# Association of genetic variations in apolipoprotein B with hypercholesterolemia, coronary artery disease, and receptor binding of low density lipoproteins

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**Abstract** To search for unique mutations in the apolipoprotein B (apoB) gene that disrupt the binding of LDL to its receptor and cause hypercholesterolemia, we examined more than 800 patients with high LDL cholesterol levels and/or coronary artery disease (CAD). Analysis of patient DNA by single-strand conformation polymorphism and allele-specific oligonucleotide hybridization of the sequence surrounding the putative receptor– binding domain of apoB (amino acid positions 2965 to 3534) revealed seven variations. LDL from heterozygotes with either Arg 3500 Gln or Arg 3531 Cys bound defectively with the LDL receptor in competitive binding assays. The Arg 3500 Gln substitution was statistically more prevalent in patients with hypercholesterolemia ($P = 0.0003$). Total cholesterol and LDL-cholesterol were significantly higher ($P < 0.0004$) in 34 apoB 3500 Gln carriers than in the controls. The allele encoding the Arg 3531 Cys substitution was more prevalent (0.8%) in the CAD group ($P = 0.05$) than in the controls. A Ser 3252 Gly variant was statistically more prevalent in the hypercholesterolemic group ($P = 0.03$), but LDL with this mutation had normal LDL receptor-binding activity. The other four variants identified (Leu 3350 Leu, Gln 3405 Glu, Val 3396 Met, and Ser 3455 Arg) were not associated with defective LDL-receptor binding, hypercholesterolemia, or CAD, nor were the apoB mutations associated with elevated lipid levels in family members. The surprising result that only two mutations of apoB in the receptor–binding domain (Arg 3500 Gln and Arg 3531 Cys) were associated with defective LDL binding, hypercholesterolemia, or CAD is in stark contrast with familial hypercholesterolemia, where nearly 150 mutations of the LDL receptor have been described that disrupt its function. This study strongly suggests that a limited number of mutations of apoB markedly influence LDL binding to its receptor.—**Ludwig, E. H., P. N. Hopkins, A. Allen, L. L. Wu, R. R. Williams, J. L. Anderson, R. H. Ward, J-M. Lalouel, and T. L. Innerarity.** Association of genetic variations in apolipoprotein B with hypercholesterolemia, coronary artery disease, and receptor binding of low density lipoproteins. *J. Lipid Res.* 1997. **38:** 1361–1373.

**Supplementary key words** mutations • variations • SSCP • coronary artery disease • genetic markers • Amish

Low density lipoprotein (LDL) particles contain a single apolipoprotein B (apoB-100) that is responsible for the binding and clearance of LDL by the LDL receptor (1). Because nearly two thirds of all circulating cholesterol is transported by the LDL particle, its interaction with the LDL receptor is critical in maintaining extracellular and intracellular cholesterol homeostasis (2). Therefore, genetic defects in either apoB-100 or the LDL receptor that diminish receptor-ligand interactions elevate plasma cholesterol levels. Familial hypercholesterolemia (FH) is caused by genetic defects in the LDL receptor and increases the risk of myocardial infarction (MI). However, genetic defects in the LDL receptor account for only 5% of coronary artery disease (CAD) before the age of 60 (2, 3). Therefore, attention

Abbreviations: apo, apolipoprotein; SSCP, single-strand conformation polymorphism; CAD, coronary artery disease; LDL, low density lipoprotein; FH, familial hypercholesterolemia; MI, myocardial infarction; CEPH, Centre d'Etudes de Polymorphisme Humaine; EDTA, ethylenediamine tetraacetic acid; PCR, polymerase chain reaction; VNTR, variable number tandem repeats; ASO, allele-specific oligonucleotides; RFLP, restriction fragment length polymorphism; ED, effective dose; TG, triglycerides; TC, total plasma cholesterol; VLDL, very low density lipoprotein; HDL, high density lipoprotein; apoB, apolipoprotein B; apoE, apolipoprotein E; HTN, hypertension; DM, diabetes mellitus.

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has been focused on other candidate genes that could influence hypercholesterolemia and CAD.

Several lines of evidence suggest that the apoB gene locus contributes significantly to the variability in plasma apoB-100 and LDL concentrations and susceptibility to CAD. Gavish, Brinton, and Breslow (4) reported that allele-specific products of the apoB gene cause variability in apoB-100 and LDL concentrations. These authors estimated that in the general population, allele-specific products of the apoB gene are responsible for 20% of the variability in apoB-100 and LDL concentrations. Using complex segregation analysis, Hasstedt, Wu, and Williams (5) ascribed 14% of the variance of LDL cholesterol concentration to the apoB locus. Similar studies by other investigators showed evidence of a single locus gene effect on apoB, LDL-apoB, or LDL cholesterol but could not definitively implicate the apoB gene as the causative locus (6–8). Using a polygenic model, Beaty et al. (9) identified the apoB locus as a cause of variance in apoB levels. In contrast, based on linkage and sibpair analysis of specific apoB polymorphisms, Coresh et al. (10) reported that the apoB gene is not the major locus influencing circulating apoB levels. By examining several genetic markers in the apoB gene, multiple additional studies have reported an association with hypercholesterolemia, CAD, or MI. However, most of the studies have not been reproduced, and there is no consensus on the association of different markers with CAD or any other cardiovascular endpoint (11–19).

One mechanism by which mutations in apoB influence the risk of heart disease is to disrupt the binding of LDL to its receptor. In subjects with familial defective apoB-100 (FDB), diminished binding of LDL to the LDL receptor results in hypercholesterolemia (20) and an increased risk for CAD and MI (21–23). This mutation has an estimated frequency of 1 in 500 to 700 in some populations (20). The substitution of a glutamine for arginine at residue 3500 (cDNA position 10708) in apoB-100 diminishes LDLs by over 90% (24). The substitution of cysteine for arginine at residue 3531 (cDNA position 10800) in apoB-100 has a lesser effect on receptor binding, occurs less frequently (estimated at 1 in 3000), and has not been shown to be associated with CAD (25). Certainly the prevalence of these two mutations in the general population does not account for the variance of apoB or LDL concentrations reported by Gavish et al. (4) or Hasstedt et al. (5). Furthermore, apoB has not been examined nearly as extensively as the LDL receptor for sequence changes that disrupt the LDL-receptor interaction (26).

The primary purpose of this investigation is to test the hypothesis that other variations are present in apoB that cause defective LDL-receptor binding and contribute significantly to the frequency of hypercholesterolemia and CAD. This study represents an extensive search for naturally occurring mutations in the region of the putative LDL receptor–binding domain of apoB in populations at elevated risk of heart disease.

## MATERIALS AND METHODS

### Patient selection

The study subjects were selected from the population of the Great Salt Lake Basin area, a population that is genetically representative of US and European Caucasians (27). The selection criteria ensured that each group was composed of unrelated patients. Cases included a hypercholesterolemic group (LDL cholesterol >90th percentile) and two CAD groups, one defined clinically and one defined angiographically. The control groups consisted of an age- and sex-matched angiographically defined control group and a random sample.

The *hypercholesterolemic group* ($n$ = 309) had LDL greater than the 90th percentile for their age and sex (28); 53 of these patients had FH. The clinically defined CAD group (n = 227) had undergone percutaneous transluminal coronary angioplasty or coronary artery bypass graft surgery. The angiographically defined CAD group (n = 411) had greater than 60% stenosis in a major coronary artery or its first division branches. The angiographically defined CAD control group (n = 383) had less than 10% stenosis in any major coronary artery or its first division branches (see Marshall et al. (19) for more details). Subjects in the latter two groups were Caucasian males and females under the age of 65 and 70, respectively, who had not had a myocardial infarction in the last 6 weeks before angiography.

Random subjects mainly from Utah (n = 280) were individuals who had entered a blood bank for blood donation (n = 130) or grandparents enrolled in the Centre d'Etudes de Polymorphisme Humaine (CEPH) Consortium (n = 150) (29). An additional 37 non-Utah CEPH grandparents were also screened.

### Blood collection, lipid analysis, and DNA purification

Blood samples were obtained from patients after an overnight fast; potassium ethylenediamine tetraacetic acid (EDTA) was used as the anticoagulant at a final concentration of 0.1%. The plasma was separated from the cells, and both plasma and cells were immediately frozen until lipid analysis could be conducted or DNA could be isolated. Lipid and apoB analysis were per-



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formed as previously described (30). Genomic DNA from leukocytes was extracted by established protocols (31).

### Detection of apoB variations by single-strand conformation polymorphism analysis and sequencing

Nucleotide variations in the vicinity of the putative LDL receptor–binding domains of apoB were detected by single-strand conformation polymorphism (SSCP) analysis of DNA fragments amplified by polymerase chain reaction (PCR) (32). Amplifications were performed on 100 ng of genomic DNA with 0.2 μl of $\alpha^{32}$P-dCTP in a final volume of 10 μl under standard conditions (33). The primer pairs for PCR amplification and SSCP analysis are shown in Fig. 1, and the primer sequences are listed in Table 1. Several of the primers for PCR-SSCP also had the priming sites for the universal (UP) and reverse primers (RP) needed for dye-primer sequencing on the 373A ABI sequencer. All amplifications were performed at 94°C, 1 min/55°C, 1 min/72°C, 1 min for 30 cycles. After PCR amplification, 40 μl of formamide loading dye (0.5% sodium dodecyl sulfate (SDS), 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol in formamide) were added, heat denatured at 90°C for 3 min, and chilled on ice before loading on gels. Three standard gel conditions were used for SSCP: *1*) MDE gel solution (Hydro-Link, AT Biochem) at room temperature with constant 8 watts for 18 h; *2*) MDE gel solution at 4°C with 12 watts for 18 h; and *3*) 5% polyacrylamide with 5% glycerol at room temperature and 8 watts for 20 h. After electrophoresis, the gels were dried onto Whatman chromatography 3M paper and exposed to autoradiographic film.

The autoradiographs were examined for band shifts, aligned to the dried gel, and used as a template to dissect the shifted band from the gel. The band was then eluted with water and reamplified with the appropriate PCR primers. Amplification products were purified with Centricon-100 filters (Amicon) and sequenced.

### Detection of apoB variations by allele-specific oligonucleotide hybridization

PCR reactions under standard conditions with PCR primer 1 and primer 2023 (Table 1) resulted in a 2023–base pair (bp) product (cDNA positions 9102 to 11124) surrounding the putative receptor-binding domains and the Arg 3500 Gln substitution (Fig. 1). Allele-specific oligonucleotide (ASO) probes (Table 1) were used to detect published sequence variations in the 2023-bp product, and the new variations were identified by SSCP and sequencing. After amplification, a solution containing 30 μl of 0.8 N NaOH/0.1 M EDTA/1% bromophenol blue was added to the 30-μl PCR reaction, mixed, incubated for 10 min, blotted in duplicate onto Hybond N+ nylon membranes (Amersham), and neutralized with 3 M sodium acetate. Membranes were subsequently hybridized with $^{32}$P-labeled probes in 7% polyethylene glycol, 0.1% SDS, and 50 mM sodium phosphate (pH 7.0) (PEG/Hyb) solution overnight at 40°C. The membranes were then washed twice at room temperature with 6× sodium succinyl citrate/0.1% SDS for 5 min and once at 45°C for 30 min. Autoradiographs of the dot blots were examined after an overnight exposure.

### Genotype, haplotype, and segregation

The genotypes of all probands and available family members were determined for three variable number tandem repeats (VNTR) in or near the apoB gene. These markers include a (TG)n short tandem repeat (STR) 3256 bases 5′ of the transcriptional start, a (TTTA)n STR present in intron 20 and a 15-base repeat 491 bases 3′ of the cDNA end. To determine the MB19 phenotype the *Apa* LI polymorphism in exon 4 was also genotyped in some patients. The protocols for PCR amplification and detection of the genotypes are detailed in Zuliani and Hobbs (34), Ludwig and McCarthy (35), Ludwig, Haubold, and McCarthy (36), and Young and Hubl (37). When family members of probands were available, haplotypes were determined to evaluate whether elevated lipids were associated with a particular apoB allele in a family.

### Lipoprotein isolation and fibroblast LDL receptor–binding studies

LDL receptor–binding studies were performed in control subjects and patients with apoB variations. The LDL (d 1.02–1.05 mg/ml) were isolated from plasma by sequential ultracentrifugation (38), recentrifuged at d 1.05 mg/ml, and dialyzed against 0.15 M NaCl, 0.01% EDTA. After protein determination (39), an aliquot of the LDL was submitted for lipid composition analysis.

Normal human fibroblasts were plated 7 days before the receptor-binding assays and incubated with 10% human lipoprotein-deficient serum 48 h before the cell culture binding experiment to up-regulate LDL as receptor expression. All LDL receptor–binding assays were conducted at 4°C described in detail elsewhere (40).

### Solid-phase competitive radioimmunoassay

The monoclonal antibody MB19 recognizes an epitope on apoB that corresponds to a common *Apa* LI restriction fragment length polymorphism (RFLP) in exon 4, which results in a threonine to isoleucine substitution at residue 71 (37). MB19 bound to LDL isolated from subjects with high, intermediate, and low affinity, in direct correlation with the frequency of the *Apa* LI



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RFLP genotype. A detailed description of the methods is provided in Young et al. (41) and Arnold et al. (42).

### Statistical analysis

Statistical analysis was performed with Statview statistical analysis software. Statistical significance for variation distributors was calculated by the Fisher exact probability test comparing the case groups to the combined control groups. Differences were considered significant at $P < 0.05$. Differences between means for lipids, body mass index, age, and LDL-receptor binding (ED[50%]) were evaluated by $t$ test.

## RESULTS

### ApoB variations

We examined the putative LDL receptor–binding domain of apoB (cDNA positions 9102 to 10828) by SSCP for unique variations. By amplifying overlapping fragments in apoB (Fig. 1), we restricted the PCR fragments to a size ranging from 186 to 360 bp. DNA corresponding to the putative LDL receptor–binding domain from more than 400 hypercholesterolemic or CAD patients was evaluated by SSCP. Seven variants were identified (**Fig. 1 and Fig. 2**). Sequencing of the variants revealed four previously undescribed unique substitutions: an A to G transition at cDNA position 9963 resulting in a Ser to Gly substitution at residue 3252; a G to A transition at position 10259 that does not change the Leu residue at residue 3350; a G to A transition at position 10397 resulting in a Val to Met substitution at residue 3396; and a C to A transversion at position 10574 resulting in a Ser to Arg substitution at position 3455. Three other variants identified at cDNA positions 10422 (Gln 3405 Glu), 10708 (Arg 3500 Gln), and 10800 (Arg 3531 Cys) have been reported (25,43,44). However, five other previously reported variations (Fig. 1 and ref. 43) were not found by SSCP in the patients screened. To determine whether we failed to detect these published sequence differences (Leu 3029 Leu, His 3212 Gln, Leu 3259 Ile, Asn 3292 His and Thr 3400 Lys) by SSCP, we designed ASO probes (**Table 1**) to detect four of the five variants (Leu 3029 Leu was not pursued) in all of the case and control samples. These four variants were not detected by ASO hybridization in any of the 1613 cases and controls.

### Distribution and frequency of apoB variants

ASO hybridization probes were used to determine the distribution and frequency of the variations identified by SSCP in all cases and controls. **Table 2** displays the distribution of these variations in the case control groups along with the probability of significance that the variation is more prevalent in the case group than in the combined control group. Of the six variants resulting in an amino acid change, the frequency of two

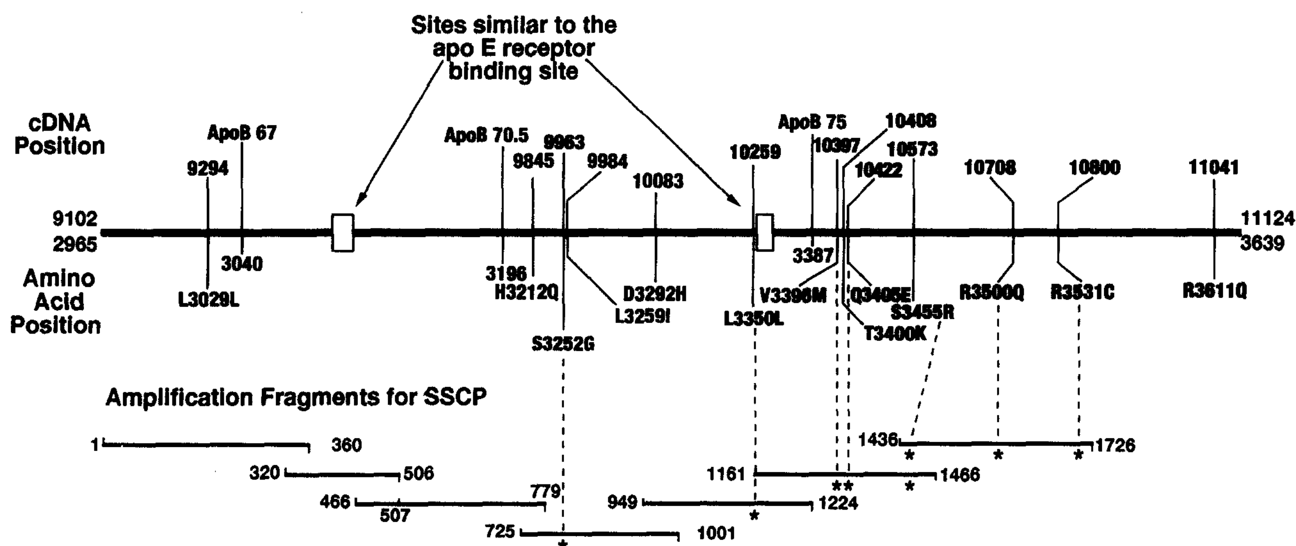


**Fig. 1.** Schematic of apolipoprotein B surrounding the putative LDL receptor–binding domain, and variations identified by SSCP. The heavy horizontal line represents the apoB sequence with cDNA and amino acid positions displayed above and below the line, respectively. The two sequences of apoB with motifs similar to the receptor–binding site of apoE are represented as open boxes. The cDNA and amino acid positions of variations identified in this study and previous studies are also presented. The lower portion of the figure displays the amplification size, amplification primers used (refer to Table 1), and range of amplification products. Each variant identified in this study is indicated by an asterisk (*).



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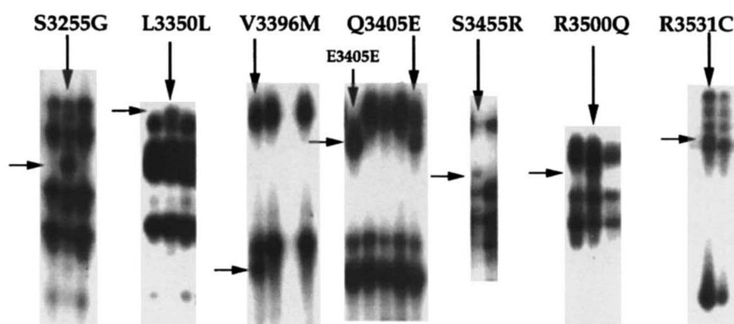


**Fig. 2.** Polyacrylamide gel electrophoresis of sequence variations by SSCP analysis. Apolipoprotein B variations were identified by amplification with internal radioactive dCTP; the amplified products were denatured by 70% formamide and elevated temperature and run on nondenaturing gels under several conditions. Patient DNA amplification products for each variation are identified by a vertical arrow, and the band shift by a horizontal arrow. Wild-type amplification products for the PCR products are in the adjacent lanes.

variants was significantly higher in one or more case groups than in controls. Of the five subjects with the Arg 3500 Gln mutation in the hypercholesterolemic groups ($P$ = 0.0003), three had been diagnosed with FH. Four subjects in the CAD group had the Arg 3500 Gln mutation. Four cases in the CAD group had Arg 3531 Cys; and five cases in the hypercholesterolemic group had Ser 3252 Gly ($P$ = 0.031). Neither the Val 3396 Met nor the Ser 3455 Arg variant was present in the control groups; however, only one proband was identified in all the case patients for each variant, and the frequency was not statistically different from that in the controls. The Gln 3405 Glu substitution has been previously identified as a sequence variation (43). However, no prior report has examined the significance of this variant in a case-control study or its biological significance. Twenty-one carriers and one homozygote were identified from all groups, ranging from 1.2–3.2% (Table 2); however, no significant difference was present between the case and control groups.

An additional 37 subjects from the CEPH consortium who were not from the Great Salt Lake Basin area and therefore not included as random controls were also screened by ASO hybridization. Two subjects in this group carried the Arg 3500 Gln substitution. Interestingly, these carriers were grandparents (one paternal and one maternal) of an extensive Amish family from Lancaster County, Pennsylvania, originally ascertained due to the prevalence of bipolar disease (45). Screening 20 family members in three generations identified seven carriers but no homozygotes. Hypercholesterolemia was also prevalent in this family but detailed lipid data and medical histories were not available or pursued at this time.

### Characteristics of patients with apoB variations

To determine the possible influence of apoB variations on plasma lipid concentrations, we determined the lipid concentration, apoB concentration, apoE phenotype, and additional clinical information for the carriers of apoB variations (**Table 3**).

The association of apoB variations and elevated lipids was evaluated by segregation analysis in families (**Fig. 3**). DNA from family members of two Ser 3252 Gly probands (kindred (K) 1044-003 and K1039-062) was available for genotyping. In addition to a Gly substitution at residue 3252, one family (K1039) also had Met 3396 Val on the same apoB allele. In both families, the variant allele was either not associated with lipids or cardiovascular disease or was uninformative because noncarriers were also hypercholesterolemic (Fig. 3). Two families with the Gln 3405 Glu variant were available (K726 and K1081) for evaluation of the segregation of the variant apoB allele with elevated lipids (Fig. 3). In addition to the 3405 Glu on one apoB allele, K1081 also had Ser 3455 Arg on the other apoB allele. However, segregation analysis of Ser 3455 Arg and Gln 3405 Glu in K1081 and of Gln 3405 Glu in K726 did not demonstrate an association with elevated lipids (Fig. 3).

The Arg 3500 Gln substitution is associated with hypercholesterolemia and heart disease (20–22). Family members from four of nine probands with this mutation were available for genetic screening, segregation analysis, and comparison of carrier and noncarrier characteristics. Elevated total cholesterol and LDL cholesterol levels were associated with the Arg 3500 Gln in three families, K27, K657, and K669; however, cholesterol values varied substantially in Arg 3500 Gln carriers in K5417. We also compared the difference of age, body mass index, total cholesterol, triglycerides, high density lipoprotein cholesterol, and LDL cholesterol by paired $t$ test in 34 carriers of the Arg 3500 Gln allele to 26 noncarriers (**Table 4**). Only the differences in total cholesterol and LDL cholesterol levels were significant ($P <$ 0.0001). In the CAD case group, four probands had Arg 3531 Cys, which has been reported to cause FDB (25). However, family members were not available for segregation analysis of the mutation with increased lipid concentrations.

### Haplotype of the Arg 3500 Gln and Arg 3531 Cys variations

The apoB genotypes of the subjects with the Arg 3500 Gln and Arg 3531 Cys mutations were determined with



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TABLE 1. Oligonucleotide sequences

| PCR and Sequence Primers | cDNA Position | | Sequence |
|---|---|---|---|
| 1 | 9102 | 9123 | 5′ gtc gat tcc cag cat gtg ggc c 3′ |
| UP-320[a] | 9461 | 9441 | 5′ cat aat gtt ctc gtt gtt tcc 3′ |
| 360 | 9483 | 9463 | 5′ cat aat gtt ctc gtt gtt tcc 3′ |
| 466 | 9567 | 9587 | 5′ ctg aaa gat ttc tct cta tgg 3′ |
| RP-506[a] | 9605 | 9588 | 5′ RP cct tca agc ctg ttt ttt c 3′ |
| UP-507 | 9606 | 9622 | 5′ UP gaa ttc ttg aaa acg ac 3′ |
| UP-725 | 9826 | 9843 | 5′ UP aca aag ctg aaa aat ctc 3′ |
| 779 | 9878 | 9859 | 5′ tat cca gga att tga aag gtc c 3′ |
| 949 | 10050 | 10070 | 5′ cat gtc cct aga aat ctc aag 3′ |
| RP-1001 | 10100 | 10083 | 5′ RP aca caa ttc ctt gaa atg 3′ |
| UP-1161 | 10262 | 10279 | 5′ UP gta caa att aga ggg cac 3′ |
| 1224 | 10323 | 10300 | 5′ cag agc tgt ggc taa ctt caa tcc cc 3′ |
| UP-1436 | 10537 | 10554 | 5′ UP act cta ccg cta aag gag 3′ |
| RP-1466 | 10566 | 10551 | 5′ RP tgt ggt caa ctg ctc c 3′ |
| RP-1726 | 10826 | 10811 | 5′ RP act gtg ctc cca gag g 3′ |
| 2023 | 11124 | 11101 | 5′ cta agg atc ctg caa tgt caa ggt 3′ |
| ASO primers | | | |
| ASO-H3212 | 9837 | 9853 | 5′ aaa tct cac gac gag ct 3′ |
| ASO-3212Q | 9837 | 9853 | 5′ aaa tct cag gac gag ct 3′ |
| ASO-S3252 | 9856 | 9871 | 5′ agc agt cag cat gcc t 3′ |
| ASO-3252G | 9970 | 9956 | 5′ ggc atg ccg act gct 3′ |
| ASO-L3259 | 9976 | 9992 | 5′ tct cca tcc tag gtt ct 3′ |
| ASO-3259I | 9976 | 9992 | 5′ tct cca tca tag gtt ct 3′ |
| ASO-N3292 | 10076 | 10092 | 5′ tct tcc aga ttt caa gg 3′ |
| ASO-3292H | 10076 | 10092 | 5′ tct tcc aca ttt caa gg 3′ |
| ASO-V3396 | 10403 | 10389 | 5′ cca ctg aca ctt cca t 3′ |
| ASO-3396M | 10388 | 10403 | 5′ tat gga aat gtc agt gg 3′ |
| ASO-T3400 | 10400 | 10416 | 5′ gtg gca aca acc aca a 3′ |
| ASO-3400K | 10400 | 10416 | 5′ gtg gca aaa acc aca a 3′ |
| ASO-E3405 | 10415 | 10431 | 5′ aaa agc cca aat tcc aa 3′ |
| ASO-3405Q | 10415 | 10431 | 5′ aaa agc cga aat tcc aa 3′ |
| ASO-S3455 | 10566 | 10582 | 5′ aag ctt agc ttg gaa ag 3′ |
| ASO-3455R | 10566 | 10582 | 5′ aga ctt aga ttg gaa ag 3′ |
| ASO-R3500 | 10716 | 10701 | 5′ ctg aag acc gtg tgc t 3′ |
| ASO-3500Q | 10701 | 10716 | 5′ agc aca cag tct tca g 3′ |
| ASO-T3531 | 10794 | 10810 | 5′ ctc caa cgc ata tat tc 3′ |
| ASO-3531C | 10810 | 10793 | 5′ gaa tat atg cat tgg agt 3′ |

[a]UP and RP refer to the universal and reverse sequence primers attached to the apoB sequence.

| | Sequence |
|---|---|
| UP | 5′ tgt aaa acg acg gcc agt 3′ |
| RP | 5′ cag gaa aca gct atg acc 3′ |

three VNTRs. Haplotypes were deduced by segregation analysis of the apoB genotypes from four families with Arg 3500 Gln. Haplotype analysis revealed one predominant haplotype ($TG_{14}$–$TTTA_{14}$–3500Gln–$HVE_{48}$) and a second haplotype differing by two repeats in the 3′ HVE ($TG_{14}$–$TTTA_{14}$–3500Gln–$HVE_{46}$). Both haplotypes have been described (35). While family members were not available for haplotype deduction of the Arg 3531 Cys carriers, the genotypes of the probands suggest that the Arg 3531 Cys is present on at least two different haplotypes. With the markers used in this study, the most probable consensus haplotype deduced from the genotypes is $TG_{14}$–$TTTA_{13}$–3531Cys–$HVE_{34}$, which agrees with one of those presented by Pullinger et al. (25). However, the second deduced haplotype in K5153-001 (assuming the least number of changes within the VNTR), $TG_{14}$–$TTTA_{13}$–3531Cys–$HVE_{32}$ or $HVE_{36}$ is unique.

TABLE 2. Distribution and percent of apolipoprotein B variations identified in cases and controls

| | All CAD | >90th Percentile LDL | All Controls |
|---|---|---|---|
| Variation | n/Total (%) | n/Total (%) | n/Total (%) |
| Ser 3252 Gly | 0/498 (0.0) | 5/250 (2.0)[a] | 2/545 (0.4) |
| Val 3396 Met | 0/498 (0.0) | 1/304 (0.3) | 0/545 (0.0) |
| Glu 3405 Gln | 6/495 (1.2) | 8/252 (3.2) | 8/419 (1.9) |
| Ser 3455 Arg | 0/509 (0.0) | 1/252 (0.4) | 0/419 (0.0) |
| Arg 3500 Gln | 4/638 (0.6)[b] | 5/304 (1.6)[c] | 0/666 (0.0) |
| Arg 3531 Cys | 4/516 (0.8)[d] | 0/252 (0.0) | 0/550 (0.0) |

The number of patients in each group varied due to the availability of DNA and the success of amplification. Probability ($P$) values are calculated by the Fisher exact probability test comparing the case groups to the combined control groups.

[a]$P = 0.031$.

[b]$P = 0.058$.

[c]$P = 0.00031$.

[d]$P = 0.055$.



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TABLE 3. Characteristics of probands with apolipoprotein B variations

| Variation | Group | Proband ID | Sex | Age | TG | TC | VLDL | LDL | HDL | ApoB | ApoE | Age at Onset of MI | HTN/DM[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *yr* | | | | | | | | *yr* | |
| S3252G | >90th % LDL | K1044-003 | M | 63 | 122 | 276 | 23 | 234 | 23 | | 3/3 | 59 | 1/0 |
| | >90th % LDL | K3120-001 | M | 54 | 160 | 283 | 50 | 198 | 33 | | 2/3 | 50 | 0/0 |
| | >90th % LDL | K726-260[b] | M | 63 | 251 | 231 | 46 | 149 | 36 | | 3/4 | | 0/0 |
| | >90th % LDL | K5036-003 | M | 45 | 149 | 270 | 34 | 199 | 39 | | 3/3 | | 1/0 |
| | CAD cont | K3090-001 | M | 60 | 158 | 157 | 26 | 95 | 32 | 69 | 3/3 | | 1/0 |
| S3252G/V3396M | >90th % LDL | K1039-062 | F | 54 | 138 | 286 | 21 | 207 | 60 | | 3/4 | | 0/0 |
| E3405E | >90th % LDL | K678-002 | F | 46 | 242 | 280 | 56 | 205 | 28 | | 3/4 | | 0/0 |
| Q3405E | >90th % LDL | K726-030 | F | 57 | 136 | 311 | 62 | 211 | 78 | | 3/4 | | 0/0 |
| | >90th % LDL | K784-107 | M | 25 | 176 | 251 | 44 | 173 | 34 | | 3/3 | | 0/0 |
| | >90th % LDL | K3000-001 | M | 54 | 68 | 236 | 14 | 188 | 34 | | 3/4 | 50 | 0/0 |
| | >90th % LDL | K774-101 | M | 40 | 150 | 266 | 44 | 191 | 34 | | 3/3 | | 0/0 |
| | >90th % LDL | K302-003 | M | 51 | 103 | 300 | 20 | 222 | 53 | | 3/4 | | 1/0 |
| | >90th % LDL[c] | K1132-001 | F | 34 | 114 | 312 | 23 | 237 | 43 | | 3/3 | | 0/0 |
| | CAD case | K3155-001 | F | 43 | 108 | 190 | 18 | 115 | 42 | 89 | 3/3 | | 0/0 |
| | CAD case | K5145-001 | M | 63 | 97 | 159 | 27 | 113 | 19 | 69 | 3/3 | | 1/1 |
| | CAD case | K3022-003 | M | 49 | 172 | 302 | 33 | 231 | 38 | | 3/3 | 36 | 0/0 |
| | CAD case | K3016-001 | F | 52 | 83 | 227 | 12 | 198 | 34 | | 3/3 | 49 | 1/0 |
| | CAD case | K3087-001 | F | 55 | 110 | 227 | 18 | 154 | 56 | | 3/3 | 29 | 0/0 |
| | CAD case | K3109-060 | M | 57 | 170 | 190 | 36 | 126 | 29 | | 3/3 | 51 | 0/0 |
| | CAD cont | K5039-001 | F | 60 | 80 | 143 | 14 | 77 | 52 | 46 | 2/3 | | 1/0 |
| | CAD cont | K5044-001 | F | 60 | 61 | 186 | 11 | 133 | 42 | 67 | 3/4 | | 0/0 |
| | CAD cont | K5168-001 | M | 46 | 61 | 180 | 1 | 142 | 37 | 89 | 3/3 | | 0/0 |
| | CAD cont | K5190-001 | F | 68 | 61 | 121 | 1 | 87 | 39 | 143 | 3/4 | | 0/1 |
| | CAD cont | K5481-001 | M | 56 | 68 | 155 | 10 | 104 | 41 | 51 | na | | 1/0 |
| | CAD cont | K3156-001 | F | 58 | 106 | 174 | 26 | 94 | 42 | 73 | 3/3 | | 1/0 |
| S3455G&Q3405E | >90th % LDL[c] | K1081-001[d] | F | 75 | 214 | 311 | 55 | 215 | 52 | | 3/3 | 74 | 0/0 |
| R3500Q | >90th % LDL[c] | K27-006 | F | 52 | 164 | 330 | 35 | 229 | 65 | | 3/3 | | 0/0 |
| | >90th % LDL | K664-002 | F | 50 | 66 | 325 | 8 | 224 | 97 | | 3/4 | | 0/0 |
| | >90th % LDL | K657-001 | F | 24 | 83 | 276 | 14 | 215 | 58 | | 3/3 | | 0/0 |
| | >90th % LDL[c] | K1120-001 | F | 27 | 92 | 354 | 15 | 273 | 61 | | | | 0/0 |
| | >90th % LDL[c] | K2087-003 | F | 55 | 193 | 278 | 51 | 183 | 44 | | 3/3 | | 0/0 |
| | CAD case | K5117-001 | F | 60 | 131 | 266 | 32 | 206 | 28 | 115 | 2/3 | 59 | 0/0 |
| | CAD case | K5417-001[d] | M | 55 | 450 | 180 | 99 | 53 | 28 | 69 | 3/3 | 44 | 1/0 |
| | CAD case | K1013-001 | M | 53 | 192 | 367 | 35 | 304 | 31 | | 3/3 | 47 | 0/0 |
| | CAD case | K669-003 | M | 48 | 248 | 319 | 71 | 203 | 38 | | 3/3 | 47 | 0/0 |
| R3531C | CAD case | K3169-001 | M | 65 | 251 | 310 | 16 | 240 | 41 | 148 | 3/3 | | 1/1 |
| | CAD case | K3635-001 | F | 64 | 96 | 261 | 25 | 190 | 46 | 112 | 4/4 | | 1/0 |
| | CAD case | K5153-001[d] | M | 51 | 203 | 244 | 32 | 173 | 39 | 119 | 3/4 | 43 | 1/0 |
| | CAD case | K5392-001 | M | 64 | 297 | 216 | 45 | 138 | 33 | 100 | 3/3 | 55 | 1/0 |

Lipid values are in mg/dl. Abbreviations: ID, identification number; TG, triglycerides; TC, total plasma cholesterol; VLDL, very low density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; HDL, high density lipoprotein cholesterol; apoB, apolipoprotein B concentration in mg/dl; apoE, apolipoprotein E phenotype; MI, myocardial infarction; HTN, hypertension; DM, diabetes mellitus.

[a]Hypertension and diabetes mellitus are scored as 0 and 1, referring to the absence and presence of disease.

[b]Identified by screening family members of K726-030.

[c]Diagnosed as FH.

[d]Patient treated with lovastatin.

### LDL receptor–binding assay

To determine whether any of the apoB mutations identified by SSCP and ASO hybridization cause LDL to have impaired receptor binding, we examined their ability to compete with normal human $^{125}$I-labeled LDL for binding to LDL receptors on cultured human fibroblasts. **Figure 4** graphically represents the competitive binding ability of LDL from patients for each variation identified (graphed as probit-log scale). The concentration of competitor LDL required to displace 50% of normal $^{125}$I-labeled LDL from the LDL receptors is indicated by the effective dose (ED) 50th percentile. In the assays represented in Fig. 4, LDL with the Arg 3500 Gln and Arg 3531 Cys variations were less effective competitors than control LDL. **Table 5** presents the ED 50% and the competitive binding ratio of the control and patient LDL (measured as the ED 50% of the control/the ED 50% of the carrier) and the mean values for all assays. The mean ED 50% of the control LDLs in these assays were 2.1 and 2.2 μg/ml. The mean ED 50% ± S.D. for control LDLs in 50 assays previously conducted was 2.0 ± 0.4 μg/ml. LDL from the Ser 3252 Gly carriers, three Gln 3405 Glu carriers, and the Gln 3405 Glu homozygote bound normally. Receptor binding was also normal for the LDL with both the Gln 3405 Glu and the Ser 3455 Arg variations and the LDL with



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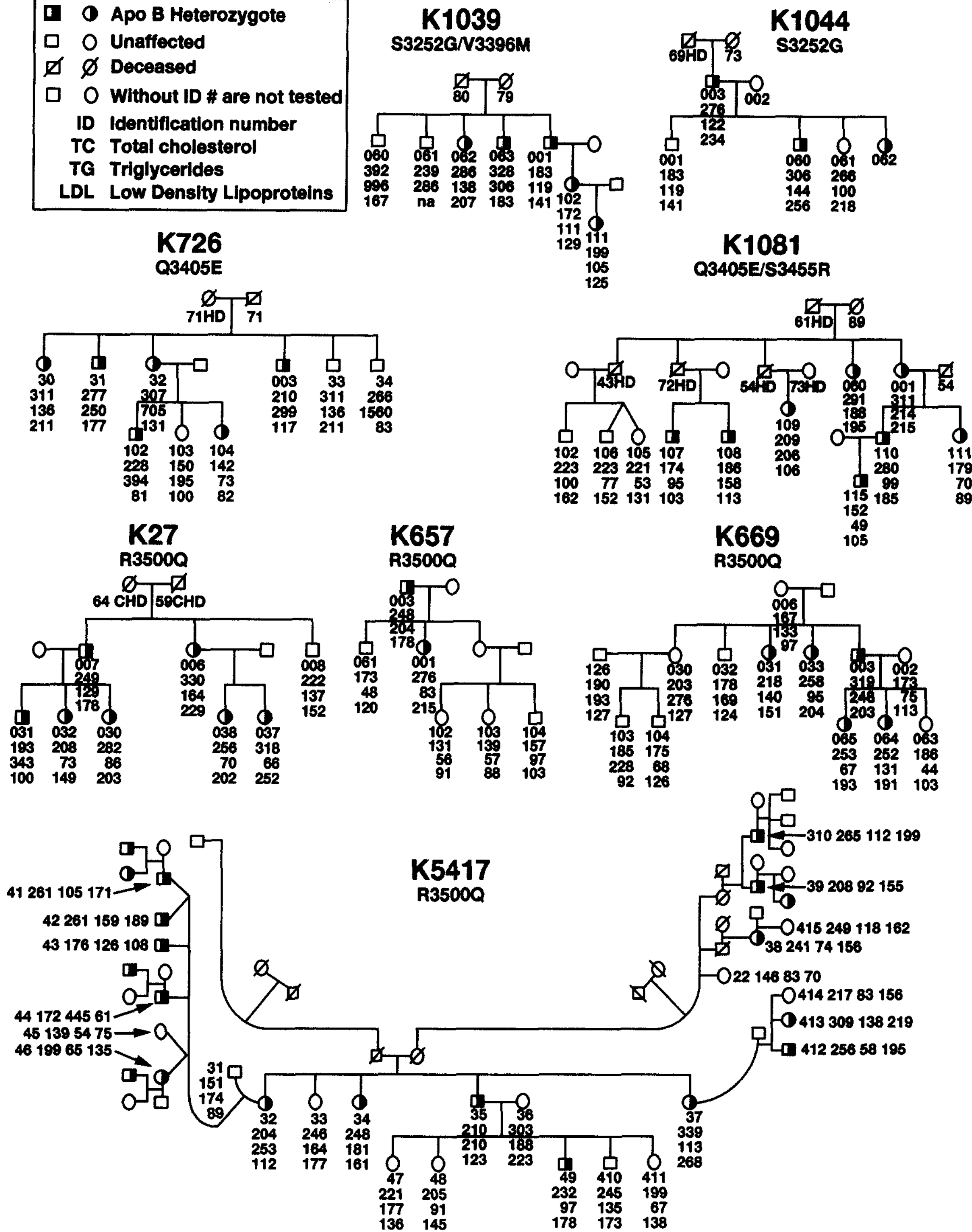


Fig. 3. Kindreds with apoB variations available for segregation analysis.

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TABLE 4. Distribution and significant differences for age, body mass index, and lipid levels in apoB Arg 3500 Gln carriers and family members without the mutation

| Variable | R3500Q (n = 34) | No Mutation (n = 26) | Significance[a] |
|---|---|---|---|
| Age | 40.23 ± 2.60 | 37.38 ± 4.10 | $P = 0.247$ |
| BMI[b] | 25.41 ± 0.83 | 24.68 ± 1.31 | $P = 0.209$ |
| Cholesterol | 259.7 ± 8.6 | 192.69 ± 8.11 | $P < 0.0001$ |
| Triglycerides | 141.2 ± 14.5 | 134.38 ± 16.19 | $P = 0.296$ |
| HDL | 46.9 ± 2.6 | 42.23 ± 2.55 | $P = 0.202$ |
| LDL | 184.3 ± 8.8 | 124.0 ± 6.93 | $P < 0.0001$ |

Lipid values are in mg/dl. Values expressed as mean ± SE.
[a]Two-tailed, paired $t$ test.
[b]Body mass index in kg/m².

the Ser 3252 Gly/Val 3396 Met variations. LDL from subjects with the Arg 3500 Gln or the Arg 3531 Cys bound defectively to the normal LDL receptors ($P = 0.011$ and $P = 0.002$, respectively) as previously shown (20, 21, 25).

### Determination of allele-specific concentrations of LDL by solid-phase radioimmunoassay

Each particle of LDL has one copy of apoB-100, and therefore subjects with two different allelic forms of apoB-100 have two populations of LDL. If the variation or mutation in the apoB-100 causes abnormal biosynthesis of VLDL or abnormal catabolism of LDL, then the LDL with the apoB-100 variant will be present in the plasma at a higher or lower concentration than the LDL from the normal apoB allele. Monoclonal antibody MB19 has been used to detect allele-specific differences in concentrations of apoB-100 (LDL) (4, 40). MB19 binds to one apoB allotype (MB19-1) with an 11-fold higher affinity than to the other allotype (MB19-2) (41). In a competitive solid-phase radioimmunoassay with MB19 LDL from an MB19-2 homozygote, an MB19 heterozygote and an MB19-1 allotype yielded distinctly different displacement curves, as shown in **Fig. 5,** resulting from different affinities of antibody MB19 for allotypes MB19-1 and MB19-2 (4, 42). This procedure has been used to estimate the percentage of MB19-2 (and thus defective-binding) LDL in FDB heterozygotes (23, 42). Also shown in Fig. 5 are the displacement curves generated through computer-assisted sigmoidal fit of the points of LDL from an Arg 3500 Gln heterozygote, an Arg 3531 Cys heterozygote, and a Gln 3405 Glu heterozygote. From the curves, the amount of apoB necessary to inhibit 50% of the antibody MB19 binding to immobilized MB19-1 LDL (ED50) could be calculated. LDL heterozygotes containing a higher percentage of low-affinity MB19-2 LDL were less effective competitors (i.e., their displacement curves shifted to the right) (Fig. 5 and Table 5). From these curves we calculated

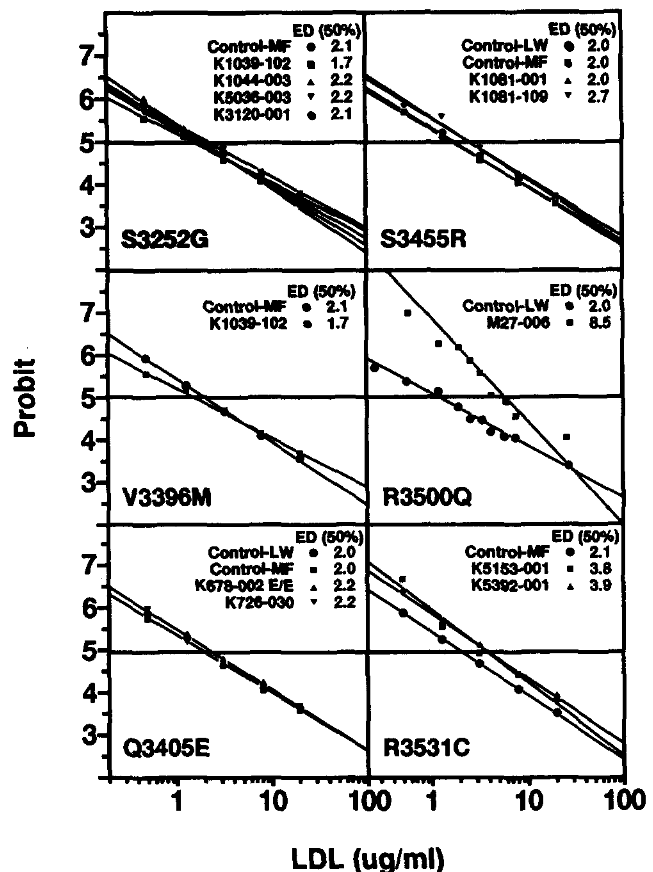


**Fig. 4.** Fibroblast LDL receptor competition assays with LDL isolated from subjects with apoB variants. The x-axis represents the concentration in µg/ml of the unlabeled competitor LDL. The y-axis represents the probit transformed percentage of $^{125}$I-labeled normal LDL bound to fibroblast LDL receptors. The effective dose (ED) 50% is the amount of competitor LDL required for 50% inhibition of the $^{125}$I-labeled LDL binding to LDL receptors.

(42) that 73% of the LDL from a Arg 3500 Gln heterozygote and 63% of the LDL from a Arg 3531 Cys heterozygote were the product of the MB19-2 allotype. Both the Arg 3500 Gln and the Arg 3531 Cys variations are on the low-affinity allele (MB19-2). These results confirm previous studies demonstrating that these apoB allotypes accumulate in the plasma because of impaired receptor-mediated clearance. In contrast, LDL from the Gln 3405 Glu heterozygotes had approximately equal amounts of the two apoB allotypes, as expected because LDL from this subject had normal receptor-binding activity.

## DISCUSSION

We searched for variants of apoB-100 in the region of apoB between cDNA positions 9102 and 10810



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TABLE 5. Summary of the LDL receptor competition assays and MB19 competitive solid-phase radioimmunoassays

| | | Competitive LDL Receptor Binding Assays | | | | | | | | MB19 Competition RIA | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | TC2055 | | TC2056 | | TC2057 | | | | | |
| Variant | Patient | ED 50% | Binding Ratio[a] | ED 50% | Binding Ratio[a] | ED 50% | Binding Ratio[a] | Mean Binding Ratio[b] | Patient | RIA-1 ED 50% | RIA-2 ED 50% |
| Control-MF | MF | 2.1 | 1.10 | 2.0 | 1.00 | nd | nd | 1.03 | C-MF MB19 High | 311 | 251 |
| Control-LW | LW | 2.5 | 0.92 | 2.0 | 1.00 | 2.0 | 1.00 | 0.97 | C-LW MB19 Inter | 534 | 446 |
| | | | | | | | | | C-CH MB19 Low | 5819 | 3921 |
| S3252G | K1044-003 | 2.2 | 1.05 | 2.2 | 0.91 | nd | nd | 0.95 | | | |
| S3252G | K3120-001 | 2.1 | 1.10 | 1.6 | 1.25 | nd | nd | 1.14 | | | |
| S3252G | K5036-001 | 2.2 | 1.05 | 2.2 | 0.91 | nd | nd | 0.95 | | | |
| S3252G | K3090-001 | 2.4 | 0.96 | 1.4 | 1.43 | nd | nd | 1.11 | | | |
| S3252G/V3396M | K1039-102 | 1.7 | 1.35 | 1.6 | 1.25 | nd | nd | 1.28 | | | |
| E3405E | K678-002 | 2.5 | 0.92 | 2.2 | 0.91 | nd | nd | 0.90 | | | |
| Q3405E | K3000-001 | 2.0 | 1.15 | 3.0 | 0.67 | nd | nd | 1.06 | K3000-001 | 622 | |
| Q3405E | K726-030 | nd | nd | 2.2 | 0.91 | nd | nd | 0.95 | K726-030 | 570 | 489 |
| Q3405E/S3455R | K1081-001 | 2.3 | 1.00 | 2.0 | 1.00 | nd | nd | 0.98 | | | |
| S3455R | K1081-109[c] | 2.4 | 0.96 | 2.7 | 0.74 | 1.8 | 1.11 | 0.92 | | | |
| R3500Q | K27-030 | nd | nd | 2.9 | 0.69 | 4.0 | 0.50 | 0.61[d] | K27-030 | 898 | |
| R3500Q | K27-006[c] | nd | nd | 3.6 | 0.56 | 8.5 | 0.24 | 0.34[d] | K27-006 | 1016 | 685 |
| R3531C | K5153-001[c] | 3.8 | 0.61 | 3.1 | 0.65 | 2.9 | 0.69 | 0.54[e] | K5153-001 | 614 | 750 |
| R3531C | K5392-001 | 3.9 | 0.59 | 2.5 | 0.80 | 2.0 | 1.00 | 0.75[e] | | | |

ED 50% is expressed as μg/ml; nd, not determined.
[a]The binding ratio is calculated as the mean ED 50% of the control LDL for that assay divided by the ED 50% for that patient's LDL.
[b]This column refers to the mean binding ratios of all three assays.
[c]Patient treated with lovastatin.
[d]$P = 0.011$ for both R 3500 Q carriers combined versus combined control data (paired $t$ test).
[e]$P = 0.002$ for both R 3531 C carriers combined versus combined control data (paired $t$ test).

(amino acid positions 2965 to 3534) (illustrated in Fig. 1). Several lines of evidence suggested to us that mutations of apoB DNA in this region could cause LDL to have defective receptor binding. First, two potential receptor-binding sites at amino acid residues 3147 to 3157 and 3359 to 3367 were identified by homology with the apoE receptor–binding site (46). Second, certain monoclonal antibodies specific for apoB abolish binding of LDL to its receptor. The epitopes of these antibodies have been mapped to this region of apoB-100. For example, the epitopes of the inhibitory antibodies MB47 and 4G3 are at amino acid positions 3429 to 3523 (47) and 2980 to 3084 (48), respectively. Third, a mutation at codon 3041 results in the formation of truncated apoB (apoB-67). LDL containing apoB-67 cannot bind to the LDL receptor (49), while LDL containing apoB-75, another truncation mutant of apoB, binds to the LDL receptor (50). Fourth, two point mutations downstream of this truncation, at cDNA positions 10708 and 10800 (residues 3500 and 3531), reduce the binding affinity of LDL to the receptor, suggesting that other mutations of amino acids in this region could reduce the receptor binding of LDL (20, 25). Taken together, the evidence suggests that the receptor-binding domain lies downstream of the stop codon for apoB-67 and upstream of the carboxyl-terminus of apoB-75, with a secondary domain surrounding the 3500 and the 3531 variants that modulates the receptor-ligand interaction.

Technically, our search for apoB mutations benefited from the experiences of Sheffield et al. (51). By using several gel conditions and optimal size fragments, they were able to detect 97% of all variations (28 of 29) in the mouse globin promoter by SSCP analysis. In our protocols, four of the seven PCR fragments for apoB were between 270 and 290 bp, one at 186 bp and two larger than 300 bp. These investigators reported that by using only one gel condition, SSCP was able to detect approximately 65–75% of all variations within this PCR size range. We used three gel conditions and overlapping fragment amplifications, so we should have been able to detect virtually all variations of apoB DNA sequence. Of the eight previously published sequence differences or bona fide variants in this region, we detected three by SSCP. The other four (L3029L was not screened) were not present in this population, as determined by ASO hybridization. These results strongly support the efficiency of the technique and suggest we were able to detect most if not all of the variants present in this region of the apoB DNA.

We identified seven variants within the 1726 bases of DNA by SSCP. Only two of these variants, Arg 3500 Gln and Arg 3531 Cys, had reduced LDL receptor binding



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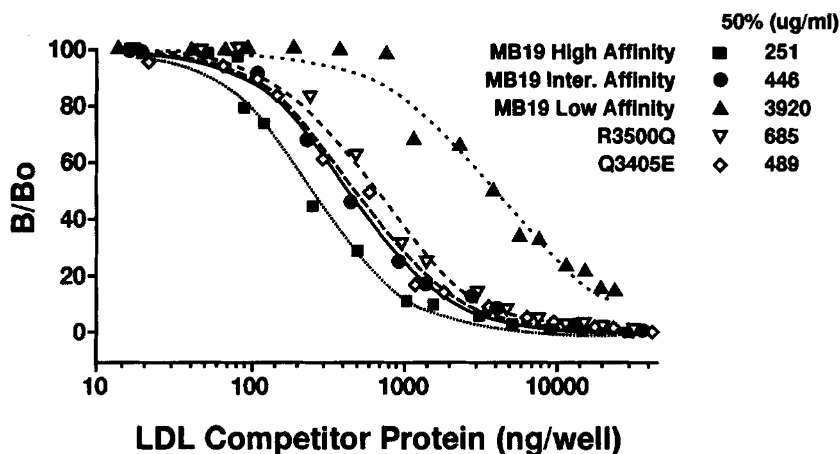


**Fig. 5.** Solid-phase competitive radioimmunoassay demonstrating the ability of various patient LDLs to compete with immobilized high-affinity (*Apa*LI +/+) LDL from control-MF for binding (B/Bo) to antibody MB19. Competitor LDLs: Control-MF, MB19 high-affinity LDL (*Apa*LI +/+); Control – LW, MB19 intermediate-affinity LDL (*Apa*LI +/–); Control-CH, MB19 low-affinity LDL (*Apa*LI –/–); K27-006, Arg 3500 Gln carrier; K726-030, Gln 3405 Glu carrier.

and were significantly associated with hypercholesterolemia and CAD. Together, these variants were found in less than 2% of our subjects with hypercholesterolemia or CAD. It is surprising that we did not find additional mutations of apoB-100 that disrupt LDL-receptor interactions. In studies similar to these, no apoB variants in this sequence were identified by SSCP in a Finnish population (52). These findings are even more striking when contrasted to the large number of mutations in the LDL receptor that disrupt its function. For example, in a comparably sized sequence encompassing the ligand-binding domain of the LDL receptor (exons 2 to 6), 48 point mutations or small deletions have been identified (26), and yet only seven variants are evident in this sequence of apoB. In addition, most of the LDL receptor variants cause FH, whereas only two variants in the putative LDL receptor–binding domain of apoB have any biological impact that is ascertainable (collectively accounting for a minimal overall contribution to CHD). Finally, the apoB locus is proposed to contribute to 14–20% of the variance of apoB and LDL levels (4, 5). Several factors could contribute to this obvious discrepancy. One possibility is that the statistical analysis may be confounded by selection biases. Potentially, however less likely, another gene influencing the receptor binding of apoB is in linkage disequilibrium with apoB. It is also possible that variations contributing to defective binding of apoB may lie elsewhere in the gene or that other variations of the apoB protein alter the synthesis of apoB or the conversion of VLDL to LDL.

Nevertheless, the discrepancy remains between the estimates of the percentage of hypercholesterolemia due to variations in the apoB locus and our findings that only two mutations in this region cause hypercholesterolemia by retarding receptor-mediated catabolism of LDL and that these mutations are present in only 1–2% of the hypercholesterolemic subjects of our case groups. It is also possible that genetic defects in apoB are present on relatively common alleles within our general population that influence receptor binding but their effect is minimal and not detectable by in vitro receptor-binding assay. In combination with other additive or interactive genetic and environmental factors, these mutations might cause hypercholesterolemia. This may explain why some complex segregation analyses find the apoB locus associated with LDL in polygenic models (9) and why other studies have failed to identify a monogenic dominant mode of transmission, possibly due to population heterogeneity resulting in multiple apoB alleles minimally influencing LDL or CAD. Such alleles might be very difficult to detect because they may present a genetic predisposition to CAD, however, additional genetic and environmental factors may be required for phenotypic expression. Within a genetically heterogeneous human population and uncontrollable environmental factors influencing phenotypes, the association of these significant defects may remain difficult to detect.

In addition to identifying variations of apoB present in subjects from the Great Salt Lake Basin area, we also found the apoB Arg 3500 Gln mutation in subjects from a Lancaster County, Pennsylvania, Amish family. The 12,500 Amish of Lancaster County are descendants of a small founding population and have remained genetically and culturally homogeneous. The distribution of the Arg 3500 Gln mutation in family members screened suggests that this allele may be more prevalent in the Amish population than in the general population. The presence of large families, clear paternity, and excellent genealogical records exemplify a unique potential resource to better understand effects of environment and other genes on the FDB phenotype.

We have confirmed the association of the apoB Arg 3500 Gln and Arg 3531 Cys mutations with hypercholesterolemia. Both FDB mutations were also identified in CAD patients. However, hypercholesterolemia is not



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necessarily present in all FDB carriers in the CAD group. This is probably due to secondary genetic or environmental factors masking the effect of FDB. In an extensive genetic search for other apoB variants encompassing the LDL receptor binding region, we did not identify any additional variants of apoB that were associated with either hypercholesterolemia or premature CAD and had any detectable impact on the in vitro binding of LDL to its receptor. This study suggests that a limited number of mutations in the region of the receptor-binding domain of apoB-100 markedly influence LDL receptor binding.

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