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# GENETIC VARIATION OF APOLIPOPROTEIN B IN THE FINNS: EFFECTS ON SERUM LIPID LEVELS

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Helsinki 1999

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# GENETIC VARIATION OF APOLIPOPROTEIN B IN THE FINNS: EFFECTS ON SERUM LIPID LEVELS

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ACADEMIC DISSERTATION

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HELSINKI 1999

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**SUMMARY**

The coding region of the apolipoprotein B (apoB) gene was screened for previously unknown mutations in hyperlipidemic Finnish patients, using single-strand conformation analysis as the detection method. ApoB gene exons 2 to 25, 27 and 28 were amplified as a whole, and exons 26 and 29 in six and three overlapping segments, respectively. Altogether, twelve new variants of apoB were detected. Four of them were silent variants, and three other were considered not likely to affect lipid levels. Possible lipid effects of the other five apoB variants were assessed in hyperlipidemic families and patients, in the normal population and patiens undergoing coronary angiography, and during diet or drug intervention. In addition, effects of earlier known apoB variants, the apoB ins/del, Thr71→Ile and Ala591→Val, on serum lipid levels were studied. The newly detected Val703→Ile polymorphism seemed to affect triglyceride levels in healthy subjects, and the Ala591→Val polymorphism serum apoB levels in hypertriglyceridemic subjects. The His1896→Arg polymorphism was shown to affect serum total and LDL cholesterol concentrations in healthy men during low-fat, low-cholesterol diet. No statistically significant lipid effects of three other apoB polymorphisms, the Asn1887→Ser, Arg4243→Thr, and Ala4454→Thr, could be detected in the subjects studied. A new immunogenetic apoB polymorphism, detectable by monoclonal antibody D7.2 and associated with both Asn1887→Ser and His1896→Arg variations was characterized.

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I Ilmonen M, Heliö T, Ebeling T, Pyörälä K, Uusitupa M, Palotie A, and Tikkanen MJ. Screening for mutations in the exon 26 of the apolipoprotein B gene in hyper-cholesterolemic Finnish families by the single-strand conformation polymorphism method. Human Mutation 4: 217-223, 1994.

II Ilmonen M, Ebeling T, Viikari J, Ojala J-P, and Tikkanen MJ. Screening of the 3´ two-thirds of the coding area of the apo B gene in Finnish hypercholesterolemic patients. Report of six new genetic variants. Atherosclerosis 128: 191-199, 1997.

III Ilmonen M, Heliö T, Bütler R, Palotie A, Pietinen P, Huttunen JK, and Tikkanen MJ. Two new immunogenetic polymorphisms of the apoB gene and their effect on serum lipid levels and responses to changes in dietary fat intake. Arteriosclerosis, Thrombosis, and Vascular Biology 15: 1287-1293, 1995.

IV Ilmonen M, Knudsen P, Taskinen M-R, and Tikkanen MJ. Genetic variation in the amino-terminal part of apolipoprotein B: studies in hyperlipidemic patients. Atherosclerosis 138: 367-374, 1998.

**ABBREVIATIONS**

| | |
|---|---|
| aa | amino acid |
| Ag | antigen group |
| AGE | advanced glycosylation end product |
| apo | apolipoprotein |
| APOBEC-1 | apoB mRNA editing enzyme catalytic polypeptide-1 |
| BMI | body mass index |
| bp | base pair |
| CAD | coronary artery disease |
| CCM | chemical cleavage of mismatch analysis |
| cDNA | complementary DNA |
| CE | cholesteryl ester |
| CETP | cholesteryl ester transfer protein |
| chol | cholesterol |
| CM | chylomicron |
| DGGE | denaturing gradient gel electrophoresis |
| DNA | deoxyribonucleic acid |
| FC | free cholesterol |
| FDB | familial defective apolipoprotein B |
| FH | familial hypercholesterolemia |
| GAG | glycosaminoglycan |
| HA | heteroduplex analysis |
| HDL | high density lipoprotein |
| HL | hepatic lipase |
| HVR | hypervariable region |
| IDL | intermediate density lipoprotein |
| kb | kilobase |
| kDa | kiloDalton |
| LCAT | lecithin-cholesteryl acyltransferase |
| LDL | low density lipoprotein |
| LDLR | low density lipoprotein receptor |
| Lp(a) | lipoprotein(a) |
| LPL | lipoprotein lipase |
| LRP | low density lipoprotein receptor-related protein |
| mAb | monoclonal antibody |
| mm-LDL | minimally modified LDL |
| mRNA | messenger RNA |
| MTP | microsomal triglyceride transfer protein |
| NIDDM | non-insulin-dependent diabetes mellitus |
| PCR | polymerase chain reaction |
| PG | proteglycan |
| Pl | phospholipid |
| RER | rough endoplasmic reticulum |
| RFLP | restriction fragment length polymorphism |
| RNA | ribonucleic acid |
| SER | smooth endoplasmic reticulum |
| SSCP | single-strand conformation polymorphism |
| Tg | triglyceride |
| TGGE | temperature gradient gel electrophoresis |
| TGRLP | triglyceride-rich lipoprotein |
| VLDL | very low density lipoprotein |
| VNTR | variable number of tandem repeats |

# 1. INTRODUCTION

The risk of coronary artery disease (CAD) is correlated with elevated serum levels of total cholesterol, low density lipoprotein (LDL) cholesterol, apolipoprotein B (apoB) and triglycerides, as well as with low levels of high density lipoprotein (HDL) cholesterol (1-5). Although heritability estimates, genetic models and modes of transmission vary between different study settings, a considerable portion of the variability of serum levels of these lipids and lipoproteins is believed to be genetically determined (6-8). One of the candidate genes possibly involved in this variation is the apoB gene.

The apoB gene encodes for two protein isoforms, apoB-100 and apoB-48, of which apoB-48 is composed of the first 2552 (approximately 48 percent) amino acids of apoB-100. ApoB-100 is the major lipid-binding protein constituent of very low density (VLDL), intermediate density (IDL) and low density (LDL) lipoproteins and functions in LDL also as a ligand for the LDL receptor (LDLR) (9,10). The main role of apoB-48 is to bind lipids in chylomicrons (CM) (9,10). The enormous size of apoB and its gene, and the variability of lipid phenotypes have made studying the pathogenetic role of apoB in dyslipidemias difficult. The most common approach has been the use of population association studies. Several genetic polymorphic variants of apoB have been described, many of which have been associated with elevated serum cholesterol, triglyceride, LDL-cholesterol or apoB levels in different populations (9,11,12). Among the most studied is the silent XbaI restriction fragment length polymorphism (RFLP), which has been associated with serum lipid levels and CAD in several populations, especially the Finns (12). To date, three mutations of apoB with major effects on serum lipid levels have been characterized, the apoB Arg3500→Gln (13,14), Arg3531→Cys (15), and Arg3500→Trp mutations (16). All have been shown to elevate serum total and LDL cholesterol levels (17-21). None of these mutations have so far

been detected in the Finnish population.

For decades, the Finns have belonged to populations with a high incidence of dyslipidemias, especially hypercholesterolemia, and thus increased risk of CAD (22,23). Although primary preventive measures have lead to major declines in both coronary risk factors and the incidence of CAD (24-26), CAD mortality in the Finns is still among the highest in the world (27,28). As a nationally and regionally isolated population, the Finns are genetically exceptionally homogeneous (29,30), a fact reflected in the existence of four common unique LDLR mutations causing familial hypercholesterolemia (FH) (31), and in the apparent absence of the apoB Arg3500→Gln mutation in the Finnish population (32). With these facts in mind, the series of studies presented in this thesis were designed to search for previously unknown apoB genetic variants characteristic to Finns which could explain part of the commonly occurring dyslipidemias in this country.

# 2. REVIEW OF THE LITERATURE

## 2.1. General outline of lipoprotein metabolism

### 2.1.1. Plasma lipids and lipoproteins

The major lipids in the body are triglycerides (Tg), cholesterol (free cholesterol, FC, and cholesterol esters, CE), and phospholipids (Pl) (10,33). Triglycerides serve as a source of energy and are stored in adipose tissue. Cholesterol serves as a component of cell membranes and as a precursor for steroid hormones and bile acids. Phospholipids are major components of cellular membranes and lipid-transporting lipoproteins. As hydrophobic compounds, cholesterol and triglycerides cannot dissolve directly in plasma but are carried in the circulation together with the amphipathic Pl as water-soluble lipoproteins. Basically, all lipoproteins are organized into a hydrophobic core of neutral lipids (Tg and CE), and a hydrophilic surface coat of polar lipids (FC and Pl) and apolipoproteins. Although lipoprotein particles, differing in their relative lipid and apolipoprotein composition, size, density and function actually form a heterogenous continuum, major classes of lipoproteins have been defined. A traditional classification, based on the density at which lipoproteins float during ultracentrifugation, divides them into chylomicrons (CM), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) (10,33). Additionally, lipoproteins can be classified on the basis of particle size, electrophoretic mobility, or apolipoprotein content. Within the classical lipoprotein fractions, especially the LDL and HDL fractions have been shown to be comprised of several distinct subclasses differing in their density, particle size or apolipoprotein composition (34-36), reflecting steps in lipoprotein metabolism. In addition to the classic five lipoprotein classes, a heterogeneous class of LDL-like lipoprotein particles termed lipoprotein(a) containing apolipoprotein(a) and apoB-100 as protein moiety has

been characterized (37,38). The major characteristics of these lipoprotein classes are presented in Table 1.

### 2.1.2. Apolipoproteins

Apolipoproteins are specific protein components of lipoproteins. They act as structural lipid binding components of lipoproteins (apoA-I, apo A-II, apoB-48, apoB-100), as ligands for lipoprotein receptors (apoA-I, apoB-100, and apoE), as inhibitors for lipoprotein-receptor interactions (apoC-I and apoC-III), as modulators of the activity of enzymes involved in lipoprotein metabolism (apoA family, apoC family), and as cofactors in lipid transport between lipoproteins (apoA-IV) (10,39). Characteristics and physiological functions of ten of the best known apoliporoteins are summarized in Table 2. Additionally five minor apolipoproteins, termed apoD, apoF, apoG, apoH ($\beta_2$-glycoprotein), apoI (serum amyloid A), and apoJ (clusterin), have been characterized (39,40). Their physiological roles are still largely unknown.

### 2.1.3. Lipoprotein metabolism

Lipid and lipoprotein metabolism can be divided into two pathways, the exogenous pathway involved in the transport of dietary lipids, and the endogenous pathway, both schematically illustrated in Figure 1. In the intestine, absorbed and re-esterified Tg, CE and Pl are packed into apoB-48-containing CM, and secreted via the lymph to the circulation (10,41). Thereafter, CM Tg are rapidly hydrolyzed by lipoprotein lipase (LPL) and to some extent also by hepatic lipase (HL), which process, together with other changes in the lipid and apolipoprotein content of the particles, results in the formation of smaller, cholesterol-enriched CM remnants. CM remnants are removed from the plasma mainly by the liver through the LDLR and to a smaller extent through the LDL receptor-related protein (LRP-1), or cell surface proteoglycans (PG) (10,42). Under normal conditions, most of the absorbed Tg

carried by the CM is used in the extrahepatic tissues whereas nearly all cholesterol is delivered to the liver. A small portion of CM remnants seems to be cleared by peripheral tissues as well (43).

The endogenous lipid transport system can be divided into two subsystems: the apoB-100 lipoprotein system (VLDL, IDL and LDL) and the apoA-I lipoprotein system (HDL). The apoB-100 system begins with the hepatic assembly and secretion of apoB-100-containing VLDL particles (10). Thereafter, VLDL Tg are hydrolyzed in peripheral tissues by LPL, and the particles converted to smaller Tg-depleted remnant particles. A part of VLDL remnants are directly cleared from the plasma by hepatic and possibly also peripheral receptor-mediated mechanisms (10,42,43). The remaining particles enter into the VLDL-IDL-LDL cascade, where most of the core Tg in the particles are hydrolyzed by LPL and HL, leading to the formation of IDL and LDL particles. Some of the IDL particles are conceivably removed by the liver via LDLR or LRP-1. The rest are converted to LDL particles. Most of the LDL particles are cleared by the liver through the LDLR, other receptors and non-receptor-mediated, still poorly defined pathways playing a smaller role in LDL clearance (10,44).

The metabolism of the apoAI-containing HDL-particles is intimately connected with both the exogenous and endogenous lipid transport pathways. HDL particles are derived from precursor complexes secreted by the liver and intestine. They are the main mediators of the reverse cholesterol transport system whereby cholesterol synthesized or deposited in peripheral cells is returned to the liver (4,10,45). This process begins with the removal of FC from cell membranes to nascent HDL particles (35,46) and esterification of FC by lecithin cholesterol acyl transferase (LCAT), after which the CE is transferred to the hydrophobic core of the HDL particle. In this process, nascent HDLs are converted to spherical lipid-rich HDL. Part of the HDL core CE is then transferred to apoB-48- or

apoB-100-containing lipoproteins in exhange for Tg by the cholesteryl ester transfer protein (CETP), whereafter these transferred CEs can either be removed from the circulation by the liver or redistributed to peripheral cells. The CEs remaining in the HDL particles are taken up by hepatocytes either via receptor-mediated endocytosis by of apoE-containing HDL particles by the LDLR, LRP-1, or the putative HDL holoparticle receptor, or through selective removal of HDL CE by the hepatic scavenger receptor BI (4,5,10). At the same time, Tg transferred from other lipoproteins to HDL are hydrolyzed by HL, leading to the conversion of Tg-rich $HDL_2$ to Tg-poor $HDL_3$ particles, and release of free apo-AI and lipid-poor HDL to be reused in the reverse cholesterol transport cycle (35,45). Besides the exchange of CE for Tg, the complex interplay of HDL with other lipoproteins during reverse cholesterol transport involves exchange of other components as well, such as apolipoproteins and Pl. Thus, HDL particles can be considered to serve in plasma as a reservoir of lipids and apolipoproteins for apoB-100 and apoB-48-containing lipoproteins (10,42).

#### 2.1.4. Factors regulating lipid and lipoprotein levels

2.1.4.1. Diet, other lifestyle factors, age, gender, and obesity

The metabolism and plasma levels of lipids and lipoproteins are influenced by several non-genetic factors, including both dietary as well as other lifestyle factors, age, gender and the degree and distribution of body fat. A summary of the effects of these factors on plasma lipid and lipoprotein levels is shown in Table 3.

Of all dietary constituents, the amount and composition of fatty acids and the amount of cholesterol seem to be the most important modulators of serum lipid and lipoprotein metabolism, and therefore have been targeted in dietary recommendations aimed at reducing lipoprotein levels and CAD risk. Dietary fats are composed of mixtures of saturated, monounsaturated or

polyunsaturated fatty acids. Of these, most saturated fatty acids raise serum total and LDL cholesterol by impairing the clearance of LDL, whereas the polyunsaturated and mono-unsaturated fatty acids, when substituted for saturated fatty acids, reduce serum cholesterol levels (47,48). Thus, reductions of total dietary fat to 30% of total calories and dietary saturated fatty acids to 10% of total calories with a moderate increase in polyunsaturated and monounsaturated fatty acids in the diet is considered beneficial (49). The influence of dietary cholesterol on plasma lipoproteins is variable (48,50,51), but usually, high levels of cholesterol consumption have been associated with elevations of cholesterol in all lipoprotein classes, an effect enhanced when cholesterol is consumed with saturated fatty acids (47,48,52). Current recommendations suggest a limitation of dietary cholesterol to 300 mg/day (49). Compared with the effects of dietary fatty acids and cholesterol on lipid and lipoprotein levels, the effects of other dietary components, such as carbohydrates (52,53), soluble fibre (54), and proteins (55,56) are more subtle and also variable and have not lead to major dietary recommendations. Dietary alcohol raises Tg and HDL cholesterol levels, and lowers LDL cholesterol (57). A high overall caloric intake increases hepatic VLDL synthesis, resulting in hypertriglyceridemia and hyper-cholesterolemia (52).

Besides diet, several other lifestyle factors such as tobacco smoking (58), physical activity (59), and psychological stress (60), are associated with variations in lipid and lipoprotein levels. However, knowledge of the relative importance of these factors on lipid levels and their variability is currently limited. Instead, the effects of age (61-63), gender (61,62), menopausal (63,64) and menstrual (65) status in women, and the degree (66-70) and distribution (66,70) of body fat are more pronounced and should be taken into account when estimating the effects of genetic variation on lipid and lipoprotein levels. In addition, endocrinological, renal and hepatic diseases, diabetes mellitus, and drugs are known to affect lipid metabolism and

have to be considered when lipid effects of other factors such as genes are estimated (52,71,72).

2.1.4.2. Genes

A considerable portion of the variability in serum levels of lipids and lipoproteins between individuals as well as the variability of changes induced in these levels by factors such as dietary modifications and weight reduction is believed to be genetically determined. Several polymorphisms in the genes encoding for various proteins involved in lipid metabolism have been characterized, some of which have been associated with serum lipid levels or responses to diet in populations. The most studied and well characterized genetic variants in this respect are located in the apoE and apoB genes.

ApoE is a protein constituent of the triglyceride-rich lipoproteins VLDL, ßVLDL, IDL, CM, their remnants, and apoE-rich HDL (39,73-76). It mediates the interaction of these particles with cell surface receptors, and occupies a central role in determining the metabolic fate of lipoproteins. A role in the reverse cholesterol transport for apoE has also been postulated (39). The apoE gene exhibits a common polymorphism with three alleles coding for three protein isoforms of apoE (E2, E3, and E4). In population studies, the apoE4 isoform has been associated with higher and apoE2 with lower serum total and LDL cholesterol and apoB levels (39,75,77). Compared with apoE3, apoE2 is defective in receptor binding, leading to reduced cholesterol delivery to the liver and up-regulation of LDLR, whereby LDL clearance is increased (39,75). ApoE4, while binding to receptors equally well as apoE3, seems to be metabolized more rapidly, leading to cholesterol accumulation in hepatic cells and down-regulation of LDLR (39,75). In addition, subjects with the apoE4 isoform absorb cholesterol more efficiently than carriers of the other two isoforms (75). Carriers of the E4 allele have also been shown to be more responsive to dietary modifications (75,76).

In addition to the apoE gene, polymorphisms in several other genes have been associated with variations in lipid and lipoprotein levels. To date, such associations have been reported from genetic variants in the LDLR gene (74,78,79), the LRP-1 gene (80), the VLDL receptor gene (79), the scavenger receptor BI gene (81), genes in the apoAI-CIII-AIV cluster (39,76,82), the apoCI gene (83), the LPL gene (79,84-86), the HL gene (87), the CETP gene (88,89), the MTP gene (90), the fatty acid binding protein-2 gene (91,92), the paraoxonase gene (91,93-95), and the haptoglobin gene (96). The magnitude of the effect of each of these polymorphisms on plasma lipid levels is probably small for the individual. Since the sequences causing these polymorphisms are common, most individuals are likely to carry several such lipid-affecting genetic variants, which, when combined, may lead to major changes in lipid levels. Similarly, if common enough, they can also have an impact on serum lipid levels at the population level as well.

Besides gene polymorphisms affecting lipid and lipoprotein levels in the population, occasionally a single mutation is capable of producing an abnormal lipoprotein phenotype genetically transmitted as a familial dyslipoproteinemia. The underlying genetic change of some forms of familial dyslipoproteinemias is known (97). Among these are mutations in the LDLR gene leading to the production of missing or defective LDLR and causing FH (44), and mutations in the apoB gene producing a LDLR-binding defective apoB-100, termed familial defective apoB-100 (FDB) (15-17). Hypobetalipoproteinemia and abetalipoproteinemia have been linked to mutations in the apoB and MTP gene, respectively (98,99). On the other hand, the exact underlying genetic defect of some other common syndromes such as familial hypertriglyceridemia or familial combined hyperlipidemia is still unknown. Of the possible candidate genes, the apoB, apoCIII and LPL genes have been excluded as causative factors for familial hyper-triglyceridemia (100), and the LPL, HL, hormone-sensitive lipase and several other candidate genes as causative for familial combined hyperlipidemia (101,102).

Recently, linkage of familial combined hyperlipidemia to two novel loci on chromosomes 1 and 11 has been reported (102,103).

### 2.2. Genetic approaches to study heritability of plasma lipid traits and hyperlipidemias

The classical approach to estimate the relative contribution of genetic and environmental factors for a particular trait has been to use biometrical methods comparing individuals who are likely to share genes and environment to different extents, such as identical and non-identical twins, sibling pairs, and parents and children. These methods have demonstrated a significant impact of genetic variation in determining the plasma levels of total, LDL, and HDL cholesterol, apoB and apoAI, with reported heritabilities in the range of 0.4-0.6 (6,7). For Tg, reported estimates of heritability are more variable, ranging from 0.2 to 0.8 (104,105), and for Lp(a), higher, over 0.9 (6,106). Family studies and complex segregation analyses have found evidence for a major gene determining levels of total, LDL and HDL cholesterol, apoB and apoAI, with both environmental factors and genes of small or intermediate effect making a contribution (6). After establishing evidence for the existence of a major gene affecting lipid metabolism, the next problem is to identify this gene. To accomplish this, two approaches can be used (107). In the candidate gene approach, an association between lipid and lipoprotein levels and a genetic marker of a known gene whose product is believed to be important in lipid metabolism is looked for. In the random search strategy, novel genes involved in lipid metabolism are searched for by screening the whole human genome with hundreds or more of random markers distributed across the genome and associating them with lipid parameters. While the majority of studies of genetic determinants of lipid metabolism published so far have been based on the study of candidate genes, recent developments in molecular genetics and automatization of genotyping have made the random search approach more feasible.

Depending on the material studied, differing strategies in searching for genes involved in lipid metabolism can be adapted (107,108). Of these strategies, classical linkage analysis is one of the most powerful methods to find the chromosomal localization and to identify major disease causing genes. It is based on calculation of the genetic distance between a genetic marker and the supposed disease locus in families where the inheritance of these two loci can be followed. If the loci cosegregate more often than expected by chance, they are said to be linked; the more tightly the loci are linked, the more close they are in the genome (108). Linkage analysis and its modifications such as the affected-pedigree-member and the sib-pair analysis have been used to identify disease loci in dyslipidemias. However, besides the need for informative families of sufficiently large size, the need to know the mode of inheritance of the disease or trait in question, and the fact that low penetrance of the trait, the multiple loci involved, and phenocopies (i.e. the same phenotype produced by different loci) weaken the strength of the analysis, the use of linkage analysis in lipoprotein genetics is further hampered by difficulties in estimating the cutoff levels for normal and abnormal lipid and lipoprotein concentrations (107).

Population association studies take advantage of the same principle of genetic closeness of a candidate locus and a supposed disease locus leading to linkage disequilibrium between these two loci. If a genetic marker and a disease or trait occur in the same individual more often than expected by chance, they are said to be associated (108). Analysis of the association of a genetic marker with a specific trait in populations has been the most widely used approach to study the effects of candidate genes on lipoprotein traits (6,107). In contrast to linkage analysis, knowledge of the mode of inheritance is not needed in association studies, neither do the latter suffer from incomplete penetrance, and families are not necessary for the study. However, large samples are needed, and the populations studied should be well stratified in terms of factors such as

age, sex, and ethnic composition (6,12,107). This approach has been most successful in genetically homogeneous populations where the effects of variation in genes other than those being studied are expected to be lower than in genetically more heterogeneous groups (30). In the case of highly polymorphic genes, such as the apoB gene, powerful association studies have also been performed using combinations of polymorphic markers lying in the same chromosome, termed haplotypes, as markers instead of single diallelic markers (12,107).

### 2.3. Apolipoprotein B: structure and function

In humans, apoB exists in two isoforms, apoB-100 and apoB-48, both always attached to lipids. ApoB-48 is normally found in fasting plasma in very low concentrations, in the range of only a few $\mu$g/ml, whereas apoB-100 concentrations range normally from 60 to 120 mg/dl (9). More than 90% of apoB-100 is found in the LDL fraction. Due to the large size of apoB, its extreme hydrophobicity and thus poor solubility in aqueous buffers after delipidation, its tendency to aggregate, and its sensitivity to degradation, direct studies of apoB structure have been difficult (109). Only after the cloning of the apoB gene and uncovering of the nucleotide and amino acid sequence of the protein have details of its structure and functional domains been elucidated.

#### 2.3.1. Structure of the apoB gene

The apoB gene is located on the short arm of chromosome 2 (110-112). The complementary DNA (cDNA) and part of intronic nucleotide sequences, the deduced amino acid sequence as well as molecular organization of the apoB gene are known (113-118). In humans, the apoB gene is expressed mainly in liver hepatocytes and intestinal epithelial cells (111); to a small extent, expression of the human gene has been found also in the heart (119), aortic endothelial cells (120), fibroblasts (121) as well as kidney, colon and stomach (122). The apoB gene, with over 43

kilobases (kb), is organized into 29 exons and 28 introns, shown schematically in Figure 2. Two of the exons, exon 26 (7572 bp) and exon 29 (1906 bp), are exceptionally large. The apoB cDNA is composed of 14121 nucleotides, with 5´ and 3´ untranslated regions of 128 and 304 bp, respectively (116). Outside the apoB gene, two 5´ and one 3´ nuclear matrix attachment regions ranging from ~5.2 kb upstream of the gene to a few hundred bp 3´ to the gene have been identified (123). This 47.5 kb domain has been suggested to represent a topologically sequestered functional unit containing both the regulatory elements and coding region of the gene (123). However, recent studies in transgenic mice expressing the human apoB gene have shown that while this 47.5 kb domain is sufficient for expression of the apoB gene in the liver (124,125) as well as in the heart (126), intestinal expression is dependent on an enhancer element located outside this matrix attachment domain and situated between 54 and 62 kb 5´ to the structural gene (127). In the 5´ part of the 47.5 kb domain, the apoB gene contains classical promotor structures such as a TATA box and a CAAT box within 60 bp upstream from the transcriptional start site (113). In addition, several other regulatory elements have been identified extending from a negative regulatory element -2738 to -1802 bp upstream from the transcription start site (128) through a promoter region between nucleotides +1 to -898 (129) to the first untranslated exon containing both positive and negative regulatory elements (130), and enhancer elements located in the second (131) and third (132) introns of the gene. The 3´ end of the apoB gene also contains two sites possibly involved in gene expression regulation: the first is localized in the 3´ untranslated region of the gene about 80 bases beyond the translational stop codon in exon 29, and the second in the middle of the AT-rich hypervariable region (133). The exon 29 site has been postulated to have a role in transcription termination, whereas the site located inside the 3´ VNTR region may provide an accessible region of DNA for the action of topoisomerase II, an enzyme that alters the topological state of DNA during transcription (133). Besides the identification of

these regulatory regions and motifs and some of the nuclear proteins binding to these regulatory elements (134-136), not much is known about the mechanisms and factors regulating the level of gene expression in apoB expressing tissues. In adult humans, the apoB gene seems to be constitutively expressed, with relatively constant steady state levels of messenger RNA (mRNA) even under conditions in which the level of apoB production changes significantly (9,137,138).

### 2.3.2. ApoB mRNA editing

ApoB-48 is produced by the same gene as apoB-100 through a mechanism termed mRNA editing. In this process, the apoB mRNA is posttranscriptionally deaminated at nucleotide 6666, substituting a uracil for a normal cytosine, changing codon 2153 from CAA to UAA, and replacing the normal glutamine with a termination codon (139-143). Thus, an apoB protein with 2152 amino acids and about 48% of the size of full length apoB is produced. The mRNA editing process involves a specific RNA binding cytidine deaminase (143-147), termed the apoB mRNA editing enzyme catalytic complex-1 (APOBEC-1), and auxiliary factors that complement APOBEC-1 in apoB mRNA editing in the so called 'editosome' complex (143,146,148). The gene for human APOBEC-1, located in chromosome 12, has been cloned (149-153). In adult humans and rabbits, APOBEC-1 expression is restricted almost exclusively to gastrointestinal epithelial cells, whereas in the rat and mouse, the gene is more widely expressed (143). The auxiliary protein factors required for apoB mRNA editing are widely distributed in mammalian tissues, including many that do not synthesize apoB (143,146-148,154). To date, one APOBEC-1 binding protein suggested to be involved in apoB mRNA editing has been cloned (155), and some others have been partly characterized (148,154). Under physiological conditions, apoB mRNA editing is a very specific process leading to the deamination of only cytidine 6666 of apoB mRNA. Several sequence elements in apoB mRNA needed for efficient editing have been identified (143,156,157). Computer modelling and ribonuclease

probing of wild type and mutant apoB mRNA substrates suggest that the secondary structure required for proper editing involves a stem loop which contains the cytidine 6666 to be edited within the loop (157). To date, it is not known whether defective apoB mRNA editing in human intestine leads to a specific disease or not. One polymorphism in the human APOBEC-1 gene has been described, but this polymorphisms seems to bd functionally silent (152). Likewise, no genetic variant of the apoB gene located close to the editing site and affecting efficient editing has been identified so far.

### 2.3.3. Structure of the apo B protein

In the liver and intestine, the mRNA of apoB codes for a 4560 to 4565 amino acid and a 2176 to 2181 amino acid protein, respectively. Before either of these peptides are translocated to the endoplasmic reticulum, an amino-terminal 24 to 29 amino acid signal peptide is cotranslationally cleaved from the growing nascent peptide. Thus, a full-length mature apoB-100 protein comprised of 4536 amino acids and with a calculated amino acid molecular weight of approximately than 550 kDaltons (kDa), and a 2152 amino acid apoB-48 with a molecular weight of 264 kDA are produced (115,117,158,159). The sequence of the apoB-100 polypeptide chain is unique, although homology with other apolipoproteins, the microsomal triglyceride transfer protein (MTP) and vitellogenin has been found (160-164). In addition, the apoB polypeptide sequence contains several long internal repeats (160). In lipoproteins, apoB is glycosylated: 4-9% of its mass is carbohydrate linked to asparagine residues (165). Of the 19 potential N-linked glycosylation sites of apoB, at least 16 have been found to be glycosylated (166). The distribution of the glycosylated asparagines is asymmetric, clustering in the vicinity of the putative LDLR binding site, but whether this clustering has any role in LDLR binding is not known (167). During apoB synthesis, glucose trimming of asparagine residues seems to be important for proper association

of apoB with endoplasmic reticulum-resident proteins such as calnexin and calreticulin (168). ApoB contains 25 cysteine residues, 16 of which are known to exist in disulfide form, forming cysteine bridges in the molecule (169,170). The distribution of the cysteines is uneven, 12 occurring within the first 500 amino acids of the protein and all of them being involved in cysteine bridge formation (170). The amino-terminal cysteine bridges are believed to stabilize the amino-terminal end of apoB into a globular domain (170). Proper folding of this domain is believed to be essential for lipoprotein assembly and secretion (171,172). Furthermore, the disulfide bond between cysteines 218 and 234 seems to have an important functional role in lipoprotein assembly separate from a structural role in apoB folding (173). A cysteine bridge between residues 3167 and 3297 takes part in the conformational organization of the LDLR binding site of apoB (158). In addition, a disulfide bond between apoB cysteine 4326 and apo(a) cysteine 4057 is involved in Lp(a) assembly (174-177).

The tertiary structure and conformation of apoB-100 in lipoprotein particles has been explored by a number of methods. Original analyses of the accessibility of apoB-100 to proteolytic enzymes such as trypsin suggested apoB-100 to have five broad domains (166). Based on combined data from trypsin accessibility studies (166), studies with monoclonal antibodies (mAb) against native and delipidated apoB-100 (178-180) and electron microscopic studies with negatively stained lipid extracted LDL (181), the following model has been put forward, with the apoB-100 suggested to have a globular amino terminal end extending away from the lipoprotein particle and a belt-like structure with apoB sequences partly inside the particle and partly on the surface of the particle (166,181). Further support for this model has come from immunoelectron (182) and cryo-electron microscopic studies (183,184). A recent cryo-electron microscopic study (185) suggests that the first 89% of apoB wraps like a ribbon once around the LDL particle, while the carboxyl-terminal 11% constitutes a bow that crosses the ribbon

and brings this part of apoB near the putative LDLR binding site. A schematic illustration of this ribbon-and-bow model is shown in Figure 3a. Based on the primary sequence of apoB, circular dichroism spectra and infrared spectroscopy analyses, LDL apoB is known to present both α-helixes and β-structure (158,186). Studies using computer-assisted methods searching for amino acid sequences in the apoB protein capable of forming amphipathic α-helixes and β-sheets to identify lipid-associating domains of apoB-100 have also suggested a pentapartite structure (Figure 3b) for apoB in LDL: $NH_2$-$\alpha_1$-$\beta_1$-$\alpha_2$-$\beta_2$-$\alpha_3$-COOH, with $\alpha_1$ representing the globular amino-terminal domain (187,188). CM apoB-48 would thus be comprised of only two domains, the globular amino-terminal $\alpha_1$ domain and a second domain of a cluster of amphipathic β-sheets. When compared, the trypsin-accessible and computer-identified domains and domains accessible or not accessible to mAbs seem to be closely correlated (189) and agree also with experimental thermodynamic data indicating a five-domain, folding organization for apoB-100 (190).

### 2.3.4. ApoB functions and functional domains

#### 2.3.4.1. Lipid binding domains

The main function of both isoforms of apoB relate to their ability to bind lipids: in the absence of apoB-48 or apoB-100, no CM or VLDL particles are formed and lipid absorption and transport is severely hampered as can be seen in homozygous abetalipoproteinemia and some forms of homozygous hypobeta-lipoproteinemia (98,191). The ability of apoB to bind lipids resides in the multiple hydrophobic domains present throughout the length of the protein (114,158). Cell cultures expressing truncated apoB proteins of varying size seem to confirm this: the smaller the apoB protein, the smaller the quantity of core lipids in the secreted lipoprotein (192). Both the amphipathic α-helices 2 and 3 and the amphipathic β-sheet clusters have high lipid binding potential, of which the α-clusters are claimed to

bind lipids reversibly and the β-clusters irreversibly (187). Analyses of the effects of progressive lipidation of apoB-100 on its immunoreactivity suggest that the β-domains represent inflexible regions of apoB structure that could act as a backbone during lipoprotein assembly, whereas the amphipathic α-helical domains would represent flexible lipid-binding regions that allow the particles to accommodate varying amounts of lipid during lipoprotein assembly and intravascular metabolism (189). The wide dispersion of lipid-binding sequences has been assumed to explain the fact that apoB is never exchanged between lipoprotein particles, in contrast to other apolipoproteins, which have only one or two putative lipid binding domains and are readily exchangable (9,10). ApoB-48 has been suggested to contain only one lipid-binding domain, a cluster of β-sheets (187). A recent study with cells expressing recombinant truncated human apoB-48 showed that these β-strands are critical determinants of lipoprotein assembly, with very short hydrophobic sequences from 152 to 237 amino acids mediating the recruitment of large quantities of Tg into the lipoprotein particle (193).

#### 2.3.4.2. Receptor binding domains

In LDL particles, apoB-100 functions as a ligand for the LDLR (44). Much interest has been focussed on the putative receptor binding region of apoB. Several kinds of observations point towards the carboxyl-terminal portion of the apoB molecule as the receptor binding region. Among these are studies with carboxyl-terminally truncated natural apoB variants (194), studies with apoB mutations causing defective binding of LDL with LDLR (14-16), studies using mAbs with epitopes in the carboxyl-terminal region of apoB blocking LDLR binding (179), apoB sequence analyses identifying positively charged regions assumed to interact with the negatively charged regions in the ligand-binding domain of the LDLR (158), indentification of sequence homology with the receptor binding region of the other LDLR ligand, apoE (158), and chemical modification of the

positively charged amino acid residues arginine and lysine of apoB leading to abolishment of LDLR binding (195,196). The LDLR binding domain of apoB has been suggested to lie somewhere between two regions enriched with arginine and lysine residues, amino acids 3147-3157 (Site A) and 3359-3367 (Site B), in a region containing a disulfide bond between cysteines 3167 and 3297 bringing the two short amino acid sequences close to each other (158). However, studies with mAbs suggest that even a larger part of the protein, spanning amino acids 2285 and 4081 (179) or 2935 and 4189 (180) could be involved. Recent observations with transgenic mice expressing mutant forms of human apoB suggest that Site B is critical for LDLR binding and forms the actual binding site (197). In favor of this hypothesis, this region is evolutionally highly conserved (198), binds heparin (199), is extremely similar to the site on apoE that binds to the LDLR (158) and seems also in vivo to be needed for LDLR binding since natural apoB truncations lacking this region cannot bind to the LDLR (194,200) whereas apoB:s truncated distally to this site show receptor binding capacity (201-203). Besides the actual LDLR binding site, the carboxyl-terminal end of apoB seems also to influence the efficiency of LDLR binding. Immunoelectron microscopic studies suggest that the carboxyl terminus of apoB beginning at amino acid residue 4050 constitutes a bow-like structure that stretches back into one hemisphere of LDL and crosses the linear ribbon-like part of apoB between residues 3000 and 3500, bringing the carboxyl-terminal part of apoB-100 near to the actual LDLR binding site (185). Since apoB truncations lacking the carboxyl-terminal sequences show increased clearance and binding to LDLR compared with full-length apoB-100 (202-204), the carboxyl-terminal sequences have been suggested to act as a negative regulator of LDLR binding. Studies with gene-targeted mice show that removal of the carboxyl-terminal 20% of apoB increases the binding activity of normally receptor-negative VLDL to the LDLR (197). In addition, this 20% of apoB-100 seems also to be necessary in order for the apoB Arg3500→Gln mutation to disrupt receptor binding (197). Thus, it has been proposed that the carboxyl-

terminus of apoB-100 normally functions to inhibit the interaction of VLDL apoB-100 with the LDLR, but after the conversion of triglyceride-rich VLDL to smaller cholesterol-rich LDL, an interaction with a region encompassing Arg 3500 modulates the conformation of the carboxyl tail allowing the interaction of the actual receptor binding site with the LDLR. Mutation in this modulator element such as the Arg3500→Gln change would abolish this interaction, resulting in disrupted LDLR binding of LDL (197).

ApoB-containing lipoproteins have been shown to bind to other receptors of the LDLR superfamily (42,205-207) as well as to the scavenger receptors (205,208), the lipolysis-stimulated receptor (209), and the asialoglycoprotein receptor (210). However, data on whether the LDLR binding region, other regions or any sites of lipoprotein apoB have any direct function in these receptor-ligand interactions is still limited. A mAb against the receptor-binding region of apoB-100 (mAb 4G3) has been shown to inhibit the interaction of LDL with and endocytosis by one member of the LDLR family, LRP-2 (211). Since a natural apoB variant, B70.5, lacking the LDLR binding site B still binds to LRP-2, the sites recognized by these two receptors seem to differ (200). The amino terminus of apoB (amino acids 547-735) has been proposed to have a specific function in the recognition of malondialdehyde-modified LDLs by class A scavenger receptors (212). Also, apoB-100 has been suggested to be one ligand for the lipolysis-stimulated receptor (213). Finally, an amino-terminal site of apoB with no heparin binding affinity located at or near the LPL-binding domain has been suggested to mediate binding of Tg-rich lipoproteins to a partly characterized receptor in human monocytes and macrophages (214).

2.3.4.3. Domains involved in lipoprotein(a) assembly

Lipoprotein(a) is a lipoprotein particle similar to LDL in terms of lipid content and composition containing apoB-100 covalently linked by a disulfide bond to apo(a) (38,106). After the

secretion of apo(a) from the liver, Lp(a) particles are assembled on the hepatocyte cell surface or in the circulation through processes involving both initial noncovalent and covalent linkages between apoB-100 and apo(a) (215,216). The noncovalent association is mediated by amino-terminal apoB sequences between residues 680 and 781 (217), and apo(a) kringle IV types 6-8 (218). For the formation of a stable Lp(a) particle, a covalent disulfide bond between cysteines 4057 and 4326 of the apo(a) and apoB proteins, respectively, is required (174-177). In addition to these sites, the carboxyl terminus of apoB, especially amino acid residues 4331-4397, and possibly also the LDLR binding region of apoB, although not absolutely required, seem however to be necessary for efficient Lp(a) assembly (219,220).

#### 2.3.4.4. LPL binding domain

Lipoprotein lipase (LPL) has multiple roles in lipoprotein metabolism (221-226). It is generally accepted to be the major enzyme responsible for hydrolysis of lipoprotein Tg molecules at the luminal surface or capillary endothelium (223,224). In addition, LPL has been suggested to facilitate cellular uptake of lipoprotein particles either by acting as a bridge anchoring the lipoproteins to cells or matrix, or by acting directly as a ligand for lipoprotein receptors (221,223,226). In vitro, enhanced receptor binding and cellular uptake of a variety of lipoproteins via LPL has been demonstrated in conjunction with essentially all known members of the LDLR superfamily as well as the scavenger receptors (226), but the biological relevance of this effect in vivo has so far not been elucidated. Within arteries, LPL bound to the subendothelial wall PGs has been shown to increase binding of both native and oxidized LDL (227-229) as well as other lipoproteins (230,231) to the arterial wall matrix, leading to entrapment of lipoproteins in the subendothelial space which is believed to be one of the key initiating events in the pathogenesis of atherosclerosis (232).

For long, LPL has been believed to bind to endothelial cells via electrostatic interactions with heparan sulphate PGs (233,234). However, studies with cultured endothelial cells suggested that LPL binding to the endothelium involves both PG and non-PG binding sites (235). The non-PG binding site was identified as a 116 kDa heparin-releasable protein (hrp-116) which was shown to confer specificity to the binding of LPL to the endothelial cells (235,236). The sequence of hrp-116 is identical with four different regions in the amino terminus of apoB, and hrp-116 is also recognized by mAbs against amino-terminal epitopes of apoB (237). Amino-terminal fragments of apoB and mAbs against the apoB amino terminus compete with LPL binding to endothelial cells (237). ApoB is expressed and synthesized in endothelial cells, and pulse-chase studies suggest initial production of full-length apoB-100 with further degradation to produce the 116-kDa apoB fragment which is thereafter released from the cells with heparin and is able to bind LPL (120). Cell-surface expression studies of the amino-terminal apoB region (apoB-17, amino acid residues 1-771) demonstrate that this domain of apoB is not only able to mediate the association of LPL with the cells but that the LPL-apoB interaction is also more stable than the LPL-PG association (238). Based on these observations, it has been suggested that apoB fragments such as the hrp-116, expressed on the surface of endothelial cells, provide a high-affinity binding site that stabilizes LPL activity and delays its release into the bloodstream and eventual catabolism in the liver (238).

LPL is known to associate with most lipoprotein classes (239), but whether this binding is mediated through lipoprotein lipids or apolipoproteins has not been fully clarified. Studies with mAbs against apoB, thrombin digested fragments of apoB, delipidated LDL, and a recombinant truncated amino-terminal fragment of apoB in a solid phase assay free of lipoprotein receptors and cell surface PGs suggest that LPL association with LDL involves protein-protein interaction with the amino-terminal end of apoB (240). The association of LDL with LPL in solution

is also diminished by antibodies against the amino-terminal region of apoB (241). These experimets, identifying the amino-terminal globular region of apoB as a mediator for the LPL-lipoprotein association, were carried out with LDL. However, since LPL has been shown to be associated with all classes of apoB-containing lipoproteins in postheparin plasma (239,242), since all apoB-containing lipoproteins are able to compete with the VLDL-LPL-interaction (243) as well as inhibit LPL-induced VLDL hydrolysis (244), and since binding of LPL with VLDL was similar to that of LDL (240), it has been suggested that the amino terminus of apoB acts as a binding site for LPL in all apoB-containing lipoprotein classes (241). This specific interaction between LPL and lipoprotein apoB has been proposed to play a role in facilitating Tg hydrolysis of circulating triglyceride-rich apoB-containing lipoproteins (240). As to the biological role for the well-documented LPL association with LDL, which is not a physiologic substrate for LPL-catalyzed Tg hydrolysis, another role for this specific protein-protein interaction has been postulated, suggesting that LDL particles could act as carriers of LPL molecules dissociated from the endothelial surface and circulating in the bloodstream, preventing LPL from readhering to the vessel wall and allowing its clearance by the liver (241). On the other hand, an interaction of LDL with LPL in the vessel wall would lead to accumulation of LDL in the subendothelial matrix (229). Both hypotheses have to be confirmed by further studies. Also, the exact LPL binding motif in the amino terminus of apoB needs to be defined.

#### 2.3.4.5. Hepatic lipase binding domain

Like LPL, hepatic lipase (HL) is a multifunctional protein exhibiting both enzymatic and other functions in lipoprotein metabolism (223,245,246). HL acts as an acylhydrolase and as a phospholipase and is essential for LDL production and conversions in the HDL fraction during lipoprotein metabolism. HL may also mediate the unloading of cholesterol from HDL to the

liver and to steroidogenic tissues such as the ovaries and adrenal glands (245). In addition, HL has been postulated to promote apoB-containing lipoprotein, especially CM remnant uptake by cells either by acting as a bridge between cell surface PGs and the lipoprotein, or by acting as a direct ligand for lipoprotein receptors, such as LRP-1 (245-248). Like LPL, the HL-lipoprotein interaction has been suggested to involve protein-protein interactions. While the interaction between CM and VLDL remnants and HL might involve apoE, HL has also been shown to associate with LDL (242). In vitro, HL enhances LDL uptake via LDLR (249), and it can also bind to hrp-116 (250). Thus, direct interaction of HL with apoB seems plausible and has recently been demonstrated in ligand blot studies which show that HL binds to apoB but not to apoE or apoAI (251). While the exact site(s) in apoB mediating these interactions have not yet been identified, the site of apoB interacting with HL and LPL seems to differ: both carboxyl-terminal and amino-terminal regions of apoB seem to be involved in the apoB-HL interaction, which is futhermore not inhibited by LPL (251).

#### 2.3.4.6. Heparin and proteoglycan binding domains

Proteoglycans (PG) are large molecules consisting of linear heteropolysaccharide chains termed glycosaminoglycans (GAG) covalently attached to a core protein through a short nonpolymeric polysaccharide linker (252-254). PGs are found both in intracellular granules, at cell surfaces and extracellularly, with different types of PGs predominating in different sites. ApoB-containing lipoproteins are known to interact with both cell surface and matrix PGs (252). Association with cell surface PGs, either directly or through a bridging action of LPL or HL, has been suggested to facilitate receptor mediated clearance of lipoproteins, but some types of heparan sulfate PGs may function also directly as lipoprotein receptors (254). On the other hand, matrix PGs have been suggested to be involved in the retention of cholesterol-rich atherogenic lipoproteins within the intima of the vessel wall (255). The lipoprotein-PG association of

apoB-containing lipoproteins is believed to be mediated through an interaction of apoE and apoB regions rich in positively charged amino acids, such as arginine and lysine, with the negatively charged polysaccharides in PGs (252,256,257). Seven heparin-binding sites evenly distributed throughout the apoB molecule have been identified (amino acids 5-99, 205-279, 875-932, 2016-2151, 3134-3209, 3356-3489, and 3659-3719), with the two sites exhibiting the highest affinity for heparin located in the putative LDLR binding region of apoB (199). Still other sites have been suggested to be involved in the binding of apoB with chondroitin sulphate PGs (amino acids 2106-2121, 3145-3157, 3359-3377, and 4230-4254) (258). The relative role of these putative sites, identified in delipidated or synthetic apoB-fragments, in the apoB-PG interaction of lipid-containing lipoproteins is not precisely known. Separate sites seem to be used for binding with differing PGs (259). Differences in PG binding between lipoprotein classes and subclasses suggest that the lipid composition and conformation of binding sites may also determine which sites are used (259-261). In addition, modulating factors such as LPL and apoE, or apoB modifications such as proteolysis or fusion of lipoproteins may affect the apoB-PG interaction (262-264). Recently, studies with transgenic mice expressing recombinant mutated human apoB identified apoB amino acids 3359-3369 as the principal site for LDL apoB-PG interaction (265). This same site has also been identified to be the major LDLR binding site of apoB (197). While an artificial mutation at this site disrupting the sequence of positive charges was shown to abolish the affinity of LDL for the PGs studied (265), no natural apoB mutations at this site interfering with apoB-PG interactions have been characterized so far. In addition to the carboxyl-terminal PG-binding region, the amino-terminal globular region of apoB has also been shown to mediate binding of both apoB-100 and apoB-48-containing lipoproteins with PGs (266). To date, no metabolic defects connected with abnormal apoB-PG interaction are known. Theoretically, an altered apoB-PG interaction might modulate both receptor-mediated metabolism and artery wall entrapment of

several kinds of apoB-containing lipoproteins.

2.3.4.7. Microsomal triglyceride transfer protein binding domain

The assembly of apoB-containing lipoproteins requires the association of lipid with the nascent apoB polypeptide while the protein is still being translated. This first step of lipidation, initiated after the folding of the amino-terminal disulfide bonded domain of apoB (172), involves a specific microsomal triglyceride transfer protein (MTP) (99,267). ApoB and MTP have been shown to associate physically through both ionic and hydrophobic interactions (268,269). The amino terminal 18% of apoB seems to be essential for optimum MTP binding capacity, with both increasing length of the apoB polypeptide and lipidation decreasing this affinity (268). In particular, lysine and arginine residues in the amino terminus of apoB differing from those associated with PG of LDLR binding seem to be critical for the binding of apoB with MTP (269). Recent studies have delineated two major amino-terminal sites on apoB for MTP binding: one located between amino acid residues 1-264, and another, centered on residues 512-592 but including flanking residues 430-511 and 640-711 (163,270,271). Several mutations of the MTP gene leading to defective hepatic lipoprotein assembly and secretion and a disease entity termed abetalipoproteinemia have been described (99). To date, no apoB mutations in the MTP binding domain leading to a comparable metabolic defect have been characterized.

2.3.4.8. Other functions and functional domains of apoB

Besides its roles in lipoprotein metabolism, several other functions for apoB still waiting for verification and detailed information concerning their mechanisms and physiological relevance have been proposed. Based on studies with genetically engineered mice, a putative role in normal fetal development, possibly relating to lipid nutrient transport to developing embryos (272-275), as well as a role in spermatogenesis

(274,276) has been suggested. ApoB has been proposed to have domains binding benzo(a)pyrene (277), thyroxin (located at amino acid residues 471-539, 1438-1481 and 3250-4536) (278), and nucleolin, a nuclear protein expressed also at the surface of cultured hepatic cells (279). Other putative apoB functions include enzymatic activities such as phospholipase A1 and A2 activity (280) and protein tyrosine kinase activity (residues 3788-4006) (281). ApoB-100 seems to inhibit the procoagulant action of tissue factor (amino acids 3147-3160) (282,283). In addition, its carboxyl terminus has recently been shown to mediate binding of the platelet-activating factor acetyl-hydrolase to LDL particles (283).

## 2.4. Metabolism of apolipoprotein B

### 2.4.1. ApoB synthesis and secretion

In humans, apoB-containing lipoproteins are synthesized mainly in liver hepatocytes and intestinal epithelial cells (143,284). To a small extent, apoB is produced in other tissues as well, some of which, such as the heart, have been shown to synthesize and secrete lipoproteins (285), and some of which, such as aortic endothelial cells, have not (120). The synthesis and secretion of apoB-containing lipoproteins is a complex process requiring the association of lipid with the apoB polypeptide in an appropriate temporal sequence (41,286-288). ApoB synthesis takes place in the rough endoplasmic reticulum (RER), where, while still being translated, the nascent polypeptide enters the RER lumen through a proteinaceous channel, the translocon, directed by the amino terminal signal peptide sequence, which is thereafter cleaved from the growing peptide chain (286,289). Soon after translocation, the amino terminus of apoB is folded into a globular domain through a process involving several ER proteins, which act either as catalysts for the formation of disulfide bonds in this region, or as chaperones protecting the growing chain from degradation (288,290). Proper folding of the amino-terminus of apoB, mediated partly through the formation of

disulfide bonds in this domain, is required to initiate MTP-dependent lipid transfer to nascent apoB in the RER (172). Especially, the bond between cysteines 218 and 234 is of importance for efficient lipoprotein assembly and secretion (173). The MTP-binding site of apoB has been located within this amino-terminal globular domain (163,270,271). During translation, the translocation of apoB into the RER lumen has been postulated to pause at certain points in a way not affecting chain synthesis. This translocational pausing, dictated by so called pause transfer sequences of apoB, has been proposed to be important for lipid binding of apoB or to allow the nascent apoB to interact with other macromolecules involved in the regulation of lipoprotein synthesis and degradation (286,291). While studies with cell-free systems have suggested that translocational pausing does occur (286,291,292), it is not known, whether this phenomenon can regulate apoB synthesis or secretion in vivo (289). After translation and translocation, apoB seems to associate with the inner leaflet of the ER membrane, but whether this newly synthesized apoB assumes a transmembrane orientation or is totally translocated into the ER lumen is not clear (284,293). The transmembrane orientation is supported by the fact that apoB associates with cytosolic proteins (294), and that two regions of apoB (amino acids 690-797 and 3221-3240) seem to be exposed to the cytosol in the secretory pathway of HepG2 cells (293). This pattern of transmembrane orientation could help in keeping the apoB protein close to ER-resident proteins, functioning both as regulators of lipidation or degradation of apoB (293). After initial apoB protein synthesis, assembly of apoB-containing lipoproteins occurs in a putative two-step process in which a primordial lipoprotein particle is formed in the RER, and expansion of the lipid core then occurs in a second step in the smooth endoplasmic reticulum (SER), and, possibly, the Golgi apparatus (41,99,143,284,295). The first lipidation step seems to be required for the protection of apoB from degradation (41,288). After formation of the primordial lipoprotein particle, more lipids are added to the partilce either sequentially as it moves

along the secretory pathway, or through a fusion of the primordial particle with preformed triglyceride-rich lipid particles in the RER/SER junction (41,287,295-297). MTP is generally accepted to mediate the co-translational first lipidation step, but its role in the second step is still a matter of dispute (41,99,295). After the second lipidation step, the particles are transported to the Golgi apparatus for further modulation where they are glycosylated, phosphorylated and aquire additional phospholipids, whereafter the mature lipoprotein particles are rapidly secreted (284,298).

Regulation of the secretion of apoB-containing lipoproteins is a co- and post-translational process. ApoB mRNA has a relatively long half life, with levels that do not usually change substantially in situations where apoB secretion is altered (284,286,287). Instead, apoB secretion is regulated through intracellular degradation of newly synthesized apoB (286). ApoB degradation has been shown to occur at every step of lipoprotein synthesis, ranging from co-translational degradation of misfolded nascent apoB peptides to degradation of preformed lipoprotein particles in the ER, Golgi or lysosomes (287,288). Two major apoB degradation pathways have been characterized. In the ubiquitin-proteasome pathway, misfolded or poorly lipidated apoB is tagged by ubiquitin to be degraded by a cytosolic multi-subunit protease complex termed the proteasome (286,299) in a process involving other cytosolic chaperone-like proteins such as the heat shock protein 70 (300). This proteasome-mediated degradation has been shown to occur both co- and post-traslationally (301,302). In this degradation pathway, the apoB chain has to be translocated back to the cytosol from the lumen of the ER, either through the translocon complex involved in early apoB chain synthesis, or other not yet characterized specialized retrotranslocons (288,302). Unassembled or aberrant apoB retained in the ER are also degraded by proteases inside the ER lumen. This degradation pathway seems to involve ER resident molecular chaperones such as calnexin or calreticulin and ER proteases such as ER 60 (284,286,290). In some species,

the main site of apoB degradation is in the Golgi or post-Golgi department; details of apoB degradation in these locations are still limited (286,287).

The most important factor regulating apoB degradation is intracellular lipid availability (41,284,287), which seems also to be the main mediator of other factors affecting lipoprotein secretion, such as hormones and diet. ApoB in lipoproteins with inadequate lipids is incorrectly folded and destined to be degraded (143,286). The major lipids secreted in the newly formed lipoproteins are Tg, FC, CE, and Pl. Of these, Pl, especially phosphatidylcholine, though required for the normal secretion of apoB-containing lipoproteins, are usually found in cells in quantities exceeding the amounts needed for lipoprotein assembly and are therefore not likely to be main regulators of lipoprotein degradation or secretion (284,303,304). However, phospholipid composition may regulate apoB degradation: in vitro, enrichment of microsomal membranes with phosphatidyl-monomethylamine impairs apoB secretion, possibly by inhibiting the restart of translocation after pausing, and directing apoB to degradation instead of secretion (292). Availability of the other main lipid components of apoB-containing lipoproteins, Tg and cholesterol, plays a major role in the efficient assembly and secretion of lipoproteins. The relative contribution of Tg and cholesterol or CE in this regulation is still a matter of discussion: studies favoring both factors have been published (41,287,296,305,306). Besides phospholipids, the composition of other lipids in the assembled lipoproteins seems to be of importance as well: in vitro, n-3 fatty acids increase apoB degradation (307), and oxidized fatty acids interfere with the assembly and secretion of TGRLP particles (308).

Apart from the lipidation-assisted folding of apoB, lipoprotein maturation involves other post-translational modifications such as glycosylation and phosphorylaton. While glycosylation appears not to be crucial in apoB secretion regulation, aberrant glycosylation has been shown to disrupt the assembly and

secretion of apoB-containing lipoproteins (286,309,310). To date, no role for the post-translational phosphorylation of apoB in lipoprotein secretion or degradation regulation is known (286,309).

The lipoprotein particles secreted by the liver are heterogeneous in terms of their lipid composition and size (36). Mainly, the liver produces lipoproteins of the VLDL class; these, in turn, can be subdivided into large, buoyant, triglyceride-rich VLDL1 particles and to small, dense, cholesterol-enriched VLDL2 particles (36,311,312). In addition, part of the LDL particles in the circulation are postulated to be secreted directly from the liver (36,311). The intracellular mechanisms regulating the lipid composition and thus the spectrum of lipoproteins secreted from the liver are largely unknown. Factors affecting apoB degradation during lipoprotein synthesis affect mainly the number of particles secreted. However, there seem to be both common elements that regulate the secretion of lipoproteins in general (such as estrogen, regulating secretion of both VLDL1 and 2), and factors with independent effects on the secretion of distinct lipoprotein subclasses (such as insulin, inhibiting VLDL1 secretion (36,312).

### 2.4.2. Intravascular metabolism of apoB-containing lipoproteins

After the secretion of CM particles into intestinal lymph, and VLDL particles into the space of Disse in the liver, both triglyceride-rich particles undergo several modifications affecting both their lipid and apolipoprotein composition before their final removal from the circulation through lipoprotein receptors (9,10,36,42,313). These modifications include hydrolysis of some of their core Tg, removal of surface components to HDL particles, changes in their apolipoprotein content, and exchange of non-hydrolyzed Tg for CE derived from HDL particles. These modifications lead to the formation of smaller, CE-enriched remnant particles and to the production of

IDL and LDL particles from VLDL precursors. Despite these considerable changes in both the lipid and protein composition of lipoproteins during their intravascular metabolism, the primary structure of their apoB moiety remains unchanged throughout the metabolic cascade. However, the tertiary organization of apoB undergoes modifications during these processes. This is reflected in differences in immunochemical reactivity of mAbs against apoB epitopes between lipoprotein classes (314) and can also be visualized as structural differences between lipoprotein classes detected by cryo-electon microscopy (315). Some of these conformational changes may have effects on apoB functions such as affinity for lipoprotein receptors (197) or PGs (259).

### 2.4.3. Receptor mediated and receptor-independent metabolism of apoB-containing lipoproteins

LDLR-mediated lipoprotein uptake to hepatic and peripheral cells is the major mechanism for lipoprotein catabolism (44,316). In this process, apoB-containing particles are specifically bound to the LDLR in a process facilitated by cell surface GAGs, and, possibly by LPL and HL (42,317). Receptor-bound lipoproteins are internalized by endocytosis and transported into lysosomes where their lipids and apolipoproteins are degraded. The cholesterol liberated from the lipoprotein particle regulates cell cholesterol homeostasis by down-regulating LDLR expression, diminishing cholesterol synthesis and increasing cholesterol esterification (44). The ligand for the LDLR in LDL is apoB; in other apoB-containing lipoproteins, apoE molecules, inhibited by apoC:s, mediate the interaction with the receptor (42). Since several molecules of apoE associate with a single lipoprotein particle, apoE-containing lipoproteins are more effectively bound and catabolized than LDL particles, which contain only one apoB-100 molecule (44). The removal of LDL particles in plasma is mediated mainly through hepatic LDLR, whereas peripheral LDLR, other lipoprotein receptors and non-receptor-mediated pathways play a less important role (10,44). The LDLR pathway is

also considered to comprise the main route for hepatic lipoprotein remnant catabolism (42,313).

In recent years, several other cellular receptors structurally related to the LDLR have been characterized (206,207). They all show high affinity for apolipoprotein E and an inhibitory protein termed the receptor-associated protein (RAP) (318), but their tissue distribution and preferred ligands vary considerably, and their physiological contribution to lipoprotein metabolism in humans has still to be determined. Of these LDLR family receptors, the LDL receptor-related protein (LRP-1) (319) may be involved in LDLR-independent lipoprotein remnant catabolism in the liver (42,320), and the VLDL-receptor (321,322) has been suggested to function in the clearance of large CM and VLDL remnants in peripheral tissues (43). The physiological functions of other LDLR family receptors such as the apoE receptor 2 (LR7/8B) (323), LR11 (324), and LRP-2 (megalin or glycoprotein 330) (325,326) are largely unknown. LRP-2 mediates LDL endocytosis in vitro, but its mainly extravascular expression seems to preclude its role in clearance of LDL directly from the blood in vivo (211). Two other non-LDLR-related receptor proteins believed to be involved in lipoprotein metabolism have been characterized. Of these, the lipolysis-stimulated receptor has been suggested to mediate the cellular uptake of large TGRLP in the liver (209). Instead, the role of the hepatic asialoglycoprotein receptor in lipoprotein metabolism is still disputable (42,320,327).

Much of the cholesterol that accumulates in atherosclerotic plaques is found within monocyte-macrophages. Uptake of lipoproteins in these cells seems to occur through specialized receptors, two types of which have been characterized. One class of these receptors mediates apoE-independent uptake of TGRLP such as CM into macrophages, leading to their conversion into foam cells (214,328-330). Still another class of macrophage receptors termed scavenger receptors and recognizing modified, such as oxidized, lipoproteins may be even more important for

foam cell formation (208,331). Besides macrophages, these receptors are expressed in other cells, such as endothelial and vascular smooth muscle cells. As opposed to the LDLR pathway, uptake of lipoproteins through scavenger receptors is not followed by down-regulation of receptor expression: in fact, some types are upregulated by oxidized LDL (208). Besides mediating selective cholesterol uptake from HDL particles to liver and steroidogenic tissues and cholesterol removal from peripheral cells to HDL, the scavenger receptor BI (SR-BI) binds also native LDL and VLDL with high affinity in vitro (332). In transgenic mice overexpressing SR-BI, LDL and apoB levels are decreased, and in vitro studies show selective uptake of LDL CE mediated by SR-BI (332). While the exact role of SR-BI in lipoprotein metabolism in humans is not known, recently, genetic variation in the human SR-BI gene was associated with both LDL and HDL cholesterol levels (81). Apart from SR-BI, other scavenger receptors seem to recognize only modified, mostly ozidized apoB-containing lipoproteins. Therefore, they are not likely to be main mediators of normal lipoprotein catabolism.

A minor part of lipoprotein particles have been suggested to be catabolized through receptor-independent pathways. Among possible mechanisms are direct internalization and eventual degradation of LDL through cell-surface heparan sulfate PGs belonging to the syndecan and perlecan family (254), and selective CE uptake from LDL bound to hepatic or peripheral cells mediated by proteins other than the SR-BI (333,334). In addition, nonspecific low affinity mechanisms such as fluid endocytosis and adsorptive endocytosis, possibly facilitated by cell surface proteoglycans, may take part in lipoprotein uptake (10,335,336). The physiological relevance of all these mechanisms is still unknown.

#### 2.4.4. Oxidative and other modifications of apoB-containing lipoproteins and their role in atherogenesis

Besides modifications involved in their lipid-carrier function,

apoB-containing lipoproteins undergo other changes in vivo, many of which are commonly regarded to be involved in the pathogenesis of atherosclerosis. Lipoprotein oxidation is among the most important of these (337-341). All lipoproteins, especially LDL, are susceptible to oxidation (337), and although oxidation mainly involves lipoprotein lipids, apoB protein is also known to be modified (340,341). Among apoB oxidative modifications are lipid derivative adduct formation with apoB amino acids such as lysine, and cross-link generation between lipids and the apoB protein or within apoB, and, in addition, direct oxidation of amino acids such as tryptophan, arginine and histidine (337,339, 341). The relative and temporal role of protein modification in the oxidation process is still a matter of discussion. While oxidative modification of apoB, leading to protein aggregation and fragmentation, is usually considered to be a relatively late phenomenon in lipoprotein oxidation, some apoB changes seem to occur already at the earliest stages of LDL oxidation (341). Depending on the extent of oxidative modifications, a heterogeneous continuum of oxidized lipoproteins ranging from minimally modified LDL (mm-LDL) to fully oxidized lipoproteins is produced. A major difference between mm-LDL and fully oxidized LDL lies in their receptor-mediated metabolism: the former are recognized by the LDLR, whereas the latter are taken up by scavenger receptors (339,341). So far, no apoB domains especially sensitive to oxidative modification, or apoB genetic variants associated with changes in lipoprotein oxidazibility have been identified.

In addition to oxidation, lipoproteins are also known to be glycated in vivo (342,343). Nonenzymatic protein glycation by glucose is a physiological process resulting either from direct interaction of glucose with serum proteins, or a modification of proteins with low molecular weight, highly reactive advanced glycation end products (AGE), formed during degradation of other glycated proteins (344). In lipoproteins, AGE modification may involve lipids as well. Sugar residues in glycated LDL are readily auto-oxidized, generating superoxide radicals which

stimulate further LDL lipid and protein oxidation (343). AGE-LDL show reduced affinity for the LDLR, but are instead taken up by vascular cells through both scavenger and specific AGE-receptors, leading to several cellular injury responses (344-347). ApoB lysine residues are known to be important in both the initial glycation and AGE-modification of the protein (344). While lysine residues within the putative apoB LDLR binding site seem to be protected from glycation, residues in close proximity to this site are modified, and may explain the reduced affinity of AGE-LDL for the LDLR (344). In addition, a domain of apoB between residues 1388 and 1453, detected by AGE-specific antibodies seems to be specifically modified (348), and still other sites, showing changes in their immunoreactivity after LDL glycation have been identified (344). Elevated amounts of circulating AGE-LDL, detected in diabetics, patients with renal insufficiency, and in hyperlipidemia, may partly explain the increased CAD risk in these subjects. So far, no apoB genetic variants associated with altered glycation sensitivity have been identified.

Sialic acid is a terminal residue of the carbohydrate chains of glycoproteins, such as lipoproteins (349). The high surface charge associated with sialic acid plays an important role in the prevention of lipoprotein aggregation (350). Desialylation of LDL particles leads to several changes in their biological behaviour, many of which have been suggested to play a role in the early steps of atherosclerosis (351). Like normal LDL, desialylated LDL are recognized by the LDLR (352,353), but seem also to be taken up through scavenger receptors (352), the hepatic asialoglycoprotein receptor (350), a galactose-specific macrophage lectin receptor (353), and through direct phagocytosis of aggregated desialylated LDL particles (351). Desialylated LDL are smaller, denser, more negatively charged, more prone to PG binding and aggregation than normal LDL particles, and more suspectible to oxidation (351). In vitro, these changes are reflected in increased uptake of desialylated LDL by fibroblast and smooth muscle cells (354) and in their

accumulation in aortic intimal cells and macrophages (352,353). In vivo, the catabolism of desialylated LDL has been shown to be accelerated (355). LDL desialylation seems to occur in vivo: LDL particles isolated from healthy subjects is partly desialylated, and even more desialylated LDL particles are found in the plasma of diabetics and CAD patients (351). However, the exact mechanism of LDL desialylation is not known, and no specific apoB sites have been shown to be involved in LDL desialylation processes.

Increased levels of plasma homocysteine have been associated with an increased risk for CAD (356,357). Homocysteine may modify lipoproteins either by facilitating LDL oxidation (356,357), or by forming adducts with apoB lysine residues, leading to particles with foam-cell formation capacity (357). Whether homocysteine-modified LDL are present in vivo is not known, and thus, the mechanism of the homocysteine and CAD association has not yet been uncovered (357). The ethanol oxidation product acetaldehyde is known to be chemically highly reactive, forming adducts with proteins in alcoholics. Modification of LDL apoB by acetaldehyde reduces its binding affinity to the LDLR (358), and in vivo, acetaldehyde modification of VLDL apoB has been shown to decrease fractional catabolic rates for both VLDL, IDL and LDL, and to reduce the fraction of VLDL converted to LDL (359). Considerable qualitative heterogeneity in these metabolic changes have however been observed and thus, the physiological relevance of lipoprotein modification by acetaldehyde remains to be determined.

Atherosclerosis is a complex and continuous process. Starting with modulations of vascular endothelial cell function, it continues by accumulation of lipids in the subendothelial cell matrix and recruitment of monocytes into the arterial wall with their transformation to machrophages, and is later followed by smooth muscle cell migration and proliferation, and the formation of a fibrous plaque, the rupture of which eventually

leads to thrombus formation (232,255,338,339,341,360,361). Oxidatively or otherwise modified lipoproteins seem to play an important role in atherogenesis. The initial endothelial changes are believed to be mediated through actions of native or only minimally modified lipoprotein particles (232,339,362), or their remnants (363,364). After their transport and entrapment into the subendothelial matrix, both normal and modified lipoproteins undergo further oxidative (339,341) and enzymatic modifications (365-368), and are thereafter taken up into arterial wall cells through scavenger and other specific receptors (361), or through non-receptor-dependent mechanisms (336). Uptake of modified lipoproteins leads to cellular activation and further lipoprotein trapping and modification (339,360). Atherosclerotic lesions contain both lipids and components of apoB. To what extent specificity of the steps leading to full blown atherosclerosis is due to lipoprotein apoB is not known; some role in the initial lipoprotein attachment to the endothelium (237), in the subendothelial matrix trapping (232,265), and receptor-mediated uptake of modified lipoproteins to macrophages (212) seem to be dependent on their apoB moiety.

## 2.5. ApoB genetic variants and lipids

### 2.5.1. Polymorphisms

Among the first polymorphisms of apoB to be characterized were the antigen group (Ag) polymorphisms detected by the use of alloantibodies produced in multiply transfused patients (369). These Ag polymorphisms were based on antigens $a_1$/d, c/g, h/i, t/z, and x/y, which appeared to be products of five closely linked allele pairs (370). Later on, DNA changes corresponding to each of the five Ag epitopes were identified, and all were found to be detectable at the DNA level by restriction endonucleases (371). Since the availability of the original alloantibodies for these Ag epitopes is limited, these polymorphisms are currently analyzed either at the DNA level as RFLPs, or at the protein level with mAbs.

Besides the Ag polymorphisms, several other apoB genetic variants have been characterized and studied in populations. Among these, the most important are the three-allele insertion/deletion polymorphism situated in the signal peptide of apoB (372), the XbaI polymorphism in exon 26 (113,373,374), and a multiallelic locus at the 3´ end of the gene termed 3´VNTR (375,376). In the following, the main characteristics and effects of the major polymorphisms of apoB, in 5´ to 3´ order, are discussed. The nucleotide and amino acid sequence and numbering used in defining apoB variants is based on the sequence published by Knott et al. (116). Therefore, some polymorphisms will be presented as a change from the actually rare allele to the common one. A summary of apoB polymorphisms as well as hypercholesterolemia-producing and neutral apoB mutations is shown in Table 4.

The apoB signal peptide insertion/deletion polymorphism was first characterized as a two-allelic genetic variant leading to the insertion or deletion of three codons coding for the signal peptide, which thus contains either 27 or 24 amino acids, the allele containing 27 amino acids designated as the insertion (ins), and the allele with 24 amino acids the deletion (del) allele (372). Later on, a third allele encoding a signal peptide with 29 amino acids was detected: this third allele seems to be present at a low frequency (0.03 to 0.08) only in Amerindians (377,378). In Caucasians, the allele frecuency of the del allele is approximately 0.30 (379-381); in non-Caucasoid populations, lower frequencies have been reported (378,380,382-388). Several studies have addressed the effects of the ins/del polymorphism on lipid levels in both healthy, hyperlipidemic and CAD patient populations. Some of these studies have shown an association of the del allele with higher serum total or LDL cholesterol, Tg or apoB levels (379-384,389-397), while in the Finns, an association between the ins allele and higher Tg levels has been detected (398), and yet some studies have found no effect of the ins/del polymorphism on lipid levels (378,385-387,399-402). Three studies have found an association between the del allele

and the extent or presence of atherosclerosis or myocardial infarction (380,391,403), two studies an association between CAD and the ins allele (404,405), while in others, no allele frequency differences between healthy or CAD populations could be seen (392,396,406-408). Concerning lipid responses to dietary change, ins/ins homozygotes have been shown to be more responsive both to modifications in dietary fat and fibre compared to del/del homozygotes (78,395). This response is variable, being more consistent in del/del homozygotes (409). The ins allele has also been associated with larger lipid changes after a fat challenge (410-412). The mechanism by which the ins/del polymorphism affects lipid levels is presently not known with certanity. The signal peptide is cleaved away from the growing apoB nascent polypeptide chain early in lipoprotein synthesis and cannot be involved directly in their metabolism after particle assembly and secretion. Studies using genetically modified yeast cells, in which the apoB signal peptide region was added to yeast invertase, have shown the del allele to be defective as opposed to the ins allele with respect to invertase secretion from the cell (413). Molecular modelling suggests that the del allele is not hydrophobic enough to be effectively translocated through the ER membrane (414). This could, hypothetically, lead to reduced lipoprotein secretion in del/del homozygotes, a hypothesis gaining some support from a recent apoB turnover study in obese men showing higher hepatic VLDL apoB secretion in ins/ins homozygotes compared with ins/del heterozygotes or del/del homozygotes (415). This hypothetical apoB secretion model contrasts with the documented associations of the del allele with higher lipid levels in population studies. One explanation for this apparent discrepancy could be that these observed associations in population studies reflect a linkage disequilibrium of the ins/del polymorphism with some other polymorphic site of the apoB gene.

The Ag(c/g) polymorphism results from a C→T change at cDNA nucleotide 421 leading to a Thr→Ile change at amino acid residue 71 of apoB (416,417). It can be detected both with restriction

endonucleases ApaLI (416) and Bsp12861 (416) and with mAbs MB19 (418,419), BIP45 (420) and D2E1 (421). The frequency of the Ag(c) (apoB 412 Ile, ApaLI-, restriction site absent) allele in Caucasian populations is estimated to be approximately 0.30 (420,422-424). In other racial groups, considerably lower frequencies have been reported (370,425). The Ag(c) allele has been associated with higher serum apoB and LDL cholesterol concentrations in Finnish children (426), the Ag(c/g) phenotype with higher serum apoB concentrations (423) and, the Ag(c) allele connected with the XbaI+ allele with higher serum total and LDL cholesterol in Finnish healthy men (427). Several other studies have not detected significant associations between the Ag(c/g) polymorphism and lipid levels (424,428-430). In Congolese blacks, the Ag(c) was associated with lower serum apoB levels (425). Only two studies have addressed the possible effects of the Ag(c/g) polymorphism on the risk of CAD, with no signs of significant association (424,428).

The Ag($a_1$/d) polymorphism, resulting from a T→C change at cDNA nucleotide 1981 and producing a Ala→Val change in amino acid 591 of the mature apoB protein (431), can be detected with restriction endonuclease AluI (431) and with mAb H11G3 (421). The allele frequency of the apoB 591 Ala (Ag(d), AluI+, restriction site present) allele in Caucasians is approximately 0.45 (370,432). In Chinese, its frequency is much lower, 0.16 (433), and in Africans, much higher, 0.93 (370). Data on lipid effects of the Ag($a_1$/d) variation are so far limited. The 591 Val allele has been associated with larger postprandial TGRLP responses after a fat load (410). In patients suffering from ischemic stroke, serum apoB concentrations have been found to be higher in the carriers of the 591 Ala allele (434). No studies addressing the effects of this polymorphism on CAD risk have been published. According to data from both Finnish and other studies, the signal peptide del and the Ag(d) (apoB 591 Ala) allele seem to be closely associated (398,410).

The XbaI polymorphism, a T→C change at nucleotide 7673 with no

change in the threonine residue 2488 of apoB (113,373,374), is one of the most studied apoB polymorphisms. In Caucasian healthy populations, the reported allele frequency of the XbaI+ allele has ranged from 0.40 to 0.60 (373,435), and a similar frequency has also been reported in healthy Finns (427,436). In non-Caucasoid populations, the frequency of the XbaI+ allele is considerably lower, from 0.01 in the Chinese (433,437) to 0.29 in a population of South Asian descent (382). A summary of XbaI population studies addressing its effects on serum cholesterol, Tg or apoB levels or on the risk of CAD is shown in Table 5. Many, but not all studies show an association of the XbaI+ allele with serum lipid and lipoprotein levels. These effects of the XbaI site on lipid levels seem to be especially clear in the Finnish population. In Asian populations, the XbaI+ allele (very rare in these populations) is associated also with lower serum HDL cholesterol or apoAI levels (382,384,406,438-440), contradictory to a few studies in Caucasians, including the Finns, which show an association of the XbaI+ allele with higher serum HDL cholesterol or apoAI levels (441,442). In addition to studies listed in Table 5, some studies have suggested an effect of the XbaI polymorphism on postprandial lipid responses (443), responses to dietary changes (395,442,444-446) and to lipid lowering drugs (447), as well as on the intra-individual lipid variability over time (448). As for CAD, several studies show an association of the XbaI- allele with the presence of CAD. Being a silent polymorphism, the XbaI change cannot be the causative factor in these associations and effects. Rather, this site is believed to be in linkage disequilibrium with another apoB genetic variant influencing lipid levels or predisposing to CAD. Regardless of the actual site of apoB mediating the association detected with the XbaI polymorphism, it does not seem to act through changes in the production of apoB-containing lipoproteins. In hepatoma-derived liver cell lines, the XbaI polymorphism has no detectable effect on cholesterol, Tg or apoB secretion (449), and kinetic studies show similar LDL production rates across different XbaI genotypes (450,451). On the other hand, kinetic studies have shown that LDL particles from XbaI+/+

homozygotes exhibit lower fractional clearance rates compared with XbaI-/- LDL particles (402,450-452). In addition, lower LDLR-dependent degradation rates by mononuclear cells (453) and fibroblasts (454) of the XbaI+/+ LDL particles have been detected. These two lines of studies point towards an association of the XbaI+ allele with a factor affecting LDL removal from the circulation.

The Ag(x/y) polymorphism has been linked to two different DNA polymorphisms of apoB: a C→T change at nucleotide 8344 (exon 26), leading to a Pro→Leu change in amino acid 2712 (455), and detectable with restriction endonucleases MaeI (371) and BfaI (456), and an A→G change at cDNA nucleotide 13141 (exon 29) with an apoB Asn4311→Ser change (457), detectable with Eco57I (456). The Ag(x) epitope represents the allele encoding both 2712 Leu and 4311 Ser (458). The Ag(x/y) and the XbaI polymorphisms are closely linked, with the Ag(x) allele associating with the XbaI- allele (459,460). In Caucasians, including the Finns, Ag(x) allele frequencies of 0.20 to 0.40 have been reported, whereas the frequency of the Ag(x) allele in non-Caucasoid populations is highly variable (422,458,461). In population studies, the Ag(x) allele has been associated with lower serum total and LDL cholesterol, apoB and Tg levels and with higher serum HDL cholesterol and apo AI levels (458,462,463). Of these associations, the most significant have been detected in the Finnish population (462). So far, no association between the Ag(x/y) polymorphism and CAD has been detected (404,463).

The Ag(h/i) polymorphism associates with a G→A change at nucleotide 11041, and leads to an Arg→Gln substitution at amino acid 3611, detectable with MspI (464,465). With an allele frequency of the 3611 Gln (MspI-, Ag(h)) allele in the range of 0.02-0.14 in Caucasian as well as non-Caucasian populations, it is the least frequent of the apoB antigenic polymorphisms (370,406,465,466). Most population studies reported to date show no association between this polymorphism and lipid levels (378,390,393,402,437,465-470) or the presence of CAD (406,407,

437,471-474). Still, in a few studies, the rare Ag(h) allele has been associated with higher serum lipid levels (79,475-477). As for CAD, associations with both the rare MspI- (478) and the common MspI+ allele (470) have been reported.

The Ag(t/z) polymorphism represents the apoB EcoRI RFLP, which is the result of an A→G change at nucleotide 12669 (exon 29) producing a Lys to Glu change at amino acid 4154 (113,479,480). Reported allele frequencies of the 4154 Lys (Ag(z), EcoRI-) allele have ranged between 0.10 to 0.20 in most Caucasian as well as African populations (370,388,404,479,481,482). In Asian and Amerindian populations, lower 4154 Lys allele frequencies have been detected (378,384,385,440,466,483). In most population studies, no effect of this polymorphism on serum lipid and lipoprotein levels have been detected (78,79,378,382,385,390, 393,400,404,406,432,437,438,440,441,466-469,476,477,484-490). Some studies show an association between the 4154 Lys allele and elevated serum cholesterol, Tg or apoB levels (470,491-495) and still others report an association of lower serum cholesterol levels with the 4154 Lys allele (402,475,483,496). The Ag(t/z) polymorphism seems not to have an effect on lipid responses to changes in diet (444,445,497). Both studies showing an increased risk for developing CAD in carriers of the 4154 Lys allele (440,441,478,485,488,491), as well as a neutral role for the Ag(t/z) polymorphism in the pathogenesis of CAD have been published (382,400,406,407,435,470,472,476,484,498). In patients with non-insulin-dependent diabetes mellitus (NIDDM), 4154 Glu homozygosity associates with an increased risk for CAD (499). There are also reports on the association of the 4154 Lys allele with NIDDM (492), with high (500) or low BMI (494), and blood pressure (501). Two studies have shown age-dependent selection towards lower prevalence of the 4154 Lys allele in very old compared with younger subjects (473,502). The biological mechanisms for all these associations are far from being clear. No relation between cholesterol, Tg and apoB secretion from hepatoma derived liver cell lines and the EcoRI genotype has been detected (449). Studies on the effects of the 4154 Lys

allele on LDL fractional catabolic rate have shown both decreased (451), increased (402,500) and invariant rates of clearance (450); the only LDL binding study reported to date could not demonstrate an effect of this polymorphism on LDL binding to fibroblasts (503).

The apoB 3´VNTR polymorphism (variable number of tandem repeats) or 3´HVR (hypervariable region) consists of a multiallelic locus with repetitive 11 to 16 bp AT-rich DNA sequences beginning 73 bp 3´ to the second polyadenylation signal (375,376). The 3´VNTR was originally defined as a simple length polymorphism, resulting from differing numbers (from 22 to 57) of repeated sequences, and it is most often genotyped based on this size variation by polymerase chain reaction-based methods in ordinary agarose (504) or denaturing acrylamide electrophoresis gels (505). Before the development of these methods, a crude estimate of the different-sized alleles was achieved with restriction endonucleases such as MspI, BamHI, and HindIII with or without SspI (485,487). The majority of length variation resides in the 5´ end of the repeat array constituting from 4 to 15 copy numbers of one specific 30 bp repeat unit containing the 15 bp sequences termed x and y (376,506,507). In addition, the number and type of other repeats which are considered derivatives of the basic x and y repeats have been shown to vary between different alleles (506-509). Usually, apoB 3´ VNTR alleles are named according to the number of repeats. Depending on whether an 11 bp sequence in the 3´ boundary of this region is included as a repeat or not, two nomenclatures with mainly even (repeat not included) (505) or odd numbers of repeats (504) have been proposed. To date, 26 different-sized 3´VNTR alleles have been characterized in humans (504,505,510,511). Despite being widely used in population association studies, definition of the 3´VNTR site on the basis of size alone is an oversimplification. The repetitive core sequences seem to exhibit considerably more genetic variation than has hitherto been known. According to careful sequence analyses, alleles with an equal number and type of repeats, formerly believed to be identical, still differ in

sequence. Structural analysis of 18 size-characterized alleles resulted in the detection of altogether 39 alleles with up to 6 alleles bearing the same number of repeats (509). As a multiallelic locus with a high grade (from 0.70 to 0.90) of heterozygosity (511), the apoB 3´VNTR is a good genetic marker to be used, for example, in forensic analyses. In lipid genetics, the high number of possible alleles leads to difficulties in associating single alleles with lipid levels or CAD. Therefore, for practical purposes, most association studies have divided the 3´VNTR alleles into groups containing large or small alleles, a definition largely based on the known bimodal distribution of different-sized alleles in populations. In most populations, alleles containing 35 and 37 repeats (odd number nomenclature) form the larger, and alleles 47 and 49 the other distribution peak (387,397,504,505,512-514). In the Koreans, an unimodal distribution with a peak on allele 35 (515), and in African populations, unimodal peaks on alleles 35 or 37 and with a considerable number of both large and small alleles as well the presence of unique alleles not detected in Caucasians (510,511) have been reported. A small number of studies seem to connect single apoB 3´VNTR alleles with serum lipid levels (381,397,514,516-519), and one study has shown an association of apoB 3´VNTR allele 35 with essential hypertension (520). However, most studies have not detected significant lipid associations (387,390,393,440,471,478,485,486,493,515,521,522). While the definition of a large allele has varied in different studies, large alleles have been associated with an increased risk of CAD in many (440,471,478,485,522,523), but not all studies (381,393,493,516,518).

In addition to these common apoB polymorphisms, several other polymorphic sites in the apoB gene have been detected (Table 4). Most of them have been reported shortly after the cloning of the apoB gene and represent classical RFLPs. For some of these, the exact site of DNA variation has later been identified. Also, intronic polymorphisms have been detected. Data on the effects of these polymorphisms on serum lipid levels or CAD risk are

limited. The apoB 5´VNTR locus has been excluded from causing familial hypertriglyceridemia (100), and it seems not to associate with serum lipid levels or CAD (524). Recently, an association of the -516 T allele located in the promoter area of the apoB gene with elevated LDL cholesterol levels and acute myocardial infarction was reported (525). Based on in vitro transfection experiments, the LDL-cholesterol elevating effect of this variant was suggested to be due to an increased rate of apoB transcription. One study has shown the apoB promoter region -265 T allele to have an LDL cholesterol and apoB lowering effect in healthy subjects (404). Neither the StyI polymorphism in intron 2 or the HincII polymorphism in intron 4 have been associated with serum cholesterol levels (475,515,526). The intron 4 PvuII polymorphism, while linked with serum apoB levels in one large kindred (517,527), seems not to be associated with either lipid levels or CAD in population studies (441,484,515). With regard to other apoB polymorphisms listed in table 4, no reported data on their effects are available.

Any attempts to measure the contribution of apoB genetic variants to hyperlipidemias and CAD have been confounded by the variable, sometimes even opposing results in association studies analyzing the effect of apoB polymorphisms on lipid levels. Several reasons for this can be suggested (12). Sample sizes have often been too small to adequately show associations between a genetic variant and lipid levels, when the effect is small. Genetic heterogeneity of the studied sample and the way in which the statistical analyses are carried out may have also affected the results of these association studies. According to one hypothesis, another explanation for the discrepant results could be that it is not the single apoB polymorphism per se, but a combination of DNA and amino acid changes in the whole apoB protein that is affecting lipid levels (528). Thus, attempts to associate lipid levels with specific combinations of several apoB polymorphic alleles, termed haplotypes, could be more informative. Some studies have analyzed partial haplotypes for a small number of apoB polymorphisms (441,475,477,488,498). More

detailed haplotypes, however, are difficult to deduce unambiguously in unrelated subjects. Therefore, while a few studies using this approach have been reported (515,528,529), large scale studies of this kind are not likely to gain wide use in apoB association analyses.

### 2.5.2. Mutations

#### 2.5.2.1. ApoB mutations causing hyperlipidemia

The first apoB heritable defect shown to be associated with hypercholesterolemia was an A→G transition at cDNA nucleotide 10708, leading to an Arg→Gln change at apoB amino acid 3500 and to the production of ligand-defective apoB-100, resulting in a disease entity termed familial defective apolipoprotein B (FDB) (13,14). Despite lying outside the putative apoB LDLR binding site, the mutation has been suggested to change the three-dimensional structure of the binding area so that LDL particles possessing mutated apoB have reduced binding affinity to the receptor (530). Proof favoring this hypothesis has recently been obtained from studies in transgenic mice expressing the human FDB mutation (197). In vivo, LDL particles from FDB heterozygotes exhibit plasma clearance rates (531,532) and LDLR binding affinities (13) of less than 50% and 33% of normal, respectively. The binding affinity of isolated 3500 Gln LDL particles is much lower, approximately 9% of normal (533). This leads to an accumulation of LDL particles carrying mutated apoB in the circulation (533,534). The receptor-mediated metabolism of VLDL and IDL particles seems to be normal, possibly because these particles can be removed by the LDLR pathway through interactions of the receptor with lipoprotein particle apoE (532,535-537). This has been suggested to explain the fact that the few 3500 Gln homozygotes detected so far do not seem to suffer from a more severe disease than heterozygotes (538-540). The phenotypic characteristics of FDB carriers range from a disease indistinguishable from FH (18,541,542) through moderate hyperlipidemia (17,543) to normal serum lipid levels (534,544-

546). Considerable variability between lipid levels in FDB carriers belonging to the same families has also been reported (544-546). The mutation can, but is not always expressed during childhood (18,547). An increased risk of CAD, possibly reflecting live-long blood lipid levels in FDB carriers has been reported (18,536,544,546,548). The Arg3500→Gln mutation has been detected in several Caucasian populations and, depending on the type of population studied, has been estimated to occur with a frequency of 1/71-1/1322 (15,17,20,548-553), with the highest prevalence reported recently in Germany. In Caucasians, most FDB carriers have identical apoB haplotypes (526,544,554-557), suggesting a common founder for the mutation. In the few FDB carriers detected in other racial groups and one German patient, haplotype analyses point towards recurrent new mutations different from the Caucasian ancestor mutation (16,551,558,559). Despite its frequency in other populations, the FDB mutation seems to be absent from the Finns (32). Based on the distribution of FDB carriers, the common haplotype of the mutation, and knowledge of early human migrations, the original FDB mutation has been suggested to have occurred in a common ancestor living in Europe about 6750 years ago (560).

Recently, two other apoB mutations have been associated with an FDB-like clinical disease. The first to be detected was the apoB Arg3531→Cys mutation (15); the second affected the same apoB 3500 codon but now with a substitution of Trp for Arg (16). These mutations are more rare than the original FDB mutation. Both have been shown to result in defective binding of LDL to the LDLR and leading to hypercholesterolemia although it has been suggested that the clinical disease produced by these mutations is less severe than that produced by the Arg3500→Gln mutation (20,21,548). So far, the Arg3531→Cys mutation has been detected in 26 families, some of which are of Celtic origin and share a unique haplotype, while others seem to have occurred separately and are associated with four separate apoB haplotypes (15,21,456,548,557,561). The Arg3500→Trp mutation has so far been detected in one Scottish and two German families and twelve

subjects of Asian descent; haplotype analyses in the latter point toward a common ancestor whereas the Scottish and the two German mutations each seem to have occurred independently (16,20,562,563).

All three hypercholesterolemia-producing apoB mutations are located in the vicinity of the putative LDLR binding site. Thus it is not surprising, that their effects on lipid metabolism are similar and seem to involve the ability of apoB to act as a ligand for the LDLR. Recently, several reports on methods to detect all these apoB mutations easily in population samples have been published (16,20,557,564-566). With these methods, the relative importance of the new apoB variants in producing hypercholesterolemia at the population level can be assessed.

#### 2.5.2.2. ApoB mutations causing hypobetalipoproteinemia

In contrast with the few hypercholesterolemia-producing apoB variants, several unique mutations associated with low cholesterol levels and a disease entity termed familial hypobetalipoproteinemia (FHBL) have been characterized (98). Most of them lead to the production of apoB variants truncated at different points of the peptide. A summary of mutations reported to date to be associated with FHBL, distributed throughout the apoB gene, is shown in Table 6. Usually, the truncated apoB protein is present in plasma in very small amounts in varying lipoprotein subclasses. Two mechanisms seem to lead to this. Firstly, kinetic studies suggest that the production of the truncated apoB is diminished (202,567-569), with the secretion rate of truncated apoB directly related to the size of the protein (567). In vitro studies suggest that the low production of truncated apoB protein results from an increased rate of cytoplasmic degradation of the mutant apoB mRNA (570). Secondly, the clearance of lipoproteins containing the abnormal apoB may be accelerated (202-204,571). A mouse model for FHBL with an apoB 83-like truncated apoB suggests that both mechanisms account for the low plasma levels of the

abnormal apoB species (572). At the same time, the plasma concentrations of full-length apoB-100 in FHBL patients is also often reduced to a level which is less than half of that expected from normal apoB allele production. This has been attributed to both diminished production (573,574) and increased catabolism (574) of apoB-100 in FHBL heterozygotes. Clinically, heterozygous FHBL carriers manifest as asymptomatic subjects with low (less than 50% of normal) plasma total and LDL cholesterol levels. Compound FHBL heterozygotes (201,575,576) and homozygotes (203,577-581) show varying signs, ranging from fat malabsorption, liver and neurological abnormalities to complete absence of symptoms. With a gene frequency of only 0.0001 in the general population, truncated apoB variants are likely to explain only a minor part of persistent hypobeta-lipoproteinemia, most cases being related to other causes, such as the E3/E3 genotype (582). However, in normo- or hypo-cholesterolemic healthy subjects, FHBL mutations are detected more often, with reported mutation frequencies from 0.002 to 0.013 (98,583).

2.5.2.3. Neutral apoB mutations

Rare apoB variants not having any effect on lipid levels may be termed neutral mutations. Many of these represent actual polymorphisms. Silent mutations producing no amino acid change are also likely to be neutral in terms of lipid effects (21,561,562). In addition, for several of them, such as the Phe1410→Leu, Glu2539→Lys, Ala3094→Thr, His3292→Asp, Lys3400→Thr, and Arg3480→Pro mutations, data on lipid levels are either totally lacking or otherwise too restricted to assess possible associations with the DNA variant (565,584,585). Among the first neutral apoB variants to be detected was the Arg4019→Trp mutation (apoB Hopkins), which did not associate with the hyperlipidemic phenotype presented in the family in which the mutation was detected (586). Another more studied neutral apoB mutation is the Glu3405→Gln mutation, which, although showing changes in LDLR binding studies has not been associated with

hyperlipidemia in family or population studies (20,561,587,588). The Val3894→Ile mutation, claimed to be associated with hypocholesterolemia (585), has been described in a single family including also one hypocholesterolemic non-carrier, pointing towards a neutral effect of this mutation on serum cholesterol levels. The Ser3252→Gly, Ala3371→Val, Val3396→Met and Ser3455→Arg mutations seem either not to be associated with hyperlipidemia or to influence the LDLR binding activity of apoB (561,588).

## 2.6. Methods to detect DNA variations

### 2.6.1. Search for new variations

#### 2.6.1.1. Southern blot hybridization

Restriction fragment length polymorphism (RFLP) analysis (589) with either genomic DNA or cloned DNA from a liver cDNA library (complementary DNA produced from liver mRNA) as the template, and the Southern blot hybridization technique as the detection method (590) was one of the earliest methods to detect apoB variations. This method is based on digestion of the DNA sample with a restriction endonuclease cutting the DNA strand at or near a specific enzyme recognition site, electrophoretic separation of the digested fragments, and detection of target sequences by hybridization with sequence-specific probes labeled with radioisotope, fluorescent, or chemiluminescent tags (591). If the sequence recognized or the site cut by the enzyme is altered by a mutation, the abolishment or creation of a new restriction site results in a change in the length of the digested fragments. Since most point mutations occur on sites not harboring restriction sites, the Southern technique is now rarely used in the search for new mutations. In direct assays of gene structure, it remains a useful method for detecting large deletions or gene rearrangements.

#### 2.6.1.2. Single-strand conformation polymorphism analysis

The polymerase chain reaction-based (592,593) single-strand conformation polymorphism analysis (SSCP) method (594,595) is one of the most widely used techniques to search for point mutations, and it has been used with success in the search for variants of the apoB gene as well (490,561,584,585). SSCP analysis is based on the tendency of single-stranded DNA molecules to adopt a specific three-dimensional conformation that is uniquely dependent on their nucleotide sequence composition under non-denaturing conditions. A change in a single base may affect this conformation, resulting in an altered electrophoretic migration pattern of the mutant DNA strand in a non-denaturing polyacrylamide gel. SSCP has been reported to be most sensitive when used to detect sequence variation in PCR amplified DNA molecules 300 bp or less in size (596). Due to constraints on the ability of small DNA fragments to form stable secondary structures, there appears to be a lower sizw limit (100 bp) as well (597). Within the optimal size range of DNA fragments (200 bp), SSCP is sensitive enough to detect up to 70-90% of single base substitutions (596). However, several reports show succesful detection of mutations in much larger, up to 700 bp, fragments, provided several differing electrophoresis conditions such as temperature, buffer concentrations, gel composition, running time and power are used (598-600). Besides the size of the segment to be analyzed and electrophoretic conditions used, the sensitivity of SSCP seems also to depend on the sequence composition of the whole DNA fragment, whereas the position and type of mutation seems to be of less importance (597). In fact, base substitutions as close as 6 bp to the 3´ end of the amplification primer have been detected by SSCP (601). As the conformation of RNA molecules is more stable than that of single-stranded DNA, a modified SSCP procedure based of the generation of a single-stranded RNA transcript has been developed (602). In spite of its efficiency, leading to up to 95% detection of variations, this ssRNA-SSCP-method seems to be too inconvenient for large scale SSCP screening. Most often,

SSCP analyses are carried out using radioactive nucleotides in the PCR and detecting the moving pattern of the fragment by autoradiography. Alternatively, non-isotopic modifications such as SSCP with silver or ethidium bromide staining (603,604), and methods using fluorescence labeling and an automated DNA sequencer (605,606) may be used. Among other modifications to the classical SSCP protocol are restriction enzyme digestion of large PCR fragments before SSCP (607,608), the use of multiplex (609), allele-specific (610) or asymmetric (611) PCR products for SSCP analysis, or dideoxy chain termination to localize the approximate position of an SSCP variation in a sequence, a method termed dideoxy fingerprinting (612). High throuhgput modifications of SSCP for mutational analyses have also been developed (596).

#### 2.6.1.3. Denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis

Double-stranded DNA molecules are typically organized into high- and low-melting domains. If a DNA molecule in solution is subjected to an increasing temperature or concentration of denaturant, such as urea or formamide, the low-temperature melting domain will eventually become single-stranded. The denaturing condition at which a domain melts is specific and sequence-dependent, so that a single base-pair substitution may alter the point at which the transition to the melted state occurs. Transition of double-stranded DNA molecules into partly dissociated strands leads to a decrease of their electrophoretic mobility. These properties of DNA form the basis of both the denaturing gradient gel electrophoresis (DGGE) method (613,614) and the temperature gradient gel electrophoresis (TGGE) techniques (615,616). Since the behaviour of a DNA fragment in a denaturing gradient gel can be predicted from the base-pair sequence (617), the melting characteristics of the fragment can be altered by addition of a GC-rich sequence termed a GC-clamp to the fragment (618,619). The sensitivity of DGGE can also be enhanced with the use of heteroduplex DNA molecules between

wildtype and mutant sequence, generated either during PCR cycling of by mixing of PCR products, for the analysis (620,621). DGGE is a reliable and sensitive method even if relatively large DNA fragments, up to several hundred bp (621) are studied, and has the advantage that mostly, non-radioactive means for the detection of difference in the moving pattern can be used. Thus, DGGE is being more and more used in screening studies, either alone or combined with SSCP. DGGE has been adapted to apoB gene mutation screening as well (16,21,457,562, 565,622,623). TGGE has been used less often: only two studies on the use of TGGE in apoB gene screening have been published (20,624).

2.6.1.4. Heteroduplex analysis

In addition to their differentation under denaturing conditions, heteroduplex DNA molecules containing internal mismatches show altered migration when compared with correctly matching homoduplexes also in nondenaturing polyacrylamide gels (625-627). Reported sensitivities of this heteroduplex analysis (HA) method lie in the same range as SSCP (627,628). Both isotopic and non-isotopic detection methods can be used for HA, and, in addition, both SSCP and HA can be performed simultaneously (627,629). A HA-based mutation detection protocol has been described and used in apoB screening (456,542,564), and has been suggested to be equally sensitive but more simple than DGGE in the detection of the apoB Arg3500→Gln mutation (623).

2.6.1.5. Other methods detecting DNA mismatches

Several mutation detection methods applying either chemical or enzymatic modification of mismatched base pairs in heteroduplex DNA strands have been developed. One of these is the chemical cleavage of mismatch (CCM) method (630,631), which is based on modification of mismathced bases by chemicals such as osmium tetroxide or hydroxylamine, cleavage of the DNA at the modified mismatch by piperidine, and electrophoretic detection of the

cleavage products. CCM has the andvantage of a relatively low size constraint in mutation scanning, being effective in detecting mutations in PCR products up to 1.7 kb in length (632). Three reports of apoB genetic screening with CCM have been published (458,633,634), but in spite of its sensitivity, CCM has not otherwise been used in large scale apoB mutation screening studies. Among other techniques used in mismatch detection are modification of mismatches with carbodiimide (635,636), cleavage of mismatched bases between a wild-type RNA probe and mutant DNA by RNaseA (637), and mismatch modification or cleavage with bacterial mismatch repair proteins (638-640) or bacteriophage resolvases (641,642). Neither of these latter methods have been used in apoB mutation screening.

#### 2.6.1.6. Direct sequencing

Compared with other mutation detection methods, sequence analysis of DNA has the advantage of simultaneously detecting and characterizing the location and nature of the DNA change. DNA sequencing protocols used in most laboratories apply the Sanger dideoxy chain termination method (643) and direct sequencing of PCR products, but cloned templates derived from genomic or amplified DNA have also been used as sequencing templates. Several procedures for direct sequencing of double-stranded PCR amplification products have been described (644). To overcome problems associated with strand annealing in double-stranded DNA sequencing, methods to produce single-stranded templates have been developed. The method used in this thesis is the solid-phase sequencing technique (645,646). In this method, one of the amplification primers is biotinylated, leading to the generation of a double-stranded amplification product with one strand biotinylated. Taking advantage of the high affinity between biotin and streptavidin, the PCR product can then be captured on an avidin-coated solid phase, after which the non-biotinylated strand can be melted and washed away, leaving the biotinylated single-strand template immobilized on the solid phase to be used for sequencing. Other modifications of this

principle use the non-biotinylated (647) or both strands (bidirectional solid-phase sequencing) (648) as the sequencing template. Single-stranded sequence templates can also be generated by other methods such as the asymmetric PCR technique (649), the thermal asymmetric PCR technique (650), and by exonuclease digestion of the PCR-product (651). Originally, direct sequencing procedures required much manual work and were therefore considered too labor intensive for large scale mutation screening. Recent developments in the use of automation and new fluorescence detection technology has greatly facilitated the use of direct sequencing in these kinds of studies (652). A semi-automated DNA mutation screening procedure to detect genetic variants in the vicinity of the apoB Arg3500→Gln mutation has been presented (653).

### 2.6.2. Screening methods for known variants

#### 2.6.2.1. Allele-specific oligonucleotide methods

Allele-specific oligonucleotides (ASO) are short nucleotide probes complementary to a specific allelic sequence. When hybridized with a DNA sample, they form stable duplexes only with the allele containing the complementary sequence with no mismatches. Mutation detection techniques based on ASOs are particularly useful when the DNA variant in question does not change restriction endonuclease sites. One of the most widely used is the ASO hybridization technique (654,655), in which the region surrounding the target DNA sequence is cloned or amplified with PCR, the samples applied to a solid support which is then hybridized with an oligonucleotide probe. Alternatively, the DNA template can be scanned for several possible sequence variants by studying its hybridization to an array of immobilized probes (656). This method has been widely used in the identification of the apoB Arg3500→Gln mutation in both population and family studies (14,15,17,32,402,534,544,549, 550,555,558,560,561,657-665), and it is still in use in some research laboratories in the detection of other apoB variants

(561,584). In addition to ASO hybridization, specific alleles can also be distinguished through the generation of allele-specific PCR products by using ASOs differing at their 3´ end as primers that allow efficient amplification only if no mismatches exist between the ASO primer and the DNA template. In the literature, this type of method has been termed as allele-specific PCR (ASPCR) (666), amplification refractory mutation system (ARMS) (667), allele-specific amplification (ASA) (668), or PCR amplification of specific alleles (PASA) (669). To overcome problems associated with unspecific DNA amplification in ASPCR, further modifications, such as the competitive oligonucleotide priming (COP) and competitive blocker oligonucleotide methods (670,671) have been developed. Some of these allele-specific PCR methods have been adapted to mutation screening of the apoB gene as well (672-676).

2.6.2.2. Restriction endonuclease recognition of mutant alleles

If a DNA variant changes the cutting or recognition site of a restriction endonuclease, the presence of this specific nucleotide change can be easily detected in PCR-amplified DNA strands surrounding the enzyme cutting site by endonuclease digestion and electrophoresis of the different-sized digestion products in ordinary agarose or polyacrylamide gels (592). This restriction fragment length polymorphism (RFLP) method is now widely used in the genotyping for apoB RFLPs such as the XbaI polymorphism (19,378,390,395,443,456,562,677,678), and the Arg3500→Trp (548,566) and Arg3531→Cys (15) mutations. As a modification of RFLP analysis, a method to artificially create new restriction sites on PCR amplification products using mutagenic PCR primers has been described (679-681), and has been used to screen for the apoB Arg3500→Gln (557,682-688), Arg3531→Cys (557), and Glu3405→Gln (20) mutations.

2.6.2.3. Solid-phase minisequencing

The solid-phase minisequencing technique (689,690) is capable of

recognizing basically any kind of nucleotide transitions. Like solid-phase sequencing, this method is based on the generation of a biotinylated single-stranded DNA template on a solid phase. Thereafter, a sequence-specific oligonucleotide primer, termed the detection primer, ending just 3´ to the nucleotide of interest, is allowed to hybridize to the immobilized DNA strand, and the detection primer is elongated by one nucleotide using a DNA polymerase. In order to identify specific alleles, separate extension reactions are performed so that each reaction contains only the complementary, either radioactively or non-radioactively labeled, nucleotide of the sequence of interest. The amount of nucleotide incorporation in each reaction reflects the presence of the complementary nucleotide at the 3´-end of the detection primer. The solid-phase minisequencing technique is easy to perform even when large numbers of samples are analyzed, and it has been succesfully used for mutation detection in several types of study settings, ranging from hereditary diseases to the analysis of mutations in solid tumors (690). Compared with ASO hybridization, minisequencing takes advantage of the specificity of the DNA polymerase to differentiate between the mutant and normal nucleotides, whereas ASO hybridization relies on the specificity of base pairing between the probe and template. Thus, less stringent reaction conditions in the minisequencing method are needed. A few reports on the use of this method in detecting apoB genetic variants have been published (691), but so far this method has not gained wide use in lipid gene research.

#### 2.6.2.4. Other methods

In principle, methods used in mutation scanning such as SSCP, DGGE, TGGE, HA, or CCM, can be used in the screening of DNA samples for known mutations as well. However, since these are not capable of identifying specific nucleotide changes, identical DNA movement patterns from differing mutations can produce false results, and they are also not particularly suitable for simultaneous screening of large numbers of samples.

In spite of this, DGGE analysis has been adapted to simultaneously screen for the apoB Arg3480→Pro, Arg3500→Gln, Arg3500→Trp and Arg3531→Cys mutations (16,565), TGGE analysis in the detection of Arg3500→Gln, Arg3500→Trp and Arg3531→Cys mutations (20), and HA analysis in the detection of the Arg3500→Gln and Arg3531→Cys mutations (564). Among other, less frequently used mutation identification methods are the oligonucleotide ligation assay (OLA) (692) and the ligation chain reaction (LCR) method (693,694). Recently, an automated OLA-based screening procedure for simultaneous screening of several LDLR mutations and the 3500 Arg→Gln mutation has been described (695).

## 3. AIMS OF THE PRESENT STUDY

The general purpose of this series of studies was to search for new variants in the apoB gene possibly causing moderate to severe hyperlipidemias in the Finnish population.

The specific aims were:

1) To set up an SSCP-based mutation screening method to cover the whole coding area of the apoB gene and to look for and characterize new apoB variants in hyperlipidemic patients.

2) To evaluate the effects of new polymorphisms detected by screening on serum lipid and lipoprotein levels in hyperlipidemic families and patients, in the normal population, in diet and drug intervention studies, and in patients undergoing coronary angiography.

3) To evaluate the effects of the signal peptide ins/del, Thr71→Ile and Ala591→Val polymorphisms on serum lipid and lipoprotein metabolism in normo- and hyperlipidemic study populations.

4) To evaluate effects of an immunogenetic polymorphism of apoB, detectable with mAb D7.2 and consisting of two apoB genetic variants, Asn1887→Ser and His1896→Arg, on lipid and lipoprotein levels during dietary intervention.

# 4. SUBJECTS AND METHODS

## 4.1. Subjects

ApoB gene screening analyses, allele frequency estimations and evaluation of lipid effects of detected genetic variants were carried out in several different study materials summarized in Table 7.

### 4.1.1. Kuopio district hypercholesterolemic subjects and families (Groups 1a and 1)

Screening of the coding region of the apoB gene (exons 2 to 29) for new variants by SSCP was carried out in twenty-nine hypercholesterolemic subjects. These subjects were selected from a group of ninety-two probands with primary non-FH hypercholesterolemia identified in Kuopio province in connection with a population-based study on familial aggregation of hypercholesterolemia originally based on the FINNMONICA 1987 population risk factor survey (696). None of the 92 probands was a carrier of the apoB Arg3500→Gln mutation, but one subject was a carrier of the FH-Helsinki and two were carriers of the FH-Pohjois-Karjala mutation. These three subjects were included in allele frequency estimations of new polymorphisms detected in this study, but were excluded from final lipid and lipoprotein calculations. The selection of probands to be screened with SSCP was based on the lipid data of the families of the remaining 89 probands, leading to the selection of 29 subjects belonging to families in which inherited hypercholesterolemia appeared most likely. In addition to lipid data, DNA samples taken from family members of the probands were used to confirm segregation of new apoB variants. Besides SSCP screening, allele frequencies and lipid effects of the apoB Val703→Ile, Arg4243→Thr, and Ala4454→Thr polymorphisms were analyzed in the whole group of 89 hypercholesterolemic non-FH subjects. In the following chapters, the Kuopio district 29 probands will be referred to as Group 1a and the whole group of 92 probands (which contains the subgroup

1a) as Group 1. (I, II, IV, unpublished).

#### 4.1.2. Non-FH hypercholesterolemic patients (Group 2)

ApoB gene exons 26 to 29 were screened for new variants in a group of 39 patients with hyperlipidemia resembling classical FH. In addition to the absence of major LDL-receptor gene rearrangements and the FH-Helsinki, FH-North Karelia and apoB Arg3500→Gln mutations in the whole group, defects in either the coding or proximal promoter area of the LDL-receptor gene had been excluded in 31 of these patients (31). Allele frequencies of the apoB Asn1887→Ser and His1896→Arg polymorphisms were also assessed in this group. (II, unpublished).

#### 4.1.3. Hypertriglyceridemic patients (Group 3)

DNA samples from 76 severely hypertriglyceridemic patients, originally studied for the effects of LPL variants 291 Asn→Ser and 477 Ser→Ter on lipid metabolism (697), were subjected to SSCP-screening for DNA variants in apoB gene exons 2 to 16 and 21. Both persistently (38 patients) and sporadically (38 patients) hypertriglyceridemic patients were studied, and since the only exclusion criterion used was intravenous alimentation at the time of the study, this group contained subjects with secondary causes of hypertriglyceridemia as well. 10 of the patients were carriers of the LPL 291 Ser, and 14 carriers of the LPL 447 Ter allele. (IV).

#### 4.1.4. Diet study participants (Group 4)

The identification and characterization of the mAb D7.2 polymorphism, located in the 5´ end of apoB exon 26, was carried out in a group of 102 apparently healthy subjects who had participated in three dietary intervention studies in North Karelia, and belonged to identical intervention groups (442). Serum and DNA samples were also available from family members of the two study participants who showed the most marked shifts in

their mAb D7.2 displacement curves. (III). DNA samples from the 102 diet study participants were also used to analyze allele frequencies and lipid effects of the apoB Val703→Ile (69 subjects), Arg4243→Thr and Ala4454→Thr polymorphisms (II,IV). Previously published data from the same group concerning the effects of the apoB Ag(c/g) and Ag($a_1$/d) polymorphisms were used for comparisons in study IV. The dietary interventions carried out in this group consisted of a 2-week baseline period, a 6- or 12-week intervention period during wich the diet was modified to provide a low-fat, low-cholesterol content as well as a ratio of polyunsaturated to saturated fatty acids approximating 1, and a 5- to 6-week switchback period. During the baseline and switchback periods, the participants were on their normal free-choice diets.

#### **4.1.5. Random population sample of healthy subjects** (Group 5)

Analyses of allele frequencies and lipid effects of apoB polymorphisms Val703→Ile, Arg4243→Thr and Ala4454→Thr were carried out in a group of eighty-eight apparently healthy subjects selected from a random sample of Finnish subjects born in 1954 (698). (II, IV). Samples from a subgroup of twenty subjects were analyzed to assess the allele frequency of the silent polymorphism at the third base of apoB codon 2285 (I).

#### **4.1.6. Drug intervention study participants** (Group 6)

Effects of the apoB Arg4243→Thr and Ala4454→Thr polymorphisms on serum lipid levels and their responses to antihyperlipidemic medication were analyzed in 220 hypercholesterolemic patients participating in two multicenter drug trials carried out at 19 centers in Southern and Eastern Finland (32,699). Both patients with FH and other primary hypercholesterolemia were included. The trials compared the effects of gemfibrozil (1200 mg/d) with different daily doses of lovastatin (20 to 80 mg/d) in a double-blind parallel study setting (32) and, as an extension, in an open-label switch study setting with patients originally

belonging to the gemfibrozil group in the parallel study (699). (II).

#### 4.1.7. Coronary angiography patients (Group 7)

Allele frequencies of the apoB Asn1887→Ser and His1896→Arg polymorphisms and their effects on serum lipid and lipoprotein levels were assessed in 327 Finnish patients undergoing coronary angiography because of suspected ischaemic heart disease (700) (unpublished). Coronary angiography verified CAD in 205 of these patients, and 122 were free of CAD. In addition, effects of the earlier known apoB immunogenetic variants, Ag(c/g) and Ag($a_1$/d) on lipid levels were assessed in a group of 160 patients collected from the same population of subjects undergoing coronary angiography (IV). Of these subjects, 76 had angiographically normal coronary arteries, and 84 were shown to have CAD.

#### 4.1.8. Normal voluntary controls (Group 8)

Allele frequencies and lipid effects of the apoB Asn1887→Ser and His1896→Arg polymorphisms were assessed in a group of 72 voluntary subjects, who had contacted community health centers for various reasons not related to CAD and who showed no symptoms of ischemic heart disease or any ECG changes indicative of CAD (701) (unpublished).

#### 4.1.9. Subjects used as SSCP homogeneity controls

To verify the homogeneity of SSCP analyses carried out at different time points, two DNA samples from apparently healthy Finnish subjects were used as standards in each SSCP-run. For the apoB polymorphism geno- or phenotypings, samples from subjects with known geno- or phenotypes were used as controls. In the case of a new apoB polymorphism, only control samples perviously genotyped by DNA sequencing were used. In SSCP analyses flanking the region coding for the apoB Arg3500→Gln

mutation, a DNA sample from a known FDB carrier (a gift from Dr Stephen Humphries, University College London Medical School, London, UK) was included.

### 4.2. Lipids, lipoproteins and apolipoproteins

All blood samples for lipid and lipoprotein analyses were drawn after an overnight fast. Cholesterol and Tg determinations in the whole plasma, serum, or lipoprotein fractions were carried out by enzymatic methods using commercial kits (Boehringer Mannheim, Germany) (702,703). LDL cholesterol concentrations were calculated according to the Friedewald formula (704), except for Groups 3, and 8, and severely hypertriglyceridemic (> 4.0 mmol/l) subjects in Groups 6 and 7, in which LDL cholesterol concentrations were determined through isolation of the LDL fraction by sequential ultracentrifugation (705). In Group 1 subjects with Tg > 4.0 mmol/l, the LDL fraction was isolated using a combined preparative ultracentrifugation and precipitation method (705,706). HDL cholesterol concentrations were determined after precipitation of the VLDL and LDL fractions by dextran sulphate - $MgCl_2$ (706), except for Groups 3 and 8, where HDL concentrations were estimated after isolation of the corresponding density fraction by sequential ultracentrifugation, and Group 5, where precipitation with heparin - $MnCl_2$ was used for HDL isolation (707). ApoB concentrations were determined by an immunoturbidic method based on the immunoprecipitation of apoB in liquid phase (Orion Diagnostica, Espoo, Finland) (Groups 1, 5, 6, 7, and 8) (708, 709), or by the radial immunodiffusion method (Behringwerke, Marburg, Germany) (Groups 3 and 4) (710).

### 4.3. DNA analyses

#### 4.3.1. DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes by phenol and chloroform extraction and ethanol precipitation

(711), or directly by a rapid cell lysis procedure (Group 3) (712).

#### 4.3.2. Polymerase chain reactions and enzyme digestions

ApoB exons were amplified either as a whole (exons 2 to 25, 27, and 28), or in six (exon 26), or three (exon 29) overlapping fragments using oligonucleotides designed according to published sequences (118) and presented in Table 8. All oligonucleotides were synthesized using standard phosphoramidite chemistry (713) by an Applied Biosystems DNA synthesizer model 392 (Foster City, CA, USA) at the Institute of Biotechnology, University of Helsinki, Finland. All amplification reactions were conducted in an automatic Perkin Elmer/Cetus Thermal Cycler (Norwalk, CT, USA) in a total volume of 50 µl with 50 ng of genomic DNA (or 5 µl of DNA mixture in samples extracted by rapid cell lysis), 50 pmol of each oligonucleotide used as primer, 0.5 µl of deoxycytidine 5´-[$\alpha$-$^{32}$P]triphosphate (Amersham International, Amersham, UK), 1.25 U of Taq DNA polymerase (Promega, Madison, WI, USA) or Dynazyme DNA polymerase (Finnzymes, Espoo, Finland), and nucleotides, amplification buffer and $MgCl_2$ as specified by the manufacturer. After an initial denaturation step of 95°C for 5 min, the PCR reaction was carried out using a cycle number, and temperature and duration of denaturation, annealing and elongation steps in accordance with the size of the fragment to be amplified. Usually, 27 to 35 cycles of a denaturation step at 95°C for 30 to 60 s, an annealing step at 52-60°C for 30 to 60 s, and an elongation step at 72°C for 45 s to 5 min were carried out. Sometimes, two to three smaller exons were amplified in the same multiplex PCR reaction in which case, in order to be able to identify individual exons later in SSCP gels, control reactions amplified with only one set of primers were included. Prior to SSCP analysis, the 1190- to 1435-bp fragments of exon 26, and the 632- to 698-bp amplified fragments of exon 29 were cut into smaller pieces with two (exon 26) or one (exon 29) restriction enzymes (New England Biolabs, Beverly, ME, USA) in reactions conducted in a volume of 30 µl at 37°C for 3 hr with 5-

10 µl of the PCR amplification product, 10-15 U of each enzyme, and the buffer recommended by the manufacturer. To identify the different-sized digestion products of exon 26 fragments in the SSCP gels, one sample was subjected to both simultaneous digestion with both enzymes and to digestion with each enzyme alone. Characteristics of the amplification products, the enzymes used, their cutting sites, and sizes of digestion products are listed in Table 9.

### 4.3.3. Single-strand conformation polymorphism analyses of apoB exons 2 to 29

For the SSCP analysis, the amplification and digestion products were diluted 1:5 with 10 mM EDTA and 0.1% SDS, and mixed 1:1 with 95% formamide, 0.05% bromphenol blue, and 0.05% xylene cyanol. The samples were thereafter denatured at 80°C for 4 to 6 min, and 2-5 µl of each sample was applied to a vertical polyacrylamide gel running apparatus (Bio-Rad Laboratories, Richmond, CA, USA) for electrophoresis. Depending on the size of the fragments to be analyzed, SSCP gels for each analysis were electrophoresed with differing concentrations of polyacrylamide (5 or 12%, 1:60 bisacrylamide:acrylamide, Bio-Rad Laboratories), glycerol (0, 5 or 10%), or Tris-borate/EDTA buffer (0.5 or 1 ×), differing running time (4 to 24 hr), power (10 to 50 W), and temperature (+8 or +20°C). At least three, usually more differing sets of electrophoresis conditions were used to achieve satisfactory resolution of the amplification and digestion products. Two or three samples with nondenatured DNA (size control), two standard samples from apparently healthy subjects (SSCP homogeneity control), as well as samples with individual exon amplification products (multiplex PCR), or individual endonuclease digestion products (exon 26 fragments) were included in each analysis. After the run, gels were dried on filter paper in a Bio-Rad vacuum gel dryer and exposed X-ray film at -80°C for 1-5 days with an intensifying screen.

#### 4.3.4. Solid-phase sequencing

DNA fragments showing mobility shifts in SSCP analysis were sequenced according to the direct solid-phase sequencing method (646). DNA segments to be sequenced were amplified with a set of primers of which one was biotinylated at its 5´ end. The biotinylation of the primers was carried out using dC-biotin phosphoramidite directly on the DNA synthesizer (714). Amplification products were purified by letting 25 µl of the PCR product bind for 1 hr to avidin-coated polystyrene particles (Baxter Healthcare Corporation, Mundelein, IL, USA), washing the particles twice with TENT buffer (40 mM Tris-HCL, 50 mM NaCl, 1 mM EDTA, 0.01% Tween 20), and denaturing the bound DNA strands with NaOH (50 mM, 5 min). After two final washes, the single-stranded avidin-bound DNA strand was sequenced by the Sanger dideoxy termination method (643) using T7 DNA polymerase (Sequenase version 2.0, United States Biochemical, Cleveland, OH, USA), [$\alpha$-$^{35}$S]dATP (Amersham International), and 50 pmol of the non-biotinylated PCR primer as the sequencing oligonucleotide. Sequencing reactions were electrophoresed on a 6% polyacrylamide/7 M urea gel, after which the gels were fixed, dried, exposed to X-ray film, and manually analyzed. The oligonucleotides used and characteristics of the PCR products for the sequencing reactions to identify the SSCP shifts seen in apoB gene exons 15, 18 and 27, in the 422-bp segment cut from the second fragment of apoB exon 26 by BanI, the 501-bp fragment cut from the same exon 26 fragment by EcoRI, the 400-bp segment in the middle of the third fragment of exon 26, in the 5´ 246-bp segment of fragment 29b, and in the 5´ 403-bp segment of fragment 29c are shown in Table 10.

#### 4.3.5. Solid-phase minisequencing

DNA changes not easily detectable by use of the PCR-RFLP analysis were genotyped with the primer guided nuclotide incorporated assay (689), carried out on streptavidin coated microtiter wells using the solid-phase minisequencing method

(690). Biotinylated PCR products were produced in amplification reactions conducted in a volume of 50 µl with 200 ng of genomic DNA, 20 pmol of the biotinylated primer, 100 pmol of the non-biotinylated primer, 1.25 U of Dynazyme DNA polymerase (Finnzymes), and with nucleotides and amplification buffer as recommended by the manufacturer. After PCR, a 10 µl aliquot of the PCR mixture was captured on microtiter wells (Streptavidin Covalent ScintiStrips™; Wallac Oy, Turku, Finland) with 40 µl of buffer (0.15 M NaCl, 20 mM $NaHPO_4$, 0.1% Tween 20 at pH 7.4) and incubated for 1.5 h at 37°C. The bound PCR products were thereafer denatured with 100 µl 50 mM NaOH for 5 min at 20°C, and the wells washed 3-5 times at 20°C with 350 µl of a buffer containing 40 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl, and 0.1% Tween 20 at pH 8.8. The minisequencing reaction was carried out by addition of the detection step primer (20 pmol), the appropriate $^{3}$H dNTP (2 pmol) (Amersham International), and 0.5 U Dynazyme DNA polymerase (Finnzymes) in 50 µl of a buffer containing 50mM KCl, 10 mM Tris-HCL, 0.1% Triton X-100, and 4 mM $MgCl_2$ to the micro-titration plate wells, and allowing the extension reaction to proceed for 10 min at 50°C with gentle shaking. After this, the wells were washed three times as described above, treated with 100 µl of 50 mM NaOH, and air-dried for 60 min at 20°C. The quantity of the the 3H-labeled dNTPs incorporated was determined thereafter in a Wallac MicroBeta™ scintillation counter. The oligonucleotides used for the PCR and minisequencing reactions and the labeled nucleotides for the wild-type and mutant alleles for the A5869→G (Asn1887→Ser), A5896→G (His1896→Arg), G12937→C (Arg4243→Thr), and G13569→A (Ala4454→Thr) changes are shown in Table 10.

**4.3.6. PCR-RFLP analyses**

Several of the newly detected DNA variants as well as the earlier known polymorphisms of apoB represent restriction fragment length changes detectable with a specific endonuclease, enabling rapid genotyping through PCR and enzyme digestion, followed by ethidium-bromide-stained agarose gel electro-

phoresis. In this study, genotyping for these RFLPs was carried out from amplified DNA fragments flanking the DNA variant to be studied in reactions conducted for 3 hr at 37°C in 30 µl with 10-20 µl of the PCR mixture, 10-15 U of the enzyme in question, and the buffers recommended by the manufacturer (New England Biolabs). The presence or absence of a cutting site was thereafter visualized in a ethidium bromide-stained 1-3% agarose gel. Details of the PCR-RFLP analyses used in this study are shown in Table 11.

#### 4.3.7. Ins/del genotyping

Genotyping for the signal peptide ins/del polymorphism of the apoB gene was carried out using PCR followed by electrophoresis in a polyacrylamide gel (372,715). The primers used are shown in Table 11. The PCR reaction was conducted in a total volume of 50 µl, with primer, DNA, nucleotide, buffer and polymerase concentrations as described earlier, except for the addition of 10% dimethylsulphoxide, and with 50 cycles of denaturation at 94°C for 1 min and simultaneous annealing and extension at 65°C for 1.5 min after the initial 6 min denaturation step at 94°C (372,715). The amplification products were thereafter electrophoresed in 8% polyacrylamide gels at 90 V for 5 hr and visualized by ethidium bromide staining.

#### 4.3.8. Apo B 3´VNTR genotyping

To confirm segregation of newly detected apoB genetic variants in family members of carriers, apoB 3´VNTR genotyping was performed using denaturing polyacrylamide gel electrophoresis (716), with primers as shown in Table 11, and an amplification mixture identical to that of the SSCP-PCR mixtures. After initial denaturation, 26 cycles with denaturation at 94°C for 1 min and simultaneous annealing and extension at 55°C for 7 min followed. The amplified samples were diluted 1:1 with a mixture containing 95% formamide, 20 mM EDTA, 0.05% bromphenol blue and 0.05% xylene cyanol, incubated at 72°C for 3 min and run on a

denaturing polyacrylamide/7M urea gel at 75W and 48°C for 3.5 hr, after which the gels were dried on filter paper and autoradiographed.

## 4.4. Antibody binding assays

### 4.4.1. Ag phenotyping

Phenotyping for the apoB antigen polymorphisms Ag c/g, $a_1$/d, x/y, h/i and t/z in study Groups 4 and 7 (Study IV) was performed by the passive hemagglutination inhibition technique (717,718). This method is based on the inhibition of the anti-Ag serum -induced hemagglutination of human erythrocytes coated with the LDL to be studied by normal human sera. The human anti-Ag serum was extracted from multiply transfused patients having developed antibodies against epitopes of the foreign LDL particles contained in the transfused blood preparations.

### 4.4.2. mAb D7.2 assays

A solid-phase enzyme-linked immunosorbent assay (419) was used to detect the apoB mAb D7.2 polymorphism (Group 4, Study III). This method detects the binding affinity of the LDL particles studied to a specific mAb against LDL-apoB, and it has been used also to detect the Ag(c/g) polymorphism (mAb 19) (419), and the apoB Arg3500→Gln mutation (mAb 47) (32). Briefly, the wells of microtiter plates were coated with 150 µl of standard-LDL (1 µg/ml), and extra binding sites were saturated by incubation with 3% albumin solution for 4 hr at room temperature. The mAb D7.2 and increasing amounts of sample LDL were loaded to each LDL-coated well and allowed to incubate for 4 hr at room temperature, after which the wells were washed three times with 1% BSA-PBS followed by the addition of a second antibody conjugated to alkaline phosphatase. The amount of antibody bound was thereafter detected by spectrophotometry. The antibody D7.2 used for these analyses was a generous gift from Dr Gus Schonfeld and Dr Elaine Krul (Lipid Research Center, Washington

University, St. Louis, MO, USA.).

### 4.5. Statistical methods

Allele frequencies of apoB polymorphisms were estimated using the gene counting method, and differences in the observed allele frequencies between study populations were tested by $\chi^2$ analysis and Fisher's exact test (II, III, IV). These tests were also used to estimate associations between the apoB Asn1887→Ser and His1896→Arg polymorphisms and the mAb D7.2 polymorphism (III). Other statistical analyses were carried out with the BMDP statistical software package (BMDP Statistical Software Inc., Los Angeles, CA). The mean values of serum lipid and lipoprotein levels between different genotypes were compared by analysis of variance (ANOVA), repeated after adjustments for BMI (III), or BMI and age (II and IV). In case of non-normal distribution, logarithmically transformed values were used for comparison. If statistically significant differences in sample variances according to Levene's test were detected, Welch and Brown-Forsythe statistics instead of ANOVA were used (IV).

# 5. RESULTS

## 5.1. Screening for and characterization of apoB genetic variants
(I, II, IV, and unpublished)

The SSCP method was used to screen for new apoB variants in the gene region encoding the apoB protein (exons 2 to 29), consisting of 13911 nucleotides (Table 9). This was accomplished through amplification of the small exons 2 to 25, 27 and 28 as a whole, and the large exons 26 and 29 in six and three overlapping fragments, respectively. Since the amplified fragments of exons 26 and 29 were too large for SSCP analysis, they were cut into smaller segments by restriction endonucleases prior to the SSCP gel run. With this strategy, SSCP analysis of altogether 50 amplified and digested segments of the apoB gene ranging from 80 to 579 bp in size was carried out. Due to the large size variation of the analyzed segments, several different electrophoretic conditions were used. All DNA samples showing a shift in the SSCP moving pattern were subjected to a second SSCP analysis before further characterization. In some cases, especially when SSCP shifts in the largest segments were detected, additional SSCP analyses, using another set of primers flanking the suspected area, and another restriction endonuclease were performed. The DNA change behind each shift was identified by direct sequencing of the segment in question. After identification, detection of new variants in families and population samples was carried out either by SSCP, direct sequencing, PCR-RFLP, or solid-phase minisequencing. Available samples from family members of carriers of the new DNA changes were used to confirm segregation in families as well as cosegregation with the apoB 3´VNTR. Identical DNA variants detected in two or more unrelated SSCP-screened subjects were suspected to represent apoB polymorphisms, in which case, especially if family data suggested lipid effects, their occurrence and allele frequencies were further assessed in Finnish normo- and hyperlipidemic population samples.

SSCP screening of the whole apoB gene coding area (exons 2 to 29) was carried out in a group of twenty-nine moderately hypercholesterolemic probands selected from a sample of 92 hypercholesterolemic families identified in the Kuopio district (Group 1a). In addition, apoB gene exons 26 to 29 were screened in a group of thirty-nine patients with severe hypercholesterolemia resembling FH, in whom both major Finnish LDLR gene mutations and the apoB Arg3500→Gln mutation had been excluded (Group 2). By choosing exons 26 to 29 for screening in this material, we aimed at covering apoB regions possibly contributing to the LDL-LDLR interactions, including sites located apart from the actual LDLR binding site, such as the carboxyl-terminal region of apoB (185,197). In severely hypertriglyceridemic patients (Group 3), SSCP screening was carried out in apoB gene exons 2 to 16 and 21, containing the apoB domain interacting specifically with LPL (240).

Since apoB is known to be a highly polymorphic protein, several SSCP moving pattern shifts were anticipated. All previously known common apoB genetic variants situated in the regions included in our analyses were detected and could be easily genotyped in the SSCP gels. The SSCP-based genotyping of these apoB polymorphisms was verified through additional PCR-RFLP analyses (ApaLI, AluI, XbaI, BfaI, MspI, and EcoRI). In addition, twelve new apoB variants, located in exons 15, 18, 26, 27 and 29, were detected. In the following, these new apoB variants will be presented according to their order in the 5´ to 3´ direction of the gene. All new apoB variants are also shown in Table 4.

In the hypertriglyceridemic patients (Group 3), analysis of apoB gene exon 15 revealed one subject with an SSCP-shift resulting from a G→A change at cDNA nucleotide 2316, changing codon 703 from GTC to ATC, and generating a Val→Ile change in the mature apoB protein. Further studies in Kuopio district probands (Group 1) led to the identification of seven more carriers of this apoB Val703→Ile change. Family studies confirmed the segregation of

this variant in each family, with the cosegregation of the 703 Ile allele with the apoB 3´VNTR allele 45. Allele frequency analyses in the Kuopio district hypercholesterolemic patients (Group 1, n=78), in the diet study participants (Group 4, n=69), and in the random sample of healthy Finns (Group 5, n=83), confirmed the polymorphic nature of the apoB Val703→Ile change with frequencies of the 703 Ile allele of 0.045, 0.036 and 0.048 in these populations, respectively (differences not significant). Compared with the other studied groups, the 703 Ile allele in the hypertriglyceridemic patients (Group 3) was slightly but not significantly underrepresentated (one carrier in 76 patients, allele frequency 0.007). During the course of this study, this Val703→Ile variant has also been detected in a family with hypobetalipoproteinemia, not however cosegregating with the FHBL phenotype (719). (IV)

Three Kuopio district Group 1a probands showed a shift in the SSCP analysis of apoB exon 18. In the families of these probands, the shift cosegregated with apoB 3´ VNTR allele 49. The DNA change producing this shift results from a C→T change in the third nucleotide of apoB codon 875 (AAC→AAT), with no change in the amino acid sequence of apoB (apoB Asn875→Asn), and with no effect on the lipid phenotype in the studied families. No further studies to determine its allele frequency in the Finnish population were therefore carried out. During the course of this study, this apoB polymorphism was also reported in a French population of thirty-five apoB 3´VNTR allele 49 carriers, with a rare allele frequency of 0.032 and a weak association with the 3´VNTR 49 allele (584). (Unpublished).

Exon 26 contains the region coding for all three FDB-producing apoB mutations, the Arg3500→Gln (14), Arg3500→Trp (16), and Arg3531→Cys (15) mutation. With this in mind, this region was especially carefully analyzed in both the moderately as well as the severely hypercholesterolemic patient groups (Groups 1a and 2). In spite of the readily identified mobility shift in the DNA sample from a known 3500 Gln carrier, no similar or other

mobility shifts in this region were detected in our SSCP analyses. Instead, four other apoB genetic variants were detected in exon 26. Of these, the A→G changes at cDNA nucleotides 5869 and 5896, leading to Asn1887→Ser and His1896→Arg changes, respectively, are more thoroughly characterized in connection with their association with the newly described mAb D7.2 polymorphism later in this section. The detection of a mobility shift between nucleotides 6506 and 7007 (the 3´ end of fragment 26b cut with EcoRI) in one Group 2 severely hypercholesterolemic proband led to the identification of a new apoB mutation, a deletion of nucleotides 6766-6768. This ATG deletion removes the second and third bases of codon 2186 (Asp) and the first of 2187 (Glu), leading to a frameshift which generates a GAG codon, produces a Glu residue at the site of the deletion, and deletes the Asp 2186. The segregation of this apoB del Asp 2186 variant in family members was confirmed, yet with no clear effects on the lipid phenotype. The fourth DNA variant detected in exon 26 was a T→C change in the third base of codon 2285 (GAT→GAC) at cDNA nucleotide 7064, with no change in the amino acid sequence of apoB (apoB Asp2285→Asp). This T7064→C change is also detectable with MaeII (113). Genotyped with SSCP, the apoB T7064→C polymorphism was shown to be closely linked with the XbaI RFLP, with the 7064 C allele occurring in conjunction with the XbaI- allele. The allele frequency of the C allele was similar to the XbaI- allele in both hyper-cholesterolemic patients (Group 1a) and a subgroup of 20 healthy subjects from Group 5 (0.500 and 0.525, respectively). (I,II).

Exon 27 analysis revealed an A→G change at cDNA nucleotide 11961, leading to a Thr3918→Ala (ACT→GCT) variation. This variant was found in one hypercholesterolemic Group 2 patient. Additional DNA was available from only the proband´s sister, who, although severely hypercholesterolemic, had not inherited this apoB mutation. Thus, this variant was considered unlikely to be the cause of the FH-like hypercholesterolemia in this family, and no further attempts to identify more carriers were considered necessary. This apoB variant has also been documented

in the French population 3´VNTR allele 49 carriers with an allele frequency of the 3918 Ala allele of 0.006 and linkage disequilibrium with the 3´VNTR 49 allele (584). No lipid data from this study population have been reported. (II).

SSCP analysis of apoB exon 29 revealed three moving pattern changes. A complex change in the 5´ end of fragment 29b was shown to be the result of three closely located DNA changes at nucleotides 12922, 12935, and 12937. The nucleotide 12922 T→C substitution produces a Val4238→Ala (GTA→GCA) change. It was detected in two normocholesterolemic subjects (control samples), of which no family data was available, and was thus not further characterized. The second change, a T→C substitution at nucleotide 12935, is silent at the protein level (Tyr4242→Tyr, TAT→TAC), but produces a new RsaI cutting site at nucleotide 12933. The segregtion of this variant was followed in five families (both hyper- and normocholesterolemic), with no clear effects on the lipid phenotype, and no further attempts to assess the allele frequency of the RsaI+ allele in other Finnish populations were carried out. The third component of this polymorphic area was a G→C change resulting in an Arg4243→Thr variation. Its segregation was followed in five hypercholesterolemic families (Group 1a), in which the 4243 Thr allele was shown to cosegregate with 3´VNTR alleles 47 or 49, but again, no clear effects on the lipid phenotype were detected. Studies in other Finnish populations (Groups 1, 4, 5, and 6) revealed an allele frequency of the 4243 Thr allele of 0.017, 0.034, 0.028 and 0.047, respectively, with no statistically significant differences between these populations. Ag haplotyping analyses in Group 4 revealed no association of the 4243 Thr allele with any common Ag haplotype. (II).

SSCP analysis in four Kuopio district Group 1a patients showed a mobility shift in the 5´ half of segment 29c. It was produced by a G→A change at nucleotide 13569 resulting in an Ala4454→Thr (GCC→ACC) variation. Later on, its segregation was followed in eight families, showing cosegregation of the 4454 Thr allele

with 3´VNTR allele 35. In other Finnish population samples (Groups 1, 4, 5 and 6), this polymorphism was detected with an allele frequency of the 4454 Thr allele of 0.022, 0.020, 0.017 and 0.022, respectively (no differences between different populations). Ag haplotyping and 3´VNTR analyses in Group 4 showed an association between the apoB Ag haplotype $xa_1gti$ and, as in families, 3´VNTR allele 35. This same apoB variant has been detected in the French population with an allele frequency of the 4454 Thr allele of 0.039 (584). In the French subjects, the 4454 Thr allele seems not to associate with the apoB 3´VNTR 49 allele. (II).

Two hypercholesterolemic Group 2 patients showed a SSCP shift in the 3´ half of fragment 29c. This shift resides in the non-coding area of exon 29, and it cannot affect the amino acid sequence of apoB. While DNA changes in non-coding regions of exons can have some effect on gene expression and are in principle of interest, this SSCP shift did not seem to associate with lipid parameters in the families of these probands and was thus not further characterized. (II).

### 5.2. Identification and characterization of the apoB D7.2 Ag polymorphism (III)

In connection with analyses of lipid and lipoprotein responses to changes in diet in the apparently healthy diet study group (Group 4), LDL samples were tested for apoB variants with a panel of mAbs. In 18 of the 102 subjects tested, a marked shift to the right of displacement curves against mAb D7.2 was detected. In two individuals, this change in binding affinity was clearly stronger than in the remaining 16. Some of the family members of these subjects showed similar shifts in mAb D7.2 displacement curves, suggesting a genetic basis for the changes in binding affinity. However, the observed changes in families were too complex to be the result of a common biallelic apoB genetic variant.

The epitope of mAb D7.2 lies between apoB amino acids 1878 and 2148 (201). The availability of the SSCP-based mutation detection system covering the DNA region coding for the mAb D7.2 epitope offered us a possibility to explore the DNA change causing the changes observed in the Ag binding assays. SSCP analysis of the 5´ end of fragment 26b showed a complex mobility shift suggesting either a three-allelic polymorphism or two closely located mutations. DNA sequencing revealed two A→G transitions at cDNA nucleotides 5869 and 5896, leading to Asn1887→Ser (AAT→AGT) and His1896→Arg (CAT→CGT) changes. The two subjects expressing the most marked shifts in their mAb D7.2 displacement curves were shown to be doubly heterozygous for both DNA variants. Family data collected from relatives of these two subjects confirmed the segregation of the DNA variants as well as a relatioship between the DNA variation and the binding affinity of LDL apoB with mAb D7.2.

Genotyping of other participants of the diet study (Group 4) was carried out by the PCR-RFLP technique. The A5896→G change removes a normal BsrDI cutting site at nucleotide 5863, whereas the A5896→G change produces a new RsaI cutting site at nucleotide 5897. Compared with binding assay data, subjects homozygous for both common alleles of these polymorphisms usually showed normal immunoreactivity with mAb D7.2. All subjects heterozygous for the 1887 Ser allele showed reduced binding irrespective of their apoB 1896 genotype. However, only 11 of the 18 subjects carrying the 1896 Arg allele and homozygous for the 1887 Asn allele showed detectable shifts in the displacement curves, suggesting a less severe effect of the 1896 His allele on the mAb D7.2 epitope. Since the displacement curves from the compound heterozygotes were shifted even more to the right than those from 1887 Ser heterozygotes alone, an additive effect of both polymorphisms on the immunoreactivity of LDL with mAb D7.2 is suggested. Genotyping data from Group 4 subjects showed no further association between the two polymorphisms, and family data indicated that they were not inherited in the same apoB allele. Ag phenotyping, 3´VNTR, and

XbaI RFLP genotyping of the new polymorphisms showed association of the 1896 Arg allele with the $xa_1$gti-3´VNTR 35-X- (XbaI- allele) haplotype in the diet study participants, and with the 3´VNTR 35-X- allele in families. With regard to the 1887 Ser allele, no common haplotype was shared by the carriers. In families, the 1887 Ser allele cosegregated with 3´VNTR alleles 33 and 37.

Allele frequency estimations carried out in Group 4 and other Finnish population samples (Groups 1a, 2, 7, 8) after either PCR-RFLP or solid-phase minisequencing genotyping showed similar frequencies for the rare alleles of both polymorphisms. For the Asn1887→Ser polymorphism, an allele frequency of the Ser allele in Groups 1a, 2, 4, 7 and 8 of 0.034, 0.013, 0.025, 0.035 and 0.011, respectively, was estimated. The observed allele frequency differences were not statistically significant, nor were differences between the allele frequencies detected in Group 7 subjects irrespectively whether they had (0.037) or had not (0.027) angiographically documented CAD. In accordance with our studies in the Finnish population, similar allele frequencies of the 1887 Ser allele have also been detected in both a French population of apoB 3´VNTR allele 49 carriers (0.032) (584), in French hypercholesterolemic subjects (0.053) (720), and a Norwegian population of hypercholesterolemic patients (0.024) (585). As for the His1896→Arg polymorphism, estimated Arg allele frequencies in Groups 1a, 2, 4, 7 and 8 were 0.034, 0.051, 0.110, 0.043 and 0.062, respectively. The allele frequency differences between these groups were not statistically significant except for the diet study (Group 4) and coronary angiography subjects (Group 7), between which the difference in observed allele frequencies became significant ($p=0.0014$, Fisher's excact test). Further analysis of Group 7 showed that this allele frequency difference was confined to patients with CAD (allele frequency 0.034, difference compared with Group 4 significant, $p=0.0006$), whereas those with angio-graphically normal coronary arteries had a 1896 Arg allele frequency (0.058) similar to and not statistically significantly

different from the other groups. In addition to the Finns, the His1896→Arg polymorphism has also been detected in Norwegian and French hypercholesterolemics with an allele frequency of 0.024 in both populations (585,720).

### 5.3. Lipid and lipoprotein effects of the new apoB variants in exons 26 to 29 in Finnish populations (II, III, unpublished)

Lipid and lipoprotein effects of the Asn1887→Ser and His1896→Arg polymorphisms were assessed in the diet study participants (Group 4), in the patients undergoing coronary angiography (Group 7, unpublished results), and a group of healthy subjects free of symptoms or signs of CAD (Group 8, unpublished results). Due to the low number of 1887 Ser allele carriers in women of all these groups, and in the healthy men (Group 8) statistical calculations were restricted to Group 4 and 7 men only. As shown in Tables 12 and 13, the Asn1887→Ser polymorphism did not show significant effects on baseline serum lipid and lipoprotein levels or their responses to dietary change in these subjects. Due to the low number of subjects heterozygous for the 1887 Ser allele, a moderate effect of this allele on serum lipid or lipoprotein concentrations cannot, however, be totally ruled out.

Effects of the His1896→Arg polymorphism on serum lipid and lipoprotein levels were estimated in both male and female subjects of Groups 4, 7 and 8. Results of these analyses are summarized in Tables 14 to 17. In the diet study, no differences in baseline or swithcback lipid or lipoprotein levels between the apoB His1896→Arg genotypes could be observed in either sex (Tables 14 and 15). During the diet intervention, men carrying the 1896 Arg allele tended to exhibit slightly higher serum total ($p=0.0792$), LDL cholesterol ($p=0.0494$), and apoB levels ($p=0.0814$), leading to smaller increases in total and LDL cholesterol and apoB levels when changing back from the intervention to the original diet. Higher serum total and LDL cholesterol levels were also observed in carriers of the apoB

1896 Arg allele when male Group 7 subjects with no signs of angiographic CAD were studied (Table 16). No differences between His1896→Arg genotypes were observed in Group 7 men with CAD or Group 8 healthy men. In Group 4, 7 and 8 women, no significant differences between genotypes were detected (Table 17).

Lipid and lipoprotein effects of the apoB Arg4243→Thr and Ala4454→Thr polymorphisms were assessed in the 89 Kuopio district hypercholesterolemic subjects (Group 1), the diet study subjects (Group 4), the healthy population sample (Group 5), and in the drug intervention participants (Group 6). Results in males and females were similar. Therefore, combined results for both sexes are presented (Tables 18 and 19). Allele frequencies of the rare Thr alleles of both polymorphisms were low, and in general, no statistically significant effects on either baseline lipid levels, diet responses or responses to drug intervention could be detected. In Groups 4 and 5, initial analyses of the lipid effects of the Ala4454→Thr polymorphism seemed to show significant differences. These differences were however towards opposite directions, and lost significance after repeated calculations. More precisely, in Group 5, 4454 Thr carriers exhibited slightly higher LDL cholesterol levels compared to 4454 Ala homozygotes (Table 19). In this group, all 4454 Thr carriers were men, and when calculations were repeated after exclusion of women and after adjusting for BMI, this difference was no longer significant (p=0.0679). Group 4 included only one female 4454 Thr carrier. Analyses of the male subjects in this group (three 4454 Thr carriers) suggested a baseline LDL cholesterol lowering effect of the rare Thr allele (4.97±1.07 and 3.59±0.57 mmol/l for the Ala/Ala and Ala/Thr genotypes, respectively, p=0.0335). In repeated calculations adjusted for age and BMI, this difference between genotypes was no longer statistically significant (p=0.0555). Based on these calculations, our conclusion was that this polymorphism is not likely to be a major determinant of LDL cholesterol levels. As mentioned earlier, no associations with the lipid phenotype and the carrier status for the apoB Arg4243→Thr or Ala4454→Val

variants in the small number of families available for study were seen.

Of the other DNA variants in apoB exons 26 to 29, the del 2186 Asp, Thr3918→Ala, Val4238→Ala, the silent Tyr4242→Tyr mutation, and the non-characterized apoB variant in the non-coding region of exon 29, were either detected in normocholesterolemic subjects or were not associated with the lipid phenotype in the families of the mutation carriers. Thus, no further attempts to characterize their possible lipid or lipoprotein effects at the population level were carried out. In view of the close connection of the new silent Asp2285→Asp polymorphism with the well known and widely studied apoB XbaI polymorphism, no additional information regarding its associations with lipids or lipoproteins was anticipated. Therefore, no population studies conserning the effects of this polymorphism were performed.

### 5.4. Lipid and lipoprotein effects of the apoB variants in the amino-terminal region of apoB in Finnish populations (IV)

Since the Val703→Ile change was originally detected in only one severly hypertriglyceridemic Group 3 patient, an analysis of its lipid or lipoprotein effects in this group was not possible. Instead, the effects of the 703 Ile allele were assessed in Kuopio district hypercholesterolemic probands (Group 1), the diet study population (Group 4), and in the random sample of healthy subjects (Group 5). A summary of these results is shown in Table 20, both sexes combined. In Groups 1 and 4, no lipid effects of the 703 Ile allele could be detected. In Group 5, lipid and lipoprotein analyses showed an association between lower serum Tg levels and the 703 Ile allele. This effect was more pronounced in males (1.41±0.31 and 0.83±0.16 mmol/l for the 33 Val/Val homozygotes and the 4 Val/Ile heterozygotes, respectively, p=0.0040); in females, the difference was not significant (0.84±0.43 and 0.67±0.21 mmol/l, p=0.4837). Although larger population samples would be needed to confirm this finding, both the association detected in Group 5 and the slight

underrepresentation of the apoB 703 Ile allele in severely hypertriglyceridemic Group 3 patients could point towards a triglyceride-lowering effect of this allele.

With the recently proposed hypothesis of a specific function for the globular apoB amino-terminal domain in the interactions between LPL and lipoproteins (240), we were interested in studying whether any lipid or lipoprotein effects of previously known apoB polymorphisms situated in this region, the Thr71→Ile and Ala591→Val, could be seen in severely hypertriglyceridemic patients. Since the apoB signal peptide ins/del polymorphism is known to be in close linkage disequilibrium with the Thr71→Ile polymorphism (398), genotyping of the ins/del polymorphism was included in the study of hypertriglyceridemic patients. For comparison, lipid and lipoprotein effects of the Thr71→Ile and Ala591→Val polymorphisms were additionally analyzed in both the diet study population (Group 4) and in subjects undergoing coronary angiography (Group 7). A summary of these analyses is shown in Tables 21 to 23, both sexes combined.

Results from the lipid and lipoprotein analyses in the hypertriglyceridemic patients (Table 21) showed that the 591 Ala allele was associated with higher serum apoB levels in both sexes, remaining statistically significant even after adjusting for age and BMI (p=0.0113, Figure 4.). The 71 Ile allele showed a non-significant trend in the same direction, and a similar trend was also detected for the signal peptide del allele. By combining the subjects with at least one of both alleles associated with elevated apoB levels, in other words, those homozygous or heterozygous for both rare alleles (apoB 71 Ile + and 591 Ala +; apoB 1.56 ± 0.44 mg/ml, n=35) and comparing them with those homozygous for both common alleles (apoB 71 Thr/Thr and 591 Val/Val; apoB 1.18 ± 0.46 mg/ml, n=20), results showed significantly higher apoB levels in the group consisting of carriers of the "apoB-elevating" alleles (p=0.0044 after adjustment for age and BMI). To analyse the effects of these "apoB-elevating" alleles in normolipidemic populations, similar

comparisons were made in the diet study and the coronary angiography populations (Groups 4 and 7). In Group 4, no significant effects on baseline lipid and lipoprotein levels of either polymorphism, whether alone or combined were detected (Table 22). The slight triglyceride-lowering effect of the 71 Ile allele in this group did not remain statistically significant when adjusted for age and BMI (p=0.0836 for the whole group, p=0.3964 and 0.0904 for males and females, respectively). Likewise, no differences in lipid and lipoprotein levels between genotypes of either polymorphism in Group 7 were detected (Table 23), regardless of whether the whole group or subjects with or without CAD were studied (data not shown). However, when hypertriglyceridemic (serum Tg ≥2.00 mmol/l) Group 7 subjects alone were analyzed, men with the 591 Ala/Ala genotype showed significantly higher (p=0.0154 after age and BMI adjustment) apoB concentrations (1.27 ± 0.20 mg/ml, n=9) when compared with the 591 Val/Ala or Val/Val men (apoB 1.01 ± 0.17 mg/ml, n=11; apoB 1.07 ± 0.20, n=10, respectively). When divided into hypertriglyceridemic men with or without CAD, this apoB-elevating effect of the 591 Ala allele was still significant in men with CAD (n=22, p=0.0464 after age and BMI adjustment). In males with healthy coronary arteries, this trend was no longer significant (n=8, p=0.1559). Unfortunately, the number of subjects with serum Tg level ≥ 2.00 mmol/l in Group 4 was too low for meaningful comparisons. Nevertheless, the results obtained in other groups could point towards an apoB-elevating role of the 71 Ile and 591 Ala alleles (or their combination), expressed only in context of hypertriglyceridemia.

# 6. DISCUSSION

## 6.1. Evaluation of the mutation screening methodology

Of the mutation detection methods, both SSCP, DGGE (457,622), TGGE (20) and HA (456,542) have been successfully used to screen and detect apoB variants; most studies have applied SSCP (490,561,584,585). Of the new apoB mutations associated with defective binding of LDL apoB to the LDLR, the Arg3531→Cys variant was originally detected by SSCP (15), and the Arg3500→Trp by DGGE screening (16). A recent SSCP analysis-based study (561) detected seven variations in the sequence surrounding the putative receptor binding domain of apoB. When DGGE, SSCP and HA were compared, DGGE was found to be the most reliable and HA the simplest method of the three for the detection and distinction between the three apoB FDB mutations (623). Developments in technical equipments, with the arrival of systems capable of casting both nondenaturing SSCP and the more demanding DGGE gels will probably result in a shift of the apoB mutation screening methods towards DGGE or a combination of different techniques.

The SSCP technique was chosen for our apoB screening studies because we wanted to screen large regions of the apoB gene in a relatively easy and reproducible way within a reasonable time scale. The ability to analyze several fragments at the same time by the use of restriction endonucleases or the multiplex PCR technique was regarded as an advantage. Some of the DNA fragments in our SSCP analyses were much larger than currently considered optimal. Thus, efforts were focussed on achievment of maximal resolution of the fragments through analysis under several differing SSCP conditions as well as repetition of the SSCP analyses either after additional PCR reactions covering smaller regions of the gene or after other restriction endonuclease digestions when a mobility shift in the original SSCP gels was suspected.

With our SSCP protocol, all the previously known common apoB variants situated in the exonic regions screened were easily identifiable, as was the Arg3500→Gln mutation in the FDB-positive control sample. In addition, altogether twelve new variants were detected, some of which resulted from complex DNA variations located very close to each other and were still distinguishable in the SSCP gels. In spite of this, the existence of yet more DNA variations, in particular in the largest fragments, not detected by us cannot be excluded.

**6.2. Rationale for the apoB screening strategy**

For long, the putative apoB LDLR binding domain was suggested to be located between two short apoB segments that contain clusters of positively charged amino acids and include amino acids 3147-3157 and 3359-3367, with a disulphide bridge between cysteines 3167 and 3297 bringing these clusters close to each other (158). Recent studies with transgenic mice expressing mutant forms of apoB have delineated the actual receptor binding site to amino acids 3359-3369 (197). Several lines of evidence indicate, that other regions of apoB surrounding this "core domain" are also involved in the receptor binding function of apoB. These studies include work using anti-apoB mAbs wich block binding of LDL to the LDLR, studies using chemically modified LDL, and studies with naturally occurring truncated apoB variants. Thus, a domain of apoB between amino acids 2285 and 4189 has been implicated to be involved in LDLR binding (179,180). The carboxyl-terminal domain of apoB has been suggested to play a negative regulatory role in this function (185,197). Therefore, to cover the domains of apoB possibly involved in either LDL binding to the LDLR or in its regulation, practically the whole 3´ two-thirds of the gene should be considered to be screened. The search for hypercholesterolemia-producing apoB mutations has so far largely concentrated on the LDLR binding region of apoB (490,561,622,633), and only few studies have spanned the screened area to other parts of the gene (457,584). In the studies presented in this thesis, the screening system for apoB

variants in hypercholesterolemic subjects was extended to exons 26 to 29 including amino acids 1384-4536.

The globular amino-terminal domain of apoB (amino acids 1-1000) has recently been suggested to play several differing roles in lipoprotein metabolism. Among these is an interaction between LPL and apoB-containing lipoproteins, taking place either in the circulation or at the vessel wall. In vitro data have shown that LPL associates with LDL and VLDL through specific protein-protein interactions involving the amino-terminal end of apoB (240). A protein shownt to be comprised of the amino terminus of apoB has been extracted from vascular endothelial cells and has been shown to affect the binding of LPL to vascular endothelium in vitro (237). ApoB expression has also been detected in vascular endothelial cells (120). While the biological relevance of these findings is still unknown, it is tempting to hypothesize, that a defect in the binding of LPL to apoB-containing lipoproteins, either in the circulation or in the tissue vasculature, could affect the effectiveness of LPL mediated Tg hydrolysis. In addition, expression of an apoB terminal fragment in vascular endothelium, if confirmed in vivo, could suggest a new function for the apoB protein in the trapping of LPL to arterial vasculature and the development of atherosclerosis not connected with the lipid carrier ability of apoB. In this thesis, an attempt to test the first hypothesis was made by looking for new variants and studying the effects of already known variants in this region of apoB in severely hypertriglyceridemic patients. The choice of exons (2 to 16 and 21) to be screened in this material was based on the original findings delineating the LPL-associating domain of apoB (240).

Based on the above delineated apoB functional regions, three different types of screening studies were carried out. Firstly, DNA variants in the region coding for the LDLR binding domain of apoB were looked for in hypercholesterolemic subjects. Two types of results were aimed at: either the detection of a Finnish FDB-producing mutation, most likely to be found in severely

hypercholesterolemic patients (Group 2), or a common apoB polymorphisms with a more moderate effect on lipid metabolism detectable in subjects with moderate hyper-cholesterolemia (Group 1a). Secondly, severely hyper-triglyceridemic subjects (Group 3) were screened to detect variants in the amino-terminal end of apoB, possibly affecting the apoB-LPL interaction. In addition, screening of all apoB exons was carried out in moderately hypercholesterolemic Group 1a subjects. To date, only one other complete screening of the apoB gene coding area has been reported (584). This French survey included 35 subjects participating in an epidemiological study to look for possible associations with CAD in subjects who were carriers the apoB 3´VNTR 49 allele, and did not report lipid data. To our knowledge, the studies presented in this thesis comprise the first attempt to screen for apoB variants in the whole gene in hyperlipidemic subjects.

### 6.3. Limitations of the studied materials

With regard to the selection of the studied materials, two questions arise: firstly, the probability of finding a lipid-level changing mutation in the patient groups included, and secondly, the power of the sample studied to detect lipid effects of the new variants at the population level.

Compared with the many polymorphisms of apoB, real apoB mutations are rare. After the description of the so far best characterized and common apoB mutation (Arg3500→Gln) (14), it took several years to find other apoB variants associated with moderate to severe hypercholesterolemia. These Arg3500→Trp and Arg3531→Cys mutations (15,16) are even more rare than the original FDB mutation and had not even been found until several hundreds of hypercholesterolemic patients had been screened. In view of this, our attempts to look for a new FDB-like mutation in a small group of 68 patients is modest. The Finnish population is however genetically uniquely homogenous. Although the otherwise fairly common FDB mutation seems to be absent from

this population (32), several Finnish LDLR mutations have been detected and characterized in only a small group of hypercholesterolemic patients (31). A subgroup (31 patients) of the hypercholesterolemic subjects screened by us were highly selected, lacking detectable LDLR defects. Thus, if a Finnish FDB-producing mutation exists, it should have been found in a patient material such as that studied here. Two mutations in this group were in fact detected, both with no lipid effects in studied families. As to the possible role of the amino terminus of apoB in triglyceride metabolism, no previous reports of apoB genetic screening in hypertriglyceridemia or apoB mutations associated with it have been published. Hypertriglyceridemia is a heterogeneous lipid disorder, often running in families, but with a poorly understood genetic basis. Therefore, in order to concentrate on DNA variants with major effects, only patients with severe hypertriglyceridemia, although relatively widely and arbitrarly selected, were included.

With respect to the new polymorphic variants of apoB detected by these studies, no earlier knowledge of their frequency in the Finnish population was available. When studying their effects at the population level in the materials available for study, we discovered that none of these polymorphic variants was frequent enough to detect moderate effects on the genotypic variance on lipid and lipoprotein levels. The problem of population sample size has been common in all apoB association studies reported so far (12). Also, the genetic heterogeneity of the studied populations has been considered to be a drawback in these studies (12). While the genetic homogeneity of the Finns is likely to overcome the last drawback, clearly, larger population samples in the range of hundreds to thousands of subjects would be needed to confirm or exclude the suggestive findings of the possible lipid effects of the new apoB variants.

When assessing the effects of any apoB polymorphism on lipid metabolism, two additional aspects should be considered. The first is the concept of a specific hyperlipidemia-producing apoB

haplotype, referring to polymorphic variations in the same apoB molecule producing the observed effect only in combination with each other. Screening for unknown variants in carriers of such specific hyperlipidemia-producing haplotypes has been suggested to improve the possibility of identifying relevant apoB variants (12). Another aspect not usually considered is the well known influence of the apoE polymorphism on lipid levels (74,75). Most apoB association or screening studies have not taken this effect into account, which might have obscured the results obtained in this study as well.

## 6.4. Detection of new variants of apo B and their characterization

In genetic research, the detection of a new mutation or polymorphism should be followed by mutation expression experiments and a thorough characterization of the mutated protein and its biological effects. The enormous size of apoB and the special features of its biological behaviour have made this task difficult. In vitro, apoB gene may be expressed in hepatic or other cells capable of producing lipids as well, leading to the production of lipoproteins, the characteristics of which, including density, lipid composition, and size, can then be analyzed. With regard to the biological functions of in vitro expressed apoB, their LDLR binding capacity is reasonably easily studied with either peripheral blood lymphocytes, fibroblasts or other cells expressing the LDLR such as the macrophage cell line U937 (44,721). However, in vitro apoB expression studies are often hampered by the low level of gene expression, and by the fact that recombinant LDL secreted by cultured cells usually lacks normal receptor-binding activity (197). Therefore, in order to analyze changes in lipoprotein metabolism caused by apoB mutations, in vivo techniques are needed. So far, the only practical approach to study in vivo effects of apoB mutations has been based on the extraction of lipoproteins from natural mutant carriers (preferrably homozygotes) (533), and on the study of their behaviour in

different in vivo kinetic study settings. With recent developments in the research of genetically modified animals such as mice, some of these difficulties of apoB mutation studies have been overcome (722,723). So far, in most studies identifying new apoB variants, including those presented in this thesis, characterization of new variants has been restricted to attempts to associate the DNA changes with serum lipid and lipoprotein levels either in families or in groups of unrelated subjects.

Altogether twelve new variants of apoB were detected by SSCP in our study populations. Four of them were silent changes, of which, the two variations at codons 875 and 2285 were easily identified as polymorphisms, whereas the Tyr4242→Tyr variant in exon 29, detected in three families, and the noncharacterized DNA variant in the noncoding area in exon 29, detected in two families, were considered mutations. Since no amino acid changes were produced by these variants and no association between the DNA change and the lipid phenotype in families was observed, no further studies to characterize these silent changes were considered necessary. Of the eight nonsense DNA changes, two mutations were seen in only single hypercholesterolemic families. Neither the del mutation at Asp 2186, nor the Thr3918→Ala mutation seemed to cosegregate with the severe hypercholesterolemia present in the probands, and no further studies to characterize their effects on lipid metabolism were carried out. The third rare variant, Val4238→Ala, was detected in two control DNA samples from normocholesterolemic subjects and thus considered not to be of sufficient interest for further study. The remaining five nonsense DNA changes identified by us were all shown to represent new apoB polymorphisms with rare allele frequencies ranging from 0.02 (Asn1887→Ser and Ala4454→Thr) to 0.11 (His1896→Arg). In spite of the relative rareness of the variant alleles, an attempt to study their effects on lipid and lipoprotein levels in different study settings was carried out.

### 6.5. Lipid and lipoprotein effects of the new carboxyl-terminal polymorphisms of apoB

Effects of the Asn1887→Ser and His1896→Arg variations, composing the mAb D7.2 polymorphism were studied in healthy subjects (Groups 4 and 8), and patients undergoing coronary angiography (Group 7). While the effect on the reactivity against mAb D7.2 was more clear with the 1887 Ser allele compared with the 1896 Arg allele, only the latter seemed to exhibit effects on lipid phenotype. Altogether, lipid data collected from the diet study participants, patients undergoing coronary angiography, and the healthy control group pointed towards a cholesterol- and apoB-raising effect of the 1896 Arg allele. In the diet study participants, this effect was observed in men and was detectable only during a low-fat, low-cholesterol diet. In coronary angiography patients, a slight total and LDL cholesterol elevating effect of the 1896 Arg allele was obesereved in men with no angiographically detectable vessel disease. This effect was not observed in men with angiographic CAD. One explanation for the fact that lipid effects were seen solely during diet intervention in the diet study, but already at baseline in the angiographically healthy males could be that subjects with suspected CAD had changed their eating habits, and were consuming a diet which in fact resembled the low-fat low cholesterol diet of the North Karelia study. Why the same effect was not observed in men with CAD is not known, but serum LDL cholesterol levels in angiographically healthy males were than thosw of the male CAD patients (3.04±0.94 and 3.71±1.00, $p=0.0001$ in the non-CAD and CAD subjects, respectively), or control males (data not shown). Thus, it might be proposed that a lower overall LDL cholesterol level is required for the LDL cholesterol-elevating effect of the 1896 Arg allele to be detected. The possible mechanism through which the His1896→Arg change exerts its cholesterol- and apoB-raising effect is not known. No signs of altered LDLR binding affinity of LDLs extracted from the carriers of the 1896 Arg allele has been detected (724), neither has mAb D7.2 been shown to inhibit the

LDL-LDLR interaction (725). No lipid effects of the apoB 1896 Arg allele were detected in women. In all groups, women were fewer in number, making a moderate effect of the 1896 Arg allele more difficult to detect, and were also not matched for their menstrual or menopausal status, which might have influenced the results (65). In addition to differences in the effects of the 1896 allele on lipid levels between studied groups, the observed 1896 Arg allele frequencies were also different, with a higher 1896 allele frequency in the diet study participants, and a low frequency in CAD subjects. While a low 1896 Arg allele frequency might partly explain the missing lipid effects of this allele in this group, it can also be suspected to relate to changes in CAD risk. Therefore, the frequency of this allele was additionally analyzed in another diet study population (228) collected from the same area (228), showing similar allele frequencies and similar statistical significances as Group 4 compared to other groups (data not shown). Therefore, the allele frequency difference was considered more likely to represent an enrichment of the 1896 His allele in North Karelia, which is known to be a genetic subisolate (726), than a sign of underrepresentation of this allele in subjects with CAD.

With regard to the apoB Asn1887→Ser polymorphism, no major lipid effects in the studied materials were detected. However, so far, only small numbers of 1887 Asn/Ser heterozygotes have been analyzed, thus not outruling moderate effects of the 1887 Ser allele on lipid levels not detectable with a small population. As with the His1896→Arg polymorphism, no changes in the LDLR binding affinity have been detected in 1887 Ser allele carriers (724). This polymorphism has been detected with an allele frequency similar to the Finns at least in the French and Norwegian populations (584,585,720). No data concerning the lipid effects of the 1887 Ser allele in these samples are available. Recently, LDL particles from 1887 Ser allele carriers were reported to show increased electrophoretic mobility as a sign of increased LDL electronegativity (720).

Lipid and lipoprotein effects of the Arg4243→Thr and Ala4454→Thr polymorphisms were analyzed both in normolipidemic Finnish population samples (Groups 4 and 5) and in hypercholesterolemic subjects (Groups 1 and 6). No differences between allele frequencies, or significant lipid effects in the different populations could be detected. Since frequencies of the rare alleles of both polymorphisms were low, the number of observed heterozygotes remained small, weakening the power of the analyses to detect moderate lipid or lipoprotein effects. The Ala4454→Thr polymorphism was tested for its effects on LDLR binding affinity and no detectable change was observed (724). To date, only the Ala4454→Thr polymorphism has been detected in other Caucasian populations, with a slightly higher allele frequency in the French (0.039) than in the Finnish population (0.020) (584).

### 6.6. Lipid and lipoprotein effects of the amino-terminal polymorphisms of apoB

The only new apoB polymorphism characterized in the amino-terminal part of apoB, Val703→Ile, was originally detected in a sample of severely hypertriglyceridemic patients (Group 3). Further studies involving both normolipidemic and hyper-cholesterolemic subjects showed that this DNA variant was a polymorphism and that it seemed to be underrepresented in the hypertriglyceridemic patients. As to its lipid or lipoprotein effects, a Tg-lowering effect was observed in normolipidemic relatively young Finnish healthy subjects (Group 5). No effects were observed either in the slightly older population of diet study participants (Group 4) or hypercholesterolemic subjects (Group 1). Again, the frequency of the rare apoB 703 Ile allele was relatively low. It would clearly be of much interest to study the effects of this apoB polymorphism in larger population samples. The possible mechanism(s) of the Tg-lowering effect of the Val703→Ile polymorphism is not known; since it is located in the apoB domain suggested to interact with LPL (240), one explanation might be an accelerating effect on the LPL mediated

hydrolysis of Tg-rich particles. So far, this apoB polymorphism has not been detected in other populations.

Two previously characterized apoB polymorphims, Thr71→Ile and Ala591→Val, are situated in the proposed LPL-binding domain of apoB. In earlier studies, the 71 Ile allele has been associated with higher serum apoB concentrations in Finnish children (426) and healthy adults (423), the 591 Val allele with larger postprandial TGRLP responses after a fat load (410), and the 591 Ala allele with higher serum apoB concentrations in ischemic stroke patients (434). In the hypertriglyceridemic patients studied by us, a signifcant association between 591 Ala carrier status and higher serum apoB concentrations was detected. The same association was also observed when the 71 Ile and 591 Ala alleles were combined. In normolipidemic populations, this association could not be detected. Thus, these results would point towards an apoB-raising effect of these amino-terminal apoB polymorphisms, expressed only in the setting of hypertriglyceridemia. These results are preliminary and need to be confirmed in other studies. Also, the mechanism(s) through which this kind of effect is brought about are not known.

## 7. SUMMARY AND CONCLUSIONS

The main points and conclusions of the present study are:

1) SSCP is a technically easy, reproducible and effective method offering the possibility of screening for mutations even in large genes such as the apoB gene. In the studies presented here, twelve new apoB variants were detected. All the previously known apoB polymorphisms and the Arg3500→Gln mutation were also easily identifiable by SSCP.

2) The amino terminus of apoB seems to have some impact in Tg and apoB metabolism, as suggested by the recent findings of an LPL-binding amino-terminal apoB domain. A new polymorphism of apoB, the Val703→Ile variation, is associated with lower serum Tg levels in healthy relatively young subjects and seems also to be underrepresented in severely hypertriglyceridemic patients. The Ala591→Val polymorphism, alone or in combination with the apoB Thr71→Ile polymorphism, is associated with higher serum apoB levels in hypertriglyceridemic subjects.

3) The His1896→Arg variation seems to have a tendency to increase serum total and LDL cholesterol concentrations in healthy men during a low-fat, low cholesterol diet. The mechanism of this effect is not known. No direct changes in LDLR binding affinity have been detected. Since this polymorphism can be detected immunologically as well, some changes of the configuration of apoB-containing lipoproteins are possible. Kinetic studies would be needed to clarify the mechanisms of this polymorphism on cholesterol metabolism in vivo.

4) The Asn1887→Ser, Arg4246→Thr, and Ala4454→Thr polymorphisms detected in this study have so far not been shown to affect any lipid or lipoprotein parameters in different Finnish populations and study settings. Allele frequencies have however been too low for the detection of moderate effects. Larger population studies would be needed.

5) No FDB-like mutation characteristic to Finns seems to be present in the highly selected severely hypercholesterolemic study population screened for DNA changes in the 3´ two-thirds of the apoB gene covering the whole LDLR binding region of apoB and its surroundings.

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**TABLES**

TABLE 1. Characteristics of the major human plasma lipoproteins.

| Lipo-protein | Density (g/dl) | Molecular weight (kDa) | Diameter nm | Lipid composition (% of lipids) | | | Apolipoproteins |
|---|---|---|---|---|---|---|---|
| | | | | Tg | Chol | Pl | |
| CM | < 0.95 | $400\times10^3$ | 75-1200 | 80-95 | 2-7 | 3-9 | B-48, AI, AII, AIV, CI-III, E |
| VLDL | 0.95-1.006 | $10\text{-}80\times10^3$ | 30-80 | 55-80 | 5-15 | 10-20 | B-100, CI-III, E |
| IDL | 1.006-1.019 | $5\text{-}10\times10^3$ | 25-35 | 20-50 | 20-40 | 15-25 | B-100, CI-III, E |
| LDL | 1.019-1.063 | $2.3\times10^3$ | 18-25 | 5-15 | 40-50 | 20-25 | B-100 |
| HDL | 1.063-1.210 | $1.7\text{-}3.6\times10^2$ | 5-12 | 5-10 | 15-25 | 20-30 | AI-II, AIV, CI-III, E |
| Lp(a) | 1.050-1.090 | $5\times10^3$ | 28 | similar to LDL | | | B-100, apo(a) |

Data from Rudel et al. 1986, Durrington 1995, Ginsberg 1998 (10,38,727).

TABLE 2. Characteristics of the major apolipoprotein

| Name | Chromo-some | Peptide length (aa)* | Molecular weight (kDa) | Lipo-protein | Functions |
|---|---|---|---|---|---|
| A-I | 11 | 243 | 28 | HDL,CM | structural (HDL), LCAT activation, receptor ligand (SR-BI), promotes cholesterol efflux |
| A-II | 1 | 77 | 17 | HDL,CM | structural (HDL), HL, LPL activation LCAT modulator |
| A-IV | 11 | 377 | 46 | HDL,CM | LCAT activator, LPL modulator, function in Tg transport? |
| (a) | 6 | 4529 † | 250-800 | Lp(a) | unknown |
| B-100 | 2 | 4536 | 540 | VLDL, IDL,LDL | structural, LDLR ligand |
| B-48 | 2 | 2152 | 264 | CM | structural |
| C-I | 19 | 57 | 6.6 | CM,VLDL IDL,HDL | LRP and LDLR interaction inhibitor, LCAT activator |
| C-II | 19 | 79 | 8.9 | CM,VLDL IDL,HDL | LPL activator |
| C-III | 11 | 79 | 8.8 | CM,VLDL IDL,HDL | LPL inhibitor apoE-mediated remnant removal inhibitor |
| E | 19 | 299 | 34 | CM,VLDL IDL,HDL | receptor ligand, reverse cholesterol transport, cell growth and immune response regulation? |

* aa = amino acids.
† based on cDNA analysis of one apo(a) isoform; otherwise highly variable (728).
Data from Patsch and Gotto 96, Tailleux and Fruchart 96, Ginsberg 98 (10,35,39).

TABLE 3. Effects of age, gender, obesity, diet and other lifestyle factors on lipid and lipoprotein levels.

| | Chol | Tg | LDL-chol | HDL-chol | Reference |
|---|---|---|---|---|---|
| Age | ↑ | ↑ | ↑ | ↑ | (61-63) |
| Sex (male) | ↑ | ↑ | ↑ | ↓ | (61) |
| Obesity | ↑ | ↑ | ↑ | ↓ | (66-70) |
| Diet | | | | | |
| Fatty acids | | | | | (47,48,729) |
| Saturated | ↑ | | ↑ | ↑ | |
| PUFA* | ↓ | ↓ † | ↓ | ↓ | |
| MUFA* | ↓ | | ↓ | ↑ | |
| Cholesterol | ↑ | | ↑ | ↑ | (48,50,51) |
| Carbohydrate # | ↓ | ↑ | ↓ | ↓ | (53,730) |
| Fibre | ↓ | | ↓ | ↓ | |
| (54) | | | | | |
| Protein (plant) | ↓ | | ↓ | ↑ | |
| (56,731-733) | | | | | |
| Alcohol | | ↑ | ↓ | ↑ | (57) |
| High caloric intake | ↑ | ↑ | | | (52) |
| Smoking | ↑ | ↑ | ↑ | ↓ | (58) |
| Physical activity | | ↓ | ↓ | ↑ | (59) |
| Stress | ↑ | | ↑ | ↑ | (60) |

* PUFA = polyunsaturated fatty acid, MUFA = monounsaturated fatty acid. Effects produced when substituted for saturated fatty acids.
† Effect of omega-3 PUFA only.
# Low-fat, high-carbohydrate diet.

TABLE 4. ApoB genetic variants reported to date. Mutations associated with hypobeta-lipoproteinemia excluded.

| Site* | Mutation | Outcome | RFLP | Ag ass † | Allele frequency # | Number of families | Reference |
|---|---|---|---|---|---|---|---|
| | -4kb | | AvaII+/- | | 0.20 | | 734 |
| 5'VNTR | -3256bp $(TG)_n$ repeat, 7 alleles | | | | $(TG)_{14}$ 0.78<br>$(TG)_{15}$ 0.19 | | 554,735 |
| Reduc | -1860/80 $(A)_n(TA)_m$ repeat, 6 alleles | | | | 0.36,0.29<br>(2 smallest) | | 584 |
| Prom | -837 G→A | | | | 0.48 | | 584,736 |
| Prom | -516 C→T | | | | 0.31 | | 584,736 |
| Prom | -265 C→T | | MspI+/-<br>MscI-/+ | | 0.21 | | 719,737 |
| Prom | +14 C→G | | AluI-/+ | | | | 719 |
| Ex 1 | del162-170 del Leu-Ala-Leu -16-14<br>signal peptide 27→24 aa | | | | **0.27** | | 372 |
| Ex 1 | ins CTGCTG 194/5 ins Leu-Leu -6/-5<br>signal peptide 27→29 aa | | | | 0.03 | | 377 |
| In 2 | A→G +722 | | StyI+/- | | 0.01 | | 634 |
| In 3 | G→T +92 | | | | 0.47 | | 734 |
| Ex 4 | C421→T | Thr71→Ile | ApaLI+/-<br>Bsp12861+/- | g/c | **0.26** | | 416,417 |
| | | | | MB19 2/1<br>BIP45, D2E1 | | | 418,419<br>420,421 |
| In 4 | C→A + 171 | | HincII-/+ | | 0.20 | | 554,734,738 |
| In 4 | G→A -523 to ex 5 | | PvuII-/+ | | 0.04 | | 554,734,738,739 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| In 5 | C→T -9 to ex 6 | MnII+/- | | | | | 719 |
| Ex 14 | C1981→T | Ala591→Val | AluI+/- | d/$a_1$<br>H11G3 | **0.56** | | 431<br>421 |
| Ex 15 | G2316→A | Val703→Ile | Alw26I+/-<br>BsmAI+/- | | **0.04** | | 719,740 |
| Ex 18 | C2834→T | Asn875→Asn | | | 0.03 | | 584 |
| In 20 | TTTA repeats and sequence variation 5' to the Alu element, 7 alleles | | | | | | 741,742 |
| In 20 | sequence variation in the Alu element, 12 alleles | | | | | | 742 |
| In 20 | A→G -146 to ex 21 | | BalI-/+ | | 0.50 | | 734 |
| Ex 26 | C4439→G | Phe1410→Leu | | | | 1 | 584 |
| Ex 26 | A5869→G | Asn1887→Ser | BsrdI+/- | D7.2 | **0.02** | | 743 |
| Ex 26 | A5896→G | His1896→Arg | RsaI-/+ | D7.2 | **0.11** | | 743 |
| Ex 26 | del6766-8 | del Asp2186 | | | | 1 | 744 |
| Ex 26 | T7064→C | Asp2285→Asp | MaeII-/+ | | **0.63** | | 113,575,745 |
| Ex 26 | T7673→C | Thr2488→Thr | XbaI+/- | | **0.63** | | 113,373,374 |
| Ex 26 | G7824→A | Glu2539→Lys | | | 0.009 | | 584 |
| Ex 26 | C8344→T | Pro2712→Leu | MaeI-/+<br>BfaI-/+ | y/x | **0.29** | | 371,455,456,458 |
| Ex 26 | G9489→A | Ala3094→Thr | | | | 1 | 584 |
| Ex 26 | A9963→G | Ser3252→Gly | | | | 7 | 561 |
| Ex 26 | C10083→G | His3292→Asp | | | | 1 | 585 |
| Ex 26 | G10259→A | Leu3350→Leu | PstI +/- | | 0.01 | | 20,561,588 |
| Ex 26 | C10321→T | Ala3371→Val | AluI +/- | | | 1 | 588 |
| Ex 26 | G10397→A | Val3396→Met | | | | 1 | 561 |
| Ex 26 | A10408→C | Lys3400→Thr | | | | 1 | 585 |
| Ex 26 | G10422→C | Glu3405→Gln | | | 0.01 | | 20,561,587 |

TABLE 4, continued.

| Site | Mutation | Outcome | RFLP | Ag ass | Allele frequency | Number of families | Reference |
|---|---|---|---|---|---|---|---|
| Ex 26 | C10574→A | Ser3455→Arg | | | | 1 | 561 |
| Ex 26 | G10648→C | Arg3480→Pro | | | | | 565 |
| Ex 26 | C10707→T | Arg3500→Trp | | | | 15 | 16 |
| Ex 26 | G10708→A | Arg3500→Gln | | | 0.001 | | 14 |
| Ex 26 | T10760→G | Leu3517→Leu | | | | 1 | 562 |
| Ex 26 | C10790→T | Ala3527→Ala | | | | 1 | 562 |
| Ex 26 | C10800→T | Arg3531→Cys | NsiI-/+ | | | 26 | 15 |
| Ex 26 | G10829→T | Thr3540→Thr | | | | | 21 |
| Ex 26 | C10835→T | Asn3542→Asn | | | | | 21 |
| Ex 26 | C10865→T | Thr3552→Thr | | | | | 21 |
| Ex 26 | G11041→A | Arg3611→Gln | MspI+/- | i/h | **0.06** | | 464,465 |
| Ex 26 | G11889→A | Val3894→Ile | RsaI-/+ | | | 1 | 585 |
| Ex 27 | A11961→G | Thr3918→Ala | | | 0.01 | 1 | 584,744 |
| In 27 | G→C +50 | - | | | 0.20 | | 584 |
| Ex 29 | C12264→T | Arg4019→Trp | MspI+/- | | | 1 | 586 |
| Ex 29 | G12510→A | Val4101→Met | | | 0.08 | | 584 |
| Ex 29 | A12669→G | Lys4154→Glu | EcoRI-/+ | z/t | **0.84** | | 113,479,480 |
| Ex 29 | T12922→C | Val4238→Ala | | | | 2 | 744 |
| Ex 29 | T12935→C | Tyr4242→Tyr | RsaI-/+ | | | 3 | 744 |
| Ex 29 | G12937→C | Arg4243→Thr | | | **0.03** | | 457,744 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ex 29 | A13141→G | Asn4311→Ser | Eco57I-/+ | y/x | **0.29** | 456-458 |
| Ex 29 | G13569→A | Ala4454→Thr | | | **0.02** | 584,744 |
| 3'VNTR | 73 bp 3' to the 2. polyadenylation signal<br>22-57 repeats of 11-16 bp sequences | | | | 26 alleles | 375,376 |

In addition, rare TaqI, EcoRV, StuI and RsaI polymorphisms have been reported. Their exact location is currently not known (9).
* Reduc = reducer segment of the apoB gene, Prom = promoter, Ex = exon, In = intron.
† Antigenic association with either monoclonal (mAb) or alloantibodies (Ag).
# Bolded numbers denote allele frequencies reported in the Finns.

TABLE 5. Associations between alleles of the apoB XbaI polymorphism and serum total cholesterol, triglycerides, apoB or CAD in population studies.

| Study population | | | | XbaI association § | | | | Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | High levels of | | | | |
| Source* | Sample † | n | S# | TC | Tg | apoB | CAD | |
| Norway | h | 56 | B | X+ | 0 | X+ | – | 746 |
| USA (c) | CAD+h | 219 | B | 0 | X– | 0 | 0 | 484 |
| USA (c) | MI/h | 84/84 | B | 0 | 0 | 0 | X– | 485 |
| GB (c) | ns | 83 | M | X+ | X+ | 0 | – | 747 |
| Italy | h | 79 | B | – | 0 | – | – | 748 |
| GB (c) | HL/h | 133/62 | B | X+ | X+ | – | – | 486 |
| Finland | h | 176 | B | X+ | 0 | 0 | – | 427 |
| Japan | HL/h | 53/54 | B | 0 | 0 | 0 | 0 | 749 |
| France | h | 53 | B | X– | – | – | – | 429 |
| GB (c) | MI/h | 100/62 | M | – | – | – | 0 | 498 |
| GB (c) | HL/h | 124/34 | B | 0 | X+ | – | – | 487 |
| Norway | HC | 64 | M | 0 | 0 | X+ | – | 750 |
| GB | ATH/h | 205/139 | B | 0 | 0 | 0 | X– | 488 |
| GB (c) | CAD+h | 290 | M | 0 | 0 | 0 | X– | 475 |
| Finland | FH | 120 | B | X+ | 0 | – | – | 751 |
| Sweden | h | 187 | B | 0 | 0 | 0 | – | 752 |
| GB (c) | CAD/h | 124/146 | M | 0 | 0 | 0 | X– | 441 |
| Sweden | MI/h | 52/52 | M | 0 | X– | 0 | 0 | 753 |
| USA (c) | CAD/h | 111/128 | M | 0 | 0 | 0 | 0 | 478 |
| Norway | h | 170 | M | 0 | 0 | 0 | – | 521 |
| Austria | CAD/h | 106/118 | M | 0 | 0 | 0 | 0 | 491 |
| Germany | FCHL/h | 321/107 | B | X+ | 0 | 0 | – | 467 |
| USA (c) | >90-y | 41 | B | 0 | 0 | 0 | – | 754 |
| Italy | h ch | 209 | B | 0 | 0 | 0 | – | 468 |
| Finland | HL | 221 | M | X+ | 0 | – | – | 447 |
| Finland | h ch | 307 | B | X+ | 0 | – | – | 436 |
| Italy | h | 82 | M | 0 | – | 0 | – | 755 |
| Finland | FH | 51 | B | X+ | 0 | – | – | 756 |
| GB (c) | NIDDM | 95 | B | 0 | 0 | 0 | 0 | 492 |
| Sri Lan | CAD/h | 95/95 | M | 0 | X+ | – | X– | 472 |
| Finland | HL | 211 | B | 0 | 0 | – | – | 757 |
| GB (a) | CAD/h | 46/107 | M | 0 | 0 | – | – | 382 |
| Denmark | MI/h | 50/39 | M | X+ | 0 | 0 | X– | 493 |
| USA (c) | CAD/h | 274/162 | B | X+ | – | X+ | – | 490 |
| Italy | HL/h | 44/35 | B | – | – | – | 0 | 758 |
| Austral | CAD/h | 122/80 | B | 0 | 0 | 0 | X– | 435 |
| Finland | CAD/h | 111/142 | B | X+ | 0 | 0 | 0 | 759 |
| Sweden | MI/h | 87/91 | M | X+ | 0 | 0 | 0 | 404 |
| China | h | 221 | B | 0 | 0 | 0 | – | 438 |
| China | CAD/h | 139/149 | B | 0 | 0 | 0 | 0 | 406 |
| Spain | h | 228 | M | X+ | 0 | X+ | – | 760 |
| Norway | MI/h | 238/621 | B | X+ | 0 | X+ | X– | 761 |
| Italy | HL/h | 76/79 | B | – | – | – | 0 | 762 |
| Italy | CAD/h | 39/40 | M | – | – | – | 0 | 763 |
| China | h | 148 | M | 0 | 0 | 0 | – | 514 |
| Israel | CAD off | 525 | B | X+ | 0 | X+ | – | 764 |
| Denmark | h | 464 | M | X+ | 0 | X+ | – | 390 |

TABLE 5, continued.

| Source | Sample | n | S | High levels of TC | Tg | apoB | CAD | |
|---|---|---|---|---|---|---|---|---|
| Singapo | h | 181 | B | X+ | X+ | 0 | – | 384 |
| GB | HC/h | 280/265 | B | X+ | – | – | – | 476 |
| Finland | NIDDM | 268 | B | X+ | X+ | – | 0 | 499 |
| Java | h | 231 | B | 0 | X+ | – | – | 385 |
| Denmark | FH | 99 | B | 0 | 0 | – | – | 765 |
| Denmark | HL+CAD | 318 | B | X+ | 0 | – | – | 393 |
| Finland | 100-y | 364 | B | 0 | 0 | 0 | – | 766 |
| USA (c) | CAD/h | 444/404 | B | – | – | – | 0 | 407 |
| Finland | CAD/h | 82/50 | M | 0 | 0 | – | 0 | 767 |
| China | CAD/h | 80/60 | B | 0 | 0 | 0 | X+ | 439 |
| Finland | h | 52 | M | 0 | 0 | – | – | 768 |
| China | CAD/h | 148/153 | B | 0 | 0 | 0 | – | 437 |
| Europe | CAD/h off | 682/312 | B | X+ | 0 | X+ | – | 381 |
| China | CAD/h | 103/100 | B | 0 | 0 | 0 | X+ | 440 |
| Nigeria | h | 1222 | B | 0 | 0 | 0 | – | 386 |
| Italy | CAD/h | 45/118 | F | X+ | 0 | 0 | 0 | 400 |
| Europe | FDB | 205 | B | 0 | 0 | – | – | 19 |
| Japan | h | 1328 | B | 0 | – | – | – | 387 |
| Chech | hl/h ch | 82/86 | B | 0 | 0 | 0 | – | 769 |
| China | ch | 93 | B | X+ | 0 | 0 | – | 770 |
| Russia | CAD/h | 94/122 | M | 0 | 0 | – | 0 | 470 |
| Asia | HL/h | 131/374 | B | 0 | 0 | 0 | – | 466 |
| Norway | hl+RR/h | 108/64 | B | 0 | 0 | 0 | 0 | 477 |
| Brazil(i)h | | 82 | B | 0 | 0 | 0 | – | 378 |
| Finland | HC | 48 | B | X+ | 0 | X+ | – | 402 |

* Country of origin; c=caucasian, a=asian, i=indian.

† h, healthy, CAD, coronary artery disease, MI, myocardial infarction, ns, not specified, HL, hyperlipidemic, HC, hypercholesterolemic, ATH, atherosclerotic (both CAD and peripheral), FH, familial hypercholesterolemia, FCHL, familial combined hyperlipidemia, y, years, ch, children, NIDDM, non-insulin-dependent diabetes mellitus, off, offspring, FDB, familial defective apoB, RR, hypertensive subjects.

\# S, sex, M, male, F, female, B, both sexes.

§ TC, total cholesterol, Tg, triglycerides, X+, association with the XbaI+ allele, X-, the XbaI - allele, 0, no significant association detected, -, not analyzed.
Significance level $p<0.05$ or less.

TABLE 6. Apolipoprotein B gene mutations associated with familial hypobetalipoproteinemia.

| Name | Site* | Mutation † | ApoB size (aa) # | Outcome § | Reference |
|---|---|---|---|---|---|
| ApoB-2(4) | in 5 | G→T, +1 to ex 5 | 152 | splicing defect<br>no apoB in plasma | 575,719 |
| ApoB-9 | ex 10 | C1443→T | 411 | Arg412→Ter<br>no apoB in plasma | 575 |
| ApoB-25 | | deletion of 694 bp, from intron 20 to intron 21 | 1085 | Gly1014→Val-72aa-Ter1086<br>no apoB in plasma | 577 |
| ApoB-27.6 | in 24 | T→C, base +2 | 1282 | Ser1254→Arg-28aa-Ter1283<br>splice site disruption<br>activation of cryptic splice site in intron 24<br>traces in plasma | 579,771 |
| ApoB-29 | ex 25 | C4125→T | 1305 | Arg1306→Ter<br>no apoB in plasma | 772 |
| ApoB-31 | ex 26 | del 4480 | 1425 | Gly1424→Val-Tyr-Ter1426 | 773 |
| ApoB-32 | ex 26 | C4557→T | 1449 | Gln1450→Ter | 774 |
| ApoB-32.5 | ex 26 | T4631→G | 1473 | Tyr1474→Ter | 775 |
| ApoB-37 | ex 26 | del 5391-4 | 1728 | Asn1728→Val-Ter1729 | 776,777 |
| ApoB-38.7 | ex 26 | C5472→T | 1754 | Gln1755→Ter | 581 |
| ApoB 38.9 | ex 26 | del 5444 | 1767 | Asn1745→Lys-4aa-Ter1768 | 778 |
| ApoB 38.95 | ex 26 | del 5478-91 | 1767 | Val1757→Thr-10aa-Ter1768 | 779 |
| ApoB-39 | ex 26 | del 5591 | 1799 | His1795→Met-4aa-Ter1800 | 772 |
| ApoB-40 | ex 26 | del 5694-5 | 1829 | Val1829→Cys-Ter1830 | 201,780 |
| ApoB-43.7 | ex 26 | C6162→T | 1984 | Arg1985→Ter | 568 |

| | | | | | |
|---|---|---|---|---|---|
| ApoB-44.4 | ex 26 | ins 11 bp 6243/4 | 2014 | Ala2012→Glu-2aa-Ter2015 | 781 |
| ApoB-45.2 | ex 26 | T6368→A | 2052 | Tyr2053→Ter | 580 |
| ApoB-46 | ex 26 | C6381→T | 2057 | Arg2058→Ter | 782 |
| ApoB-48.4 | ex 26 | del 6686 | 2166 | Lys2159→Lys-7aa-Ter2167 | 783 |
| ApoB-50 | ex 26 | C6963→T | 2251 | Gln2252→Ter | 578 |
| ApoB-52 | ex 26 | del 7279-83 | 2361 | Val2357→Asp-4aa-Ter2362 | 784 |
| ApoB-52.8 | ex 26 | del 7295 | 2395 | Tyr2362→Tyr-33aa-Ter2396 | 775 |
| ApoB-52.8 | ex 26 | del 7359 | 2395 | Thr2364→His-11aa-Ter2396 | 775 |
| ApoB-54.8 | ex 26 | C7665→T | 2485 | Arg2486→Ter | 785 |
| ApoB-55 | ex 26 | C7692→T | 2494 | Arg2495→Ter | 781,786,787 (independent mutations) |
| ApoB-61 | ex 26 | del 8525-61 | 2784 | Ser2772→Ser-12aa-Ter2785 | 788 |
| ApoB-67 | ex 26 | del 9327 | 3040 | Lys3040→Arg-Ter3041 | 789 |
| ApoB-70.5 | ex 26 | ins A9754/60 | 3196 | Asn3184→Lys-12aa-Ter3197 | 790 |
| ApoB-75 | ex 26 | del 10366 | 3386 | Thr3386→Met-Ter3387 | 202 |
| ApoB-82 | ex 26 | C11411→A | 3733 | Cys3734→Ter | 775 |
| ApoB-83 | ex 26 | C11458→A | 3749 | Ser3750→Ter | 791 |
| ApoB-86 | ex 26 | del 11840 | 3896/4536 | Asn3877→Lys-19aa-Ter3897 reading frame restoration yields 10% of normal apoB-100 | 576 |
| ApoB-87 | ex 28 | del 12032 | 3978 | Glu3942→Asn-36aa-Ter3979 | 203 |
| ApoB-89 | ex 29 | del 12309 | 4039 | Glu4034→Arg-5aa-Ter4040 | 201,780 |

* In = intron, ex = exon. † Del = deletion, ins = insertion.
# Aa = amino acids. § Ter = termination codon.

TABLE 7. Summary of the studied materials used in SSCP screening, allele frequency analyses and estimations of lipid effects of newly detected DNA variants.

| Group | n | M/F* | Type of analysis † | Description | Reference |
|---|---|---|---|---|---|
| 1 | 89 | 48/41 | Allele frequency and lipid effects of codon 703,4243,4454 pm:s | Kuopio district subjects<br>Primary hypercholesterolemia<br>Cholesterol ≥ 8 mmol/l<br>ApoB Arg3500→Gln excluded<br>Three subjects (2/1) with FH excluded | (696) |
| 1a | 29 | 12/17 | Exon 2-29 SSCP screening<br>Allele frequency of codon 1887,1896 pm:s | Kuopio district subjects<br>Subgroup based on family data | (696) |
| 2 | 39 | 23/16 | Exon 26-29 SSCP screening<br>Allele frequency of codon 1887,1896 pm:s | Non-FH hypercholesterolemia<br>Cholesterol ≥ 8 mmol/l and tendom xanthomas in the proband and a first-degree relative<br>LDLR defects and apoB Arg3500→Gln excluded | (31) |
| 3 | 76 | 66/10 | Exon 2-16 and 21 screening | Severe hypertriglyceridemia<br>Triglycerides > 6 mmol/l | (697) |
| 4 | 102 | 48/54 | Characterization of mAb D7.2 polymorphism<br>Allele frequency and lipid effects of codon 703, 1887,1896,4243,4454 pm:s<br>Lipid effects of Ag(c/g) and Ag($a_1$/d) pm:s | North Karelia diet study<br>Apparently healthy | (442) |
| 5 | 88 | 41/47 | Allele frequency and lipid effects of codon 703,4243,4454 pm:s | Random sample of Finns born in 1954<br>Apparently healthy | (698) |

| | | | | | |
|---|---|---|---|---|---|
| 6 | 220 | 112/108 | Allele frequency and lipid effects of codon 4243,4454 pm:s | Drug intervention study<br>Cholesterol ≥ 6.2 mmol/l<br>Triglycerides ≤ 4.0 mmol/l<br>FH not excluded | (32,699) |
| 7 | 327 | 217/110 | Allele frequency and lipid effects of codon 1887,1896 pm:s | Coronary angiography patients<br>CAD 205 ( M/F 162/43)<br>Non-CAD 122 (55/67) | (700) |
| | 160 | 98/62 | Lipid effects of Ag(c/g) and Ag($a_1$/d) pm:s | Coronary angiography patients<br>Ag(c/g) and Ag($a_1$/d) data available<br>CAD 84 (64/20)<br>Non-CAD 76 (34/42) | (700) |
| 8 | 72 | 45/27 | Allele frequency and lipid effects of codon 1887,1896 pm:s | Normal voluntary controls<br>No symptoms or signs of CAD | (701) |

* M = male, F = female.
† pm = polymorphism.

TABLE 8. Oligonucleotides used as primers in SSCP screening of exons 2 to 29 of the apoB gene.

| Primer | Upstream (sense) | Downstream (antisense) |
|---|---|---|
| Exon 2 | 5´ CTC ACA GAA TTT CTT TCT CC | 5´ AGA TGC CTT ACT TGG ACA G |
| Exon 3 | 5´ TGT GGC TGA CGT ATT TCT C | 5´ TCC CTC CTG CCT GCA TC |
| Exon 4 | 5´ AAC CTC AAT GCT CTG CTA CC | 5´ TGC GTG TGC TCA TGT ACA AC |
| Exon 5 | 5´ ATT TCC GTG ACC ATC CTC TC | 5´ ACT GCT ATC AGC TTT CTA AAT C |
| Exon 6 | 5´ GTG TTG AAT ACA TGT GGT TGC | 5´ TAA TAA GAG GAT GCT CCT TGC |
| Exon 7 | 5´ GAT CAA AAT GCT CGT CTC C | 5´ AGG GTT GCA TCA CAT GAC |
| Exon 8 | 5´ AAT AGT ATG TTC TGG CCA TC | 5´ ATT TTC CAG CAA CTA TGT GG |
| Exon 9 | 5´ CAC TTT CCA TCT TCC AGG T | 5´ AAG TTC AGT CAG TTA CCA TC |
| Exon 10 | 5´ TGA AGG TGG TCT GTA TAA CT | 5´ AGA AAT ACA TGT GAA ACT CAC |
| Exon 11 | 5´ AAG TCC TGA CTC TCT TCT C | 5´ AAA AGT GCT TCT GAA ATG ATG |
| Exon 12 | 5´ CCT GAT GGG TTC TTG TTT C | 5´ AAC TTT CAC TTT CAG ACC TC |
| Exon 13 | 5´ GGA CAG TGA TAA CCA TCT CC | 5´ AGT GGT ATA TGG GGT GAA TAG |
| Exon 14 | 5´ AAT AAC ATG GTG TGT CAG CTC | 5´ CTA GAG AAC CTC AAA CTC TTC |
| Exon 15 | 5´ CCA TTT TCT TGT CTG ATT TTC | 5´ CTT TCC TTA AGA AGA TAC TTC |
| Exon 16 | 5´ CTC ACT GGC CAT TTT ATT AC | 5´ AAC CAA CTC TGG TCT CAT G |
| Exon 17 | 5´ TTA TTT GCT CTC CCT TGT TG | 5´ CTA AGA AAT CAA AAG GCA AAC |
| Exon 18 | 5´ GAA TCT GAA TAG GTT GTT TTC | 5´ GGC TGA AAG AAT TAC CCT C |
| Exon 19 | 5´ GAC TTG GCA ATA ACT CAG G | 5´ GGA AGG TGA GAA AAT GCT G |
| Exon 20 | 5´ ACA TTA TGC ATG TCT TCA TTG | 5´ ATG AGG CAG CTG TGT TTT G |
| Exon 21 | 5´ TAC CAC ACA TCT CTT GAT TC | 5´ CAG TGC AGG TCA GAT GAC |
| Exon 22 | 5´ ATT GGT GCC AAC TGA TTT TC | 5´ ACT TTG GAA GTG CTC ACA C |
| Exon 23 | 5´ AGT ACC ATT CAC AAC TAT TTC | 5´ ATC CAT GTA TTT ATT GAC TGG |
| Exon 24 | 5´ TTA CCT TAA AAA TCT CCT GTC | 5´ AAG AAG CCT TGC TGC TTT C |
| Exon 25 | 5´ TGA CTG TGG GGA TGT TAT C | 5´ TGC ACC CTT TAC CTG AGC |
| Exon 26a | 5´ ACA TAT GAC CAC AAG AAT ACG | 5´ TGA ACC TTA GCA ACA CTG TC |
| Exon 26b | 5´ ACA TCT ATG CCA TCT CTT CTG | 5´ ATC AAT AGC CTC AAT GTG TTG |
| Exon 26c | 5´ AAG AGA CAC ATA CAG AAT ATA G | 5´ ACA AAG TCA ATT GTA AAG GAA G |
| Exon 26d | 5´ GTT TTC CAC ACC AGA ATT TAC | 5´ TAT ACT GAT TGA ACC TAG CAC |
| Exon 26e | 5´ TAA CTA TGC ACT GTT TCT GAG | 5´ GAG TAC AGC ATT GAA GAA TTG |
| Exon 26f | 5´ AGT CAA AAC CTA CTG TCT CTT | 5´ TCA TAT TCT AGG AAC TGT ACG |

| | | |
|---|---|---|
| Exon 27 | 5´ ACA AAA TCT CTC CTA TAC AG | 5´ TCA ATA AAA GCT CCA TAC TG |
| Exon 28 | 5´ TCT GTT TTT CTG CTT TCA GG | 5´ CAT TAG GTG GTA TTT ACC TG |
| Exon 29a | 5´ TCC TCT CCA GAT AAA AAA CTC | 5´ TCT GAA CCA TTA TGG ACT TTC |
| Exon 29b | 5´ ATG TTC ATA AGG GAG GTA GG | 5´ TTC CTG AAT ATT TCT GTG CAG |
| Exon 29c | 5´ TAC TTC CCA ACT CTC AAG TC | 5´ TAT GAT ACA CAA TAA AGA CTC C |

Sequence data from Ludwig et al. 1987 (118).

TABLE 9. Characteristics of PCR-amplified fragments of the apoB gene, restriction enzymes used, and sizes of digestion products screened with SSCP.

| Fragment* | Nucleotide position in cDNA † | Amino acids | Product size (bp) # | Restriction enzymes | Cutting sites (cDNA) | Digestion product size (bp) |
|---|---|---|---|---|---|---|
| 2 | 211-249 | 1-14 | 80 | - | - | - |
| 3 | 250-365 | 14-52 | 170 | - | - | - |
| 4 | 366-511 | 53-101 | 207 | - | - | - |
| 5 | 512-665 | 101-152 | 211 | - | - | - |
| 6 | 666-821 | 153-204 | 244 | - | - | - |
| 7 | 822-946 | 205-246 | 180 | - | - | - |
| 8 | 947-1032 | 246-275 | 170 | - | - | - |
| 9 | 1033-1252 | 275-348 | 293 | - | - | - |
| 10 | 1253-1480 | 348-424 | 277 | - | - | - |
| 11 | 1481-1598 | 424-463 | 191 | - | - | - |
| 12 | 1599-1745 | 464-512 | 217 | - | - | - |
| 13 | 1746-1957 | 513-583 | 278 | - | - | - |
| 14 | 1958-2195 | 583-662 | 311 | - | - | - |
| 15 | 2196-2372 | 663-721 | 239 | - | - | - |
| 16 | 2373-2564 | 722-785 | 267 | - | - | - |
| 17 | 2565-2732 | 786-841 | 227 | - | - | - |
| 18 | 2733-2944 | 842-912 | 258 | - | - | - |
| 19 | 2945-3127 | 912-973 | 264 | - | - | - |
| 20 | 3128-3249 | 973-1014 | 183 | - | - | - |
| 21 | 3250-3460 | 1014-1084 | 283 | - | - | - |
| 22 | 3461-3636 | 1084-1143 | 243 | - | - | - |
| 23 | 3637-3824 | 1143-1205 | 259 | - | - | - |
| 24 | 3825-3970 | 1206-1254 | 205 | - | - | - |
| 25 | 3971-4344 | 1254-1379 | 437 | - | - | - |
| 26a | 4359-5707 | 1384-1833 | 1348 | BanI | 4713 | 354 + 579 + 417 |
| | | | | HincII | 5291 | |
| 26b | 5641-7007 | 1811-2266 | 1366 | BanI | 6063 | 422 + 443 + 501 |
| | | | | EcoRI | 6506 | |
| 26c | 6936-8125 | 2243-2639 | 1190 | HindIII | 7335 | 400 + 577 + 213 |
| | | | | AvaII | 7912 | |
| 26d | 8060-9417 | 2617-3070 | 1358 | AseI | 8584 | 524 + 398 + 436 |
| | | | | EcoO109I | 8982 | |
| 26e | 9344-10540 | 3045-3444 | 1197 | EcoO109I | 9859 | 515 + 402 + 280 |
| | | | | PstI | 10261 | |
| 26f | 10474-11908 | 3422-3900 | 1435 | EcoO109I | 10850 | 377 + 541 + 518 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | BglII | 11390 | |
| 27 | 11916-12031 | 3903-3941 | 153 | - | - | - |
| 28 | 12032-12215 | 3941-4003 | 220 | - | - | - |
| 29a | 12216-12847 | 4003-4213 | 632 | PstI | 12454 | 239 + 393 |
| 29b | 12783-13478 | 4192-4423 | 696 | EcoO109I | 13028 | 246 + 450 |
| 29c | 13424-14121 | 4405-4536 | 698 | DraI | 13826 | 403 + 295 |

* Fragment numbering refers to apoB gene exons.
† Nucleotide numbering according to Knott et al., 1986 (116).
# Exons 2 to 25, 27 and 28 amplified with intronic primers flanking the exons (118).

TABLE 10. Oligonucleotides used as primers in solid-phase sequencing and minisequencing reactions, and in apoB ins/del and 3´VNTR genotyping.

| Fragment | Upstream (sense) | Downstream (antisense) |
|---|---|---|
| 15 | 5´ CCA TTT TCT TGT CTG ATT TTC | 5´ CTT TCC TTA AGA AGA TAC TTC (b)* |
| 18 | 5´ GAA TCT GAA TAG GTT GTT TTC | 5´ GGC TGA AAG AAT TAC CCT C (b) |
| 26b/422 † | 5´ CCT ACC AAA ATA ATG AAA TAA AAC (b) | 5´ TCT TGA GTT TCC AGG TGC CT |
| 26b/1887/1896 # | 5´ TGC ATT TCA GCA ATG TCT TCC (b) | 5´ TGG AGC CTT TGT AAT CAT GTG |
| 1887 D § | 5´ CCC AGA GAG CGA GTT TCC CA | |
| 1896 D | 5 TGC TAT ACA GCT GCC CAG TA | |
| 26b/501 | 5´ GGA AAA CTC CCA CAG CAA G | 5´ ATC AAT AGC CTC AAT GTG TTG (b) |
| 26c/400 | 5´ AAG AGA CAC ATA CAG AAT ATA G | 5´ TCT TGA CAG CAT CAT CAA TAA A (b) |
| 27 | 5´ ACA AAA TCT CTC CTA TAC AG (b) | 5´ TCA ATA AAA GCT CCA TAC TG |
| 29b/246 | 5´ ATG TTC ATA AGG GAG GTA GG | 5´ TCT ATT AGT TGG AAA ATG AAT TG (b) |
| 29b/4243 # | 5´ CTA GGG AGG AAC TTT GCA C (b) | 5´ TCT ATT AGT TGG AAA ATG AAT TG |
| 4243D | 5´ GAT AAA TCT TTC AAC AGT TCC | |
| 29c/403 | 5´ ATA TTG TCA GTG CCT CTA AC | 5´ GTG AAA GTT CAA TTG GAA AAG (b) |
| 29c/4454 # | 5´ ATA TTG TCA GTG CCT CTA AC (b) | 5´ TGG TCT GAA AAA TCT TGC AG |
| 4454D | 5´ TAT TTT CTT CGT CGC AAT GG | |
| ins/del | 5´ CAG CTG GCG ATG GAC CCG CCG A | 5´ ACC GGC CCT GGC GCC CGC CAG CA |
| 3´VNTR | 5´ ATG GAA ACG GAG AAA TTA TG | 5´ CCT TCT CAC TTG GCA ATA AC |

Sequence data from Ludwig et al. 1987 (118).
* b = biotinylated.
† 26b/422 denotes the 422 bp digestion product of fragment 26b.
# Primers used to produce template for minisequencing of the apoB 1887, 1896, 4243 and 4454 polymorphisms.
§ D = detection step primer.

TABLE 11. PCR-RFLP analyses of apoB polymorphisms studied.

| ApoB polymorphism | SSCP fragment | Nucleotide position in cDNA* | Product size (bp) † | Restriction enzyme | Cutting site (cDNA) | Digestion product sizes when site present (bp) # |
|---|---|---|---|---|---|---|
| Thr71→Ile | 4 | 366-511 | 207 | ApaLI | 416 | 82 + 124 |
| Ala591→Val | 14 | 1958-2195 | 311 | AluI | 1980 | **49** + **201** + 43 |
| Val703→Ile | 15 | 2196-2372 | 239 | Alw26I | 2321 | 155 + 84 |
| Asn1887→Ser | 26b | 5641-6081 | 441 | BsrDI | 5873 | 173 + **60** + **208** |
| His1896→Arg | 26b | 5641-6081 | 441 | RsaI | 5897 | 256 + 185 |
| Thr2488→Thr | 26c | 6936-8125 | 1190 | XbaI | 7682 | 747 + 443 |
| Pro2712→Leu | 26d | 8060-9417 | 1358 | BfaI | 8343 | 176 + **108** + **488** + 570 |
| Arg3611→Gln | 26f | 10474-11908 | 1435 | MspI | 11039 | 566 + 869 |
| Lys4154→Glu | 29a | 12216-12847 | 632 | EcoRI | 12669 | 453 + 178 |

* Nucleotide numbering according to Knott et. al. 1986 (116).
† Exons 4, 14 and 15 amplified with intronic primers flanking the exons.
# Amplified segment contains both variant and invariant cutting sites. Bolded figures denote digestion products involved in the polymorphic variation.

TABLE 12. Serum lipid and lipoprotein levels in Group 4 men with and without the apoB Asn1887→Ser DNA change during dietary intervention.

| | Asn/Asn (n=44) | Asn/Ser (n=3) | P |
|---|---|---|---|
| Tot-chol (mmol/l) | | | |
| B | 6.57 ± 1.22 | 6.19 ± 0.80 | 0.5924 |
| I | 5.20 ± 0.85 | 5.29 ± 0.30 | 0.8608 |
| S | 6.30 ± 0.96 | 5.98 ± 1.39 | 0.5931 |
| LDL-chol (mmol/l) | | | |
| B | 4.90 ± 1.14 | 4.68 ± 0.64 | 0.7371 |
| I | 3.80 ± 0.81 | 3.94 ± 0.17 | 0.7728 |
| S | 4.64 ± 0.95 | 4.47 ± 1.25 | 0.7772 |
| Tot-tg (mmol/l) | | | |
| B | 1.43 ± 1.10 | 1.30 ± 0.38 | 0.8345 |
| I | 1.25 ± 0.64 | 1.14 ± 0.16 | 0.7742 |
| S | 1.30 ± 0.81 | 0.97 ± 0.22 | 0.4876 |
| HDL-chol (mmol/l) | | | |
| B | 1.38 ± 0.34 | 1.25 ± 0.31 | 0.5093 |
| I | 1.16 ± 0.26 | 1.13 ± 0.25 | 0.8601 |
| S | 1.40 ± 0.31 | 1.32 ± 0.30 | 0.6479 |
| ApoB (mg/ml) | | | |
| B | 1.25 ± 0.27 | 1.34 ± 0.03 | 0.5457 |
| I | 1.09 ± 0.26 | 1.25 ± 0.21 | 0.3064 |
| S | 1.28 ± 0.26 | 1.36 ± 0.21 | 0.6010 |

B=baseline, I=intervention, S=switchback period.
Values expressed as mean ± S.D.

TABLE 13. Serum lipid levels in Group 7 men with angiographically detected CAD or with normal coronary arteries with or without the apoB Asn1887→Ser change.

| | CAD | | | Non-CAD | | |
|---|---|---|---|---|---|---|
| | Asn/Asn (n=150) | Asn/Ser (n=10) | P | Asn/Asn (n=52) | Asn/Ser (n=3) | P |
| Tot-chol (mmol/l) | 5.74 (±1.12) | 5.12 (±0.89) | 0.0861 | 5.09 (±0.91) | 5.17 (±1.64) | 0.8868 |
| LDL-chol (mmol/l) | 3.74 (±1.00) | 3.16 (±0.78) | 0.0767 | 3.04 (±0.94) | 3.01 (±1.23) | 0.9599 |
| Tot-tg (mmol/l) | 1.89 (±0.95) | 1.67 (±0.59) | 0.4689 | 1.66 (±0.94) | 0.93 (±0.26) | 0.2055 |
| HDL-chol (mmol/l) | 1.13 (±0.28) | 1.20 (±0.24) | 0.4805 | 1.30 (±0.38) | 1.73 (±0.49) | 0.0638 |
| ApoB (mg/ml) | 1.01 (±0.20) | 0.90 (±0.11) | 0.0658 | 0.89 (±0.21) | 0.89 (±0.15) | 0.9630 |

Values expressed as mean ± S.D.

TABLE 14. Serum lipid and lipoprotein levels in Group 4 men with and without the apoB His1896→Arg DNA change during dietary intervention.

| | His/His (n=37) | His/Arg (n=10) | P |
|---|---|---|---|
| Tot-chol (mmol/l) | | | |
| B | 6.52 ± 1.23 | 6.67 ± 1.10 | 0.7141 |
| I | 5.10 ± 0.77 | 5.62 ± 0.95 | 0.0792 |
| S | 6.31 ± 0.98 | 6.16 ± 1.00 | 0.6723 |
| LDL-chol (mmol/l) | | | |
| B | 4.84 ± 1.14 | 5.08 ± 1.02 | 0.5392 |
| I | 3.69 ± 0.74 | 4.24 ± 0.82 | 0.0494 |
| S | 4.64 ± 0.98 | 4.58 ± 0.95 | 0.8807 |
| Tot-tg (mmol/l) | | | |
| B | 1.42 ± 1.14 | 1.45 ± 0.80 | 0.9292 |
| I | 1.19 ± 0.57 | 1.44 ± 0.77 | 0.2604 |
| S | 1.26 ± 0.81 | 1.37 ± 0.74 | 0.6969 |
| HDL-chol (mmol/l) | | | |
| B | 1.40 ± 0.37 | 1.30 ± 0.18 | 0.4378 |
| I | 1.17 ± 0.28 | 1.09 ± 0.12 | 0.3857 |
| S | 1.43 ± 0.34 | 1.30 ± 0.16 | 0.2777 |
| ApoB (mg/ml) | | | |
| B | 1.13 ± 0.27 | 1.34 ± 0.24 | 0.2226 |
| I | 1.06 ± 0.26 | 1.22 ± 0.21 | 0.0814 |
| S | 1.29 ± 0.28 | 1.27 ± 0.19 | 0.8364 |

B=baseline, I=intervention, S=switchback period. Values expressed as mean ± S.D.

TABLE 15. Serum lipid and lipoprotein levels in Group 4 women with and without the apoB His1896→Arg DNA change during dietary intervention.

| | His/His (n=43) | His/Arg (n=10) | P |
|---|---|---|---|
| Tot-chol (mmol/l) | | | |
| B | 5.89 ± 1.12 | 5.78 ± 0.45 | 0.7527 |
| I | 4.88 ± 1.03 | 4.61 ± 0.84 | 0.4512 |
| S | 5.79 ± 1.20 | 5.65 ± 0.84 | 0.6945 |
| LDL-chol (mmol/l) | | | |
| B | 4.20 ± 1.05 | 4.04 ± 0.43 | 0.6195 |
| I | 3.41 ± 0.91 | 3.12 ± 0.74 | 0.3455 |
| S | 4.04 ± 1.05 | 3.90 ± 0.78 | 0.9398 |
| Tot-tg (mmol/l) | | | |
| B | 0.94 ± 0.27 | 0.90 ± 0.30 | 0.7308 |
| I | 0.98 ± 0.38 | 0.91 ± 0.38 | 0.5829 |
| S | 0.99 ± 0.44 | 0.98 ± 0.45 | 0.9398 |
| HDL-chol (mmol/l) | | | |
| B | 1.50 ± 0.34 | 1.56 ± 0.17 | 0.5965 |
| I | 1.27 ± 0.32 | 1.31 ± 0.13 | 0.6864 |
| S | 1.55 ± 0.32 | 1.55 ± 0.14 | 0.9710 |
| ApoB (mg/ml) | | | |
| B | 1.11 ± 0.30 | 1.11 ± 0.27 | 0.8132 |
| I | 0.95 ± 0.26 | 0.94 ± 0.38 | 0.9686 |
| S | 1.12 ± 0.29 | 1.07 ± 0.22 | 0.6056 |

B=baseline, I=intervention, S=switchback period. Values expressed as mean ± S.D.

TABLE 16. Serum lipid levels in Group 7 men with angiographically detected CAD or with normal coronary arteries, and in healthy control men (Group 8) with or without the apoB His1896→Arg change.

| | CAD | | | Non-CAD | | | Controls | | |
|---|---|---|---|---|---|---|---|---|---|
| | His/His (n=151) | His/Arg (n=11) | P | His/His (n=48) | His/Arg (n=7) | P | His/His (n=40) | His/Arg (n=5) | P |
| Tot-chol (mmol/l) | 5.73 (±1.13) | 5.46 (±0.88) | 0.4385 | 5.00 (±0.91) | 5.71 (±0.98) | 0.0610 | 5.88 (±0.93) | 6.21 (±0.27) | 0.4258 |
| LDL-chol mmol/l) | 3.73 (±1.01) | 3.43 (±0.92) | 0.3600 | 2.94 (±0.89) | 3.72 (±1.10) | 0.0400 | 4.04 (±0.87) | 4.41 (±0.41) | 0.3522 |
| Tot-tg (mmol/l) | 1.85 (±0.90) | 2.18 (±1.21) | 0.2622 | 1.62 (±0.97) | 1.40 (±0.56) | 0.5471 | 1.12 (±0.36) | 1.21 (±0.37) | 0.6405 |
| HDL-chol (mmol/l) | 1.13 (±0.28) | 1.17 (±0.31) | 0.6876 | 1.32 (±0.37) | 1.36 (±0.52) | 0.7583 | 1.54 (±0.40) | 1.58 (±0.42) | 0.8328 |
| ApoB (mg/100ml) | 1.01 (±0.19) | 0.99 (±0.31) | 0.7117 | 0.88 (±0.20) | 0.96 (±0.24) | 0.3420 | 0.97 (±0.20) | 1.01 (±0.12) | 0.6294 |

Values expressed as mean ± S.D.

TABLE 17. Serum lipid levels in Group 7 women with angiographically detected CAD or with normal coronary arteries, and in healthy control women (Group 8) with or without the apoB His1896→Arg change.

| | CAD | | | Non-CAD | | | Controls | | |
|---|---|---|---|---|---|---|---|---|---|
| | His/His (n=40) | His/Arg (n=3) | P | His/His (n=58) | His/Arg (n=7) | P | His/His (n=23) | His/Arg (n=4) | P |
| Tot-chol (mmol/l) | 6.03 (±1.32) | 5.49 (±0.58) | 0.4927 | 5.15 (±1.07) | 5.36 (±0.80) | 0.6157 | 5.85 (±1.20) | 5.70 (±1.35) | 0.8318 |
| LDL-chol (mmol/l) | 3.94 (±1.29) | 3.42 (±0.59) | 0.5010 | 3.06 (±0.96) | 3.13 (±0.78) | 0.8390 | 3.93 (±1.15) | 3.85 (±1.04) | 0.8911 |
| Tot-tg (mmol/l) | 1.95 (±0.91) | 1.78 (±0.75) | 0.7450 | 1.33 (±0.53) | 1.48 (±0.77) | 0.4961 | 1.16 (±0.34) | 1.02 (±0.30) | 0.4547 |
| HDL-chol (mmol/l) | 1.20 (±0.31) | 1.26 (±0.31) | 0.7519 | 1.47 (±0.44) | 1.55 (±0.28) | 0.6457 | 1.68 (±0.30) | 1.70 (±0.46) | 0.8862 |
| ApoB (mg/ml) | 1.10 (±0.38) | 0.95 (±0.10) | 0.5094 | 0.85 (±0.18) | 0.84 (±0.19) | 0.8006 | 0.93 (±0.22) | 0.84 (±0.23) | 0.4896 |

Values expressed as mean ± S.D.

TABLE 18. Serum lipid and lipoprotein levels in Group 4 subjects with and without the apoB Arg4243→Thr or Ala4454→Thr DNA changes during dietary intervention.

| | 4243 Arg/Arg (n=95) | 4243 Arg/Thr (n=7) | P | 4454 Ala/Ala (n=98) | 4454 Ala/Thr (n=4) | P |
|---|---|---|---|---|---|---|
| Tot-chol (mmol/l) | | | | | | |
| B | 6.16 ± 1.12 | 6.72 ± 1.58 | 0.2411 | 6.20 ± 1.15 | 5.97 ± 1.31 | 0.6941 |
| I | 5.00 ± 0.92 | 5.26 ± 1.13 | 0.5010 | 5.01 ± 0.93 | 4.98 ± 0.97 | 0.9435 |
| S | 6.02 ± 1.09 | 6.03 ± 1.14 | 0.9815 | 6.02 ± 1.09 | 6.03 ± 1.02 | 0.9799 |
| LDL-chol (mmol/l) | | | | | | |
| B | 4.47 ± 1.05 | 5.09 ± 1.46 | 0.1771 | 4.52 ± 1.07 | 4.30 ± 1.50 | 0.6918 |
| I | 3.55 ± 0.85 | 3.88 ± 1.05 | 0.3661 | 3.57 ± 0.86 | 3.55 ± 1.03 | 0.9605 |
| S | 4.31 ± 1.02 | 4.36 ± 1.08 | 0.8860 | 4.31 ± 1.02 | 4.26 ± 1.19 | 0.9278 |
| Tot-tg (mmol/l)* | | | | | | |
| B | 1.15 ± 0.77 | 1.42 ± 1.22 | 0.5858 | 1.18 ± 0.80 | 0.77 ± 0.34 | 0.1891 |
| I | 1.08 ± 0.51 | 1.34 ± 0.72 | 0.2826 | 1.11 ± 0.52 | 0.72 ± 0.34 | 0.1030 |
| S | 1.11 ± 0.60 | 1.32 ± 1.09 | 0.6366 | 1.14 ± 0.65 | 0.80 ± 0.37 | 0.2631 |
| HDL-chol (mmol/l) | | | | | | |
| B | 1.45 ± 0.33 | 1.35 ± 0.41 | 0.4629 | 1.44 ± 0.33 | 1.52 ± 0.30 | 0.6714 |
| I | 1.23 ± 0.28 | 1.11 ± 0.26 | 0.3251 | 1.22 ± 0.28 | 1.29 ± 0.15 | 0.6374 |
| S | 1.49 ± 0.31 | 1.40 ± 0.34 | 0.4643 | 1.48 ± 0.31 | 1.60 ± 0.34 | 0.4136 |
| ApoB (mg/ml) | | | | | | |
| B | 1.16 ± 0.29 | 1.25 ± 0.26 | 0.4490 | 1.17 ± 0.28 | 1.07 ± 0.46 | 0.5069 |
| I | 1.01 ± 0.28 | 1.08 ± 0.32 | 0.5436 | 1.02 ± 0.28 | 0.95 ± 0.28 | 0.6022 |
| S | 1.19 ± 0.28 | 1.20 ± 0.28 | 0.9391 | 1.20 ± 0.28 | 1.16 ± 0.34 | 0.8186 |

B=baseline, I=intervention, S=switchback period. Values expressed as mean ± S.D.
* Statistical calculations were made using log-normalized values.

TABLE 19. Serum lipid and lipoprotein levels in Kuopio district hyperlipidemics (Group 1), the random sample of healthy subjects (Group 5), and drug intervention participants (Group 6, baseline levels) with and without the apoB Arg4243→Thr or Ala4454→Thr changes.

| | Genotype | Kuopio (n) | Random sample (n) | Drug intervention (n) |
|---|---|---|---|---|
| Tot-chol | Arg/Arg | 8.32±1.07 (81) | 5.10±0.90 (83) | 9.0±1.7 (192) |
| | Arg/Thr | 7.82±0.56 (3) | 5.43±1.00 (5) | 9.4±1.9 (19) |
| | | P=0.4293 | P=0.4374 | P=0.2938 |
| | Ala/Ala | 8.27±1.07 (83) | 5.09±0.90 (85) | 9.0±1.7 (211) |
| | Ala/Thr | 8.78±0.43 (4) | 5.94±0.56 (3) | 9.1±1.9 (9) |
| | | P=0.3467 | P=0.1104 | P=0.7774 |
| LDL-chol | Arg/Arg | 5.82±0.94 (81) | 2.84±0.88 (83) | 6.8±1.7 (192) |
| | Arg/Thr | 5.48±0.88 (3) | 3.16±1.03 (5) | 7.2±2.1 (19) |
| | | P=0.5349 | P=0.4382 | P=0.2979 |
| | Ala/Ala | 5.79±0.94 (83) | 2.82±0.87 (85) | 6.8±1.8 (211) |
| | Ala/Thr | 6.27±0.31 (4) | 3.99±0.66 (3) | 7.0±2.0 (9) |
| | | P=0.3184 | P=0.0227 | P=0.7140 |
| Tot-tg* | Arg/Arg | 2.40±1.72 (81) | 1.02±0.64 (83) | 2.08±1.09 (192) |
| | Arg/Thr | 2.62±0.83 (3) | 1.67±1.03 (5) | 1.93±0.79 (19) |
| | | P=0.5765 | P=0.2062 † | P=0.5676 |
| | Ala/Ala | 2.42±1.70 (83) | 1.05±0.68 (85) | 2.07±1.06 (211) |
| | Ala/Thr | 1.70±1.05 (4) | 1.35±0.60 (3) | 1.79±0.65 (9) |
| | | P=0.3459 | P=0.3478 | P=0.4127 |
| HDL-chol | Arg/Arg | 1.41±0.48 (81) | 1.78±0.50 (83) | 1.26±0.30 (192) |
| | Arg/Thr | 1.17±0.26 (3) | 1.52±0.37 (5) | 1.31±0.39 (19) |
| | | P=0.3817 | P=0.2400 | P=0.4904 |
| | Ala/Ala | 1.39±0.46 (83) | 1.78±0.49 (85) | 1.27±0.31 (211) |
| | Ala/Thr | 1.74±0.62 (4) | 1.34±0.38 (3) | 1.33±0.52 (9) |
| | | P=0.1444 | P=0.1261 | P=0.5509 |
| ApoB | Arg/Arg | 1.56±0.34 (81) | 1.03±0.23 (83) | 1.74±0.37 (192) |
| | Arg/Thr | 1.53±0.20 (3) | 1.34±0.65 (5) | 1.78±0.44 (19) |
| | | P=0.9084 | P=0.3499 † | P=0.6824 |
| | Ala/Ala | 1.55±0.33 (83) | 1.04±0.27 (85) | 1.74±0.39 (211) |
| | Ala/Thr | 1.66±0.39 (4) | 1.24±0.04 (3) | 1.70±0.39 (9) |
| | | P=0.5063 | P=0.2291 | P=0.7690 |

Values expressed as mean ± S.D. Tot-chol, LDL-Chol, Tot-tg, and HDL-chol, mmol/l; and apoB, mg/ml.
* Statistical calculations were made using log-normalized values.
† Welch and Brown-Forsythe statistics.

TABLE 20. Effects of the apoB Val703→Ile polymorphism on serum lipid and lipoprotein concentrations in the hypercholesterolemic Kuopio district patients (Group 1), random sample of healthy subjects (Group 5), and diet study participants (Group 4, baseline levels).

| | | Hypercholesterolemic | | Normolipidemic groups | | | |
|---|---|---|---|---|---|---|---|
| | Genotype | Kuopio | n | Random healthy | n | Diet study | n |
| Tot-chol (mmol/l) | Val/Val | 8.27 ± 1.08 | 69 | 5.14 ± 0.95 | 75 | 6.23 ± 1.12 | 64 |
| | Val/Ile | 8.73 ± 1.14 | 6 | 4.92 ± 0.64 | 8 | 5.52 ± 1.04 | 5 |
| | | P=0.3249 | | P=0.5332 | | P=0.1771 | |
| LDL-chol (mmol/l) | Val/Val | 5.76 ± 0.91 | 69 | 2.86 ± 0.92 | 75 | 4.53 ± 1.07 | 64 |
| | Val/Ile | 6.46 ± 0.97 | 6 | 2.79 ± 0.45 | 8 | 3.94 ± 1.05 | 5 |
| | | P=0.0768 | | P=0.7075 † | | 0.2324 | |
| Tot-tg* (mmol/l) | Val/Val | 2.32 ± 1.76 | 69 | 1.09 ± 0.70 | 75 | 1.18 ± 0.87 | 64 |
| | Val/Ile | 2.36 ± 0.59 | 6 | 0.75 ± 0.19 | 8 | 0.91 ± 0.42 | 5 |
| | | P=0.5687 | | P=0.0140 † | | P=0.4106 | |
| HDL-chol (mmol/l) | Val/Val | 1.46 ± 0.50 | 69 | 1.76 ± 0.49 | 75 | 1.46 ± 0.32 | 64 |
| | Val/Ile | 1.20 ± 0.17 | 6 | 1.79 ± 0.32 | 8 | 1.40 ± 0.25 | 5 |
| | | P=0.2146 | | P=0.8810 | | P=0.6959 | |
| ApoB (mg/ml) | Val/Val | 1.51 ± 0.33 | 69 | 1.06 ± 0.28 | 75 | 1.19 ± 0.28 | 64 |
| | Val/Ile | 1.73 ± 0.27 | 6 | 0.94 ± 0.26 | 8 | 1.01 ± 0.30 | 5 |
| | | p=0.1444 | | p=0.2669 | | p=0.1550 | |

Values expressed as mean ± SD.
* Statistical calculations were made using log-normalized values.
† Welch and Brown-Forsythe statistics.

TABLE 21. Effects of the apoB ins/del, Thr71→Ile, and Ala591→Val polymorphisms on lipid and lipoprotein levels in severely hypertriglyceridemic subjects (Group 3).

| | Ins/Ins (n=40) | Ins/Del (n=29) | Del/Del (n=5) | Thr/Thr (n=39) | Thr/Ile (n=32) | Ile/Ile (n=5) | Val/Val (n=22) | Val/Ala (n=37) | Ala/Ala (n=17) |
|---|---|---|---|---|---|---|---|---|---|
| Tot-chol (mmol/l) | 7.41 (±1.92) | 7.72 (±2.79) P=0.8511 | 7.57 (±1.78) | 7.43 (±1.94) P=0.8553 | 7.54 (±2.70) | 8.03 (±1.52) P=0.6330 | 7.17 (±1.40) | 7.57 (±2.20) | 7.86 (±3.17) |
| LDL-chol mmol/l | 3.02 (±1.32) | 3.19 (±1.26) P=0.8448 | 2.98 (±1.05) | 2.99 (±1.32) P=0.4423 | 3.24 (±1.22) | 2.53 (±0.92) P=0.5609 | 2.84 (±1.31) | 3.11 (±1.29) | 3.26 (±1.14) |
| Tot-tg (mmol/l)* | 8.34 (±7.36) | 7.59 (±6.20) P=0.9552 | 8.28 (±7.13) | 8.47 (±7.41) P=0.4792 | 7.15 (±6.03) | 9.92 (±6.28) P=0.9369 | 8.06 (±6.49) | 8.23 (±7.22) | 7.47 (±6.39) |
| HDL-chol (mmol/l) | 0.97 (±0.36) | 0.92 (±0.25) P=0.6050 | 1.05 (±0.21) | 0.97 (±0.36) P=0.4535 | 0.90 (±0.24) | 1.05 (±0.21) P=0.3576 | 0.98 (±0.44) | 0.90 (±0.25) | 1.01 (±0.19) |
| ApoB (mg/ml) | 1.37 (±0.56) | 1.47 (±0.46) P=0.3399 | 1.71 (±0.33) | 1.37 (±0.57) P=0.2911 | 1.47 (±0.44) | 1.73 (±0.30) P=0.0372 | 1.20 (±0.44) | 1.54 (±0.57) | 1.51 (±0.33) |

Values expressed as mean ± S.D.
* Statistical calculations were made using log-normalized values.

TABLE 22. Effects of the apoB Thr71→Ile and Ala591→Val polymorphisms, alone or combined, on baseline lipid and lipoprotein levels in the diet study participants (Group 4).

| | Thr/Thr (n=57) | Thr/Ile (n=45) | Ile/Ile (n=5) | Val/Val (n=32) | Val/Ala (n=54) | Ala/Ala (n=21) | Val/Val Thr/Thr (n=27) | Ala+/Ile+ (n=45) |
|---|---|---|---|---|---|---|---|---|
| Tot-chol (mmol/l) | 6.29 (±1.05) | 6.04 (±1.24) | 6.60 (±1.44) | 6.11 (±1.00) | 6.18 (±1.19) | 6.38 (±1.28) | 6.16 (±1.03) | 6.12 (±1.30) |
| | | P=0.4083 | | | P=0.6942 | | P=0.9076 | |
| LDL-chol (mmol/l) | 4.59 (±1.10) | 4.40 (±1.09) | 4.77 (±0.96) | 4.46 (±1.02) | 4.49 (±1.14) | 4.68 (±1.08) | 4.50 (±1.05) | 4.46 (±1.10) |
| | | P=0.5830 | | | P=0.7445 | | P=0.8595 | |
| Tot-tg (mmol/l)* | 1.35 (±0.98) | 0.98 (±0.42) | 0.79 (±0.24) | 1.17 (±0.74) | 1.18 (±0.90) | 1.12 (±0.59) | 1.25 (±0.77) | 0.99 (±0.42) |
| | | P=0.0209 † | | | P=0.9856 | | P=0.0771 | |
| HDL-chol (mmol/l) | 1.43 (±0.32) | 1.45 (±0.31) | 1.67 (±0.54) | 1.42 (±0.25) | 1.46 (±0.34) | 1.48 (±0.40) | 1.41 (±0.26) | 1.47 (±0.35) |
| | | P=0.2737 | | | P=0.7751 | | P=0.4122 | |
| ApoB (mg/ml) | 1.22 (±0.29) | 1.09 (±0.27) | 1.20 (±0.26) | 1.15 (±0.27) | 1.17 (±0.31) | 1.18 (±0.25) | 1.17 (±0.26) | 1.12 (±0.27) |
| | | P=0.0759 | | | P=0.8856 | | P=0.3853 | |

Values expressed as mean ± S.D.
* Statistical calculations were made using log-normalized values.
† Welch and Brown-Forsythe statistics.

TABLE 23. Effects of the apoB Thr71→Ile and Ala591→Val polymorphisms, alone or combined, on lipid and lipoprotein levels in subjects undergoing coronary angiography (Group 7).

| | Thr/Thr (n=73) | Thr/Ile (n=73) | Ile/Ile (n=14) | Val/Val (n=38) | Val/Ala (n=74) | Ala/Ala (n=41) | Val/Val Thr/Thr (n=33) | Ala+/Ile+ (n=76) |
|---|---|---|---|---|---|---|---|---|
| Tot-chol (mmol/l) | 5.22 (±1.09) | 5.38 (±0.91) | 5.63 (±1.11) | 5.24 (±0.92) | 5.31 (±1.05) | 5.41 (±1.05) | 5.16 (±0.91) | 5.36 (±0.96) |
| | | P=0.3134 | | | P=0.7477 | | P=0.2910 | |
| LDL-chol (mmol/l) | 3.18 (±0.99) | 3.32 (±0.88) | 3.60 (±0.98) | 3.20 (±0.90) | 3.28 (±0.97) | 3.34 (±0.95) | 3.12 (±0.83) | 3.31 (±0.88) |
| | | P=0.2846 | | | P=0.8107 | | P=0.2963 | |
| Tot-tg (mmol/l)* | 1.87 (±1.14) | 1.60 (±0.84) | 1.79 (±0.82) | 1.95 (±1.19) | 1.63 (±0.93) | 1.74 (±0.89) | 2.04 (±1.25) | 1.67 (±0.88) |
| | | P=0.2910 | | | P=0.2583 | | P=0.0957 | |
| HDL-chol (mmol/l) | 1.21 (±0.37) | 1.32 (±0.41) | 1.22 (±0.24) | 1.21 (±0.35) | 1.29 (±0.38) | 1.29 (±0.42) | 1.16 (±0.30) | 1.30 (±0.39) |
| | | P=0.1851 | | | P=0.5500 | | P=0.0814 | |
| ApoB (mg/ml) | 0.94 (±0.20) | 0.94 (±0.22) | 0.99 (±0.32) | 0.94 (±0.20) | 0.93 (±0.19) | 0.97 (±0.30) | 0.95 (±0.20) | 0.95 (±0.25) |
| | | P=0.6873 | | | P=0.6714 | | P=0.9549 | |

Values expressed as mean ± S.D.
* Statistical calculations were made using log-normalized values.

**FIGURES**

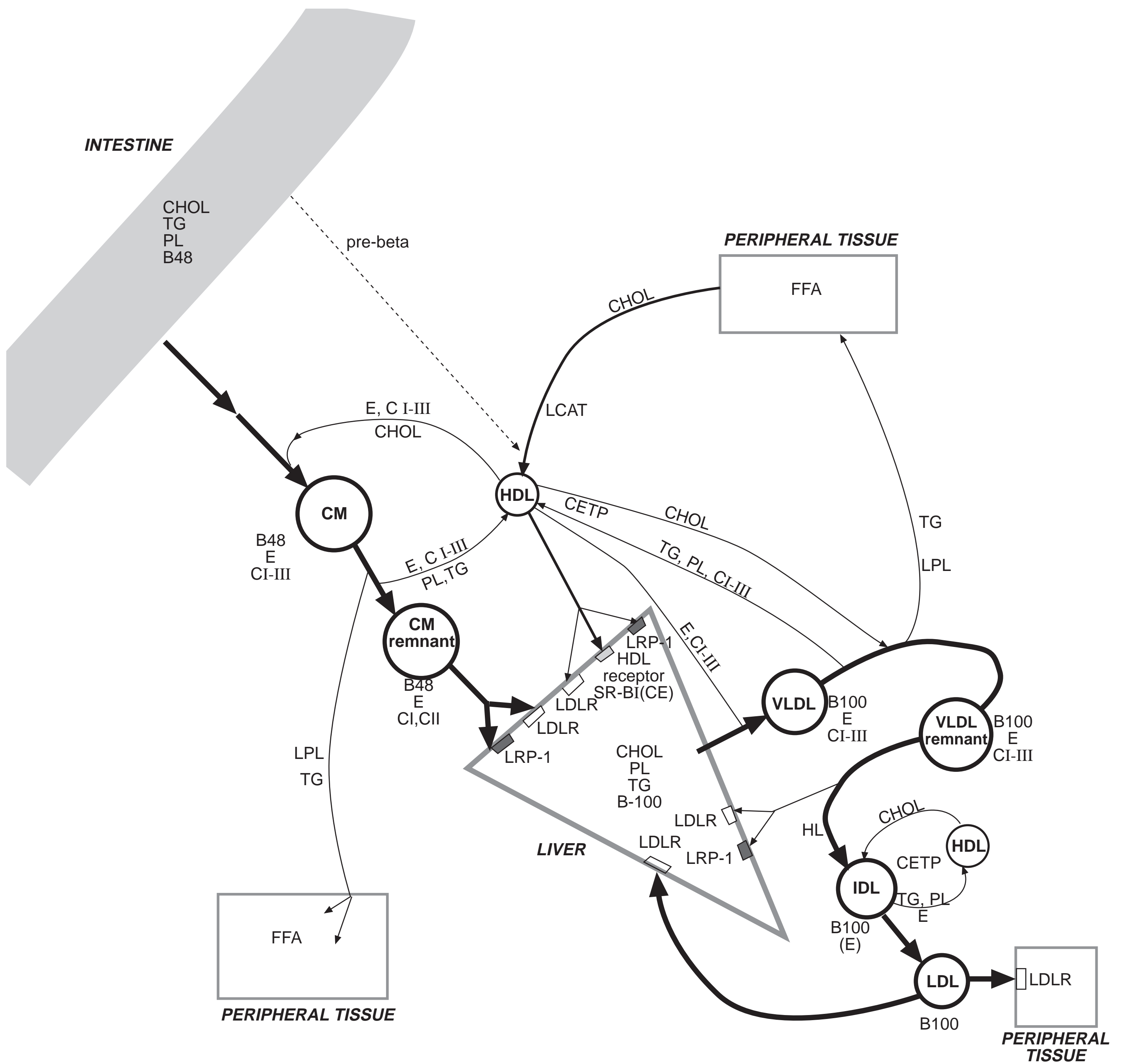



**Figure 1.** Schematic illustration of lipoprotein metabolism: endogenous and exogenous pathways: See chapter 2.1.3. for details.

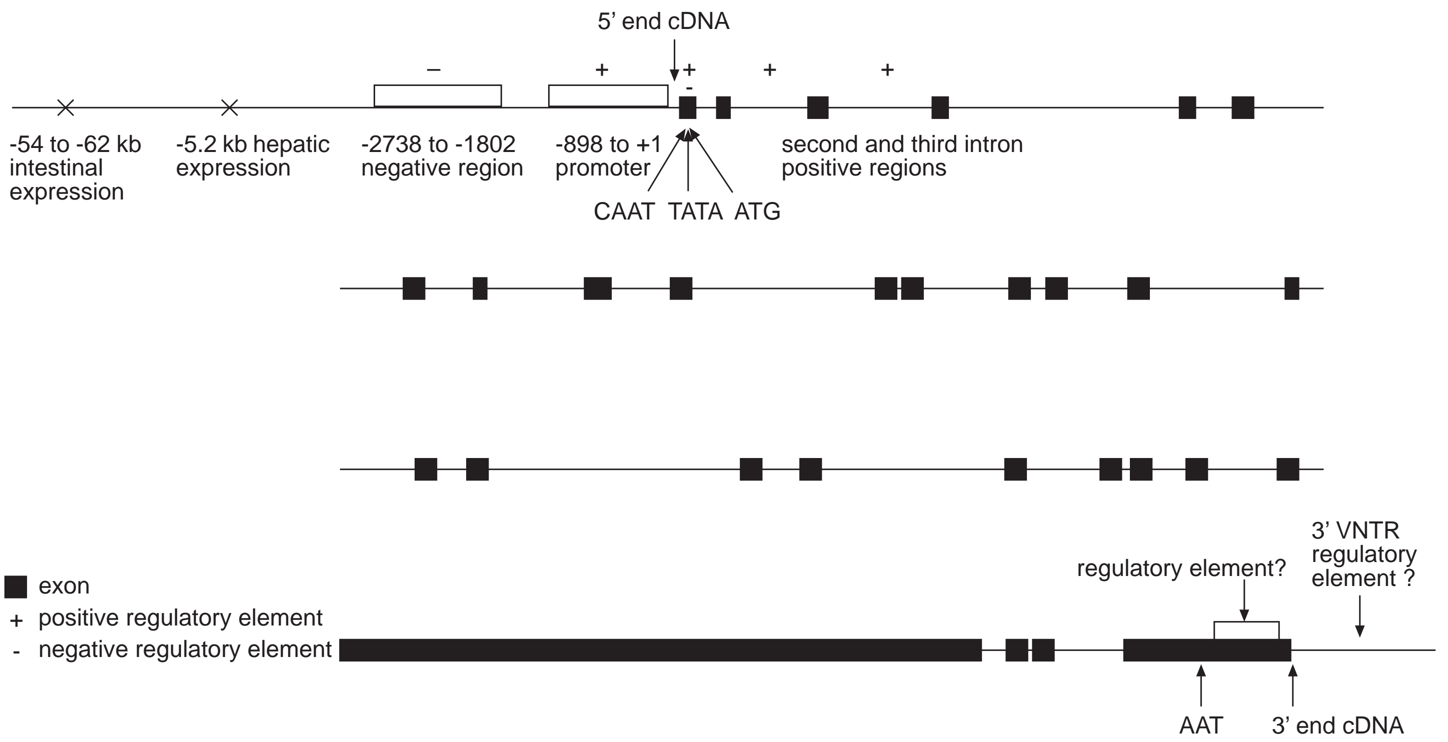
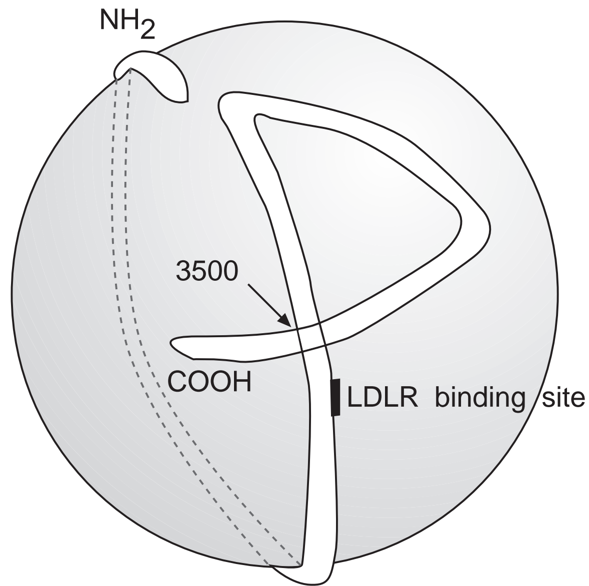



**Figure 2.** Schematic representation of the apoB gene and localization of its regulatory motifs.

**3a)**




**3b)**

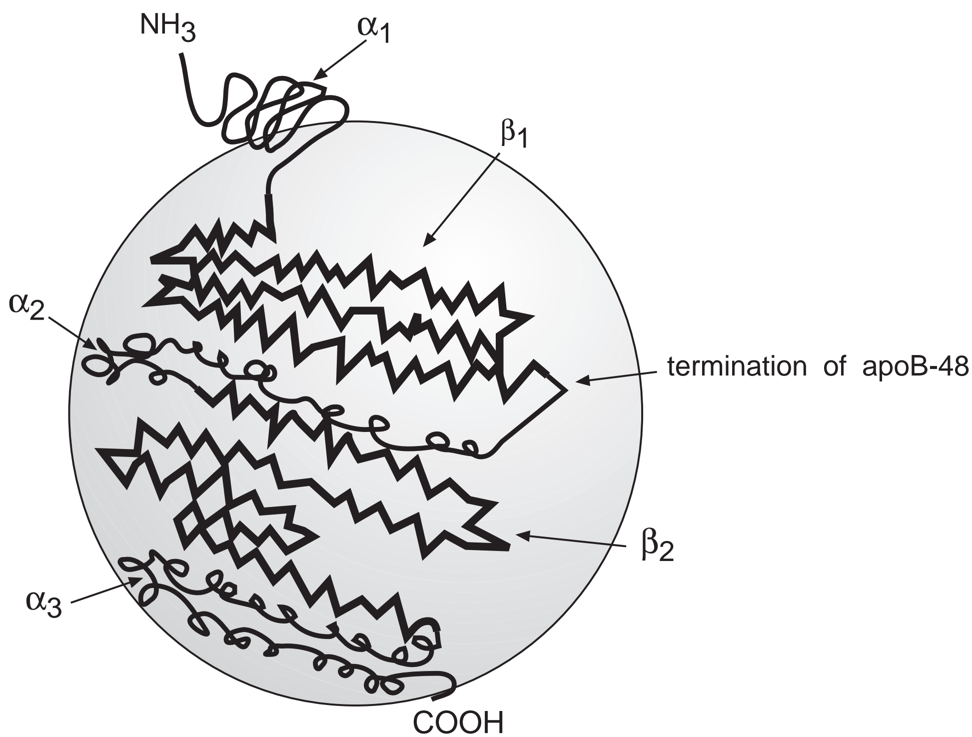



**Figure 3.** (a) Schematic illustration of the ribbon-and-bow model for the organization of apoB on lipoprotein particles. (b) Schematic diagram of the five-domain structure of apoB. $\alpha_1$, amphipathic helix cluster 1, $ß_1$ and $ß_2$, amphipathic ß-sheet clusters 1 and 2 (irreversible lipid binding domains), $\alpha_2$ and $\alpha_3$, amphipathic helix clusters 2 and 3 (reversible lipid binding domains).

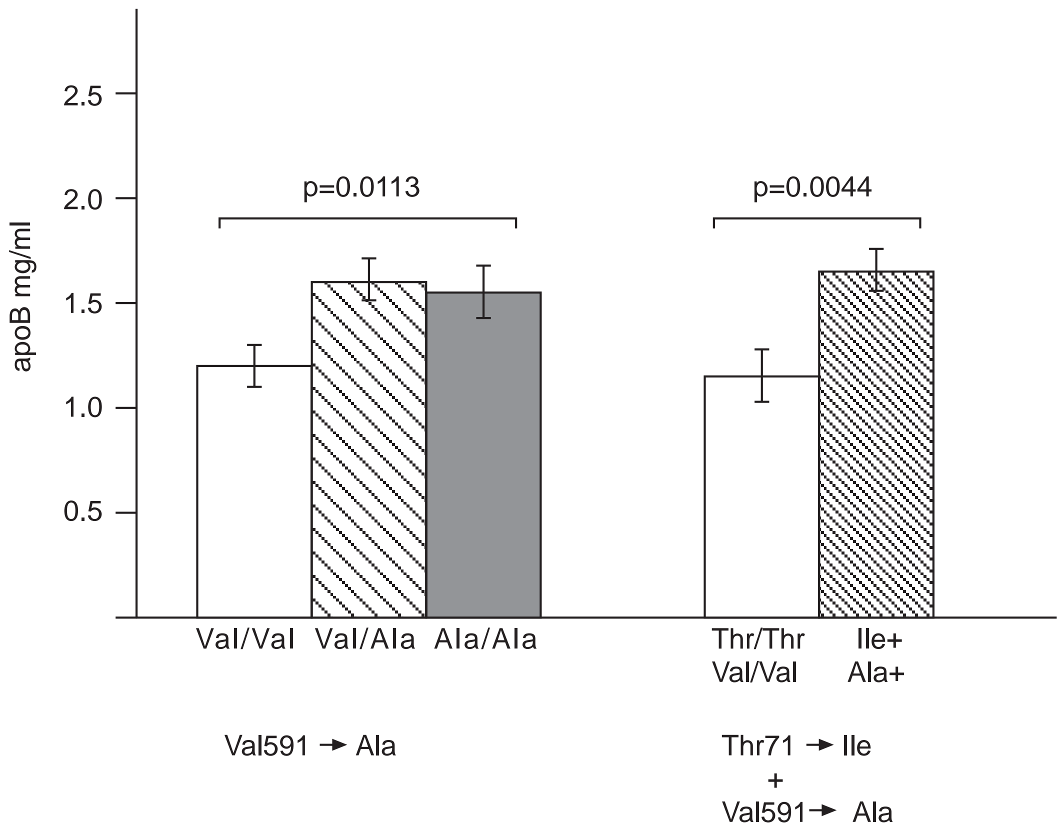



**Figure 4.** Effects of the apoB Val591 → Ala polymorphism alone, and the apoB Thr71 → Ile and Val591 → Ala polymorphisms combined on serum apoB levels in severely hypertriglyceridemic Group 3 patients.