Evidence for a third genetic locus causing familial hypercholesterolemia: a non-LDLR, non-APOB kindred[1]

L. Haddad,[2,*] I. N. M. Day,[3,*] S. Hunt,[†] R. R. Williams,[†] S. E. Humphries,[*] and P. N. Hopkins[4,†]

Centre for Cardiovascular Genetics,* Department of Medicine, University College London Medical School, The Rayne Institute, 5 University Street, London WC1E 6JJ, United Kingdom, and Cardiovascular Genetics,[†] Department of Internal Medicine, University of Utah, 410 Chipeta Way, Room 167, Salt Lake City, UT 84108

Abstract **Monogenically inherited hypercholesterolemia is most commonly caused by mutations at the low density lipoprotein receptor (LDLR) locus causing familial hypercholesterolemia (FH) or at the apolipoprotein B (APOB) locus causing the disorder familial defective apoB (FDB). Probands from 47 kindreds with a strict clinical diagnosis of FH were selected from the Cardiovascular Genetics Research Lipid Clinic, Utah, for molecular genetic analysis. Using a combination of single-strand conformation polymorphism (SSCP) and direct sequencing, 12 different LDLR gene mutations were found in 16 of the probands. Three of the probands were carriers of the APOB R3500Q mutation. In five of the remaining 28 pedigrees where no mutation had been detected, samples from enough relatives were available to examine co-segregation with the LDLR region using the microsatellite marker D19S221, which is within 1 Mb centromeric of the LDLR locus, and D19S394, sited within 150 kb telomeric of the LDLR locus. In four of the families there was strong evidence for co-segregation between the LDLR locus and the phenotype of hypercholesterolemia, but in one large family with 18 living affected members and clear-cut bimodal hypercholesterolemia, there were numerous exclusions of co-segregation. Using length polymorphic markers within and outside the APOB gene, linkage of phenotype in this family to the APOB region was similarly excluded. In this large family, the degree of hypercholesterolemia, prevalence of tendon xanthomata, and occurrence of early coronary disease were indistinguishable from the other families studied. In summary, the data provide unequivocal evidence that a third locus can be etiological for monogenic familial hypercholesterolemia and should be reinvigorating to research in this field.**—Haddad, L., I. N. M. Day, S. Hunt, R. R. Williams, S. E. Humphries, and P. N. Hopkins. **Evidence for a third genetic locus causing familial hypercholesterolemia: a non-LDLR, non-APOB kindred.** ***J. Lipid Res.*** **1999.** 40: **1113–1122.**

Supplementary key words familial hypercholesterolemia • LDLR gene • APOB gene • microsatellites

Segregation of severely elevated plasma low density lipoprotein cholesterol levels, tendon xanthomata, and premature coronary disease occur in the well-recognized disorder of familial hypercholesterolemia (FH). FH has provided a paradigm in the study of genetics of atherosclerosis, including a detailed understanding of the major pathway of low density lipoprotein (LDL) clearance from plasma via the LDL receptor (1). A wide range of different defects in this receptor accounts for the majority of (if not all) FH homozygotes (2) and for many FH heterozygotes also (3). Ligands for the LDL receptor include apoB, present as a single protein molecule per LDL particle, and apoE, present in multiple copies in very low density lipoprotein (VLDL) and in intermediate density lipoprotein (IDL). ApoB defects in receptor binding remained a plausible cause of familial hypercholesterolemic phenotype (1), and an example was presented (4) and subsequently shown to involve a specific mutation, R3500Q in APOB (5). Extensive studies since have shown this single mutation to be specific to western European populations and their descendants, to have a population prevalence up to 1/1,000 and to account typically for 2–5% of patients with the familial hypercholesterolemic phenotype (6). The apoB mutation was designated familial defective apoB-100 (FDB). Other apparently rarer APOB mutations have since been identified in hypercholesterolemic patients (7–9).

The picture of receptor defects and ligand defects ap-

Abbreviations: APOB, gene encoding apolipoprotein B; APOE, gene encoding apolipoprotein E; BMI, body mass index (kg/m^2); BP, blood pressure (mm Hg); FDB, familial defective hypercholesterolemia; HDL, high density lipoprotein; HVR, hypervariable repeat; LDL, low density lipoprotein; LDLR, gene encoding low density lipoprotein receptor; PCR, polymerase chain reaction; SSCP, single-stranded conformational polymorphism; TG, triglyceride; VLDL, very low density lipoprotein; VNTR, variable number tandem repeats.

[1] This paper is dedicated to Roger R. Williams, our dear colleague and friend, who brought together the co-authors and this study. He died in the Swissair disaster September 1998.

[2] Present address: Complex Traits Analysis Group, Imperial College School of Medicine (Hammersmith Campus), DuCane Road, London W12 0NN, UK.

[3] Present address: Wessex Human Genetics Institute. Duthie Building (MP808), Southampton University Hospital, Tremona Road, Southampton S016 6YD, UK.

[4] To whom correspondence should be addressed.

pears complete, and to our knowledge little attention has been paid to the possibility of further loci causing hypercholesterolemia. However, there is a case that other loci could be involved.

1. Direct mutation detection has not identified mutations in all FH patients whether homozygous, heterozygous, proven LDLR deficient, or not (2, 3 and references therein, 10); International Workshop on Familial Hypercholesterolemia and the LDL-receptor, Amsterdam, Holland 1997). It is assumed but not proven that in each instance the failure is technical, due, for example, to insensitive methods or not all of the gene having been examined, e.g., introns.

2. All homozygotes appear to be LDLR deficient. However, this could be secondary to another genetic defect, e.g., absence of a transcriptional activator.

3. LDLR mutation homozygotes are rare (10^{-6}) representing an LDLR mutant allele frequency of 10^{-3} in the general population (11). If another locus with an allele frequency of 10^{-4} were present in the general population, the homozygote frequency would be 10^{-8}. Such homozygotes would thus be too rare to be present in existent homozygote collections.

4. Homozygosity for another locus may not be expressed in the profound and characteristic way that homozygous LDLR defects are. This is indeed the case for apoB R3500Q homozygotes who can remain free of symptoms until a late age (12, 13). Atypical cases of hypercholesterolemia have been reported (14).

5. In no study of heterozygous FH from genetically heterogeneous populations, such as the UK or US, has more than 70% of probands been shown directly to have LDLR mutations. Usually in heterozygous FH patients LDL receptor activity is low, but receptor assays are complex and may not always represent hepatic receptor function or overall in vivo activity. Some heterozygous FH probands have been reported with LDL receptor activity within or near the reference range (10, 15, 16). However, the lack of concordance between cellular and clinical phenotypes cannot always be invoked as the explanation (17); Sirtori et al. (18) described a case of increased LDL biosynthesis.

One approach to confirm the involvement of the LDL receptor locus in causing FH in a kindred is by co-segregation studies using DNA markers spanning the LDL receptor locus. Co-segregation studies are rarely undertaken because kindred collection is difficult and laborious. Additionally, until recently, only intragenic haplotyping using RFLPs has been available for the LDL receptor locus, itself labor intensive and often uninformative or laborious (19 and references therein). For this reason, comparatively few LDLR co-segregation studies have been published and these have often been reports on small families with markers of low informativeness. Although it seems likely that LDLR mutations obscure to current mutation scanning will turn out to be etiological in many FH families, up to 30% of uncharacterized autosomal dominant heterozygous FH could be attributable to autosomal dominant mutations other than in LDLR and other than apo R3500Q. Many genes would be plausible candidates including those known from studies of cell function (20).

In order to search for non-LDLR FH we first established a rapid approach to co-segregation based on highly informative, microsatellite polymorphisms closely flanking the LDLR gene (21). The physical locations of these markers are now known (22). We have examined genetic recombination closely and have also demonstrated very strong linkage disequilibrium between microsatellite locus D19S394 and LDLR (23). D19S394 is a tetranucleotide repeat sited 150 kb telomeric to LDLR; it shows 90% heterozygosity, amplifies well by PCR, and is readily genotyped. Furthermore, it is compatible with buccal wash sampling by mail for rapid kindred review. As a one-step primary evaluation of FH kindreds, this is a powerful tool. Here we describe its use in conjunction with D19S221 (22), an APOB intron 20 dinucleotide length polymorphism (24), APOB 3′ VNTR (25), and polymorphic microsatellite D2S220 (26), to exclude co-segregation of clear-cut bimodal hypercholesterolemic phenotype with either the LDLR gene or the APOB gene in a large Caucasian kindred. We have designated the gene causing hypercholesterolemia in this family as FH-3.

METHODS

Ascertainment of FH families in Utah

In 1976 the University of Utah Cardiovascular Genetics Research Clinic was founded and began to identify and examine persons with early familial coronary heart disease (CHD) and their close and distant relatives including persons with heterozygous familial hypercholesterolemia (27, 28). From 1985 to the present, expanded ascertainment of FH families was carried out as a humanitarian public health project called "Med Ped FH" (Make Early Diagnoses to Prevent Early Deaths from Familial Hypercholesterolemia) in collaboration with the Utah Department of Health, the U.S. Centers for Disease Control, the World Health Organization, and Merck Human Health (29, 30).

Possible FH index cases were found by contacting highrisk persons from computer files. These included those with total cholesterol above 300 mg/dl from records of state and local health department screening of 75,000 persons, hospital data identifying over 5,000 persons discharged with diagnosis of CHD before age 55, shopping mall cholesterol screening of 7,000 persons, referrals from responses to a letter sent to 720 primary care physicians in Utah asking them to identify patients with FH, and "Health Family Tree" questionnaires collected from the parents of 80,000 high school students in Utah (31). Follow-up mail questionnaires and reminder letters were sent to 3,598 persons who were considered at risk for FH with follow-up phone contacts. These included probable FH index cases and relatives traced to first, second, and third degree relationships (siblings, parents, offspring, aunts, uncles, grandparents, nieces and nephews, and first cousins). Details regarding highest pretreatment fasting cholesterol, as well as triglyceride, HDL cholesterol, and calculated LDL were obtained from 2,038 persons, including 502 FH heterozygotes (32). DNA testing for FH began in Utah in 1986 with successful genetic linkage of one large seven generation Utah pedigree to a biallelic RFLP in the LDL receptor gene (33). Clinical criteria for the diagnosis of FH in relatives and new index cases were derived using mathematical analysis of cholesterol distributions published for the general US population and persons

with FH. These criteria were validated by comparing clinical versus DNA diagnosis of FH for persons whose status could be unambiguously determined from their status with respect to the RFLP linked to the LDL receptor (29).

When these criteria were applied to serum cholesterol results obtained from the 2,038 persons traced, 101 index cases and 502 living relatives were identified, meeting criteria for FH. Genealogical tracing of ancestors in 4–8 previous generations and computer-assisted matching found common ancestors for about half of the index cases, merging 101 small pedigrees into 48 unrelated multigenerational pedigrees. An LDL receptor mutation was identified in the laboratory of Dr. Helen Hobbs, Dallas, Texas for one pedigree (34), leaving an additional 47 pedigrees from which samples were taken for the present study.

Blood samples were collected in the morning after a 12–16 h fast, and prepared according to guidelines of the Lipid Research Clinic's program manual of Laboratory Operations (35). Lipids were measured by a microscale procedure developed in the laboratory (36). Briefly, high density lipoprotein (HDL) was measured in the supernatant after precipitation of apolipoprotein B-containing particles with dextran sulfate–$MgCl_2$ and centrifugation in an Eppendorf microcentrifuge. Triglyceride-rich lipoproteins, primarily VLDL, were separated from LDL and HDL by use of a Beckman TL 100 ultracentrifuge. The value for VLDL cholesterol was taken as the measured cholesterol in the top fraction. This value was compared to the total cholesterol minus the cholesterol in the bottom fraction containing LDL and HDL and verified to yield identical results. Cholesterol and triglycerides in total plasma and subfractions were measured with a Roche FARA II automated analyzer. DNA was obtained from seven families (86 individuals) for analysis of co-segregation of phenotype with the LDL receptor gene by the phenol/chloroform extraction based on that of Kunkel et al. (37).

PCR conditions

Two polymorphic microsatellites, D19S394 and D19S221, were examined, telomeric and centromeric of the LDLR gene, respectively. D19S394 is a tetranucleotide (GAAG)n repeat approximately 150 kb from the gene, with a heterozygosity of 0.9. Oligonucleotide sequence and optimal conditions are as described in Day et al. (23). D19S221 is a dinucleotide (CA)n repeat, with a heterozygosity of 0.87. PCR primer sequences were: 5′-GCAAGA CTCTGACTCAACAAAA-3′ and 5′-CATAGAGATCAATGGCATG AAA-3′. One primer of each pair contained the fluorophor 6-carboxyfluorescein (6-FAM) coupled to the 5′-end by phosphoramidite linkage. For PCR amplification of D19S221, the reaction was performed in 20 ml total volume, containing 40–100 ng of genomic DNA, 8 pmol of each PCR primer, 0.3 U Taq polymerase (Gibco-BRL Ltd, Paisley UK), 200 mmol/l each dNTP, 50 mmol/l Tris-HCL (pH 8.3), W1 (Gibco BRL Ltd, Paisley, UK), and overlaid with 20 ml of paraffin oil. Thermal cycling conditions were: 95°C for 5 min, 55°C for 5 min, 72°C for 5 min, 96°C for 1 min, 55°C for 1 min, and 72°C for 3 min for 30 cycles; and finally 72°C for 10 min.

To exclude the involvement of the apoB locus as a cause of FH, three markers were analyzed for haplotype analysis of one family (1000/1173): D2S220 (CA)n (25), heterozygosity 0.82, a polymorphic (TTTA)n tandem repeat located in intron 20 of the apoB gene with a heterozygosity of 0.66 (24), and a variable number tandem repeat located at the 3′ end of the apoB gene, heterozygosity 0.75 (25). The intron 20 tetranucleotide repeat sense strand PCR primer was FAM labeled as above and one D2S220 PCR primer was HEX-labeled for fluorescent genotyping. Other components of the PCR reaction mixes for the intron 20 repeat and D2S220 and the 3′ hypervariable repeat were as for D19S221. Cycling conditions for D2S220 were: 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min repeated for 27 cycles then 72°C for 6 min. Thermal cycling conditions for the intron 20 repeat were 95°C for 4 min, 95°C for 1 min, 68°C for 3 min repeated 25 cycles. For the 3′ hypervariable repeat conditions were as follows: 95°C for 5 min, 58°C for 5 min; 92°C for 1 min, 58°C for 5 min repeated for 35 cycles. Thermal cycling was performed in an MJ Tetrad (Genetic Research Instrumentation Ltd (GRI), Felsted, Essex, UK).

Amplified fragments of fluorescently labeled products were electrophoresed on an ABI Prism 377 automatic sequencer (Foster City, CA), and separated on standard 12 cm well-to-read 6% denaturing SequaGel-6™ polyacrylamide gels (National Diagnostics, Atlanta, GA) (21). The 3′ hypervariable repeat was sized by electrophoresis on a 36 cm 6% polyacrylamide denaturing gel (acrylamide–bis-acrylamide 19:1 (Severn Biotech Ltd), 1 × TBE) for 4 h at a constant power of 65 W. The gel was subsequently silver stained (38, 39).

FDB R3500Q direct test

Seven affected family members of Kindred 1000/1173 and suitable positive controls were tested for the presence of the mutation R3500Q in the apoB gene by introduction of a restriction site (Sau 96I) during PCR. PCR amplification was carried out on an MJ Tetrad thermal cycler using conditions as described (40). Briefly, the reaction mixture contained 40 ng genomic DNA, 0.2 mm dNTPs, 10 mm Tris-HCL (pH 8.3), 50 mm KCl, 0.001% gelatin, 0.05% w/v W1, and 8 pmol of each primer. PCR amplified products (8 ml) were tested for the presence of FDB R3500Q by digestion with 3 U Sau96I (Boehringer Mannheim), 1 ml manufacturer's recommended 10× buffer, incubation at 37°C for 3 h. Restriction digest patterns were analyzed by using the microtiter array diagonal gel electrophoresis (MADGE) system (41). A Sau96I restriction site was induced in the PCR of normal alleles only, not of FDB R3500Q alleles. Sau96I digest is therefore 100% sensitive, although not specific to R3500Q.

APOE testing

ApoE genotypes were determined by a genotyping method routinely performed in the laboratory involving PCR-RFLP, described by Wu, Wu, and Hopkins (42).

RESULTS

Forty seven probands with a clear cut clinical diagnosis of FH were analyzed for mutations in the LDLR gene by a combination of SSCP and direct sequencing (**Fig. 1**). Three of these probands were found to have the FDB R3500Q substitution. As shown in **Table 1**, twelve different mutations were found, two of which are novel, in 16 apparently unrelated patients, a detection rate of 36.2%.

Of the remaining 28 probands in whom no mutation was seen, five pedigrees were selected on the basis of large kindred size and possible sample availability, for co-segregation studies using microsatellite markers D19S394 and D19S221. Eighteen allele lengths were observed for D19S394 (range 211–279 mobility units, equivalent to nucleotides), and ten for D19S221 (range 192–210 mobility units, equivalent to nucleotides). As data were gathered for co-segregation studies, four families showed no inconsistency with co-segregation of phenotype with LDLR gene, i.e., although small numbers of relatives had been examined, the lod score for each family was above zero (**Table 2**) for markers D19S394 and D19S221. However, one medium-

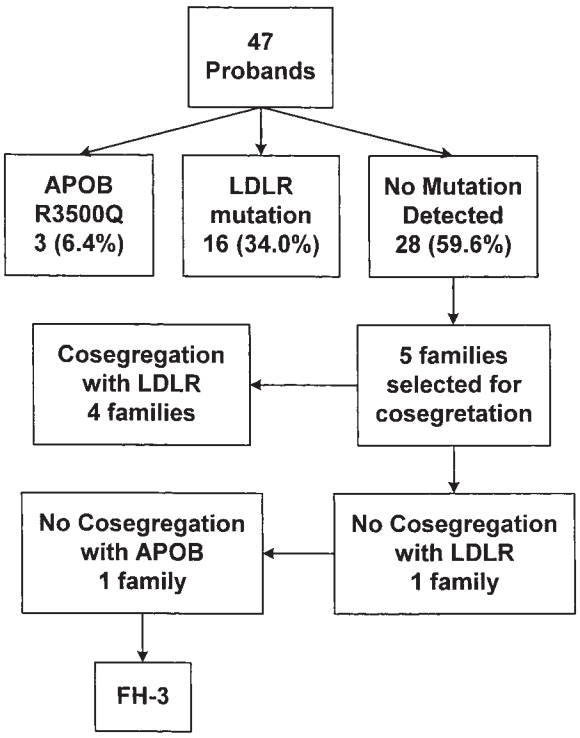

Fig. 1. Flow chart of molecular genetic analysis in 47 kindreds with familial hypercholesterolemia.

sized mutigenerational pedigree, formed from the merging into common ancestry of two smaller kindreds (K1000 and K1173), was excluded from linkage with either LDLR gene or APOB loci. Detailed results for all persons tested in these two kindred are presented in **Table 3**. Most of the affected members of this pedigree had multiple lipid determinations. These are also presented in Table 3. This family was then examined in considerably greater detail. In this kindred, with 18 haplotyped, affected individuals and 13 unaffected individuals (with blood available for genotyping), numerous inconsistencies with co-segregation with LDLR gene were evident both by genotypes and haplotypes (**Fig. 2**).

TABLE 1. Mutations found in kindreds from Utah with FH

Exon/Gene	SSCP/Mutation	Molecular event (ref)	Family ID
2	C6W	81T>G (2)	1103
3	S78X	296C>G (26)	1174
4	C139Y	478A>G (2)	1069
4	C163Y	551G>A (27)	1161
4	D200G	662G>A (3)	1066
4	D206E	681C>G (28)	1060
4	SSCP[a]	SSCP 3′ exon 4[a]	1169
8	Fs352	1116del4 (29)	625, 656
9	R395W	1246C>T (3)	613, 1110, 1167
10	P505S	1576C>T (2)	1050
11	G528D	1646G>A (2)	1106
11 + 17	N543H + 777del LLV	1690A>C (30); 2392del9 (3)	1142, 1068
APOB	R3500Q	10708G>A (5)	1013, 1168, 27

[a]Sequence change not available.

The clinical characteristics of the "FH-3 kindred" are compared with those with FH due to LDLR mutations in **Table 4**. Classic features of clinical FH were observed among these individuals: *1*) early coronary heart disease in affected male and female relatives; *2*) tendon xanthomata; *3*) very high total and LDL cholesterol levels in approximately half of the relatives; *4*) bimodality with normal cholesterol levels in the other half of the relatives and a large separation of about 100–150 mg/dl for LDL cholesterol between normal and affected siblings; and *5*) absence of features of familial combined hyperlipidemia (normal triglycerides and normal frequency of diabetes, hypertension, and obesity). Most of the family members with very high cholesterol levels have been resampled several or many times and, like classic heterozygous familial hypercholesterolemia, in the absence of powerful lipid-lowering drugs their total and LDL-cholesterol levels have been confirmed to remain persistently high. Body mass index (BMI), diastolic and systolic blood pressure (BP), and triglycerides were significantly lower in this kindred, compared to those kindreds in whom FH was due to LDLR mutations.

Figure 3 demonstrates the clear-cut bimodality of highest measured LDL cholesterol levels seen in this kindred comparing affected and unaffected individuals. Total and LDL cholesterol levels in heterozygotes were considerably more elevated than in their unaffected relatives. Untreated LDL cholesterol levels were not available for all affected individuals. Further, there were several instances in which the most recent total or LDL cholesterol values were lower than prior levels due to treatment with a strict lipid-lowering diet. An example of this is individual 1000-151 (male, aged 41.7 years) with a total cholesterol level of 303 mg/dl. This individual, now on a strict diet, was reported to have had an untreated highest total cholesterol level of 479 mg/dl and another LDL cholesterol of 292 at age 34.

Genotypes and haplotypes at the APOB locus, the only other gene known to cause this phenotype, were defined using three markers in the family 1000/1172. As for the LDLR gene, numerous inconsistencies with co-segregation of phenotype with the APOB gene were identified either by genotype or haplotype. These are also considered for their robustness below. Amplification of the intron 20 polymorphism gave three fragments with sizes of 151 nt, 147 nt, and 143 nt. This marker showed an exclusion between both branches of the family (1000 and 1173). D2S220 (size from 155–167 mobility units, equivalent to base pairs) genotypes gave inconsistencies both within and between the two family branches. Although the 3′ hypervariable repeat (HVR) is highly polymorphic (heterozygosity 0.75 (25)), in this family the majority of alleles were either 33, 35, or 37 repeat units. Nevertheless, by haplotype analysis it was possible to unambiguously rule out the involvement of the APOB gene in the causation of FH (Fig. 2). APOE genotypes were also examined (Fig. 2). Again there are numerous inconsistencies with co-segregation of affected status with an allele at the APOE locus.

To assure correct assignment of genotypes and proper

TABLE 2. Results of co-segregation studies using two markers flanking the LDLR, D19S394, and D19S221

Family ID	No. of Affecteds Examined	No. of Individuals Examined	Co-seg Allele D19S394	LOD at θ = 0	Co-seg Allele D19S221	LOD at q = 0.1
307	5	6	259 nt	0.9	196 nt	0.3
1116	3	4	259 nt	0.4	202 nt	0.7
20	5	5	259 nt	0.6	206 nt	0.2
1107	6	16	259 nt	6.6	206 nt	1.4
1000/1173	18	31	multiple exclusions	−9.1	exclusions	−14.0

sample handling, all members of pedigree 1000/1173 were independently retyped for markers D2S220 and D19221 by a different, outside laboratory. The allele assignments agreed with those shown in Fig. 2 in every instance. Furthermore, several other anonymous markers at various locations in the genome were, in all instances, consistent with the pedigree as drawn with no apparent non-paternity.

DISCUSSION

Using a combination of direct mutation scanning and SSCP, 19/47 (40.4%) of FH probands were found to have a mutation either in the LDLR gene or the APOB gene (as R3500Q). Some studies suggest that SSCP is sensitive to 70–95% of base changes for fragments of 200 bp or less, although this may depend on optimization and use of

TABLE 3. Clinical characteristics of kindred 1000/1173

Kindred	Rel #	Age	Sex	FH	CHD	Chol	Trig	HDL	LDL	ApoE	Comments, prior LDL levels
1000	1	39.4	F	1	30	450				2-2	Historical untreated total cholesterol only. Small Achilles tendon xanthomas. 10 measured LDL, all on treatment, most recent 144 on multiple medications. Also expresses type III phenotype.
1000	2	52	F	0		178	161	43	108	3-2	
1000	5	77.9	M	0	49	187	187	41	137	3-3	
1000	30	67.3	M	1	50	468				3-4	Historical untreated total cholesterol only. Small Achilles tendon xanthomas. Most recent LDL 116 on multiple medications.
1000	60	32.6	F	0		146	146	54	62	2-2	
1000	61	28.7	F	0		203	63	63	122	3-4	
1000	101	34.7	M	0		168	100	31	115	—	
1000	102	19.9	F	1		342	78	41	280	2-4	On diet. Prior LDL: 297 age 11.2, 346 age 11.5, 416 age 13.0, 315 age 13.6, 355 age 15, 292 age 16.
1000	103	11.6	M	1		344	181	34	274	2-3	Prior LDL: 252 age 9, 238 age 9
1000	104	7.9	M	0		136	101	43	80	2-3	Prior LDL 84 age 7.6
1000	105	6.6	F	1		345	125	32	280	2-3	Prior LDL 236 age 4.2, 247 age 4.5
1000	108	54.6	M	0		276	178	33	191	3-4	
1000	109	54.7	F	0		257	142	31	193	3-3	
1000	144	34.3	M	0		201	120	45	124	2-3	
1000	145	9.2	M	0		200	126	50	118	2-3	
1000	147	7.8	M	0		148	44	51	88	2-3	
1000	150	39.3	M	1		357	87	42	280	4-4	On diet. Historical highest total cholesterol 370. Prior LDL 244 on diet age 32. Multiple treated LDL, most recent 189 on multiple medications.
1000	151	41.7	M	1		303	88	51	244	3-4	On diet. Historical highest total cholesterol 479. Prior LDL: 244 age 34.0, 292 age 34.4
1000	155	55.6	F	0		251	128	81	147	3-4	
1000	156	30.4	F	0		170	49	74	93	—	
1000	157	18.3	M	1		267	78	42	205	4-4	On diet. Prior LDL: 295 age 10.1, 284 age 10.9, 329 age 11.4, 316 age 11.9, 326 age 12.5, 323 age 12.9
1000	158	8.4	F	0		169	68	63	94	3-4	Repeated total cholesterol 143 age 8, 135 age 9.
1173	1	33.6	F	1		487	203	23	381	2-3	On careful diet. Prior LDL 441 age 33.5
1173	5	63.3	M	1	49					2-4	No untreated values. LDL 162 on full dose statin and careful diet.
1173	6	59	F	0		144					Reflotron total cholesterol only.
1173	7	25.0	F	1		313	112	29	268	3-4	On diet. Prior LDL 325 age 24.4
1173	9	28.1	F	1		408	102	35	331	3-4	
1173	17	57.8	M	1	54	275	150	37	143	2-2	On careful diet. Prior LDL 209 on statin age 50.
1173	41	57	F	0		156	54	49	96		
1173	43	32.1	F	1		353	128	36	268	2-3	On careful diet. Moderate size Achilles tendon xanthomas. Prior LDL: 333 age 25.7, 244 age 26.0, 262 age 26.5, 240 age 26.9, 211 age 27.4, 220 age 30.3
1173	47	20.7	F	1		294	59	61	222	—	On diet. Prior LDL 199 age 13.
1173	48	18.2	F	1		211	78	33	161	—	On diet. Prior LDL 209 age 11.
1173	49	29.9	M	1		435	124	36	365	—	Prior LDL: 262 age 23, 372 age 25.0. On diet and low dose statin LDL 262 age 29.9.
1173	50	25.0	M	1		316	175	32	244	—	On diet. Prior LDL: 202 age 22.5, 158 age 22.8

Lipid levels (mg/dl) are most recent values without lipid-lowering medication. LDL and VLDL levels are measured values after ultracentrifugation. Historical values reported by patients are so indicated. Prior LDL cholesterol are measured, untreated values unless so stated. Abbreviations: Chol, total cholesterol; Trig, triglycerides; HDL, HDL cholesterol; LDL, LDL cholesterol.

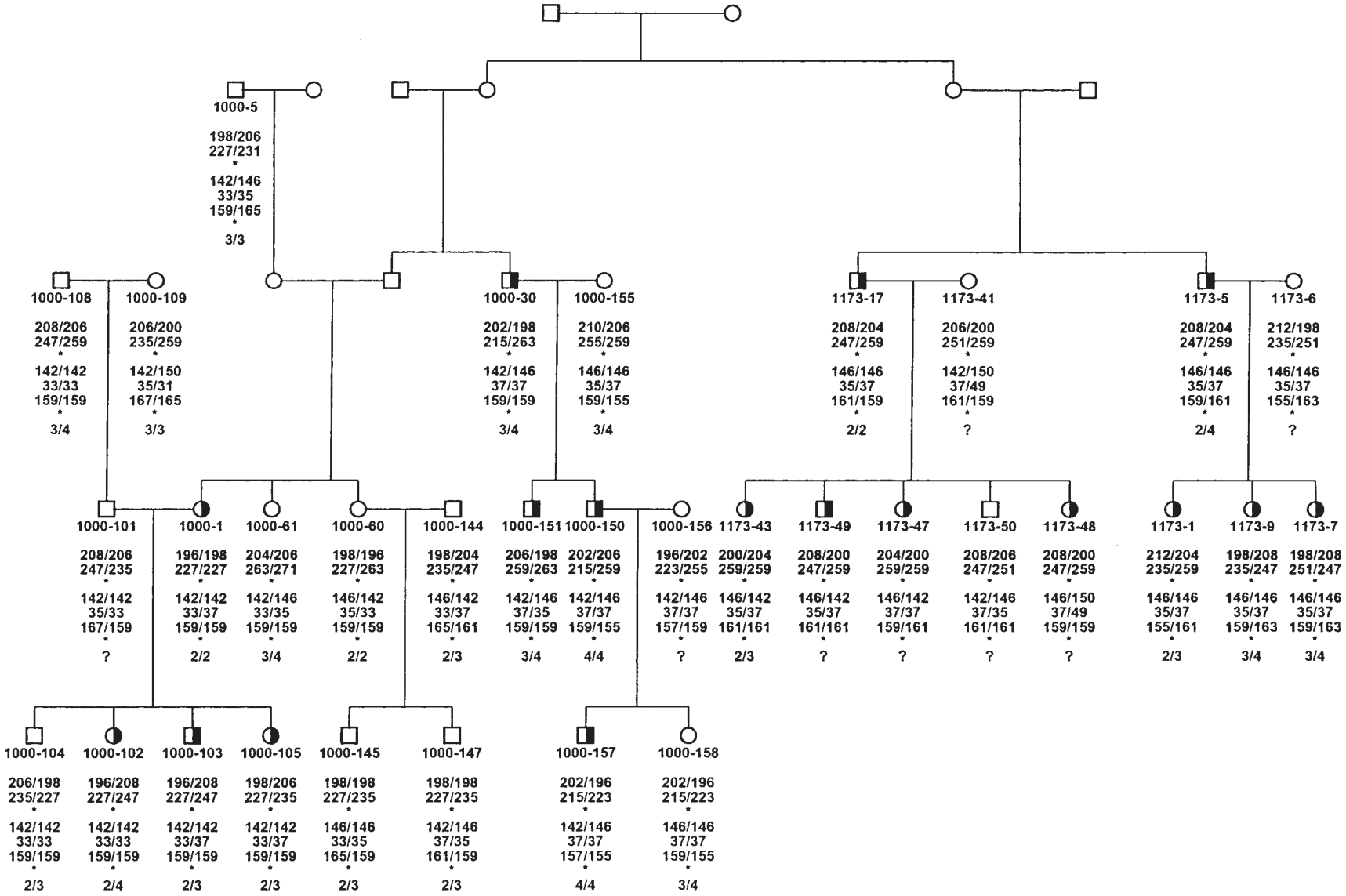

Fig. 2. Family drawing of FH kindred 1000/1173. Clear symbols, unaffected; half filled symbols, affected. Numbers on the first line directly below the symbols are the individual ID numbers in the pedigree. Marker values are in the following order: D19S221, D19S394 (representing LDLR region); intron 20 VNTR in APOB gene, 3′ VNTR in APOB gene, D2S220 (representing APOB region); APOE. Markers for each genomic region are grouped, each group is separated in the figure by the symbol *. Unaffected individuals who were not genotyped are not shown.

TABLE 4. Clinical characteristics of patients diagnosed with heterozygous FH in pedigrees linked to the LDL receptor or for whom an LDL receptor mutation is present (n = 198) compared to phenotypically affected members of kindreds 1000 and 1173 (n = 16 with available information)

	Receptor Linked Pedigrees		Kindreds 1000 and 1173		
	Mean ± SD	Range	Mean ± SD	Range	*P* Value
Percent male (%)	46.0		50.0		0.8
CAD present (%)	18.7		25		0.5
Early CAD present (%)	17.7		25		0.7
Tendon xanthoma (%)	28.8		18.8		0.3
Hypertension present (%)	10.1		0		0.2
Diabetes present (%)	2.0		0		0.7
Ever smoked (%)	8.6		6.2		0.6
Alcohol use (ever) (%)	5.4		0		0.5
Age (years)	41.3 ± 18.6	4.9–78.8	34.9 ± 15.8	18.2–67.3	0.2
BMI (kg/m^2)	27.7 ± 5.1	18.1–42.0	22.7 ± 2.1	20.5–28.1	<0.0001
Systolic BP (mm Hg)	116 ± 15	71–169	100 ± 10	84–115	0.0002
Diastolic BP (mm Hg)	69.5 ± 10.1	47–100	60.1 ± 7.0	43–68	0.0006
Total cholesterol	327 ± 64	194–510	310 ± 77	210–487	0.4
Triglycerides	171 ± 99	52–545	102 ± 44	58–203	0.0004
HDL cholesterol	43.1 ± 11.8	17–77	39.5 ± 10.3	23–61	0.3
LDL cholesterol	240 ± 59	107–393	237 ± 70	143–381	0.9

Differences between the two groups were tested by *t*-test for continuous variables and Fisher's exact test for categorical variables. Lipid levels include only persons not receiving lipid-lowering medications at the time of blood draw (11 members of kindreds 1000 and 1173 and 101 members of other pedigrees). All lipid values are given in mg/dl.

multiple conditions for given PCR fragments. Under non-optimized or single conditions, fewer than 40% of mutations may be detected (43). In FH, no SSCP study, except of founder populations, has achieved greater than a 70% find rate for LDLR mutations. An additional 2.5–5% of mutations are due to major gene rearrangements (44), not detectable by SSCP; and intron–exon splice junctions which may amount for a further 15% (45) have not always been analyzed. Absence of a mutation in the screened probands may thus be due to base changes outside the gene regions examined, base substitutions/variations in the APOB gene other than R3500Q (such as R3500W and R3531Q), or to mutations not involving the LDLR gene.

Clear cut co-segregation of specific alleles of markers D19S394 and D19S221 with hypercholesterolemia was seen in four of the five pedigrees, consistent with the presence of as yet undetected mutations involving the LDLR locus. However, in one family, 1000/1173, with classical features of familial hypercholesterolemia, D19S394 and D19S221 genotypes and haplotypes provided unequivocal information excluding involvement of the LDLR gene region. This was confirmed using additional anonymous markers. Considered by genotype, at microsatellites flanking the LDLR gene, there is effectively random distribution of allele sizes among the 18 characterized affected individuals in the kindred. For D19S221, 1 Mb centromeric

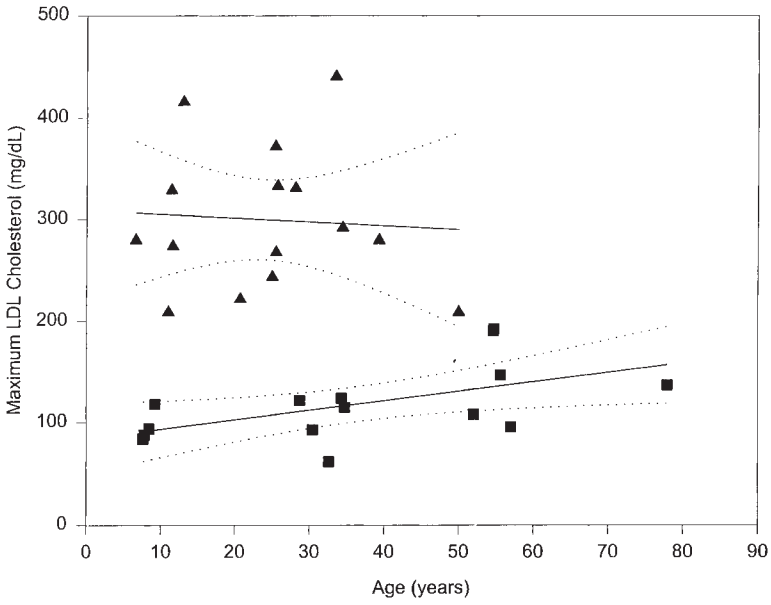

Fig. 3. Bimodality graph of highest measured LDL cholesterol in affected and unaffected individuals in 1000/1173 kindred. Triangles represent affected family members; squares are unaffected family members. The two outer dotted lines for each group indicate the 95% confidence intervals, and the middle line is the mean cholesterol level. Note the lack of change with age in the affected pedigree members.

to LDLR, allele sizes of 196 (3), 198 (6), 200 (4), 202 (3), 204 (5), 206 (5), 208 (8), and 212 (1) mobility units were observed. The most frequent allele was 208 with 8 out of 34 alleles, whereas there should be a shared allele size among all/almost all 18 affected individuals were there co-segregation of a particular allele size with affected status. Similarly for D19S394, located 150 kb telomeric to LDLR, allele sizes of 215 (3), 223 (1), 227 (5), 235 (3), 247 (8), 251 (1), 259 (11), and 263(2) mobility units were observed. From study of a common mutation R329X (exon 7 of the LDLR gene), found in the south of England (23), we have previously shown that an allele of D19S394 remains in total linkage disequilibrium with an LDLR mutation over many generations. In addition, careful measurement of genetic distance between D19S394 and LDLR in the CEPH families has shown that recombination is negligible, far less than 1% (21). The absence of a common allele size in more than 11 out of 18 affected individuals for D19S394 is further convincing evidence against co-segregation of phenotype with LDLR. In most individuals, haplotypes can be unequivocally assigned for both markers together. Consideration of transmissions, both within each small sub-branch of the family and between branches, both by genotype and haplotype, leads to inconsistencies with segregation of hypercholesterolemia with LDLR in each of the four sub-branches arising from the sibship containing four affected males and also between these sub-branches.

Direct testing for the common APOB mutation, R3500Q, was unequivocally negative in 7 affected members from family 1000/1173. However, other mutations are known although apparently much rarer and less penetrant. APOB is a large gene though, with a 14 kb mRNA, mostly coding sequence, and 29 exons, and therefore it was simplest to examine for APOB gene region involvement by linkage study. Using three markers, both genotypes and deduced haplotypes (Fig. 2) were inconsistent with segregation of hypercholesterolemia with the APOB region. By haplotype, there is at least one inconsistency in each of the four sub-branches, with further inconsistencies between branches.

The APOE locus on chromosome 19 is another plausible but less likely candidate for being the etiological locus in kindred 1000/1173. It encodes the other major ligand for the LDL receptor, but apoE mutations have a strong impact on IDL clearance as well as LDL clearance and therefore usually result in concomitant hypertriglyceridemia. However, it is not the etiological gene in kindred 1000/1173, again ruled out by the co-segregation study alone.

The clinical phenotype in kindred 1000/1173 is indistinguishable from pedigrees living in the same geographical region with apparent LDLR linkage. The statistically significant lower mean BMI, systolic and diastolic blood pressure, and triglyceride levels in members of kindred 1000/1173 are likely to reflect their lower mean age. However, within this family, BMI, TG, and BP did not differ between affected and unaffected individuals (as do cholesterol values). Further clinical and biochemical studies, e.g., LDL receptor assays, will be the subject of a future report.

The conclusion that familial hypercholesterolemia in family 1000/1173 represents autosomal dominant transmission that is not linked either to the LDLR gene or to the APOB gene is very robust. The assignment of phenotype was unambiguous as plasma LDL levels were unequivocally bimodal and most of the affected subjects had multiple, clearly elevated lipid measurements in the absence of lipid-lowering drugs. The genotyping data was based on length polymorphisms typed by highly reliable methods, and with secondary checks including consistency of genotype and haplotype inheritance in the family and also reconfirmations in pertinent meioses by adjacent track or same track co-runs of relative pairs. In the light of initial observations suggesting inconsistency with linkage with LDLR or APOB, we examined closely for possible sample swaps and miscoding at every point between the kindred members and laboratory data acquisition, and these were ruled out. The confirmation of genotype assignment for markers near both the LDLR and APOB loci by an independent laboratory provides strong reassurance of accuracy. The structure of the pedigree as shown in Fig. 2 was further confirmed with multiple additional anonymous markers. The internal consistencies of stated family relationships with genotypes and deduced haplotypes are very powerful confirmation of the several multiallelic genotypes examined. The multiple exclusions of co-segregation of phenotype with LDLR or APOB cannot be explained by any apparent non-paternities. Furthermore, an inconceivable number of double recombinations within very short genetic intervals, or an inconceivable number of length slippages of polymorphisms (also remaining consistent with parental, sib and offspring other allele sizes) during meioses, would have to be involved to support the hypothesis of co-segregation of phenotype with either LDLR or APOB. With the markers used at both loci, only one or two single recombinations would have been expected in the whole sample of seven families, and only one was observed, between markers D19S221 and D19S394 in transmission from mother 1173-6 to daughter 1173-9. Unlike the situation with smaller kindreds, less informative markers, and the other possible reasons for an apparent exclusion of linkage considered above, we have a relatively large kindred, highly informative markers with assignment verified by an outside laboratory, and high quality phenotyping confirmed at multiple time points. This study therefore provides the first unequivocal evidence for the presence of a third and largely unsuspected locus exerting a major gene effect on normotriglyceridemic LDL–hypercholesterolemia, and early coronary disease.

The application of co-segregation analysis using linkage markers for LDLR and APOB provides a definitive approach to the identification of potential new loci with a megaphenic (single gene) effect on plasma LDL-cholesterol levels and early coronary disease. By contrast, the study of intermediate phenotypes such as LDLR function and LDL function and turnover studies would fail to identify some plausible mechanisms, such as those to which a change in LDL or receptor function is secondary. Additionally, future genome scanning and positional cloning

will offer a secure strategy to identify the FH-3 gene, known or new. Nevertheless, identification of candidate loci may be guided by information about cellular LDL receptor function and LDL turnover status in this large family, and this information is currently unknown. The demonstration that a third locus must be etiological for the FH phenotype, and establishment of an effective approach to exclusion of LDLR and APOB genes, is reinvigorating for the investigation of hypercholesterolemic kindreds for other genetic loci with major impact on LDL cholesterol levels and coronary disease.

INMD thanks the British Heart Foundation (BHF) for support (Intermediate Fellowship FS/92030), also the Lister Institute for a Senior Fellowship. LH is a BHF student, and SEH thanks the BHF for both Chair Award and programme grant support. Support for RRW, PNH, and SH was from NIH grants HL47561 and HL21088.

Manuscript received 4 June 1998 and in revised form 19 January 1999.

REFERENCES

1. Goldstein, J. L., and M. S. Brown. 1989. Familial hypercholesterolemia. *In* C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. The Metabolic Basis of Inherited Disease. McGraw-Hill, New York. 1215–1250.
2. Hobbs, H. H., M. S. Brown, and J. L. Goldstein. 1992. Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. *Hum. Mutat.* **1:** 445–466.
3. Day, I. N. M., R. A. Whittall, S. D. O'Dell, L. Haddad, M. K. Bolla, V. Gudnason, and S. E. Humphries. 1997. Spectrum of LDL receptor gene mutations in heterozygous familial hypercholesterolemia. *Hum. Mutat.* **10:** 116–127.
4. Vega, G. L., and S. M. Grundy. 1986. In vitro evidence for reduced binding of low density lipoproteins to receptors as a cause of primary moderate hypercholesterolemia. *J. Clin. Invest.* **78:** 1410–1414.
5. Innerarity, T. L., R. W. Mahley, K. H. Weisgraber, T. P. Bersot, R. M. Krauss, G. L. Vega, S. M. Grundy, W. Fried, J. Davignon, and B. J. McCarthy. 1990. Familial defective apolipoprotein B-100: a mutation of apolipoprotein B that causes hypercholesterolemia. *J. Lipid Res.* **31:** 1337–1349.
6. Myant, N. B. 1993. Familial defective apolipoprotein B-100: a review, including some comparisons with familial hypercholesterolemia. *Atherosclerosis.* **104:** 1–18.
7. Pullinger, C. R., L. K. Hennessy, J. E. Chatterton, W. Liu, J. A. Love, C. M. Mendel, P. H. Frost, J. M. Malloy, V. N. Schumaker, and J. P. Kane. 1995. Familial ligand-defective apolipoprotein B. Identification of a new mutation that decreases LDL receptor binding affinity. *J. Clin. Invest.* **95:** 1225–1234.
8. Gaffney, D., J. M. Read, I. M. Cameron, K. Vass, M. J. Caslake, J. Shepard, and C. J. Packard. 1995. Independent mutations at the codon 3500 of the apolipoprotein B gene are associated with hyperlipidemia. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1025–1029.
9. 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. 1997. Association of genetic variations in apolipoprotein B with hypercholesterolemia, coronary artery disease, and receptor binding of low density lipoproteins. *J. Lipid Res.* **38:** 1361–1373.
10. Sun, X. M., D. D. Patel, B. L. Knight, and A. K. Soutar, with the FH Regression Study Group. 1997. Comparison of the genetic defect with LDL receptor activity in cultured cells from patients with a clinical diagnosis of heterozygous FH. *Arterioscl. Thromb. Vasc. Biol.* **17:** 3092–3101.
11. Myant, N. B., and J. Slack. 1976. Type II-hyperlipoproteinaemia. *In* G. Schettler, H. Greten, G. Schlierf, and D. Seidel, editors. Handbuch der inneren Medizin: Fettstoffwechsel. Springer-Verlag, Berlin. 275–300.
12. Marz, W., M. W. Baumstark, H. Scharnagl, V. Ruzicka, S. Buxbaum, J. Herwig, T. Pohl, A. Russ, L. Schaaf, A. Berg, H. J. Bohles, K. H. Usadel, and W. Gross. 1993. Accumulation of 'small dense' low density lipoproteins in a homozygous patient with familial defective apolipoprotein B-100 results from heterogenous interaction of LDL-subfractions with the LDL receptor. *J. Clin. Invest.* **92:** 2922–2933.
13. Gallagher, J. J., and N. B. Myant. 1995. The affinity of low-density lipoprotein remnants for the low-density lipoprotein receptor in homozygous familial defective apolipoprotein B-100. *Atherosclerosis.* **115:** 263–272.
14. Uauy, R., G. L. Vega, and S. M. Grundy. 1991. Coinheritance of two mild defects in low density lipoprotein receptor function produces severe hypercholesterolemia. *J. Clin. Endocrin. Metab.* **72:** 179–187.
15. Koivisto, P. V. I., U. M. Koivisto, P. T. Kovanen, H. Gylling, T. A. Miettinen, and K. Kontula. 1993. Deletion of exon 15 of the LDL receptor gene is associated with a mild form of familial hypercholesterolaemia. *Arterioscler. Thromb.* **13:** 1680–1688.
16. Moorjani, S., M. Roy, A. Torres, C. Betard, C. Gagne, M. Lambert, D. Brun, J. Davignon, and P. Lupien. 1993. Mutations of low-density-lipoprotein-receptor gene, variation in plasma cholesterol, and expression of coronary heart disease in homozygous familial hypercholesterolaemia. *Lancet.* **341:** 1303–1306.
17. Emi, M., R. M. Hegele, P. N. Hopkins, L. L. Wu, R. Plaetke, R. R. Williams, and J. M. Lalouel. 1991. Effects of three genetic loci in a pedigree with multiple lipoprotein phenotypes. *Arteriosclerosis.* **11:** 1349–1355.
18. Sirtori, C. R., A. L. Catapano, G. Franceschini, A. Corsini, G. Noseda, C. Fragiacomo, E. Panzeri, V. Vaccarino, S. Guenzi, G. Casari, and S. Baralle. 1991. Aortic and coronary atheromatosis in a woman with severe hypercholesterolemia without LDL receptor alterations. *Eur. Heart. J.* **12:** 818–824.
19. Miserez, A. R., H. Schuster, N. Chiodetti, and U. Keller. 1994. Polymorphic haplotypes and recombination rates at the LDL receptor gene locus in subjects with and without familial hypercholesterolemia who are from different populations. *Am. J. Hum. Genet.* **52:** 808–826.
20. Kingsley, D. M., and M. Krieger. 1984. Receptor-mediated endocytosis of low density lipoprotein: somatic cell mutants define multiple genes required for expression of surface-receptor activity. *Proc. Natl. Acad. Sci. USA.* **81:** 5454–5458.
21. Haddad, L., L. B. Day, J. Attwood, S. Povey, S. E. Humphries, and I. N. M. Day. 1997. Development of a microsatellite-based approach to co-segregation analysis of familial hypercholesterolaemic kindreds. *Ann. Hum. Genet.* **61:** 497–506.
22. Ashworth, L. K., M. A. Batzer, B. Brandriff, E. Branscomb, P. de Jong, E. Garcia, J. A. Garnes, L. A. Gordon, J. E. Lamerdin, G. Lennon, H. Mohrenweiser, A. A. Olsen, T. Selzak, and A. V. Carrano. 1995. An integrated metric physical map of chromosome 19. *Nat. Genet.* **11:** 422–427.
23. Day, I. N. M., L. Haddad, S. D. O'Dell, L. B. Day, R. Whittall, and S. Humphries. 1997. Identification of a common low density lipoprotein receptor mutation (R329X) in the South of England: complete linkage disequilibrium with an allele of microsatellite D19S394. *J. Med. Genet.* **34:** 111–116.
24. Zuliani, G., and H. H. Hobbs. 1990. Tetranucleotide repeat polymorphism in the apolipoprotein B gene. *Nucleic Acids Res.* **18:** 4299.
25. Boerwinkle, E., W. Xiong, E. Fourest, and L. Chan. 1988. Rapid typing of tandemly repeated hypervariable loci by the polymerase chain reaction: application to the apolipoprotein B 3′ hypervariable region. *Proc. Natl. Acad. Sci. USA.* **85:** 1462–1466.
26. Buetow, K. H., J. L. Weber, L. Ludwigsen, T. Scherpbier-Heddema, G. M. Duyk, V. C. Sheffield, Z. Wang, and J. C. Murray. 1994. Integrated human genome-wide maps constructed using the CEPH reference panel. *Nat. Genet.* **6:** 391–393.
27. Williams, R. R., S. J. Hasstedt, D. E. Wilson, K. O. Ash, F. G. Yanowitz, G. E. Reiber, and H. Kuida. 1986. Evidence that men with familial hypercholesterolemia can avoid early coronary death: an analysis of 77 gene carriers in four Utah pedigrees. *J. Am. Med. Assoc.* **255:** 219–224.
28. Williams, R. R., and S. C. Hunt. 1987. Recruitment of members of high-risk Utah pedigrees. *Controlled Clin. Trials.* **8:** 105S–114S.
29. Williams, R. R., S. C. Hunt, M. C. Schumacher, R. A. Hegele, M. F. Leppert, E. H. Ludwig, and P. N. Hopkins. 1993. Diagnosing heterozygous familial hypercholesterolemia using new practical criteria validated by molecular genetics. *Am. J. Cardiol.* **72:** 171–176.
30. Williams, R. R., I. Hamilton-Craig, G. M. Kostner, R. A. Hegele,

M. R. Hayden, S. N. Pimstone, O. Faergeman, H. E. S-T. Schuster, U. Beisiegel, C. Keller, A. E. Czeizel, E. Leitersdorf, J. C. Kastelein, J. J. P. Defesche, L. Ose, T. P. Leren, H. C. Seftel, F. J. Raal, A. D. Marais, M. Eriksson, U. Keller, A. R. Miserez, T. Jeck, D. J. Betteridge, S. E. Humphries, I. N. M. Day, P. L. Kwiterovich, R. S. Lees, E. Stein, R. Illingworth, J. Kane, and V. Boulyjenkov. 1996. MEDPED: An integrated genetic strategy for preventing early deaths. *In* Genetic Approaches to Noncommunicable Diseases. K. Berg, V. Boulyjenkov, and Y. Christen, editors. Springer-Verlag Heidelberg. 35–45.

31. Williams, R. R., S. C. Hunt, G. K. Barlow, R. M. Chamberlain, A. D. Weinberg, H. P. Cooper, J. P. Carbonari, and A. M. Gotto. 1988. Health Family Trees: a tool for finding and helping young family members of coronary and cancer prone pedigrees in Texas and Utah. *Am. J. Pub. Health.* **78:** 1283–1286.
32. Williams, R. R., M. C. Schumacher, G. K. Barlow, S. C. Hunt, J. L. Ware, M. Pratt, and B. D. Latham. 1993. Documented need for more effective diagnosis and treatment of familial hypercholesterolemia according to data from 502 heterozygotes in Utah. *Am. J. Cardiol.* **72:** 18D–24D.
33. Leppert, M. F., S. J. Hasstedt, T. Holm, P. O'Connell, L. L. Wu, K. O. Ash, R. R. Williams, and R. L. White. 1986. A DNA probe for the LDL receptor gene is tightly linked to hypercholesterolemia in a pedigree with early coronary disease. *Am. J. Hum. Genet.* **39:** 300–306.
34. Hobbs, H. H., D. W. Russell, M. S. Brown, and J. L. Goldstein. 1990. The LDL receptor locus in familial hypercholesterolemia: mutational analysis of a membrane protein. *Annu. Rev. Genet.* **24:** 133–170.
35. Lipid Research Clinics Program Manual of Laboratory Operations. 1974. DHEW (NIH) Publication No. 75-628.
36. Wu, L. L., G. R. Warnick, J. T. Wu,, R. R. Williams, and J. M. Lalouel. 1989. A rapid micro-scale procedure for determination of total lipid profile. *Clin. Chem.* **35:** 1486–1491.
37. Kunkel, L. M., K. D. Smith, S. H. Boyer, D. S. Borgaonkar, S. S. Wachtel, O. J. Miller, W. R. Breg, H. W. Jones, and J. M. Rary. 1977. Analysis of human Y-chromosome-specific reiterated DNA in chromosome variants. *Proc. Natl. Acad. Sci. USA.* **74:** 1245–1249.
38. Laywood, A., R. Whittall, V. Gudnason, S. E. Humphries, and I. N. M. Day. 1994. Handling of large (300 × 400 mm), thin (0.4 mm) polyacrylamide gels and recovery as dried gels. *Biotechniques.* **17:** 850–854.
39. Marz, W., V. Ruzicka, E. Fisher, A. P. Russ, W. Schneider, and W. Grob. 1993. Typing of the 3′ hypervariable region of the apolipoprotein gene: approaches, pitfalls, and applications. *Electrophoresis.* **14:** 169–173.
40. Mamotte, C. D. S., and F. M. van Bockxmeer. 1993. A robust strategy for screening and confirmation of familial defective apolipoprotein B-100. *Clin. Chem.* **39:** 118–121.
41. Day, I. N. M., and S. E. Humphries. 1994. Electrophoresis for genotyping: microtitre array diagonal gel electrophoresis (MADGE) on horizontal polyacrylamide (H-PAGE) gels, Hydrolink or agarose. *Anal. Biochem.* **222:** 389–395.
42. Wu, L. H., J. T. Wu, and P. N. Hopkins. 1994. Apolipoprotein E. Laboratory determination and clinical significance. *In* N. Rifai and G. R. Warnick, editors. Laboratory Measurement of Lipids, Lipoproteins and Apolipoproteins. AACC Press, Washington, DC. 279–305.
43. Liu, Q., and S. S. Sommer. 1994. Parameters affecting the sensitivities of dideoxy fingerprinting and SSCP. *PCR Methods. Appl.* **4:** 97–108.
44. Sun, X. M., J. C. Webb, V. Gudnason, S. Humphries, M. Seed, G. R. Thompson, B. L. Knight, and A. K. Soutar. 1992. Characterization of deletions in the LDL receptor gene in patients with familial hypercholesterolemia in the United Kingdom. *Arterioscler. Thromb.* **12:** 762–770.
45. Krawczak, M., J. Reiss, and D. N. Cooper. 1992. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Hum. Genet.* **90:** 41–54.
46. Leren, T. P., K. Solberg, O. K. Rodningen, S. Tonstad, and K. Berg. 1994. Two founder mutations in the LDL receptor gene in Norwegian familial hypercholesterolaemia subjects. *Atherosclerosis.* **111:** 175–182.
47. Ward, A. J., M. O'Kane, I. Young, D. P. Nicholls, N. C. Nevin, and C. A. Graham. 1995. Three novel mutations in the EGF precursor homology domain of the low-density lipoprotein receptor gene in Northern Irish patients with familial hypercholesterolaemia. *Hum. Mutat.* **6:** 254–256.
48. Kotze, M. J., E. Langenhoven, L. Warnich, M. P. Marx, and A. E. Retief. 1989. Molecular characterisation of a low frequency mutation in exon 8 of the low-density lipoprotein receptor gene. *S. Afr. Med. J.* **76:** 402–405.
49. Solberg, K., O. K. Rodningen, S. Tonstad, L. Ose, and T. P. Leren. 1994. Familial hypercholesterolaemia caused by a non-sense mutation in codon 329 of the LDL receptor gene. *Scand. J. Clin. Lab. Invest.* **54:** 605–609.
50. Tricot-Guerber, F., B. SainJore, K. Valenti, T. Foulon, M. Bost, and A. J. Hadjian. 1995. Identification of a mutation, N543H, in exon 11 of the low-density lipoprotein receptor gene in a French family with familial hypercholesterolaemia. *Hum. Mutat.* **6:** 87–88.