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A new molecular defect in the lecithin:cholesterol acyltransferase (LCAT) gene associated with fish eye disease[1]

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Abstract: We report a new genetic defect in the lecithin:cholesterol acyltransferase (LCAT) gene associated with classical clinical and biochemical features of fish eye disease. The 63-year-old Australian female proband also suffers from non-insulin-dependent (type II) diabetes mellitus. She presented with corneal opacities, markedly reduced HDL-cholesterol (0.1 mmol/L; < 10% of normal controls), and elevated plasma triglycerides. The presence of diabetes did not explain the lipoprotein profile, which differed markedly in comparison to two female hypertriglyceridemic diabetic subjects. Cholesterol esterification in HDL-like particles was minimal but plasma cholesterol esterification was maintained due to LCAT activity in non-HDL-containing lipoprotein fractions. DNA sequence analysis of the proband's LCAT gene showed two C to T transitions resulting in the substitution of Thr_{123} with Ile and Tyr_{144} with Cys. Allele-specific PCR amplification procedures were used to confirm the presence of the mutations in this proband and to screen for additional carriers in her family. Three first degree relatives (mother, brother, son) were heterozygous for the $Thr_{123} \rightarrow$ Ile mutation and her daughter had the $Tyr_{144} \rightarrow$ Cys mutation. Apart from a reduction in HDL-cholesterol levels to half the normal concentration and a 20% reduction in apoA-I levels, their plasma lipids were unremarkable. The proband's son and daughter were further investigated. Both had normal cholesterol esterification rates in plasma and VLDL/LDL-depleted plasma, but reduced LCAT activity (50% that of normal). Thus, the biochemical and phenotypic expression for fish eye disease in the heterozygote subjects was similar, irrespective of the underlying LCAT mutation.—**Contacos, C., D. R. Sullivan, K-A. Rye, H. Funke, and G. Assmann.** A new molecular defect in the lecithin:cholesterol acyltransferase (LCAT) gene associated with fish eye disease. *J. Lipid Res.* 1996. **37:** 35–44.

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Epidemiologic studies have shown that low plasma HDL-cholesterol (HDL-C) levels are a major predictor for the development of coronary artery disease (CAD) (1, 2). However, in some cases, despite severely reduced HDL-C levels, patients are not at increased risk of premature CAD (3, 4). Low HDL-C concentration may be caused by genetic disorders of HDL metabolism (3, 4). Two such anomalies, fish eye disease (FED) and familial lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.4.3) deficiency, result from the partial or total inability of the enzyme LCAT to esterify cholesterol in plasma lipoproteins (4, 5). LCAT esterifies cholesterol with the *sn*-2 fatty acid derived from phosphatidylcholine (6). In the normal state, 95% of plasma cholesteryl esters are produced by the LCAT reaction on HDL, termed αLCAT, but LCAT may also act on apoB-containing lipoproteins, termed βLCAT activity (7–9).

Classically, subjects with FED and familial LCAT deficiency cannot esterify cholesterol on artificial HDL-like proteoliposomes, but differ in that subjects with FED maintain βLCAT activity (8). Thus, subjects with FED have near normal ratios of plasma unesterified to total cholesterol, whilst in familial LCAT deficiency the plasma unesterified cholesterol levels are markedly elevated (4, 5). Although subjects with either disorder present with corneal opacities, the clinical presentation

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; HDL-C, high density lipoprotein-cholesterol; MS-PCR, mutagenically separated-PCR; CAD, coronary artery disease; FED, fish eye disease; LDL-C, low density lipoprotein-cholesterol; CER, cholesterol esterification rate; PCR, polymerase chain reaction.

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of LCAT deficiency and FED differs in that the former can also develop anemia and renal disease (4).

Several molecular defects of the LCAT gene causing familial LCAT deficiency (10, 11) and FED (12, 13) have been identified. In the classical definition of FED (βLCAT but absent αLCAT), subjects have been identified as homozygous for a $Thr_{123} \rightarrow$ Ile mutation (12) or a $Pro_{10} \rightarrow$ Leu mutation (13), while one compound heterozygote with mutations at codons 123/347 (14) on the LCAT gene has been described. By contrast, subjects with clinical features of fish eye syndrome who nevertheless exhibit HDL-associated αLCAT activity have recently been identified (15–17). In in vitro experiments, these reports showed that reduced amounts of functional mutant LCAT were produced that maintained αLCAT specific activity.

We have investigated an Australian family with classic clinical and biochemical characteristics for FED. The proband is compound heterozygote for $Thr_{123} \rightarrow$ Ile and the previously undescribed C to T exchange in codon 144, causing the substitution of tyrosine with cysteine ($Tyr_{144} \rightarrow$ Cys). The proband does not have clinical symptoms or signs of cardiovascular disease despite the presence of non-insulin-dependent (type II) diabetes mellitus for the past 13 years.

METHODS

Subjects

The 63-year-old female proband (IIa, see **Fig. 1**), was originally referred to the hospital in 1964 with corneal opacities and again in 1971 with mild hypertriglyceridemia, obesity, bilateral arcus senilus, and xanthelasma. Her HDL-C was extremely low (0.1 mmol/L) and agarose gel electrophoresis showed an absent α and increased β band. She developed non-insulin-dependent diabetes mellitus in 1981 and subsequently commenced insulin therapy in 1983. At the time of this study, her diabetes was managed with insulin (neutral insulin 8 units mane, 6 units midi, 4 units nocte; isophane insulin 11 units nocte) and an oral hypoglycemic agent (metformin hydrochloride 500 mg TDS). Her body mass index was 31.0 kg • m^{-2}, hematological parameters and renal function were normal, and there was no evidence of proteinuria or hypertension. There was no clinical evidence of CAD. The proband's four living first degree relatives (mother, brother, daughter, and son) were also studied. Apart from the 88-year-old mother, (Ia) who had a cerebrovascular accident in 1993, the relatives had no known health problems. All subjects were born in Australia.

Two hypertriglyceridemic female subjects with non-insulin-dependent diabetes mellitus were used as diabetic controls for the proband. Neither subject was on lipid-lowering medications and their diabetes was managed on diet alone. Five healthy volunteers, two males and three females, were used as normal controls. The diabetic and normal controls were not related to each other or to the proband and her family.

Lipid, apolipoprotein analyses and lipoprotein isolation

EDTA blood (final concentration, 1.7 mg/mL disodium-EDTA) was collected from all subjects after a 12-h overnight fast and immediately centrifuged (2,500 rpm) for 20 min at 4°C to recover plasma. HDL-C was determined in plasma after precipitation of apoB-containing lipoproteins by dextran sulphate–magnesium chloride (18). LDL-cholesterol (LDL-C) in the proband was determined in the d > 1.006 g/mL plasma fraction by subtracting HDL-C (measured in the d > 1.006 g/mL fraction as above) from the total cholesterol in the d > 1.006 g/mL fraction. In other subjects, where plasma triglyceride was less than 4 mmol/L and LDL composi-

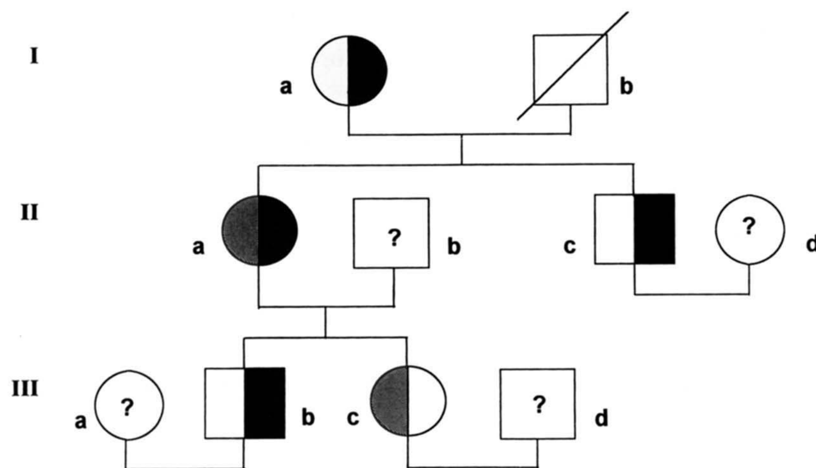

Fig. 1. Pedigree of the Australian FED family: ○, female; □, male; dark-solid area, heterozygous for T123I mutation; shaded area, heterozygous for Y144C mutation; diagonal bar, deceased; ?, family members not available for analysis.

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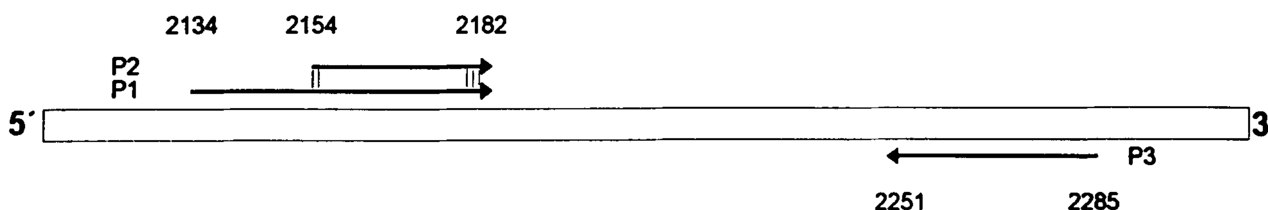

Fig. 2. Primers used for the allele specific detection of the FED mutation (LCAT:T123I) with MS-PCR. The box represents exon 4 of LCAT and adjacent sequence. The arrows indicate the location and lengths of the primers used in a single tube PCR amplification. Nucleotide positions are numbered according to McLean et al. (27). Primers P1 and P2 differ in length by 20 nucleotides and in sequence at five positions: two mutations at the 5′-end of P2 and three positions at the 3′-end of P1 and P2. The difference at the most 3′ position (nt 2182) represents the difference in the allelic sequence. P1 is wild type (C) at this position, P2 has the mutant nucleotide (T) at this position. The four other mutations are present to assure specificity of allele detection (25). The presence of a wild type allele in the analyzed sample is detected by a 152 bp long PCR product, whereas a 132 bp long fragment is indicative of the mutant allele.

tion was not grossly altered, LDL was calculated by the Friedewald equation (19).

VLDL of d < 1.006 g/mL and the d > 1.006 g/mL fraction were isolated from plasma by single spin ultracentrifugation (50.4 rotor; 40,000 rpm, 4°C, 16 h in a Beckman ultracentrifuge, Palo Alto, CA). VLDL was recovered in the top fraction after tube slicing. LDL (1.019 < d < 1.063 g/mL) was isolated from fasted plasma by sequential ultracentrifugation.

Total cholesterol (TC), triglycerides (TG), unesterified cholesterol (UC), and plasma phospholipids (PL) were determined with standard enzymatic techniques. Other routine biochemical indices were measured on a Hitachi 747 autoanalyser. Cholesteryl ester (CE) was calculated as TC minus UC. ApoA-I and apoB were measured by nephelometry using commercially available antibodies (Behringwerke AG, Marburg, Germany). LDL composition was determined by the percentage weight of constituents, with 651 and 860 as the molecular weights for CE and TG, respectively. Plasma and lipoprotein fractions were stored at -70°C until assayed.

Gradient gel electrophoresis

LDL and HDL particle size was determined in the d < 1.25 g/mL plasma fraction isolated by ultracentrifugation (100,000 rpm, 16 h, 4°C) in a Beckman TL 100 ultracentrifuge equipped with a 100.1 rotor. LDL and HDL were separated by non-denaturing gradient gel electrophoresis (GGE) (20) on 3–13% and 3–30% gradient gels (Gradipore Ltd, Sydney, Australia), respectively. Reference proteins (Pharmacia high-molecular-weight proteins; thyroglobulin diameter, 17 nm; ferritin, 12.2 nm; lactate dehydrogenase, 8.2 nm; and bovine serum albumin, 7.1 nm) were included in the electrophoretic run for particle diameter determination. An additional lane of standards (calibrated latex beads, diameter 38 nm; Duke Scientific, Palo Alto, CA) was included in the 3–13% gels. Gels were stained for protein (0.04% Coomassie blue and 3.5% perchloric acid) and scanned (633 nm) with an LKB Ultroscan XL laser densitometer.

Plasma cholesterol esterification rate (CER) and LCAT activity

The CER was measured by determining the rate of esterification of [^{3}H]cholesterol in autologous plasma as a substrate as previously described (21). In addition, the rate of esterification of [^{3}H]cholesterol was measured in VLDL/LDL-depleted plasma as a substrate (18). As autologous sample was used, the CER was influenced by the amount of substrate (lipoproteins) and LCAT in the sample and therefore represented the total cholesterol esterification and total LCAT activity in plasma or VLDL/LDL-depleted plasma. The sample (plasma or VLDL/LDL-depleted plasma) was labeled by an overnight 4°C incubation with [^{3}H]cholesterol. The labeled sample was incubated at 37°C for 30 min and aliquots were taken before and after the 37°C incubation. Lipids were extracted with ethanol and the protein precipitate was removed by centrifugation. CE and UC were separated by TLC (21), the respective bands were cut after being visualized by iodine vapors, and radioactivity was quantitated in a liquid scintillation counter (Tri-carb 4000 series, Packard Instruments, Zurich, Switzerland). The molar esterification rate was determined by multiplying the fractional esterification rate by the concentration of unesterified cholesterol in the sample and expressed per h • mL^{-1}. The interassay coefficient of variation for this procedure was 4.6% (n = 10) on an aliquot of plasma stored at -70°C for 7 months.

The LCAT activity was measured in the lipoprotein-free (d > 1.25 g/mL) plasma fraction and measured the rate of esterification of [^{3}H]cholesterol using an exogenous substrate of discoidal reconstituted HDL (22, 23). The discs were prepared with egg phosphatidylcholine (POPC), UC, and apoA-I by the cholate dialysis method (23). The POPC and UC were purchased from Sigma Chemical Co., St. Louis, MO. ApoA-I was isolated from HDL exactly as described elsewhere (24). The final POPC:UC:apoA-I molar ratio was 80:5:1. As an exogenous HDL-like substrate was used, measurement of LCAT activity by this method was specific for HDL-as-

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sociated or α-LCAT activity in the d > 1.25 g/mL plasma fraction. LCAT activity was expressed as nmoles CE formed in the d > 1.25 g/mL fraction per h • mL^{-1}.

Gene sequencing and genotype determination

The LCAT gene of the compound heterozygous patient was sequenced directly from single-stranded DNA as previously described (11, 12). Sequencing templates were prepared by PCR amplification of all exons and by subsequent single-strand preparation using streptavidin-covered magnetic beads (Dynabeads M-280-Streptavidin; Dynal, Oslo, Norway). This technique facilitates phase separation by the use of a strong magnet. Beads (35 μL) were washed twice with 50 μL washing buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 M NaCl) and were resuspended in 50 μL washing buffer. Then 50 μL of PCR product was added and incubated at 37°C for 30 min with occasional gentle mixing. After incubation, one washing step with washing buffer was added and the beads were then incubated twice for 5 min with 50 μL 0.15 M NaOH. After these incubations, beads were briefly washed with 50 μL 0.15 M NaOH, with 50 μL washing buffer and with 50 μL 1 × TE, respectively. Beads were then suspended in water to yield a template volume required by the sequencing protocol.

Carriers of the classic FED mutation were detected with mutagenically separated PCR (MS–PCR), a previously described allele-specific mutation-detection system (25). In brief, two allele specific PCR reactions are carried out simultaneously in a single test tube. Each PCR reaction uses an allele-specific primer and an opposite strand primer, which is identical in both reactions. The specific primers differ in the nucleotide at their 3′ ends, which correspond to the mutant and wild-type alleles, respectively. Additional mutations at noncorresponding base positions have been introduced into the primers to increase sequence differences between them. This increases allele-specificity and prevents cross-amplification (25). To allow separation of the allele-specific amplification products upon subsequent agarose electrophoresis, the wild type specific primer is 20 bases longer than the FED-specific primer. The sequences of the specific primers are the following: 5′ TGGCCCAAGCCCCCGGTGCTCATGGTCCCCCA CAGGGTACCTGCAAAC 3′ (wild type) and 5′ GCTGGTCCCCCCACAGGGTACCTGCACTT 3′ (mutant). Positions carrying mutations are underlined. The opposite-strand primer sequence is 5′ TCATCCGCA-GAGACACTCACCGGGCTCCAGCCGCC 3′. A graphic description of the amplification strategy is given in **Fig. 2.**

The opposite-strand primer used in the allele-specific PCR amplification of the mutation in codon 123 was placed at a position that included codon 144, the second mutant position found in this family, in the PCR product. Because the mutation in codon 144 destroyed a Mae III restriction site, a site which did not occur in the DNA between the two mutations, incubation of the PCR fragment with this restriction endonuclease was used to determine the *cis* or *trans* location of the mutations in codons 123 and 144.

RESULTS

Lipid and lipoprotein analyses

The proband and her relatives. The lipid and apolipoprotein analyses for the proband and her relatives are shown in **Table 1,** and the family pedigree in Fig. 1. The proband had elevated plasma TC, TG, LDL-C, and plasma apoB levels, and an extremely low HDL-C (0.10

TABLE 1. Plasma lipid, apolipoprotein concentrations and basic genetic defect in LCAT gene in the proband and her first degree relatives

		Concentration				Concentration		
Family Member	Age/Sex	TC	TG	HDL-C	LDL-C	ApoA-I	ApoB	Mutation Amino Acids in LCAT Gene
		mmol/L				*g/L*		
Comp. heterozygote								
IIa	63/F	6.6	3.8	0.10	6.0	0.23	2.46	$Thr_{123} \rightarrow Ile$ $Tyr_{144} \rightarrow Cys$
Heterozygote								
Ia	88/F	3.9	1.6	0.56	2.6	ND	ND	$Thr_{123} \rightarrow Ile$
IIc	65/M	4.2	1.7	0.50	2.9	1.08	1.24	$Thr_{123} \rightarrow Ile$
IIIb	36/M	4.6	0.8	0.63	3.6	1.02	1.12	$Thr_{123} \rightarrow Ile$
IIIc	33/F	5.5	1.6	0.64	4.1	1.18	1.60	$Tyr_{144} \rightarrow Cys$
Normal controls; mean ± SD (n = 5)[a]	38 ± 5 2M, 3F	4.6 ± 0.6	0.9 ± 0.3	1.58 ± 0.40	2.6 ± 0.6	1.53 ± 0.19	1.03 ± 0.21	ND

Abbreviations: M, male; F, female; TC, plasma total cholesterol; TG, plasma triglycerides; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol calculated as described in Methods; apo, apolipoprotein; Comp., compound; ND, not determined.

[a]Values for normal controls represent the mean and standard deviation from five individuals from an unrelated family.

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TABLE 2. Characteristics of FED subject and control female diabetic subjects

Subject	Age	BMI	ApoE	Fasting Lipids: TC	TG	HDL-C	LDL-C	VLDL-TC	VLDL-TG	Apoproteins: ApoA	ApoB
	yr	*kg • m⁻²*		*mmol/L*						*g/L*	
IIa[a]	63	31	E4/E4	6.6	3.8	0.10	6.0	0.5	0.6	0.23	2.46
JW	57	28	E3/E4	5.3	3.1	0.69	3.4	1.2	2.8	1.47	1.24
HT	66	34	E2/E3	6.7	2.4	1.08	4.7	0.9	2.2	1.27	1.84

Abbreviations: BMI, body mass index; Apo, apolipoprotein; TC, plasma total cholesterol; TG, plasma triglyceride; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol determined in the d > 1.006 g/mL plasma fraction as described in Methods; VLDL-TC, very low density lipoprotein-cholesterol; VLDL-TG, very low density lipoprotein-triglyceride. JW and HT are the initials of the control female diabetic subjects.

[a]Proband.

mmol/L; < 10% of normal controls) and plasma apoA-I (0.23 g/L; < 20% of normal controls). Apart from HDL-C levels, other lipid levels for her relatives were unremarkable (daughter's TC marginally elevated at 5.5 mmol/L). All the relatives had HDL-C levels less than half the normal concentration (0.5–0.64 mmol/L compared to 1.58 mmol/L in normals), and plasma apoA-I levels were 20% lower than the normal control subjects. The phenotypic expression of plasma lipids and apolipoproteins was indistinguishable between the two LCAT mutations in this family.

The proband and female diabetic control subjects. The characteristics and fasting lipids for the proband and two diabetic control subjects are listed in **Table 2.** The proband had an elevated plasma apoB and reduced HDL-C and apoA-I concentration compared to the diabetic controls. There was a marked difference in the concentration and distribution of lipoprotein triglycerides between the proband and the diabetic control subjects. All subjects had elevated plasma TG, but the proband had normal VLDL-TG concentration while the diabetic controls had elevated VLDL-TG, typically seen in hypertriglyceridemia. The proband's elevated plasma TG was due to an increased TG concentration in the d > 1.006 g/mL plasma fraction (3.8 mmol/L compared to 0.4–0.7 mmol/L in the diabetic controls).

Stokes' diameter of HDL and LDL; composition of LDL

The Stokes' diameter of HDL and LDL and composition of LDL for the proband is shown in **Table 3.** As judged by GGE, the proband had only small HDL (7.6 nm) that were smaller than the normal range for HDL Stokes' diameters between 7.9–8.5 nm (HDL_3) and 8.7–12.5 nm (HDL_2). Her LDL was larger (30.8 nm) compared to both normal (29.3 nm) and diabetic LDL (27.3 nm). The composition of the proband's LDL was grossly abnormal, primarily due to a 5-fold increase in LDL-TG, and a reduced amount of cholesteryl ester per LDL, compared to both normal and diabetic controls.

Plasma CER and LCAT activity

As the concentration of UC and PL, specifically phosphatidylcholine, in a sample affect the plasma CER, these variables were measured in the plasma of the FED proband to determine the validity of comparative CER measurements with control subjects. Plasma UC and PL were markedly elevated in the FED proband. Plasma UC

TABLE 3. LDL (1.019 < d < 1.063 g/mL) composition, LDL and HDL Stokes' diameter for the proband, normal and diabetic control subjects

Constituent	LDL; Percentage Composition by Mass: Compound Heterozygote (IIa)	Normal Controls[a]	Diabetic Controls
Phospholipid	23.3	21.5 ± 0.6	20.4
Cholesteryl ester	19.1	41.7 ± 1.9	44.0
Unesterified cholesterol	9.0	8.8 ± 0.4	7.8
Triglyceride	23.2	5.1 ± 1.7	3.3
Protein	25.4	22.9 ± 0.6	24.5
LDL Stokes' diameter (nm)	30.8	29.3 ± 0.5	27.3
HDL_2 Stokes' diameter (nm)	Not detectable	8.7–12.5	ND
HDL_3 Stokes' diameter (nm)	7.6	7.9–8.5	ND

Abbreviations: LDL, low density lipoprotein; ND, not determined.

[a]Values for normal controls represent the mean and standard deviation from five individuals from an unrelated family. Values for diabetic controls represent the mean of the two diabetic controls.

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TABLE 4. Cholesterol esterification and LCAT activity in the proband, her relatives and diabetic controls

Subject	UC:TC	Plasma CER	LCAT Activity
		nmol • h⁻¹ • mL⁻¹	
Compound heterozygote			
IIa	0.46	44 [0]	0.8
Heterozygote			
Ia	ND	ND	ND
IIC	ND	ND	ND
IIIb	0.29	65 [18]	7.3
IIIc	0.30	84 [22]	9.3
Normal controls (n=5)[a]	0.27 ± 0.01	87 ± 23 [28 ± 6]	15.1 ± 1.3
Diabetic controls	0.29	108 [26]	25.1 ± 3.8

Abbreviations: UC, plasma unesterified cholesterol; CER, plasma cholesterol esterification rate; LCAT, lecithin:cholesterol acyltransferase; ND, not determined; numbers in brackets represent CER in VLDL/LDL-depleted plasma.

[a]Values for normal controls represent the mean and standard deviation from five unrelated individuals. Values for diabetic controls represent the mean of the two diabetic controls.

was 3.0 mmol/L (result not shown) which was equivalent to 46% of total plasma cholesterol in the proband (Table 4, normal is between 25–30% of total plasma cholesterol). Plasma PL was 5.8 mmol/L (result not shown) while the normal reference range is between 2.03 and 3.55 mmol/L (26). The proband's phospholipid profile showed that 71.7% of the total plasma phospholipid was as phosphatidylcholine (result not shown). These results confirm both adequate substrate levels of UC and phosphatidylcholine for CER in the FED proband.

The plasma CER and LCAT activity for the proband, her relatives and the normal and diabetic controls are shown in **Table 4.** The proband's son and daughter had different LCAT mutations; her son, IIIb, was heterozygous for the $Thr_{123} \rightarrow$ Ile mutation while her daughter, IIIc, was heterozygous for the new $Tyr_{144} \rightarrow$ Cys mutation. The plasma unesterified cholesterol to total cholesterol ratio was elevated in the proband (0.46) compared to her heterozygote children (0.29–0.30), the normal controls (0.27), and diabetic controls (0.29), but not to the extent observed in familial LCAT deficiency (0.8–0.9) (11). This result was reflected in the plasma CER where the proband's plasma CER was reduced to approximately 50% of the normal and 40% of the diabetic controls, but the plasma CER values for her two children were comparable to the normal controls.

The CER was also measured in VLDL/LDL-depleted plasma. In the diabetic and normal controls, between 24 and 32% of the total plasma CER, respectively, was due to HDL in this plasma fraction. In contrast, the proband had no CER associated with HDL in this fraction but both children had normal CER in VLDL/LDL-depleted plasma.

The LCAT activity of the d > 1.25 g/mL fraction of the patient's plasma was markedly reduced (0.8 nmol • h^{-1} • mL^{-1}) compared to the LCAT activity for normal and diabetic controls, 15.1 and 25.1 nmol • h^{-1} • mL^{-1}, respectively. The proband's son and daughter both had a 40–50% reduction in LCAT activity compared to the normal controls.

LCAT gene sequencing and genotype determination

Sequence analysis of the LCAT gene of the proband showed deviations from the reported wild-type sequence (27). The proband had two heterozygous mutations, the first at codon 123 ($Thr_{123} \rightarrow$ Ile) (**Fig. 3A**) and the second, previously unknown mutation at codon 144 causing the substitution of tyrosine with cysteine (Fig. 3B).

Carriers in the family were identified by a combination of MS-PCR and allele-specific restriction site cleavage. The electrophoretogram showing the genotypes in codon 123 is shown in **Fig. 4A.** The 154 bp long fragment detected the wild type and the 134 bp long fragment indicated the presence of the T123I-mutation in LCAT. The proband, her mother, brother, and son were heterozygous for the $Thr_{123} \rightarrow$ Ile mutation (see also Table 1). Figure 4B shows the same DNA fragments as in Fig. 4A after digestion with the restriction endonuclease Mae III (GTNAC). The enzyme specifically cuts the MS-PCR-derived fragment after nucleotide 2244 when codon 144 is mutant (TGT). The presence of a 111 bp long fragment in the proband and her daughter (IIIc)

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identified these two individuals as carriers of the Y144C mutation (see also Table 1) and demonstrated that the mutations in codons 123 and 144 were located in *trans.*

DISCUSSION

In recent years, several molecular mutations in the LCAT gene causing HDL deficiency, hypoalphalipoproteinemia, and corneal opacities have been identified. Subjects with various LCAT mutations are identified as having either familial LCAT deficiency or FED according to their clinical and biochemical presentation. Classic familial LCAT-deficient patients present with normochromic anemia, glomerulosclerosis, and markedly increased plasma unesterified cholesterol levels due to an inability of LCAT to esterify cholesterol on all lipoproteins (4). Subjects with FED do not develop anemia or renal disease and maintain total plasma cholesterol esterification due to LCAT activity on apoB-containing lipoproteins and thus have a near normal plasma cholesteryl ester/unesterified cholesterol ratio (8).

Until recently, all subjects with the clinical features of fish eye syndrome were reported as having βLCAT but an absence of αLCAT activity, and an inability to esterify cholesterol in artificial HDL-like particles (8, 9, 12, 14). However, subjects with corneal opacity and normal unesterified to esterified cholesterol ratio who maintain LCAT activity on HDL and HDL-like particles have since been reported by Klein et al. (15). The siblings were homozygous for an $LCAT_{300} \rightarrow _{del}$ mutation and had an αLCAT activity that was 14% of normal controls, but normal levels of LCAT activity per unit mass LCAT (specific αLCAT activity). They also reported a markedly reduced plasma CER, when compared to that of previous FED subjects. The authors attributed the clinical presentation to an overall reduction in both β and αLCAT activity, rather than selective loss in αLCAT. Similarly, two more LCAT mutations have been reported whereby the mutant LCAT retained some cholesterol esterification on HDL and HDL-like particles. One subject was described as homozygous for an Arg_{158} → Cys mutation (17) while another was compound heterozygous for mutations at the 252/391 codons, with only the 391 mutation expressing an ability to esterify cholesterol in HDL-like proteoliposomes (16). These

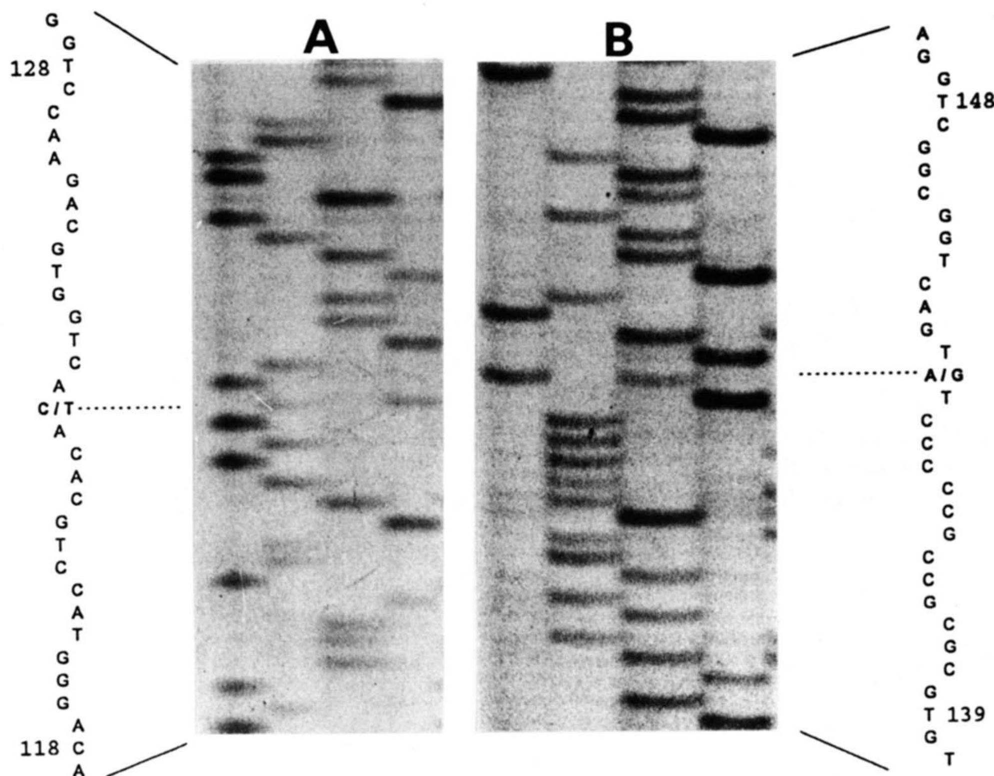

Fig. 3. Identification of the basic defects in a subject with fish eye disease. Panel A shows coding strand sequence of the region coding for amino acids 118 to 128 and a heterozygous mutation in codon 123 that causes a Thr → Ile change in the encoded amino acid sequence. Panel B shows the sequence between codons 139 and 148 and a heterozygous mutation in codon 144 causing a Tyr → Cys exchange in the encoded amino acid sequence.

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cases manifest the clinical features of the fish eye syndrome at present, however, it may be more appropriate in future to reclassify them as a form of LCAT deficiency if they show any progressive tendency to develop anemia or renal impairment.

Our subject typifies the former definition of FED in that she had β but minimal α LCAT activity. However, she maintained a residual level of total LCAT activity (also seen in the latter cases of FED) that resulted in the phenotypic expression of FED as opposed to familial LCAT deficiency.

The proband also has non-insulin-dependent diabetes mellitus (type II) and is therefore the first case of FED to be reported with intercurrent diabetes. However, despite the presence of an extremely low concentration of HDL-C (comprised solely of smaller than normal HDL) and diabetes mellitus, this 63-yr-old female proband has no evidence of cardiovascular disease. The d > 1.25 g/mL fraction of the proband's plasma afforded minimal esterification of UC in discoidal, reconstituted HDL. There was no cholesterol esterification in VLDL/LDL-depleted plasma but plasma cholesterol esterification was maintained by βLCAT activity. Although her plasma CER was approximately 50% of the normal level and her plasma unesterified to total cholesterol ratio was higher than normal, these levels were sufficient to prevent any renal or hematological disorders commonly seen in familial LCAT deficiency.

DNA sequence analysis of the proband's LCAT gene identified two mutations. Haplotyping located the mutations on different chromosomes. No other mutations have been found in the exons, the splice donor and acceptor sites and 55 bases of 5′-untranslated region. This, together with half-normal values for HDL-C and LCAT activity in the plasma of heterozygote mutation carriers, previous reports on the T123I-mutation (12, 14), and the reported absence of functionally irrelevant mutations from the LCAT gene (11) establish the two mutations of the compound heterozygote proband as causative for the observed phenotypic changes.

The proband's first degree relatives were also studied. Only the proband's daughter inherited the $Tyr_{144} \rightarrow Cys$ mutation, while the other members of the family inherited the $Thr_{123} \rightarrow Ile$ mutation. All subjects had HDL-C levels 50% of normal and plasma apoA-I levels 80% of normal, but otherwise unremarkable plasma lipid and apolipoprotein levels. The CER in plasma and VLDL/LDL-depleted plasma was normal in the proband's son and daughter and both had approximately a 50% reduction in LCAT activity. Hence, the biochemical and phenotypic expression in these subjects was the same, irrespective of the underlying genetic mutation.

The functional significance of the $Tyr_{144} \rightarrow Cys$ mutation in the LCAT gene remains unknown. However, several factors indicate that this mutation, seen in the proband and her daughter, is probably involved in the LCAT reaction on HDL-associated cholesterol. The proband's daughter had a low HDL-C concentration (50% of normal) as well as a 50% reduction in LCAT activity associated with reconstituted HDL. Her LCAT activity and plasma lipids were comparable to her brother as well as other heterozygotes for FED with the $Thr_{123} \rightarrow$ Ile mutation (12, 14). Expression of these two LCAT mutations in the compound heterozygous proband re-

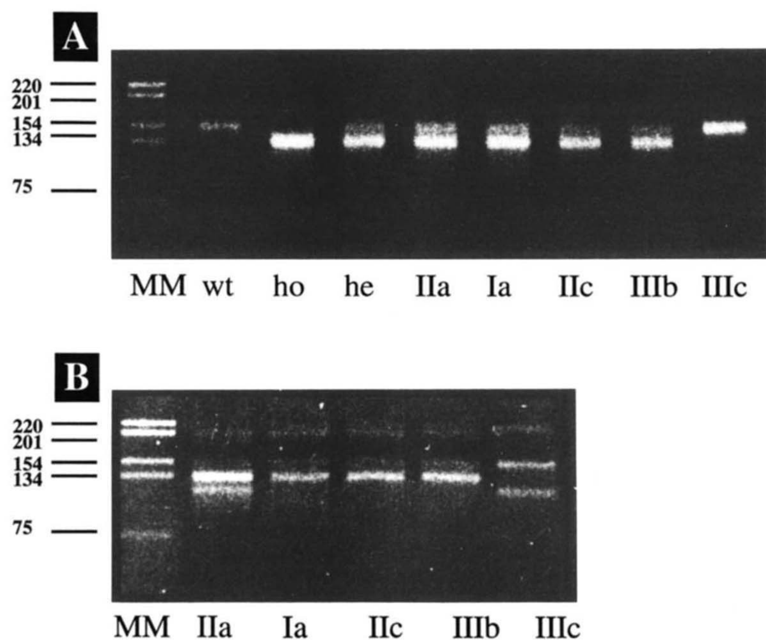

Fig. 4. Agarose gel electrophoresis of MS-PCR for allele specific detection of LCAT mutations in a family with FED. Panel A shows the genotypes in codon 123 of LCAT as analyzed by MS-PCR. A 154 bp long fragment detects wild type and a 134 bp long fragment indicates the presence of the T123I-mutation in LCAT. The lane marked MM contains a molecular weight marker with the fragment sizes indicated on the left, wt, ho and he denote wild type, homozygote and heterozygote control DNA taken from file (12). IIa, Ia, IIb, IIIb, and IIIc represent members of the Australian FED family and show that the proband, her mother, brother, and son are heterozygous for the T123I defect. Panel B shows the same DNA fragments from the same individuals as in panel A after incubation with the restriction endonuclease Mae III (GTNAC). The mutation Y144C forms a recognition site for the restriction endonuclease Mae III, which is absent from the wild type sequence. The presence of a 111 bp long fragment in the compound heterozygote and her daughter (IIIc) identifies these two individuals as carriers of the Y144C mutation and demonstrates that the mutations in codons 123 and 144 are located in trans.

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sulted in an HDL-C level 10% that of normal and minimal LCAT activity. The significance of this mutation could be addressed in an in vitro system in which transfected cells express mutant $LCAT_{Tyr144 \rightarrow Cys}$, therefore enabling the determination of the effect of this mutant enzyme on the esterification of cholesterol in different lipoprotein fractions.

Although we have yet to determine whether this mutation is causative for FED, we note the proximity of the $Tyr_{144} \rightarrow Cys$ mutation to two mutations in the LCAT gene known to cause familial LCAT deficiency. The first mutation occurs as an insertion of glycine between $arginine_{140}$ and $alanine_{141}$ (28) and the second at codon 147 causing a substitution of arginine with tryptophan (29). However, we are unable to determine which LCAT deficiency disorder this mutation may be associated with because subjects heterozygous for familial LCAT deficiency or FED have similar reductions in HDL-C concentration and LCAT activity, but otherwise normal levels of other plasma lipids and plasma unesterified cholesterol (11, 12, 30).

In order to determine the effect of FED on the diabetic lipoprotein profile, we compared the results of our proband with two hypertriglyceridemic female diabetic subjects of similar age. Diabetics in general are at increased risk of premature CAD (31, 32), and have small dense LDL in association with elevated plasma TG and low HDL-C (33, 34). However, the lipoprotein profile of the proband was typical of that seen in FED but not diabetes. She maintained large LDL, mainly due to a 5-fold increase in LDL-TG, which was reflected in an elevated plasma TG. In contrast, diabetic hypertriglyceridemia was solely due to an elevated VLDL-TG. The size and composition of LDL for the two diabetic controls was similar to normal LDL even though diabetics generally have small, dense, cholesteryl ester-depleted LDL (35). The plasma unesterified cholesterol to total cholesterol ratio was normal in the two diabetic controls, but the plasma CER and LCAT activities were higher than the normal controls. As there were only two diabetic controls, who were also obese and hypertriglyceridemic, it is difficult to determine whether this is significant as reports on LCAT activity in diabetes have varied according to lipid status and differing methodology (36, 37).

In summary, we have identified a female diabetic subject with clinical and biochemical characteristics for FED. She is compound heterozygous for LCAT mutations at codons 123/144. Her daughter is heterozygous for the previously undescribed $Tyr_{144} \rightarrow Cys$ mutation, and the biochemical and phenotypic presentation of this mutation is indistinguishable from the $Thr_{123} \rightarrow Ile$ mutation.

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