Familial defective apolipoprotein B-100: a mutation of apolipoprotein B that causes hypercholesterolemia

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Abstract   Familial defective apolipoprotein B-100 is a genetic disorder of apolipoprotein B-100 that causes moderate to severe hypercholesterolemia. A single amino acid mutation in apolipoprotein B diminishes the ability of low density lipoproteins to bind to the low density lipoprotein receptor. Low density lipoproteins accumulate in the plasma because their efficient receptor-mediated catabolism is disrupted. This mutation has been identified in the United States, Canada, and Europe and is estimated to occur at a frequency of ~ 1/500 in these populations. Thus, it appears that this newly described disorder may be a significant genetic cause of hypercholesterolemia in Western societies. — **Innerarity, T. L., R. W. Mahley, K. H. Weisgraber, T. P. Bersot, R. M. Krauss, G. L. Vega, S. M. Grundy, W. Friedl, J. Davignon, and B. J. McCarthy.** Familial defective apolipoprotein B-100: a mutation of apolipoprotein B that causes hypercholesterolemia. *J. Lipid Res.* 1990. **31:** 1337–1349.

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In Western societies, atherosclerosis is the most common cause of death. Numerous epidemiological studies have demonstrated that elevated levels of plasma cholesterol, particularly cholesterol transported in low density lipoproteins (LDL), are associated with increased coronary heart disease (1–4). The fact that approximately 50% of the adult population of the United States possesses LDL levels that place them at risk of atherogenesis demonstrates the magnitude of the problem (1–5). In addition to environmental factors such as diet, genetic factors play a major role in determining the risk for atherosclerosis (6–8). Investigations of genetic abnormalities that affect plasma cholesterol levels have led to a greater understanding of the function of apolipoproteins and the LDL receptor and have underscored their importance in regulating plasma lipoprotein metabolism (6–11). Well-studied examples of these abnormalities are genetic defects in the LDL receptor and apolipoprotein (apo) E, one of the ligands of this receptor. Patients with familial

hypercholesterolemia (FH) have defective or absent LDL receptors and extremely elevated plasma levels of cholesterol and LDL and develop premature cardiovascular disease (9). Patients with type III hyperlipoproteinemia have apoE molecules with one of several amino acid substitutions that prevent normal binding to lipoprotein receptors (10, 11).

In this review article, we discuss a prevalent point mutation in the human apoB-100 gene that also causes hypercholesterolemia. This genetic disorder has been designated familial defective apoB-100 (FDB). Apolipoprotein B-100 is the exclusive protein constituent of LDL and is the ligand on LDL recognized and bound by the LDL receptor. This mutation in apoB-100 disrupts the binding of LDL to the LDL receptor; thus, in one sense the disorder is a counterpart to FH. Both disorders are characterized by mutations that lead to a disruption of normal function of either the LDL receptor (FH) or the ligand (FDB), and both mutations disrupt LDL receptor-mediated catabolism and result in hypercholesterolemia.

STRUCTURE OF APOLIPOPROTEIN B-100

In humans, most plasma cholesterol is transported in LDL. This lipoprotein consists of a central core composed of apolar neutral lipids (triglycerides and cholesteryl esters) and an outer shell of phospholipids, unesterified cholesterol, and a single apoB-100 molecule as its protein
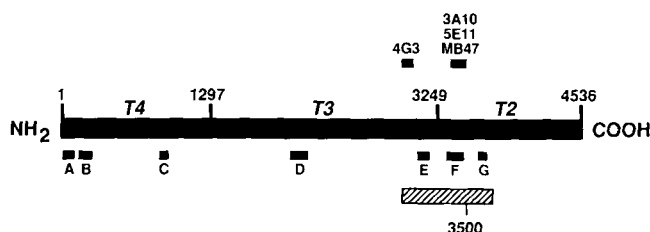
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component. Because the large size and insolubility of apoB-100 in the absence of lipid and detergents make it refractory to analysis by protein chemistry (12, 13), its structure was elusive for many years. More recently, a number of laboratories determined the structure of human apoB-100 by cloning and sequencing apoB cDNA (14-17). Human apoB mRNA is 14.5 kilobases (kb) in length and codes for a mature protein of 4536 amino acids that has a molecular weight of about 550,000, including about 10% carbohydrate (14-17). The signal peptide contains either 24 or 27 amino acids (18). Thrombin cleaves apoB-100 of LDL at three sites, which serve as useful landmarks in analyzing the functional domains of apoB-100 (**Fig. 1**) (19). The apoB gene is 43 kb in length, contains 28 introns and 29 exons, and is located near the tip of the short arm of chromosome 2 (15, 20-22). The distribution of the introns within the gene is unusual in that 24 of the 29 introns occur in the 5' terminal one-third of the gene (22). Over half of the protein is coded by the extremely long exon 26, whose 7572 base pairs make it one of the largest exons yet reported in the human genome (22).

FUNCTIONAL DOMAINS OF APOLIPOPROTEIN B-100

Apolipoprotein B-100 has four well-recognized functional activities. First, it is required for the synthesis, assembly, and secretion of hepatic triglyceride-rich lipoproteins. Second, it binds to lipids and is a structural component of very low density lipoproteins (VLDL) and LDL. Third, it binds to heparin and various proteoglycans found in the arterial wall that may be involved in binding LDL within atherosclerotic plaques. Finally, apoB-100 interacts with the LDL receptor, mediating the clearance of LDL from the plasma. Elucidation of the functional domains involved in these activities has been accelerated by the determination of the amino acid sequence of apoB-100.

Apolipoprotein B-100 is obligatory for the secretion of VLDL from the liver (12, 13). Humans with the genetic disorder familial abetalipoproteinemia or homozygous hypobetalipoproteinemia secrete little (if any) apoB-100 in the form of VLDL. Moreover, LDL, a metabolic product of VLDL, is absent in these patients. The genetic defect that causes familial abetalipoproteinemia is unknown, but it is not linked to the gene for apoB (23). Recent work by several laboratories has indicated that hypobetalipoproteinemia can be caused by mutations in the coding region of the apoB-100 gene that result in premature translation termination codons (24-31). As a consequence, greatly reduced amounts of VLDL and LDL are found in the plasma of these individuals, and their lipoproteins possess a truncated form of apoB. From the analysis of a number of these mutations, it appears that a minimum length of apoB is necessary before any truncated apoB-containing lipoproteins are detected in the plasma. Apparently, a normal or nearly full-length apoB-100 is necessary to achieve normal VLDL secretion (27, 29, 30).

A basic function of all apolipoproteins, including apoB-100, is lipid transport. However, apoB-100 differs from other apolipoproteins in its interactions with lipids. Most apolipoproteins, such as A-I, A-II, and E, transfer freely among various lipoprotein particles. The association of these apolipoproteins with lipids is thought to be mediated through amphipathic α-helices. In contrast, apoB-100 does not exchange among lipoprotein particles and has limited amphipathic helical structure. Rather, apoB-100 may interact with lipids via its hydrophobic β-sheet structure and by segments of hydrophobic amino acids resembling abbreviated membrane-spanning regions of receptors (14-17). These regions are located throughout the apoB-100 molecule, and all three thrombolytic fragments of apoB-100 have been shown to recombine with lipids in microemulsions (32).

In atherosclerotic lesions, the association of apoB-containing lipoproteins with acidic glycosaminoglycans or specific proteoglycans suggests that this interaction may be a contributing factor in the pathogenesis of atherosclerosis (33, 34). In vitro studies have demonstrated that the apoB-100 of LDL particles binds with high affinity to specific proteoglycans and to heparin (34-36). Recently, those apoB-100-binding domains that interact with heparin and proteoglycans have been located (34-36). The seven distinct heparin-binding sites are situated throughout the molecule (Fig. 1). Two of these heparin-binding sites also interact with chondroitin-6-sulfate arterial proteoglycans (34).

One of the most interesting functional regions of apoB-100 is the receptor-binding domain. Of the approaches used in an attempt to locate this domain, the most informative has been derived from the use of monoclonal antibodies (37-40). Several antibodies raised

Fig. 1. Linear representation of apoB-100 showing the locations of the heparin-binding sites (the boxes lettered A-G), the epitopes of four monoclonal antibodies that inhibit LDL receptor binding (4G3, 3A10, 5E11, MB47), and the putative location of the receptor-binding region (hatched box). The "3500" represents the amino acid substitution of glutamine for arginine that is believed to cause familial defective apolipoprotein B-100 (FDB).

against LDL or apoB-100 have the ability to abolish the binding of LDL to its receptor. Mapping the epitopes of these inhibitory antibodies has helped to narrow down the region of apoB-100 involved in mediating receptor binding. Four monoclonal antibodies to apoB-100 that totally inhibit LDL receptor binding have epitopes near the thrombin cleavage site at residue 3249 (T3/T2 junction) of apoB-100 (Fig. 1). Monoclonal antibodies to apoB-100 with epitopes outside this region had little or no effect on LDL receptor binding (37-40). Another approach examined which monoclonal antibodies to apoB-100 bound to LDL that were already bound to the LDL receptor. These experiments demonstrated that apoB-100 monoclonal antibodies with epitopes in the region of residues 3000-4000 bound poorly, if at all, to receptor-bound LDL. While these studies broadly defined the receptor-binding region of apoB-100, its precise location remains to be determined (40).

MUTATIONS OF APOLIPOPROTEIN B-100

The sequencing of five complete and several partial apoB-100 DNA sequences, in conjunction with direct protein sequencing, has permitted an estimation of the naturally occurring variations of sequence in apoB-100 (41). The latest compilation of the number of variants of apoB-100 lists 75 nucleotide differences, 54 of which would cause amino acid substitutions (42). Of these 75 differences, 12 have been confirmed by at least two laboratories and therefore almost certainly represent bona fide variants. Studies of apoB-100 gene variants by restriction fragment length polymorphism or by monoclonal antibodies (see below) have confirmed that several of the variants defined by sequencing represent different apoB alleles. On the other hand, it appears that some apparent nucleotide differences may reflect sequencing errors (42).

Before the structure of apoB-100 was established, genetically determined polymorphisms of LDL-associated apoB-100 were evident from immunological studies. An immunochemical polymorphism designated as the antigen group (Ag) has been detected in apoB-100 by human antisera obtained from multiply transfused patients. These antisera identify five antithetical pairs of epitopes on LDL-associated apoB-100: Ag(a$_1$/d), Ag(x/y), Ag(t/z), Ag(c/g), and Ag(h/i) (43-45). Recently developed monoclonal antibodies recognize two of these sites (46, 47). The monoclonal antibodies MB19 and H11G3 detect apoB polymorphisms that are identical to the Ag(c/g) and the Ag(a$_1$/d) polymorphisms, respectively. Four of the Ag polymorphisms have recently been identified as single amino acid substitutions in the primary structure of apoB-100. The Ag(c/g)/MB19 polymorphism is associated with an interchange of isoleucine for threonine at amino acid 71 (46), while the Ag(a$_1$/d)/H11G3 polymorphism is caused by a substitution of alanine for valine at residue

591 (47). The Ag(h/i) epitope corresponds to a substitution of glutamine for arginine at residue 3611 (48, 49), and the Ag(t/z) immunochemical polymorphism exhibits perfect association with a glutamic acid-for-lysine substitution at residue 4154 (50).

A number of studies have examined whether common DNA or antigenic polymorphisms of apoB-100 are associated with altered lipid levels or atherosclerosis (51-59). For example, an association between an *Xba*I restriction fragment length polymorphism of apoB-100 and myocardial infarction was found by Hegele et al. (51), and in a Finnish population the Ag(c/g) polymorphism was associated with elevated cholesterol levels (55). While many studies have demonstrated an association between a particular apoB-100 polymorphism and elevated lipid levels (51-56), in other populations this association was not evident (57-59). Therefore, none of these association studies demonstrate that the apoB-100 polymorphism examined is the cause of the altered phenotype (elevated lipid levels or increased risk for atherosclerosis). Instead, in the population studies in which a positive association is observed, the apoB-100 polymorphism is probably in linkage disequilibrium with a functionally important mutation elsewhere within the apoB gene. However, we have identified a mutation within the apoB-100 gene that appears to disrupt the function of apoB-100 in humans and to cause hypercholesterolemia.

FAMILIAL DEFECTIVE APOLIPOPROTEIN B-100

Screening studies were undertaken to determine whether mutations in the apoB gene could directly affect plasma lipid levels. Low density lipoproteins were isolated from a large number of individuals and tested for functional differences—in vivo receptor-mediated catabolism or in vitro binding of LDL to the LDL receptor. The first evidence of defective-binding LDL came from in vivo turnover studies (60). This functional test measured the rate at which intravenously injected radioactive LDL were cleared from the plasma by the LDL receptor pathway. Clearance rates of autologous and normal homologous LDL from the plasma were determined in 15 subjects who had moderate primary hypercholesterolemia. In 10 of these subjects, the autologous and homologous LDL were cleared from the plasma at the same rate, whereas in the remaining 5, the subjects' own LDL were catabolized significantly more slowly than were normal LDL. The LDL from one of these latter individuals (G.R.) was striking in that it had a plasma clearance rate of about 50% of normal (**Fig. 2A**). Since between two-thirds and three-fourths of normal LDL are cleared from the plasma by the hepatic LDL receptor pathway, these results suggested that the LDL from these five patients (especially G. R.) were poor ligands for the LDL receptor (60).
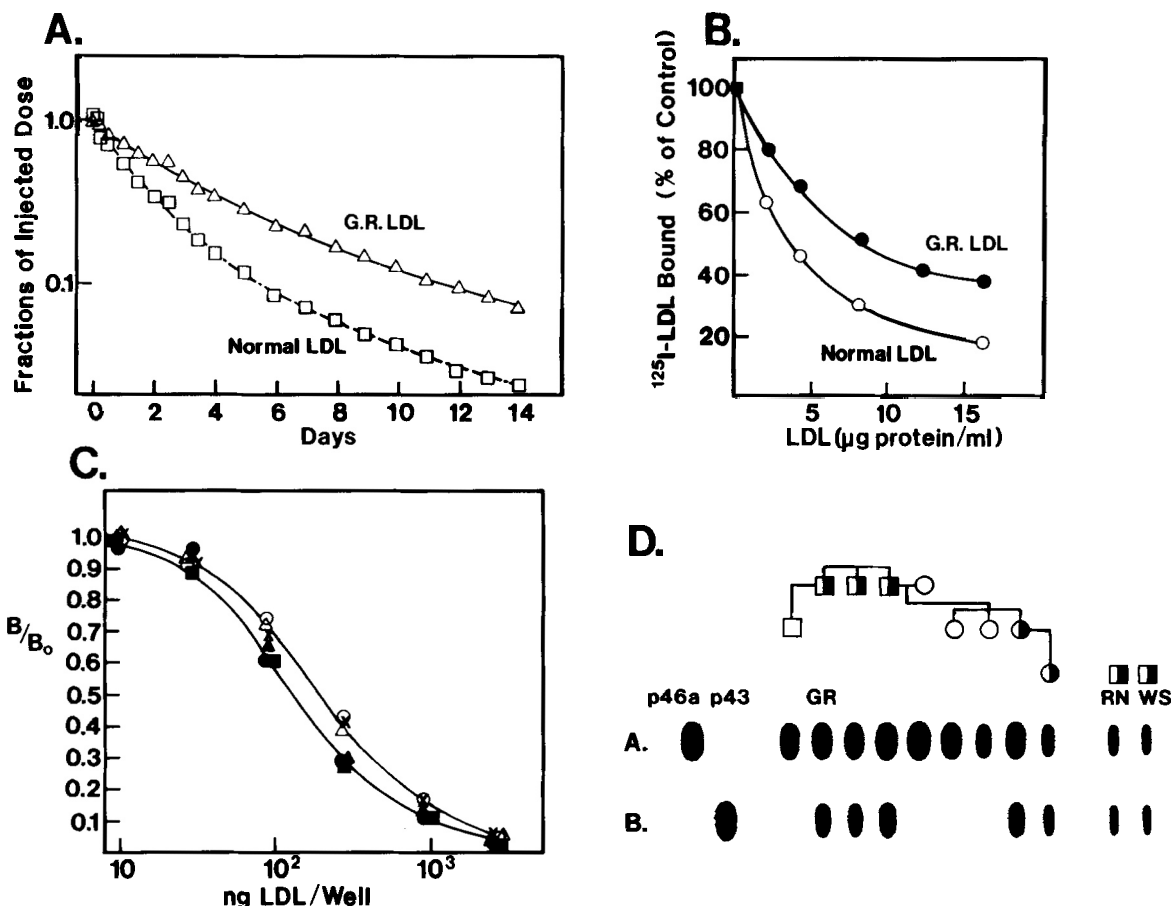
Fig. 2. Detection of FDB. (A) Plasma decay curves of G.R. LDL and normal LDL intravenously injected into subject G.R. (reproduced from the *Journal of Clinical Investigation.* 1986. **78:** 1410–1414; by permission of the American Society for Clinical Investigation. (B) Ability of G.R. LDL and normal LDL to compete with normal ^{125}I-labeled LDL for binding to LDL receptors on normal human fibroblasts (reproduced from ref. 61). (C) Ability of G.R. LDL and affected family members' LDL (lower curve) and normal LDL (upper curve) to compete with normal LDL for binding to apoB-100 monoclonal antibody MB47 (reproduced from ref. 62). (D) Differential hybridization of polymerase chain reaction-amplified DNA from the G.R. kindred with allele-specific oligonucleotide probes. Probe A identifies wild-type DNA; probe B recognizes DNA with the mutation in the codon for amino acid 3500 of the human apoB gene. Also included are DNA of plasmids p46a and p43, representing the wild-type and mutant allele sequences, respectively (modified from ref. 63).

To test this possibility directly, LDL from these five subjects were tested for their ability to bind to LDL receptors on cultured human fibroblasts in a competitive binding assay. As shown in Fig. 2B, the LDL from subject G.R. were not as effective as normal LDL in competing with normal human ^{125}I-labeled LDL for receptor binding. The LDL from subject G.R. had only about 32% of normal receptor-binding activity. In addition, the abnormal LDL were much less effective than normal LDL in competing with ^{125}I-labeled normal LDL for cellular uptake and degradation and in stimulating intracellular cholesteryl ester formation in normal human fibroblasts. Thus, in vitro tissue culture assays for receptor binding agreed with the in vivo turnover studies, indicating that the LDL from subject G.R. bind defectively to the LDL receptor. Studies of this proband's relatives indicated that the disorder, which we designated familial defective apolipoprotein B-100 (FDB), is transmitted as an autosomal co-dominant trait and that the affected subjects are heterozygotes (61).

We characterized the LDL and the apoB-100 from FDB heterozygotes in an attempt to detect any major structural abnormalities. No significant difference in lipid composition was observed between normal LDL and FDB LDL. Nor was there any detectable difference in the size or shape of these particles, either by electron microscopy of negatively stained LDL or by nondenaturing polyacrylamide gradient gel electrophoresis. Likewise, density gradient ultracentrifugation of the normal and abnormal LDL demonstrated no major differences. Furthermore, an examination of intact apoB-100 and its thrombolytic and tryptic fragments by SDS-PAGE and immunoblots using monoclonal antibodies to apoB-100 provided no evidence of any major deletions or insertions in the apoB-100 from FDB heterozygotes (T. L. Innerarity and R. M. Krauss, unpublished data).

Based on this evidence, the LDL from the FDB heterozygotes were found to be essentially normal, except for the defect in receptor binding. Thus, it seemed likely that the mutation was located in or near the receptor-binding domain of apoB-100. We probed for the mutation in intact LDL by using four apoB-100-specific monoclonal antibodies, all of which block LDL binding to the LDL receptor. Three of these reacted equally well with normal and defective LDL, whereas the monoclonal antibody MB47 bound with an enhanced affinity to LDL from G.R. and other FDB heterozygotes (62). In a solid-phase radioimmunoassay, the abnormal LDL were more effective competitors for MB47 than normal LDL were (Fig. 2C). This MB47 assay was refined so that the isolation of LDL was not necessary and plasma samples could be examined directly (62). In every individual with abnormal LDL, the plasma radioimmunoassay demonstrated that the MB47 antibody bound to these LDL with a higher affinity than to normal LDL. The absolute association of this enhanced MB47 binding with abnormal LDL suggested that the mutation in apoB-100 occurs in the region of the MB47 epitope. The MB47 epitope is definitely located in the cyanogen bromide fragment containing residues 3442 to 3569 (K. H. Weisgraber, unpublished data), and most likely in the vicinity of residues 3490 to 3510 (39).

To identify the mutation responsible for the receptor-binding defect, genomic DNA clones spanning nucleotides 7500 to 11916 of apoB-100 from the original proband were sequenced. This region, which codes for amino acids 2488 to 3901, includes the receptor-binding domain identified by the foregoing monoclonal antibody studies and the MB47 epitope. Only one unique mutation was found: in codon 3500 of apoB-100, CGG was changed to CAG, causing a glutamine-for-arginine substitution at this site within one allele but not the other (63). A rapid assay for examining DNA was developed to examine the kindred for this mutation. This procedure, using the polymerase chain reaction combined with allele-specific oligonucleotide probes, distinguishes between the mutant and normal alleles and provides a reliable assay to detect the point mutation in apoB-100 at codon 3500. Analysis of the R. family kindred with this assay detected the mutant allele only in family members who were both hypercholesterolemic and possessed binding-defective LDL (Fig. 2D) (63). In every case in which this mutation has been identified in apoB-100 genomic DNA (11 probands, see below), in vitro receptor binding experiments have shown that the isolated LDL bind defectively to the LDL receptor. It should be pointed out that even though the concordance between this mutation and the functional defect is absolute, the final proof that the relationship is causal awaits the functional testing of expressed full-length apoB-100 gene constructs differing only in this codon.

CONSEQUENCES OF THE MUTATION IN APOLIPOPROTEIN B-100 ON BINDING TO THE LOW DENSITY LIPOPROTEIN RECEPTOR

Because there is only one copy of apoB-100 per LDL particle, the FDB heterozygotes have two populations of LDL: one containing normal apoB-100 and the other containing defective apoB-100. We devised a procedure to purify the population of LDL particles containing defective apoB-100 by taking advantage of the observation that several of the FDB subjects were heterozygous for the MB19 polymorphism. The monoclonal antibody MB19 detects a common, functionally neutral apoB polymorphism and binds to the LDL of one apoB allotype with an 11-fold higher affinity than to the other (64, 65). Thus, using MB19 immunoaffinity chromatography and LDL from a subject heterozygous for both FDB and the MB19 polymorphism made it possible to isolate an LDL fraction enriched in defective receptor-binding activity. The enriched defective LDL possessed less than 10% of the receptor-binding activity of normal LDL. Because the defective LDL used in the procedure were contaminated with a small amount of normal LDL (5-7%), these results indicate that the defective LDL actually displayed only about 3-5% of the binding activity of normal LDL (66). Thus, somewhat surprisingly, if the single amino acid substitution of glutamine for arginine is the responsible mutation, as all the evidence indicates, then in a protein consisting of 4536 amino acids this substitution is sufficient to virtually abolish the receptor binding of LDL to the LDL receptor.

Results from physical biochemistry measurements indicate that the 3500 mutation causes a localized perturbation of the structure of apoB-100. The average secondary structure of normal and FDB LDL are identical as determined by circular dichroism. However, carbon-13 NMR studies have indicated that the microenvironments of about six lysines on LDL-associated apoB-100 from FDB subjects are altered so that their pK values change from 8.9 to 10.5. Lysine residues are known to be involved in apoB-100 binding to the LDL receptor (67). It is therefore possible, and perhaps even likely, that the glutamine-for-arginine substitution at residue 3500 alters the pK of lysine residues that lie in the vicinity of the mutation and the receptor-binding domain (68). These results, taken together with observations that monoclonal antibodies with epitopes that span about a thousand amino acids (approximately residues 3000 to 4000) of the apoB-100 molecule can disrupt receptor binding (40), suggest that an elaborate geometry may be involved in the binding of LDL to the LDL receptor. It follows that the disruption of receptor binding by this mutation probably involves an alteration in the conformation of the secondary or tertiary structure of the apoB-100 receptor-binding domain.

GENETIC DISORDERS ANALOGOUS TO FAMILIAL DEFECTIVE APOLIPOPROTEIN B-100

Genetic disorders analogous to FDB have been described in swine. Hypercholesterolemia and atherosclerosis in these animals have been shown to be associated with variant alleles of apoB-100 (69–71). For example, pigs homozygous for the immunologically defined marker for and apoB polymorphism termed Lpb5 possess a two- to fourfold elevation of plasma LDL. The LDL from these pigs had an abnormal composition and, when injected into either control or Lpb5 mutant pigs, had a retarded clearance from the plasma. In addition, the Lpb5 pigs had reduced in vitro LDL receptor-binding activity. However, LDL from these animals exhibit less defective binding to LDL receptors than do LDL from FDB heterozygotes, indicating that the mutation in the pig LDL is not as functionally severe as in the 3500 mutation. Moreover, when the receptor-binding region of pig Lpb5 apoB-100 DNA was sequenced, no mutations were found that were unique to this allele (69–71). Presumably, the mutation that causes the functional abnormalities and resulting hypercholesterolemia in these animals is in some other region of the molecule. If that is true, then mutations outside the receptor-binding domain of apoB-100 have the potential to alter the receptor-mediated catabolism of LDL significantly (69–71).

PREVALENCE OF FAMILIAL DEFECTIVE APOLIPOPROTEIN B-100

The development of rapid immunoassays and DNA detection assays has permitted the screening of the general population to determine prevalence of heterozygosity for this disorder. Our screening efforts have centered on four widely dispersed geographical regions: San Francisco, Dallas, Montreal, and Salzburg, Austria. Not only were the samples from different regions, but the plasma cholesterol levels of the subject population were dramatically different (**Fig. 3**). The ethnically homogenous Salzburg population was part of a coronary study and consisted of 110 subjects with coronary disease (as determined by EKG or coronary angiography) and 130 controls, of whom 117 were matched for age and sex (72). Most of the San Francisco and Dallas sample groups were moderately hypercholesterolemic, whereas a majority of the French Canadians from Montreal were moderately to severely hypercholesterolemic, and many were FH heterozygotes. A total of 1100 individuals were screened for FDB by one or more of the following procedures: the competitive binding of plasma LDL to the LDL receptors on cultured human fibroblasts, the binding of monoclonal antibody MB47 to LDL in a competitive radioimmunoassay using whole plasma, and the detection of a single base change at codon 3500 using allele-specific probes on polymerase
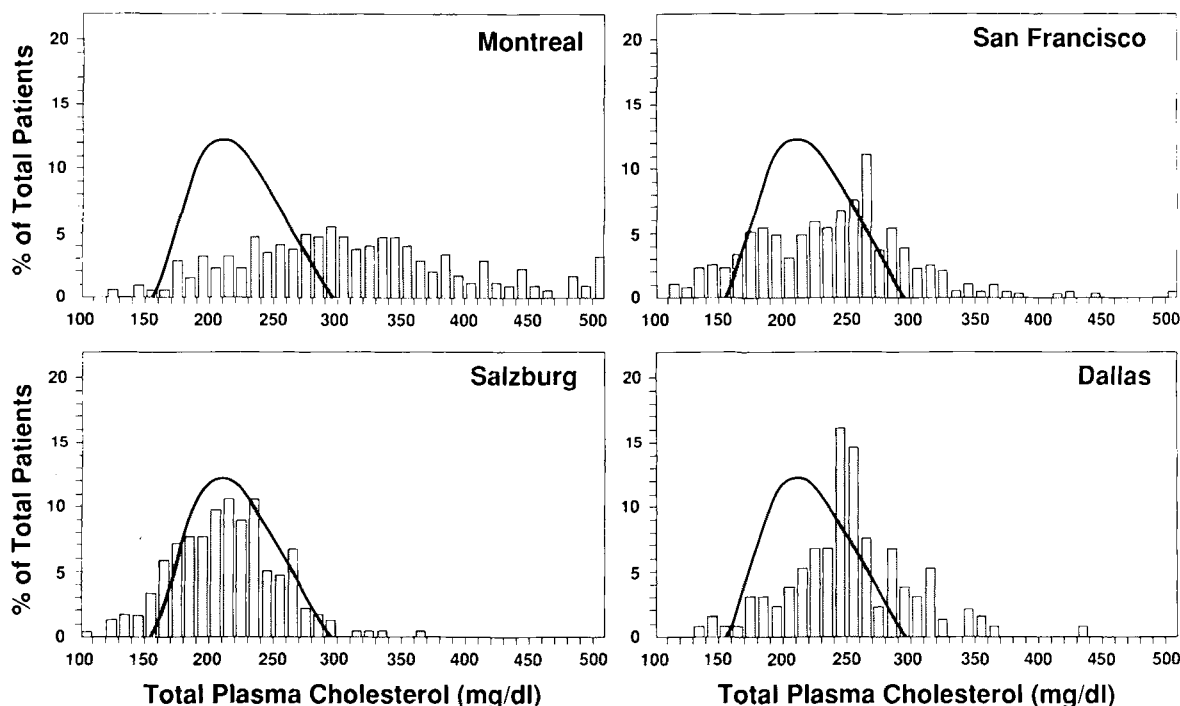
Fig. 3. The plasma cholesterol distribution of the subjects tested for FDB from the different geographic regions. The average cholesterol distribution in the United States is shown as the curve in each panel.

TABLE 1. Prevalence of familial defective apolipoprotein B-100 in four populations

Region	Number of Samples	MB47	Assay DNA	Tissue Culture	Affected Probands	Total Affected Subjects
Montreal	343	143	333	33	1	1
Salzburg	236	12	224	13	2	8
San Francisco	388	165	212	261	4	18
Dallas	133	38	80	119	4	14
Total	1100	358	849	426	11	41

chain reaction-amplified genomic DNA.

This extensive screening resulted in the discovery of 11 probands who were heterozygous for FDB; all four regions were represented in the 11 (**Table 1**). Family studies of these probands identified a total of 41 individuals heterozygous for FDB. No homozygotes for FDB have thus far been found. Since the populations sampled were for the most part hypercholesterolemic, it is not possible to calculate the frequency of this disorder with precision. However, it is possible to obtain a rough estimate of the frequency of this disorder in the populations sampled. From the extensive, multi-centered Lipid Research Clinic (LRC) Population Studies, the cholesterol distribution of a random group of 48,482 Americans has been compiled and grouped by age and sex (5). In terms of cholesterol levels, it is possible to correct the bias of our sample group by comparing them with this large, random population (**Table 2**). This cholesterol frequency normalization depends on the reasonable assumption that the cholesterol distribution of the total unselected population

in Salzburg, Dallas, San Francisco, and Montreal is similar to that of the LRC population. Based on this assumption, the derived frequency of this disorder is about 1/500 in our sampled population (Table 2). Consistent with this approximation are the results from a study of 745 hyperlipidemic individuals from the United Kingdom and Scandinavia, which identified 10 probands with FDB. The frequency of this disorder in this group was 3%, and the overall frequency in the population was estimated to be 1/600. If this estimate is correct for the general population, then the prevalence of this disorder is equivalent to that of FH (1/500), making it one of the most common single-gene mutations responsible for causing a clinical abnormality.

The apparent relatively high frequency of this mutation and the occurrence of the mutation in several different populations raise questions concerning its origin. Furthermore, the fact that the mutation occurs at a hypermutable CG dinucleotide suggests that the mutation could be recurrent, as has been shown for mutations at CG

TABLE 2. Procedure used to estimate the frequency of familial defective apolipoprotein B-100 heterozygotes in the populations sampled

Group[a] (Age/Sex/Chol)	Number of Individuals				Frequency[b] (FG)
	This Study		LRC Study[a]		
	Probands (I)	Total in Group (II)	Total in Group (III)	Study Total (IV)	
30–34/M/>254	1	22	122	48,482	0.000114
45–49/M/234–257	1	32	344	48,482	0.000222
45–49/M/>276	1	30	115	48,482	0.000079
55–59/M/262–275	1	15	81	48,482	0.000111
65–69/M/210–232	1	5	188	48,482	0.000775
65–69/M/>274	1	9	38	48,482	0.000087
35–39/F/>245	1	18	101	48,482	0.000116
50–54/F/>285	1	17	100	48,482	0.000121
60–64/F/>296	1	30	54	48,482	0.000037
60–64/F/>296	1	30	54	48,482	0.000037
65–69/F/277–296	1	6	41	48,482	0.000141
				FTP =	0.00184

[a]From ref. 5.
[b]Frequencies (FG, frequency of the defect in each age/sex/cholesterol group) were calculated according to the following formula: (I/II) × (III/IV) = FG; where I = number of probands from our study in each LRC age/sex/cholesterol group; II = number of individuals from our study in the same age/sex/cholesterol group as the proband; III = number of individuals in the LRC study in the same age/sex/cholesterol group as the proband; IV = total number of individuals in the LRC study. FTP = $\Sigma FG_1 \dots FG_{11}$; FTP = frequency of FDB in the total population we sampled, which is the sum of the frequencies of all of the age/sex/cholesterol groups. FG_1 is the frequency in age/sex/cholesterol group 1, e.g., 0.000114. If no proband occurs in an age/sex/cholesterol group, the frequency for that group is zero.

doublets in other genes, e.g., factor VIII (73). For these reasons, we have conducted a detailed haplotype analysis in eight kindreds in which the proband is heterozygous for the mutation in codon 3500.
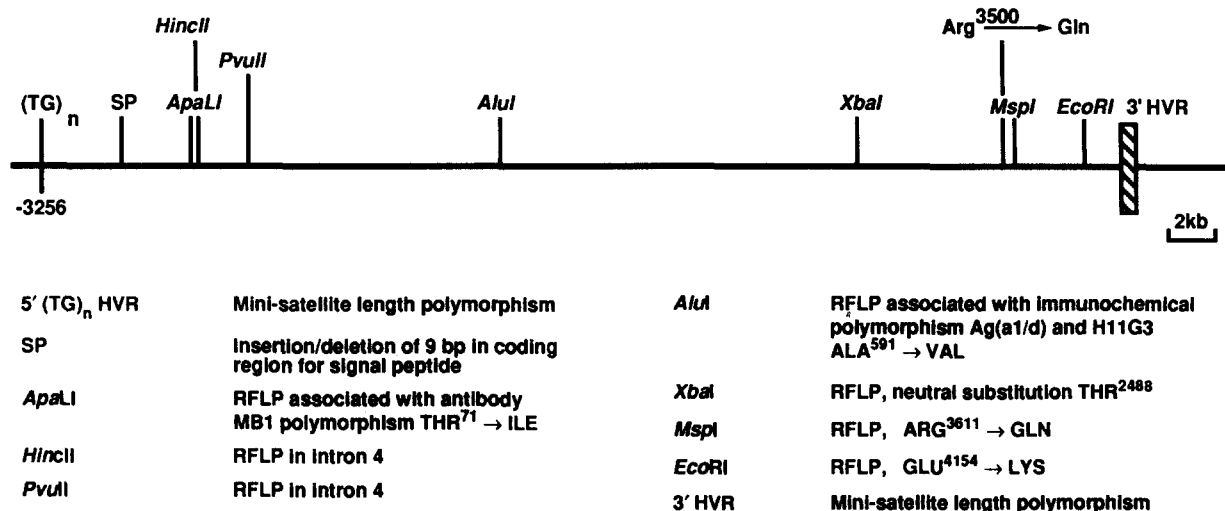
The haplotype markers used for this analysis are illustrated in **Fig. 4**. Eight of the 10 DNA markers used are conventional diallelic markers, i.e., restriction fragment length polymorphisms or insertion/deletion polymorphisms, and the other two are minisatellite hypervariable multi-allelic markers. The hypervariable region at the 3' end of the gene comprises 17 resolvable alleles containing different numbers of 15-base pair (bp) repeats (18, 74). That at the 5' end comprises seven alleles of the sequence $d(TG)_n$, where n is between 12 and 18. The use of eight diallelic markers and these two hyperviariable regions provides very high resolving power for distinguishing many haplotypes. In the absence of linkage disequilibrium, the theoretical number of alleles that can be distinguished is more than 30,000 (i.e., $2^8 \times 7 \pm 17$). In a study of eight kindreds, all 85 subjects proved to be heterozygous for at least one marker, and all but two were heterozygous for two or more markers (75).

An example of a haplotype analysis of one kindred is illustrated in **Fig. 5**. Of the 18 individuals studied, five were heterozygous for the $Arg_{3500} \rightarrow Gln$ mutation. In this particular family, all individuals were heterozygous for at least three of the 10 markers, and thus the inheritance of each haplotype can be unequivocally deduced. Furthermore, the haplotype upon which the 3500 mutation is superimposed may be deduced from the data. In this family and in six others studied by the same methods, the haplotype of the mutant allele proved to be identical (**Table 3**). The haplotype of the eighth proband examined

differed only for the 3' HVR marker, where the number of repeats was 46 rather than 48. We attribute this either to recombination between the 3500 mutation and the 3' HVR or, what is more likely, to slippage during DNA replication, which has been observed in studies of the inheritance of other minisatellite markers (76). Genotype analysis of six other mutant probands in the absence of pedigree data yielded data consistent with this same major haplotype for the mutant allele (75).

Given the high resolution of this haplotype analysis, the fact that seven of eight probands carry identical mutant haplotypes argues strongly against the possibility of recurrent mutations. These data are much more consistent with a founder effect, although it does not rigorously prove that this is the case. Furthermore, the fact that the populations from which these probands were drawn are predominantly, if not exclusively, Caucasian implies that the putative "founder" was a member of this ethnic group.

Although it is likely that other mutations that disrupt LDL receptor binding and cause familial defective apoB-100 will be found, we predict that the mutation in the codon for residue 3500 will be by far the most common one that causes a ligand-binding abnormality. We base this prediction on the following observations. Using an in vitro receptor binding assay, we examined LDL from over 400 individuals from our population study for binding activity. We found six unrelated probands with severely binding-defective LDL. The DNA from these individuals was examined for the glutamine-for-arginine substitution at residue 3500, and all six were heterozygous for this mutation. Thus, unlike the situation with FH, in which no single mutation predominates in the general population (9), it appears that in FDB the 3500 mutation is by

5' $(TG)_n$ HVR	Mini-satellite length polymorphism
SP	Insertion/deletion of 9 bp in coding region for signal peptide
ApaLI	RFLP associated with antibody MB1 polymorphism $THR^{71} \rightarrow ILE$
HincII	RFLP in intron 4
PvuII	RFLP in intron 4

AluI	RFLP associated with immunochemical polymorphism Ag(a1/d) and H11G3 $ALA^{591} \rightarrow VAL$
XbaI	RFLP, neutral substitution THR^{2488}
MspI	RFLP, $ARG^{3611} \rightarrow GLN$
EcoRI	RFLP, $GLU^{4154} \rightarrow LYS$
3' HVR	Mini-satellite length polymorphism

Fig. 4. Haplotype markers for the human apoB gene. The 5' hypervariable region $(TG)_n$ is 3256 bp 5' of the transcription start site, and the 3' hypervariable region (3' HVR) is 491 bp 3' of the translational termination site. SP represents a 9-bp insertion/deletion in the signal peptide. All other sites are restriction fragment length polymorphisms (RFLP) caused by single-base substitutions. (Modified from ref. 75).
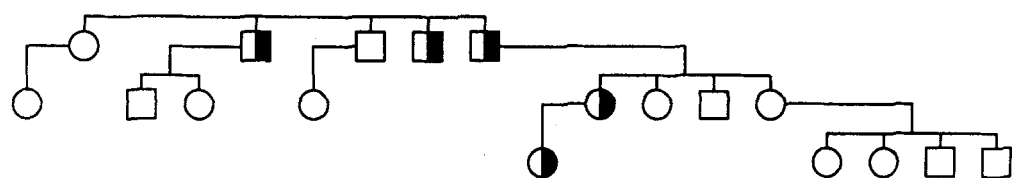
ID	JG	EB	RS	CL	LeS	JM	LS	MS	WS	KL	JL	TN	BS	SS	KJ	SJ	JJ	RW
5'(TG)$_n$	14/15	14/14	14/15	15/15	15/14	14/14	14/15	14/14	15/14	14/14	15/14	14/15	14/15	15/15	15/15	15/14	14/15	14/15
SP	+/+	+/-	+/-	-/+	-/+	+/-	+/-	-/+	-/+	+/+	+/+	+/-	+/-	+/-	+/-	-/-	-/-	-/-
ApaLI	+/+	+/-	+/-	-/+	-/+	+/-	+/-	-/+	-/+	+/+	+/+	+/-	+/-	+/-	+/-	-/-	-/-	-/-
HincII	-/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	-/-	+/-	+/-	+/-	-/-	-/-	-/-
PvuII	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	-/-	+/-	+/-	+/-	-/-	-/-	-/-
AluI	-/-	-/+	+/-	-/-	-/-	-/+	-/-	+/-	-/-	-/-	+/-	-/-	+/-	+/-	+/+	+/-	+/-	+/-
XbaI	-/-	-/+	+/-	-/-	-/-	-/-	-/-	+/-	-/-	-/-	-/-	+/-	-/-	-/-	-/+	+/-	+/-	+/-
Arg → Gln	-/-	-/-	-/-	-/-	-/+	-/-	-/-	-/+	-/+	-/+	-/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-
MspI	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
EcoRI	+/-	+/+	+/-	-/-	-/-	+/+	+/-	+/-	-/-	+/-	+/-	+/-	+/-	+/-	+/+	+/-	+/-	+/-
3' HVR	34/46	34/36	36/38	38/48	38/48	34/34	34/38	36/48	38/48	34/48	30/48	36/38	30/38	30/38	30/36	36/38	36/38	36/38
C (mg/dl)	208	271	213	210	197	188	172	257	231	253	247	141	243	182	174	154	171	219
LDL-C (mg/dl)	122	193	146	136	122	112	102	168	183	190	171	78	178	105	115	86	98	147
LDL-IC$_{50}$ (µg/ml)	3.1	2.3	2.3	2.8	6.6	1.9	1.8	5.4	9.9	7.4	9.4	2.7	2.3	2.2	3.2	2.8	2.4	2.4
Age	40	69	42	39	68	40	64	52	66	19	42	21	37	38	21	18	19	23

Fig. 5. Haplotype analysis of one of the kindreds with Arg$_{3500}$→Gln mutation. The half-solid symbols represent subjects heterozygous for the Arg$_{3500}$→Gln mutation. For the seven single-base substitution restriction fragment length polymorphisms, the (+) represents digestion at the restriction site. For the single-peptide (SP) insertion/deletion, the (+) represents the presence of the 9-bp insertion/deletion and the (−) its absence. The numbers for the 5' (TG)$_n$ and the 3' HVR represent the number of TG dinucleotides or 15-bp repeats, respectively. C (mg/dl), total plasma cholesterol. LDL-C (mg/dl), LDL cholesterol. LDL-IC50 (mg/ml), the concentration of unlabeled LDL needed to displace 50% of normal ^{125}I-labeled LDL from LDL receptors on normal human fibroblasts (see ref. 61 for the procedure).

far the most common one. It is also possible that mutations of apoB-100 that cause less severe receptor-binding abnormalities would not be readily detected by an in vitro receptor binding assay.

CLINICAL FEATURES

The identification of 41 FDB heterozygotes has given us an opportunity to examine the impact of this defect on plasma lipid levels and other clinical features. Most FDB heterozygotes did not possess the characteristic clinical features of FH heterozygotes, which include cholesterol deposition in the tendons (xanthomas) and the eyes (arcus cornea). Only the proband from the Montreal population had arcus corneae and tendon xanthomas in the extensor tendons of the hand. However, eight of 10 individuals identified as FDB heterozygotes from a hyperlipidemic population from the United Kingdom and Scandinavia had arcus corneae and xanthomas (77).

Except for one family in our study, there were no major differences in the mean plasma triglyceride, VLDL, and high density lipoprotein levels between affected and unaf-

TABLE 3. Summary of the haplotype analysis of eight kindreds in which the proband was heterozygous for the Arg$_{3500}$ → Gln mutation

Kindred Number	5' (TG)$_n$	SP	ApaLI	HincII	PvuII	AluI	XbaI	MspI	EcoRI	3' HVR
1, 2, 3, 4, 5, 6, 7	14	+	+	−	−	−	−	+	−	48
8	14	+	+	−	−	−	−	+	−	46

fected family members. The most readily detected manifestation of the FDB gene defect is hypercholesterolemia. As shown in **Fig. 6A**, the hypercholesterolemia in the subjects identified in our study is moderate. The average plasma cholesterol level of 269 mg/dl for the 41 FDB heterozygotes we studied is considerably lower than the mean plasma cholesterol levels of large groups of FH heterozygotes ($\sim$360 mg/dl) (9). The plasma cholesterol level of each FDB subject was compared with the average plasma cholesterol level of the age- and sex-matched controls from the LRC Population Studies (Fig. 6B). Familial defective apoB-100 heterozygotes had an average plasma cholesterol level 81 mg/dl higher than the 50th percentile levels of the age- and sex-matched LRC controls. For comparison, 44 unaffected family members of the proband families had an average plasma cholesterol level only 7 mg/dl higher than that of the LRC controls. In addition, FDB heterozygotes had an average plasma LDL cholesterol level of 199 mg/dl. This was 71 mg/dl higher
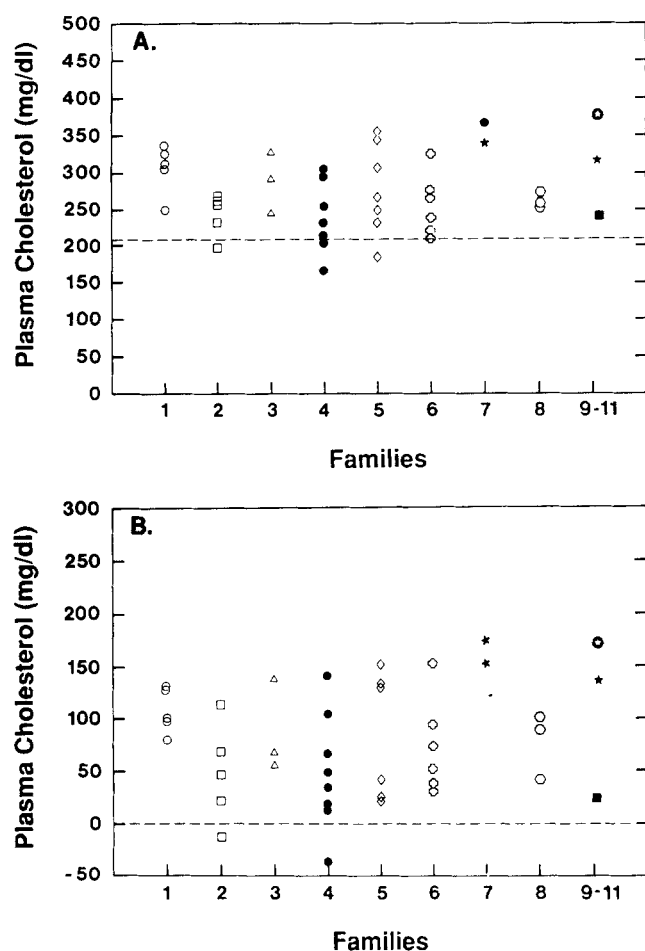
than that of the LRC controls, whereas the average plasma LDL level of unaffected family members was only 4 mg/dl higher than that of the controls. Therefore, the increased plasma cholesterol level of these FDB heterozygotes was due to an elevation of plasma LDL, as expected. Whether a comparison is made with the large LRC control group or with unaffected family members, it is clear that this single amino acid mutation causes a major increase in plasma LDL cholesterol. Moreover, the 10 FDB heterozygotes in the Tybjaerg-Hansen et al. study (77) had a mean plasma cholesterol level of 369 mg/dl, which is remarkably similar to the mean cholesterol level of FH heterozygotes. Their average plasma cholesterol levels were 163 mg/dl higher than those of the LRC age- and sex-matched controls. From these two studies, it is clear that, with the exception of mutations in the LDL receptor, no other genetic mutation causes such a large increase in plasma LDL cholesterol.

The impact of this mutation on atherosclerosis cannot be determined with certainty from the limited number of FDB subjects identified. However, Tybjaerg-Hansen et al. (77) reported that seven of 10 of their FDB heterozygotes had coronary heart disease. Because all these subjects had previously been classified as hypercholesterolemic, and hypercholesterolemia is clearly multifactorial, it cannot be taken as prima facie evidence that the FDB mutation alone predisposes these individuals to accelerated atherosclerosis. Thus, a direct causal relationship between this mutation and atherosclerosis has not been established. Nevertheless, despite these caveats, it is not unreasonable to propose that the considerable increase in cholesterol level associated with this mutant allele does increase the risk of premature heart disease.

Fig. 6. The effect of FDB on cholesterol levels. (A) The plasma cholesterol levels of all FDB heterozygotes. The dashed line represents the average cholesterol level in the United States. (B) The plasma cholesterol concentration of each FDB heterozygote was compared with the 50th percentile of his or her age- and sex-matched LRC controls. Each point represents the plasma concentration above (or below) the control, which is shown as a dashed line.

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