisms. Three of these alleles, APOE*2, APOE*3 and APOE*4, are common in nearly all populations, and have their origin in single-nucleotide polymorphisms in codon 112 and 158. In addition to polymorphisms in the coding region there are many polymorphisms in non-coding regions including the promotor region. Some of these are common.

The apolipoprotein E protein (apoE)

ApoE was identified as a constituent of VLDL in 1973 and first became known as the »arginine-rich apoprotein; ARP« because about 11% of the amino acid residues in the polypeptide chain is this diamino, monocarboxylic amino acid [Shore and Shore, 1973]. A few years later Utermann suggested the name »apoE« in accordance with the convention of using alphabetic nomenclature in this field [Utermann, 1975] and Rall et al. described the complete amino acid sequence in1982 [Rall et al., 1982]. The protein is synthesized with an 18-residue signal peptide that is removed cotranslationally, leaving a mature protein with 299 amino acids and molecular weight of 34.2 kDa. Additionally, about 20% of the apoE in plasma has a variable amount of N-acetylneuramidate (sialic acid) attached as O-linked carbohydrate to threonine 194 [Wernette-Hammond et al., 1989].

ApoE consists of two independently folded structural domains separated by a 'hinge' region of random structure. The amino-terminal domain (encompassing residues 1 to 119) exists as a stable globular bundle of four helices arranged in an antiparallel fashion with a short additional helix connecting two of them. The fourth helix in the domain (residue 130 to 164) contains the receptor-binding region and also the region responsible for the protein's ability to bind to glycosylaminoglycans (including heparin). The carboxy-terminal domain is elongated and consists of three amphipathic helical stretches, which contain the lipid-binding region of the protein. It tends to form tetrameric structures when cleaved from the other domain by thrombin and mediates a tetramerization of the intact delipidated protein [Weisgraber, 1994].

Although they are independently folded in the lipid-free state, the amino- and carboxy-terminal domains influences each other's biochemical properties in the intact protein, and thus also the properties of apoE as such. The subject is worth dwelling on because it is a fine illustration of how difficult it can be to anticipate the effects of a genetic polymorphism on functional properties of the encoded protein isoforms — and the consequent influences on phenotypic traits that are discernible only at a clinical or epidemiological level. In short, the impact of the common APOE polymorphism illustrates the unfeasibility of a through and through deterministic endeavor to "calculate" the macroscopic phenotypic effects of a simple genetic polymorphism:

- The gene coding for apoE shows a common polymorphism in codon 112 and the two corresponding protein isoforms, apoE3 and apoE4, have either a cysteine residue in this position or an arginine residue (see page 12; the apoE2 isoform is similar to apoE3 in this regard).
- Residue 112 is in helix 3 in the amino-terminal domain. In apoE3 the cysteine residue is close to arginine in position 61 in helix 2, whereas in apoE4 the arginine residue forms a salt-bridge with a glutamine residue in position 109 and causes the arginine in position 61 to adopt a different, more exposed position [Dong et al., 1994].
- The exposed arginine in position 61 in apoE4 interacts with a glutamic acid residue in position 255 in the carboxy-terminal domain [Dong and Weisgraber, 1996].
- This interaction somehow influences the properties of the lipid-binding domain so that apoE4 preferentially binds to the larger triglyceride-rich VLDL, whereas apoE3 preferentially binds to HDL [Gregg et al., 1986; Steinmetz et al., 1989b; Weisgraber, 1990].
- ApoE3 but not apoE4 can form disulfide-linked homodimers and heterodimers with apoA-II and this contributes to stabilizing the binding of apoE3 to HDL [Weisgraber and Mahley, 1978] [Innerarity et al., 1978; Weisgraber and Shinto, 1991] [Borghini et al., 1991].
- The preferential binding of apoE4 to VLDL is presumably an important reasons why plasma lipoprotein metabolism in carriers of the APOE*4 allele is different from metabolism in other individuals (see page 13).

The apolipoprotein E gene (APOE)

The APOE gene is located on chromosome 19q13.2 in a 45-kb cluster with the genes for apoC-I, apoC-II and apoC-IV, as well as an apoC-I pseudogene [Olaisen et al., 1982; Myklebost and Rogne, 1988; Smit et al., 1988b; Allan et al., 1995]. The gene has four exons and three introns, and a complex promotor region with many regulatory elements located in the proximal 5' flanking region and in the first intron [Paik et al., 1985; Smith et al., 1988; Paik et al., 1988; Chang et al., 1990; van den Maagdenberg, 1993; Artiga et al., 1998; Bullido and Valdivieso, 2000].

APOE shares several structural characteristics with the other three apolipoprotein genes in the cluster on chromosome 19, as well as with the cluster of genes for apoA-I, apoC-III and apoA-IV on chromosome 11 and with the apoA-II gene on chromosome 1. Therefore, the genes are probably evolutionarily related and form a so-called multigene family that arose from a common ancestral gene that existed about 680 million years ago. According to a scenario proposed by Luo et al., the ancestral gene was very similar to the present day apoC-I gene, whereas the genes for apoA-I and apoA-IV, and apoA-II and apoC-III are the most recently evolved genomic structures. They were formed about 280 million years ago. The APOE gene diverged from the ancestor of the apoA-I and apoA-IV genes about 420 million years ago, so APOE is not present in birds [Luo et al., 1986; Li et al., 1988]. In contrast, the APOB gene, which codes for the other ligand of the LDL receptor, is probably a member of a wholly different family of genes coding for large lipid transfer proteins (LLTP). They include the genes coding for insect apolipophorin, human apolipoprotein B, invertebrate and vertebrate vitellogenins and the large subunit of mammalian microsomal triglyceride transfer protein (MTP), and presumably emerged from an ancestral molecule which played a pivotal role in the intracellular and extracellular transfer of lipid soluble substances [Babin et al., 1999].

APOE mutants and polymorphisms

About 30 variant alleles coding for different transcription products have been described [de Knijff et al., 1994]. Four rare alleles code for protein isoforms, which differ in size from other isoforms. One has a 21 bp insertion in exon 4, one has a single-nucleotide deletion in exon 3 causing a frame shift and a premature stop codon, one has a single-nucleotide mutation in intron 3 creating abnormally spliced mRNA forms, and the last has a single-nucleotide mutation creating a premature stop codon in exon 3. All other allelic variants code for apoE isoforms that only differ from each other with respect to the amino acid residue in one or more positions.

Three of these alleles have their origin in single-nucleotide polymorphisms (SNPs) at codon 112 and 158 in exon 4 and are common in nearly all populations (see page 21) — APOE*4 (Arg112; Arg158), APOE*3 (Cys112; Arg158), and APOE*2 (Cys112; Cys158). Those are the alleles that were originally detected by isoelectric focusing of apoE [Utermann et al., 1977]. Five other alleles are not uncommon, at least not in some populations, and are due to SNPs in codon 3 (Glu;Lys), codon 28 (Leu;Pro), codon 84 (Pro;Arg), codon 142 (Arg;Cys), and in codons 244 and 245 (Glu;Lys). All other coding variants seem to be rare [de Knijff et al., 1994; Nickerson et al., 2000].

In addition to the polymorphisms (mutations) in the coding regions of APOE there are at least 18 polymorphisms in the noncoding regions [Nickerson et al., 2000]. Some are in the 5' region to exon 1, involve regulatory elements in the promoter region and are common. Other polymorphisms are in the introns and in the 3' end of the gene, and some of these are also common [Artiga et al., 1998; Bullido et al., 1998; Lambert et al., 2000; Nickerson et al., 2000; Bullido and Valdivieso, 2000].

It is important to note that the alleles defined by the common SNPs in the promoter region are in linkage dis-equilibrium with the common APOE*2 - APOE*3 - APOE*4 alleles (at least in Caucasoid populations), meaning that observed phenotypic effects of the SNPs in the promoter region are confounded by the effects of a polymorphism affecting the structure of the encoded protein, and *vice versa* [Bullido and Valdivieso, 2000]. Or, to envision the case in an all-inclusive biological framework: it is very likely that we may encounter phenotypic effects of variations in the APOE gene at large that are due to the joint effects of differences in quantitative expression of an allele *and* of the properties of the encoded protein [Lambert et al., 1998]. Conversely, one may reasonably question the proposition that a polymorphisms in the promoter region of APOE can have effects on a phenotypic trait independent of effects of a polymorphism in a coding region, or *vice versa* [Lambert et al., 2000].

2. Apolipoprotein E, lipoproteins and atherosclerosis

Summary of the chapter

ApoE plays several important roles in the catabolism of chylomicrons and VLDL, and is involved also in HDL-mediated reverse cholesterol transport. The different functional properties of apoE2, apoE3 and apoE4 have profound influences on lipoprotein metabolism, and produce a characteristic pattern of differences in plasma lipid and apolipoprotein levels among APOE genotypes in human populations at large, e.g. with lower plasma cholesterol levels in APOE*2 carriers than in APOE 3-3 genotypes, and with higher levels in APOE*4 carriers. The pattern is not universally invariant, however, and the epidemiological effects of the APOE polymorphism are more complex than indicated simply by genotype specific differences in e.g. average plasma cholesterol. Apart from these 'normal' or 'common' effects of the APOE polymorphism, APOE is also associated with familial dysbetalipoproteinemia (type III hyperlipidemia), which is associated with a markedly increased cardiovascular risk. Some cases have rare APOE alleles, but far the majority has the APOE 2-2 genotype and only develops hyperlipidemia when lipoprotein metabolism is burdened by other factors.

The role of apoE and APOE polymorphism for development of atherosclerosis and clinical cardiovascular disease may not only relate to plasma lipoproteins, but also to involvement of apoE in important anti-atherogenic processes in arterial walls including binding of metallic ions, stimulation of cholesterol efflux, stimulation of cellular proteoglycan production, and inhibition of proliferation of various cells. ApoE4 appears to be less effective than apoE2 and apoE3 in some processes.

Autopsy studies indicate that APOE*2 carriers have fewer atherosclerotic lesions in the aorta and coronary arteries than APOE 3-3 genotypes, and that APOE*4 carriers have more lesions. Many studies of patients with coronary artery disease as determined by angiography have failed to support a relationship between APOE genotypes and risk of disease. However, studies that specifically considered the severity of disease suggest that the frequency of APOE*2 is decreasing with increasing numbers of diseased vessels, whereas the frequency of APOE*4 is increasing. Some ultrasonographic studies have suggested that intima-media thickness in the carotid arteries is related to APOE genotype, with less thickness in APOE*2 carriers than in APOE 3-3 genotypes, and more APOE*4 carriers. Other studies have failed to find any association, and some have found that APOE*2 is positively associated with atherosclerotic carotid disease.

Role of apoE in plasma lipoprotein metabolism

ApoE has multiple functions in this sophisticated transport system, and although triglycerides, cholesterol and phospholipids are the quantitatively dominant lipophilic substances carried in plasma lipoproteins, one must not forget that the system also serves to distribute many other lipophilic compounds that exert critical biochemical functions, e.g. lipophilic vitamins. Thus, any systemic dysfunction or perturbation related to apoE may have biological consequences that reach beyond a visible effect on e.g. plasma total cholesterol concentration.

Metabolism of triglyceride rich lipoproteins

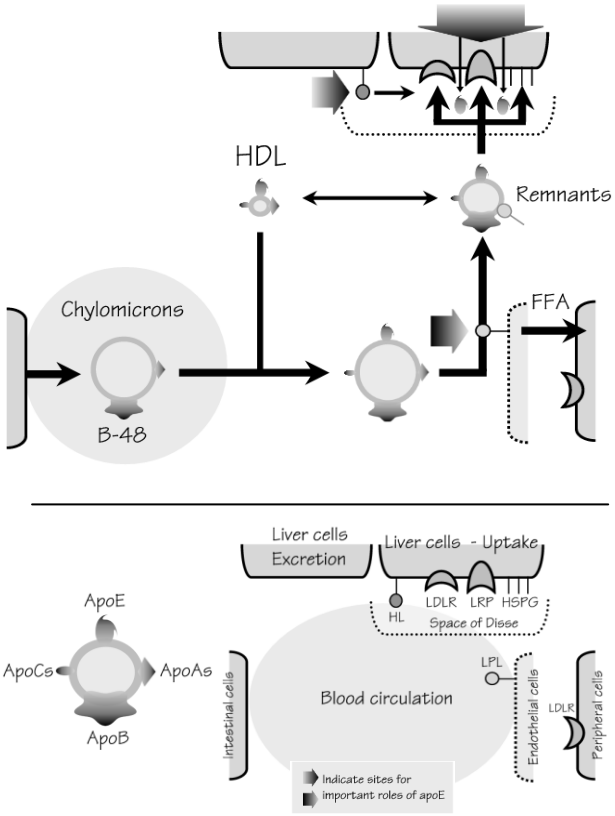
ApoE is a component of VLDL particles as they are secreted from the liver and is acquired by chylomicrons by transfer from HDL soon after their secretion from the small intestine, which is one of the few tissues that does not synthesize apoE. These triglyceride-rich lipoproteins, VLDL with apoB-100 and chylomicrons with apoB-48, are attacked by lipoprotein lipase (LPL) on the surface of endothelial cells in the capillary vascular bed in e.g. muscles and adipose tissue and lose most of their triglycerides. The resulting remnant particles are enriched in apoE and are cleared from the circulation by the liver, except that approximately 50% of the VLDL remnants are processed to form LDL particles. This metabolic pathway involves mostly smaller particles with relatively few apoE molecules (intermediate density lipoproteins, IDL) and with hepatic lipoprotein lipase (HL) playing a role [Havel and Kane, 1995].

ApoE has several important roles in the metabolism of chylomicrons and VLDL (Figure 1 and 2 on the next page summarize the main pathways of the metabolisms of these lipoproteins):

- VLDL production by the liver is apparently stimulated by apoE synthesis and secretion, or by apoE accumulation in the plasma [Huang et al., 1998; Huang et al., 1999; Mensenkamp et al., 1999].
- ApoE inhibits LPL-mediated lipolysis of triglyceride-rich lipoproteins, presumably by displacing or masking apoC-II [Rensen and van Berkel, 1996; Jong et al., 1997; Huang et al., 1998; Huang et al., 1999]. Consequently, apoE

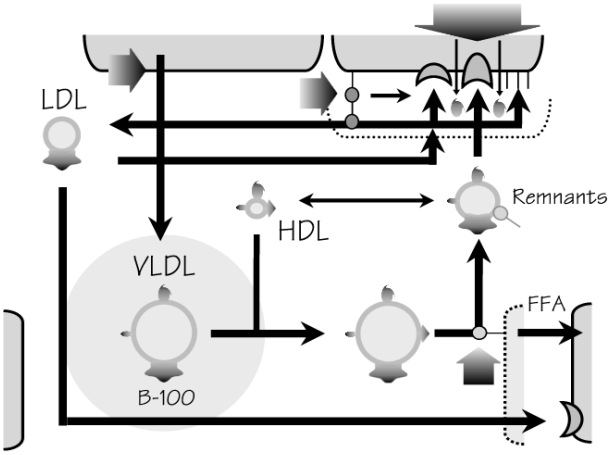
accumulation on the surface of the particles during lipolysis put a brake on the process. All apoE isoforms have this effect on LPL, but since apoE4 has a particular preference for associating with VLDL rather than HDL [Gregg et al., 1986; Steinmetz et al., 1989b; Weisgraber, 1990], the effect becomes more marked or occurs at an earlier stage during lipolysis of the particles [Mahley et al., 1999].

Figure 1. Metabolism of chylomicrons. The used symbols and outlines of this figure and of Figure 2 and 3 are shown in the lower panel.



- ApoE plays a pivotal role for the hepatic processing and uptake of chylomicron and VLDL remnants. This is a multifaceted process, which is initiated by the sequestration of remnants in the space of Disse. The space is rich in heparan sulfate proteoglycans (HSPG) and contains an abundance of apoE and HL, which is secreted by hepatocytes, and LPL, which is carried in with the remnants. Hence, the remnants are captured, further degraded and enriched with apoE in the space of Disse [Havel, 1998; Mahley and Ji, 1999; Medh et al., 2000].
- ApoE activates HL and thus speeds up the processing of chylomicrons remnants and the conversion of IDL to LDL. It appears that apoE3 and apoE4 enhance HL activity much more than apoE2 does [Mahley et al., 1999].
- Internalization of the remnants may be mediated by either LDLR, LRP in combination with HSPG, or by HSPG alone (a number of other receptors have also been suggested to be involved, but their role has not been established) [Mahley and Ji, 1999]. Both apoE3 and apoE4 have high affinities for the LDLR, whereas apoE2 has a low affinity for this receptor. Interestingly, it appears VLDL with apoE4 is more effective than VLDL with apoE3 in competing the LDLR in hepatocytes (but not in fibroblasts)[Mamotte et al., 1999]. In contrast, apoE2 binds well to HSPG (50-90% of the apoE3 binding activity) and the three isoforms do not seem to differ in their affinities for LRP [Mahley et al., 1999; Mahley and Ji, 1999].
- The relative importance of the three pathways *in vivo* has not been fully established. Interestingly, however, advanced experiments with transgenic mice indicate that whereas extrahepatic secretion of apoE by macrophages permits efficient hepatic clearance of remnants when the LDLR is present, hepatic expression of apoE is necessary for remnant uptake via the LRP pathway, i.e. this pathway involves a local apoE secretion-capture principle [Linton et al., 1998]. Moreover, it appears that the LRP pathway is important only for the hepatic clearance of apoB-48 containing lipoproteins (i.e. chylomicron remnants) and not for the clearance of apoB-100 containing remnants. The LDLR, on the other hand, does not appear to be quantitatively important for the clearance of chylomicron remnants, although it does mediate some uptake [Véniant et al., 1998].

Figure 2. Metabolism of VLDL and LDL



The overall impact *in vivo* of the APOE polymorphism on the metabolism of chylomicrons, VLDL, IDL and LDL in humans is substantial, but also complex, as evidenced by the results from kinetic studies [Weintraub et al., 1987; Gregg and Brewer, 1988; Demant et al., 1991; Boerwinkle et al., 1994; Bergeron and Havel, 1996; Welty et al., 2000].

- Compared to APOE 3-3 genotypes, APOE*2 carriers have a reduced clearance rate of chylomicron remnants, a reduced production rate of larger VLDL, an increased direct catabolism of smaller VLDL and IDL and a reduced conversion of these particles to LDL.
- Conversely, APOE*4 carriers have a lower VLDL production rate, but an increased conversion of VLDL to LDL and a lower catabolism of LDL. ApoE isoform dependent differential uptakes of the various lipoprotein species by the liver most probably plays a central role and could be associated with differences in the hepatic cholesterol pool and receptor activity, i.e. a lower pool and a higher activity in APOE*2 carriers and vice versa in APOE*4 carrier.
- Alternatively, differential lipoprotein lipase and hepatic lipase activities could also play an important role, at least in APOE*2 carriers [Mahley et al., 1999] and it is also possible that differential secretion-capture rates of VLDL play a role [Knouff et al., 1999].

Anyway, the findings partly explain why the plasma levels of APOB containing lipoproteins show some characteristic differences among APOE genotypes (see below), and in particularly why APOE*2 carriers tend to have lower levels of LDL cholesterol than APOE 3-3 genotypes and APOE*4 carriers to have higher levels [Davignon et al., 1988].

ApoE, high density lipoproteins and reverse cholesterol transport

The metabolic pathways of HDL and triglyceride-rich lipoproteins are linked in important parts that involve apoE, and the protein is also directly involved in HDL metabolism (Figure 3 summarizes the main pathways):

- HDL particles serve as a plasma reservoir for the apoE that is transferred to chylomicrons, VLDL and their remnants. This reservoir is lower in APOE*4 carriers (see above); however, the inability of apoE4 to form heterodimers with apoA-II may increase the availability of transferable protein.
- ApoE is secreted by many peripheral cells (in particular macrophages), and apoE increases local cellular efflux of cholesterol to HDL, apoA-I particles or to small lipoprotein particles with apoE as the sole apoprotein [Huang et al., 1994; Mazzone, 1996; von Eckardstein, 1996; Davignon, 1999]. HDL particles may receive cholesterol while they are docked at the scavenger receptor class B type I (SR-BI) [Krieger, 1999] and transfer cholesterol to VLDL, IDL and LDL particles in exchange for triglyceride in a process involving lecithin:cholesterol acyltranferase (LCAT) and cholesterol ester transfer protein (CETP), thereby mediating reverse cholesterol transport (i.e. cholesterol transport from peripheral cells to the liver) [Eisenberg, 1984; Reichl and Miller, 1989; Fielding, 1991; Barter, 1993; Schmitz and Lackner, 1993].

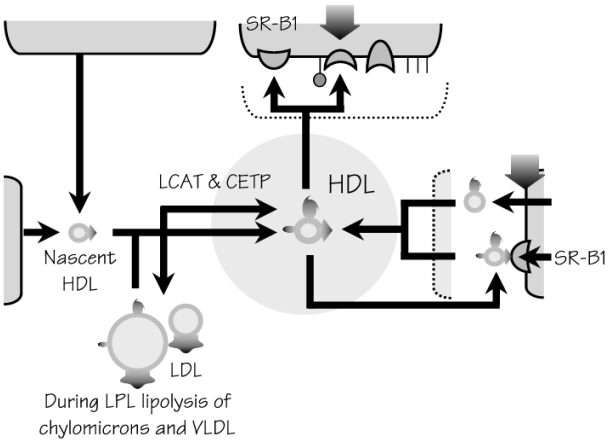


Figure 3. HDL and reverse cholesterol transport

- In addition to the reverse cholesterol transport that involves VLDL, IDL and IDL, some transport is directly mediated by HDL binding to SR-BI in the liver and by uptake of apoE enriched HDL particles via the LDLR [Innerarity et al., 1980; Funke et al., 1984; Hennessy et al., 1997]. Hepatic lipase seems to play a central role in these processes [Thuren, 2000]. The importance of apoE-mediated HDL uptake is presumably small under normal conditions, but increases with cholesterol or fat feeding. Due to the low LDLR affinity of apoE2 and the preference of apoE4 for VLDL rather than HDL, the pathway may be less efficient in both APOE*2 and APOE*4 carriers.

ApoE enriched HDL is a prominent subclass of plasma lipoproteins in species with low or no CEPT activity (e.g. rats, mice, dog and pigs) and serves a more general role as a cholesterol transporter in these species, compared to its role in man and other species with high CEPT activity (e.g. other primates and rabbits) [Weisgraber, 1994]. Interestingly, however, apoE enriched HDL possible play a prominent role for lipid transport in fetuses [Innerarity et al., 1984; Blum et al., 1985; Averna et al., 1991] and apoE enriched HDL-like lipoproteins play a central role for lipid transport in the central nervous system (see page 17).

The common APOE polymorphism and normal variation in plasma lipoprotein levels

All the differences, smaller or larger, in the functional properties of apoE2, apoE3 and apoE4 in various processes of plasma lipoprotein metabolism add up to produce a pattern of differences in plasma lipid and apolipoprotein levels

among APOE genotypes that is fairly uniform across different populations, and across subpopulations by gender and age:

- Compared to individuals with the most common genotype, APOE 3-3, carriers of the APOE*2 allele tend to have 5-15% lower mean concentrations of total cholesterol, LDL cholesterol and apoB, and higher concentrations of VLDL cholesterol, triglyceride and apoE.
- In contrast, carriers of APOE*4 tend to have 5-10% higher mean concentrations of total cholesterol, LDL cholesterol, apoB, VLDL cholesterol and triglyceride, but lower apoE concentrations, than individuals with APOE 3-3.

No other single genomic polymorphism has been identified with such a large contribution to the general inter-individual variability in plasma lipoprotein concentrations [Davignon et al., 1988; Boerwinkle and Utermann, 1988; Smit et al., 1988a; Steinmetz et al., 1989a; Lehtimäki et al., 1990; Hallman et al., 1991; Xhignesse et al., 1991; Xu et al., 1991; Hanis et al., 1991; Dallongeville et al., 1992; James et al., 1993; Schaefer et al., 1994; Gajra et al., 1994b; Louhija et al., 1994; Luc et al., 1994; Kamboh et al., 1995; Mahley et al., 1995; Matsunaga et al., 1995; Braeckman et al., 1996; Kataoka et al., 1996; Gerdes et al., 1996b; Sanghera et al., 1996; Pablos-Mendez et al., 1997; Salah et al., 1997; Hegele et al., 1997; Zaman et al., 1997; Gomez-Coronado et al., 1999; Kamboh et al., 1999; Frikke-Schmidt et al., 2000b].

Then again, the influence of the common APOE polymorphism on plasma lipid and apolipoprotein concentrations is certainly not universally invariant, but vary among populations and ethnic subgroups [Hallman et al., 1991; Luc et al., 1994; Pablos-Mendez et al., 1997] and is even absent in some populations [Deiana et al., 1998; Aguilar et al., 1999; Kamboh et al., 1991b; Sandholzer et al., 1995; Kamboh et al., 1996]. Moreover, many of the above studies showed considerable differences with respect to both the quantitative and qualitative phenotypic effects among population subgroups defined by e.g. gender, age and health status. This includes a particular difference with respect to the influence of APOE polymorphism on plasma HDL cholesterol and apoA-I levels which is mostly seen in women — the levels are a little higher in APOE*2 carriers and lower in APOE*4 carriers, compared to individuals with APOE 3-3 [Frikke-Schmidt et al., 2000b; Mahley et al., 2000].

A number of important studies have specifically addressed the issue of evident complexity in the field, based (by and large) on the sensible view that the phenotypic effects of a common genomic polymorphism cannot be adequately described by focusing solely on averages of some defined quantitative traits, when the case is a protein involved in a dynamic metabolic system whose functional characteristics are also influenced by environmental perturbations, by internal regulative forces, by other genomic polymorphisms, and also by the overall metabolic state of an individual (as determined for instance by age)[Sing et al., 1992; Sing and Reilly, 1993; Sing et al., 1994]. The studies have revealed that:

- APOE genotype has an influence not only on means but also on variances and covariances (correlations) of plasma lipids and apolipoproteins, i.e. that APOE is “variability gene” as much as a “level gene” [Berg, 1990],
- APOE genotype has an influence on regression relationships between plasma lipids and apolipoproteins and concomitants such as smoking and body size (corresponding to statistical interactions between APOE genotype and the concomitants on the lipid traits), and
- the influences of APOE genotype on the parameters (i.e. means, variances, covariances or regression coefficients) are mostly gender- and age-specific [Boerwinkle et al., 1987; Kaprio et al., 1991; Reilly et al., 1991; Reilly et al., 1992; Reilly et al., 1994; Haviland et al., 1995; Zerba et al., 1996; Lussier-Cacan et al., 2000; Frikke-Schmidt et al., 2000b].

APOE and familial dysbetalipoproteinemia (type III hyperlipidemia)

The pioneering studies of apoE by Utermann and associates were focused on its role for development of dysbetalipoproteinemia [Utermann et al., 1975; Utermann et al., 1977; Utermann et al., 1979] and eventually led to unraveling of the basis of its common genetic polymorphism [Zannis et al., 1981]. Dysbetalipoproteinemia is characterized by combined and often severe hypercholesterolemia and hypertriglyceridemia due to the accumulation of β-VLDL particles in plasma, and also IDL particles. This results in a very characteristic profile when the plasma lipoproteins are separated by e.g. size exclusion chromatography [Gerdes et al., 1992b].

Beta-VLDL are remnant lipoproteins that derive from chylomicrons and VLDL, and differ from normal VLDL by being more cholesterol-enriched and relatively depleted of triglycerides, and also by containing much less C apolipoproteins and more apoE. Patients often have pathognomonic yellowish lipid deposits in the palmar creases (xanthoma striata palmaris), and sometimes other xanthomas. Premature or accelerated atherosclerosis occurs in

one-third to more than one-half of the patients and notably involves the arteries in the lower extremities and the coronary tree [Mahley and Rall, 1995].

By far the most patients with dysbetalipoproteinemia have the APOE 2-2 genotype, i.e. homozygotes for the isoform with a very low affinity for the LDLR. However, less than 10% of the individuals with the genotype (e.g. about 1% in most European populations) ever develop overt hyperlipidemia. In fact, individuals with APOE 2-2 usually have lower plasma cholesterol than other APOE genotypes, and the precipitation of hyperlipidemia therefore involves additional genetic, hormonal or environmental factors, such as hypothyroidism, estrogen status, obesity, diabetes and age. In contrast, a number of rare (mutant) APOE alleles are associated with dominant inheritance of type III hyperlipidemia (i.e. one mutant allele is sufficient), which often also become manifest already in childhood [de Knijff et al., 1994; Mahley and Rall, 1995]. Thus, the pathogenesis of the overt dysbetalipoproteinemia has been enigmatic, and only recent studies in transgenic animals have resolved the mechanisms [Mahley et al., 1999].

ApoE and lipid transport in the central nervous system

The lipoproteins in cerebrospinal fluid (CSF) are mostly of the size and density of plasma HDL and contain apoA-I, apoA-II, apoA-IV, apoD, apoE and apoJ (clusterin), whereas apoB is absent. ApoE is the dominant apolipoprotein in CSF and both this protein and apoJ are produced within the blood-brain barrier by astrocytes and microglia, whereas apoA-I and apoA-II may originate from the plasma. ApoE containing lipoproteins play a central role for distant lipid transport within the blood-brain barrier, but apoE is also intimately involved in local exchange and recycling of lipids in neural tissue, e.g. to supply lipids for axonal growth and during repair of myelin sheaths [Boyles et al., 1985; Pitas et al., 1987; Boyles et al., 1989; Mahley et al., 1989; Boyles et al., 1990; Borghini et al., 1995; Ladu et al., 2000; Vance et al., 2000].

As in plasma, the common APOE polymorphism is associated with differential distributions of apoE among lipoproteins of different sizes, i.e. apoE2 and apoE3 preferentially bind to the smaller (dense) lipoproteins in CSF whereas apoE4 binds to the larger particles [Yamauchi et al., 1999]. Interestingly, mean CSF concentrations of total cholesterol and phospholipid appear to be considerably lower in APOE*4 carriers than in other individuals. Yamauchi et al. have suggested that the ability of apoE2 and apoE3 to form homodimers and heterodimers with apoA-II may reduce the affinity of these isoforms to lipoprotein receptors, whereas apoE4 is entirely monomeric and thus may mediate a more effective clearance of CSF lipoproteins [Yamauchi et al., 1999; Yamauchi et al., 1999].

Systemic lipoprotein metabolism primarily involves the LDLR and LRP, whereas three other members of the LDLR family, namely the VLDL receptor (VLDLR), the apoE receptor 2 (ApoER2, or LRP8) and LR11, play more prominent roles in nervous tissue [Schneider et al., 1997; Willnow et al., 1999; Ladu et al., 2000; Vance et al., 2000]. A sixth member of the family, megalin, is expressed in small amounts in cells of the choroid plexus and ependyma as well as in endothelial cells in capillaries at the blood-brain barrier [Chun et al., 1999]. It binds apoE but is also the only known apoJ receptor, and its localization suggests that it may be involved in exchange of lipids and associated components across the blood-brain barrier [Ladu et al., 2000].

Role of apoE for maintaining a local anti-atherogenic milieu in arterial walls

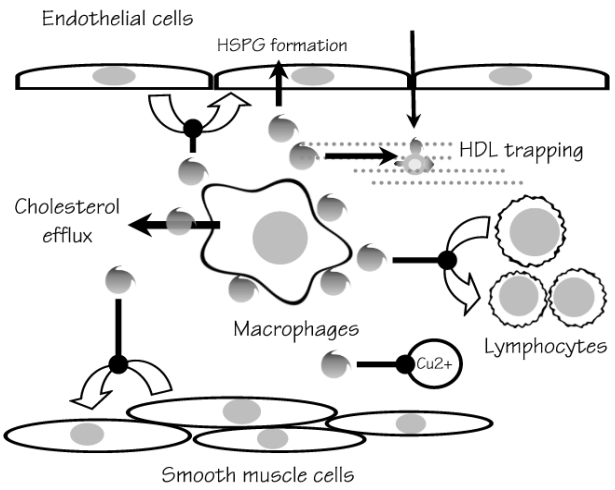
The associations of the common APOE genotypes with different tendencies to develop atherosclerosis and clinical manifest cardiovascular disease may obviously relate to the characteristic differences in the genotype-specific plasma lipoprotein profiles (as described above), i.e. we believe that APOE*2 carriers have a lower risk of disease than APOE 3-3 genotypes because they tend to have a less atherogenic lipoprotein profile, whereas APOE*4 carriers have a higher risk because they tend to have a more atherogenic profile.

However, apoE is also directly involved in local physiological processes in arterial walls that determine their integrity, or, if anomalous conditions prevail, result in development of atherosclerosis. In fact, some studies suggest that local functions of apoE may be more important for atherosclerosis than the functions related to plasma lipoprotein metabolism. For example, establishing apoE-producing macrophages or adrenal gland cells in APOE null mice can markedly inhibit the development of atherosclerotic lesions despite persistence of hypercholesterolemia [Curtiss and Boisvert, 2000; Thorngate et al., 2000].

Macrophages are the dominant (if not only) source of apoE in arterial walls [O'Brien et al., 1994] and the protein is apparently involved in many important places [Mazzone, 1996; Larkin et al., 2000; Curtiss and Boisvert, 2000] — see Figure 4:

- ApoE binds cupric, ferrous, ferric and zinc ions and has anti-oxidative effects that may relate to this ability. A study using rat neuronal cells showed apoE2 to be the most effective in protecting cells from oxidative death, apoE3 moderately effective and apoE4 the least effective [Miyata and Smith, 1996]. It is thus possible that local apoE can reduce the formation of oxidized LDL trapped in the intima, which is believed to be an important early pathogenic process in the development of atherosclerosis [Ross, 1993; Ross, 1999].
- The production and secretion of apoE by macrophages is associated with cholesterol efflux from these cells, and thus may slow the formation of foam-cells resulting from uptake of modified LDL. ApoE production is modulated by inflammatory cytokines, e.g. stimulated by tumor necrosis factor α [Duan et al., 1995] and macrophage APOE mRNA expression is particularly prominent in atherosclerotic plaques [O'Brien et al., 1994].

Figure 4. Functions of apoE in arterial walls.



- The effect of apoE to increase cholesterol efflux is not simply due to the protein acting as an extracellular cholesterol acceptor, since endogenous apoE is more effective than exogenous apoE [Lin et al., 1999]. Interestingly, it appears that the net efflux of cholesterol (excretion minus reuptake) is higher with apoE2 than apoE3 and lower with apoE4 [Cullen et al., 1998].
- Macrophages keep a cell surface pool of apoE bound to HSPG and the LDLR. Its exact function is not settled, but it may be important for binding of HDL, modulation of the uptake of other lipoproteins by macrophages and interactions with other cells or with cell-derived matrix components [Zhao and Mazzone, 1999].
- ApoE binds to biglycan, a small extracellular dermatan sulfate proteoglycan, which is synthesized by smooth muscle cells and accumulates in atherosclerotic lesions. Biglycan also binds apoB and may thus contribute to trapping of apoE and apoB containing lipoproteins in the intima, i.e. VLDL and LDL [Camejo et al., 1993]. However, apoE colocalizes with apoA-I in atherosclerotic vessels, suggesting that apoE may act as a bridging molecule to also trap HDL in atherosclerotic intima [O'Brien et al., 1994]. It is not clear whether this HDL can bind extracellular lipid and promote its removal from the intima.
- Proteoglycan production in various cell types is enhanced by apoE [Obunike et al., 2000] including HSPG formation in endothelial cells [Paka et al., 1999]. The latter appears to be a specific effect of apoE associated with HDL (e.g. not with VLDL) and has a general bearing on the potential role of apoE as an “athero-protective” substance because abundant pericellular HSPG is a property of healthy intact endothelium [Paka et al., 1999; Ross, 1993].
- ApoE stimulates HSPG production in smooth muscle cells and inhibit their platelet-derived growth factor (PDGF) stimulated proliferation [Ishigami et al., 1998; Paka et al., 1999]. Interestingly, this effect is clearly evident only with apoE3, and not with apoE2 and apoE4 [Paka et al., 1999].
- ApoE can inhibit basic fibroblast growth factor stimulated proliferation and migration of endothelial cells [Vogel et al., 1994].
- Activation and proliferation of T-lymphocytes contribute to the inflammatory response of atherosclerosis and apoE containing lipoproteins have long been known to have an inhibitory effect on mitogen-induced lymphocyte proliferation [Mahley, 1988]. The effect of apoE is very specific although the mechanism including the involved receptor or binding site has not been identified [Pepe and Curtiss, 1986; Kelly et al., 1994; Mistry et al., 1995; Mahley and Rall, 2000].

APOE polymorphism and atherosclerosis in humans

Autopsy studies

Results from human autopsy studies leave the general impression that APOE*2 carriers tend to have fewer atherosclerotic lesions (fatty streaks, plaques and complicated lesions) in the aorta and the coronary arteries than individuals with the APOE 3-3 genotypes, whereas APOE*4 carriers tend to have more lesions [Hixson, 1991; Scheer et al., 1995; Ilveskoski et al., 1999; Boudreau et al., 1999]. However, the findings are heterogeneous in several respects and not very consistent among studies. Thus, the effect of APOE genotype is either only apparent in

some regions of the arterial tree, or it only has a bearing on specific kinds of atherosclerotic lesions, or it is only seen in some ethnic groups or in younger individuals. It is interesting to note that the significant findings in the larger of these autopsy studies, the PDAY study of younger men who died of external causes, were largely unaffected by adjustment for cholesterol levels, suggesting that the effects of the APOE polymorphism is partly unrelated to lipoprotein metabolism [Hixson, 1991].

Coronary artery disease determined by angiography

While there are only few studies on the association between APOE genotypes and directly visualized atherosclerotic vascular lesions, many studies have aimed at evaluating the role of the common APOE polymorphism for developing significant coronary artery disease as determined by angiography, including studies on progression of coronary disease and restenosis after angioplasty [Menzel et al., 1983; Reardon et al., 1985][Schmidt et al., 1985; Kuusi et al., 1989; Stuyt et al., 1991; Nieminen et al., 1992; Patsch et al., 1992; Köhler et al., 1992; Payne et al., 1992; de Knijff et al., 1992b; van Bockxmeer and Mamotte, 1992; Waters et al., 1993; Marshall et al., 1994; Nikkilä et al., 1994; Damaraju et al., 1995; Lehtinen et al., 1995; Wang et al., 1995; van Bockxmeer et al., 1995; Regis-Bailly et al., 1996; Ou et al., 1998; Dzimiri et al., 1999].

The study findings are very different, presumably for the same reasons that determine the variability of results in studies of acute coronary syndrome (see page 41), i.e. that APOE has a variable influence on risk in different populations and subpopulations, and that different study design characteristics may cause variable results. Nevertheless, Wilson and coworkers included the results from 5 of the above studies in a meta-analysis, and concluded that the data did not support a relation of either APOE*2 or APOE*4 with angiographically determined coronary artery disease [Wilson et al., 1996; Menzel et al., 1983; Kuusi et al., 1989; Stuyt et al., 1991; van Bockxmeer and Mamotte, 1992; Lehtinen et al., 1995].

However, there is an important quality of the pathological condition that was not considered in most studies, namely the severity of the coronary artery disease. Hence, in a study of 500 German men under the age of 45 years with coronary artery disease, APOE*4 carriers were particularly common among those with two or three diseased vessels, and among those with disseminated rather than localized lesions, while there were relatively few APOE*2 carriers in these subgroups of patients [Köhler et al., 1992]. A similar tendency was observed in a Finnish study of 256 elderly men [Lehtinen et al., 1995]; and in a study of 424 Euro-American patients under the age of 65 years, Wang and coworkers observed a significant increase in the frequency of APOE*4 in patients with increasing numbers of diseased coronary arteries (0 to 3), and a decreasing frequency of APOE*2 [Wang et al., 1995].

Atherosclerosis determined by ultrasonography

Some ultrasonographic studies have suggested that the intima-media thickness in the carotid arteries differ among APOE genotypes, with less thickening in APOE*2 carriers than in individuals with APOE 3-3- and more in APOE*4 carriers [Cattin et al., 1997; Terry et al., 1996], i.e. results corresponding to the findings reviewed above. However, several studies have failed to find any association between APOE genotype and atherosclerosis determined by ultrasonography [Kogawa et al., 1997; Sass et al., 1998; Hillen et al., 2000] and others have found that APOE*2 is positively associated with atherosclerotic carotid disease [de Andrade et al., 1995; Hanon et al., 2000]. The findings for APOE*2 may appear odd at first sight, considering the effects on plasma lipoproteins and the findings in the autopsy studies (above), but several recent case-control studies of cerebrovascular events actually points to an increased risk in APOE*2 carriers (see page 39).

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3. APOE allele distributions in the world

Summary of the chapter

The frequencies of the common APOE alleles in the Danish population have been estimated in three larger studies and the results agree well. Nevertheless, it is important to recognize potential errors in estimated APOE allele frequencies for a population (or subpopulation). Inconsiderate sampling procedures may cause both random and systematic errors, and errors may also result from using apoE phenotyping to deduce APOE genotypes.

The APOE allele frequencies in aboriginal populations around the world show some interesting patterns. First, there is a conspicuous south-to-north APOE*4 gradient in Europe, with frequencies rising from 5-10% in south to 20-30% in the north. It could be an 'admixture gradient' that arose in the Neolithic Age, but selective forces may also have been in play. The APOE*4 gradient may contribute to the south-to-north gradient in the burden of coronary heart disease on this continent, but only little. Secondly, the APOE*2 allele is (nearly) absent in Inuit and peoples in America, whereas the APOE*4 frequency is high in Inuit and otherwise vary as much among Americans as among Europeans. Thirdly, the frequencies of APOE*4 is low in Asia (with few exceptions), at the level seen in Southern Europe, but 25-50% in Australia, and in Papua New Guinea, where also the frequency of APOE*2 appears to be high (over 15%). Finally, the frequencies of APOE*4 is high in ancient African peoples and also in other Africans, except Arabs.

In the late 1980s and early 1990s, accumulated data from studies in various populations around the world revealed some differences in APOE allele frequencies. It was recognized, that the APOE*4 frequency is lower in Japanese and Chinese than in northern Europeans (including Euro-Americans and Euro-Australians), and higher in Africans (including Afro-Americans), whereas APOE*2 appeared to be absent in aboriginal Americans and have a relatively low frequency in Japanese. However, it had also been discovered that the APOE*4- and APOE*2 frequencies are higher and lower, respectively, in Finns than in other European populations and in Euro-Americans [Asakawa et al., 1985; Ehnholm et al., 1986; Eto et al., 1986; Utermann, 1987; Davignon et al., 1988; Sepehrnia et al., 1988; Kamboh et al., 1989; Hallman et al., 1991; Kamboh et al., 1991b].

Danish studies

We conducted the first study in Denmark to estimate the APOE allele frequencies in this population, using blood samples from 466 randomly selected men born in 1948 and residing in the city of Aarhus in 1988 (RisiKalk) and apoE phenotyping. To report the results with appropriate indications of imprecision of the study estimates as population values, we introduced a normal approximation method to calculate confidence intervals (see page 57)**[Gerdes et al., 1992a]**.

Table 1 shows the estimated APOE allele frequencies in this study and the results based on direct APOE genotyping **[Hansen et al., 1994a]**. The table also shows recent results from a study of participants in the Danish Osteoporosis Prevention Study (DOPS) recruited in Aarhus [Gerdes et al., 2000c] and the results from a large study of participants in the Copenhagen City Heart Study [Frikke-Schmidt et al., 2000a]. All results agree well, although the APOE*4 frequency estimated in Copenhagen (0.166 for the combined data for men and women) is about 5% lower than our estimates. It is possible due to the different ages of the study populations (see page 22).

Table 1. APOE allele frequencies in Danish Studies; point estimates and 95% confidence intervals

Study	Population sample	N	APOE*2	APOE*3	APOE*4
RisiKalk, phenotyping	Men, 40 years	466	0.085 (0.068 - 0.105)	0.741 (0.712 - 0.769)	0.174 (0.150 - 0.200)
RisiKalk, genotyping		460	0.087 (0.070 - 0.108)	0.738 (0.708 - 0.766)	0.175 (0.151 - 0.201)
DOPS Aarhus	Women, 45 - 58 years	479	0.089 (0.072 - 0.109)	0.737 (0.708 - 0.764)	0.174 (0.151 - 0.200)
Copenhagen City Heart	Men, 20 - 90 years	4,219	0.081 (0.075 - 0.087)	0.757 (0.747 - 0.766)	0.162 (0.154 - 0.170)
Copenhagen City Heart	Women, 20 - 90 years	5,112	0.082 (0.077 - 0.087)	0.750 (0.741 - 0.758)	0.169 (0.161 - 0.176)

We presented our results with a compilation of data from studies in 45 other populations and discussed two subjects: (1) Possible sources of errors when estimating APOE allele frequencies in a population, and (2) Differences in APOE

allele frequencies in populations around the world. The discussion is expanded with additional viewpoints and inclusion of more recent data in the two following sections.

Errors in estimated APOE allele frequencies for a population

Anyone who reads the literature concerning APOE has noticed that reported allele frequencies in population samples, blood donors, healthy volunteers etc. vary much more than can possibly be attributed to known differences in ethnicity. And as for all other endeavors aiming at measuring quantities, unexplained variability in the results signals problems with the quality of the employed methods [Dybkjær, 1995]. In the present context 'methods' include the procedures for sampling individuals and the laboratory techniques to determine APOE genotypes. Systematic and random errors can have an impact on interpopulation studies, and can give rise to biases when APOE allele (or genotype) frequencies are compared with findings in studies of e.g. patients with cardiovascular disease.

Potential errors due to technical problems with determining APOE genotypes have been discussed in the literature, notably in relation to the use of protein phenotyping (see below), whereas problems with sampling have not. In fact, when reading literature on APOE, it appears to be a widespread belief, that because APO genotype is a fixed trait of an individual, it doesn't matter much how individuals for a study are sampled. This belief is definitely wrong: genetic epidemiological studies are as challenging as other epidemiological studies in this regard [Khoury et al., 1993; Rothman and Greenland, 1998].

Sample size as a source of random error (imprecision)

Sample size is the only determinant of precision and its importance should never be underestimated — even studies involving several hundred individuals can easily result in estimated allele frequencies that deviate 10% from the true population frequencies. However, samples can be small because the corresponding target populations are small (e.g. some peoples in remote places on the Earth). Moreover, it is also important to remember that increasing sample size usually also increases the risk of errors in the laboratory and during processing of data (notably if resources are limited), and that an inconsiderate heading for a large sample size may introduce sources of systematic error.

Sampling procedure as a source of systematic error (bias)

The probability than an individual has a particular APOE genotype depends on his familial origin, ethnicity, health status and age. Sampling procedures that do not consider and control these factors are likely to inflict biases on a study.

- For instance, if the target is a particular aboriginal population in a geographical region, the results will obviously be biased if the investigated sample includes resident members of other ethnic groups with different allele frequencies. Conversely, if the aim is to estimate the allele frequencies in a geographically defined multiethnic population, the results will be biased if the sampling method causes preferential selection of members of some ethnic subgroups, or of only younger people. This may often occur if one resorts to recruiting for example blood donors or hospital staff members.
- Selection for health will inevitably produce systematic error or 'particular results' in a neutral term. For instance, we determined that if our study population of 40 years old men had been selected by the (approximate) criteria used in an American study of people selected for health [Xhignesse et al., 1991], the estimated APOE*4 frequency would have been 0.165 (and not 0.174). Similarly, an exclusion of men with total plasma cholesterol > 6 mmol/L and/or triglycerides > 2.5 mmol/L would have resulted in an estimated frequency of 0.157 **[Gerdes et al., 1992a]**. Exclusion of patients with cardiovascular disease and Alzheimer's disease will also give 'particular results' if the diseases are common in the target population, and non-attendance on grounds of diseases may also have this effect.
- The importance of selection by age follows from the evidence showing that APOE*4 frequency is decreasing with advancing age (over 50 years) and that APOE*2 and APOE*3 frequencies are increasing. This seems to be independent of population frequency levels (see page 35), and may cause particular biases in estimated relative risks for e.g. cardiovascular disease if data for elderly patients are matched with data from younger controls (see page 41).

APOE genotyping method as a source of systematic error (bias)

Practically all results published until the early 1990s stem from using a large variety of isoelectric focusing techniques to determine apoE phenotypes, see e.g. [Warnick et al., 1979; Utermann et al., 1982; Menzel and Utermann, 1986; Havekes et al., 1987; Ordovas et al., 1987; Steinmetz, 1987; Baumstark et al., 1988; Kamboh et al., 1988; Hill and Pritchard, 1990; Kamboh et al., 1990; Mailly et al., 1990].

Posttranslational *in vivo* sialylation and glycosylation of apoE and *in vitro* deamidation of the protein create a general problem with these techniques (unless two-dimensional electrophoresis is employed), because the modifications cause anodal shifts in the genetic isoforms isoelectric points and superimposition of modified apoE4 on unmodified apoE3 and apoE2, and of modified apoE3 on unmodified apoE2 in the gels. Pretreating samples with neuraminidase can alleviate confounding problems due to sialylation, but it does not prevent a systematic tendency for the APOE*4 frequency to be underestimated and the APOE*2 frequency to be overestimated when using phenotypes to deduce genotypes [Snowden et al., 1991; Wenham et al., 1991]. In some cases the frequency of APOE*4 can also be overestimated, namely if a study population includes individuals with high serum concentrations of the acute-phase protein serum amyloid A (SSA) and the phenotyping technique does not include specific immunoblotting to determine the localization of apoE bands. This is because SSA binds to lipoproteins and the SSA_2 isoform has the same isoelectric point as apoE4 [Menzel and Utermann, 1986; Steinmetz et al., 1989c].

We examined the extent of possible errors (misclassifications) from using apoE phenotypes in our study of 40-years old Danish men **[Gerdes et al., 1992a]** by also determining their APOE genotypes **[Hansen et al., 1994a]**. The results are shown in Table 2 and indicate a very modest extent of errors — discrepant results were seen in only 9 out of 460 cases (2%; DNA was not available for 6 men) — or, after the original phenotyping immunoblots were critically reviewed, in only 4 cases. Similar consistencies were found in four smaller comparative studies [Kontula et al., 1990; Mailly et al., 1992; Tsukamoto et al., 1993; James et al., 1994] and in one larger study [Lahoz et al., 1996].

Table 2. Apolipoprotein E genotypes and phenotypes in 40-year old Danish men

		Genotyping							
		2-2	**2-3**	**3-3**	**2-4**	**3-4**	**4-4**	**Total**	NA
Phenotyping	**2-2**	**6**	2					8	
	2-3		**53**					53	1
	3-3		3	**252**		3		258	2
	2-4				**9**			9	
	3-4		1			**113**		114	3
	4-4						**18**	18	
	Total	6	59	252	9	116	18	460	6

Such findings are reassuring for the use of apoE phenotyping to determine the common APOE genotypes, notwithstanding that other studies have shown alarming extents of discrepancies [Snowden et al., 1991; Wenham et al., 1991]. With respect to »verified discrepancies« (e.g. the 4 in our study) one should note, that they may be due to the presence of rare APOE alleles [de Knijff et al., 1994; Lahoz et al., 1996].

Differences in APOE allele frequencies in populations around the world

To put our findings in the Danish study and our findings in a later study of Inuit in Greenland in perspective, I compiled data from the literature on estimated APOE allele frequencies in samples of various ethnical or geographically defined populations around the world **[Gerdes et al., 1992a; Gerdes et al., 1996b]**. The resulting world map of APOE allele frequencies revealed some interesting patterns.

A south-to-north APOE*4 gradient in Europe

We discovered that European populations are much more heterogeneous with respect to their APOE allele frequencies than first believed, and noted a possible existence of a south-to-north gradient in the frequency of APOE*4 on this continent **[Gerdes et al., 1992a]**. The existence of the gradient was confirmed when more data became available [Corbo et al., 1995; Gerdes et al., 1996b; Lucotte et al., 1997].

Figure 5A and 5B, which are based on estimated allele frequencies in 69 European population samples (data available in July 2000; see Appendix I), show the overall heterogeneity of the APOE allele frequencies and the allele frequencies as functions of latitude north, respectively.

The APOE*4 gradient is the most conspicuous, with frequencies rising from 5-10% in the south (samples of Basques, Spaniards, Portuguese, Sardinians, Southern Italians and Greeks) to 20-30% in the north (samples of Swedes, Finns and Saami). The APOE*2 frequency also exhibits some variation, being 3-7% in most populations in the south and upper north, and 7-12% in the populations in between.

Figure 5A. APOE allele frequencies in European population samples

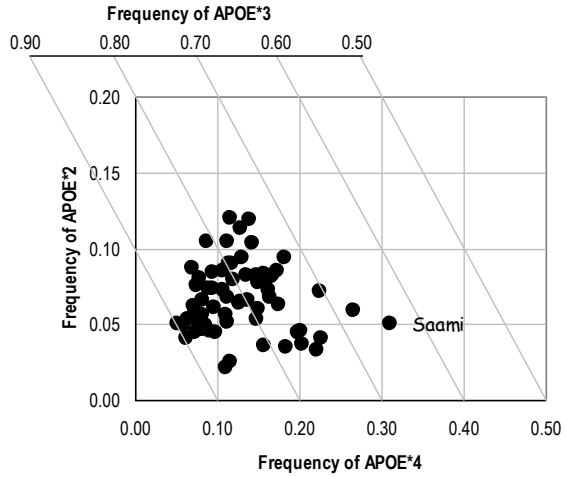
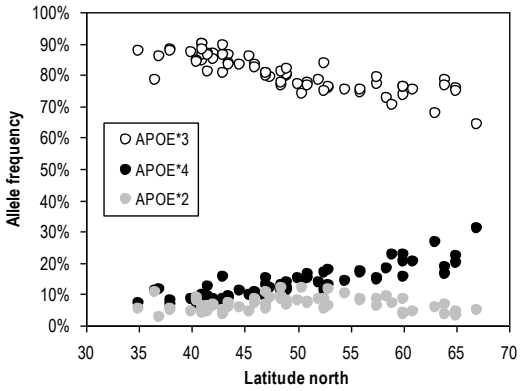


Figure 5B. APOE allele frequencies in Europe as functions of latitude north



How did this European APOE*4 gradient arise?

Cavalli-Sforza and co-workers have estimated that about 40% of the total variation in 95 genes in 26 European population samples can be explained by two components in the shapes of south-to-north gradients. The first (main) gradient originates in the Middle East and was presumably generated by a migration of Neolithic farmers from the area of origin of agriculture in Anatolia, followed by admixture of the expanding farmers with local hunter-gatherers in the west and northwest. The second gradient is a more vertical south-to-north gradient directed from the Iberian Peninsula to the northern parts of Scandinavia. It cannot be ascribed to known migrations, but may be interpreted on a climatic basis [Cavalli-Sforza and Piazza, 1993; Cavalli-Sforza et al., 1994].[Piazza et al., 1995].

Data for the APOE gene were not included in the analyses, but they clearly fit into the picture, suggesting that the European APOE*4 gradient is primarily a simple 'admixture gradient' generated by migration of Neolithic farmers with a low frequency of APOE*4 towards the north, where the local peoples had a high frequency of the allele. However, selective forces may also have been in play, at least for maintaining the APOE*4 gradient. The arguments for this closely relate to the discussion of whether APOE*3 or APOE*4 is the ancestral allele, and the possible reasons why APOE*4 seems to be more common in (recent) cultures of hunters/gatherers or nomads than in long-established agricultural communities (see page 30).

Does the APOE*4 gradient contribute to the gradient of coronary heart disease?

The epidemic rise of CHD in the beginning of the past century hit the populations in northern Europe much more severely than the populations in the south. The markedly declining disease rates in Western Europe since the 1970s have caused some levelling-out of the south-to-north gradient in the burden of CHD, but it is still conspicuous [Thom and Epstein, 1994; Beaglehole, 1999]. For instance, in the late 1980s and early 1990s, the annual coronary event rates for men aged 35 to 65 covered a 4-fold range from 835 per 100,000 in North Karelia, Finland, to 210 per 100,000 in Catalonia, Spain. For women, rates covered a 7.5-fold range from 265 per 100,000 in Glasgow, Scotland, to 35 per 100,000 in Catalonia [Tunstall-Pedoe et al., 1999]. The impressive rises and declines in regional CHD event rates during time spans of only few decades demonstrate that modifiable environmental factors play an important role, and different life styles in Southern and Northern Europe undoubtedly contribute to the European CHD gradient [Menotti et al., 1990; Gibney, 1999].

However, inter-population differences in genetic susceptibility to CHD may also play a role, and we pointed to the potential role of the APOE*4 gradient **[Gerdes et al., 1992a]**. Stengård, Weiss and Sing recently examined the ecological association between CHD mortality rates in men and the frequencies of APOE*4 in eight European populations and one Chinese, using matched data from the WHO MONICA Project and independent studies of APOE allele frequencies in the same populations [Stengård et al., 1998]. Linear regression analysis suggested that

75% of the inter-population variation in CHD mortality rates was associated with variation in the frequency of APOE*4 (75% = 100 X r^2, i.e. the squared linear correlation coefficient). This is a conspicuously high percentage, considering that the risk for CHD in APOE*4 carriers is only modestly increased compared to the risk in individuals with other genotypes (see page 41). One may therefore suspect the presence of ecological bias, i.e. that the relative risk in APOE*4 carriers derived from the ecological analysis is much higher than ever observed within any population [Morgenstern, 1982] [Morgenstern, 1998].

We did at similar study, using the annual acute coronary event rates for men and women aged 35-64 years during 1985-91 in 17 European WHO MONICA populations [Tunstall-Pedoe et al., 1999] and matched studies with data on APOE genotype frequencies in the populations (Gerdes LU, Corder EH; the results have been presented at The 6th Annual Scandinavian Athersclerosis Conference, Humlebæk 1999). Data for men and women were analysed separately, and we used the proportion of APOE*4 carriers in a population as the dependent variable instead of the APOE*4 allele frequency.

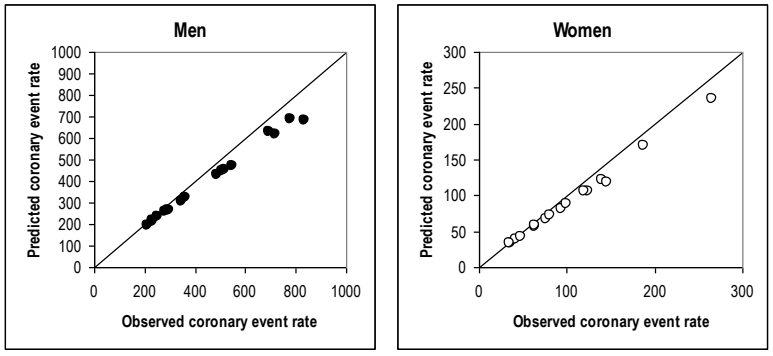
- We first noticed that linear regression models gave a negative value of the intercept with data for men. This result is clearly meaningless, since the intercept should estimate the predicted event rate in a hypothetical population without APOE*4. Similarly, the intercept for women was unrealistically low — only 10 per 100,000 — as compared to the observed 35 per 100,000 in Catalonian women with a proportion of APOE*4 carriers of only 14%.
- Therefore, log-linear regression models were fitted to the data for CHD rates (CHDR), with the proportions of APOE*4 carriers (P_{APOE*4}) in the populations, i.e. $\ln(CHDR) = \beta_0 + \beta_1 x\, P_{APOE*4}$. With this model, the predicted CHD rate in a hypothetical population without APOE*4 is $\exp(\beta_0)$ and the predicted rate in a hypothetical population of only APOE*4 carriers is $\exp(\beta_0+\beta_1)$. Hence, $\exp(\beta_0+\beta_1)/ \exp(\beta_0) = \exp(\beta_1)$ is an ecological estimate of the relative risk (at the individual level) in APOE*4 carriers compared to individuals with other genotypes [Morgenstern, 1998].
- The results showed that for r^2 values about 60% for men and about 40% for women, respectively, the relative risk of CHD in APOE*4 carriers should be about 100. This risk estimate is clearly unrealistic and proves that the seemingly strong association between coronary event rates and APOE*4 allele frequencies in Europe is not causal.

The APOE*4 gradient may nevertheless contribute to the variation in CHD rates and we tried to estimate (or at least visualize) its potential contribution. This was done by considering that:

- the overall coronary event rate in a population is the sum of the rates for APO*E-carriers and non-APOE*4-carriers, weighted by their proportions, i.e. $CHDR = P_{APOE*4} \times CHDR_{APOE*4} + (1- P_{APOE*4})CHDR_{notAPOE*4}$,
- knowing the relative risk of CHD in APOE*4 carriers (RR) permits one to calculate the event rate in non-APOE*4 carriers, i.e. since $RR = CHDR_{APOE*4}/ CHDR_{notAPOE*4}$ the above formula can be rearranged to give $CHDR_{notAPOE*4} = CHD[RR \times P_{APOE*4} + (1- P_{APOE*4})]^{-1}$,
- for a given population the estimated CHD rate in non-APOE*4 carriers predicts what the CHD rate would be if the increased risk in APOE*4 was somehow abolished, i.e. it is associated with the concept of attributable risk ($AF = P_{APOE*4}(RR-1) \times [P_{APOE*4}(RR-1) + 1]^{-1}$) [Rothman and Greenland, 1998].

Figure 6 shows that such predicted CHD rates deviate little from the observed rates when the calculations are based on a relative risk of 1.5 in APOE*4 carriers, indicating that the APOE*4 gradient only contribute little to the CHD gradient.

Figure 6. Predicted versus observed acute coronary event rates per 100.000 in 17 European populations, if an increased relative risk of 1.5 in APOE*4 carriers is abolished



We have not been able to derive a single, aggregated and yet unambiguous measure for the contribution. However, one may notice, that for a relative risk of 1.5, the attributable risks for APOE*4 vary from 6% in Catalonia to 18% in North Karelia.

The APOE*2 allele is (nearly) absent in Inuit and aboriginal peoples in America

We determined the APOE allele frequencies in population samples of Inuit from Nuuk on the south-west coast of Greenland (n=100) and from the Ammassalik region on the south-east coast (n=78) **[Gerdes et al., 1996b]**. The results are shown in Table 3 along with the results from two other studies of Inuit in Greenland [de Knijff et al., 1992a; Boudreau et al., 1999] and a study of Inuit in Alaska [Scheer et al., 1995]. The frequency of APOE*4 is relatively high in Inuit (e.g. at the level only found in Finns and Swedes in Europe), whereas the frequency of APOE*2 is very low. In fact, the allele was absent in the sample of 78 Inuit from the Ammassalik region. People living here presumably characterises Inuit better than people living on the west coast or in Alaska, where admixture with Europeans has been more extensive.

Table 3. APOE allele frequencies in studies of Inuit: point estimates and 95% confidence intervals

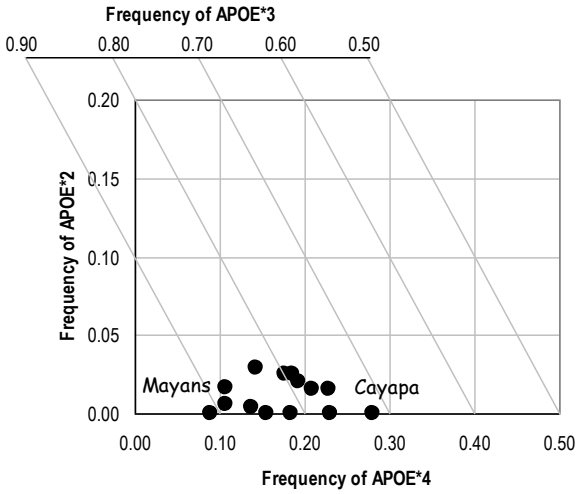
Study	Population sample	N	APOE*2	APOE*3	APOE*4
Nuuk	People aged 12 to 70 years	100	0.025 (0.009 to 0.061)	0.790 (0.726 to 0.843)	0.185 (0.135 to 0.247)
Ammasalik	People aged 16 to 62 years	78	0.000 (0.000 to 0.030)	0.769 (0.694 to 0.831)	0.231 (0.169 to 0.306)
Nanortalik*	People aged 30 to 34 years	133	0.015 -	0.756 -	0.229 -
Nuuk and Illulissat	People aged 18 to 86 years	98	0.015 (0.004 to 0.048)	0.776 (0.709 to 0.831)	0.209 (0.156 to 0.274)
Alaska	People aged 9 to 85 years	127	0.020 (0.007 to 0.048)	0.787 (0.732 to 0.836)	0.193 (0.146 to 0.247)

* Confidence intervals could not be calculated because the numbers of individual genotypes were not presented

Figure 7 shows that a low frequency of APOE*2 (or the absence of the allele) is a characteristic not only of Inuit, but also of aboriginal peoples in America including Indians in the subarctic [Hegele et al., 1997], Indians in North- and South Dakota, Oklahoma and Arizona [Kataoka et al., 1996], Mayans [Kamboh et al., 1991b] and other Indians in Mexico [Aguilar et al., 1999], and Indians from Central- and South America [Asakawa et al., 1985; Crews et al., 1993; Scacchi et al., 1997]. Estimated APOE*4 frequencies are generally higher than in Europeans, but vary as much, from 9% in Mayans to 28% in Cayapa in Ecuador.

The low frequency of APOE*2 in Inuit and Americans indicates that the allele was absent in the humans from northern Asia (Siberia) who settled in the Arctic and peopled America 10,000 to 35,000 years ago. Interestingly, APOE*2 is absent in Evenki herders of central Siberia [Kamboh et al., 1996].

Figure 7. APOE alleles frequencies in samples of Inuit and Americans



APOE allele frequencies in Asia, Australia and Papua New Guinea

The samples from Asia include three of Turks [Mahley et al., 1995; Malle et al., 1996; Brega et al., 1998], one of Evenki herders in Siberia [Kamboh et al., 1996], three of Indians [Hallman et al., 1991; Mastana et al., 1998], three of Chinese [Hallman et al., 1991; Kao et al., 1995; Gerdes et al., 1992a], one of Koreans [Kim and Kamboh, 1998], 10 of Japanese [Asakawa et al., 1985; Eto et al., 1986; Tsuchiya et al., 1986; Utermann, 1987; Kobori et al., 1988; Sano et al., 1988; Miida, 1990; Yamamura et al., 1990; Matsunaga et al., 1995; Zaman et al., 1997], two of Malaysians [Hallman et al., 1991; Gajra et al., 1994a] and one of Javanese [Gajra et al., 1994b].

Figure 8 shows that the APOE*4 frequency is relatively high in Evenki herders (15.3%), Javanese (16.8%) and Orang Asli of Malaysia (24.2%), but low in all other Asian populations studied. The lowest frequency of APOE*4 was

Figure 8. APOE allele frequencies in Asian samples

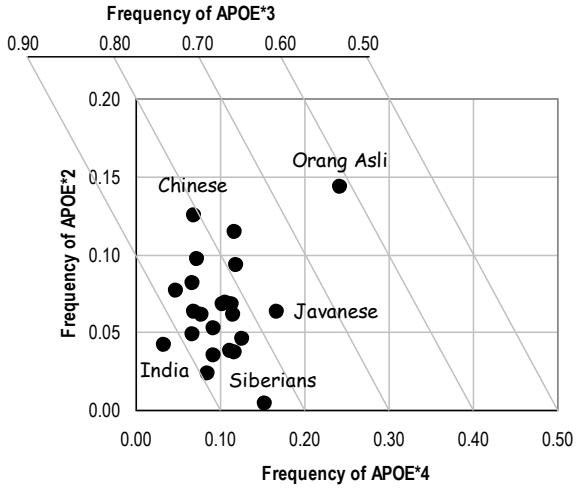
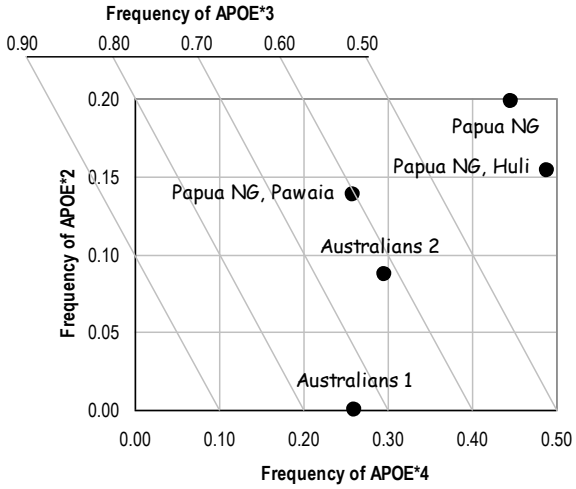


Figure 9. APOE allele frequencies in samples of Australians and Papuans



estimated in a small study of a tribal (Baiga) population in India (3.3%) [Mastana et al., 1998]. The estimates in Chinese are 4.9%, 7.0% and 7.4%, and the in Turks they are 6.7%, 6.9% and 7.9%, i.e. values in the lower end of the range seen in Southern Europe. Nine of the 10 studies of Japanese estimated an APOE*4 frequency between 8.6% and 12%, i.e. values in the upper end of the range in Southern Europe, and one estimated a value of 6.7% [Utermann, 1987]. Interestingly, perhaps, the higher frequencies were found in the northern parts of Japan.

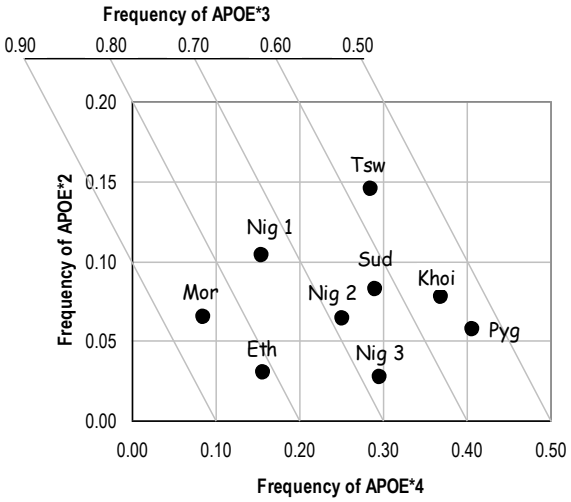
Relatively high frequencies of APOE*2 are seen in Chinese (7.6%, 9.7% and 12.4%) and Malaysians (11.4% and 14.3%), whereas the allele is absent in Evenki herders. The estimated allele frequency in Japanese ranges from 2.3% to 9.3%, and in Turks the estimates were 4.8%, 6.1% and 6.3%.

Figure 9 shows the data for Australia and Papua New Guinea. Both available studies of Australians indicate that the frequency of APOE*4 is high in these people (26.0% and 29.7%), but whereas APOE*2 was not seen in the smaller study of the two [Kamboh et al., 1991a], its frequency was 8.7% in the other[Shaw et al., 1999]. Papuans seem to have very high frequencies of both APOE*4 and APOE*2, and are the only people with a lower frequency of APOE*3 than APOE*4.

A high APOE*4 allele frequency in ancient African populations

Khoisan-speaking peoples and Aka (Biaka) pygmies presumably represent the most ancient populations of modern humans [Cavalli-Sforza et al., 1994; Chen et al., 2000]. Figure 10 shows that the frequency of APOE*4 is high in these peoples ('Khoi' and 'Pyg') [Sandholzer et al., 1995; Zekraoui et al., 1997]. The frequency is also high in Tswana-speaking Bantu of South Africa ('Tsw') and in sub-Saharan Africans and Bantus of the north (Sudanese and Nigerians; 'Sud', 'Nig1', 'Nig2' and 'Nig3')[Hallman et al., 1991; Sepehrnia et al., 1988; Corbo et al., 1999; Kamboh et al., 1999], but low in Caucasoid peoples of Morocco ('Mor') [Valveny et al., 1997] and intermediate in Ethiopians ('Eth') [Corbo et al., 1999].

Figure 10. APOE allele frequencies in African samples



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4. On the evolution of the APOE polymorphism

Summary of the chapter

Despite that APOE*3 is the most common allele in the world, there are several arguments for APOE*4 being the ancestral allele from which first APOE*3 and then APOE*2 arose. But is there also a fourth allele — a 'reverse' APOE*3 allele (Arg_{112}-Cys_{158}) somewhere? Genetic drift could possibly account for the present-day differences in APOE allele frequencies around the world, but selection forces may also have contributed, and indirect evidence including the different APOE alleles frequencies in various populations suggests some possible mechanisms: First, APOE*4 may confer a selective advantage when food supplies are scarce or irregular. Secondly, APOE*3 may confer a lower susceptibility to many common infectious diseases. Thirdly, APOE*4 carriers may be less fertile than APOE 3-3 genotypes, or APOE*3 may have been reproduced more efficiently following the evolution of 'grandmothering' in humans. Fourthly, APOE*4 carriers appear to tolerate head injuries less well than individuals with other genotypes. Finally, it is speculated that APOE*4 may protect against vitamin D deficiency, because the higher frequency of APOE*4 in dark-skinned humans and the higher frequency in people living in places where the intensity of solar radiation is relatively low.

APOE*3 is the most frequent allele in all populations studied except Papuans (see page 26) and is therefore often considered to be the ancestral or 'wild-type' allele. Moreover, the APOE*2 and APOE*4 alleles can each arise from APOE*3 by a single nucleotide change, APOE*2 by a C → T transition in codon 158 and APOE*4 by a T → C transition in codon 112.

The case for APOE*4 being the ancestral allele

Despite the seemingly obvious case for APOE*3, there are several arguments for APOE*4 being the ancestral allele:

- The APOE allele in non-human primates does not show a common polymorphism corresponding to the one seen in humans [Zannis et al., 1985] and the monkey and ape APOE alleles are similar to human APOE*4, in that they code for arginine at the positions equivalent to residues 112 and 158 in humans [Hanlon and Rubinsztein, 1995]. Nevertheless, one needs to consider that even though most animals have 'E4-like' apoE, the proteins do not display the property that distinguishes human apoE4 from human apoE3 — preferential binding to triglyceride-rich lipoproteins. This is because animal apoE does not have arginine (but threonine) in the position equivalent to residue 61 that is crucial for domain interaction in human apoE4 (see page 11). It is possible, however, that the Thr-61 to Arg-61 change occurred in early hominids and was the first step in establishing particular functions of apoE in humans [Finch and Sapolsky, 1999].
- APOE*4 is very frequent in ancient African populations (see page 27).
- The two polymorphic sites at codons 112 and 158 are both in CpG sequences, which are 'hot spot' mutation sites. The methylation of C in CpG sequences and their ready deamination to T suggest that C → T transitions would be favoured over the reverse T → C transition, which would be necessary to obtain APOE*4 from APOE*3.
- Different degrees of linkage disequilibrium between the common APOE alleles and a common Hpa I polymorphism in the adjacent APOC-I gene in Europeans (and Euro-Americans) and Africans (and Afro-Americans) suggests that APOE*4 is the ancestral allele from which first APOE*3 and then APOE*2 arose [Seixas et al., 1999; Xu et al., 1999].
- Finally, a recent study of the relationships between 31 haplotypes defined by 22 diallelic sites in APOE strongly suggests that APOE*4 is the ancestral allele and that the variation including APOE*3 and APOE*2 has evolved over the past 200,000 years [Fullerton et al., 2000].

Is there a fourth APOE allele in the APOE*2, *3 *4 family?

One may speculate whether there is not a fourth member in this family of alleles — one coding for an apoE3 isoform with Arg_{112}-Cys_{158}, i.e. a 'reverse' APOE*3 allele. If APOE*4 (Arg_{112}-Arg_{158}) is the ancestral allele this allele could have arisen as readily as APOE*3 (Cys_{112}-Arg_{158}) and could also be a parent allele for APOE*2 (Cys_{112}-Cys_{158}). On the other hand, if APOE*3 is the ancestral allele, it could have arisen from secondary mutations in either APOE*4 or (less likely) APOE*2.

The allele has not been described in the literature, but it would also be difficult to discover — 'reverse apoE3' obviously cannot be distinguished from apoE3 by isoelectric focusing, and the most probable genotype including this putative allele (heterozygosity with APOE*3 as the other allele) would give a band pattern similar to the genotype APOE 2-4 with the commonly used genotyping method of Hixson and Vernier [Hixson and Vernier, 1990].

If the allele exists, it most probably does not exist in Europeans (or is very rare). It follows from the fact that all published studies with informative comparisons of apoE phenotypes and APOE genotypes were conducted with European samples (a total of approximately 1,700 individuals), and that none of the observed discrepancies were of the kind 'apoE 3-3 phenotype but APOE 2-4 genotype' [Kontula et al., 1990; Hansen et al., 1994a; James et al., 1994; Lahoz et al., 1996].

One could surmise that 'reverse APOE*3' does not exist, because the functional properties of the encoded apoE isoform are somehow disastrous and even incompatible with life. However, a strain of transgenic mice with human 'reverse APOE*3' has very recently been generated at the Gladstone Institute of Cardiovascular disease (Robert W. Mahley and Yadong Huang; personal communication). The animals thrive but have hyperlipidemia, as expected perhaps, considering that 'reverse apoE3' presumably has a preference for binding to triglyceride-rich lipoproteins (as apoE4 does) and a low LDL receptor affinity (as apoE2 has), i.e. properties that are shared by the products of many rare APOE alleles that associate with dominant type III hyperlipidemia in humans [Mahley et al., 1999].

Has natural selection contributed to the present-day APOE allele frequencies?

Genetic drift could possibly account for the present-day differences in APOE allele frequencies around the world, but as for other functionally important genetic polymorphisms [Cavalli-Sforza et al., 1994; Vogel and Motulsky, 1986] one may reasonably speculate if selection forces have not contributed? The speculation is particularly obvious if APOE*4 is indeed the ancestral allele, because it is difficult to imagine how APOE*3 could have achieved such high frequencies in all populations without having a selective advantage. Or conversely, if APOE*3 is the ancestral allele, whether APOE*4 has a selective advantage under some conditions?

The following is a review of some possible selective mechanisms that could favor the survival of either APOE*3 or APOE*4 under the variable environmental conditions that humans have faced in various places and at various times (and still do).

Is APOE*4 advantageous when food supplies are scare or irregular?

Many studies indicate that APOE genotype modulates the effects of dietary fat (quantity and quality) on plasma lipoprotein levels. And notwithstanding that the studies are extremely heterogeneous in all aspects of their design, and that there are several studies with discrepant results [Marshall et al., 1996; Dixon et al., 1997; Pasagian-Macaulay et al., 1997; Ordovas and Schaefer, 2000], the overall impression is that APOE*4 carriers are generally more 'responsive' to dietary fat than individuals with other genotypes [Miettinen, 1991; Xu et al., 1992] [Gylling et al., 1995; Lehtimäki et al., 1995; Ordovas et al., 1996; Kallio et al., 1997; Gylling et al., 1997; Tso et al., 1998].

This phenotypic functional trait could reflect a selective advantage of APOE*4 when food supplies are scarce or irregular if it is related to better intestinal absorption of lipids [Miettinen, 1991; Miettinen et al., 1992; Gylling et al., 1995] including the fat-soluble vitamins A, D, E and K. In seeming accordance with this speculation, APOE*4 appears to be more frequent in (recent) cultures of hunters/gatherers or nomads than in long-established agricultural communities, e.g. in Southern Europe, South-east Asia and Central America [Gerdes et al., 1996b; Corbo and Scacchi, 1999].

APOE and differential susceptibility to infectious disease

The beginning of agriculture over 10.000 years ago was associated with very profound changes in living conditions. Not only did the main sources of food change to products from cultivated plants and domesticated animals, but the sources also became more stable and stimulated population growth and formation of large communities [Cavalli-Sforza et al., 1994]. This may have changed the selective forces on genetic traits that relate to susceptibility to those infectious diseases, which either originated from the domesticated animals or only become endemic in larger communities (e.g. smallpox, tuberculosis, measles, mumps, rubella, influenza and viral gastroenteritis). APOE*3 is particularly common in long-established agricultural communities and this could reflect a lower susceptibility of APOE*3 carriers to these "new" diseases.

Mahley and Rall recently reviewed the evidence for an involvement of apoE in immunoregulation and susceptibility to infections [Mahley and Rall, 2000].

- ApoE can modulate antigen- and mitogen-induced lymphocyte activation.

- The protein may be able to interfere with some viruses binding to cell surfaces (as their initial means of gaining entry).
- Studies of apoE-knockout mice indicate that they are more susceptible than normal mice to infections with *Listeria momocytogenes* and *Klebsiella pneumoniae*.
- Finally, there is some evidence that apoE plays a role in protection against malaria.

There are only few reports on apoE isoform-specific effects in relation to infections. Amouyel et al found an increased proportion of APOE*4 carriers in 61 French patients with Creutzfeldt-Jacob disease (33%, as compared to 22% among controls)[Amouyel et al., 1994] and Gérard et al found a very high proportion of APOE*4 carriers (68%) in a study of 30 patients with synovial infection with *Chlamydia pneumonia*. Two studies indicate that neurological symptoms are more common or severe in APOE*4 carriers with Herpex simplex and HIV infections, respectively [Itzhaki et al., 1997; Corder et al., 1998].

The last studies point to an important matter to consider in this context, namely that APOE genotype need not necessarily influence susceptibility to infection in order to become associated with differential mortality. A genotype-specific influence on the course and prognosis of an infectious disease is equally important, and such an influence need not even have bearings on specific pathological mechanisms, but may relate to unspecific robustness for instance to the effects of diarrhea or fever.

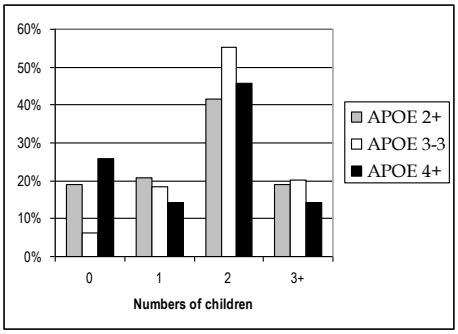
Are APOE*4 carriers less fertile than APOE 3-3 genotypes?

ApoE is involved in local transport of lipids in all steroid-producing tissues including the gonads and also participates in transplacental lipid transport [Olson et al., 1995; Rindler et al., 1991]. Although it has not been investigated, the possibility exists that the apoE isoforms have different functional properties in these tissues, as elsewhere, so that the APOE polymorphism could have an impact on reproductive efficiency. Another focal point in an association between APOE polymorphism and reproduction could reside in the development of the embryo. In mice, the transcription of APOE begins already in the one-cell embryo and the gene appears to be imprinted during preimplantation and early stages of postimplantantion, with stronger expression from the paternal allele [Mann et al., 1995]. Interestingly, the APOE gene at 19q13.2 is presumably one of the human homologous chromosome areas involved in imprinting [Hall, 1990]. Again, however, there is no direct evidence for differential effects of APOE genotypes on survivability of human embryos; the only indication of such effects stems from the observation that the frequency of APOE*4 is unexpectedly high in cases of trisomy 13 and 18 [Nagy et al., 2000b]

Nevertheless, if APOE genotype does have an impact on reproductive efficiency (whatever the mechanism), it is presumably modest and will only be reflected as changing allele frequencies in populations with little restrictions on multiplication and growth. This case only arose with establishment of agriculture [Cavalli-Sforza et al., 1994], and we hypothesized that the high frequency of APOE*3 in the long-established agricultural centers in the world could relate to a higher fertility in APOE 3-3 genotypes than in other individuals **[Gerdes et al., 1996a]**.

To examine that possibility we compared the numbers of children in 40-year-old married Danish men and found that the distributions of children by APOE genotype groups were statistically significantly different (p=0.0026). On the average, men with the APOE 3-3 genotype (n=212) had 1.93 children, whereas men with the APOE 3-4 or 4-4 genotype (n=105) had 1.50 and men with the APOE 3-2 and 2-2 genotype (n=53) had 1.66 children.

Figure 11. Distribution of married Danish men with different APOE genotypes by the numbers of their biological children



As shown in Figure 11, the most conspicuous finding was that 26% of the APOE*4 carriers were childless at the age of 40 years, as compared to 19% of the APOE*2 carriers and only 6% of the men with genotype 3-3. We considered several sources of confounding, but we were unable to confirm the importance of any, suggesting that this indication of a differential fertility in Danish men is real. A subsequent study of patients attending a fertility clinic indicates that the frequency of APOE*4 is increased among men in couples where the cause of infertility is unknown, in contrast to couples where the infertility problems could be ascribed to tubal disease or inferior semen quality (Gerdes, LU & Ingerslev, J.; in preparation).

The observed ratio of average numbers of children in APOE*4 carriers and in men with APOE 3-3 corresponds to a relative fitness coefficient of about 0.75 in a simple natural selection model, which predicts that the frequency of

APOE*3 in a growing population could raise from 1-2% to 100% in 100-300 generations (i.e. over 2.500-7.500 years). Hence, the proposition that APOE*3 could achieve its dominant status through mechanisms directly related to fertility is certainly not unrealistic. It is nevertheless important to point out that the net cumulative maternity function (fitness) associated with a particular APOE genotype can be highly variable among populations, depending on wholly different mechanisms **[Gerdes et al., 1996a]**. Thus, the above simple estimation can neither strengthen nor weaken the credibility of our findings, and it certainly does not imply that APOE*4 (or APOE*2) should have been extinct in humans by now.

Finch and Sapolsky have outlined a fascinating hypothesis for a wider implication of APOE genotypes in the reproductive schedule in humans [Finch and Sapolsky, 1999]. They suggest, first, that APOE*3 arose from APOE*4 in relation to increases in the brain size of *Homo sapiens* and concurrently delayed maturation, with a postponement of the age of puberty and a prolongation of the duration of the reproductive age in females. Secondly, they hypothesize that APOE*3 may have been selected during the evolution of 'grandmothering' in humans. This social behavior has to do with the prolonged dependence of maternal care during years of growth and the necessity of learning skills, which are critical for the adult competence (and often are instinctual in other species), including nursing of infants; and it also embrace grandmothers (and in particular maternal grandmothers) participating in rearing their grandchildren. Clearly, this can be important for the survival of the young, so that there could be a selective advantage in families in which members survive to an old age and have well-preserved cognitive functions, i.e. favouring APOE*3 over APOE*4 (see page 39).

Do APOE*4 carriers have less robust brains?

Some studies have shown that APOE*4 carriers recover less well after head injuries, and that they are more likely, for instance, to develop chronic brain injury in boxing than individuals with other genotypes [Teasdale et al., 1997; Friedman et al., 1999; Jordan et al., 1997]. This could obviously have a negative effect with respect to selection, notably in wartimes.

Does APOE*4 protect against vitamin D deficiency?

A putative association of APOE with bone metabolism has been ascribed to an impact of APOE polymorphism on the transport of vitamin K (see page 40), but it could also relate to vitamin D metabolism and embrace a very strong selection pressure. Hypovitaminosis D in childhood (rickets) causes bone deformations, which can reduce the probability of surviving to adulthood, and perhaps more importantly, can cause pelvic deformations in girls that later may cause their death during delivery under primitive conditions, and also the death of their offspring. Inadequate endogenous production of vitamin D_3 can be due to insufficient dietary supplementation or reduced intestinal uptake of the vitamin, or to low exposure to sunlight (UVB-radiation). The latter can be a particular problem to people with dark skin, because melanin blocks for ultraviolet photons and thus limits the synthesis of previtamin D_3 [Vogel and Motulsky, 1986].

Mourant and co-workers showed that the frequency of 'Gc-2-allele' for the gene coding for vitamin-D-binding protein (DBP; previously known as the group-specific component, Gc, of the α_2-globulins of human plasma) was high in populations living in areas with low levels of sunlight and vice versa (with some exceptions). They suggested that the distribution could be explained by means of natural selection if the encoded isoform were more efficient in binding vitamin, and so in protecting Gc-2 carriers from rickets [Mourant et al., 1976]. This may be true, although the concept has been weakened by an analysis including more detailed climatic data [Cavalli-Sforza et al., 1994].

Interestingly, a very consistent pattern appears if one correlates the frequency of APOE*4 in aboriginal peoples around the world to their skin pigmentation, while also considering the intensity of solar radiation in their habitats:

- The APOE*4 frequency is generally higher in dark-skinned humans than in humans with less melanin, and the frequency is particularly high (40-50%) for instance in Papuans, Pygmies and Khoisan, who are dark peoples and whose (recent) habitats are tropical forests where the intensity of sunlight is relatively low.
- High APOE*4 frequencies (20-30%) are also found in Saami and Inuit, who are moderately pigmented humans living in regions with low average solar radiation, and in peoples living in South American rain forests.
- Conversely, the lowest frequencies of APOE*4 (5-10%) is found among lightly or moderately pigmented humans living in areas of high insolation, i.e. around the Mediterranean Sea, in East Asia, in the southern parts of North America and in Central America.
- The APOE*4 gradient in Europe (and possible also in Japan) could be interpreted to indicate natural selection for this allele with decreasing solar radiation.

The putative advantage of APOE*4 could be related to better intestinal absorption of vitamin D (see page 30), but could also be related, somehow, to the fact that apoE and DBP both binds to megalin. This receptor plays a central

role in vitamin D metabolism, since it binds and internalizes DBP on the luminal surface in the renal proximal tubuli. The function prevents systemic loss of vitamin D through the urine and is also a step in the conversion of 25-hydroxy-vitamin D_3 to the biologically active 1,25-dihydroxy-vitamin D_3 [Willnow et al., 1999].

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5. APOE genotypes in centenarians

Summary of the chapter

Compared with 40 years old men, there were more APOE*2 carriers among Danish centenarians (21% versus 13%) and less APO*4 carriers (15% versus 29%), indicating that the relative mortality risks in APOE*2 carriers and APOE*4 carriers are decreased and increased, respectively, in old age. We developed a method to estimate a meaningful measure of the impact of APOE genotype on mortality risk over a broad range of ages — genotype-specific average relative mortality risk, *R*. The values of *R* indicate only modest differences in average mortality risk, from 0.95 in APOE*2 carriers to 1.13 in APOE*4 carriers, with APOE 3-3 genotypes as the reference group. Data from the literature and an unpublished study of supercentenarians indicate that these averages may include differences that are largest in the lower end of the age interval and vanish in the oldest old. The diseases that are 'producing' the differences in mortality risk must be common mortal diseases, and coronary heart disease, stroke and Alzheimer's disease are likely candidates. Some evidence suggests that osteoporosis and cancer could also contribute, but the cases are weak.

A comparison of the APOE genotype distributions across age groups may give an impression of the influence of the individual APOE genotypes on total mortality in the age intervals considered. For instance, since APOE*4 and APOE*2 have opposite effects of on risk of several common mortal diseases, it is not unexpected that the proportions of APOE*4 carriers are lower, and the proportions of APO*2 carriers are higher among octogenarians, nonagenarians and centenarians than among younger people [Davignon et al., 1987; Kervinen et al., 1994; Rebeck et al., 1994; Louhija et al., 1994; Schächter et al., 1994; Asada et al., 1996].

A study of Danish centenarians

Table 4 shows the distribution of APOE genotypes in 177 Danish centenarians and the distribution in 40 years old men **[Gerdes et al., 2000b]**.

Table 4. APOE genotypes in Danish centenarians and in 40-year old men

	Centenarians						40 years old men	
	Women		Men		Total			
	n	%	n	%	n	%	n	%
APOE 2-2	0	0.0	0	0.0	0	0.0	8	1.7
APOE 2-3	26	19.5	11	25.0	37	20.9	54	11.6
APOE 3-3	81	60.9	25	56.8	106	59.9	260	55.8
APOE 2-4	5	3.8	3	6.8	8	4.5	9	1.9
APOE 3-4	20	15.0	5	11.4	25	14.1	117	25.1
APOE 4-4	1	0.8	0	0.0	1	0.6	18	3.9
Total	133	100.0	44	100.0	177	100.0	466	100.0

There are more APOE*2 carriers among the centenarians (although none had the APOE 2-2 genotype) than among the 40 years old men (21% versus 13%; ignoring APOE 2-4) and fewer APOE*4 carriers (15% versus 29%; ignoring APOE 2-4). Correspondingly, the estimated allele frequencies in the centenarians are 0.127 (0.095 to 0.167) for APOE*2, 0.774 (0.726 to 0.816) for APOE*3, and 0.099 (0.071 to 0.136) for APOE*4, i.e. the frequencies of APOE*2 and APOE*4 are about 40% higher and lower, respectively, than the frequencies in 40 years old men (see page 21).

Analyzing and interpreting the data

How should such data be analyzed to estimate the influences of APOE genotypes on mortality risk (or survival chance)? The data resemble data from cumulative case-control studies and some have therefore used odds ratios to estimate relative risks (chances) [Louhija et al., 1994; Schächter et al., 1994]. However, odds ratios fail to reflect relative mortality risks in a meaningful way with such data and we therefore developed a method to obtain a different

measure of association from cross-sectional genetic studies of old people versus young people **[Gerdes et al., 2000b]**.

This *genotype-specific average relative mortality risk* in an age interval, *R*, was derived as follows:

- Let $\mu_1(x)$ be the hazard of death at age *x* in carriers of a genotype (group 1) and let $\mu_2(x)$ be the hazard in individuals without the genotype in question (group 2). Under the proportional hazard assumption $\mu_1(x) = R \cdot \mu_2(x)$, where *R* denotes the relative risk of group 1 compared with group 2.
- Let $s_1[a,b]$ and $s_2[a,b]$ denote the probabilities of survival in the two groups from some starting age *a* to some final age *b*.
- Then because $s_i[a,b] = \exp(-\int_a^b \mu_i(x)dx)$, it follows that $s_1[a,b] = (s_2[a,b])^R$
- Let $N(a)$ be the total size of a population at starting age *a* and let $p_1(a)$ and $p_2(a)$ be the proportions in the two groups. Similarly, let $N(b)$, $p_1(b)$ and $p_2(b)$ be the size, and proportions among survivors at age *b*, and let $S[a,b] = N(b)/N(a)$ be the probability of surviving from age *a* to age *b* in the population as a whole.
- Then, with $s_1[a,b]$ defined as above, the following identity holds: $N(a) \cdot p_1(a) \cdot s_1[a,b] = N(b) \cdot p_1(b)$.
- Using $N(b) = N(a) \cdot S[a,b]$, rearranging terms and canceling $N(a)$ yields $s_1[a,b] = p_1(b) \cdot S[a,b] / p_1(a)$. Similarly, $s_2[a,b] = p_2(b) \cdot S[a,b] / p_2(a)$.
- Inserting these expressions in $s_1[a,b] = (s_2[a,b])^R$ and solving for *R* yields the formula we used:

$$R = \frac{\ln p_1(b) - \ln p_1(a) + \ln S[a,b]}{\ln p_2(b) - \ln p_2(a) + \ln S[a,b]}.$$

The four values of *p* can be calculated from the data. The values $p_1(b)$ and $p_1(a)$ are the proportions of APOE*2 carriers (or APOE*4 carriers) in centenarians and in 40-years old men, respectively, and $p_2(b)$ and $p_2(a)$ are the proportions of APOE 3-3 + APOE 2-4 genotypes (the reference group) in the two age groups.

In theory the four values of *p* should be weighted to account for ignoring APOE*4 carriers when calculating *R* for APOE*2 carriers (and vice versa), because it is assumed that $p_1(b) + p_2(b) = 1$ and $p_1(a) + p_2(a) = 1$. However, the errors introduced by not doing this are minimal. *S[a,b]* is the probability of surviving from age 40 to age 100 in the population as a whole. Data from the Danish Mortality Database shows that for Danes who were 40 years old some 60 years ago, the chance of surviving from 40 to 100 (i.e. *S[a,b]* in the formula) was about 0.002.

Approximate 95% confidence intervals for *R* were calculated as $R_L, R_U = R^{(1 \pm 1.96/\chi)}$, where χ is the square root of the null χ^2 for the observed distribution of individuals in group 1 and 2 at ages *a* and *b*. The formula was derived for rate ratios or odds ratios [Miettinen, 1985] and gave the same results with *R* as with a sampling method.

Results

As shown in Table 5, the values of *R* indicate only modest differences in average relative mortality risks over the age interval from 40 to 100 years, i.e. from 0.95 in APOE*2 carriers to 1.13 in APOE*4 carriers.

Table 5. Proportions of APOE genotype categories in 40- and 100-year old Danes, and estimated genotype-specific average mortality risks

	AGE				Relative risk		
	40 years		100 years		*R*	95% CI	
APOE*2 carriers	62	13%	37	21%	**0.95**	(0.88	- 1.02)
APOE 3-3 and 2-4	269	58%	114	64%	1.00		
APOE*4 carriers	135	29%	26	15%	**1.13**	(1.05	- 1.22)
Total	466	100%	177	100%			

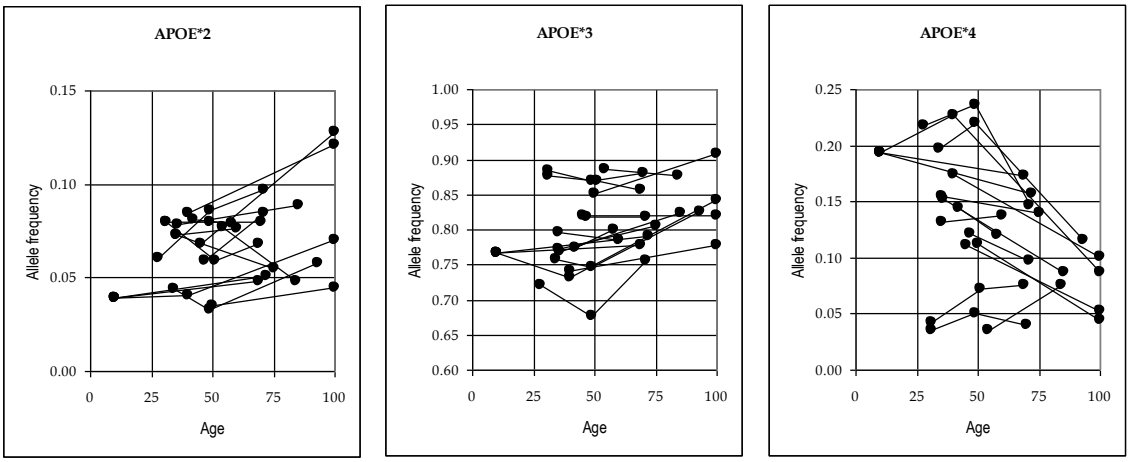
We also applied the method to published data from a Finnish and a French study of centenarians [Louhija et al., 1994; Schächter et al., 1994] and obtained similar results, except that the *R*-values for APOE*2 carriers tended to be lower (0.88 and 0.89, respectively)**[Gerdes et al., 2000b]**.

The results associate with the view that APOE is a 'frailty gene' — a gene that has a modest influence on average mortality over a broad range of ages, rather than being a 'longevity gene' with a particular effect on survival in extreme old age. This viewpoint raises some interesting questions, which are addressed in the next sections.

Do APOE genotype-specific relative mortality risks vary across age groups?

The above *R*-values estimates genotype-specific *average* relative mortality risks over a broad age interval from about 40 years of age to 100 years, but relative mortality risks can obviously vary within the interval. The following data suggest that they do, and they specifically suggest that the differential effect of APOE genotype on mortality vanish in people over 90 years of age.

Figure 12. Observed frequencies of APOE*2, APOE*3 and APOE*4 in population subgroups of different ages



APOE allele frequencies in elderly, old, and very old people

Figure 12 is based on data from 15 studies of APOE genotypes in two or more age groups within the same population including our study of centenarians [Davignon et al., 1987; Ordovas et al., 1987; Cauley et al., 1993; Eggertsen et al., 1993; Kervinen et al., 1994; Schaefer et al., 1994; Louhija et al., 1994; Schächter et al., 1994; Feskens et al., 1994; Kuusisto et al., 1995; Kao et al., 1995; Stengård et al., 1995; Asada et al., 1996; Bader et al., 1998; Gerdes et al., 2000b]. Lines connect data points from corresponding age groups, which are positioned by the group's mean or median age (or the midpoint of a range of ages in some cases).

The figure shows that APOE allele frequencies in centenarians are not peculiar, but appear as extensions of almost linear trends that are visible also in data for 70-, 80- and 90 year olds (the conspicuous exceptions for APOE*4 were seen in a study of Chinese in Taiwan, with separate data for men and women [Kao et al., 1995] and in a small study of Italians [Bader et al., 1998]). This seems to indicate that the APOE genotype-specific relative mortality risks are more or less constant over a broad age interval from about 70 years and onwards. However, since total mortality rates show nearly exponential increases with age, such linear trends may in fact indicate that genotype-specific relative mortality risks are converging toward unity in the oldest old.

Relative mortality risks in cohort studies of older people

We only reviewed the results from two cohort studies in our paper **[Gerdes et al., 2000b]**, but there are more:

- We used the published data from a 5-years follow study of two cohorts of Finnish men aged 65-84 years [Stengård et al., 1995; Stengård et al., 1996] to estimate genotype-specific relative mortality risks (rate ratios) to compare with our *R*-values. The rate ratios for APOE*2-carriers, with genotype 3-3 as the reference, were 3.5 (1.8 to 6.8) in one cohort and 1.1 (0.6 to 2.2) in the other, and for APOE*4 carriers they were 1.9 (1.2 to 3.1) and 1.4 (0.9 to 2.1). Hence, the values for APOE*2 carriers differed markedly from the *R*-values we calculated with data from the Danish, Finnish and French studies of centenarians. The estimated relative mortality risk in APOE*4-carries were also higher, notably in the first cohort. These differences can be due to the very different approaches to estimate relative mortality risks, but they may also indicate that the differential mortality risks associated with APOE are closer to 1 in people under the age of 65 and over the age of 90 years.

- Corder et al determined relative mortality hazards in APOE 2-3 and APOE 3-4 genotypes, with genotype 3-3 as the reference, in a Swedish cohort of men and women aged 75 years or older, followed for up to seven years [Corder et al., 1996]. The relative hazards (risks), controlling for age and sex, were 0.8 (0.6 to 1.1) in APOE*2 carriers and 1.3 (1.0 to 1.6) in APOE*4 carriers, respectively; i.e. values that are not very different from the *R*-values we found. However, the study indicated a stronger influence of APOE polymorphism on mortality in people aged 85 years and older than in the younger cohort members.
- Vogt et al studied the mortality experience in Euro-American women aged 65 to 93 years, followed for up to 7.6 years, and compared APOE*4 carriers with APOE 3-3 genotypes [Vogt et al., 1997]. The relative all cause mortality risk was 1.2 (0.8 to 1.8), controlling for multiple covariates, i.e. close to *R*-value we found. In contrast to the findings by Corder et al, the relative risk associated with APOE-4 was seemingly higher in women aged 65-69 years than in women aged 70-93 years, suggesting that the effect of APOE*4 on mortality is decreasing with advancing age.
- Similarly, a Finnish study with 11 years follow-up of people aged 65 years and older showed that the adjusted relative mortality risk for both vascular and nonvascular deaths were over 2 in APOE*4 carriers in individuals initially under 80 years of age, but close to 1 for older people [Räihä et al., 1997].
- Another Finnish study with 5 years follow-up of people initially aged 75, 80 and 85 years also showed that the relative mortality risk associated with APOE*4 decreased from 1.9 (1.1 to 3.2) to 1.5 (1.1 to 2.1), and to 1.0 (0.7 to 1.3) [Tilvis et al., 1998].
- In a third Finnish study with 3 years follow-up study of Finns aged 85 years and over, the crude mortality odds ratio for APOE*4 carriers was close to 1 [Juva et al., 2000].

Thus, despite some inconsistencies among these studies, they seem to indicate that APOE genotype-specific relative mortality risks are converging toward unity in the oldest old, i.e. that the differential mortality vanishes.

APOE genotypes in supercentenarians

That suggestion is also supported by data from a small Danish study of supercentenarians (Andersen-Ranberg K, Jeune B, Gerdes LU, Petersen NE, Vaupel JW; unpublished data). As shown in Table 6, the genotype distribution in these very old people does not differ much from the distribution in centenarians (considering the small numbers). The estimated allele frequencies are 0.147 (0.073 to 0.254) for APOE*2, 0.750 (0.630 to 0.847) for APOE*3, and 0.103 (0.042 to 0.201) for APOE*4.

Table 6. APOE genotypes in Danish centenarians and supercentenarians

	Centenarians		Supercentenarians					
	N	%	101-105	%	105+	%	Total	%
APOE 2-2	0	0.0	1	6.7	0	0.0	1	2.9
APOE 2-3	37	20.9	4	26.7	4	21.1	8	23.5
APOE 3-3	106	59.9	7	46.7	11	57.9	18	52.9
APOE 2-4	8	4.5	0	0.0	0	0.0	0	0.0
APOE 3-4	25	14.1	3	20.0	4	21.1	7	20.6
APOE 4-4	1	0.6	0	0.0	0	0.0	0	0.0
Total	177	100.0	15	100.0	19	100.0	34	100.0

But is it not what one should expect? We believe (or at least I do) that APOE genotypes are associated with different mortality risks because they are associated with different risks for developing relatively well-defined serious diseases. However, very old people mostly die of a little of everything, and no single genomic polymorphism can have an influence on that.

Which diseases are 'producing' the genotype-specific relative mortality risks?

The candidates are relatively few, since they must be common mortal diseases in elderly people, and have some known or suggested relationship to APOE — cardiovascular diseases, Alzheimer's disease, cancer and osteoporosis. The following indicates that cardiovascular diseases and Alzheimer's disease are those to bet on, whereas the evidence for the importance of osteoporosis and cancer is weak.

Cardiovascular diseases

These include first and foremost ischemic heart diseases with acute coronary syndrome as the deadly flagship. The relationships between APOE genotypes and atherosclerosis, risk of coronary heart disease, prognosis after myocardial infarction, and aortic aneurysms are dealt with elsewhere (page 18 ff, page 41 ff and page 49), but important differential associations with risk of cerebrovascular disease and prognosis have also emerged:

- APOE*4 carriers have an approximately two-fold increased relative risk of ischemic cerebrovascular disease and also an increased risk for intracerebral and subarachnoid hemorrhage [McCarron et al., 1999; Kokubo et al., 2000].
- APOE*2 carriers also appear to have an increased risk of cerebral infarction, intracerebral hemorrhage and embolism compared to individuals with APOE 3-3 [Kokubo et al., 2000].
- APOE*4 is associated with improved survival in patients with ischemic cerebrovascular disease, but a reduced survival in patients with intracerebral hemorrhage [Alberts et al., 1995; McCarron et al., 1998; McCarron et al., 1999].
- Both APOE*4 and APOE*2 carriers have a markedly increased risk of recurrent hemorrhagic strokes compared to patients with APOE 3-3 [O'Donnell et al., 2000].

Taken as a whole, the associations of APOE*4 with increased risk of both coronary heart disease and cerebrovascular disease, and also associations of the allele with worse prognoses may certainly contribute to explain the increased total mortality risk in APOE*4 carriers. The case for APOE*2 is less obvious. It is possibly associated with a reduced risk of coronary heart disease (see page 44), but then with an increased risk of stroke.

Nevertheless, it is interesting here also to contrast the altered APOE allele frequencies in the centenarians with the observation that the allele frequencies associated with common polymorphisms in several other putative candidate genes for cardiovascular risk — namely coagulation factors V and VII, fibrinogen, plasminogen activator inhibitor type 1, tissue plasminogen activator, platelet receptor glycoprotein IIb/IIIa, prothrombin, methylene tetrahydrofolate reductase, angiotensin converting enzyme, and angiotensinogen — were practically identical in the centenarians and in blood donors aged 20-64 years, or newborns [Kristensen et al., 1998; Bladbjerg et al., 1999]. Does this mean that these other genes are unimportant for risk of mortal cardiovascular risk? Or does it mean that the altered APOE allele frequencies in centenarians are not chiefly due to differential mortality risks for cardiovascular disease?

Alzheimer's disease

The discovery by Roses and associates that APOE*4 is associated with risk of Alzheimer's disease [Strittmatter et al., 1993; Corder et al., 1993; Saunders et al., 1993b; Saunders et al., 1993a; Schmechel et al., 1993] gave a starting signal to an immense research activity in that field in the past 7 years. It also motivated ongoing basic research into the role and functions of apoE in neurobiology including the influences of the APOE polymorphism. The results are breathtaking since they not only point to apoE isoform-specific inferences with the properties and metabolism of the amyloid-beta peptide that aggregate to form the characteristic amyloid plaques in the brains of patients with Alzheimer's disease, but also to effects on neuron cytoskeletal stability and functions, on growth of neurite extensions, and on the ability of neurons to recover from injurious exposures [Weisgraber et al., 1994; Mahley and Huang, 1999; Saunders, 2000; Mahley and Rall, 2000].

The risk of Alzheimer's disease is markedly increased in APOE*4 carriers of European ancestry (odds ratios are 2.5 to 3.5 for individuals with APOE 2-4 or 3-4, and 10-15 for individuals with APOE 4-4, relative to individuals with APOE 3-3), whereas APOE*2 carriers are protected (odds ratios 0.6 to 0.7) [Farrer et al., 1997; Tang et al., 1998]. However, ethnicity, gender and age are important modifiers of these effects:

- The relative risk associated with APOE*4 is substantially lower in people of African ancestry and in Hispanics, whereas it is higher in Japanese (OR > 30 in individuals with APOE 4-4). In contrast, APOE*2 appears to be equally protective in all ethnic subgroups [Farrer et al., 1997; Tang et al., 1998].
- The relative risk in women with APOE*4 is higher than in men [Farrer et al., 1997].
- The relative risk in APOE*4 carriers is increasing between ages 40 and 60 years but then decline with age hereafter [Farrer et al., 1997]. This also appears when comparing for instance people under the age of 65 years with other people [Rubinsztein and Easton, 1999] The protective effect of APOE*2 is unaffected by age, except perhaps that the allele is 'inverted' to a risk factor in individuals with familial early-onset Alzheimer's disease [Rubinsztein and Easton, 1999].

Nevertheless, the clear association of APOE*4 with increased risk of Alzheimer's disease and the opposite effect of APOE*2 most probably contribute to APOE genotype-specific mortality risks in elderly people.

Osteoporosis and bone fracture risk

Several recent studies have shown that APOE*4 carriers tend to have lower bone mineral density (or higher rates of bone mineral losses) than other individuals, and also an increased risk of bone fractures including hip fracture [Shiraki et al., 1997; Kohlmeier et al., 1998; Johnston et al., 1999; Cauley et al., 1999]. Such effects could undoubtedly contribute to increased morbidity and mortality in elderly APOE*4 carriers. However, other studies have failed to corroborate the above findings [Heikkinen et al., 2000; Booth et al., 2000] or have instead pointed to a beneficial effect of APOE*2 [Gerdes et al., 2000c], and it definitely appears, that if APOE genotype does have an influence on bone metabolism, then it is complex. Thus, the putative influence is probably dependent on gender, age and the presence of other risk factors for osteoporosis including in particular estrogen status in women [Cauley et al., 1999; Salamone et al., 2000; Gerdes et al., 2000c]. Moreover, the influence of APOE on bone metabolism may possibly be regional, i.e. with effects on of only some parts of the skeleton, or different effects in different regions [Cauley et al., 1999; Gerdes et al., 2000c] (results from this study were presented at an Workshop on Growth and Regeneration of Bone, University of Aarhus, Aarhus 2000)..

A recent communication has shown that APOE is expressed in the skeleton in rats and is regulated by estrogen and parathyroid hormone (Hefferan et al.; abstract from the September 2000 meeting of the American Society for Bone and Mineral Research), indicating some central function of apoE in bone metabolism. However, it is also possible that the APOE polymorphism has an effect on bone metabolism via genotype-specific differences in the metabolism of vitamin K (see page 45) or vitamin D (see page 32). Overall, however, the case for a differential risk of osteoporosis and bone fractures as important contributors to APOE genotype-specific mortality risk is weak.

Cancer

Only few studies indicate an association of the APOE polymorphism with differential risk of cancer. A Finnish case-control study showed that APOE*4 carriers have a reduced risk of adenoma and carcinoma of the proximal colon (odds ratios of about 0.4 compared individuals with other APOE genotypes), whereas their risk of tumors of the distal colon was not reduced [Kervinen et al., 1996]. An recent American study indicates that women with APOE*4 who also have high serum triglycerides run a four-fold increased risk of developing breast cancer, whereas neither APOE*4 nor elevated triglycerides alone was associated with significant risk [Moysich et al., 2000]. In their follow-up study of women aged 65 years and older, Vogt et al. found odds ratios of about 2 for both cancer and cardiovascular deaths in women aged 65-69 years, but not in older women [Vogt et al., 1997]. Cancer is a very common cause of death in late life, and it is clearly important to investigate the possibility that APOE genotypes may confer differential risks. At present, however, the case is weak.

Did the APOE genotype frequencies in centenarians change when they were young?

When we use APOE data from e.g. 40 year olds and centenarians to estimate genotype-specific relative mortality risks in late life, we assume that the observed genotype frequencies in the 40 year olds today represent the distribution in the centenarians' birth cohort some 60 years ago. The assumption may not hold in places where immigrations or emigrations of larger ethnic subgroups have occurred, e.g. in some regions in Finland [Stengård et al., 1995]. Moreover it cannot be excluded that APOE genotypes have conferred differential survival under the circumstances that prevailed when today's centenarians were children and young, and where notably infectious diseases and nutritional disorders took a large toll, so that the distribution in the centenarians' birth cohort when they reached adulthood may have differed from the distribution we observe in adults today. However, since the middle-aged people today are the centenarians' grandchildren, the differences obviously cannot have been substantial (unless we imagine a complex scenario which also includes differences in fertility).

6. APOE genotype and acute coronary syndrome

Summary of the chapter

Many studies have aimed at determining differences in risk of cardiovascular disease among APOE genotypes, but the findings vary considerably. This may reflect a variable influence of APOE genotype on risk due to e.g. gene-by-environment interactions as illustrated in a simulation study. Varying findings may also be due to study design characteristics. An overview of studies of APOE and acute coronary syndrome suggests that studies with proper matching of cases and controls by age and recruitment of incident cases show higher relative risks for APOE*4 carriers than found in other studies, and the overview also points to an increased relative risk in APOE*2 carriers. The influence of estimated relative risks of recruiting either prevalent or incident cases could relate to different risks of thrombotic events among APOE genotypes, and an influence of APOE genotype on prognosis. We examined the latter in a follow-up study of survivors of myocardial infarction who participated in the Scandinavian Simvastatin Survival Study and found that APOE*4 carriers had a nearly two-fold higher risk of dying compared with other patients. We also found that a high plasma Lp(a) level was independently associated with a worse prognosis, so that APOE*4 and that Lp(a) can be combined to define a subgroup of patients with a nearly four-fold increased of dying following a myocardial infarction. The excess mortality in APOE*4 carriers (including those with high Lp(a) levels) could be abolished by treatment with simvastatin, but other findings in the study nevertheless indicate that the effect of APOE*4 on prognosis may be unrelated to plasma lipoprotein metabolism. A summary model suggesting several mechanisms for the effect of APOE polymorphism on risk of acute coronary syndrome is presented.

Why has it been so difficult to assess the effect of APOE polymorphism on risk?

The association of the common APOE polymorphism with a 'normal variation' in plasma lipoprotein levels led to the obvious speculation in the 1980s that APOE*4 is a genetic risk factor for atherosclerosis and clinical cardiovascular disease in humans, and conversely, that APOE*2 is protective. One could perhaps also expect that it would be relatively easy to settle this matter, using epidemiological studies of various designs. However, the extensive literature on the subject that has emerged during the past two decades tells a different story. Notwithstanding that some studies stand out with seemingly strong evidence suggesting that APOE*4 is indeed a risk factor and APOE*2 offers protection (and are frequently cited for that reason), the published results at large show very considerable variation.

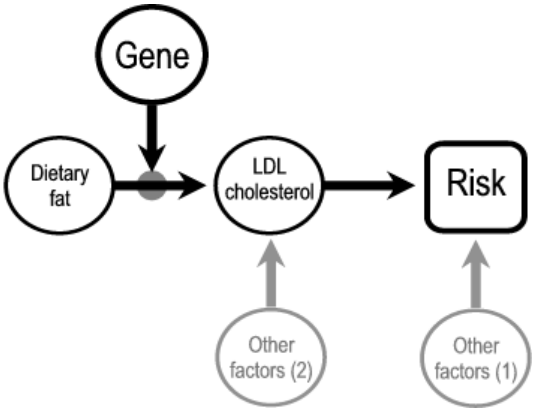
There are two likely explanations for this. One is that APOE has a variable influence on risk. The other has to do with the characteristics of the studies on the subject.

APOE has a variable influence on cardiovascular risk

The influence of the APOE gene on cardiovascular risk, measured as differences or ratios between the genotype-specific incidence rates etc., may not be universally invariant, but subject to modification by gene-gene interactions (ethnicity), genotype-by-environment interactions, and by gender, age, body size and other traits that characterize an individual's overall 'biological status' at a given time. With reference to the variable influence of the APOE polymorphism on plasma lipid levels conditional on e.g. ethnicity, gender and age (see page 15), one would actually *expect* studies conducted in different target populations to obtain quantitatively different results for e.g. estimated genotype-specific relative risks of disease.

This was illustrated in a simulation study based on a simple population model **[Gerdes, 1995]**. The model described in the original paper is a little more complex, but the essential structure is shown in Figure 13:

Figure 13. Simulation model – see text for explanation



The distribution of risk for coronary heart disease in the populations was modelled as a function of the distribution of serum LDL cholesterol and the distribution of a sum of other factors (unmeasured risk factors, e.g. proportion of smokers). The distribution of LDL cholesterol was in turn modelled as a function of the distribution of dietary fat intake and the distribution of a sum of other factors (e.g. proportion of obese individuals). The effect of a putative genetic polymorphism defining only two genotypes ("Gene" in Figure 13) was modelled as a modifier of the effect of dietary fat on LDL cholesterol, so that those with one genotype have higher mean LDL cholesterol on a given dietary fat intake than those with the other genotype (e.g. as might be the case for APOE*4 carriers compared to non-APOE*4 carriers).

The model generated cases from the upper end of the distribution of risk and calculated the relative risk (incidence density ratio) in those with the one genotype compared to those with other genotype. The parameters of the model were fitted so that the total incidence density of cases was held within reasonable limits (e.g. less than 250 per 1000 person-years).

Simulations showed that although individuals with the one genotype were "doomed" to have higher mean LDL cholesterol than other individuals, the calculated relative risk for the genotype was surprisingly alterable. Thus, the relative risk could be made to increase from about 1 (i.e. indicating no excess risk) to about 3 by increasing the mean dietary fat intake, or could be made to decrease from about 3 to about 1 by increasing the population load of risk factors not related to dietary fat intake and LDL cholesterol (i.e. "Other factors (1)" in Figure 13).

One may question the value of mathematical models and simulated scenarios of this kind, since models are crude by nature (no matter how elaborated), and the obtained results strongly dependens on the premises and parameters they are fed with. However, in my experience, working with such models can give a basic understanding of the importance of the many sources of variability that can be met with in genetic epidemiological studies of common multifactorial diseases, and can help to recognize that it is virtually impossible to describe "an independent effect" of a gene in this context.

Incidentally, a recent study of interactions between APOE genotypes and other factors in determining the risk of dying from coronary heart disease in two cohorts of Finnish men shows that predictions of the kind made with the above model are realistic. For instance, in one of the cohorts, the relative risk in men with APOE 3-4 compared to APOE 3-3 was seemingly increased in lean men, but decreased in obese men [Stengård et al., 1999].

The characteristics of studies vary in important ways

The other explanation for the variable results in the literature could relate to variability in study design characteristics including properties that have a bearing on the quality of a study. The used designs are dissimilar in practically all aspects including type (e.g. cohort, case-referent or case-control studies), and many studies are of questionable quality with regard to their real informative potential. For instance, the kind of studies where APOE genotypes are determined in accessible blood samples from groups of loosely specified and superficially characterised 'patients with coronary heart disease' and 'healthy controls' of different origins, may not give results that contribute much to clarification.

The impact of two distinct study design characteristics is illustrated in following section. The overview is restricted to studies of patients with acute coronary syndrome, because this is a distinct clinical entity that usually forces itself to be recognized and whose occurrence can be dated with reasonable accuracy. This excludes many studies of patients with various other (and often mixed) clinical manifestations of coronary heart disease [Miida, 1990; Katzel et al., 1993; Miettinen et al., 1994; Kuusisto et al., 1995; Nakata et al., 1996; Corbo et al., 1997; Weber et al., 1997; Biggart et al., 1998; Shen et al., 1998; Benes et al., 2000; Frikke-Schmidt et al., 2000a], including two studies that are frequently cited for showing that APOE*4 is associated with an increased risk [Eto et al., 1989; Wilson et al., 1994], as well as a number of studies involving only patients with diabetes mellitus [de Tommaso et al., 1991; Laakso et al., 1991; Ukkola et al., 1993; Koistinen et al., 1994; Boemi et al., 1995] or patients with familial hypercholesterolemia [Eto et al., 1988; Yamamura et al., 1990; Gylling et al., 1991; Hill et al., 1991; Hansen et al., 1994b]. Results from studies on angiographically defined coronary artery disease were briefly reviewed on page 19.

An overview of studies of APOE genotypes and acute coronary syndrome

A total of 29 different data sets were analysed (see Appendix II on page 56; data available in 1994 were includied in a meta-analysis presented at the 3rd annual meeting of the International Genetic Epidemiology Society, Paris 1994). They originate from the following sources:

I Two cohorts with follow-up for coronary death [Stengård et al., 1995].

II Two case-referent studies nested within cohort studies considering myocardial infarction and coronary death [Mänttäri et al., 1991; Eichner et al., 1993]

III Six case-referent studies [Luc et al., 1994; Scaglione et al., 1999](and unpublished data from a Danish study).

IV Four case-control studies with matching by age and gender [Peacock et al., 1992; Wilson et al., 1993; Joven et al., 1998; Corbo et al., 1999]; a fifth published study could not be included because details on APOE genotypes were missing [Inbal et al., 1999].

V 15 case-control sets without matching (studies where controls had been sampled independently of the cases, or participants in population studies etc. were used as controls) [Cumming and Robertson, 1984; Utermann et al., 1984; Lenzen et al., 1986; Shiina, 1989; Yamamura et al., 1990; Krantz et al., 1990; Köhler et al., 1992; Eggertsen et al., 1994; Lehtinen et al., 1995; Nakai et al., 1998](Gerdes LU; data from the Danish participants in the 4S compared with data from 40-years old men). Four sets included myocardial infarction patients who had also angiographically defined coronary artery disease [Lenzen et al., 1986; Köhler et al., 1992; Lehtinen et al., 1995].

The following characteristics were recorded for each data set: (A) Young cases = 1 if all cases were ≤ 60 years of age; (B) Proportion of men; (C) Incident cases = 1 if cases originated from cohort studies (i.e in I and II above) or were recruited on admission for myocardial infarction, in contrast to patients (survivors) who had been recruited months to years after an incident; (D) Selected cases = 1 if cases were selected on criteria including plasma lipid levels (and thus possibly also APOE genotype); (E) Matched controls = 1 if controls were specifically matched to cases by gender and age (i.e. in I to IV above); (F) High-low age mismatch = 1 if Young cases = 0 and the mean age of the cases was ≥ 10 years higher than the mean age of the controls; (G) Selected controls = 1 if controls were selected on criteria including plasma lipid levels; (H) Proportion of APOE*4 carriers among controls; and (I) Proportion of APOE*2 carriers among controls.

Data analyses and results

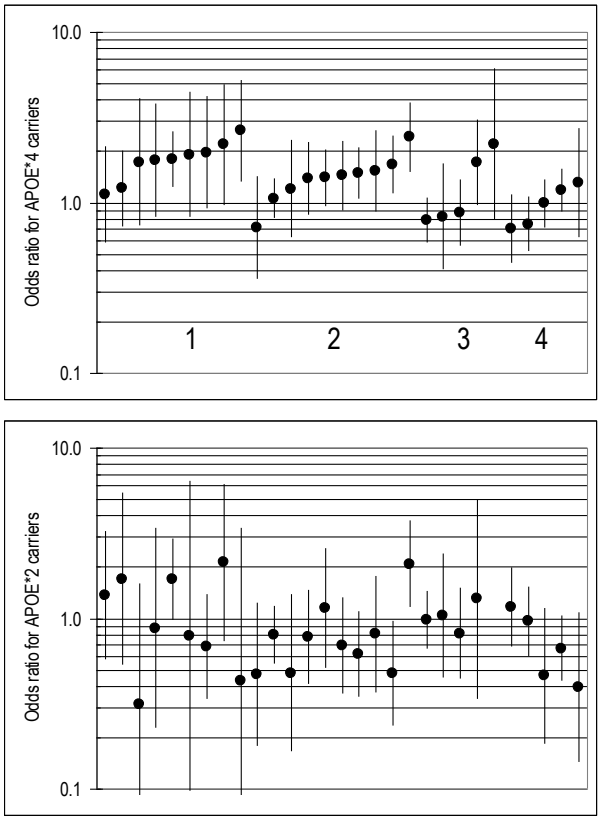
For each case-control set, odds ratios (OR) with 95% confidence intervals for APOE*4 carriers (genotypes APOE 4-4, 3-4 and 2-4) and for APOE*2 carriers (genotypes 2-2 and 2-3) were calculated with APOE 3-3 genotypes as the reference. General linear regression models (with and without weighting of the individuals studies by numbers of cases) were used to examine the potential impacts on the above study characteristics on the odds ratios (logarithmically transformed values).

For APOE*4 about one third (32%) of the total variation in odds ratios could be explained by three variables: (F) High-low age mismatch, (C) Incident cases, and (H) Proportion of APOE*4 carriers among controls. The coefficient for (F) indicates a significantly lower average odds ratio for studies where the cases included individuals over the age of 60 and the mean age of the cases was ≥ 10 years higher than the mean age of the controls (β= -0.35 for ln(OR); p=0.009). The coefficient for (C) indicates a higher odds ratio for studies of incident cases (β= 0.21 for ln(OR); p=0.086). And the coefficient for (H) indicates that the odds ratio is decreasing with increasing proportions of APOE*4 carriers among the controls (β= -0.016 for ln(OR) for a 1% change; p=0.116).

For APOE*2 one fifth (20%) of the total variation could be explained by (C) Incident cases and (I) Proportion of APOE*2 carriers among controls. The coefficient for (C) indicates a higher odds ratio for studies of incident cases (β=0.24 for ln(OR); p=0.146) and the coefficient for (I) indicates that the odds ratio is decreasing with increasing proportions of APOE*2 carriers among the controls (β= -0.047 for ln(OR) for a 1% change; p=0.031).

Figure 14 shows the odds ratios (with 95% confidence intervals) for the 29 data sets, sorted by the High-low age mismatch and Incidence cases variables, and the odds ratios for APOE*4 carriers:

Figure 14. Odds ratios for APOE*4 and APOE*2 in studies of APOE genotypes and acute coronary syndrome



1. The 9 sets to the left are those without age mismatch and with incident cases. The two low values are from a Scottish case-control study [Wilson et al., 1993] and a Finnish nested case-controls study [Mänttäri et al., 1991], respectively, whereas the highest value is from a Finnish cohort study (Southwest cohort) [Stengård et al., 1995].
2. The next 10 sets are those without age mismatch, but with prevalent cases. The two low values are from a French case-referent study in Lille [Luc et al., 1994] and a German study of angiographed myocardial infarction patients [Köhler et al., 1992], respectively, whereas the highest value is from a recent Spanish case-control study [Joven et al., 1998].
3. The next 5 are those with age mismatch and incident cases. The two high values are from two Japanese studies [Shiina, 1989; Yamamura et al., 1990].
4. The last 5 are those with age mismatch and prevalent cases.

The grand mean of the odds ratio for APOE*4 carriers is 1.3 (1.1 to 1.5) as evaluated for a proportion of APOE*4 of 24% among controls and using an unweighted analysis. The estimated marginal means for studies with and without age mismatch are 1.0 (0.9 to 1.3) and 1.5 (1.3 to 1.8), respectively, and for studies with incident cases and prevalent cases the estimated means are 1.4 (1.2 to 1.7) and 1.2 (1.0 to 1.4), respectively. By using the above 'meta-regression' coefficients for the impacts of age mismatch and time schedule for recruitment of cases, the predicted mean is 1.7 for studies without age mismatch and with incident cases. Incidentally, this value is identical to the pooled odds ratio obtained by using meta-analysis to summarise the 9 available data sets of this kind with a total of 867 cases and 1539 controls: 1.7 (1.4 to 2.1).

Possible explanations for the findings for APOE*4

It is not very surprising that estimated relative risks based on odds ratios tend to be biased towards the null value (i.e. 1) if cases are much older than the controls in a study. As shown previously (page 37) the APOE*4 frequency is declining with advancing aged in most populations, so that the APOE*4 frequency in young controls does not represent what it is supposed to represent, namely the estimated frequency in the population *at risk*. The frequency is overestimated, and a simple numerical example can illustrate the consequences of using such data. Suppose that the proportion of APOE*4 carriers among healthy young people is 30% and only 20% among healthy seventy-year-olds. Now, if APOE*4 is associated with a relative risk of myocardial infarction of about 1.7 in the elderly people, the proportion of APOE*4 carriers among elderly cases would be about 30%, i.e. at the level found in young healthy people. Hence, if these were used as controls, the false conclusion would be that APOE*4 is not a risk factor for myocardial infarction in the elderly. This source of bias can perceptibly also invalidate studies of other diseases including Alzheimer's disease [Jarvik, 1997]

The importance of recruiting either incident cases or prevalent cases for a study of APOE genotypes and risk of coronary heart disease may relate to particular malignant courses of the disease in APOE*4 carriers. Some studies suggest that APOE*4 carriers are particularly prone to develop severe or disseminated coronary lesions [Köhler et al., 1992; Lehtinen et al., 1995; Wang et al., 1995]. This, and possibly also an increased propensity to coronary thromboses (see page 45), may cause APOE*4 carriers to have a higher risk of dying from a heart attack either immediately [Eichner et al., 1993] or during the following years **[Gerdes et al., 2000a]** (see page 45). Clearly, the consequence would be that the proportion of APOE*4 carriers is smaller among prevalent cases with coronary heart disease (e.g. patients attending outpatient clinics) than among incident cases (e.g. patients admitted to a intensive care unit), so that the estimated relative risk of acute coronary syndrome is underestimated if prevalent cases are used in a study. The bias can be particularly important in studies of patient subgroups with an overall high mortality rate (e.g. older patients).

The tendency for the odds ratio for APOE*4 carriers to decrease with an increasing proportion of APOE*4 carriers among controls could reflect true differences in the relative risks across populations (e.g. a higher relative risk in Southern Europeans than in Northern Europeans). However, the tendency can also be a numerical artefact, which is inflated because many studies are relatively small.

What is the effect of APOE*2 on coronary risk?

As seen in Figure 14 most odds ratios for APOE*2 carriers are below 1 (indicating protection) and this is also apparent in overviews of studies of other clinical manifestations of coronary heart disease and in studies of angiographically defined coronary heart disease (not shown). However, there are several studies suggesting the opposite including some well designed, larger studies [Mänttäri et al., 1991; Eichner et al., 1993; Eto et al., 1989; Wilson et al., 1994]. These discrepancies are curious, but may in some cases relate to differences between the effects of APO*2 in men and women [Wilson et al., 1994; Frikke-Schmidt et al., 2000a], and possibly also different ages of the patients considered [Moore et al., 1997].

It may also relate to differences in the effects of APOE 2-2 and 2-3, and in particular the fact the few percent of APOE*2 homozygotes who develop dysbetalipoproteinemia very often have premature or accelerated atherosclerosis and a high risk of coronary heart disease [Mahley and Rall, 1995], and therefore tend to be relatively frequent in case-control studies [Utermann et al., 1984; Frikke-Schmidt et al., 2000a].

ApoE and thrombosis

Could the higher relative risk of acute coronary syndrome and the worse prognosis in APOE*4 carriers relate to an increased propensity to coronary thromboses, or possibly more acutely propagating thromboses? To my knowledge, the subject has never been directly addressed in any study, but substantial indirect evidence suggests that apoE is involved in central biological processes and that the APOE polymorphism could be important:

- Phospholipid vesicles with apoE suppress platelet agonist induced aggregation by binding to apoER2 receptors (LRP8) and activating the L-arginine:nitic oxide pathway [Riddell et al., 1997; Riddell et al., 1999]. The suppression is dose-dependent and probably does not involve receptor-mediated endocytosis of apoE and lipids, but a signal transmission involving the cytoplasmic tail of the apoE2R. All members of the LDLR family have a asparagine-proline-x-tyrosine motif (NPxY; where x is any amino acid) which binds and interacts with intracellular signaling components [Willnow et al., 1999; Herz et al., 2000].
- Equal concentrations of apoE2, apoE3 and apoE4 in phospholipid vesicles inhibited platelet aggregation to a similar degree *in vitro* [Riddell et al., 1999], but if HDL with apoE are involved *in vivo* [Desai et al., 1989b; Desai et al., 1989a; Higashihara et al., 1991], then suppression (or regulation) of platelet aggregation may be less effective in APOE*4 carriers, because they have HDL particles with less apoE than other individuals (see page 15).
- The ability of HDL with apoE to enhance HSPG formation in endothelial cells is associated with increased amounts of luminal antithrombin [Paka et al., 1999]. Again, one could surmise a relative disadvantage in APOE*4 carriers in this regard, which, in concert with other unfavorable local circumstances, could increase the likelihood of having a propagating arterial thrombus.
- Plasma lipoproteins bind several coagulation factors and inhibitors, and recent a study of postprandial responses in patients with hyperlipidemia showed that activated factor VII increases with increasing triglycerides. This was particularly evident in APOE*2 carriers and may possibly increase the risk of acute thrombotic events in these individuals [Nordøy et al., 2000].
- Vitamin K is important for γ-carboxylation of glutamylresidues in prothrombin, factors VII, IX and X, and protein C and S. The highly lipophilic vitamin is transported with lipoproteins and the effect of APOE polymorphism on lipoprotein metabolism also influences vitamin K concentrations in plasma. Thus, the concentrations are higher in APOE*2 carriers than in individuals with the APOE 3-3 genotype, and lower in APOE*4 carriers [Saupe et al., 1993; Kohlmeier et al., 1996].
- These differences appear to have functional links, since (1) there are corresponding differences between APOE genotypes in plasma hydroxylapatite-binding capacities of osteocalcin (another protein whose activity is dependent on vitamin K for γ-carboxylation) [Saupe et al., 1993; Kohlmeier et al., 1996], and (2) APOE*4 carriers have lower mean plasma prothrombin than other individuals when treated with similar doses of vitamin K antagonists [Kohlmeier et al., 1995]. This indicates that APOE*4 carriers may have a reduced propensity to thrombosis, but the opposite may be the case in untreated patients if the reduced availability of vitamin K affects the production of the inhibitors protein C and S.

Thus, apoE most probably has some regulatory effects on the haemostatic system, but differential effects of the genetic isoforms are still only speculative.

APOE genotypes and survival after myocardial infarction

If APOE*4 carriers are particularly prone to develop disseminated or severe coronary lesions as some studies suggest [Köhler et al., 1992; Lehtinen et al., 1995; Wang et al., 1995] and have a particular high risk of fatal primary events of coronary heart disease [Eichner et al., 1993], the allele could possibly also have an adverse influence on the prognosis in survivors of myocardial infarction.

We examined this matter by determining the APOE genotypes in a subset of Danish and Finnish participants in the Scandinavian Simvastatin Survival Study — a secondary intervention trial with a median follow-up period of 5.4 years of men and women aged 35 to 70 years with a history of myocardial infarction or angina. Additionally, we examined if the benefit of treatment with simvastatin differed between APOE*4 carriers and other patients **[Gerdes et al., 2000a]**.

Patients with and without APOE*4 did not differ with respect to baseline characteristics including distributions by nationality, gender and age groups, frequencies of concurrent diseases, smoking habits and mean plasma lipid and apolipoprotein levels.

APOE genotype and prognosis in patients on placebo

Four-hundred-seventy-eight patients were treated with placebo and 166 of these were APOE*4 carriers.

Figure 15 shows the Kaplan-Meier survival curves for patients with and without APOE*4. Sixteen percent of the APOE*4 carriers died during follow-up, compared to only 9% of other patients.

The crude hazard (risk) ratio for APOE*4 was 1.8 (1.1 to 3.1), and as shown in Table 7, a similar estimate was obtained in a multivariable analysis.

Variables in the multivariable models emerged from backward selections with either death, coronary death or a major coronary event (MCE) as outcome. The APOE*4 allele, male sex, age > 60 years, concurrent angina, diabetes and high Lp(a) were all associated with total mortality risk ratios > 1.5, whereas the effects of smoking and high LDL cholesterol were smaller and not statistically significant. The same pattern was seen for coronary deaths, except that male sex and age seemed to be less important and diabetes and smoking more important. Risk ratios for a major coronary event were generally lower than for death, except for high LDL cholesterol. Only this trait and male sex and diabetes were statistically significant predictors of a MCE.

Figure 15. Kaplan-Meier curves for all-cause mortality in myocardial infarction patients with and without APOE*4 who were treated with placebo

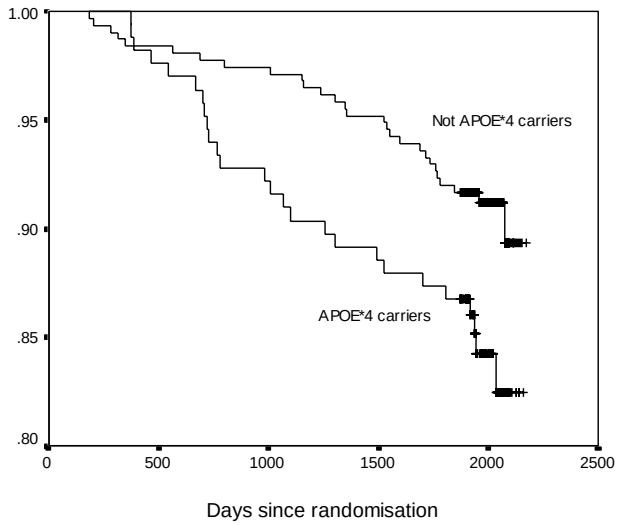


Table 7. Results of multivariate Cox regression analysis of data from patients treated with placebo: estimated relative hazards (with 95% confidence intervals) associated with various outcomes

Variable	Death (all causes)	Coronary death	Major coronary event
APOE*4	1.9 (1.1 to 3.3)	1.7 (0.9 to 3.2)	1.1 (0.8 to 1.6)
Male sex	2.3 (1.0 to 5.5)	1.4 (0.6 to 3.4)	1.9 (1.1 to 3.3)
Age 60-70 years	2.8 (1.5 to 5.1)	2.1 (1.0 to 4.2)	0.9 (0.6 to 1.2)
Angina	1.8 (1.1 to 3.2)	1.6 (0.8 to 3.2)	1.3 (0.9 to 1.9)
Diabetes	3.5 (1.6 to 7.5)	4.1 (1.7 to 9.9)	2.2 (1.2 to 4.0)
Smoker	1.5 (0.9 to 2.7)	1.9 (1.0 to 3.8)	1.2 (0.9 to 1.8)
LDL > 4.75 mmol/L	1.1 (0.7 to 2.0)	0.9 (0.5 to 1.8)	1.4 (1.0 to 2.0)
Lp(a) > 30 mg/dL	2.0 (1.1 to 3.4)	2.0 (1.0 to 3.8)	1.3 (0.9 to 1.8)

Combined effect of APOE genotype and Lp(a) level

Serum Lp(a) above 30 mg/dL was associated with a two-fold increased mortality risk, and it appeared that the presence or absence of APOE*4 and a high or low Lp(a) could be combined to define three groups of patients with markedly different risks of death when treated with placebo:

Compared to patients without APOE*4 and Lp(a) ≤ 30 mg/dL (42% of the patients),

- those with either APOE*4 or a high Lp(a) level (45% of the patients) had a relative risk of 2.3 (1.1 to 4.5), and
- those with both APOE*4 and a high Lp(a) level (13% of the patients) had a relative risk of 3.7 (1.7 to 8.1).

Similarly, one could define a still smaller subgroup of patients who were also over the age of 60 years (6%), whose relative risk was 8.4 (2.8 to 25.1; there were 26 patients of which 10 (39%) died (Gerdes LU, presented at Genetics and Atherosclerosis Satellite Symposium of the XIIth International Symposium on Atherosclerosis, Aarhus 2000).

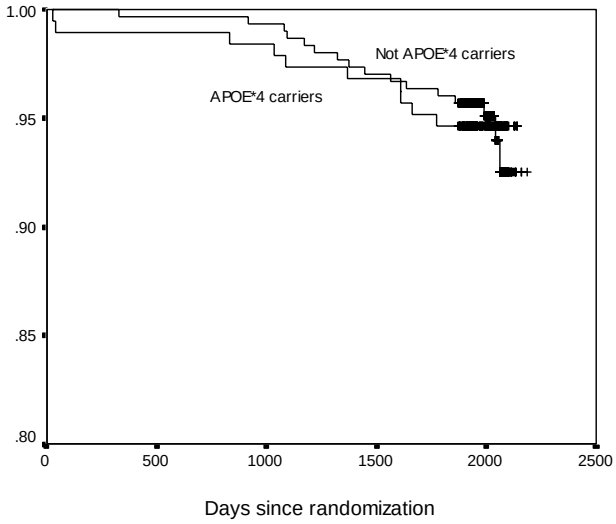
Importantly, one should consider if the effects of APOE genotype and Lp(a) level on risk are truly independent. This will *not* be the case if APOE genotype determines Lp(a) level. A Dutch study of 303 healthy middle-aged men and women suggested this — that APOE*2 is associated with lower Lp(a) levels and APOE*4 with higher levels compared to individuals with APOE 3-3 [de Knijff et al., 1991]. A similar association was also observed in a large Danish study, but only in women [Frikke-Schmidt et al., 2000b].

Nevertheless, Lp(a) levels did not differ between APOE genotypes within this study and a recent overview of published data on the subject also failed to find general support for an association [Klausen, 2000]. The data included results from a study of 40-years old Danish men which is particularly informative in this case because it considered the strong effect of the apo(a) gene size polymorphism on Lp(a) levels **[Klausen et al., 1996]**. It permitted an analysis of the effect of APOE genotype on the relatively high Lp(a) levels in individuals with small apo(a) phenotypes, i.e. carriers of small KpnI alleles at the apo(a) gene [Lemming et al., 1997]. It appeared that APOE*2 carriers in this subgroup of men had lower Lp(a) compared to men with APOE 3-3, whereas the level in APOE*4 carriers was the same. Hence, the effect of APOE genotype on risk may have some relation to Lp(a) level, although it will be of very little quantitative importance in comparisons of APOE*4 carriers and individuals with other genotypes.

APOE genotype and effect of simvastatin on mortality hazard

The survival of treated patients with and without APOE*4 was nearly the same (Figure 16). Comparisons with the data in Figure 11 therefore suggest that treatment is particularly beneficial for APOE*4 carriers. This concept was supported by different relative mortality hazard ratios due to treatment. Hence, whereas the ratio for all myocardial infarction patients was 0.49 (0.31 to 0.79; adjusted for the covariates shown in Table 7) it was 0.66 (0.35 to 1.24) in patients without APOE*4 and only 0.33 (0.16 to 0.69) in patients with APOE*4.

Figure 16. Kaplan-Meier curves for all-cause mortality in myocardial infarction patients with and without APOE*4 who were treated with simvastatin



Interestingly, the subgroup of patients with APOE*4 and a high Lp(a) level (13% of the patients) had an even greater benefit from the treatment; the mortality hazard ratio due to treatment was reduced to 0.22 (0.06 to 0.77). The results were even more impressing for the smaller subgroup of patients (6%) who were also over the age of 60 years (see above). There were 35 of the patients in the simvastatin group and none of them died during the follow-up period! (Gerdes LU, presented at Genetics and Atherosclerosis Satellite Symposium of the XIIth International Symposium on Atherosclerosis, Aarhus 2000).

Is the effect of APOE*4 on prognosis unrelated to plasma lipoprotein metabolism?

The discordant findings for risks of fatal and nonfatal coronary events suggested that apoE is involved in some particularly malignant pathogenic mechanism that increased the risk of dying. As previously discussed, APOE*4 carriers seem particularly prone to develop disseminated coronary lesions [Köhler et al., 1992; Lehtinen et al., 1995; Wang et al., 1995] and to die from coronary heart disease [Eichner et al., 1993]. We speculated if APOE*4 is specifically associated with an increased progression rate of coronary lesions or the occurrence of vulnerable plaques, and if the ability of treatment with simvastatin to abolish the increased mortality risk in APOE*4 carriers relates to inhibition of progression of lesions or stabilization of plaques **[Gerdes et al., 2000a]**.

Naturally, our speculations were extended to consider the effect of APOE*4 on LDL cholesterol and triglyceride levels as an important element in this setting (see page 15). However, there were three observations, which suggested that the effect of APOE*4 may involve pathogenic mechanisms unrelated to serum lipoproteins:

- Baseline lipid levels did not differ between patients with and without APOE*4 in our study (probably due to truncation of the distributions of lipid variables in the recruitment phase).
- Inclusion of lipid variables in multivariable models did not influence the estimated mortality risk ratio in APOE*4 carriers.
- Finally, APOE*4 carriers and patients with other genotypes were equally responsive to simvastatin treatment in terms of LDL cholesterol lowering. We therefore suggested that apoE might be involved in pathogenic mechanisms related to coronary vascular reactivity or thrombosis (see page 45).

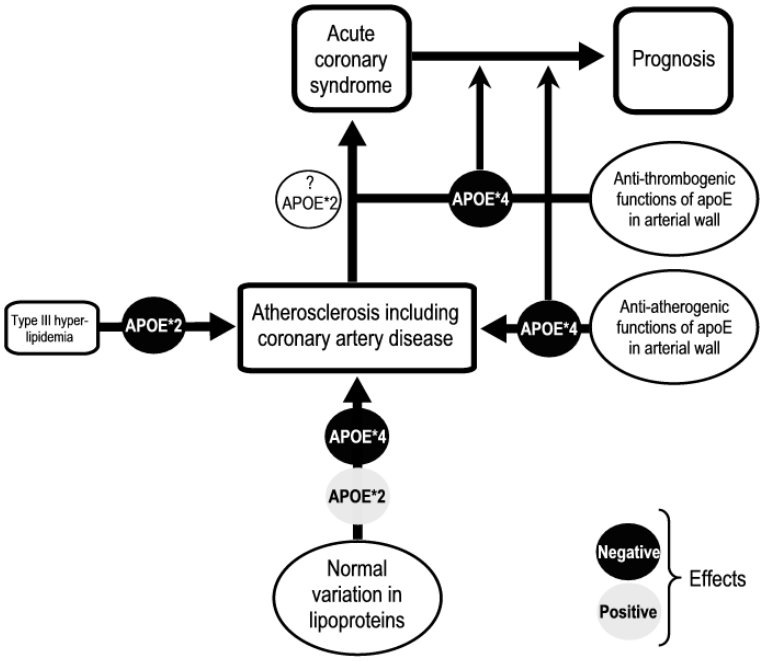
The observations that APOE*4 determines prognosis and the effect on prognosis of simvastatin treatment are exciting, since they demonstrate that molecular genetic information can be used to improve the clinical management of patients with myocardial infarction, e.g. by making decisions about prescriptions of medicine that are better tailored to individual patients.

More studies on the subject are clearly warranted, and the first results have appeared. A recently published follow-up study of 106 Italian myocardial infarction patients aged 23 to 45 years during about 4 years showed that APOE*4 was associated with an odds ratio of 6.8 (2 to 22) for a new coronary event including coronary death, myocardial infarction and coronary surgery or angioplasty [Brscic et al., 2000].

A summary model

As discussed in several places, the different biological functions of the apoE isoforms in processes related to atherosclerosis and thrombosis (page 13 ff and page 45), and the findings in various epidemiological studies (page 18 ff, page 42 ff and page 45 ff) suggest that the APOE polymorphism can influence the risk and prognosis of a coronary syndrome through several mechanisms. These influences are summarized in this figure:

Figure 17. Summary of the possible pathogenic mechanisms underlying the association of the APOE polymorphism with differential risk and prognosis of acute coronary syndrome.



7. APOE genotype and abdominal aortic aneurysm

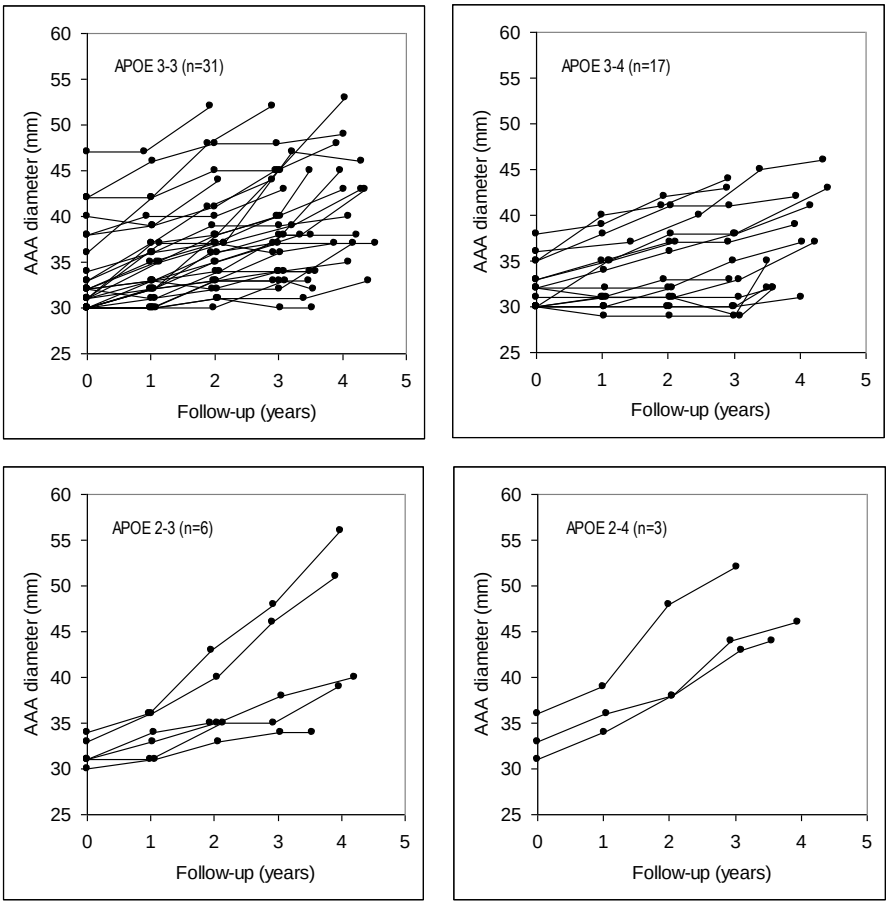
Summary of the chapter

The propensity to develop abdominal aortic aneurysms is associated with atherosclerosis and also tends to cluster in families. We examined whether APOE polymorphism could play a role for the expansion rate of aneurisms in elderly men with small aneurysms and had expected to find high rates in APO*4 carriers. However, those with APOE 3-4 appeared to have lower rates than men with other genotypes. This finding could be a result of bias, e.g. due to a relatively higher proportion of APOE*4 carriers having been referred to surgery, but it may also be real and further studies on this subject are warranted.

Abdominal aortic aneurysms (AAA) tend to cluster in families suggesting that there is a genetic component in the aetiology of the disease [Powell and Greenhalgh, 1987; Adams et al., 1993; Baird et al., 1995; Salo et al., 1999; Wilmink and Quick, 1998]. This putative genetic component could obviously relate to atherosclerosis and the above evidence suggests that APOE could be in play. The subject has never been addressed in the literature and we therefore determined APOE genotypes in 57 men aged 65-75 years with a small AAA, who had been followed with annual ultrasonographic re-examinations over 2 to 4.5 years. The specific aim of the study was to investigate if aneurysm expansion rates differed between APOE genotypes **[Gerdes et al., 2000d]**.

The crude results from this study are shown in Figure 18. Compared to men with the APOE 3-3 genotype (n=31) the mean expansion rate in those with APOE 3-4 (n=17) was significantly lower — 1.3 (0.7 to 1.9) mm/year versus 2.1 (1.7 to 2.6) mm/year (p=0.040). In contrast, the mean expansion rates appeared to be higher in the two small subgroups of men with APOE 2-3 (n=6) or APOE 2-4 (n=3) — 3.1 (2.0 to 4.1) mm/year (p=0.102) and 4.2 (2.7 to 5.6)

Figure 18. Expansion of abdominal aortic aneurysms in patients with different APOE genotypes



mm/year (p=0.009), respectively.

Owing to small numbers, one cannot attach much importance to the results for the APOE 2-3 and APOE 2-4 genotypes, whereas the results for the two larger genetic subgroups are more substantial; they indicate that patients with APOE 3-4 have a lower mean aneurysm expansion rate that APOE 3-3 genotypes. The opposite had been expected because APOE*4 tend be associated with more atherosclerotic lesions in the abdominal aorta (and elsewhere), and it was assumed that aneurysms would expand faster in patients with more advanced atherosclerosis. We discussed the possibility that the results have been influenced by bias **[Gerdes et al., 2000d]**. For instance, since about one third of the 191 men with an abdominal aortic aneurysm ≥ 30 mm identified in the screening programme either died or were referred for surgery before they completed at least two annual ultrasonographic examinations, the results could have been influence by selection bias if many of these “lost patients” had the APOE 3-4 genotype.

The findings can nevertheless be real. Aortic aneurysm expansion rate may not simply be a function of the extent of aortic atherosclerosis, but may depend on other processes in the aortic wall [Sterpetti et al., 1988; Sonesson et al., 1997]. We concluded that our preliminary findings suggested that the common polymorphism of APOE is associated with differential expansion rates of small abdominal aortic aneurysms, but that larger studies of the subject are warranted.

Summary

Apolipoprotein E (apoE) is a small glycoprotein with surprisingly many different biochemical functions. They include several important functions related to systemic long-distance transport of lipids. ApoE is particularly involved in the control of the heavy traffic of triglyceride-rich lipoproteins in blood, and play a prominent part in lipid transport in the cerebrospinal fluid. ApoE is also functioning as a local lipid carrier within tissues, and appears for instance to be an important participant in those processes that cells and tissues use to get rid of excessive cholesterol. These different functions of apoE are clearly important for the metabolism of triglycerides, cholesterol and phospholipids, but can also play a direct and indirect role for the metabolism of lipids and lipophilic molecules with special functions, e.g. the lipophilic vitamins.

The apoE molecule has a lipid-binding domain in one end, and a domain in the other end which both has high affinity to the omnipresent sulfated proteoglycans and to all members of the LDL receptor gene family. It is those properties that underlie apoE's central functions in lipoprotein metabolism, and possibly also play a role for some of the proteins other functions including its ability to stimulate and inhibit different types of cells. On the other hand, it may be other properties of the molecule that are important for some of apoE's functions in nervous tissue.

Very many polymorphisms in the APOE gene have been described, but most are rare or located in non-coding regions. However, there are two single nucleotide polymorphisms in a coding region (exon 4) that result in three common alleles. They are designated APOE*2, APOE*3 and APOE*4, and code for apoE isoforms whose amino acid sequences only differ in position 112 and 158 — apoE2 has cysteine in both positions, whereas apoE3 has cysteine and arginine, and apoE4 has arginine in both positions. Despite these modest differences in the primary structures, there are evident differences in the isoforms secondary and tertiary structures, and marked differences in their functional properties.

The APOE polymorphism is associated with differences in plasma lipoprotein metabolism and with differences in plasma concentrations of lipids and apolipoproteins. Compared with individuals with the most common genotype, APOE 3-3, carriers of APOE*4 (about 30% in the Danish population) have a more atherogenic lipoprotein profile, e.g. with a little higher average plasma concentration of LDL cholesterol, whereas APOE*2 carriers (about 15% in the Danish population) usually have a less atherogenic profile. Accordingly, there are many studies of different kinds suggesting that APOE*4 carriers have more atherosclerosis and an increased risk of clinical manifest cardiovascular disease — both coronary and cerebrovascular disease — whereas APOE*2 carriers have a lower prevalence of disease (although this is more uncertain). APOE*4 seems specifically associated with an increased risk of acute coronary syndrome, and it is also associated with a worse prognosis after myocardial infarction. However, the differences among APOE genotypes in disease risk and prognosis may also be related to other differences in the functional properties of the apoE isoforms, which mean, for instance, that a series of anti-atherogenic processes in the arterial walls are more or less effective, or which may involve the coagulation system. APOE*4 is associated with a markedly increased risk of developing Alzheimer's disease, whereas APOE*2 gives protection. The pathogenesis is related to particular functions of apoE in nervous tissue, and APOE*4 carriers generally seem to have a less robust central nervous system than individuals with other APOE genotypes. A number of recent studies suggest that the APOE polymorphism also has an effect on bone metabolism, and that APOE*4 carriers are particularly prone to have problems with osteoporosis and fractures. Moreover, some studies suggest an association between APOE*4 and risk of cancer, reduced fertility, incidence of certain chromosomal aberrations and increased susceptibility to certain infectious diseases.

All this may seem overwhelming and may surely leave the impression, that an individuals APOE genotype is a very important determinant of disease risk, and in particular, that the presence of an APOE*4 allele is a cause for serious concern. That is wrong! One has to realize, first, that the differences in disease risk among the APOE genotypes are relatively modest — e.g. under the level of the differences in smokers and nonsmokers if we speak of cardiovascular diseases — and, secondly, that the importance of a given APOE genotype depends of the context including environmental factors. It holds for all the associations with diseases studied so far, and the results for APOE are brilliant examples of the fact, that a close interaction between genetic and environmental factors is the rule rather than the exception. Finally, comparisons of the distributions of APOE genotypes in young and in very old people are reassuring — there *are* differences in the distributions corresponding to an increased mortality in APOE*4 carriers and a lower mortality in APOE*2 carriers over the years, but the differences are modest, however. And it is possible to live more that 105 years with an APOE*4 allel!

The frequencies of the three APOE alleles in aboriginal populations around the world show several interesting patterns. First, there is a conspicuous south-to-north APOE*4 gradient in Europe, with frequencies rising from 5-10% in the South to 20-30% in the North. The gradient may contribute to the south-to-north gradient in the burden of coronary heart disease on this continent, but only little. Secondly, APOE*2 is practically absent in Inuit and in

Americans, whereas the frequency of APOE*4 is high in Inuit and otherwise vary as much among Americans as among Europeans. Thirdly, the frequency of APOE*4 is low in Asia, but very high in Australians and in the peoples of New Guinea, where also the frequency of APOE*2 is high. Finally, the frequency of APOE*4 is high in Africans and notably in peoples such as Khoi San and Pygmies. That observation is one of several arguments for APOE*4 being the ancestral allele, although APOE*3 is much more common in the world today. This indicates that selection forces have played a role, and thus also may have contributed to the differences in the frequencies of the APOE alleles around the world. One can point to mechanisms regarding differences in the ability to absorb fats when food supplies are scarce, differences in susceptibility to infectious diseases, differences in fertility or in the efficiency of reproduction following the evolution of 'grandmothering' in humans, i.e. that the elderly participate in the upbringing of their grandchildren. There is also an interesting pattern in the frequency of APOE*4 in various places in the world, which suggests that this allele may protect against vitamin D deficiency, and thus that apoE is involved in the vitamin D metabolism.

This is clearly a subject that should be explored, and I will also point to the influence of the APOE polymorphism on the functions of the coagulation system as a field that should be examined more closely. On the other hand, I will not be the least surprised, if it appears that apoE is also involved elsewhere.

Dansk resumé

Apolipoprotein E (apoE) er et lille glykoprotein med forbavsende mange forskellige biokemiske funktioner. De omfatter flere vigtige funktioner i relation til systemisk fjerntransport af lipider, hvor apoE især er involveret i styringen af den tunge trafik af triglyceridholdige lipoproteiner i blodbanen, og har en fremtrædende rolle i lipidtransporten i cerebrospinalvæsken. ApoE fungerer også som lokal lipidtransportør i vævene, og synes bl.a. at være en vigtig deltager i de processer der skaffer celler og væv af med overskud af kolesterol. Disse forskellige funktioner af apoE er selvsagt vigtige for omsætningen af triglycerider, kolesterol og fosfolipider, men kan også spille en direkte og indirekte rolle for omsætningen af lipider og lipofile molekyler med specielle funktioner, fx de lipofile vitaminer.

ApoE molekylet har et lipidbindende domæne i den ene ende og et domæne i den anden ende, som både har en høj affinitet til de allestedsnærværende sulfatede proteoglykaner og til alle medlemmer af LDL receptor gen familien. Det er disse egenskaber der ligger til grund for apoE's centrale funktioner i lipoproteinstofskiftet, og som muligvis også spiller en rolle for nogle af proteinets andre funktioner, herunder evnen til at stimulere og hæmme forskellige celletyper. Derimod kan der være andre egenskaber ved molekylet, som er vigtige for nogle af apoE's funktioner i nervevæv.

Der er beskrevet mange polymorfier i APOE genet, men de fleste er sjældne eller lokaliseret i ikke-kodende regioner. Der er imidlertid to enkelt-base polymorfier i en kodende region (exon 4), som resulterer i tre almindeligt forekommende alleler. De kaldes APOE*2, APOE*3 og APOE*4, og koder for apoE isoformer hvis aminosyresekvenser kun er forskellige i position 112 og 158 — apoE2 har cystein i begge positioner, mens apoE3 har henholdsvis cystein og arginin, og apoE4 har arginin i begge positioner. Trods disse beskedne forskelle i primærstrukturen, er der tydelige forskelle i isoformernes sekundær- og tertiærstrukturen, og markante forskelle i deres funktionelle egenskaber.

APOE polymorfismen er associeret med forskelle i plasmalipoproteinernes omsætning og med forskelle i plasmakoncentrationerne af lipider og apolipoproteiner. Sammenlignet med individer med den almindeligste genotype, APOE 3-3, har bærere af APOE*4 (omkring 30% i den danske befolkning), en mere aterogen lipoproteinprofil, fx med en lidt højere gennemsnitlig plasmakoncentration af LDL-kolesterol, mens APOE*2 bærere (omkring 15% i den danske befolkning) almindeligvis har en mindre aterogen profil. Der er da også mange studier af forskellig art, som peger på, at APOE*4 bærere har en øget forekomst af aterosklerose og øget risiko for klinisk manifest kardiovaskulær sygdom — både koronar og cerebrovaskulær sygdom — mens APOE*2 bærere har en lavere forekomst af sygdom (skønt dette er lidt mere usikkert). APOE*4 synes især knyttet til en øget risiko for akut koronarsyndrom, og er også forbundet med en dårligere prognose efter et myokardieinfarkt. Forskellene mellem APOE genotyper i sygdomsrisiko og prognose kan imidlertid også være knyttet til andre forskelle i apoE isoformernes funktionelle egenskaber, som fx betyder, at en række anti-aterogene processer i arterievæggene er mere eller mindre effektive, eller som kan involvere koagulationssystemet. APOE*4 er forbundet med en klart øget risiko for at udvikle Alzheimers sygdom, mens APOE*2 yder beskyttelse. Patogenesen er knyttet til apoE's særlige funktioner i nervevæv, og bærere af APOE*4 allelet synes i det hele taget at have et mindre robust centralnervesystem end individer med andre APOE genotyper. En række studier fra de seneste år peger på at APOE polymorfismen også har en effekt på knoglemetabolismen, og at det især er bærere af APOE*4 der udvikler problemer med osteoporose og knoglebrud. Enkelte studier har endvidere antydet at der kan være en sammenhæng mellem APOE*4 og risiko for cancer, nedsat fertilitet, forekomst af visse kromosomfejl og øget modtagelighed for visse infektionssygdomme.

Alt dette kan virke meget overvældende og kan givetvis også efterlade det indtryk, at et individs APOE genotype er en meget betydningsfuld determinant for sygdomsrisiko, og især, at tilstedeværelsen af et APOE*4 allel må give anledning til alvorlig bekymring. Det er forkert! Man skal være klar over, for det første, at de forskelle i sygdomsrisiko der findes mellem APOE genotyperne er forholdsvis beskedne — fx på et niveau lidt under forskellene mellem at være ryger og ikke-ryger hvis vi taler om kardiovaskulære sygdomme — og for det andet, at betydningen af en given APOE genotype afhænger af sammenhængen, herunder miljømæssige faktorer. Det gælder alle de associationer til sygdomme der hidtil er studeret, og resultaterne for APOE er fornemme eksempler på det faktum, at tætte samspil mellem genetiske og miljømæssige faktorer er reglen, snarere end undtagelsen. Endeligt er sammenligninger af APOE genotypefordelingerne blandt unge og meget gamle mennesker beroligende — der *er* forskelle i fordelingerne, som svarer til en øget dødelighed blandt APOE*4 bærere og en lavere dødelighed blandt APOE*2 bærere over årene, men forskellene er beskedne, trods alt. Og det er muligt at leve mere end 105 år gammel med et APOE*4 allel.

Hyppighederne af de tre APOE alleler i oprindelige befolkninger rundt omkring i verden viser flere interessante mønstre. For det første er der en påfaldende syd-til-nord gradient af APOE*4 i Europa, med hyppigheder der stiger fra 5-10% i syd til 20-30% i nord. Gradienten kan bidrage til syd-til-nord gradienten i forekomsten af koronarsygdom på dette kontinent, men kun lidt. For det andet er APOE*2 praktisk taget fraværende hos Inuit og i Amerika, mens hyppigheden af APOE*4 er høj hos Inuit og ellers varierer ligeså meget blandt amerikanere som blandt europæere.

For det tredje er hyppigheden af APOE*4 lav i Asien, på omkring samme niveau som i Sydeuropa, men meget høj hos australiere og i befolkningen på Ny Guinea, hvor også hyppigheden af APOE*2 er høj. Endeligt er hyppigheden af APOE*4 høj blandt afrikanere og især blandt folk som fx Khoi San og Pygmæer. Denne observation er et af flere argumenter for at APOE*4 må være det oprindelige allel, selvom APOE*3 er langt hyppigere forekommende i verden i dag. Dette tyder på at selektionskræfter har spillet ind, og følgelig også kan have bidraget til forskellene i hyppighederne af APOE allelerne rundt omkring i verden. Man kan pege på mekanismer der vedrører forskelle i evnen til at optage fedtstoffer når mængden af føde er begrænset, forskelle i modtagelighed for infektioner, forskelle i fertilitet eller i den reproduktiv effektivitet der knytter sig udvikling af 'grandmothering' hos mennesker, dvs. at de ældre deltager i opdragelsen af deres børnebørn. Der tegner sig også et interessant mønster i hyppigheden af APOE*4 forskellige steder i verden, som kunne tyde på, at dette allel beskytter mod udvikling af vitamin D mangel, og altså at apoE er involveret i vitamin D metabolismen.

Dette er selvsagt et emne som bør udforskes, og jeg vil også pege på APOE polymorfiens betydning for koagulationsystemets funktioner, som et område der bør undersøges nærmere. Omvendt vil jeg ikke blive spor overrasket, hvis det viser sig at apoE også er involveret andre steder.

Appendices

Appendix I. Data on APOE genotypes in Europe

The table does not include studies (or substudies), which specifically selected people over the age of 65 years.

Nation	Region (city)	Size	Ages	Men	Source	Method	APOE*2	APOE*3	APOE*4	Reference
-	Tyrol	469	NA	NA	Blood bank	Phenotyping	9.0%	79.2%	11.8%	[Hallman et al., 1991]
Belgium	Ducth speaking part	760	35-59	100%	Working place study	Phenotyping	7.2%	76.5%	16.3%	[Braeckman et al., 1996]
Belgium	Luxembourg	782	Newborns	NA	Screening programme	Genotyping	7.8%	77.0%	15.2%	Descamps (abstract, 2000)
Cyprus	West (Greek part)	335	16-65	46%	Thallasssaemia Referral Center	Genotyping	5.4%	87.6%	7.0%	[Cariolou et al., 1995]
Czech Republic	Brno	222	avg 48	100%	Healthy volunteers	Genotyping	7.2%	82.7%	10.1%	[Benes et al., 2000]
Denmark	Jutland (Aarhus)	466	40	100%	Population study	Phenotyping	8.5%	74.1%	17.4%	[Gerdes et al., 1992]
Denmark	Seeland (Copenhagen)	9241	20-80	45%	Population study	Genotyping	8.1%	75.3%	16.6%	[Frikke-Schmidt et al., 2000]
Finland	Kuopio, Oulu	729	9-24	50%	Population study	Phenotyping	3.5%	78.1%	18.4%	[Lehtimäki et al., 1990]
Finland	North Karelia	111	30-50	NA	Dietary intervention study	Phenotyping	5.9%	67.6%	26.6%	[Tikkanen et al., 1990]
Finland	Oulu (Oulu)	124	21-39	57%	Hospital staff and other volunteers	Phenotyping	4.4%	75.8%	19.8%	[Kervinen et al., 1994]
Finland	Oulu (Oulu)	136	40-64	63%	Hospital staff and other volunteers	Phenotyping	3.3%	74.6%	22.1%	[Kervinen et al., 1994]
Finland	Saarni in Northern parts	69	23-85	100%	Heath survey	Phenotyping	5.0%	64.0%	31.0%	[Lehtinen et al., 1994]
Finland	Tampere, Turku	482	9-24	50%	Population study	Phenotyping	4.5%	75.3%	20.2%	[Lehtimäki et al., 1990]
Finland	Uudenmaan (Helsinki)	366	9-24	50%	Population study	Phenotyping	3.7%	76.0%	20.4%	[Lehtimäki et al., 1990]
Finland	Uudenmaan (Helsinki)	615	20-66	NA	Institute staff and volunteers	Phenotyping	4.1%	73.3%	22.7%	[Ehnholm et al., 1986]
France	Alsace (Strasbourg)	172	25-64	100%	Population study	Phenotyping	8.5%	80.8%	10.7%	[Luc et al., 1994]
France	Artois (Lille)	150	25-64	100%	Population study	Phenotyping	11.9%	74.1%	14.0%	[Luc et al., 1994]
France	Haute-Garonne (Toulouse)	182	25-64	100%	Population study	Phenotyping	5.6%	86.3%	8.2%	[Luc et al., 1994]
France	Ile de France (Paris)	161	20-70	NA	Pedigree study	Genotyping	6.8%	82.0%	11.2%	[Schächter et al., 1994]
France	Ile de France (Paris)	237	avg 41	50%	Study of obese patients	Phenotyping	6.5%	79.7%	13.7%	[Furneron et al., 1988]
France	Ile de France (Paris)	498	NA	NA	Blood bank	Phenotyping	7.9%	80.1%	12.0%	[Bailleul et al., 1993]
France	Lorraine (Nancy)	303	24-67	50%	Parents in family study	Phenotyping	12.0%	76.4%	11.6%	[Gueguen et al., 1989]
France	Lorraine (Nancy)	1101	30-50	48%	Population study (selected)	Genotyping	9.4%	77.6%	13.0%	[Salah et al., 1997]
France	Lyonnais (Lyon)	191	NA	NA	Outpatients (selected)	Phenotyping	8.4%	83.0%	9.4%	[Cartier and Sassolas, 1992]
Germany	Hessen (Marburg, Giessen)	1031	NA	NA	Blood banks	Phenotyping	7.7%	77.3%	15.0%	[Utermann et al., 1984]
Germany	Nordrhein (Münster)	1557	NA	62%	Factory empleyees	Phenotyping	8.2%	78.2%	13.6%	[Assmann et al., 1984]
Great Britain	England, Cambridge	107	NA	NA	Healthy volunteers	Genotyping	5.1%	83.7%	11.2%	[Loktionov et al., 1999]
Great Britain	England, Mid- & north-east	465	NA	NA	Unknown	Phenotyping	11.3%	75.8%	12.9%	[Mastana et al., 1998]
Great Britain	England, Scotland (Bristol, Glascow)	192	18-26	64%	University students	Phenotyping	6.3%	76.3%	17.5%	[Tiret et al., 1994]
Great Britain	Scotland, Grampain	100	40-65	100%	Health centres	Phenotyping	6.0%	79.0%	15.0%	[Wilson et al., 1993]
Great Britain	Scotland, Grampain	400	45-60	47%	Population study	Phenotyping	8.3%	77.0%	14.8%	[Cumming and Robertson, 1984]
Great Britain	Ireland (Belfast)	176	25-64	100%	Population study	Phenotyping	10.3%	75.4%	14.3%	[Luc et al., 1994]
Greece	Nikea (Athen)	216	19-64	68%	Blood bank	Genotyping	5.3%	88.2%	6.5%	[Sklavounou et al., 1997]
Hungary	Not specified	104	NA	NA	Unknown	Genotyping	5.3%	79.8%	14.9%	[Benkmann et al., 1996]
Hungary	Not specified	202	NA	NA	Blood bank	Phenotyping	6.4%	80.7%	12.9%	[Hallman et al., 1991]
Hungary	Not specified	302	18-62	50%	Blood bank	Genotyping	10.4%	80.7%	8.7%	[Nagy et al., 1999]
Island	All island	185	30-64	NA	Population study	Phenotyping	6.8%	76.8%	16.5%	[Hallman et al., 1991]
Italy	Campagnia	157	NA	NA	Blood bank	Phenotyping	6.1%	84.4%	9.6%	[Corbo et al., 1995]
Italy	Lazio (Rome)	417	NA	NA	Blood bank	Phenotyping	6.6%	85.1%	8.3%	[Corbo et al., 1995]
Italy	Lazio (Sezze Romano, Rome)	195	8-11	55%	Health project	Genotyping	6.2%	86.7%	7.2%	[Xu et al., 1991]
Italy	Marche (Ancona)	296	20-64	81%	Blod banks	Phenotyping	7.3%	83.8%	8.9%	[Boerni et al., 1993]
Italy	Marche (Ancona)	365	20-64	74%	Blood bank	Phenotyping	7.3%	83.3%	9.4%	[James et al., 1993]
Italy	Puglia	95	NA	NA	Blood bank	Phenotyping	5.8%	86.3%	7.4%	[Corbo et al., 1995]
Italy	Reggio Emilia	304	NA	NA	Blood bank	Genotyping	5.6%	83.4%	11.0%	[Corbo et al., 1999]
Italy	Sardinia	633	6-89	46%	Healthy outpatients (selected)	Genotyping	4.0%	89.7%	6.3%	[Deiana et al., 1998]
Italy	Sardinia (Olbia)	280	NA	NA	Blood bank	Phenotyping	5.0%	89.8%	5.2%	[Corbo et al., 1995]
Italy	Sicilia, Calabria, Lucania	115	NA	NA	Blood bank	Phenotyping	4.8%	87.4%	7.8%	[Corbo et al., 1995]
Italy	Veneto (Padova)	352	NA	NA	Unknown	Phenotyping	4.4%	85.7%	9.8%	Gabelli et al (abstract, 1988)
Norway	Akerhus (Oslo)	156	42-61	44%	Twin study	Phenotyping	8.3%	76.0%	15.7%	[Pedersen and Berg, 1989]
Norway	Not specified	239	31-66	50%	Spouses in twin study	Phenotyping	9.0%	79.5%	11.5%	[Pedersen and Berg, 1990]
Portugal	North	149	NA	NA	Unknown	Genotyping	4.4%	88.2%	7.4%	[Seixas et al., 1999]
Spain	Alpujarra	99	25-54	50%	Unknown	Genotyping	2.5%	85.9%	11.6%	[Valveny et al., 1997]
Spain	Andalusia (Málaga)	566	NA	NA	Outpatients	Phenotyping	10.4%	78.5%	11.2%	[Fiol et al., 1991]
Spain	Basque Contry (Bilbao)	143	NA	NA	Unknown	Genotyping	4.9%	89.2%	5.9%	[Mastana et al., 1998]
Spain	Basque Country	111	22-56	52%	Unknown	Genotyping	4.9%	86.5%	8.6%	[Valveny et al., 1997]
Spain	Canary Islands, Tenerife	72	NA	NA	Unknown	Genotyping	2.1%	86.8%	11.1%	[Valveny et al., 1997]
Spain	Canary Islands, Tenerife	399	30-64	85%	Workers annual check-up	Phenotyping	7.5%	84.9%	7.5%	[Muros and Rodríguez-Ferrer, 1996]
Spain	Castilla la Nueva (Madrid)	415	NA	NA	Hospital staff	Phenotyping	8.7%	84.5%	6.9%	[Fiol et al., 1991]
Spain	Castilla la Nueva (Madrid)	614	24-72	36%	Hospital staff	Phenotyping	8.0%	84.2%	7.8%	[Gomez-Coronado et al., 1999]
Spain	Catalonia	88	18-65	38%	Unknown	Genotyping	4.5%	86.4%	9.1%	[Valveny et al., 1997]
Spain	Catalonia	226	avg 36	NA	Paternity testing procedure	Genotyping	6.4%	81.0%	12.6%	[Gene et al., 1997]
Spain	Catalonia (Barcelona)	512	35-65	48%	Population study	Phenotyping	5.4%	86.9%	7.7%	[Fiol et al., 1991]
Spain	Catalonia, Tarragona (Reus)	250	avg 48	100%	Controls in case-control study	Genotyping	5.0%	87.6%	7.4%	[Joven et al., 1998]
Spain	Central Spain	120	18-60	58%	Unknown	Genotyping	4.6%	87.1%	8.3%	[Valveny et al., 1997]
Spain	Montes de Pas	70	19-93	50%	Unknown	Genotyping	3.6%	80.7%	15.7%	[Valveny et al., 1997]
Sweden	Stockholm (Huddinge)	288	17-60	60%	Hospital staff and students	Phenotyping	7.1%	70.3%	22.6%	[Eggertsen et al., 1993]
Sweden	Östergotland (Linköbing)	223	45-69	100%	Controls in case-control study	Phenotyping	9.4%	72.4%	18.2%	Gerdes, LU (unpublished)
Switzerland	Vaud Fribourg (Geneve)	173	NA	NA	Unknown	Phenotyping	7.2%	82.1%	10.7%	[James et al., 1987]
The Netherlands	All country	2018	35	100%	Population study	Phenotyping	8.2%	75.0%	16.7%	[Smit et al., 1988]

Appendix II. Studies on APOE genotypes and acute coronary syndrome

The studies are shown in the same order as in Figure 14 on page 43. Please see the text for an explanation of the variable considered. Data from the study by Yamamura et al. (1990) were split by gender and ages of the cases, data from the study by Lenzen et al. (1986) were split by ages of the cases, and data from the study by Utermann et al. (1984) were split by the recruitment site of the cases (intensive care unit or rehabilitation center*).

Study	Cases and controls	Design	Numbers	Split data	Percent men	Average age	Upper age limit	Age mismatch	Incident cases	Selected	APOE 2-2	APOE 2-3	APOE 3-3	APOE 2-4	APOE 3-4	APOE 4-4
Wilson et al., 1993	Myocardial infarction	IV	100		100%	54	65	No	Yes	No	0	14	60	1	23	2
Scotland	Referent controls (approximation)		100		100%	52	65			Yes	0	11	64	1	19	5
Mänttäri et al., 1991	Myocardial infarction	II	136		100%	55	55	No	Yes	Yes	0	8	77	1	48	2
Finland	Noncases (nested)		132		100%	55	.			No	1	4	82	3	35	7
Yamamura et al., 1990	Myocardial infarction	V	57		100%	.	60	No	Yes	No	0	2	37	1	14	3
Japan	Healthy people		60	*	72%	46	.			No	0	7	42	1	11	1
Scaglione et al., 1999	Myocardial infarction	III	98		96%	40	45	No	Yes	No	0	4	73	1	19	1
Italy	Referent controls		98		96%	41	45			Yes	0	5	80	1	12	0
Eichner et al., 1993	Myocardial infarction	II	207		100%	59	60	No	Yes	Yes	1	24	110	4	61	6
USA	Noncases (nested)		412		100%	59	60			Yes	2	35	276	4	85	10
Stengård et al., 1995	Coronary deaths	I	26		100%	74	85	No	Yes	No	0	1	15	2	7	1
Finland, East	Noncases (follow-up)		255		100%	72	.			No	0	15	178	4	56	2
Corbo et al., 1999	Myocardial infarction	IV	148		100%	70	93	No	Yes	Yes	1	16	104	5	21	1
Italy	Referent controls (approximation)		114		100%	69	91			No	0	20	83	0	9	2
Gerdes and Færgeman, unpub.	Myocardial infarction	III	55		100%	49	55	No	Yes	Yes	2	8	23	0	21	1
Denmark	Referent controls		64		100%	49	54			Yes	0	8	39	1	13	3
Stengård et al., 1995	Coronary deaths	I	40		100%	75	85	No	Yes	No	0	1	16	2	21	0
Finland, South-West	Noncases (follow-up)		304		100%	73	.			No	0	26	180	12	86	0
Luc et al., 1994	Myocardial infarction	III	64		100%	54	65	No	No	No	0	6	43	0	13	2
France, Lille	Referent controls		150		100%	54	64			No	2	23	84	6	32	3
Köhler et al., 1992	Myocardial infarction + coronary artery disease	V	509		100%	41	45	No	No	No	8	40	324	9	115	13
Germany	Population sample		624		100%	37	.			No	6	67	393	20	124	13
Peacock et al., 1992	Myocardial infarction	IV	86		100%	41	45	No	No	Yes	0	6	46	5	24	5
Sweden	Referent controls (approximation)		83		100%	40	.			Yes	0	12	44	4	20	3
Luc et al., 1994	Myocardial infarction	III	187		100%	54	65	No	No	No	1	20	113	5	42	6
France, Strasbourg	Referent controls		172		100%	54	64			No	2	24	109	1	32	4
Nakai et al., 1998	Myocardial infarction	V	254		82%	60	None	No	No	No	0	10	178	2	52	6
Japan	Population sample		422		35%	62	.			No	0	16	327	4	74	1
Luc et al., 1994	Myocardial infarction	III	183		100%	54	65	No	No	No	2	17	102	4	49	9
Ireland	Referent controls		176		100%	54	64			No	2	26	104	6	32	6
Cumming and Robertson, 1984	Myocardial infarction	V	239		81%	54	65	No	No	No	0	18	128	10	77	6
Scotland	Population sample		400		50%	53	60			No	2	51	233	11	99	4
Luc et al., 1994	Myocardial infarction	III	140		100%	54	65	No	No	No	0	11	94	5	29	1
France, Toulouse	Referent controls		182		100%	54	64			No	0	19	131	1	30	1
Lenzen et al., 1986	Myocardial infarction + coronary artery disease	V	239		100%	.	50	No	No	Yes	0	12	136	4	82	5
Germany	Population sample		262	*	100%	37	.			No	3	28	165	8	52	5
Joven et al., 1998	Myocardial infarction	IV	250		100%	49	55	No	No	Yes	0	34	152	5	59	0
Spain	Referent controls (approximation)		250		72%	48	55			Yes	0	21	195	4	27	3
Utermann et al., 1984	Myocardial infarction	V	355		96%	.	None	Yes	Yes	No	5	43	226	8	65	8
Germany	Healthy people		700	*	96%	Young	.			No	7	84	419	10	160	20
Yamamura et al., 1990	Myocardial infarction	V	103		100%	.	None	Yes	Yes	No	2	11	73	0	13	4
Japan	Healthy people		109	*	100%	46	.			No	1	12	75	1	19	1
Eggertsen et al., 1995	Myocardial infarction	V	180		100%	.	None	Yes	Yes	No	2	18	111	5	40	4
Sweden	Population sample		235		100%	47	.			No	3	27	136	10	38	21
Shiina et al., 1988	Myocardial infarction	V	125		100%	57	None	Yes	Yes	No	0	5	77	0	43	0
Japan	Healthy people		110		100%	47	.			No	0	4	80	0	26	0
Yamamura et al., 1990	Myocardial infarction	V	39		0%	.	None	Yes	Yes	No	0	0	24	1	11	3
Japan	Healthy people		41	*	0%	46	.			No	0	5	28	0	7	0
Utermann et al., 1984	Myocardial infarction	V	168		100%	.	None	Yes	No	No	2	25	107	3	27	4
Germany	Healthy people		331	*	100%	Young	.			No	3	40	198	5	76	9
Lenzen et al., 1986	Myocardial infarction + coronary artery disease	V	331		100%	.	None	Yes	No	Yes	1	39	224	6	54	7
Germany	Population sample		362	*	100%	37	.			No	3	39	228	12	72	8
Lehtinen et al., 1995	Myocardial infarction + coronary artery disease	V	184		100%	58	None	Yes	No	Yes	0	5	112	0	59	8
Finland	Population sample		1577		72%	.	18			No	5	85	926	28	483	50
Gerdes et al (Danish 4S), unpub.	Myocardial infarction	V	435		96%	59	70	Yes	No	Yes	2	36	240	10	133	14
Denmark	Population sample		466		96%	40	40			No	8	54	260	9	117	18
Krantz et al., 1990	Myocardial infarction	V	83		100%	59	None	Yes	No	No	0	6	56	1	14	6
Germany	Healthy people		92		100%	27	.			No	0	16	59	1	15	1

*These data were kindly supplied by Professor Utermann.

Appendix III. Notes on methodology

APOE genotyping

The description in our original paper is very brief [Hansen et al., 1994a]; so here is a more elaborated description of the method. We used a modification of the method described by Hixson and Vernier [Hixson and Vernier, 1990]. DNA was isolated from blood leucocytes by phenol/chloroform extraction and ethanol precipitation, and a 244 bp sequence including the polymorphic sites of APOE coding for the amino acids in positions 112 and 158 of the protein was amplified by PCR using the oligonucleotide primers 5'-TTA GCT TGG CAC GGC TGT CCA AGG A-3' and 5'-ACA GAA TTC GCC CCG GCC TGG TAC AC-3' (DNA Technology, Aarhus, Denmark) and *Taq* polymerase (AmpliTaq Gold, Perkin Elmer (Roche), New Jersey, USA). The amplified material was cleaved with the *Hha* I isoschizomer *Cfo* I (Boehringer Mannheim, Germany) and the fragments were separated by polyacrylamide gel electrophoresis. *Cfo* I recognizes the sequence GCG/C which is present at six sites in the amplified 244 bp sequence for APOE*4 including the two codons coding for arginine in position 112 and 158. The GCG sequence at position 112 is absent in both APOE*3 and APOE*2 and is also missing at position 158 in APOE*2, so that the cleavage maps are unique for the six common APOE genotypes.

Estimation of confidence intervals for APOE allele frequencies

The limits of a 95% confidence intervals for a APOE allele frequency, CI_{Low} and CI_{High}, can be calculated as $p \pm (1.96 \times SE)$, where p is the point estimate based on gene counting and $SE = \sqrt{p(1-p)/n}$, with n = the total number of alleles. This is a simplified method based on normal approximation to the binomial distribution and works well if np and $n(1-p)$ are both above 10 [Gardner and Altman, 1989; Armitage and Berry, 1987]. A less reduced approximate method, which also includes a continuity correction for small samples, gives these two formulae for the limits[Nielsen et al., 1976]:

$$CI_{Low} = (n+1.96^2)^{-1}\left[r-0.5+\frac{1.96^2}{2}-1.96\sqrt{\frac{(r-0.5)(n-r+0.5)}{n}+\frac{1.96^4}{4}}\right], \text{ and}$$

$$CI_{High} = (n+1.96^2)^{-1}\left[r+0.5+\frac{1.96^2}{2}+1.96\sqrt{\frac{(r+0.5)(n-r-0.5)}{n}+\frac{1.96^4}{4}}\right],$$

where r is number of counted alleles, i.e. np. We used this method in most studies **[Gerdes et al., 1992a; Hansen et al., 1994a; Gerdes et al., 1996b; Gerdes et al., 2000b]**

However, with modern computer programs (e.g. Microsoft Excel 2000) it is not difficult to calculate exact limits by using the mathematical link between the binomial and the F distributions [Armitage and Berry, 1987]. The formulae are:

$$CI_{Low} = \frac{r}{r+(n-r+1)\cdot F_{0.025;2n-2r+2;2r}}, \text{ and}$$

$$CI_{High} = \frac{r+1}{r+1+(n-r)\cdot (F_{0.025;2n-2r+2;2r})^{-1}},$$

where $F_{0.025;v1;v2}$ are the percentage points of the F distribution for a probability of 0.025 and the specified degrees of freedom.

It should be noted, that all the above formulae are derived for the case where there are only 2 groups (i.e. the binomial case), so that the estimated frequency (or proportion) of the members in one of the groups, and the confidence interval for this frequency, is mirrored in the results for the other group, e.g. CI_{Low} (group A) = 1-CI_{High} (group B). The case for APOE alleles is trinomial, however, and the use of the binomial model means that the sum of the upper limits for the three estimated confidence intervals > 1. It can be confusing, notably in small studies where the confidence intervals become wide.

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