THE ROLE OF APOLIPOPROTEIN E IN GALLSTONE DISEASE, COLORECTAL CANCER AND GASTROINTESTINAL CELL REGULATION

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Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in Auditorium 10 of the University Hospital of Oulu, on January 26th, 2000, at 12 noon.

OUlUN YLIOPISTO, OULU 2000
Niemi, Mari, The role of apolipoprotein E in gallstone disease, colorectal cancer and gastrointestinal cell regulation
Biocenter Oulu, Department of Internal Medicine and Department of Pathology, University of Oulu, P.O.Box 5000, FIN-90014 University of Oulu, Finland
2000
Oulu, Finland
(Manuscript received: 5 January 2000)

Abstract

Apolipoprotein E (apo E) is one of the key regulatory proteins in cholesterol and lipoprotein metabolism. The present research focuses on the role of apo E in gastrointestinal diseases. The polymorphism of apo E has been suggested to be associated with the cholesterol content in gallstones and the crystallization rate of gallbladder bile. The possible effect of apo E polymorphism on the susceptibility to gallstone disease at the population level was examined in comparison with the classical risk factors for gallstone disease. The data suggest that the apolipoprotein E2 isoform is a genetic factor that provides protection against gallstone disease in women.

The alterations in plasma lipoprotein levels and bile acid metabolism observed in patients with colorectal adenoma and carcinoma may reflect a genetic background predisposing to tumors through altered lipid metabolism. To determine, whether the polymorphism of apo E is associated with proximal or distal colonic neoplasia, the apo E phenotype was determined in 135 patients with colorectal carcinoma, and 199 randomly selected control subjects. The frequency of the e4 allele of apo E was low in the patients with proximal adenoma and those with carcinoma, respectively, compared with the control subjects. The patients with distal tumors showed no alteration in e4 frequency. The data suggest that the e4 allele of apo E provides protection against the development of adenoma and carcinoma of the proximal colon. The association of apo E polymorphism with tumors is not a generalized phenomenon as is shown by the lack of association with breast or prostate cancers.

To further study the mechanisms by which apo E might affect colon cancer, the expression of apo E in human intestine and the localization of apo E in normal and malignant gastrointestinal tract was studied using immunohistochemistry and in situ hybridization. Both immunoreactive apo E protein and apo E mRNA were present throughout the stomach, small intestine and colon. The phagocytes of lamina propria were positive for apo E, but the number of positive cells and the staining intensity varied according to localization. Macrophages in the superficial lamina propria of normal colon were more strongly positive for apo E than those in the small intestine, where the most positively stained cells were dendritic cells and macrophages in the germinal centers of lymphoid follicles. In samples from colorectal carcinomas intensely positive macrophages surrounded the tumor area, suggesting that apo E might play a role in the proliferation of malignant cells.

Apo E binds with very high affinity to heparin and proteoglycans and inhibits the proliferation of several cell types, but the antiproliferative mechanism of apo E is still largely unknown. The effects of apo E at the cellular levels were studied in cell culture experiments. The effect of recombinant human apo E3 on cell polarity and the distribution of β-catenin were examined in undifferentiated (G+) and differentiated (G+ reversed) HT29 human colon adenocarcinoma cell lines. In cultured undifferentiated HT29 cells, treatment with apo E improved cell polarity and translocated β-catenin from the cytoplasm to cell-cell adhesion sites. Apo E may thus modulate epithelial integrity and contribute to cell growth and malignant transformation.

Keywords: Apo E, cholesterol, bile, intestine.
Acknowledgements

This work was carried out as a collaborative effort between the Departments of Internal Medicine, Pathology and Surgery at Oulu University and Biocenter Oulu, during years 1993-1999.

I have been privileged to grow up in a companion of passionate scientists and good friends:

My supervisor, Professor and the Head of the Department, Antero Kesäniemi deserves my deepest gratitude for arousing my interest in the fascinating world of research work, and for providing excellent research facilities. His expert knowledge of lipoprotein research and his enthusiasm for science have been of importance.

I am grateful to my second supervisor, Docent Kari Kervinen for his tireless encouragement and guidance. I also wish to express my sincere gratitude to Professor Markku Savolainen for his advice on gene technology, optimism and support during these years.

I wish to thank Professor Timo Strandberg and Docent Martti Färkkilä, who reviewed the manuscript of this thesis and provided constructive criticism. I also owe my thanks to Sirkka-Liisa Leinonen, Phil.Lic., for revising the English language of the manuscript with skill and promptness.

I am greatly indebted to Professor Seppo Ylä-Hertuala for a fruitful co-operation and an opportunity to continue research work in his productive team. My warmest thanks go to all members of Seppo’s group. Doctors Tomi Häkkinen and Antti Kivelä are acknowledged for collaboration as well as good company. I also want to thank scientists Marja Laitinen, Pauliina Lehtolainen, Anna-Leena Heikkilä, Anna-Mari Turunen, Johanna Laukkanen and Jonna Koponen for the therapeutic “Girls’ Sessions” in our room. Researchers Mikko Laukkanen, Mikko Turunen, Mikko Hiltunen and Tuomas Rissanen are acknowledged for teaching me invasive cardiovascular techniques and laboratory methods.

I would like to thank Docent Sinikka Eskelinen, Docent Tuomo Karrtunen and Doctor Markus Mäkinen, who introduced me to the field of cell biology and pathology. I was shown amazing fluorescent images, which were more beautiful than I have ever been able
to create on canvas - and again I realized I had chosen the right career when deciding not to study arts (which actually was my adolescent dream).

I wish to thank Professor and Head of the Department of Surgery, Matti Kairaluoma, Professor Olavi Lukkarinen, Docent Heikki Kiviniemi, Docent Juhani Lehtola, Docent Jyrki Mäkelä, the whole staff of the Department of Surgery and the Gastroenterological Unit of the University for helping me to collect the samples for the study. In addition, my wholehearted thanks go to thousands of study participants.

All the senior and junior researchers, and the whole staff in the Research Laboratory of the Department of Internal Medicine deserve my thanks for their help. Whatever was the matter, we got through it together. Special thanks are to Ms. Anne Salovaara for helping me with all the paperwork. There simply wasn’t such a problem Anne could not resolve (and you all know what it is like when researchers are dealing with financial and bureaucratic matters). Ms. Erja Tomperi and Ms. Marja Tolppanen are acknowledged for the guidance in the field of histochemistry, and Ms. Liisa Kärki and her photographic laboratory staff deserve my thanks for their excellent work.

I wish to thank my senior colleagues in the Kokkola Central Hospital for introducing me to the speciality of internal medicine. The Beverly Hills Barbecue Association of Kokkola, founded by doctors Katja Simonsson, Jussi Sia, Pasi Karjalainen et al. is acknowledged for supplying wine, food and social education.

Finally, I would like to thank my parents Pirkko and Erkki for the love, support and “häjy” genes they gave me, and my friends Veera and Jaana for dealing with the consequences. And there are many other persons who have meant a lot to me. You are not forgotten, I wish to thank you all. But like Holden Caulfield would say: “Don’t ever tell anybody anything. If you do, you start missing everybody.”

This work was supported financially by grants from the Cancer Society of Northern Finland, The Finnish Medical Society Duodecim and the Medical Council of the Academy of Finland. This support has been of great value.

Oulu, January 4th, 2000

Mari Niemi
### Abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ</td>
<td>amyloid beta</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AEC</td>
<td>3-aminoethyl-carbazole</td>
</tr>
<tr>
<td>AIDS-KS</td>
<td>acquired immunodeficiency syndrome-associated Kaposi’s sarcoma</td>
</tr>
<tr>
<td>ALCAM</td>
<td>activated leukocyte-cell adhesion molecule</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>ASO</td>
<td>allele-specific oligonucleotide</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>apo</td>
<td>apolipoprotein</td>
</tr>
<tr>
<td>BBD</td>
<td>benign breast disease</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BPH</td>
<td>benign prostate hyperplasia</td>
</tr>
<tr>
<td>CETP</td>
<td>cholesterol ester transfer protein</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified essential medium</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FAP</td>
<td>familial adenomatous polyposis</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>GSD</td>
<td>gallstone disease</td>
</tr>
<tr>
<td>HBP</td>
<td>high density lipoprotein binding protein</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HNPCC</td>
<td>hereditary non-polyposic colorectal cancer</td>
</tr>
<tr>
<td>IDL</td>
<td>intermediate-density lipoprotein</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LCAT</td>
<td>lecithin:cholesterol acyltransferase</td>
</tr>
</tbody>
</table>
LDL  low-density lipoprotein
LDLR  low-density lipoprotein receptor
LEF  lymphoid enhancer binding factor
LPL  lipoprotein lipase
LRP  low-density lipoprotein receptor-related protein
mAb  monoclonal antibody
MAP  microtubule-associated protein
M-CSF  macrophage colony-stimulating factor
mRNA  messenger ribonucleic acid
MSH 2,3,6  mutator S homolog 2, 3, and 6 genes
NFM  160 kDa neurofilament protein
NSAID  non-steroidal anti-inflammatory drug
OGTT  oral glucose tolerance test
p  chromosome short arm
PBS  phosphate-buffered saline
PCR  polymerase chain reaction
PDGF  platelet derived growth factor
RFLP  restriction fragment length polymorphism
PIPED  1,4-piperazinebis(ethanesulfonic acid)
PLTP  phospholipid transfer protein
q  chromosome long arm
RT  reverse transcription
SAS  Statistical Analysis System
SDS  sodium dodecylsulphate
SR-B1  scavenger receptor class B1
SSCP  single-strand conformation polymorphism
TCF  T cell-specific factor
UTP  uridine5’triphosphate
VLDL  very low-density lipoprotein
Wg  Wingless
WHHL  Watanabe heritable hyperlipidemic
WHO  World Health Organization
List of original articles

The present thesis is based on the following original articles, which are referred to in the text by their Roman numerals.


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Introduction

Apolipoprotein E (apo E) is a central regulatory protein in lipid and lipoprotein metabolism. It has been known for many years that defective expression of apo E (either absent expression or expression of variant forms) is associated with an increased risk for atherosclerotic vascular disease, the leading cause of death in western societies (Schaefer et al. 1984, Mahley 1988).

Apo E binds with very high affinity to heparin and proteoglycans (Cardin et al. 1988, Mahley 1988), and it participates in plasma lipoprotein metabolism through its high-affinity interaction with cell surface receptors, including the low-density lipoprotein (LDL) receptor and the LDL receptor-related protein (LRP) (Herz et al. 1988; Lund et al. 1989; Yamada et al. 1989). Apo E has three common isoforms, E2, E3 and E4, which are coded by the alleles ε2, ε3 and ε4, respectively. Apo E4 has been associated with several diseases, such as coronary heart disease (Mahley 1988, Laakso et al. 1991), macro- and microangiopathy in patients with diabetes (Ukkola et al. 1993), and Alzheimer’s disease (Kuusisto et al. 1994, Strittmatter & Roses 1995), which means that the possibility of subjects with apo E4 to reach extreme old age is low (Kervinen et al. 1994, Tilvis et al. 1998, Jian-Gang et al. 1998). The ε2 allele is associated with low and the ε4 allele with high serum total and low-density lipoprotein (LDL) cholesterol levels in various populations (for a review, see Mahley 1988) and with altered enterohepatic metabolism of cholesterol and bile acids (Kesäniemi et al. 1987). It was previously shown that the cholesterol content of gallstones tends to be low in subjects with the ε2 allele and high in subjects with the ε4 allele (Juvonen et al. 1995). The role of apo E4 isoform as a risk factor for cholelithiasis was suggested by a subsequent study (Bertomeau et al. 1996). Apo E polymorphism may also be related to the expression of primary biliary cirrhosis and the response of the disease to ursodeoxycholic acid treatment (Vuoristo et al. 1997).

In addition to being synthesized by hepatocytes as components of lipoprotein particles, apo E is also synthesized by a number of cell types not directly involved with cholesterol metabolism (Mahley 1988). Apo E also exhibits biological activities that are not obviously related to lipid transport. For example, apo E is a potent suppressor of lymphocyte activation (Cardin et al. 1988), and it inhibits cell proliferation and tumor growth (Vogel et al. 1994). However, the functions of peripherally produced apo E are not fully understood.

The present work was carried out to investigate the potential functions of apolipoprotein E in the gastrointestinal tract, particularly in colon tumors and gallstone formation. Firstly,
the role of apo E in comparison with the conventional risk factors for gallstone disease was assessed. Secondly, the possible associations between the apo E phenotype and cancer of colon, breast and prostate were investigated. Thirdly, the expression of apo E in normal human intestine and colon cancer was studied. Fourthly, the effect of apo E on human colonic adenocarcinoma cells was explored.
2. Review of the literature

2.1. Lipoprotein classes and lipoprotein metabolism

Lipoproteins mediate lipid transport in plasma (for a review, see Gotto et al. 1986). They have a spherical structure with a hydrophobic core of nonpolar triglyceride and cholesterol ester molecules surrounded by an amphipathic surface of apolipoproteins and phospholipids. Apolipoproteins are generally responsible for the interaction of the lipoprotein with specific cell membrane receptors and enzymes (Mahley et al. 1984). Plasma lipoproteins are usually divided into five major categories: chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). This classification is related to the physiological and physical characteristics of the lipoproteins and their isolation with ultracentrifugation based on their density (Havel et al. 1955, Schumaker & Puppiene 1986).

Chylomicrons (d <0.93 g/ml), which are formed in the intestine, are the largest and lightest lipoprotein particles. They are rich in triglycerides, contain apo B48, and, in the circulation, receive apo Cs (C-I, C-II and C-III) and apo E from HDL particles. Exogenous lipid from the gut and lipid secreted to the gut in bile are absorbed and packaged into chylomicrons. Chylomicrons carry cholesterol esters formed by acyl CoA:cholesterol acyltransferase (ACAT) in the endothelial cells of the intestine. Chylomicrons are secreted into lymph, and their triglycerides are lipolysed into free fatty acids by the action of lipoprotein lipase (LPL), which is activated by apo CII. Concomitantly, surface materials of chylomicrons (phospholipids, free cholesterol, apo Cs and apo A-I) are transferred to the HDL pool and the liberated fatty acids are used as an energy source or taken up by adipocytes. The triglyceride-depleted, cholesteryl ester-enriched chylomicron remnant particles are taken up by the liver mainly via remnant receptors. A blood sample taken from a healthy subject after an overnight fast contains no chylomicrons.

VLDL (d=0.94-1.006 g/ml), IDL (d=1.007-1.019 g/ml) and LDL (d = 1.020-1.063 g/ml) constitute a group of interrelated particles which function in the transport of endogenous triglycerides and cholesterol from the liver to other tissues (Lusis 1988, Brewer et al. 1988, Sehayek & Eisenberg 1990, Fielding 1992). VLDL, secreted by the liver, carries the endogenously synthesized triglyceride and cholesterol. The major protein of LDL is apoB_{100}, but VLDL also contains apoC1-I, C-II, C-III and and apo E. The VLDL triglycerides are lipol-
ysed in the capillaries of the adipose tissue of skeletal muscle by LPL. The VLDL remnants are reduced in size, enriched in cholesterol esters and retain the apolipoprotein components apo B-100 and apo E, giving rise to IDL particles. About half or the circulating IDL particles are removed quickly by the LDL receptors in the liver cells, due to the high affinity of the IDL apo E molecules to these receptors. The other half of the IDL particles develop into apo B-100 containing LDL particles, which deliver cholesterol to tissues (Babiak 1988).

The apo B-100 molecules are recognized by the LDL receptors of tissue and liver cells, which leads to endocytosis of whole LDL particles into the cells.

HDLs (d=1.063-1.210) are a heterogeneous group of particles. Newly formed HDL particles appear as spherical particles containing neutral lipids or as discoidal particles of apolipoproteins and phospholipids (for a review, see Jonas 1991). Spherical HDLs are synthesized in the liver and intestine and discoid HDLs probably as a result of lipolysis of triglyceride-rich lipoproteins and the subsequent shedding of their surface material (Eisenberg 1984). HDL particles differ with respect to size, density and composition, and are modified by CETP, PLTP, LCAT, LPL and hepatic lipase. HDL is divided by ultracentrifugation into two major subfractions, HDL$_2$ and HDL$_3$, of densities 1.063 to 1.125 g/ml and 1.125 to 1.210 g/ml, respectively. Using different methods, HDL can also be divided into various subspecies, depending on size or apolipoprotein content. HDL particles are the key components in reverse cholesterol transport, the process in which cholesterol is transported from extrahepatic tissues to the liver (for a review, see Glomset 1968, Gotto et al. 1986). HDL particles function as a receptacle for excess phospholipids and cholesterol derived from the membranes of tissue cells or from the surfaces of other lipoprotein particles. HDL particles also serve as a reservoir for apo E and apo C. The cholesterol esters that accumulate in HDL particles are further transferred to apo B-containing lipoproteins by CETP in exchange for triglycerides. HDL also delivers cholesterol esters directly to the liver and steroidogenic cells via type B1 scavenger receptors (SR-B1), which recognize both apoptotic cells and negatively charged lipoproteins (Yamada et al. 1996). Several HDL binding proteins, quite disparate in structure, have recently been cloned and their role in HDL metabolism is currently being assessed (for a review, see Fidge 1999). One candidate HDL receptor, HB$_2$ shows high sequence homology with adhesion molecules, particularly the activated leukocyte-cell adhesion molecule (ALCAM). High-density lipoprotein binding protein, HBP (vigilin), which lacks a transmembrane domain, is responsive to the cell cholesterol levels, but its physiological significance is still unknown. The ligand specificity of HDL receptors, confounded by nonspecific lipid interactions, remains controversial. It appears that several biochemical pathways are affected by HDL or its apolipoproteins, but the elements of complete signal transduction triggered by HDL are yet to be confirmed. (For a review, see Fidge 1999.)

### 2.2. Cholesterol homeostasis

LDL and HDL particles are the key components of cholesterol transport (figure 1). Cholesterol in the circulation is acquired either via an exogenous route (dietary cholesterol) or an endogenous route (cholesterol synthesis). The rate-limiting enzyme in endogenous cholesterol biosynthesis is the 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase.
In addition to cholesterol synthesis, cells acquire cholesterol by receptor-mediated endocytosis from LDL (Brown and Goldstein 1986). LDL is internalized and delivered to lysosomes, and cholesterol esters are then hydrolyzed into unesterified cholesterol. In hepatocytes cholesterol is converted into bile acids or is secreted into blood in lipoprotein particles, whereas in steroidogenic cells, cholesterol is oxidized into steroid hormones. About 600-750 mg of cholesterol is lost each day in fecal bile acids, and about 500-600 mg in fecal neutral sterols; 75-100 mg is shed by skin and 35-50 mg is converted into steroid hormones.

HMG-CoA reductase and LDL receptors are the main proteins controlling the cellular levels of cholesterol. ACAT controls the intracellular formation and deposition of cholesterol esters. LDL receptor and HMG-CoA reductase expressions are up-regulated and ACAT activity down-regulated when the cellular cholesterol levels are low, whereas a rise of cellular free cholesterol activates ACAT and suppresses LDL receptor levels and HMG-CoA reductase (Brown & Goldstein, 1986).

Cholesterol is an important compound; it is present in almost all cellular membranes and it is a precursor of bile acids and steroid hormones, such as estrogens, testosterone and corticosteroids. On the other hand, an excess of exogenously or endogenously derived lipoproteins, most frequently in relation to a diet high in saturated fat or to genetic determinants, predisposes to atherosclerosis, the leading cause of death in the western countries.

2.3. Apolipoproteins

Apolipoproteins have three main functions: Firstly, they help to solubilize cholesterol esters and triglyceride by interacting with phospholipids. Secondly, they regulate the reaction of these lipids with enzymes such as LCAT, lipoprotein lipase and hepatic lipase. Thirdly, they bind to cell surface receptors and thus determine the sites of uptake and the rate of degradation of other lipoprotein constituents.

Apolipoproteins bind lipids primarily upon hydrophobic bonding between the fatty acyl chains of phospholipids and the non-polar regions of apolipoproteins, with ionic interaction between the polar head groups of phospholipids and pairs of oppositely charged amino acids in the (-)helical regions of the apolipoprotein playing a secondary, stabilizing role. The apolipoproteins A, B, C and E are known to play major roles in cholesterol homeostasis (Eisenberg 1984, Brown & Goldstein 1986, Mahley 1988). The molecular weights, chromosomal origin and plasma concentration of apolipoproteins are shown in table 1. Apolipoproteins also exhibit several functions other than those directly involved with lipid metabolism, and the literature overview will concentrate on the potential functions of apolipoprotein E.
Figure 1.
Tissue sites of origin and degradation and the intravascular metabolism of the lipoprotein classes.
FC and CE refer to free cholesterol and cholesterol ester, respectively.
<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>Molecular Weight (kD)</th>
<th>Lipoprotein association</th>
<th>Other function*</th>
<th>Chromosomal location</th>
<th>Plasma concentration (mg/dl)</th>
</tr>
</thead>
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<tr>
<td>A-I</td>
<td>28</td>
<td>HDL, Chylomicrons</td>
<td>LCAT activator</td>
<td>11</td>
<td>100-150</td>
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<tr>
<td>A-II</td>
<td>17</td>
<td>HDL</td>
<td>HPL regulation</td>
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<td>30-40</td>
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<tr>
<td>A-IV</td>
<td>46</td>
<td>VLDL, HDL</td>
<td>LCAT activator</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>B_{100}</td>
<td>512</td>
<td>VLDL, LDL</td>
<td>TG secretion</td>
<td>2</td>
<td>90</td>
</tr>
<tr>
<td>B_{48}</td>
<td>6.6</td>
<td>HDL, VLDL, Chylomicrons</td>
<td>TG secretion (amino-terminal part of B_{100})</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>C-I</td>
<td>8.8</td>
<td>Chylomicrons</td>
<td>LCAT activator</td>
<td>19</td>
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<td>C-II</td>
<td>8.7</td>
<td>HDL, VLDL, HDL</td>
<td>LPL activator</td>
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<tr>
<td>C-III</td>
<td>33</td>
<td>HDL, VLDL, chylomicrons</td>
<td>Modulation of chylomicron and VLDL remnant clearance</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>D</td>
<td>33</td>
<td>HDL</td>
<td>LCAT activator, stabilizer</td>
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<tr>
<td>E</td>
<td>34</td>
<td>VLDL, HDL</td>
<td>Chylomicron and VLDL remnant clearance</td>
<td>19</td>
<td>3-7</td>
</tr>
<tr>
<td>F</td>
<td>28</td>
<td>HDL, LDL</td>
<td>Transport and/or esterification of cholesterol</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>G</td>
<td>72</td>
<td>VHDL, HDL</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>43-54</td>
<td>VLDL, Chylomicrons LDL, HDL</td>
<td>May prevent activation of the intrinsic blood coagulation cascade by binding to phospholipids on the surface of damaged cells</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>J</td>
<td>70</td>
<td>VHDL, HDL</td>
<td>Role in HDL metabolism Cell protection</td>
<td>8</td>
<td>10</td>
</tr>
</tbody>
</table>

* Adapted or proposed, in addition to lipid transport. ADP - adenosine diphosphate; VHDL - very-high-density lipoprotein. (Modified from Jordan-Starck et al. 1992)
2.4. Apolipoprotein E

2.4.1. Structure

Apolipoprotein E (apo E) is a 34 kDa arginine-rich glycoprotein. which is an integral surface component in circulating chylomicrons, chylomicron remnants, VLDL, IDL and some cholesterol-rich subclasses of HDL; including apo E-HDL and HDL1 (in humans and some rodents) and HDLC (in cholesterol-fed animals). LDL in the circulation does not contain apo E as opposed to arterial wall LDL (Ylä-Herttuala et al. 1988). Human apo E contains 317 amino acids, of which 18 represent a signal peptide (Breslow et al. 1982, Zannis et al. 1984).

The gene for apo E is located on chromosome 19 where it is closely linked to the genes for apo C-I and apo C-II and more distantly linked to the gene for the low density lipoprotein (LDL) receptor (Scott et al. 1985). In humans, apo E presents a genetic polymorphism (Utermann et. al. 1987, Rall & Mahley 1992). This was first suggested by isoelectric focusing (IEF) of apo E and subsequently confirmed by direct sequencing of cDNA. Apo E has three common isoforms, E2, E3 and E4, which are coded by the alleles ε2, ε3 and ε4, respectively. Apo E3 is the most frequent isoform in the general population. The molecular basis of apo E polymorphism consists of cysteine arginine interchanges. Apo E3 contains a single cysteine at residue 112 and an arginine at position 158; apo E2 contains cysteine residues at both positions 112 and 158; apo E4 contains arginine residues at both positions. Although apo E is one of the best-characterized apolipoproteins in terms of the structure-function relationships, the particular structural properties of each isoform that explain the differences in reactivity remain poorly understood.

Apo E differs from the other apolipoproteins in its tertiary structure. The Chou-Fasman algorithm predicts apo E to be highly helical and segregated into two fragments separated by a large segment whose structure is predicted to be random (Nolte & Atkinson., 1992). It is re-folded into two independent structural domains (Fig 2) corresponding to the two functional moieties of the protein: an amino-terminal and a carboxyl-terminal domain, which contain most of the ordered structure, and are divided by a region of random structure (residues 165-200). The carboxyl-terminal domain contains a region of strongly amphipathic α-helical character, with the helices having one face composed of apolar residues and the other of polar residues. These structures have been postulated to be involved in lipid binding, and the carboxyl-terminal domain thus appears to represent the major lipid-binding region of the apo E molecule.
2.4.2. Role of apo E in lipoprotein metabolism

The amino-terminal domain contains the region of apo E that binds to the LDL receptor. Apo E-rich lipoproteins also act as ligands for the LDL receptor-related protein (LRP), which is expressed mainly in the liver and brain (for a review, see Brown et al. 1991). The major role of LRP is the removal of β-VLDL and intestinal chylomicron remnants. The three common apo E isoforms have different affinities for the receptors, which is why apo E2 shows defective receptor binding affinity. VLDL and the remnants containing apo E2 are slowly removed from the plasma and induce an upregulation of the liver LDL receptor and thus a low concentration of plasma cholesterol. VLDL-apo E4 particles are removed faster from plasma than VLDL-apoE3 particles, inducing a down-regulation of the LDL receptor. Accordingly, apo E4 phenotype is thus associated with a higher concentration of circulating cholesterol. (For a review, see Mahley 1988.) The differences in the intestinal absorption of cholesterol between E2, E3 and E4 seem to contribute to the regulation of the LDL cholesterol level (Kesäniemi & Miettinen 1987).

The amount of apo E as well as the location of apo E on the lipoprotein particle are important for the interaction between apo E and its receptor (Mazzone 1996). The specific affinity of the apo E isofrom with cellular surface proteoglycans facilitates the accumulation of lipoproteins on the cell surface, which thereby reach a critical concentration for optimal receptor-mediated uptake.

Consistent with the hyperlipidemia associated with defective or deficient apo E, one major role of apo E appears to be the clearance of apo B-containing remnants. Accelerated catabolism of such remnants occurs when rabbits are injected or infused with apo E (Mahley et al. 1989). Moreover, apo E overexpression in transgenic mice is associated with lower concentrations of VLDL and more efficient clearance of apo B-containing lipopro-
teins. Conversely, apo E-null mice and transgenic mice overexpressing dysfunctional apo E develop severe hyperlipidemia resembling type III hyperlipidemia in humans (Fazio et al. 1993, 1994).

Apo E plays an important role in HDL metabolism and reverse cholesterol transport. Apo E facilitates the expansion of the HDL core, enhancing its cholesterol-carrying capacity, and seems to mediate hepatic removal and catabolism of choleseryl-ester-enriched HDL (Leblond & Marcel 1992). The expression of apo E in macrophages facilitates cholesterol efflux from cholesterol-loaded macrophages to exogenous acceptors (e.g. HDL2)(Lin et al. 1998), and apo E-rich HDL reduces LDL retention in the subendothelial matrix (Saxena et al. 1993, 1995). Intravenous administration of purified apo E into Watanabe heritable hyperlipidemic (WHHL) rabbits decreases cholesterol deposition in the aortas of the atherosclerosis-prone animals (Yamada et al. 1992). Apo E-mediated enhancement of cholesterol efflux from macrophages could therefore have some significance for modulating the formulation of vessel wall atherosclerotic lesions.

2.4.3. Apo E synthesis and regulation

In humans, apo E is synthesized primarily by the liver and the brain, but also by other tissues, including monocytes/macrophages (Basu et al. 1982). Apo E synthesis has also been detected in the human kidney (Blue et al. 1983), lung (Blue et al. 1983), skin keratinocytes (Fenjives et al. 1989), and steroid-producing tissues, such as the adrenal glands, testes and ovaries (Blue et al. 1983, Driscoll et al. 1985, Beckmann et al. 1991), and in many tumors (Pfalzer et al. 1992, Lichtor et al. 1992, DeWille et al. 1993). Particles containing only apo E, cholesterol, and cholesterol ester may also exist, at least transiently, in interstitial fluid.

Approximately 60 to 80 percent of the apo E is synthesized in the liver by hepatocytes and Kupffer cells. Accounting for the cellular RNA content and the population size of each cell type in the liver, Kupffer cells contain about 0.7% of liver apo E mRNA, while hepatocytes account for most of the remainder (Dawson et al. 1989). Separation of secreted apolipoproteins by density gradient ultracentrifugation has shown that apo E associated with lipids floats in the HDL region, but small amounts of apo E are also found in the VLDL, LDL and IDL regions. Fifty percent of apo E is secreted in a lipid-poor form. Recent studies have suggested that excess dietary cholesterol up-regulates the apo E gene by a transcriptional mechanism and dietary saturated fat by a post-transcriptional mechanism (Srivastava 1996). In addition, estrogen (Srivastava et al. 1997), growth hormone (Sjöberg et al. 1994) and insulin (Ogbonna et al. 1993) have been shown to affect apolipoprotein E synthesis in hepatocytes.

In humans, the brain is another major site of apo E synthesis apart from the liver, accounting for mRNA expression that amounts the one-third of that measured in the liver. Astrocytes are its major cellular site of synthesis (Shao et al. 1997). Apo E-containing lipoproteins are also found in the cerebrospinal fluid and appear to play a major role in lipid transport in the central nervous system (Pitas et al. 1987, Mouchel et al. 1997). Apo E may also serve as a targeting protein for local redistribution of cholesterol within neural tissues undergoing repair or remodeling. (Boyles et al. 1990, Arendt et al. 1997)
Macrophage apo E production is regulated both transcriptionally by cholesterol, even by small changes in cellular free cholesterol, and post-transcriptionally by extracellular cholesterol acceptors (Mazzone et al. 1989). Moreover, macrophage apo E expression can be stimulated by oxidized LDL as well as by other modified forms of LDL and by cytokines (Lin et al. 1995, Zhang et al. 1994, Duan et al. 1995, Cader et al. 1997). Because macrophages are rather ubiquitous cells they could be a significant source of apo E at many tissue sites.

Steroid-producing tissues, such as adrenal glands, ovaries and testes require large amounts of cholesterol, and they also synthesize apo E. Studies on human and nonhuman primate tissues have indicated that the rates of apo E synthesis and mRNA concentration in the adrenal gland are similar to those in the liver (Williams et al. 1989). In testis, apo E is synthesized in Leydig cells, and its production is also down-regulated by gonadotropin (Schleicher et al. 1993), and in human ovaries, apo E production is under the control of gonadotropin and cholesterol (Polacek et al. 1992).

Keratinocytes form a protective barrier through the formation of squamous epithelium in the epidermis. Apolipoprotein E is secreted by the small basal keratinocytes in the epidermis. The cellular localization of apo E suggests that apo E may be involved with the lipid uptake and redistribution within the epidermis (Fenjives et al. 1989, Barra et al. 1994).

Since apo E has been shown to be highly expressed in many tumors, including brain, mammary and lung tumors (Pfalzer et al. 1992, Lichtor et al. 1992, DeWille et al. 1993), it has been suggested as a possible marker for neoplasms. The proliferation of tumor cells is increased, and greater quantities of lipid are therefore required for the cellular membrane construction. The abundant expression of apo E in tumors may be a response to an increased demand for cholesterol, or alternatively, apo E may have other functions related to tumor growth.

The intestine is one of the major sites of apolipoprotein metabolism. The results on apo E expression in intestine have been contradictory, since some animal studies have suggested that apo E is not produced in the intestine (Williams et al. 1984), whereas others have shown intestinal apo E expression (Lin-Lee et al. 1985, Zannis et al. 1985, Yamada et al. 1994). Since apo E polymorphism seems to affect the cholesterol absorption efficiency (Kesäniemi et al. 1987), it would be plausible to assume that apo E also has a biologically relevant function in human intestine.

Little is known about the apo E contents in different tissues and the way in which these tissues release apo E into the extracellular fluid and other body fluids. Apo E polymorphism is, however, an important determinant of the apo E plasma concentration: the ε2 allele is associated with higher concentrations and the ε4 allele with lower apo E values. Overall, the apo E polymorphism accounts for 12% of the total apo E variability (Boerwinkle & Utermann 1988). The apo E concentrations in healthy people might also vary as a function of different biological factors (for a review, see Siest et al. 1995). Some studies suggest that women have higher apo E plasma concentrations than men, but a Finnish study showed the mean apo E concentration to be 12.7% lower in women than in men (Kaprio et al. 1991). Age, smoking, alcohol consumption (Braeckman et al. 1998) and estrogen therapy (Srivastava 1996) have also been shown to affect the apo E plasma concentration.
2.4.4. Apo E phenotyping and genotyping

As a consequence of its biological importance, apo E polymorphism has been investigated extensively (Menzel & Utermann 1986, Ehnholm et al. 1986). The apo E phenotype is usually determined by isoelectric focusing (IEF), which allows the detection of charge variations consequent upon the minor sequence differences between the principal isoforms. Originally, IEF was done on delipidated VLDL followed by protein staining. VLDL was usually isolated either by ultracentrifugation or by a specific precipitation method followed by the delipidation step. This has been replaced by IEF of whole serum followed by immunoblotting using apo E antibody and a species-matched secondary antibody. This method requires only small amounts of plasma and is applicable to large-scale studies.

Genotyping methods may complement phenotyping when certain structural variants of apo E have the same net charge as the three common isoforms. Currently, several different Apo E genotyping techniques have been described, including HhaI endonuclease digestion (Hixson et al. 1990), the use of allele-specific oligonucleotides (ASO) (Weisgraber et al. 1998), the amplification refractory mutation system (ARMS) (Wenham 1991), the single-strand conformation polymorphism (SSCP) technique (Aozaki et al. 1994), and the minisequencing (Svännen et al. 1989). The ARMS technique has many advantages compared to these and other genotyping methods: it is fast, easy to perform, and nonisotopic. Recently, a new ARMS method that requires only two PCR reactions instead of four for each instance of Apo E genotyping was designed (Donohoe et al. 1999). Of these methods, only sequencing and SSCP are able to detect rare mutations. Restriction isotyping, which can be performed in a single day (Crook et al. 1994) may be acceptable as a general method for studying apo E polymorphism. Each genotype is determined by the presence of specific HhaI fragments of different sizes, and genotypes are directly visualized by gel staining. Furthermore, the restriction isotyping used in conjunction with the protein phenotype may identify rare alleles of apo E. Due to the high GC content of the apo E gene, genotyping for apo E is a very demanding procedure. Unsuccessful amplification is a frequent problem if the PCR conditions are not rigorously controlled. The hybridization step using ASO probes may also be problematic. Thus, DNA methods may not always be superior to protein separation (phenotyping) in large-scale studies.

Genotyping is particularly helpful when the phenotype is difficult to interpret due to post-translational modification of apo E molecules, as in distinguishing phenotype E3/3 from E3/2. The intralaboratory discrepancy between the phenotype and the genotype has been reported to vary between 0.2% and 24%, depending on the method employed and the patient groups studied. (For a review, see Siest et al. 1995.)

2.4.5. Ethnic variations and apo E polymorphism

Apo E phenotype distribution is highly variable among different populations (Hallman et al. 1991), but no specific studies on the factors responsible for the difference in apo E allele frequencies among populations have been carried out. The frequency of allele (4 is low in Asian populations (Evans et al. 1993), and high in black African groups (Sephernia et al. 1988) and in Papua New Guineans (Benkmann et al. 1996). In Europe, the (4 allele fre-
quency is highest in Northern Europe and lowest in Southern Europe (Ehnholm et al. 1986, Gerdes et al. 1992, et al. Rosseneu et al. 1994). This may be one factor explaining the high incidence of coronary heart disease in Northern Europe.

2.4.6. Apo E and cardiovascular diseases

The link between apo E polymorphism and atherosclerosis was first established with the observation that patients with type III hyperlipoproteinemia and with the apo E 2/2 phenotype had premature coronary heart disease (Utermann 1975), and the effect of apo E polymorphism on serum cholesterol levels and atherosclerosis has thereafter been under extensive research. In a meta-analysis of studies that included 45 population samples from 17 countries, total cholesterol was clearly increased in subjects with the E 4/3 and E 4/4 phenotypes, while significantly lower values were seen in those with the E3/2 and E2/2 phenotypes (Dallongeville 1992). A number of investigators have reported similar effects of apo E alleles on plasma lipid concentrations (Table 2.) (Boerwinkle et al. 1987, Ehnholm et al. 1986, Sing & Davignon 1985, Eto et al. 1986a, Reilly et al. 1991). The average effect of the ε2 allele is to reduce total plasma cholesterol by 0.52 mmol/L, whereas the ε4 allele increases it by 0.26 mmol/L. Similar results have also been obtained in Finnish men (Porkka et al. 1994) for total and LDL cholesterol. There was no effect of apo E phenotype on HDL cholesterol.

Apo E polymorphism also has an effect on the apo B concentration (Boerwinkle & Utermann 1988, Porkka et al. 1994), ε2 being associated with lower values and the ε4 allele being associated with higher apo B values. In total, apo E polymorphism accounts for about 12% of the total apo B variability. Apo A-I concentrations do not show significant variations according to apo E phenotype. It has also been suggested that the ε2 and ε4 alleles may be associated with an increase of the plasma triglyceride concentrations (Boerwinkle et al. 1987). This effect was also seen in the meta-analysis by Dallongeville et al. (Dallongeville 1992), although the results obtained in different populations have been contradictory.

Several clinical studies have reported the frequency of the ε4 allele to be higher in individuals with cardiovascular disease than in control subjects (reviewed by Davignon et al. 1988). Furthermore, the ε4 allele is associated with an increased risk for silent myocardial ischemia in healthy normocholesterolemic men (Katzel 1996). In patients with non-insulin-dependent diabetes mellitus, the ε4 allele is related to coronary atherosclerosis (Laakso et al. 1991) and macro- and microangiopathy (Ukkola et al. 1993). On the other hand, the ε2 allele may have a protective effect on the development of coronary atherosclerosis.

In healthy individuals, 5 to 15% of the normal interindividual variation in plasma cholesterol levels can be attributed to the common apo E polymorphism (Davignon et al. 1988), although this effect has not been observed in all of the populations studied. The explanations for the differences between populations regarding the effect of apo E are unknown, but gender, ethnic origin, lifestyle and diet are often suggested as contributory factors. Previous calculations have suggested that the variation of serum cholesterol according to the apo E phenotype covers only 2.8% of the variation in the overall CHD risk (Davignon et al. 1988).
Apo E is clearly overexpressed in atherosclerotic lesions (Vollmer et al. 1991), and numerous studies have suggested that apo E plays an important direct role in the atherosclerotic process (Yamada et al. 1989). Transgenic mice overexpressing rat apo E show marked resistance to diet-induced hypercholesterolemia and do not develop atherosclerosis (Shimano et al. 1992), whereas mice with targeted disruption of the apo E gene develop spontaneous atherosclerosis even under basal low fat/low cholesterol conditions. (Zhang et al. 1992, Plump et al. 1992). Atherosclerosis was found to be more severe in chow-fed apo E-null mice than in cholesterol-fed apo E (+/+ ) mice, despite the relatively similar plasma cholesterol levels in the two groups (Zhang et al. 1994). In another study, transgenic expression of apo E in the arterial wall inhibited atheroma formation and severity without affecting the plasma cholesterol level or the lipoprotein profile in cholesterol-fed mice (Shimano et al. 1995). Taken together, apo E confers a major factor modulating the risk for vascular occlusive diseases, although the underlying mechanisms are not clearly understood.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>No. of subjects</th>
<th>% of the apo E3 value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Cholesterol</td>
<td>LDL</td>
</tr>
<tr>
<td>2/2</td>
<td>2-6</td>
<td>-15.5 to +3.6</td>
</tr>
<tr>
<td>2/3</td>
<td>37-54</td>
<td>-12.5 to -3.6</td>
</tr>
<tr>
<td>2/4</td>
<td>5-15</td>
<td>-18.0 to +1.0</td>
</tr>
<tr>
<td>3/4</td>
<td>29-251</td>
<td>+1.7 to +6.4</td>
</tr>
<tr>
<td>4/4</td>
<td>5-43</td>
<td>+2.0 to +9.8</td>
</tr>
</tbody>
</table>
2.4.7. Apo E, Alzheimer’s disease and neuronal cytoskeleton

Macrophages, their activation and lipoprotein oxidation are involved in the progression of atherosclerotic lesions (Steinberg et al. 1989, Ross 1993). Similar features are also seen in Alzheimer’s disease, such as activation of microglia and oxidative damage (Itagaki et al. 1989, Subbarao et al. 1989). The histological diagnosis of Alzheimer’s disease (AD) is characterized by extracellular senile plaques, neurofibrillary tangles in neuronal cells and dendrites, and vascular amyloid deposits. The amyloid peptide of 39 to 42 amino acids (Aβ) is one of the main components of senile plaques, while the Tau protein is the main component of tangles. Four types of AD can be distinguished: early and late-onset (before and after age 65, respectively), sporadic and familial. The genetic background of these diseases is linked to chromosome 21 (Aβ), chromosome 19 (apo E) and chromosome 14.

One of the first clues showing that apo E functions in the nervous system (for a review, see Mahley, 1995) came from a tissue survey of apo E mRNA expression. Apo E mRNA is abundant in the brain and is synthesized and secreted primarily by astrocytes. Secondly, apo E-containing lipoproteins are found in the cerebrospinal fluid and appear to play a major role in lipid transport in the central nervous system. Thirdly, apo E, together with a source of cholesterol, promotes neurite extension in cultured dorsal root ganglion cells, and its expression increases after peripheral nerve injury. Apo E is associated with both deposits of Aβ peptide and neurofibrillary tangles, which represent filaments of a microtubule-associated protein (MAP) called tau.

The apo E4 gene dose is a major risk factor for late-onset and sporadic Alzheimer’s disease, with 50% of homozygous patients developing the disease by age 70, whereas apo E2 seems to protect individuals against Alzheimer’s disease (Corder et al. 1993). In a Finnish population-based study, the prevalence of Alzheimer’s disease was 2.9% in subjects with no ε4 alleles, 7.6% in subjects with one ε4 allele, and 21.4% in subjects with two ε4 alleles of apolipoprotein E in a randomly selected elderly population (Kuusisto et al. 1994). The apo E ε4 allele was also associated with impaired cognitive function and excess mortality resulting from dementia and all causes in another Finnish study (Tilvis et al. 1998). Inheritance of the ε4 allele may increase the risk of developing Alzheimer’s disease due to the direct involvement of apo E in neuronal function and pathology or, alternatively, due to a linkage disequilibrium with the Alzheimer’s disease susceptibility gene located close to apo E.

In vitro studies have shown that apo E3 and apo E4 interact differentially with the Aβ peptide. Apo E4 and the Α( peptide interact readily in vitro to form a very stable complex, whereas apo E3 displays less avid interaction with the Α( peptide. Morphological studies of the apo E- Aβ peptide complexes have revealed differences in the ultrastructures of the complexes formed with apo E3 and apo E4: apo E4 initiated a massive network of long monofibrils, similar to those seen in senuratic plaques, whereas in the presence of apo E3 the network was less dense.

Because apo E is present in the cytoplasm of neurons, it could potentially interact with and regulate cytoskeletal proteins. (Strittmatter et al. 1994). Previous studies have shown that apo E3, but not apo E4, binds the microtubule-associated proteins tau and MAP-2 (Huang et al. 1994). In mammalian cells that transiently express tau and the LDL receptor, apo E is taken up from cerebrospinal fluid, and apo E3, but not apo E4, reaches the cytoplasm. This isoform difference in the intracellular distribution of apo E is dependent upon
tau expression (Lovestone et al. 1996). The findings on the association on the apo E3 and apo E4 with tau were complemented by examining the interactions of the apo E isoforms with the other cytoskeletal proteins. In a gel shift assay, apo E3 forms SDS-stable complexes with the longest isoform of human recombinant tau (T4L), the shortest isoform of human recombinant tau (T3), and the 160kDa neurofilament protein (NFM). Apo E4 does not bind T3, T4L or NFM in this assay. In an overlay assay with T4L, actin or tubulin, apo E3 and apo E4 bind T4L and tubulin equally well, whereas apo E3 binds actin with a significantly greater affinity than apo E4. These results indicate that apo E isoforms interact with cytoskeletal proteins with at least two different binding affinities. The more avid interaction results in the formation of complexes which are SDS-stable and occurs almost exclusively with apo E3, while the other interactions between apo E and cytoskeletal proteins are not specific to apo E3. The mechanisms and consequences of apo E binding to cytoskeletal proteins remain to be elucidated, and further research is necessary to determine if apo E binding is isoform-specific and functionally consequential.

2.4.8. Apo E and immunoregulation

ApoE is a potent inhibitor of mitogen-driven human lymphocyte proliferation, independent of the type of the activation signal (Cardin et al. 1988), and both CD4 and CD8 cells are inhibited. Monomeric peptides representing the residues 130-149 or 130-155 are shown to inhibit cell proliferation without causing a loss of cell viability, whereas cytostasis by a peptide representing the extended 130-169 domain or the dimeric peptides of the amino acids 141-155 or 141-149 is accompanied by potent cytotoxic activity (Clay et al. 1995).

The administration of apo E blocks growth factor-responsive lymphocytes in the GI A phase of the cell cycle (Mistry et al. 1995). As the interleukin-2 (IL2) and IL4-dependent modes of lymphocyte proliferation are equally inhibited, suppression by apo E is likely to be independent of growth factors. Apo E has no effect on IL2-augmented killing of target cells by cytotoxic T cells, indicating that it has no direct effect on signaling via interleukin receptors.

Atherosclerosis is considered to be a chronic inflammatory disease, and activated T lymphocytes are also detectable in atherosclerotic lesions (Østerud 1997, Pasterkamp et al. 1999). As apo E suppresses the production of IL2 and lymphocyte proliferation, macrophage-derived apo E could regulate local lymphocyte function in the inflammatory process. Changes in the distribution of mononuclear phagocyte subsets have been shown in atherosclerosis (Schmitz et al. 1997), other forms of acute and chronic inflammation, cancer and HIV infection (Ziegler-Heitbrock 1996). The apo E 4/4 phenotype influences the differentiation of monocytes toward a more CD16a-positive phagocytic phenotype (Stöhr et al. 1998) and is associated with a reduced amount of macrophage-derived apo E (Stöhr et al. 1998). The apo E phenotype may thus affect different inflammatory processes through its effect on monocyte differentiation.
2.4.9. Apo E and cell proliferation

Abnormal cell proliferation is a major contributor to the development of atherosclerosis. Since apo E inhibits both atherosclerotic lesion formation and lymphocyte proliferation, recent studies have explored the possibility that apo E may also have general functions related to cell signaling and proliferation.

Previous reports have shown that human apo E3 inhibits the proliferation of several other cell types, including endothelial cells, breast cancer cells and melanoma cells in a dose and time-dependent manner (Vogel et al. 1994). Apo E inhibits both de novo DNA synthesis and proliferation as assessed by an increase in cell number. Maximal inhibition of cell growth is achieved under conditions where proliferation is dependent on heparin-binding growth factors. The inhibition of cell proliferation is reversible and dependent on the time of the apo E addition to the culture. In addition, apo E inhibits the chemotactic response of endothelial cells.

Apo E has been used in an in vitro and in vivo model system for acquired immunodeficiency syndrome-associated Kaposi’s sarcoma (AIDS-KS) (Vogel et al. 1994). Apo E blocks the proliferation and chemotaxis of AIDS KS cells in response to activated lymphocyte-conditioned medium and Oncostatin M. Apo E also inhibits the formation of neoangiogenic lesions induced in BALB/c nu/nu mice by AIDS-KS cells (Browning et al. 1994). Thus, apo E may be one factor regulating angiogenesis.

Recently, apo E was shown to inhibit smooth muscle cell migration directed to platelet-derived growth factor (PDGF) or oxidized LDL (Ishigami et al. 1998) and smooth muscle cell proliferation. These inhibitory effects of apo E were not due to the suppression of PDGF binding to its receptors in smooth muscle cells, but the inhibition correlated with a significant reduction in agonist-stimulated mitogen-activated protein kinase activity. PDGF-induced cyclin D1 mRNA expression was also inhibited, suggesting that the apo E effect is mediated by a growth arrest at the G0 to G1 phase. Thus, apo E may have cytostatic functions in the vessel wall and it may protect against vascular diseases through inhibition of cell signaling events.

Both the exposure of cells to apo E protein from exogenous sources and endogenous apo E expression have been shown to exert inhibitory effects on cell regulation (Vogel et al. 1998). Free apo E is likely to be present in some tissues, such as arterial wall, and it is very efficiently internalized and metabolized in cultured cells (Al-Haideri et al. 1998). There is no difference in the lipid-free apo E uptake and degradation between LDL receptor-positive and LDL receptor-negative fibroblasts, and since the LDL receptor-negative fibroblasts express little or no LRP activity, it is suggested that lipid-free apo E is internalized and degraded via cell surface heparan sulfate proteoglycans. Thus, lipid-free apo E, after cell internalization, may be available to mediate a number of intracellular pathways.
2.5. Gallstone disease

2.5.1. Pathogenesis of gallstone disease

Gallstones may present as a surgical emergency in a patient with right upper quadrant pain, fever, chills and jaundice (Diehl et al. 1990), but they are often an incidental radiological finding in an asymptomatic patient. In fact, at least 10% of adults may have gallstones in western societies. In principle, a patient may be afflicted by gallstones of two types (Johnston & Kaplan 1993): pigment stones or cholesterol gallstones. The main component of pigment stones is calcium bilirubinate, while cholesterol accounts for less than 20% of them (Trotman & Soloway 1982). Black pigment stones are common in patients with cirrhosis or chronic hemolytic conditions, and brown pigment stones are associated with infection (Johnson & Kaplan, 1993). Cholesterol stones are composed of pure cholesterol or have cholesterol as the major chemical constituent. Cholesterol gallstones are the most common gallstones found in western patients with cholelithiasis, and 70-80% are classified as cholesterol stones (Busch & Matern 1991). A prerequisite for the development of cholesterol gallstones is lithogenic bile, which is often the result of enhanced cholesterol synthesis or a reduced bile acid pool size or both (Grundy et al. 1972). Because the formation of cholesterol stones is frequently associated with changes in biliary lipid composition, the bile usually being supersaturated with cholesterol, (Admirand & Small 1968), the research into the etiology and pathogenesis of gallstones has mainly focused on cholesterol and bile acid metabolism.

2.5.2. Epidemiology of cholesterol gallstones

The etiology of cholesterol gallstones is multifactorial, involving interactions of genes and the environment. The main conventional risk factors for GSD are sex, obesity, age and ethnic background (Bennion et al. 1978, Maclure et al. 1989, 1992 Attili et al. 1997). The prevalence of gallstones in females is 2- to 3fold compared males and increases with age (Maclure et al. 1989). Low plasma HDL cholesterol and high LDL cholesterol and triglycerides may also be associated with the risk of gallstones (Petitti et al. 1981, Thijs et al. 1990). A lipid profile of this kind as well as obesity are actually part of the risk factor cluster called the metabolic syndrome, which is associated with hypertension, non-insulin-dependent diabetes and coronary heart disease. Indeed, associations between diabetes and GSD have been suggested (De Santis et al. 1997).

Cholecystographic and ultrasonographic epidemiological studies show very different prevalences of gallstone disease in different populations. The highest prevalence has been found among North American Indians (Sempliner et al. 1970), Chileans (Nervi et al. 1988) and Mexican Americans (Maurer et al. 1987, Hanis et al. 1993), who are followed by non-Mexican Hispanics from North America (Maurer 1989), Europeans (Barbara et al. 1987, Jørgensen et al. 1987, Heaton et al. 1991, Muhrbeck & Ahlbeck 1995), and Asians from India (Khuroo 1989). The lowest prevalence has been observed among the Japanese (Nomura et al. 1988). The high prevalence of gallbladder disease in certain ethnic groups supports the view that there are dominant lithogenic genes modulate the susceptibility to GSD,
and the molar percentage of biliary cholesterol, bile acid composition, cholesterol synthesis, bile cholesterol saturation, and gallstone formation showed pairwise correlations within monozygotic twin pairs in a previous study (Kesäniemi et al. 1989). The complex interaction of a number of environmental and genetic factors in cholesterol GSD has made it difficult to identify and establish the role of putative lithogenic genes. However, some epidemiological studies have indicated that the increased risk for gallstone disease in Mexican Americans persists even after adjustment for the known risk factors for GSD, such as age, body mass index (BMI), body weight distribution, parity, dietary intake and glucose intolerance (Diehl et al. 1980 and 1989, Haffner et al. 1990, Hanis et al. 1985).

2.5.3. Lipid metabolism and gallstone disease

The association between altered plasma lipoprotein levels and GSD is controversial. Many studies have failed to detect any consistent association between plasma lipids and gallstones (Janson et al. 1985), whereas some studies have shown that decreased levels of plasma HDL cholesterol (Miguel et al. 1998) and increased levels of LDL cholesterol and triglycerides are related to an enhanced risk of gallstones (Petti et al. 1981, Thijs et al. 1990). The bile cholesterol saturation index (Thornton et al. 1981) and the cholesterol nucleation time (Janowitz et al. 1992) have also been shown to correlate positively with plasma triglycerides. On the other hand, Hoffman et al. (1982) did not find any association between the bile cholesterol saturation index and plasma lipids.

Although the plasma lipoprotein profile of patients with gallstones differs markedly from that of healthy subjects (Thijs et al. 1990), the similar serum lipid pattern in patients with pigment gallstones and with cholesterol gallstones suggests that the altered plasma lipoprotein levels could actually be a consequence of the gallstones. In the case of small gallstones, which may represent newly formed stones, a change from a negative to a positive association between gallstones and both LDL cholesterol and total cholesterol is seen (Jörgensen et al. 1989).

The mechanisms by which alterations in lipoprotein metabolism are related to the risk of gallstone disease are largely unknown. Several human studies have looked for candidate genes related to lipid metabolism that might favor cholesterol gallstone formation. These studies include the search for the expression of abnormalities in the structure of apo E, apo B, apo AI and the cholesterol ester transfer protein (Juvonen et al. 1995). Most of the studies have suggested an association between cholesterol gallstone formation and specific apo E polymorphism (Juvonen et al. 1993, Bertomeau et al. 1996), whereas no differences in the apo B, apo A-I and cholesterol ester transfer proteins genes were found between GSD patients and healthy control subjects (Juvonen et al. 1995).

2.5.4. Apo E and gallstone disease

The apo E isoform E4 has been identified as a risk factor for gallstone formation (Bertomeu et al. 1996) and recurrence (Portincasa et al. 1996). However, the mechanisms for the increased risk of gallstone formation in patients carrying the e4 allele are not well understood.
The previous reports have suggested, that apo E4 is associated with an altered enterohepatic metabolism of cholesterol and bile acids (Kesäniemi & Miettinen 1987, Miettinen 1991, Gylling & Miettinen 1992), which is why influx of cholesterol into hepatocytes is high in subjects with the e4 allele of apolipoprotein E, and the de novo synthesis of cholesterol is reduced. The high influx of cholesterol to the liver in E4 subjects seems to be related to the enhanced absorption of dietary cholesterol in these individuals (Kesäniemi & Miettinen 1987, Miettinen 1991, Gylling & Miettinen 1992). This, in turn, might lead to increased biliary cholesterol saturation, as has been shown following cholesterol feeding in subjects with and without gallstones (Lee et al. 1985). It was also suggested by Juvonen et al. that patients with the E4 isoform have increased numbers, sizes and cholesterol contents of gallstones, a higher frequency of cholesterol crystals and much faster crystallization in gallbladder bile (Juvonen et al. 1995). However, the effect of apo E on gallstone cholesterol content has not been confirmed by all studies: In one study the patients carrying the e4 allele had similar stone numbers and crystallization as the patients without the e4 allele, their cholesterol saturation index was lower, and their total protein and bile salt concentrations tended to be higher with preferential taurine-conjugation (Van Erpemc et al. 1998). The observed differences in bile salt concentration and the preferential taurine conjugation of bile salts in the case of apo E4 are of uncertain significance, but might theoretically contribute to gallstone formation.

2.6. Adenocarcinoma of the colon and rectum

2.6.1. Pathogenesis of colorectal cancer

Colorectal cancer is one of the most common cancers in the western societies and its incidence rate is increasing. Of the colorectal cancers, over 90% are sporadic and a maximum of 10% are of hereditary origin: hereditary non-polyposis colorectal cancer (HNPPC; 2-10%) or familial adenomatous polyposis (FAP; 1%) (de la Chapelle & Peltomäki 1995, Mecklin et al. 1995, Kinzler & Vogelstein 1996, Lynch et al. 1997). In HNPPC, the susceptibility to cancer is based on an inherited mutation of one of the known DNA mismatch repair genes; MLH1 (Papadopoulos et al. 1004), MSH2 (Leach et al. 1993), PMS1 or PMS2 (Nicolaides et al. 1994). Recently, a new candidate gene, a mismatch repair gene, MSH6 was shown to be associated with HNPPC in some cases (Lynch 1998, Akiyama et al. 1997, Miyaki et al. 1997). In FAP, the susceptibility to colorectal cancer is caused by germ line mutations in the adenomatous polyposis coli (APC) gene (Groden et al. 1991, Nishishio et al. 1991).

In sporadic colorectal cancer, the progression of an adenomatous lesion into cancer is known to be based on an accumulation of mutations in specific genes controlling cell division, apoptosis and DNA repair (Kinzler & Vogelstein. 1996). Studies of colon carcinogenesis have also identified agents which may either promote or protect from the development of colon cancer. Such agents include bile acids and non-steroidal anti-inflammatory drugs (NSAID). The evidence that high concentration of neutral and acid steroids in the colon lumen may have a promoting role in colon cancer stems from experimental carcinogenesis
studies in animals (Hori et al. 1998, Rigas et al. 1994). Non-steroidal anti-inflammatory drugs (NSAID) appear to have a protective effect on the development of colon cancer both in experimental animal models and in epidemiological studies (Rigas et al. 1994, Muscat et al. 1994, Wünsch et al. 1998).

It is widely accepted that dietary factors, especially dietary fat and fiber, influence the development of colorectal cancer. However, little is known of the possible interactions between dietary and genetic factors. Recent studies on the function of the APC gene and the modification of its effects by phospholipase A2 and cyclooxygenase - two enzymes involved in lipid metabolism - have shed light on these interactions (Watson & DuBois, 1996, Tsuji et al. 1998). The APC gene undergoes somatic mutations in up to 50% of sporadic colorectal cancers and is commonly mutated early in the transition from normal epithelium to adenoma. The expression of wild-type APC may induce apoptosis in colorectal cancer cells which contain a defective APC gene (Morin et al. 1996). Thus, APC may act as a “gatekeeper” ensuring that cell division is balanced by cell death. On the other hand, the APC mutation seems to require an external factor, such as digestive secretions, dietary components or intestinal flora before colon tumors develop (Gould & Dove, 1996).

Cancer located in the proximal part of the colon appears to have a different kind of pathogenesis compared to distal carcinomas. Alterations in chromosome number, aneuploidy, are common in distal cancers, whereas proximal cancer often appear diploid (Delattre et al. 1989, Böttger et al. 1993). Distal carcinomas frequently exhibit allelic losses in the chromosomal locations 17p, 18q and 5q, indicating inactivation of the tumor suppressor genes known to reside in these areas (Knudson 1985, Solomon et al. 1987, Baker et al. 1989, Delattre et al. 1989, Thiagalingam et al. 1996), whereas allelic losses are less frequent in proximal cancers. Microsatellite instability is more common in proximal than in distal cancers (Thibodeau et al. 1993, Ionov et al. 1993). The differences in the pathogenesis of proximal and distal cancer may be related to differences in the carcinogenic environment in the proximal and distal colon (Burnstein et al. 1993, Breivik et al. 1987), or due to differences in the developmental and biological properties of different parts of the colorectum (Bufill et al. 1990).

2.6.2. Epidemiology of colorectal cancer

High dietary animal fat and low fiber intakes have generally been considered environmental risk factors for colorectal tumors (Willett et al. 1990, Giovannucci et al. 1992, Sandler et al. 1993), possibly because they favor a high fecal concentration and colonic absorption of secondary bile acids. According to large-scale population studies, the risk of colon cancer increases with age (Chyou et al. 1996), heavy smoking (Hsing et al. 1998, Chyou et al. 1996), alcohol intake (Hsing et al. 1998, Chyou et al. 1996) and low level of physical activity (Hsing et al. 1998, Colditz et al. 1997, Longnecker et al. 1995). Dietary consumption of vegetables and grains and regular use of NSAIDs are shown to provide protection against colorectal carcinoma (Thun et al. 1992, Sandler et al. 1993, Sandler et al. 1997).

The data on the relation between cholesterol levels and colorectal adenoma or carcinoma are contradictory. The etiologic factors of colon cancer, such as a diet high in saturated fat and low level of physical activity tend to increase the serum cholesterol level, and accord-
ingly, some studies have shown a positive association between colon carcinoma (Mannes et al. 1986, Törnberg et al. 1986) or adenoma (Bayerdorffer et al. 1993) and serum cholesterol levels. On the other hand, several prospective cohort studies have reported an inverse association between total serum cholesterol and cancer, which is frequently attributed to colon cancer (Kark 1980, Williams et al. 1981, Law & Thompson 1991, Salonen 1982).

2.7. Cholesterol metabolism and cancer

In acute leukemia, ovarian cancer and colon cancer, serum cholesterol levels appear to reflect disease activity (Vitols et al. 1984, 1990 and 1992, Rudling et al. 1986 and 1990). The uptake of cholesterol via LDL receptors is increased in many tumors (Peterson et al. 1985), and the content of LDL receptors inversely correlates with survival time in breast carcinoma (Rudling et al. 1986). Moreover, increased LDL receptor mRNA expression in colon cancer correlates with a rise in plasma cholesterol after curative surgery (Niendorf et al. 1995). These findings provide evidence that the tumor tissue itself contributes to the decreased plasma cholesterol levels in cancer patients. This supports the hypothesis that the low cholesterol levels in cancer patients are a consequence, rather than the cause of the malignancy. On the other hand, the total absence of LDL receptors predicts a shorter survival in colon carcinoma (Caruso et al. 1998).

Despite the several epidemiological studies on the possible role of cholesterol in cancer (Kark 1980, Williams et al. 1981, Law & Thompson 1991, Salonen 1982), little is known about its effects on carcinogenesis. ‘Atherogenic’ low-density lipoproteins, especially their modified forms, have been shown to possess a cell-transforming potential (Zwijsen et al. 1992), whereas ‘non-atherogenic’ high-density lipoproteins did not induce cell transformation by themselves or in an initiation-promotion model. These results suggest that LDL could act as a (co)carcinogen.

2.8. Cell differentiation and intercellular communication

2.8.1. Polarization of epithelial cells

One of the key features of differentiated epithelial cells is their polarized structure. The generation of the polarized phenotype is a multi-stage process, which requires extracellular cues in the form of cell-cell and cell-matrix contacts and the re-organization of cell surface proteins and cytoplasmic proteins. Once established, the phenotype must be maintained by the segregation and retention of specific proteins and lipids in distinct plasma membrane domains (Caplan 1997). Attachment to the substratum occurs through interactions between integrins and the extracellular matrix (Hynes 1992). Analysis of the distribution of membrane proteins in newly adherent cells reveals that attachment initiates the process of polarization (Vega-Salas et al. 1987). One initial requirement for the generation of cellular polarity appears to be the establishment of cell-cell contacts and tight junctions, which delineate

2.8.2. Cadherin-catenin function in cell adhesion and signal transduction

The adhesion and development of cell surface polarity are regulated by calcium-dependent cell adhesion proteins called cadherins. Cadherin function is modulated by a class of proteins, catenins, which regulate cadherin function in cell-cell adhesion (Nathke et al. 1993). Apart from their direct role as physical linkers of the actin cytoskeleton to cadherins, catenins also play a central role in signal transduction and the regulation of gene expression. Free pools of β-catenin in the cytoplasm may enter the nucleus and induce transcription process (Ben-Ze’ev & Geiger 1998), and it is also needed for the induction of endothelial cell proliferation and angiogenesis (Lewalle et al. 1997). Nuclear localization of β-catenin is a common event in colorectal tumorigenesis (Tsuji et al. 1998).

2.8.3. Effect of lipoproteins on cell-cell junctions in proliferative disorders

Cancer is a disease of growth and differentiation. Oncogenes and tumor-suppressor genes are implicated in growth regulation, and their respective activation or inactivation transforms a normal tissue into a benign, i.e. noninvasive, tumor. Invasion suppressor and invasion promoter genes are more closely related to differentiation than growth regulation. Their respective inactivation or activation transforms a benign tumor into an invasive and metastasizing, i.e. malignant, one (Mareel et al. 1993). The ability of carcinomas to invade and metastasize largely depends on the degree of epithelial differentiation within the tumors. E-cadherin-mediated cell-cell adhesion has been shown to prevent the invasiveness of human carcinoma cells (Fri xen et al. 1991), and mechanisms that lead to a loss of cell-cell adhesion (mutation, loss of catenin expression, alterations in phosphorylation) are thought to contribute to a more metastatic phenotype (Sommers et al. 1994).

Abnormal proliferation of vascular smooth muscle cells is a key event in the formation of atherosclerotic plaques in humans. LDL cholesterol and especially oxidized LDL cholesterol have the potential to inhibit gap-junctional communication (Zwijsen et al. 1994). As intercellular communication is considered to play an essential role in maintaining and controlling cell growth, cell differentiation and homeostasis, this finding is in accordance with their atherogenic potency. HDL and antioxidants, such as alpha-tocopherol, butylated hydroxytoluene and glutathione are able to diminish the LDL-induced inhibition of cell-cell communication (Zwijsen et al. 1998). LDL also possesses some cell-transforming potential (Zwijsen et al. 1992), whereas HDL does not induce cell transformation either by itself or in an initiation-promotion model (Zwijsen et al. 1992).
3. Outlines of the present study

3.1. Background

The mechanisms by which alterations in lipoprotein metabolism are related to the risk of gastrointestinal diseases are largely unknown. As the polymorphism of apo E seems to be associated with enterohepatic sterol metabolism, the cholesterol content in gallstones and the prevalence of gallstone disease, the effect of apolipoprotein E polymorphism on the susceptibility to gallstone disease at the population level in comparison to classical risk factors was investigated.

It is widely accepted that dietary factors, especially a high fat intake, influence the development of colorectal cancer. However, little is known of how dietary and genetic factors may interact. As apo E polymorphism affects the enterohepatic cholesterol and bile acid metabolism and bile acid composition in the colon lumen, which may have a significant role in the development colon cancer, the possible effect of apo E polymorphism on susceptibility to colon cancer was investigated. In addition, the possible associations between two common cancer types, cancers of breast and prostate, were studied.

Apo E has both cytostatic and antimigratory properties. As intercellular communication is considered to play an essential role in maintaining and controlling cell growth, cell differentiation and homeostasis, the role of apo E and lipoprotein subfractions in cell-cell interaction were further examined.

3.2. Aims of the study:

The following questions were specifically addressed:
1) What is the role of apo E in comparison with the conventional risk factors in the susceptibility to gallstone disease at the population level (I)?
2) Is the apo E allele distribution in patients with colorectal adenoma or carcinoma different compared to random populations (II) or patients with other malignancies (III)?
3) Is apo E produced in the normal human intestine (IV)?
4) Is apo E production altered in colon tumors (IV)?
5) What is the effect of apo E on intestinal carcinoma cells (IV)?
4. Subjects and methods

4.1. Subjects

The hypertensive cohort in study I consisted of 600 (300 male and 300 female) subjects living in the city of Oulu, whose age on September 1, 1990 was between 40 and 50 years, and who, according to the register of the Social Insurance Institution were entitled to a special refund (higher reimbursement class) of hypertensive medication endorsed later than August 1980 (Rantala et al. 1999). The subjects were selected by the Social Insurance Institution in 1990. To ensure adequate recruitment of younger subjects, the randomization was age-stratified, i.e. for each year of birth (1931-1950), 15 men and 15 women were selected. An age- and sex-matched control subject was randomly selected from the whole population of the city of Oulu (106500 inhabitants at the time of selection). The selected subjects were invited by a letter to participate in the study, and one reminder letter was sent when necessary. The first invitations were mailed at the end of 1990. Five hundred and nineteen hypertensive and 526 control subjects visited the research laboratory of the Department of Internal Medicine between January 1991 and March 1993, first the men (during 1991 and early 1992) and then the women. The visits were made in the morning after an overnight fast. At this visit, anthropometric measurements (weight, height, waist, hip), blood pressure measurements and standard ECG were performed. A standard oral glucose tolerance test was made, blood samples for lipid and DNA analyses were obtained, and other tests were performed. All measurements were carried by two trained study nurses. After that, the subjects were interviewed and examined by a physician. The interviews were conducted using standard forms, and special attention was given to the past medical history, current medication, family history of diseases, smoking habits, alcohol consumption and physical activity. Alcohol consumption was determined in an extensive interview concerning the amount of beer, wine and strong alcoholic beverages consumed (Khavari & Farber 1978), and the total amounts of absolute alcohol consumed in a week (g/wk) were calculated. The past and current smoking status were inquired in a questionnaire and the amount of smoking was calculated as the number of cigarettes smoked per day. In addition, the lifetime smoking burden was calculated as pack-years (1 pack-year=20 cigarettes smoked/day in one year). Physical activity was estimated separately as leisure time and occupational activity, and the activities were scored on a 1-5 scale (where 1 was sedentary and 5 highly
active (Grimby 1986). Three trained physicians with special competence in internal medicine interviewed and examined all the study subjects. All the blood pressure measurement were recorded with an automatic oscillometric blood pressure recorder (Dinamap, Critikon Ltd., Ascot, UK). Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared. An abdominal ultrasound examination was carried out using a Toshiba SSA 270 ultrasound system with a scanning frequency of 5 MHz. Gallstones were diagnosed as mobile intraluminal echodensities with posterior shadowing. In the case of patients with prior cholecystectomy (n=83), the presence of gallstones was confirmed from the operative records. Gallstones were verified in 74 (89%) of these patients.

One hundred and twenty-two consecutive Caucasian patients undergoing treatment of colorectal adenocarcinoma were recruited in study II. This group consisted of 54 men and 68 women treated at the Unit of Gastroenterological Surgery, Oulu University Hospital, between June 1989 and September 1992. Practically all new patients with colorectal carcinoma in the province of Oulu are treated in this unit. Patients with FAP or HNPCC were excluded from the study. The biopsy specimens for histological classification (Morson & Sobin 1976) were taken during surgery or, in the case of an inoperable tumor, by endoscopy. In addition, 156 consecutive patients with colorectal polyps found in total colonoscopy at the Department of Internal Medicine were examined. The patients with prior polypectomy were excluded. The main indications for colonoscopy were abdominal symptoms or a positive fecal occult blood test. The polyps were removed or biopsied during the colonoscopy and the histological diagnosis was confirmed by the Department of Pathology, Oulu University Hospital. The adenomas were histologically classified as tubular, tubulovillous or villous according to the criteria of the World Health Organization (Morson & Sobin 1976). Epithelial dysplasia was graded as mild, moderate, or severe. Patients with familial polyposis (n=3), patients with inflammatory bowel disease (n=4), and patients with nonadenomatous polyps (hyperplastic or hamartomatous polyps (n=14) were excluded from the study; hence, the total series consisted of 135 patients (84 men and 51 women) with adenomatous polyps. One hundred and ninety-nine individuals aged 54-61 years from the city of Oulu were randomly selected as control subjects by age matching from the social insurance register covering the whole population of Oulu.

To further examine whether apo E polymorphism might be generally associated with the risk of cancer, consecutive series of 211 women undergoing surgery for breast carcinoma, 299 women treated for benign breast disease (BBD), 130 men undergoing treatment for prostate cancer and 201 men treated for benign prostate hyperplasia (BPH) in the Department of Surgery, Oulu University Hospital between January 1996 and December 1997 were included in study III. Practically all new patients with a disease of breast or prostate were examined. Patients with cancer residues were excluded from the study. Another retrospective patient group consisting of seventy-seven women with breast carcinoma and sixty-six women with benign breast disease operated on during the year 1994 was also studied. The histological diagnoses of all the patients were confirmed by the Department of Pathology, Oulu University Hospital. Of 288 patients with breast carcinoma, 177 (61%) had invasive ductile carcinoma, 48 (17%) had lobular carcinoma, 41 (14%) had carcinoma in situ, and 21 (8%) had other types of carcinoma such as papillary carcinoma, mucinous carcinoma or a combination of different cancer types. Of the 265 patients with BBD, 115 (43%) had fibroadenoma, 97 (37%) had fibrocystic mastopathy, 27 (10%) had fibrosis, 19 (7%) had normal mammary gland and 12 (5%) had mastitis. The remaining patients had other benign
breast diseases, e.g. lipoma, ductectasia, abscess, tubular adenosis or adenomyoepithelioma. The diagnosis of prostatic carcinoma or BPH was confirmed by histological analysis. Of the 130 patients with prostatic adenocarcinoma, 117 (90%) were diagnosed in the Oulu University Hospital. Of these, 61 (52%) had grade I carcinoma, 47 (40%) had grade II carcinoma and 9 (8%) had grade III carcinoma. For 13 (10%) patients, the histological diagnosis had been made in another hospital before the treatment in the Oulu University Hospital.

For study IV, 28 samples of carcinomatous and normal-appearing intestinal mucosa were obtained from a total of 14 patients admitted to the Oulu University Hospital for bowel resection. Nineteen of the patients were men and 15 women, and their ages ranged from 25 to 85 years (mean 55). Their cancer distribution was as follows: stomach 4, pancreas 2, papilla Vater 1, jejunum 3, and colon 4. In addition, 10 specimens of normal-appearing human esophagus, stomach, duodenum, ileum and proximal, transversal, and distal colon were obtained by endoscopic biopsy performed for diagnostic reasons at the Department of Internal Medicine. All the biopsies from patients with any pathological condition were excluded from the endoscopy group, i.e. the diagnosis of functional gastrointestinal disorder was set on the control subjects. The histologic confirmation of the diagnosis for all the samples was performed by pathologists with special competence in gastrointestinal pathology at the Department of Pathology, Oulu University Hospital.

All these subjects gave informed consent for the investigations, which were approved by the Ethical Committee of the University of Oulu.

4.2. Methods

4.2.1. Analysis of lipids and lipoproteins

Venous blood samples were drawn into ethylenediaminetetraacetic acid (EDTA) tubes after an overnight fast. Plasma was separated by centrifugation at 2000 rpm for 10 min and kept at 4°C until further analyses. Most of the analyses were done within two days after the blood samples had been drawn. Part of the plasma was frozen and kept at -20°C for apolipoprotein E phenotyping. The routine clinical laboratory tests were carried out in the Central Laboratory of the Oulu University Hospital and the lipid and lipoprotein analyses in the research laboratory of the Department of Internal Medicine.

An oral glucose tolerance test was performed on all the study participants in study I, except those previously known to have insulin-treated diabetics. After fasting blood had been drawn, the subjects were given a 75-g glucose load. Both 1-hour and 2-hour glucose and insulin concentrations were determined. The glucose concentrations were measured with the glucose dehydrogenase method (Diagnostics, Merck, Darmstadt, Germany). The serum insulin levels were measured using a two-site immunoenzymometric assay (AIA-PACK IRI, Tosoh Corp., Tokyo, Japan). The AUC for glucose (or insulin) was calculated according to Simpson’s rule (Winocour et al. 1992); AUC = (fasting glucose + (4x glucose

60 min.) + glucose

120 min.) /3. Diabetes mellitus was defined according to the WHO criteria (World Health Organisation 1980), i.e. known diabetes mellitus or fasting blood glucose ≥
6.7 mmol/L or 2-hour blood glucose ≥ 10.0 mmol/L at the oral glucose tolerance test (OGTT), and impaired glucose tolerance as fasting blood glucose <6.7 mmol/L, but 2-h blood glucose 6.7-9.9 mmol/L.

The VLDL fraction was separated from plasma by ultracentrifugation in a Kontron TFT 45.6 rotor at 105000 g for 18 h. The VLDL fraction was removed from the ultracentrifuged preparation by tube slicing. The plasma HDL cholesterol concentration was determined by mixing 1 ml of the VLDL-free fraction with 25 μl of 2.8% (w/v) heparin and 25 μl of 2 mol/L manganese chloride and by measuring the cholesterol concentration in the supernatant after centrifugation at 1000 g and for 30 min. (Manual of Laboratory Operations, Lipid Research Clinics Program 1974). The plasma LDL cholesterol concentration was calculated by subtracting the cholesterol concentration in HDL from that in the VLDL-free fraction. Cholesterol and triglycerides were analyzed enzymatically with kits of Boehringer Diagnostica, Mannheim GmbH, Germany, using a Kone Specific Selective Chemistry Analyzer (Kone Instruments, Espoo, Finland).

4.2.2. Apo E phenotyping

The apo E phenotype was determined from plasma with isoelectric focusing and immunoblotting techniques (Menzel & Utermann 1986, Ehnholm et al. 1986) using commercial antibodies (Daiichi Pure Chemical, Tokyo, Japan; Bio-Makor, Rehovot, Israel). The apo E genotype was also analyzed in a blind manner in a separate group of 16 diabetics and 44 controls with a solid-phase minisequencing kit (Affigene ApoE, Orion Pharmaceutica Biotechnology, Espoo, Finland). This test is based on PCR reaction amplification of DNA using a primer biotinylated at its 5’-end resulting in the production of 5’-biotinylated DNA fragments (Svänen et al. 1989) and the phenotype and genotype matched in all cases. Thus, the possible glycation of apo E did not affect the distribution of bands or the apo E phenotype classification.

4.2.3. Statistical methods

In the studies I and II, data storage and statistical analysis were performed using the statistical software package SAS, version 6.08 (Statistical Analysis System Institute, Inc., Cary, NC, USA). In study IV, the statistical analysis was carried out with the software package SPSS for Windows (Release 6.1, ©SPSS Inc.).

Continuous variables are expressed as means (95% confidence interval, (CI)) calculated with the CIA program (Gardner & Altman, 1989), though not in the case of logistic regression analysis. Unpaired t-test was used to assess the differences of the means between two groups and ANOVA to assess the differences between more than two groups. If a significant difference in ANOVA was obtained, Bonferroni’s method for multiple comparisons was applied. The triglyceride and insulin values were particularly skewed, and the analyses were performed after logarithmic transformation. When the number of subjects in one or more classes was small, the non-parametric equivalent for ANOVA (Kruskal-Wallis’ test) was used.
Allele frequencies were estimated with the gene counting method. The \( \chi^2 \) method was used to assess the distribution of apo E phenotypes to the Hardy-Weinberg equilibrium and the difference in genotype distribution between the groups. The odds ratio and 95% CI were computed between the subjects with at least one apo E \( \varepsilon4 \) allele and the subjects without the \( \varepsilon4 \) allele.

The stepwise multiple logistic regression analyses were performed to investigate the associations between the different variables with gallstone disease (study I). Estimated logistic coefficients, standard errors and odds ratios with 95% confidence intervals were given for each factor. The analyses were performed with the LOGISTIC procedure of SAS.

### 4.2.4. In situ hybridization

Detection of mRNA molecules by in situ hybridization is based on the hybridization of a nucleic acid probe to a complementary target mRNA (Zeller & Rogers 1989).

In study III, the localization of apo E mRNA was determined by the in situ hybridization procedure as previously described (Ylä-Herttuala et al. 1990).

Nucleotides 582-872 of human apo E (Zannis et al. 1984) were used for apo E antisense and corresponding sense riboprobe synthesis. Apo E cDNA was subcloned in the pBlue-scrpt SK (Stratagene, La Jolla, CA, USA) vector using standard techniques (Maniatis et al. 1989). Antisense and sense riboprobes were synthesized using T3 and T7 polymerases with \(^{35}\)S-UTP (NEN Life Science Products, Boston, MA, USA) as described (Ylä-Herttuala et al. 1990).

The samples for in situ hybridization were fixed in 4% buffered formaldehyde, sectioned (5\( \mu \)m), deparaffinized and rehydrated. The sections were treated with proteinase K, dehydrated and dried in vacuo. In situ hybridizations were performed on pretreated sections using \( 6 \times 10^5 \) cpm/ml of the labeled probe. The hybridization solution contained 50% formamide (Fluka Chemie AG, Buchs, Switzerland), 2 x SSC, 20 mM Tris, pH 7.4, 1 x Denhardt’s solution, 1 mM Na-citrate, pH 7.0. Denhardt’s solution contains 0.02 % Ficoll and 0.02 % bovine serum albumin (all from Sigma Chemical Co., St. Louis, MO, USA). After hybridization, the sections were washed three times (once for 30 min. and two times for 5 min.) in 4 x SSC at 37°C. Unspecifically bound probes were digested using RNase treatment. The sections were then washed at 37°C in 2 x SSC and 1 x SSC (15 min. each). The final wash for riboprobes was at 42°C in 1 x SSC for 15 min. The sample slides were then dehydrated, dried, dipped in Kodak NTB-2 autoradiographic emulsion (Eastman-Kodak, Rochester, NY, USA) and exposed for 3 weeks. The slides were counterstained with the hematoxylin and eosin stain. Nonhybridizing sense riboprobes were used as controls.

### 4.2.5. Immunocytochemistry

Immunocytochemical methods have been used to detect specific proteins in target tissues. Since mRNA does not always lead to protein synthesis (Ylä-Herttuala et al. 1991), it is necessary to use immunocytochemistry as a control to show the presence of the synthesized protein in the area expressing its mRNA.
In study IV, immunostainings were carried out with the following antibodies: goat polyclonal antibody against human apo E (Genzyme, West Malling, Kent, UK), mouse monoclonal antibody (mAb) against apo B-100 (MB47) (Young et al., 1986), mouse mAbs against human macrophages (KPI and PGM-1, DAKO, Copenhagen, Denmark), mAb against chromogranin A to detect endocrine cells in the epithelium (DAKO), mouse mAb against bovine and human LDL receptor (clone C7/RPN 537; Amersham Intl, New Hampshire, USA). Anti-goat and anti-mouse Ig G biotin conjugates (diluted 1:400, DAKO) and an avidin-biotin-horseradish peroxidase system (Zymed, San Francisco, CA, USA) were used for signal detection.

Tissue samples were fixed in 4% buffered formaldehyde, sectioned (5 μm) deparaffinized in xylene and rehydrated in a graded series of ethanol. Endogenous peroxidase activity was blocked by placing the slides in 3% H₂O₂ for 10 minutes. Thereafter, the slides were rinsed in distilled water and phosphate-buffered saline (PBS), and incubated in citrate buffer for 5 minutes at 95°C in a microwave oven to unmask the antigenic sites. The sections were then rinsed in PBS, and non-specific binding was blocked with fetal calf serum (FCS) for 20 minutes. The specimens were incubated with primary antibodies, diluted with PBS, and applied with biotinylated secondary antibody, after which, enzyme-labeled streptavidin was added.

The peroxidase reaction was performed using 3,3′diamino-benzidine (Sigma Chemical Co., St. Louis, MO, USA) or 3-aminoethyl-carbazole (AEC Single Solution® chromogen, Zymed) as a substrate, and counterstained with hematoxylin.

For the identification of apo E-positive cells, we used a double immunostaining procedure on the same sections: 3-aminoethyl-carbazole, which produces a red stain, was used in the apo E immunostaining, the Double Stain Enhancer® (Zymed) was then added to separate between the two chromogenic reactions, and the Vector SG substrate kit for peroxidase® (Vector Laboratories Inc., Burlingame, CA), which yields a blue-gray stain, was used in the macrophage and endocrine cell immunostaining.

Several staining methods were first compared in preliminary studies. The best morphological preservation and the clearest images were obtained with the streptavidin method. Endogenous peroxidase was blocked to remove non-specific reactivity, and control specimens showed no reduction in sensitivity. Permeabilization was carried out using different pretreatment protocols, and citrate treatment clearly enhanced the staining specificity, whereas pepsin treatment gave less specific staining. Additional blocking with 1% human serum did not improve the specificity of staining. Double staining with blue and red chromogens gave better contrast and specificity in comparison with 3,3′diamino-benzidine-based brown and black stains. Specimens from human liver and brain were used as positive controls, and irrelevant class- and species-matched immunoglobulins and incubations without the primary antibody were used as negative controls for the immunostainings.

### 4.2.6. Cell culture

HT29 human colonic adenocarcinoma cells were kindly provided by Dr. Jan Willem Kok, Groningen, the Netherlands. The differentiated HT29 G+ reversed cell line was obtained through isolation of a subpopulation of cells by growing the cells in medium with no sugars
(HT29 G⁻), followed by growth in medium containing glucose for more than 8 passages. The G⁻ reversed cells have - in contrast to undifferentiated G⁺ cells - morphological characteristics of intestinal epithelial cells (Babia et al. 1993). Both cell types express functional LDL receptors (Viallard et al. 1990, Viallard et al. 1992).

Undifferentiated G⁺ and differentiated G⁺ reversed HT29 colonic adenocarcinoma cells were cultured as previously described (Babia et al. 1993) in DMEM supplemented with 10% fetal bovine serum (Cibco, Gaithersburg, MD, USA), 2mM glutamine and antibiotics (penicillin, 100 units/ml; streptomycin sulfate, 100 mg/ml; amphotericin B, 0.25mg/ml). The cells were grown in a humidified atmosphere containing 5% CO₂, at 37°C. The media were changed every 2-3 days and the cells were used for the experiments in the passages 5-15.

4.2.7. Cell experiments

HT29 cells were grown on glass coverslips for immunofluorescence microscopy, and sparse monolayers were used for experiments. The cells were serum-starved for 24 h and human recombinant apo E3 (Biodesign, Denmark, Copenhagen) was then added to the culture to a final concentration of 0.5 μM. After 24 h incubation, the cells were washed with Hank’s balanced salt solution (Cibco, Gaithersburg, MD) and rapidly fixed.

4.2.8. Immunostaining and fluorescence microscopy of the cells

For staining with monoclonal anti-β-catenin (Zymed, San Francisco, CA) cells were grown on glass coverslips and fixed with 4 % formaldehyde in a cytoskeleton-stabilizing buffer (100 mM PIPES, 4 mM EDTA, 2 mM MgCl₂, pH 6.8) containing 0.1 % Triton X-100 (Wielkind and Swierenga, 1989). After several washes with phosphate-buffered saline (PBS; 145 mM NaCl, 10 mM phosphate, pH 7.4), the cells were post-fixed with cold methanol (-20°C) for 5 min. After repeated washings with PBS, the cells were incubated with 10 % FCS to saturate the non-specific protein-binding sites. This was followed by incubation with the primary antibody at +4°C, for 30 min., and then with Texas Red-conjugated goat anti-mouse antibody (Molecular Probes Eugene, OR). The cells were then mounted in Shandon mounting liquid (Immu-Mount, Pittsburgh, PA) and viewed under a Zeiss 405M microscope. Kodak TMAX 3200 ASA film was used for photography.

5. Results

5.1. Gallstone disease

The overall participation rate of hypertensive subjects was 87% (261 men, 258 women) and that of controls 88% (259 men, 267 women). In both genders, the hypertensive subjects had
significantly higher BMI and blood pressure values than the control subjects. The women were about one year older, because they were examined about one year later than the men.

The apo E phenotype distributions were in agreement with the Hardy-Weinberg distribution in the whole cohort and in men and women separately, and no significant differences in apo E distribution between the controls and hypertensives were observed (Table 3). The prevalence rate was 59% for hypertensive men using beta-blockers, 37% for diuretic users, 24% for calcium channel blockers and 46% for angiotensin-converting enzyme inhibitors. For hypertensive women, the corresponding rates were 57%, 48%, 25%, and 38%. Twenty-six men and 32 women among the control subjects were on chronic medication affecting blood pressure, since blood pressure lowering medication is commonly also used for other indications apart from hypertension. In a smaller portion (7 men and 14 women) of these 58 subjects, the same drugs were used to treat hypertension, although the subjects were not listed as special refund recipients at the time of randomization, because they had been entitled to the refund after the randomization, or had not applied for the right to refunding.

The prevalence of GSD was 9% in men and 19% in women. The basic characteristics and plasma lipoprotein levels of the subjects with and without gallstones among the hypertensive and control women and men are shown in the Table 4. Among both sexes, the prevalence of GSD tended to be higher in the patients with treated hypertension than in the controls, so that the relative risk for GSD was 1.2 (95% CI 0.8-1.7) for hypertensive women and 1.8 (1.0-3.1) for hypertensive men compared with the age- and sex-matched control groups. The type of hypertensive or hypolipidemic medication or was not associated with the risk of GSD.

Thirty-seven subjects (24%) in the GSD group and 133 subjects (15%) in the control group showed impaired glucose tolerance, and 25 subjects (17%) in the GSD group and 72 (8%) of the controls were diabetics. The prevalence of GSD was significantly higher in diabetics than in the normoglycemic group in both sexes (37% vs. 15% for women, respectively; OR 3.3, 95% CI 1.7-6.5; and 17% vs. 7% for men, OR 2.6, 95% CI 1.2-6.0). The prevalence of GSD was also higher in the impaired glucose tolerance group than in the normoglycemic group (25% vs. 15% for women, respectively; OR 2.2, 95% CI 1.3-3.8; and 23% vs. 15% for men, OR 2.1, 95% CI 1.0-4.4).

Triglycerides were higher in subjects with GSD than in those without GSD among both control and hypertensive women. In the female subjects within the highest BMI tertile (BMI over 28.6 kg/m²), the prevalence of gallstones was higher (27%) than in the lowest and middle tertiles (17 and 14%, respectively) ($\chi^2 = 10.7, p < 0.005$). In addition, the waist-hip ratio was higher in the women with GSD compared to those without gallstones (0.82 vs. 0.79, respectively, $p < 0.005$). In men, the prevalence of gallstones also tended to increase along with BMI, being 7, 9 and 11% in the lowest, middle and highest tertiles, respectively.

The apolipoprotein E phenotype distributions are presented in study I. In all females with at least one $\varepsilon2$ allele (E2/2, E2/3 and E2/4 phenotypes), the presence of gallstones (7%) was lower than in females without the $\varepsilon2$ allele (21%) ($\chi^2 = 5.0, p < 0.025$). The same trend was seen among both hypertensives and controls (Fig. 3). For females with vs. without the $\varepsilon2$ allele the odds ratio for GSD was 0.3 (95% CI 0.1-0.9). In the women with the $\varepsilon2$ allele, plasma cholesterol and LDL cholesterol were lower than in the women without the $\varepsilon2$ allele (Table 5). In men, no association between apo E and the presence of gallstones was observed. There were no differences in glucose and insulin levels or in the prevalence of diabetes between the individuals with or without the $\varepsilon2$ allele (data not shown).
In the stepwise multiple logistic regression model (including 2-hour glucose and insulin levels in OGTT, fasting blood glucose and serum insulin, apo E2 isoform, age, BMI, waist-hip ratio, plasma total, LDL and HDL cholesterol and plasma triglycerides as variables) elucidating the risk for GSD in women, the odds ratio for a phenotype with the E2 isoform (phenotypes E2/2, 2/3 and 2/4) was 0.28 (95% CI 0.08-0.93), for an elevation of one mmol/L in blood glucose in 2-hour OGTT 1.1 (1.0-1.2), one mU/L in serum insulin 1.1 (1.0-1.1), one mM in serum triglycerides 1.5 (1.2-2.0) and for one additional year of age 1.1 (1.0-1.1). The most powerful and consistent predictor for GSD among women was the 2-hour glucose level in OGTT, followed by fasting serum insulin, an apo E2 isoform, serum triglycerides and age. In men, the odds ratio for an elevation of one mmol/L in blood glucose in 2-hour OGTT was 1.1 (1.1-1.2), and LDL and HDL cholesterol provided protection against GSD with odds ratios of 0.6 (0.4-0.9) and 0.3 (0.1-0.8), respectively. For men, the strongest predictor of GSD was the 2-hour blood glucose in OGTT, followed by LDL and HDL cholesterol.

Table 3. Apo E phenotype distribution and allele frequencies in the control subjects and hypertensives

<table>
<thead>
<tr>
<th>Apo E Phenotype</th>
<th>Controls n=526 (100)</th>
<th>Hypertensives n=519 (100)</th>
<th>( \chi^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2/2</td>
<td>2 (0.4)</td>
<td>4 (0.8)</td>
<td></td>
</tr>
<tr>
<td>E2/3</td>
<td>37 (7.0)</td>
<td>22 (4.2)</td>
<td></td>
</tr>
<tr>
<td>E2/4</td>
<td>7 (2.3)</td>
<td>7 (1.3)</td>
<td></td>
</tr>
<tr>
<td>E3/3</td>
<td>301 (57.2)</td>
<td>319 (61.5)</td>
<td></td>
</tr>
<tr>
<td>E4/3</td>
<td>163 (31.0)</td>
<td>151 (29.1)</td>
<td></td>
</tr>
<tr>
<td>E4/4</td>
<td>16 (3.1)</td>
<td>16 (3.1)</td>
<td></td>
</tr>
</tbody>
</table>

Allele frequency

\( \varepsilon 2 \) 0.046 0.036 5.42 (p<0.37)
\( \varepsilon 3 \) 0.762 0.781
\( \varepsilon 4 \) 0.192 0.183

Percentage values in parentheses
**Table 4. Clinical characteristics of control and hypertensive women and men with and without gallstones.**

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Hypertensives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSD</td>
<td>No GSD</td>
</tr>
<tr>
<td><strong>Women:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>47</td>
<td>220</td>
</tr>
<tr>
<td>Age / years</td>
<td>54 (52-55)</td>
<td>51 (51-52)</td>
</tr>
<tr>
<td>BMI / kg/m²</td>
<td>28 (26-30)</td>
<td>26 (25-26)</td>
</tr>
<tr>
<td>Systolic blood pressure / mmHg</td>
<td>145 (138-153)</td>
<td>138 (135-141)</td>
</tr>
<tr>
<td>Diastolic blood pressure / mmHg</td>
<td>84 (81-88)</td>
<td>82 (80-83)</td>
</tr>
<tr>
<td>Alcohol consumption / g/week</td>
<td>24 (13-36)</td>
<td>23 (18-27)</td>
</tr>
<tr>
<td>Smoking / pack-years</td>
<td>13 (9-17)</td>
<td>11 (10-13)</td>
</tr>
<tr>
<td>Smokers</td>
<td>n=15</td>
<td>n=58</td>
</tr>
<tr>
<td>Physical activity / score</td>
<td>2.8* (2.5-3.0)</td>
<td>3.1 (3.0-3.2)</td>
</tr>
<tr>
<td>fB-glucose / mmol/L</td>
<td>4.5* (4.3-4.8)</td>
<td>4.3 (4.2-4.4)</td>
</tr>
<tr>
<td>fS-insulin / μU/L</td>
<td>12* (10-14)</td>
<td>9 (8-10)</td>
</tr>
<tr>
<td>2-hr glucose / mmol/L</td>
<td>6.0* (5.4-6.8)</td>
<td>5.3 (5.0-5.5)</td>
</tr>
<tr>
<td>2-hr insulin / mmol/L</td>
<td>78 (55-101)</td>
<td>53 (5-59)</td>
</tr>
<tr>
<td>AUC (glucose)</td>
<td>12.4* (11.1-13.7)</td>
<td>10.9 (10.4-11.4)</td>
</tr>
<tr>
<td>AUC (insulin)</td>
<td>142* (111-171)</td>
<td>105 (96-113)</td>
</tr>
<tr>
<td>Plasma cholesterol / mmol/L</td>
<td>5.7 (5.4-6.1)</td>
<td>5.5 (5.4-5.6)</td>
</tr>
<tr>
<td>LDL cholesterol / mmol/L</td>
<td>3.4 (3.1-3.7)</td>
<td>3.3 (3.2-3.4)</td>
</tr>
<tr>
<td>HDL cholesterol / mmol/L</td>
<td>1.5 (1.4-1.6)</td>
<td>1.6 (1.5-1.7)</td>
</tr>
<tr>
<td>Triglycerides / mmol/L</td>
<td>1.5* (1.2-1.9)</td>
<td>1.1 (1.0-1.2)</td>
</tr>
<tr>
<td><strong>Men:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>17</td>
<td>242</td>
</tr>
<tr>
<td>Age / years</td>
<td>54 (51-57)</td>
<td>51 (50-51)</td>
</tr>
<tr>
<td>BMI / kg/m²</td>
<td>28 (26-30)</td>
<td>26 (26-27)</td>
</tr>
<tr>
<td>Systolic blood pressure / mmHg</td>
<td>153 (141-165)</td>
<td>147 (145-149)</td>
</tr>
<tr>
<td>Diastolic blood pressure / mmHg</td>
<td>91 (86-96)</td>
<td>89 (87-90)</td>
</tr>
<tr>
<td>Alcohol consumtpion / g/week</td>
<td>56 (8-103)</td>
<td>92 (79-105)</td>
</tr>
<tr>
<td>Smoking / pack-years</td>
<td>26 (8-50)</td>
<td>17.3 (15-19)</td>
</tr>
<tr>
<td>Smokers</td>
<td>n=5</td>
<td>n=97</td>
</tr>
<tr>
<td>Physical activity / score</td>
<td>3.1 (2.5-3.66)</td>
<td>3.1 (3.0-3.2)</td>
</tr>
<tr>
<td>fB-glucose / mmol/L</td>
<td>5.0 (3.9-6.14)</td>
<td>4.5 (4.4-4.7)</td>
</tr>
<tr>
<td>fS-insulin / μU/L</td>
<td>12 (9-16)</td>
<td>13 (12-15)</td>
</tr>
<tr>
<td>2-hr glucose / mmol/L</td>
<td>6.0 (3.4-7.9)</td>
<td>5.2 (5.0-5.5)</td>
</tr>
<tr>
<td>2-hr insulin / mmol/L</td>
<td>64 (42-84)</td>
<td>54 (47-60)</td>
</tr>
<tr>
<td>AUC (glucose)</td>
<td>14.0 (10.0-18.0)</td>
<td>11.9 (11.4-12.5)</td>
</tr>
<tr>
<td>AUC (insulin)</td>
<td>169 (95-243)</td>
<td>123 (111-134)</td>
</tr>
<tr>
<td>Plasma cholesterol / mmol/L</td>
<td>5.1 (4.5-5.4)</td>
<td>5.8 (5.7-5.9)</td>
</tr>
<tr>
<td>LDL cholesterol / mmol/L</td>
<td>3.2 (2.8-3-6)</td>
<td>3.8 (3.6-4.0)</td>
</tr>
<tr>
<td>HDL cholesterol / mmol/L</td>
<td>1.1 (1.0-1.2)</td>
<td>1.2 (1.1-1.3)</td>
</tr>
<tr>
<td>Triglycerides / mmol/L</td>
<td>1.6 (1.2-2.0)</td>
<td>1.6 (1.5-1.7)</td>
</tr>
</tbody>
</table>

The values are presented as means (95% confidence interval).
The a: p < 0.05 for the difference compared with control subjects without GSD
b: p < 0.05 for the difference compared with hypertensive subjects without GSD
*) Standard deviation 7.3, 95% confidence interval calculation not valid.
Table 5. Age, BMI, plasma total cholesterol, LDL and HDL cholesterol and triglycerides in subjects with the ε2 allele of apo E (phenotypes E2/2, 2/3 and 2/4) and subjects without the ε2 allele (E3/3, 4/3 and 4/4).

<table>
<thead>
<tr>
<th></th>
<th>Women:</th>
<th>Men:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E2/2, 2/3 and 2/4</td>
<td>E2/2, 2/3 and 2/4</td>
</tr>
<tr>
<td>Age / Years</td>
<td>52 (49-53)</td>
<td>52 (51-52)</td>
</tr>
<tr>
<td>BMI / kg / m²</td>
<td>26 (25-27)</td>
<td>28 (27-28)</td>
</tr>
<tr>
<td>Cholesterol / Mmol/L</td>
<td>4.9 (4.6-5.2)</td>
<td>5.7 (5.6-5.8)</td>
</tr>
<tr>
<td>LDL cholesterol / mmol/L</td>
<td>2.6 (2.3-2.8)</td>
<td>3.5 (3.4-3.6)</td>
</tr>
<tr>
<td>HDL cholesterol / Mmol/L</td>
<td>1.6 (1.5-1.7)</td>
<td>1.5 (1.4-1.5)</td>
</tr>
<tr>
<td>Triglycerides / Mmol / L</td>
<td>1.3 (1.1-1.5)</td>
<td>1.4 (1.3-1.5)</td>
</tr>
</tbody>
</table>

The values are presented as means (95% CI). P-values over 0.05 are considered not significant (NS).

Fig. 3. Prevalence of gallstone disease in control, hypertensive and all women with at least one ε2 allele (phenotypes E2/2, 2/3 and 2/4, black bars) and without the ε2 allele (phenotypes E3/3, 4/3 and 4/4, shaded bars). For a: OR=0.28, 95% CI 0.08-0.92.
5.2. Cancer

5.2.1. Apo E, lipoproteins and colon cancer

In the group of 135 patients with adenomas, altogether 217 adenomas were found. Eighty-seven patients (64%) had a solitary adenoma, the mean number of adenomas per patient being 1.65 (range 1-9). Fifty-nine percent of the patients had only tubular adenomas, whereas the rest of the patients had adenomas with tubulovillous or villous growth. Mild, moderate or severe histological dysplasia was found in 55%, 37% and 8% of the patients, respectively. The classification of the patients according to the location of their most proximal adenoma or carcinoma is shown in paper II. For further analysis, the patients were divided into a group (n=40) with the most proximal tumor in the caecum or the ascending or transverse colon (proximal tumors) and a group (n=95) with the most proximal tumor in the descending colon, the sigmoid colon, or the rectum (distal tumors). Fourteen of the 135 patients with adenomas had tumors in both the proximal and the distal colorectum and were thus classified into the proximal tumor group. No difference was observed in the diameters of the proximal and distal carcinomas (47.5 vs. 46.8 mm, respectively). The age and sex of the patients and the age- and sex-adjusted plasma cholesterol, triglyceride, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol levels of the patients with proximal and distal tumors and the control subjects are shown in Table 6.

In the patients with proximal carcinoma of the colon, the total, LDL and HDL cholesterol levels were lower than in the control subjects, and the total cholesterol level was also lower than in the patients with distal carcinoma. In patients with distal carcinoma, the total and HDL cholesterol levels were lower than in the control subjects.

The apo E phenotypes for the study groups are shown in paper II. When the location of the tumor was not taken into account, the allelic frequencies of apo E did not differ between the patients with carcinomas (with frequencies of 0.029, 0.836, and 0.135 for e2, e3 and e4 alleles, respectively), the patients with adenomas (0.052, 0.822 and 0.126 ) and the control subjects (0.033, 0.786 and 0.181). However, the frequency of the e4 allele tended to be lower in the patients with carcinomas and adenomas than in the control subjects. When the location of tumor was taken into account, the allelic frequency of the e4 allele was significantly lower in patients with proximal tumors than in those with distal tumors (0.156) ($\chi^2$ =6.6; p < 0.01) or the control group (0.181) ($\chi^2$=10.9; p < 0.01). This was observed consistently in both sexes: the frequency of the e4 allele was low (0.059) in the men with proximal tumors compared with the control men (0.172) ($\chi^2$ = 7.4; p < 0.01) and tended to be low in the women with proximal tumors (0.094) compared with the control women (0.189)($\chi^2$ = 3.19; p =0.07). Among the patients 65 years or younger (n=130), the frequencies of e4 were 0.079 and 0.172 in the patients with proximal or distal tumors, respectively. Among the patients older than 65 years, (n=127), the frequencies of e4 were 0.067 and 0.140 in the patients with proximal and distal tumors, respectively. The frequency of e4 was lower in the patients with proximal adenomas (0.075) compared with control subjects (0.181)($\chi^2$=5.5; p < 0.05). Also, the frequency of e4 was low in the patients with proximal carcinomas (0.073) compared with the control subjects (0.181)($\chi^2$=5.8; p <0.05). Furthermore, no single patient with a proximal tumor was homozygous for the e4 allele, but the difference from the patients with distal tumor was not statistically significant, possibly due to the low overall
incidence of the E4/4 phenotype. In the subjects with the e4 allele of apo E compared with the subjects without the e4 allele, the odds ratio for proximal carcinoma of the colon was 0.35 (95% CI, 0.14-0.86) (compared with control subjects), and the odds ratio for proximal adenoma of the colon was 0.36 (95% CI, 0.14-0.89).

Table 6. Sex, age and age-sex-adjusted lipids and lipoproteins in patients with proximal or distal colon adenoma or carcinoma

<table>
<thead>
<tr>
<th></th>
<th>Carcinoma (n=122)</th>
<th></th>
<th>Adenoma (n=135)</th>
<th></th>
<th>Controls (n=199)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proximal (n=41)</td>
<td>Distal (n=81)</td>
<td>Proximal (n=40)</td>
<td>Distal (n=95)</td>
<td></td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>23/18</td>
<td>31/50</td>
<td>27/13</td>
<td>57/38</td>
<td>93/106</td>
</tr>
<tr>
<td>Age (years)</td>
<td>67.5 (62.8-72.1)</td>
<td>66.8 (64.4-69.1)</td>
<td>64.3 (60.6-68.0)</td>
<td>61.4 (59.3-63.5)</td>
<td>57.8 (57.5-58.1)</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.62 (4.29-4.95)</td>
<td>5.27 (5.01-5.54)</td>
<td>5.53 (5.20-5.87)</td>
<td>5.62 (5.41-5.82)</td>
<td>5.82 (5.68-5.97)</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.32 (1.15-1.49)</td>
<td>1.54 (1.37-1.72)</td>
<td>1.64 (1.40-1.89)</td>
<td>1.48 (1.30-1.66)</td>
<td>1.50 (1.40-1.61)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.95 (2.64-3.26)</td>
<td>3.33 (3.11-3.54)</td>
<td>3.57 (3.27-3.86)</td>
<td>3.65 (3.46-3.85)</td>
<td>3.71 (3.58-3.83)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.07 (0.96-1.17)</td>
<td>1.13 (1.6-1.20)</td>
<td>1.20 (1.08-1.32)</td>
<td>1.24 (1.17-1.31)</td>
<td>1.35 (1.30-1.39)</td>
</tr>
</tbody>
</table>

Values are expressed as means with 95% CI in parentheses.

*P < 0.01 for the difference between the group and the controls after analysis of variance.

**P < 0.01 for the difference between the group and the patients with proximal adenoma after analysis of variance.

*P < 0.01 for the difference between the group and the patients with distal carcinoma after analysis of variance.

5.2.2. Apo E, serum lipids, apo E and cancer of the breast and prostate

The allelic frequencies of apo E did not differ between the patients with breast cancer (with frequencies of 0.040, 0.784, and 0.175 in a prospective group; 0.026, 0.805 and 0.169 in a retrospective group for e2, e3 and e4 alleles, respectively), the patients with BBD, (0.036, 0.780 and 0.182 in a prospective group; 0.045, 0.826 and 0.129 in a retrospective group) and the control women (0.0054, 0.764 and 0.182). In addition, the apo E allelic frequencies were similar in the men with prostate cancer (0.031, 0.303 and 0.665), BPH (0.037, 0.760 and 0.203) and in the control men (0.037, 0.760 and 0.203). The apolipoprotein E phenotype distributions are presented in paper III.

The serum levels of cholesterol and its subfractions in cancer and non-cancer patients are presented in study III. The women with breast cancer were older and had higher mean
plasma cholesterol and LDL cholesterol than patients with benign breast disease, but after adjustment for age, there were no differences between the patients with breast cancer and those with BBD. The men with prostate cancer had significantly higher mean values of total cholesterol, LDL cholesterol and HDL cholesterol than the men with BPH. There were no differences in age or body mass indexes between the patients with prostate cancer and BPH. The male prostate cancer patients with the apo E isoforms E3 and E4 had significantly higher serum total, LDL and HDL cholesterol than the controls with E3 and E4, whereas the group of men with the apo E2 isoform showed no significant differences in the serum cholesterol levels between prostate cancer and BPH.

5.3. Experimental studies

5.3.1. Apolipoprotein E expression in human gastrointestinal tract

Both immunoreactive apo E protein and apo E mRNA were present throughout the stomach, small intestine and colon (study IV). The phagocytes of lamina propria were positive for apo E (Fig. 4 A and B), but there was some variation in the number of positive cells and in the staining intensity. The colonic macrophages in superficial lamina propria were more strongly positive for apo E than those in the small intestine, where the most positively stained cells were dendritic cells and macrophages in the follicular centers of lymphoid nodules (Fig.5 B and C). Of the epithelial cells, gastric chief cells (Fig 5A), some areas of intestinal metaplasia and a subpopulation of granulated endocrine cells (Fig 4C and D) were also positive. The solid carcinomas contained intensely positive macrophages lining the tumor (Fig 4C and D) in the same area where the LDL receptor expression was most intense. Apo E was colocalized with KP1 and Chromogranin A antibodies (paper IV). Control immunostainings with irrelevant class- and species-matched immunostainings and in situ hybridizations with control probes were negative (paper IV).
Figure 4. In situ hybridization with a $^{35}$S-labeled antisense riboprobe for apo E mRNA in colon. Figures A-D: Apo E is present in macrophages in the lamina propria of normal colon (A,D; arrows), and in the endocrine cells at the crypt base (C,D; arrows). Figures E-F: Expression of apo E mRNA in colon cancer. The tumor area is lined by apo E-positive cells. A, C, and E were photographed using polarized light epiluminescence and B,D and E are phase contrast micrographs from the same region.
Figure 5. Apo E in gastric epithelium and in small intestine. A: Immunostaining for apo E in gastric epithelium (antibody dilution 1:100). Apo E is present in chief cells. B: Immunostaining for apo E in follicular centers of small intestine. Apo E is present in macrophages and dendritic cells. C: In situ hybridization with a $^{35}$S-labeled antisense riboprobe for apo E from a corresponding region. D: Control hybridization with a sense oligonucleotide probe from a corresponding region.
5.3.2. Effect of apo E on the HT29 human colon adenocarcinoma cell line

The organization of b-catenin was analyzed by immunofluorescence microscopy in study IV. In apo E-treated, undifferentiated HT29 G+ cells, b-catenin appeared as an intense line in the areas of cell-to-cell interaction (Fig. 6A), whereas in control cells cultured without apo E, the distribution of b-catenin was weaker and more diffuse (Fig 6B). The polarity marked by the formation of lateral cell-cell contacts seemed to be enhanced in apo E-treated HT29 G+ cells (Fig 6A). There was no clear effect of apo E on differentiated HT 29 G+ reversed cells (data not shown).

Fig. 6. Immunofluorescence micrographs of HT29 G+ cells incubated for 24 h with human recombinant apo E. Apo E-treated cells (A) and control cells. (B) Bar = 10 μm.
6. Discussion

6.1. Validity of the study population and patient selection

There are four potential explanations for the positive association observed in a genetic association study (Cooper & Clayton 1988, Lander & Schork 1994). First, the gene has a causative role in the development of the disease. In that case, it is expected that the same positive association will also occur in other populations. Second, another gene with a causative role for the disease is in linkage disequilibrium with the marker allele. It is therefore expected that the same association will or will not be seen in other studies. Third, a selection or another bias has lead to a false positive association. Fourth, incidental findings may be made if the a priori hypothesis is not carefully evaluated and if there are no mechanistic studies to explain the possible association.

The control cohort used in the present studies was randomly selected among a middle-aged population not entitled to a special refund for antihypertensive medication. It is estimated that no more than 10% of the Finnish middle-aged population have this benefit. It is likely that the control cohort represents the general middle-aged population as a whole, particularly as the participation rate was excellent. Furthermore, the apo E allele frequency in the present study population is comparable to those previously reported for the Finnish general population. It is also to be emphasized that no significant deviation from the Hardy-Weinberg equilibrium was seen in the genotype distributions either in the whole study population or in any of the subgroups presented. One limitation in the present studies was the small number of apo E2 subjects, which makes association studies prone to false positive findings.

The population-based control group was not routinely screened for cancer, which could naturally bias the study, but a careful clinical examination was made on all of these patients. There was no overlap between the study groups, i.e. the subjects with carcinoma in their patient history were excluded from the benign patient group. As the patients with breast cancer were older than their counterparts, the lipid parameters in these groups may not be fully comparable.
6.2. The observed role of apo E phenotype in comparison with the established risk factors for the prevalence of gallstone disease

According to the present findings on the control (non-hypertensive) subjects, the prevalence of GSD is 17.6% in Finnish middle-aged women and 6.6% in men. The prevalences of GSD in neighboring Sweden have recently been reported to be 11% and 25% in women aged 40 and 60 years, respectively, and 4 and 15% in men (Muhrbeck & Ahlbeck 1995), which prevalences are quite similar to those observed in the present study.

The classical risk factors for gallstone disease, e.g. age, gender and obesity were included in study I to estimate the power of potential new risk factors. Since all the classical risk factors appeared important in the analysis, the present population-based study is not biased and is applicable to the search for potential new risk factors. The etiology of cholesterol and pigment gallstones is somewhat different, and it is notable that the subjects with cholesterol and pigment stones were not analyzed separately. Cholesterol gallstones are overwhelmingly the most prevalent gallstones (74%) in the Western countries (Juvonen et al. 1993, Cetta et al. 1995, Mendez-Sanchez 1995), and it seems probable, that the risk profile described in study I reflects the risk profile of cholesterol cholelithiasis. The prevalence of GSD in men was less than half of that in women, and a larger population of men would have been needed to assess all the risk factors for GSD.

The hypertensive subjects had higher BMI, triglyceride, glucose and insulin levels than the control group, showing the classical risk factor cluster called the metabolic syndrome. All of these risk factors for coronary heart disease also seem to be risk factors for GSD according to the present and previous studies (Bennion et al. 1978, Diehl et al. 1987, MacClure et al. 1989, Muhrbeck & Ahlbeck 1995, Attili et al. 1997). Some in-patient and autopsy studies on the association of diabetes and GSD have been controversial (Honoré et al. 1980, Hayes et al. 1992), but the present results are in agreement with an earlier epidemiological study performed on a free-living population sample (Santis et al. 1997).

According to study I, the ε2 allele of apolipoprotein E may protect middle-aged women from the development of GSD. Impaired glucose tolerance, high plasma triglycerides and advancing age are risk factors for GSD in a stepwise multiple logistic regression model. In men, the risk factor profile seems to be somewhat different, as low LDL and low HDL cholesterol, but not the apo E allele ε2 are significant risk factors. In both men and women, GSD seems to be associated with impaired glucose metabolism, obesity and hypertension.

The association between altered plasma lipoprotein levels and gallstone disease is controversial. Many studies have not detected any association between plasma lipids and gallstones, whereas decreased plasma HDL and increased LDL cholesterol and triglycerides are shown to be related to the risk for GSD in several studies. Therefore, the protective role of LDL and HDL against GSD in men in the logistic regression model seems rather questionable and may be incidental finding due to the small number of men with GSD.

6.3. Apolipoprotein E, gallstone composition and crystallization rate

Previous studies have revealed significant genetic components in biliary cholesterol saturation and biliary lipid composition (Kesäniemi et al. 1989), both of which may be affected
by apo E (Juvonen et al. 1993), as the Finnish gallstone patients with the E4 isoform have been found to exhibit increased numbers, sizes and cholesterol contents of gallstones, a higher frequency of cholesterol crystals, and much faster crystallization in their gallbladder bile compared with patients without the E4 isoform (Juvonen et al. 1993). These findings suggest that fast crystallization caused by altered cholesterol metabolism and biliary cholesterol hypersecretion might mediate the high risk of gallstone formation in case of apo E4. On the other hand, the recent findings on the similar bile crystallization, cholesterol contents and number of stones, but a lower cholesterol saturation index in the case of apo E4 (Van Erpecum et al. 1998) are consistent with the data reported from Chile (Mella et al. 1997) and Spain (Bertomeau et al. 1996). Although impaired gallbladder emptying leads to an increased risk of gallstone formation and recurrence (Portincasa et al. 1996), no association was found in this respect between subjects with and without the 4 allele (Portincasa et al. 1996). In the present study, no association between the apo E4 isoform and GSD was found in a population level. There are possible explanations for the contradictory results: First, the frequency of the apo E4 isoform in northern Europe is higher than in other populations studied. Second, the number of pigment stones in these studies may have caused different results, because Juvonen et al. did not report the number of pigment stones (Juvonen et al. 1993). Van Erpecum et al. suggested that possible differences may also be related to errors in apo E phenotyping, since no apo E genotyping was performed in the Finnish study (Van Erpecum et al. 1998). As the phenotyping method used in the present and previous studies in our laboratory was compared with apo E genotyping in a blinded manner and no differences were found, the methodological difference may not explain the different results.

If the high ratio of cholesterol to bile salts in the bile of subjects with the apo E4 isoforms does not explain the observed association between the apo E polymorphism and the prevalence of GSD, what other explanations can be given for the phenomenon? Apo E, which is present in gallbladder bile (Juvonen et al. 1993), may also act as a nucleating or antinucleating factor in itself. For example, apo E binds to monosodium urate crystals (Terkeltaub et al. 1991) and promotes polymerization of the 42-amino acid amyloid β-peptide (Ma et al. 1994). Because the serum apo E levels are highest in the case of the apo E2 isoform, intermediate for the apo E3 isoform, and lowest in the case of the apo E4 isoform (Reilly et al. 1991, Havekes et al. 1988, Boerwinkle & Davignon 1988), possibly due to the amounts of apolipoprotein E excretion in bile in the rank order: apo E4 > apo E3 > apo E2, the lowest biliary apolipoprotein E levels in the case of apo E2 might be protective against GSD. Apo E may also interact with proteins that control the nucleation of cholesterol crystals and their growth into stones.

6.4. Apo E and enterohepatic cholesterol metabolism: a possible link between associations of apo E polymorphism on hypercholesterolemia, gallstone disease and colon cancer?

Under normal conditions, bile acid synthesis is negatively related to cholesterol absorption (Gylling & Miettinen 1992), and a high cholesterol intake thus enhances bile acid synthesis (Lin & Connor 1980, Miettinen 1988). It has also been shown that apo E polymorphism has
an effect on cholesterol absorption in such a way that the absorption efficiency is lowest in the apo E2 subjects and highest in the apo E4 subjects (Kesäniemi et al. 1987, Gylling & Miettinen 1992, ). By contrast, fecal bile acid output is greatest in the subjects with the apo E2 allele and lowest in those with the apo E4 allele (Kesäniemi et al. 1987). As the effect of apo E polymorphism on gallstone formation is further confirmed by the present study, the observed genotype effect on bile acid composition appears to be clinically important.

Alterations in intestinal cholesterol and bile acid metabolism have long been postulated to contribute to the pathogenesis of colon cancer. Abundant fecal excretion of lithocholic acid and deoxycholic acid has been reported in patients with carcinoma and adenomatous polyps of the colon (Reddy 1977), and experimental carcinogenesis studies in animals (Hori et al. 1998, Rigas et al. 1994) have supported the theory that high concentrations of neutral and acid steroids in the colon lumen have a promoting role in colon cancer. In patients with the e4 allele of apo E, the levels of biliary deoxycholic acid are low (Miettinen 1991), which may be related to the low prevalence of adenoma and carcinoma.

According to study IV, macrophages and endocrine cells are the main source of apolipoprotein E in the gastrointestinal tract. Expression of apo E in macrophages facilitates cholesterol efflux from the cholesterol-loaded macrophages in exogenous acceptors (Lin et al. 1998), and transgenic mice overexpressing apo E in the intestine have markedly reduced plasma cholesterol levels and post-prandial hypertriglyceridemia (Shimano et al. 1994). Apo E synthesized in the intestine might thus influence the cellular cholesterol homeostasis in humans. However, the possibility, that apo E polymorphism operates via differences in luminal cholesterol and bile acid delivery must be taken cautiously, since no obvious mechanism by which apo E polymorphism would affect intestinal cholesterol uptake has been established. Recent studies on apo E knockout mice have even shown that apo E -/- and apo E +/- mice have similar cholesterol absorption values (Woollett et al. 1995), suggesting that apo E may not have a direct effect on cholesterol uptake from the lumen of the small intestine. The cholesterol synthesis rates in the liver and extrahepatic tissues were also identical in both genotypes, suggesting that the differences between the apo E phenotypes in cholesterol absorption result from alterations in LDL receptor-dependent uptake of lipoproteins by the liver (Woollett et al. 1995). Since different species have marked differences in their lipoprotein metabolism, no animal model is fully applicable to the study of human cholesterol homeostasis, but due to the contradictory results discussed above, the effects of apo E phenotype on intestinal cholesterol metabolism will need to be confirmed in different populations.

6.5. Apo E polymorphism, serum cholesterol levels and the risk of cancer

Apo E has many potential functions which may be related to carcinogenesis, as it inhibits proliferation of endothelial, melanoma and breast cancer cells and modulates steroid production (Dyer & Curtiss 1988) and the inflammatory process (Mistry et al. 1995, Stöhr et al. 1999). Both the apo E4/4 phenotype and hypercholesterolemia enhance the differentiation of monocytes towards phagocytically active macrophages (Rothe et al. 1996, Stöhr et al. 1998), and this might theoretically cause systemic immunological abnormalities, which
could explain the association between low cholesterol and the risk of cancer. The enhanced host defense might be protective against cancer, whereas the cellular differentiation toward a phagocytically active macrophage might accelerate the atherosclerotic process in the vessel wall.

However, the present data indicate that apo E polymorphism may not be a general link between low cholesterol and an increased risk of cancer, as the apo E phenotype distribution was not altered in instances of cancer of the breast or prostate. The possible association between apo E polymorphism and colon cancer may be due to differences in biliary lipid composition and bile acid distribution between the apo E phenotypes, or to a local effect of macrophage-derived apo E on cell transformation and angiogenesis, as no accumulation of apo E-positive macrophages was seen in the breast or prostate tissue samples, although occasional apo E-positive cells were present in the samples (data not shown).

No differences in serum total, LDL and HDL cholesterol and triglycerides between women with breast carcinoma and benign breast disease were found in the present study. The absence of an association between serum cholesterol and cancer in women was also observed in other studies (Salonen 1982, Kagan et al. 1981), and may be explained by a hormonal mechanism. In addition, the patient selection may have influenced the study results. First, in this consecutive series of patients with breast disease, the women with breast carcinoma were older than the patients with benign breast disease and the control population, and the results may be biased in spite of adjustment for age. Second, mortality may have influenced the study population in the retrospective study group.

The basis for the relation between low serum cholesterol and cancer observed in several studies is unclear, and the reduced serum cholesterol may be secondary to the presence of the tumor and may be related to enhanced elimination of LDL in malignancy (Henriksson et al. 1989). No correlations between serum cholesterol and tumor size was reported in papers II and III. However, LDL receptor activity was not taken into account. In immunocytochemical samples from colorectal carcinomas, the expression of LDL receptors was strongest in the tumor margin, which is in agreement with previous studies (Peterson et al. 1985).

On the other hand, comparisons of cancer rates internationally have revealed correlations between diets high in saturated fat and animal fat and the cancer risk, primarily for tumors of the colon and breast (Willet 1989), but it has also been suggested, that the high plasma level of HDL cholesterol could be a biochemical marker of women at increased risk for breast cancer. In our study population, however, the serum cholesterol levels were not altered in the women at the time of the breast cancer diagnosis was made, or a year after the breast surgery, suggesting that serum cholesterol might not be a major risk factor for breast cancer.

A significant association between high plasma cholesterol and prostate cancer was observed. The total, LDL and HDL cholesterol levels were high in the patients with prostate cancer compared to the patients with benign prostate hyperplasia. In a comparison between the apo E isoforms, serum total, LDL and HDL cholesterol and triglycerides were highest in the E4 phenotype group, and differences in cholesterol levels between the patients with prostate cancer and benign prostate hyperplasia were seen in the apo E phenotype groups E3 and E4. The lack of association between prostate cancer and high cholesterol levels in the apo E phenotype group E2 is presumably due to the small number of cancer patients having the apo allele e2 (n=7) and can therefore be interpreted as incidental. Overall, the present study does not provide support for the hypothesis that the apo E phenotype catego-
ries related to low cholesterol levels, namely those with E2, might be more susceptible to common cancers, such as breast and prostate carcinoma.

6.6. Potential functions of apo E in cell regulation

The growth and behavior of a malignant tumor is governed by the interaction of macrophages, lymphocytes, mast cells and cancer cells, i.e. the efficiency of the host defense elements. Apo E has been shown to have potent effects on lymphocyte function, i.e. to suppress the production of interleukin-2 and to inhibit lymphocyte proliferation (Mistry et al. 1995). The immunomodulatory properties of apo E seem to be influenced by apo E polymorphism, since the apo E 4/4 phenotype correlates with the altered macrophage colony-stimulating factor (M-CSF) dependent differentiation of monocytes based on a more CD16a-positive phenotype that allows IgG-dependent phagocytosis (Stöhr et al. 1998) and further accelerates the inflammatory process. However, this theory is mainly based on experimental models, and the clinical significance of the immunological differences between the apo E phenotypes is not known. Our recent study on lipoprotein changes during acute and chronic inflammation in rheumatoid arthritis revealed no differences in the apo E phenotype distribution between patients with rheumatoid arthritis and healthy controls. Furthermore, there were no correlations between the apo E phenotypes, apo E serum levels and acute phase proteins (Niemi et al. unpublished data).

Apo E efficiently inhibits the growth of endothelial cells and smooth muscle cells in cell cultures (Vogel et al. 1994, Ishigami 1998), and it has been suggested that apo E suppresses angiogenesis by inhibiting the effects of angiogenic growth factors, such as bFGF. Angiogenesis is an important compensatory mechanism of tissue ischemia in occlusive vascular diseases, but it is also essential for progressive tumor growth. Accordingly, angiogenesis constitutes a very promising therapeutic target both in atherosclerosis and in cancer. However, the present and previous studies (Vogel et al. 1994) have shown that the antiproliferatory effect of apo E is not specific to endothelial cells, and it seems probable that apo E is a modulator of cell differentiation rather than a novel angiogenic factor.

Apo E is capable of inhibiting the growth of several tumor cell lines (Vogel et al. 1994), including breast carcinoma cells, melanoma cells and Kaposi’s sarcoma cells. (Browning et al. 1994) The underlying mechanisms are not known, but due to its high affinity to heparan sulfate proteoglycans and numerous receptors (e.g. LDL receptor and LRP), apo E may affect cellular homeostasis in many ways. The ability of carcinomas to invade and metastasize largely depends on the degree of epithelial differentiation within the tumor. E-cadherin-mediated cell-cell adhesion has been shown to prevent invasiveness of human carcinoma cells (Frixen et al. 1991), and mechanisms that lead to a loss of cell-cell adhesion (mutation, loss of catenin expression, alterations in phosphorylation) are thought to contribute to a more metastatic phenotype (Sommers et al. 1994) As our results suggest that apo E may enhance cell polarity, we hypothesize that apo E might improve the assembly of intercellular junctions visible as alterations in β-catenin distribution, and thus induce contact-dependent growth inhibition of cell growth and migration.
7. Conclusions

1. Genetically determined Apo E polymorphism affects gallstone formation in the population. The ε2 allele seems to protect at least women against the development of gallstones. This is probably related to the inhibitory effects of the ε2 allele of apo E on cholesterol crystallization and bile composition. The present findings suggest that impaired glucose tolerance and diabetes can also be added to the list of risk factors for GSD.

2. The association between low cholesterol and colorectal carcinoma may be at least partly caused by genetic factors, such as the apo E phenotype, which affect both cholesterol metabolism and the susceptibility to carcinoma of the proximal colon. Furthermore, the findings emphasize the need to distinguish between proximal and distal tumors when evaluating the risk factors for colorectal adenoma and carcinoma, as the risk factor profile may be somewhat different. However, apo E polymorphism does not seem to be a general link between low cholesterol and an increased risk of cancer, as the apo E phenotype distribution was not altered in cancer of the breast or prostate. The possible association between apo E polymorphism and colon cancer may be due to differences in biliary lipid composition, e.g. bile acid distribution between apo E phenotypes, or to a local effect of macrophage-derived apo E on cell transformation and immunologic response.

3. Apo E is widely expressed throughout the human intestine. The macrophages and endocrine cells are the main source of apo E in normal human colon and the dendritic cells and macrophages in the germinal centers of lymphoid follicles are the main source of apo E in the small intestine.

4. In solid intestinal tumors, apo E is located in the macrophages surrounding the tumor area, whereas the tumor epithelium does not produce apo E. Although the clinical significance of the phenomenon is not known, it is hypothesized that macrophage-produced apo E may modulate the local immune response in the tumor area.

5. Treatment with apo E seemed to improve epithelial cell polarity in a cultured human colonic adenocarcinoma cell line HT29. Apo E may modulate epithelial integrity and thus contribute to cell growth and malignant transformation.
8. References


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