Severe Hypercholesterolemia in Four British Families With the D374Y Mutation in the PCSK9 Gene

Long-Term Follow-Up and Treatment Response

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Objective—Analysis of long-term (30 years) clinical history and response to treatment of 13 patients with the D374Y mutation of PCSK9 (PCSK9 patients) from 4 unrelated white British families compared with 36 white British patients with heterozygous familial hypercholesterolemia attributable to 3 specific mutations in the low-density lipoprotein (LDL) receptor gene (LDLR) known to cause severe phenotype.

Methods and Results—The PCSK9 patients, when compared with the LDLR patients, were younger at presentation (20.8±14.7 versus 30.2±15.7 years; P=0.003), had higher pretreatment serum cholesterol levels (13.6±2.9 versus 9.6±1.6 mmol/L; P=0.004) that remained higher during treatment with simvastatin (10.1±3.0 versus 6.5±0.9 mmol/L; P=0.006), atorvastatin (9.6±2.9 versus 6.4±1.0 mmol/L; P=0.006), or current lipid-lowering therapy, including LDL apheresis and partial ileal bypass in 2 PCSK9 patients (7.0±1.6 versus 5.4±1.0 mmol/L; P=0.001), and were affected >10 years earlier by premature coronary artery disease (35.2±4.8 versus 46.8±8.9 years; P=0.002). LDL from PCSK9 patients competed significantly less well for binding to fibroblast LDL receptors than LDL from either controls or LDLR patients.

Conclusions—These British PCSK9 patients with the D374Y mutation have an unpredictably severe clinical phenotype, which may be a unique feature for this cohort, and requires early and aggressive lipid-lowering management to prevent cardiovascular complications. (Arterioscler Thromb Vasc Biol. 2005;25:2654-2660.)

Key Words: coronary disease ■ atherosclerosis ■ familial hypercholesterolemia ■ statins ■ ezetimibe

Familial hypercholesterolemia (FH) is a common autosomal dominant disorder caused by mutations in the low-density lipoprotein (LDL) receptor gene (LDLR) leading to defective catabolism of plasma LDL by the liver and characterized by elevated levels of LDL cholesterol, tendon xanthomas, and excessive deposition of cholesterol in the arterial wall, causing premature atherosclerosis.1 An almost identical clinical syndrome to FH, called familial defective apolipoprotein B (apoB), can occur as a result of a dominantly inherited mutation of the ApoB gene, which encodes the ligand for the LDL receptor, causing impaired catabolism of LDL.2 Recently, heterozygous missense variants in a gene named PCSK9 (protein convertase subtilisin/kexin9) have been described to cosegregate with hypercholesterolemia in families of European origin.3–6

PCSK9 encodes a putative protease, which is a member of the subtilisin–like protein convertase family.7,8 Its physiological role has not yet been elucidated, but there is substantial evidence that it is involved in cholesterol homeostasis.9–12 PCSK9 is responsive to sterols and is a putative sterol regulatory element-binding protein (SREBP) target in mice.11,12 Adenovirus-mediated overexpression of wild-type PCSK9 in mice led to severe hypercholesterolemia by decreasing the amount of LDL receptor protein in the liver without reducing LDL receptor mRNA levels.13 However, similar overexpression of 2 naturally occurring PCSK9 missense mutants decreased hepatic LDL receptor protein content to the same extent as wild type.14 Further evidence that PCSK9 is involved in normal cholesterol metabolism comes from 2 recent observations: decreased plasma LDL cholesterol and apoB and increased LDL receptor protein in PCSK9 knockout mice,15 and 40% reduction in LDL cholesterol levels in human subjects heterozygous for nonsense mutation in PCSK9.16 These studies suggest that PCSK9 might function to reduce LDL receptor protein levels in liver but offer no convincing explanation for how missense mutations in PCSK9 cause hypercholesterolemia.

We have shown recently that expression of the missense D374Y and S127R variants (but not wild-type or F216L variant, nor a catalytically inactive mutant S386A) of PCSK9...
in stably transfected rat liver cells increases secretion of apoB100-containing lipoproteins by 2- to 4-fold.6 Our findings are consistent with lipoprotein turnover data in 2 French patients heterozygous for the S127R mutation of PCSK9, which showed a 3-fold increase in very low-density lipoprotein–apoB production rate, with a 2-fold increase in LDL–apoB production rate, along with 30% reduction in fractional clearance of LDL compared with controls;17 increased content of apoB in triglyceride-rich lipoproteins in these patients has also been reported.18 Based on studies published so far, it appears that, unlike FH attributable to mutations in LDLR, in which the catabolic defect is the leading cause of hypercholesterolemia, missense (gain-of-function) mutations in PCSK9 may cause severe hypercholesterolemia by a dual mechanism: decreased LDL receptor activity and apoB100 overproduction. Further data on this important question are needed.

Only 10 families with autosomal dominant hypercholesterolemia attributable to PCSK9 missense mutations have been described in the literature to date.3–6 However, there have been no published reports on long-term follow-up of patients with this condition that elucidate the natural history of the disease and their response to lipid-lowering treatment compared with “classical” FH patients. In this article, we describe 4 unrelated white British families comprising 13 affected individuals with the D374Y mutation of PCSK9 who have been followed up for up to 30 years. We compare the clinical characteristics and response to lipid-lowering treatment in these patients with 3 well-characterized groups of British whites with heterozygous FH attributable to 3 specific mutations in LDLR known to cause a severe phenotype. In addition, we investigated whether LDL from patients with mutations in PCSK9 was able to compete effectively with labelled LDL from healthy volunteers for binding to skin fibroblast LDL receptors.

Materials and Methods

Study Participants

**Patients With Mutation of PCSK9**

Four index patients with autosomal dominant FH from unrelated white British families were referred to the Hammersmith Hospital Lipid Clinic because of difficulty in achieving target serum cholesterol concentrations on lipid-lowering medication. They have been followed up regularly by us for 12 to 30 years. After the 4 index patients were identified to be heterozygous for D374Y in PCSK9, screening of available family members identified 13 affected individuals, comprising 5 men and 8 women (mean age 20.8 ± 14.7 years at presentation; range 22 months to 57 years). The basic clinical characteristics of the 12 individuals from the first 3 families have been described briefly.5 The nuclear family 4 (Figure 1D) has only 1 affected member (index patient II,1), whose details are presented in the online supplement (available at http://atvb.ahajournals.org).

**Patients With Mutation of LDLR**

Patients Involved in the Retrospective Response to Treatment Study

Thirty-six British whites with “classical” heterozygous FH attributable to 1 of 3 types of LDLR receptor mutation were selected from the Hammersmith Hospital Lipid Clinic database. They comprised 17 women and 19 men, with a mean age at presentation of 30.2 ± 15.7 years (range 5 to 68 years). These patients were selected solely because they were carriers of well-characterized mutations known to cause a severe phenotype in heterozygous individuals. The 3 groups comprised patients with (1) a single amino acid substitution in exon 4 (n=14); (2) a point mutation in the 3′ splice site of exon 3 that results in exclusion of exon 3 from the mRNA (n=8); and (3) a premature stop codon that results in undetectable amounts of LDLR protein (n=14).

**Patients Involved in Competition for Binding of LDL to Normal Skin Fibroblast LDL Receptors and in the Assessment of LDL Particle Size**

Three groups of patients took part in these studies: (1) all living patients with mutations in the PCSK9 shown in Figure 1, excluding 1 6-year-old girl (family 3; patient III,3) and 1 patient with partial ileal bypass (family 1; patient III,1). This PCSK9 group comprised 5 men and 4 women, with a mean age of 43.1±16.8 years, mean on-treatment serum LDL cholesterol at the time of study 5.4±3.1 mmol/L, triglyceride 1.40±1.30 mmol/L, and total apoB 132.0±41.4 mg/dL; (2) 4 patients with different LDLR mutations (D200G; W66G; deletion/frameshift in exon 9; deletion of exons 2 to 6); mean age 51.2±19.0 years, year on-treatment serum LDL cholesterol at the time of study 3.1±0.7 mmol/L, triglyceride 0.9±0.7 mmol/L, and total apoB 81.5±15.1 mg/dL; (3) 3 patients with familial defective apoB48 (mean age 60.1±16.0 years, mean on-treatment LDL cholesterol 4.2±1.6 mmol/L, triglyceride 1.0±0.3 mmol/L, and total apoB 124.0±6.6 mg/dL). At the time of the study, all patients were on treatment consisting of statins with or without ezetimibe. A control group comprised 5 normolipidemic volunteers (mean age 38.1±12.7 years, mean serum LDL cholesterol 3.1±0.6, triglyceride 1.2±0.2, and total apoB 69.5±8.5 mg/dL), who were not on any lipid-modifying drugs.

None of the patients were obese or had diabetes mellitus, hypothyroidism, or any other conditions known to influence cholesterol levels or to require treatment with medication (other than lipid-lowering drugs) affecting serum lipid levels.

Ethics research committee approval was obtained for this study, and all subjects gave informed written consent.

**Study Protocol and Methods**

Fasting venous blood samples were obtained for measurement of standard serum lipid parameters using automated enzymatic assays; LDL cholesterol was calculated using the Friedewald formula.

**Retrospective Assessment of Treatment Response**

The information about response to treatment with statins was analyzed after careful retrospective assessment of medical case notes of all affected individuals. The serum total cholesterol values on treatment of each patient had been derived as a mean of 2 measurements ≥3 months apart after the patient had been on treatment with the same statin and dose for ≥3 months and when no physiologic or other pathological causes that might have interfered with lipid levels had been identified.

Results

**Clinical Characteristics of Patients With the D374Y Mutation in PCSK9 and FH Patients With Defined LDLR Receptor Mutations Involved in Retrospective Analysis of Treatment Response**

The main clinical characteristics of patients with D374Y mutation of PCSK9 (PCSK9 patients) and the 3 groups of “classical” FH patients with severe LDLR mutations (LDLR patients) are summarized in Table I (available online at http://atvb.ahajournals.org). Of the 13 affected PCSK9 individuals, 6 had premature coronary heart disease (CHD) and 8 had tendon xanthomas; 13 of the LDLR patients had CHD, and 18 had tendon xanthomas. In all affected individuals,
D374Y was on an allele of PCSK9 with the same haplotype (please see online supplement).

Despite the much younger age at presentation of the PCSK9 patients compared with the 36 LDLR patients (20.8±14.7 versus 30.2±15.7 years; \( P = 0.003 \)), they had significantly higher serum total cholesterol concentrations (13.6±2.9 versus 9.6±1.6 mmol/L; \( P = 0.004 \)) and were affected at a much earlier age by premature CHD (35.3±4.8 versus 46.8±8.9 years; \( P = 0.002 \)). Although within the normal range, mean fasting serum triglyceride was significantly higher in the PCSK9 group (1.7±0.7 versus 1.08±0.57 mmol/L; \( P = 0.002 \); high-density lipoprotein (HDL) cholesterol levels (1.2±0.4 versus 1.2±0.27; \( P = \text{NS} \)) were similar in the 2 groups.

Figure 1 shows the pedigrees of the 4 PCSK9 families and summarizes the response to lipid-lowering management in each index patient over a long period of time. Because this is a disease for which the molecular basis was unraveled only recently and about which very limited clinical information is available, we describe in some detail the long-term medical history of the index patients and their affected relatives who have been followed up for up to 30 years (please see online supplement).

Comparison of Response to Treatment Between Patients With PCSK9 and LDL Receptor Mutations

Figure 2 shows the response to treatment in the PCSK9 patients and the 3 groups of LDLR patients. Because there
were no significant differences between the 3 LDLR patient groups in either pretreatment serum total cholesterol levels, or in absolute or percentage decreases in total serum cholesterol during treatment with simvastatin and atorvastatin, the LDLR patients have been analyzed as a single group. Mean serum total cholesterol concentrations remained significantly higher in the PCSK9 patients during treatment with either simvastatin (10.1 ± 3.9 mmol/L; P = 0.006) or atorvastatin (9.6 ± 2.9 mmol/L; P = 0.006; Figure 2A), despite the fact that the PCSK9 and LDLR groups received similar mean doses of simvastatin (48 versus 37.1 mg daily) or atorvastatin (64.0 versus 58.8 mg daily). Furthermore, 2 of the PCSK9 patients required, in addition to treatment with statins, a partial ileal bypass in 1 instance (family 1; patient III,1) and long-term LDL apheresis in the other (family 3; patient II,1) to improve serum cholesterol levels. However, although a trend was observed, the percent reduction in total cholesterol during treatment was not significantly lower in the PCSK9 group than in the 3 groups of LDLR patients (Figure 2B).

When serum total cholesterol levels were compared while all patients were on their current lipid-lowering therapy (Figure 2C), comprising statins plus ezetimibe or bile acid sequestrants, again, the values in the PCSK9 patients remained higher despite their younger age (7.0 ± 1.6 versus 5.4 ± 1.0 mmol/L; P = 0.001); similarly, the total cholesterol to HDL cholesterol ratio remained significantly higher (5.4 ± 0.95 versus 4.3 ± 1.5; P = 0.046) in the PCSK9 group. All the PCSK9 patients were on maximal doses of atorvastatin (80 mg) or rosuvastatin (40 mg in all adults and 10 mg in a minor) plus ezetimibe (10 mg daily); 2 patients, as already mentioned, had, in addition, partial ileal bypass and chronic treatment with LDL apheresis. The statin doses of the LDLR patients were substantially smaller, varying between 20 and 80 mg daily for atorvastatin (mean 54.1 ± 23.1 mg) and between 10 and 40 mg daily for rosuvastatin (mean 25.0 ± 21.1 mg); not all patients in this group were on concomitant treatment with ezetimibe or bile acid sequestrants. Despite the smaller statin doses, the LDLR FH patients achieved lower total cholesterol levels, and there was no need for additional interventions.

### LDL Binding and Competition Assays

To assess whether LDL from PCSK9 D374Y patients was able to bind to the LDL receptor with the same affinity as LDL from normolipidemic individuals, we determined its ability to compete for binding of normolipidemic 125I-labelled LDL to human skin fibroblasts in culture. In preliminary experiments with 2 different preparations of labelled LDL from a normolipidemic donor, we found that competition by LDL from a PCSK9 patient was significantly impaired compared with LDL from a normolipidemic donor (Figure 3A). To determine whether this impaired binding was common to all PCSK9 patients, LDL samples from 9 PCSK9 patients were then compared with LDL from 5 healthy volunteers, 4 LDLR patients who were on similar treatment regimes to the PCSK9 patients, and 3 patients with heterozygous familial defective apoB100 (FDB; Figure 3B). As expected, LDL from patients with FDB showed the weakest ability to compete and bind to the LDL receptors. LDL from the LDLR patients and the controls competed equally well for binding, whereas LDL from the PCSK9 patients did indeed show significantly impaired competition for binding when compared with LDL from either normolipidemic controls or LDLR patients on similar lipid-lowering treatment to the PCSK9 patients. Thus, PCSK9 LDL appears to have reduced affinity for binding to the LDL receptor.

To investigate the underlying reason for this, we determined the composition of the LDL and found that the mean cholesterol:protein ratio of PCSK9 LDL was significantly lower than that of the control subjects (Figure 3C, left). As expected, LDLR and FDB LDL had higher cholesterol:protein ratios than normal LDL. The triglyceride contents of the various LDL samples were not significantly different (data...
In this article, we describe 4 unrelated white British families comprising 13 individuals with severe autosomal dominant hypercholesterolemia attributable to the D374Y variant of the PCSK9 gene. When these patients are compared with typical heterozygous FH patients with known mutations in LDLR, even those selected as having null mutations, this group of PCSK9 patients seems to be more severely affected in that their pretreatment serum total cholesterol concentrations were higher, and levels on treatment with statins of total cholesterol and total cholesterol to HDL cholesterol ratio remained higher, and levels on treatment with statins of total cholesterol and total cholesterol to HDL cholesterol ratio remained higher. Possibly as a consequence of these 2 phenotypic features, the PCSK9 patients with the D374Y variant developed premature CHD >10 years earlier compared with the group of heterozygous carriers of severe mutations in LDLR. Unusually, 2 women 30 and 31 years of age, had total serum cholesterol levels of 13.6 mmol/L. Their pretreatment serum total cholesterol concentrations were measured before dietary advice had been provided. Indeed, like the severely affected British and Norwegian patients, there are family members in the Utah pedigree reported to have total serum cholesterol levels >11 mmol/L at 9 and 16 years of age.

In contrast, the French families carrying the F216L or S127R variants do not appear to have very severe hypercholesterolemia. Thus, it is likely that in patients with mutations in the PCSK9 gene, as is the case with patients with FH attributable to mutations in LDLR,1,20 the nature of the molecular defect has an impact on the severity of hypercholesterolemia. It is also possible that heterozygotes with the same missense D374Y mutation in the PCSK9 gene can have different phenotypic expression, as already observed in FH patients with the same LDL receptor mutation,21 and that a combination of environmental and genetic factors promotes the unusual severity of hypercholesterolemia in the British pedigrees.

Long-term management of the 13 patients described in this article has proved to be difficult. Despite maximal lipid-lowering medication, none of the patients have so far achieved currently acceptable “target” cholesterol levels.22 It is of interest that in some, but not all, PCSK9 patients, the addition of ezetimibe led to pronounced reduction in serum cholesterol, as did a very strict low-cholesterol low-fat diet; these effects are most probably attributable to drastically reduced absorption of cholesterol in the small intestine and not directly related to LDL receptor activity. Because there is no published data yet on long-term management and response to treatment in patients with PCSK9 mutations, we cannot compare our observations with other cohorts of patients with this particular mutation.

The mechanism underlying this severe autosomal dominant hypercholesterolemia associated with the D374Y mutation in PCSK9 in this group of British patients is not yet

Figure 3. Competition for binding of 125I-labelled LDL to human skin fibroblasts by unlabelled LDL. A and B, Cells were incubated for 1 hour at 4°C with 125I-labelled LDL (10 μg protein/mL; 750 cpm/μg) alone (amount bound was 57.8 ± 9.5 ng/mg cell protein) or with unlabelled LDL. A, Mean of 3 experiments with LDL from 1 normolipidemic control (normal) and 1 patient heterozygous for familial defective apoB3500; LDLR, patients with LDLR, patients with known LDLR mutations (Nor, patients with normal LDLR gene). B, LDL from hyperlipidemic patients (FDB indicates patients heterozygous for familial defective apoB3500; LDLR, patients with known LDLR mutations) and normolipidemic controls (Nor), with the number in each group shown in brackets. C, lipid:protein ratio of LDL (P, protein; TG, triglyceride; Chol, total cholesterol).
understood, nor is it clear why they should be so difficult to manage clinically compared with other FH patients. It could be speculated that the dual pathophysiological mechanism involved, namely decreased LDL receptor protein and increased apoB100 secretion, documented to occur in vivo and in vitro studies, may aggravate the phenotype. The lipid-lowering action of statins is via inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A, leading to reduced cholesterol biosynthesis and cellular cholesterol levels, which activates SREBP-2 and leads to transcriptional activation not only of the LDLR, which results in increased LDL receptor activity and lowering of plasma LDL, but also of PCSK9, which appears to have the opposing effect of reducing LDL receptor protein.12,14,15 Thus, treatment of PCSK9 patients with statins will presumably also increase the activity of the dominant-negative mutant form, further attenuating the beneficial effects of statin treatment and not allowing them to achieve target serum cholesterol levels22 even when maximal statin doses or a combination of lipid-lowering drugs are implemented, as observed in this study.

Our results indicate that LDL from PCSK9 patients have impaired capacity to compete for binding to normal fibroblast LDL receptors when compared with LDL from LDLR patients on similar lipid-lowering therapy and also with control subjects. The LDL from LDLR patients behaved similarly to those of control subjects, suggesting that the lipid-lowering medication is not responsible for the difference observed between PCSK9 and LDLR patients. The abnormal composition of PCSK9 LDL suggests that the conformation of apoB on the surface of the particles may be different from that of normal LDL and contribute to poor binding affinity. Our observations provide further evidence for the complexity of the phenotype in PCSK9 patients and raises the question as to the possible involvement of impaired LDL binding in addition to overproduction of apoB in causing their hypercholesterolemia.

The strengths of the study presented here include the fact that all the patients are British whites who have been attending our Lipid Clinics for many years and have received similar management. The PCSK9 patients all carry the same D374Y variant of PCSK9 on the same haplotype, suggesting that they share a common ancestor, whereas the LDLR patients were purposely selected to carry specific mutations in LDLR known to cause a severe hypercholesterolemic phenotype. Although the conclusions are limited by the relatively small number of patients and the retrospective nature of the study, the results clearly show that this group of British patients with the D374Y mutation of the PCSK9 have an unpredictably severe clinical phenotype, which requires early and aggressive lipid-lowering management to prevent cardiovascular complications. Currently available lipid-lowering drugs do not achieve adequate control of their hypercholesterolemia. The decreased plasma cholesterol levels and hypersensitivity to statins observed in mice lacking Pcsk915 suggests that in the future, compounds that lead to inhibition of PCSK9 activity may have synergistic if not additive effects when combined with statins. Adequate management of this disorder is expected to improve the prognosis of these patients, as has been shown for those with more typical heterozygous FH.19

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References

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Severe hypercholesterolaemia in four British families with the D374Y mutation in the PCSK9 gene: long term follow up and treatment response

Naoumova – Severe phenotype linked to PCSK9 D374Y mutation

Supplementary information

1. Clinical description of four index patients with PCSK9 mutation and affected family members from four unrelated British families

Family 1. The index patient (II,2 in Figure 1A) developed angina aged 39 yr; his cholesterol was 13.9 mmol/l and premature corneal arcus and very large tendon xanthomas on the dorsum of his hands, Achilles tendons and pre-tibial tuberositas were noted. Two brothers and his father had died of myocardial infarction aged 32 to 63 yr. Coronary artery by-pass graft (CABG) operation was performed when he was aged 42 yr and treatment with colestipol was initiated with poor response. In 1985, at the age of 51 yr he was referred to the Lipid Clinic at Hammersmith Hospital with hypercholesterolaemia that was unresponsive to treatment with maximal doses of currently available lipid-lowering drugs, as shown in Figure 1A. On a combination of colestipol 30 g/day, bezafibrate 600 mg/day and nicotinic acid 3 g daily, his fasting total cholesterol was 19.2 mmol/l and triglyceride 3.8 mmol/l, and remained exceptionally high until the introduction of potent statins. During the subsequent 19 years maximal doses of statins in combination with bile acid sequestrants and lately with ezetimibe led to a better control of serum cholesterol and to a decrease in size of his tendon xanthomata; fasting serum triglyceride ranged between 1.5-4.72 mmol/l and HDL cholesterol between 0.7-1.1 mmol/l. It is of interest that the patient now follows an exceptionally strict low cholesterol, low fat diet. He underwent re-do CABG operation in 1994 and has mild
peripheral vascular disease and asymptomatic carotid disease. At present the patient remains well with stable angina.

His daughter (III,3), who also carries the D374Y variant, was diagnosed at the age of 11 years. Her total cholesterol on treatment with colestipol and nicotinic acid was 14.3 mmol/l (pre-treatment levels are not known). Currently on treatment with rosuvastatin 40mg, her total cholesterol is 10.0 mmol/l. The proband’s niece (III,1) was referred to the Lipid Clinic at Hammersmith Hospital in 1996 at the age of 38 yr with very severe hypercholesterolaemia resistant to treatment with statins. She had large Achilles tendon xanthomas and bilateral xanthelasmas on her upper and lower lids, and a soft right carotid bruit. On simvastatin 40 mg/day and acipimox 750 mg daily, total cholesterol was 15.5 mmol/l, triglyceride 1.58 mmol/l, HDL cholesterol 1.28 mmol/l, and LDL cholesterol 13.5 mmol/l; on atorvastatin 80 mg her cholesterol remained at 11.3 mmol/l. The patient developed angina in 1997 and coronary angiography showed moderately severe premature CHD, and because of her resistance to treatment with statins she underwent partial ileal bypass, which in combination with atorvastatin 80 mg and weight loss of 15 kg led to a marked decrease in total cholesterol to 5.4 mmol/l, with triglyceride of 1.47 mmol/l and HDL cholesterol of 0.94 mmol/l. Although the patient subsequently regained 12 Kg over the next 4 yr, atorvastatin 80 mg maintained her total cholesterol between 6.2 and 7.1 mmol/l, with LDL cholesterol between 4.2 and 4.8 mmol/l. Recent addition of ezetimibe reduced her total cholesterol to 5.8 mmol/l. She remains well with stable angina.

**Family 2.** The index patient (III,2 on Figure 1B) was screened for hypercholesterolaemia at the age of 22 months because of the premature death of his mother (II,2) at age 31 yr from myocardial infarction. His non-fasting lipids were as follows: total cholesterol 13.4 mmol/l, triglyceride 4.04 mmol/l, HDL cholesterol 0.88 mmol/l.
Examination showed early tendon xanthomata on the index and middle fingers of both hands. Treatment with pravastatin 5 mg on alternate days reduced his cholesterol to 8.91 mmol/l, and triglyceride to 1.14 mmol/l, and increased HDL cholesterol to 1.80 mmol/l initially. Subsequently the dose of pravastatin was increased to 10 and 15 mg daily and colestipol was added (Figure 1B). Following referral to the Lipid Clinic at Hammersmith Hospital, pravastatin was substituted with simvastatin in combination with colestipol. Simvastatin doses were later increased to 20mg and 40 mg daily but total cholesterol levels remained high between 7 and 7.5 mmol/l. The recent introduction of rosuvastatin in combination with ezetimibe did not produce any significant further reduction in serum cholesterol. It should be noted that his serum cholesterol is very much influenced by dietary changes despite the potent statins and ezetimibe he is currently treated with. Triglyceride levels and HDL cholesterol have remained normal over the last 10 years. The patient has high Lp (a) levels ranging between 83 and 195 mg/dl (normal range: 0-30 mg/dl). At the age of 13 yr exercise ECG, echocardiography and Doppler carotid ultrasound were normal.

The index patient’s mother (II,2) was diagnosed with severe hypercholesterolaemia at the age of 13 years, with cholesterol ranging between 13 and 16 mmol/l and was treated with clofibrate and cholestyramine, followed by bezafibrate with no significant effect on her cholesterol. She developed premature CHD leading to her death at the age of 31 yr. Her father (I,1) had a myocardial infarction in his early 40s and a fatal one at 63 yr, and his sister (I,2) also died of premature CHD at 51 yr. A maternal aunt of the index patient (II,1) also has hypercholesterolaemia, which on simvastatin 40 mg decreased to 6 mmol/l from an initial 11 mmol/l. She was not known to have CHD disease until the age of 40 yr but is now lost to follow up.
**Family 3.** Following the death of his father (I,2) of MI at 47 yr (having survived one at the age of 36 yr), the index patient (II,1), who was then 21 yr of age, was found to have a total cholesterol of 18 mmol/l and treatment with diet was initiated (Figure 2C). In 1988 he was referred to the Lipid Clinic at Aintree Hospital in Liverpool, with serum total cholesterol of 12.1 mmol/l, triglyceride 2.6 mmol/l and HDL-cholesterol 1.2 mmol/l and large Achilles tendon xanthomatas and bilateral xanthelasmas. Treatment with cholestyramine 8 g daily and bezafibrate 400 mg (Bezalip Mono) resulted in total cholesterol of 13.7 mmol/l, but increasing cholestyramine to 36 g daily reduced total cholesterol to 11.8 mmol/l. In 1992, treatment with simvastatin was started with modest effect. In 2002 at the age of 41 yr, CABG was carried out and treatment with atorvastatin 80 mg, cholestyramine 8 g and fenofibrate 267 mg daily was commenced, again with little further effect on the lipid profile. Treatment with regular LDL apheresis was initiated, and, with atorvastatin 40 mg and ezetimibe 10 mg daily, his pre-LDL apheresis serum total cholesterol level remains between 7.0 and 7.5 mmol/l.

The proband’s sister (II,2) was diagnosed with severe hypercholesterolaemia in her early twenties (total serum cholesterol ~ 19 mmol/l) and died aged 30 yr while awaiting CABG. The proband’s paternal aunt (I,3) was found to have total cholesterol of 12.9 mmol/l at the age of 57 yr and on treatment with atorvastatin 80 mg and ezetimibe her total cholesterol is 7.6 mmol/l. Two children of the paternal aunt are also affected: pre-treatment total cholesterol of the daughter (II,4) was 12.0 mmol/l and of the son (II,5) 10.6 mmol/l. Treatment with rosuvastatin 40 mg and ezetimibe 10 mg daily has reduced total cholesterol to 7.6 (II,4) and 5.2 mmol/l (II,5).

**Family 4.** After the death of his father of acute myocardial infarction at the age of 52 yr, the index patient (II,1 on Figure 1D) was noted to have corneal arcus, xanthomata on
dorsum of his hands, pre-tibial and Achilles tendons at the age of 30 yr and his cholesterol was found to be 11.5 mmol/l, HDL cholesterol 0.5 mmol/l and triglyceride was normal. Treatment with simvastatin was initiated, followed by this in combination with clofibrate. Two years later he developed angina, severe triple vessel coronary disease was confirmed and he underwent successful angioplasty. In 1997, aged 35 yr, he was referred to the Lipid Clinic at Hammersmith Hospital because of poor response to treatment with lipid-lowering drugs and for consideration for treatment with LDL apheresis, which he declined to consider. Over the next 8 yr maximal doses of potent statins in combination with bile acid sequestrants or lately ezetimibe, a cholesterol absorption inhibitor, improved but did not fully normalize his lipid profile and his total cholesterol remains above 6 mmol/l. It is of interest that his HDL cholesterol remained low and ranged between 0.59 and 0.98 mmol/l over the years. There are no other affected family members.

2. Identification of the PCSK9 haplotype co-segregating with the D374Y mutation.

Fragments of PCSK9 bearing known common polymorphisms\(^1\) were amplified by PCR and their nucleotide sequence determined as described previously\(^2\). The trinucleotide repeat marker D1S417 was analysed as described previously\(^3\). Haplotypes were assigned by inspection of the pedigrees. The haplotype was defined by the following polymorphisms:

D1S417 (0.2Mb upstream of PCSK9) 187bp PCR product, exon 4 +9 G (f=0.97, 0.95), exon 5 -7 T (f=0.42, 0.28), exon 5 +3 G (f=0.38, 0.24) and exon 9 I474 (f=0.85, 0.86), exon 9 V460 G (f=0.21, 0.13), exon 12 E670 (f=0.93,0.92), where f is the reported frequency of each polymorphism in two different Caucasian populations\(^1,4\). In one individual a recombination appeared to have occurred between D1S417 and PCSK9.
The mutation D374Y occurs in the catalytic domain of the protein; when expressed in rat hepatoma cells, the mutation does not impair autoprocessing of the proprotein, nor does it affect the stability or subcellular localisation of the mature protein.

3. LDL binding and competition assays

Human skin fibroblasts were maintained in culture, seeded into 6cm diameter dishes and pre-incubated with lipoprotein-deficient serum for experiments. LDL (hydrated density 1.020-1.055 g/ml) was isolated from human EDTA-plasma by ultracentrifugation and labelled with ¹²⁵iodine monochloride. The protein concentration was assayed using the BioRad Dc Protein Assay kit, with bovine serum albumin as a standard. Binding of ¹²⁵I-labelled LDL to cells at 4°C was determined using methods described by Goldstein and Brown. The total cholesterol and triglyceride content of the LDL was determined with commercially available kits (Randox laboratories Ltd, Crumlin, co Antrim, UK).

4. Assessment of plasma LDL particle size

The analysis was carried out by non-denaturing gradient gel electrophoresis, as previously described and validated on whole EDTA-plasma, obtained on the same day as for the LDL binding and competition assay. Plasma samples were frozen immediately and sent to Cape Town on dry ice for assay within two weeks.

5. Statistical analyses

Two-way ANOVA analyses were performed using the subgroup of patients for whom data were available on pre-treatment TC levels and levels during treatment with simvastatin and atorvastatin (17 such patients with LDLR mutations and 5 with mutations in PCSK9). While the two groups were not homoscedastic, they did satisfy the so-called “Rule of two”. The factors considered in these analyses were the type of gene in which the mutation is
observed (binary coding) and treatment drug, with an interacting factor of dosage. Welch’s two sample t-tests were carried out to assess significance of difference between group means. Similarly, two-way ANOVAs were performed to assess (pairwise) differences in competition for binding of labeled LDL by LDL from normal, PCSK9, FDB and FH patients and one-way ANOVA and Welch two sample t-tests performed on the lipid/protein ratios. Analyses were performed using the functions “aov” and “t.test” from the R base package\textsuperscript{10}. All data are presented as mean and standard deviation.
### TABLE I. Clinical characteristics of patients with mutations in the LDL-receptor and PCSK9 genes

<table>
<thead>
<tr>
<th>Type of mutation</th>
<th>N</th>
<th>M/F</th>
<th>Age at presentation (yr)</th>
<th>Tendon Xanthomas (number of patients)</th>
<th>Number of Patients with CHD (mean age)</th>
<th>Current or Ex-smokers (number of patients)</th>
<th>Pre-Treatment Total Cholesterol (mmol/l)</th>
<th>Triglyceride (mmol/l)</th>
<th>HDL-Cholesterol (mmol/l)</th>
<th>LDL-Cholesterol (mmol/l)</th>
<th>Total Cholesterol / HDL-Cholesterol ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCSK9 D374Y</td>
<td>13</td>
<td>5/8</td>
<td>20.8 (14.7)†</td>
<td>8</td>
<td>6 (35.3)†</td>
<td>1</td>
<td>13.6* (2.9)‡</td>
<td>1.7 (0.5)§</td>
<td>1.2 (0.4)</td>
<td>11.3 (4.1)‡</td>
<td>11.8 (6.2)</td>
</tr>
<tr>
<td>LDLR Exon 4</td>
<td>14</td>
<td>7/7</td>
<td>26.3 (15.3)</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>9.1 (1.8)</td>
<td>0.8 (0.3)</td>
<td>1.14 (0.16)</td>
<td>7.0 (1.5)</td>
<td>7.6 (2.3)</td>
</tr>
<tr>
<td>LDLR Intron 3</td>
<td>8</td>
<td>5/3</td>
<td>35.5 (18.4)</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>10.6 (1.7)</td>
<td>1.3 (0.5)</td>
<td>1.20 (0.20)</td>
<td>8.3 (1.0)</td>
<td>8.3 (1.4)</td>
</tr>
<tr>
<td>LDLR null</td>
<td>14</td>
<td>7/7</td>
<td>31.1 (14.7)</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>9.6 (1.2)</td>
<td>1.2 (0.67)</td>
<td>1.30 (0.36)</td>
<td>8.0 (1.37)</td>
<td>8.1 (2.4)</td>
</tr>
<tr>
<td>All pts with LDLR mutations</td>
<td>36</td>
<td>19/17</td>
<td>30.2 (15.7)</td>
<td>18</td>
<td>19 (46.8)</td>
<td>9</td>
<td>9.6 (1.6)</td>
<td>1.1 (0.6)</td>
<td>1.2 (0.27)</td>
<td>7.7 (1.4)</td>
<td>8.0 (2.0)</td>
</tr>
</tbody>
</table>

* In two of the patients, pre-treatment levels were unknown and values when on treatment with colestipol and nicotinic acid (in one of the patients) and on simvastatin 40mg plus nicotinic acid (in the second patient) have been used.

Data are mean ± SD. Comparisons are between the group with PCSK9 D374Y mutation and all patients with LDLR mutations; †p=0.003; ‡p=0.004; §p=0.002
Table II. Mean pre-treatment total cholesterol and age in patients with mutations in the PCSK9 and LDL-receptor genes

<table>
<thead>
<tr>
<th>Group of heterozygous FH patients</th>
<th>No.</th>
<th>Mean pre-treatment total chol* (mmol/l)</th>
<th>Mean age (yr)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCSK9 D374Y</td>
<td>13</td>
<td>13.6±2.9</td>
<td>20.8±14.75</td>
<td>This study</td>
</tr>
<tr>
<td>Known LDL-receptor mutations</td>
<td>36</td>
<td>9.6±1.6</td>
<td>30.2±15.7</td>
<td>This study</td>
</tr>
<tr>
<td>Clinical diagnosis of definite FH:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>605</td>
<td>8.5±2.1</td>
<td>40.3†</td>
<td>11</td>
</tr>
<tr>
<td>Women</td>
<td>580</td>
<td>9.0±2.4</td>
<td>43.9†</td>
<td></td>
</tr>
<tr>
<td>Known LDLR mutation:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any</td>
<td>118</td>
<td>10.7±1.9</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>Null</td>
<td>12</td>
<td>11.3±2.1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CHD positive/known LDLR mutation</td>
<td>22</td>
<td>10.4±1.9</td>
<td>50±7</td>
<td>13</td>
</tr>
<tr>
<td>Refractory hyperchol*/ known LDLR</td>
<td>17</td>
<td>11.6±1.3</td>
<td>51±9</td>
<td>14</td>
</tr>
<tr>
<td>LDLR mutation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoB 3500</td>
<td>6</td>
<td>9.9±1.8</td>
<td>51±8</td>
<td>13,14</td>
</tr>
<tr>
<td>Fr. Canadian with LDLR10Kb</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>del‡: All</td>
<td>105</td>
<td>9.8±1.9</td>
<td>45±11</td>
<td>15</td>
</tr>
<tr>
<td>“statin non-responders”</td>
<td>9</td>
<td>9.9±4.0</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*Chol, cholesterol; †Median age; ‡del, deletion.
Supplementary Reference List


Response to HMG CoA reductase inhibitors in heterozygous familial
hypercholesterolemia due to the 10-kb deletion ("French Canadian mutation") of the LDL