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Research Article **Genetics**

While type 1 hyperlipidemia is associated with lipoprotein lipase or apoCII deficiencies, the etiology of type 5 hyperlipidemia remains largely unknown. We explored a new candidate gene, *APOA5*, for possible causative mutations in a pedigree of late-onset, vertically transmitted hyperchylomicronemia. A heterozygous Q139X mutation in *APOA5* was present in both the proband and his affected son but was absent in 200 controls. It was subsequently found in 2 of 140 cases of hyperchylomicronemia. Haplotype analysis suggested the new Q139X as a founder mutation. Family studies showed that 5 of 9 total Q139X carriers had hyperchylomicronemia, 1 patient being homozygote. Severe hypertriglyceridemia in 8 heterozygotes was strictly associated with the presence on the second allele of 1 of 2 previously described triglyceride-raising minor *APOA5* haplotypes. Furthermore, ultracentrifugation fraction analysis indicated in carriers an altered association of Apoa5 truncated and WT proteins to lipoproteins, whereas in normal plasma, Apoa5 associated with VLDL and HDL/LDL fractions. APOB100 kinetic studies in 3 severely dyslipidemic patients with Q139X revealed a major impairment of VLDL catabolism. Lipoprotein lipase activity and mass were dramatically reduced in dyslipidemic carriers, leading to severe lipolysis defect. Our observations strongly support in humans a role for *APOA5* in lipolysis regulation and in familial hyperchylomicronemia.

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Apoa5 Q139X truncation predisposes to late-onset hyperchylomicronemia due to lipoprotein lipase impairment

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While type 1 hyperlipidemia is associated with lipoprotein lipase or apoCII deficiencies, the etiology of type 5 hyperlipidemia remains largely unknown. We explored a new candidate gene, *APOA5*, for possible causative mutations in a pedigree of late-onset, vertically transmitted hyperchylomicronemia. A heterozygous Q139X mutation in *APOA5* was present in both the proband and his affected son but was absent in 200 controls. It was subsequently found in 2 of 140 cases of hyperchylomicronemia. Haplotype analysis suggested the new Q139X as a founder mutation. Family studies showed that 5 of 9 total Q139X carriers had hyperchylomicronemia, 1 patient being homozygote. Severe hypertriglyceridemia in 8 heterozygotes was strictly associated with the presence on the second allele of 1 of 2 previously described triglyceride-raising minor *APOA5* haplotypes. Furthermore, ultracentrifugation fraction analysis indicated in carriers an altered association of Apoa5 truncated and WT proteins to lipoproteins, whereas in normal plasma, Apoa5 associated with VLDL and HDL/LDL fractions. APOB100 kinetic studies in 3 severely dyslipidemic patients with Q139X revealed a major impairment of VLDL catabolism. Lipoprotein lipase activity and mass were dramatically reduced in dyslipidemic carriers, leading to severe lipolysis defect. Our observations strongly support in humans a role for *APOA5* in lipolysis regulation and in familial hyperchylomicronemia.

Introduction

Raised plasma triglyceride (TG) levels are an independent risk factor for coronary artery disease (1) and are influenced by both genetic and environmental factors. Severe hypertriglyceridemia (HTG) is a general condition with a few well-documented genetic contributors, including lipoprotein lipase (*LPL*), *APOC2*, and *APOE*, as well as environmental factors such as diet and/or conditions such as pregnancy and diabetes (2–5). While genetic factors account for a large proportion of the rare type 1 hyperlipidemia, the complex interaction between genetics and environment is only partly understood in the more common type 5 hyperlipidemia.

A strong candidate for severe HTG is the recently discovered human *apolipoprotein A-V* (*APOA5*) gene based on its profound modulation of plasma TG concentration. In mice, apoa5 overexpression lowered plasma TG concentration (6–8) whereas mice lacking Apoa5 had a 4-fold increase in plasma TG concentration (6). In humans, independent studies have demonstrated that variant haplotypes with either the S19W or the *c.A-3G APOA5* polymorphisms are strong determinants of plasma TG concentration variability across human populations (9–11). To address whether *APOA5* is a genetic contributor to TG metabolism and disease, we sequenced this candidate gene in a pedigree of vertically transmitted hyperchylomicronemia without LPL or apoCII deficiencies.

Nonstandard abbreviations used: APOA5, human apolipoprotein A-V; Apoa5, murine apolipoprotein A-V; FCR, fractional catabolic rate; HSPG, heparan sulfate-proteoglycan; HTG, hypertriglyceridemia; LPL, lipoprotein lipase; PR, production rate; SNP, single nucleotide polymorphism; TC, total cholesterol; TG, triglyceride.

Conflict of interest: The authors have declared that no conflict of interest exists.

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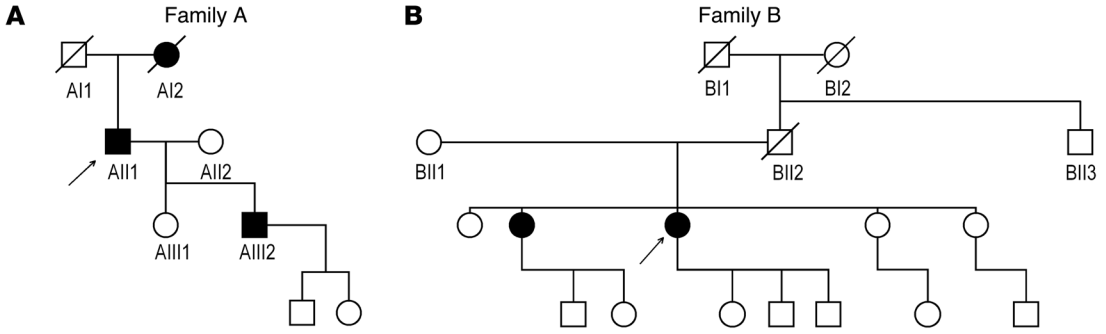
Results

APOA5-Q139X gene mutation in hyperchylomicronemia

The *APOA5* gene was studied in family A (Figure 1) because of its unusual features, late onset of hyperchylomicronemia with vertical transmission and resistance to diet and lipid-lowering treatments, whereas *LPL* and *APOC2* deficiencies were excluded. Sequence analysis of *APOA5* in the proband from family A (AII1) revealed he was heterozygous for a unique *C1047T* transition at the first nt of codon 139 (*CAG*) in exon 3 generating a Q139X nonsense mutation (Figure 1).

The presence of the Q139X allele was subsequently studied in a group of 200 unrelated control subjects who all scored negative (allele frequency < 0.25%) and in 140 patients with a history of documented HTG (TG > 15 mM/l), 2 of which were found to be positive. One heterozygous patient (BIII3) led to the identification of the second larger family B (Figure 1) and the other (CI1) was homozygous, but his death impeded further phenotype studies. Haplotype study following subcloning and sequencing of large 7-kb PCR products indicated in the 2 probands (AII1,



C

	AII1	AIII1	AII2	AIII2	AIV1	AIV2	BII1	BIII1	BIII2	BIV1	BIV2	BIII3	BIV3	BIV4	BIV5	BIII4	BIV6	BIII5	BIV7	BII3
Age (y)	63	40	65	35	7	4	77	58	57	35	38	54	32	30	24	54	33	52	33	84
TG (mM)	41.3	1.1	1.0	16.9	0.7	0.6	2.7	2.3	24.0	0.7	1.6	80.0	1.6	4.8	1.7	1.4	1.5	2.1	0.8	1.7
TC (mM)	16.7	4.9	6.1	11.8	6.4	5.5	5.8	5.6	9.7	5.3	5.8	21.3	5.2	7.2	4.2	5.3	4.8	6.9	4.8	6.4
apoAV (μg/l)	172.6	33.7	38.4	31.5	nd	nd	240.1	16.3	70.3	10.4	40.5	211.8	52.3	74.6	21.4	6.6	6.7	17.1	7.0	nd
Q139X	**+/-**	-/-	-/-	**+/-**	-/-	-/-	-/-	-/-	**+/-**	**+/-**	-/-	**+/-**	-/-	-/-	-/-	**+/-**	**+/-**	**+/-**	-/-	-/-
S19W	**+/-**	+/-	-/-	-/-	-/-	-/-	+/-	-/-	**+/-**	-/-	+/-	**+/-**	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-
c.A-3G	-/-	+/-	+/-	**+/-**	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
SNP2	-/-	+/-	+/-	**+/-**	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
SNP3	-/-	+/-	+/-	**+/-**	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Type 2 diabetes	**+**	-	-	-	-	-	**+**	-	**+**	-	-	**+**	-	-	-	-	-	-	-	**+**

Figure 1
Pedigree charts of family A (**A**) and family B (**B**). Arrows, probands; filled circles or filled squares, patients with confirmed severe type 5 hyperlipoproteinemia. (**C**) Results for the Q139X carriers are indicated in boxes. Black boxes, patients with severe chylomicronemia; gray boxes, carriers without severe chylomicronemia; –/–, homozygote genotype for the major allele; +/–, heterozygote genotype; +/+, homozygote genotype for the minor allele; ND, not determined. SNP and mutations are detailed in Table 1.

BIII3) and the homozygote (CI1) that all 3 patient had an identical Q139X-bearing haplotype from nt –2700 to +2000. This haplotype also had the minor g.A-2200T and g.C-1464T alleles, suggesting that Q139X is a founder mutation (Table 1). In the heterozygote patients AII1 and BIII3, the second *APOA5* allele was shown to harbor only previously reported polymorphisms, specifically the S19W variant which was absent from the Q139X-bearing haplotype (Table 1).

Case reports

Family A. The index case (AII1), a 63-year-old white male, was referred for a severe unmanageable HTG. His first episode (TG: 22 mM/l) was discovered when he was 38. His lipid profile was initially normalized by dietary restriction, but he relapsed in his 40s and 50s despite optimal dietary adherence. Severe HTG became permanent when he reached 60, fluctuating between 15 and 40 mM/l TG with transiently higher concentrations of over 60 mM/l. He never suffered from acute pancreatitis and showed no evidence of coronary heart disease (negative maximal treadmill test); however, his carotid intima media thickness was increased (0.90 mm), and he had atheromatous plaques both in the carotid and the aorta. He had no cause of secondary dyslipidemia except for a mild type 2 diabetes diagnosed when he was 63 (HbA1c = 6.5%; BMI = 26; waist circumference = 96 cm). Fibrates and high doses of long-chain omega-3 FFAs were unsuccessfully tested. His mother, who died at 94, had a history of plasma TG concentrations reaching 18 mM/l (AI2). His 34-year-old son of normal wt (AIII2) was found severely hypertriglyceridemic at 29 (TG = 28 mM/l; total cholesterol [TC] = 8.4 mM/l) although at 24, his fasting plasma TG levels had been found normal (1.46 mM/l). Under dietary advice and intensive exercise training, he was able to normalize his plasma TG concentrations but sometimes reached 8.3 and 11 mM/l. However, he has become permanently hypertriglyceridemic for the past 4 years (TG = 10 mM/l; TC = 6 mM/l) although he has no identified cause of secondary dyslipidemia.

PAGE of plasma lipoproteins from both cases in family A showed a type 5 hyperlipoproteinemia with accumulation of chylomicrons and VLDL with low LDL and HDL. Plasma apoCII and apoCIII concentrations were found elevated with a mild increase in the apoCII/apoCIII ratio. *APOE* genotype was determined to be *E2E3* in both AII1 and AIII2. Direct sequencing of the *LPL* gene in both patients did not reveal deleterious mutations. Overall, the presentation was a severe HTG with hyperchylomicronemia, resistant to treatment over at least 3 generations.

Family B. A second, unrelated larger family with a similar phenotype was subsequently discovered (Figure 1B). The index case was a 54-year-old white female (BIII3) exhibiting severe HTG (TG = 68 mM/l; TC = 30.3 mM/l). She had a history of hypertension, glucose intolerance, and moderate HTG (TG < 8.5 mM/l) for 13 years with abdominal obesity (BMI = 34; waist circumference = 102 cm). Subsequently, she has presented type 2 diabetes efficiently treated by metformine (HbA1c = 6.7%). She had no additional secondary cause of HTG. It took more than 2 weeks to lower her plasma TG levels to less than 10 mM/l despite intensive dietary intervention with transient insulinotherapy followed by a combination of metformine (1 g/d), fenofibrate (300 mg/d), and pioglitazone (15 mg/d). Five months after, she exhibited another acute episode of type 5 lipidemia (TG = 80

Table 1
APOA5 haplotypes in patient with severe hyperchylomicronemia

SNP/mutation	dbSNP identifier[A]	Apoa5	Haplotype I	Haplotype II APOA*2[B]	Haplotype III APOA*3[B]
g.A-2200T	rs1787680		T	A	A
g.C-1464T	SNP in AII1 genome[C]		T	C	C
g.T-1131C (*SNP3*)	rs662799		T	C	T
g.A-3G (Kozak)	rs651821		A	G	A
g.C170G	rs3135506	S19W	C	C	G
g.C245A	rs12287036	I44I	C	C	A
g.G751A (*SNP2*)	rs2072560		G	A	G
g.C1047T	Mutation in AII1 genome[C]	Q139X	T	C	C
Patients					
	AII1		+	–	+
	AIII2		+	+	–
	BIII2		+	–	+
	BIII3		+	–	+
	CI1		+	–	–

[A]As shown on the Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov/projects/SNP/). [B]APOA5 haplotype as defined in ref. 9 on the basis of genotypes at SNP1, SNP2, and SNP3. [C]Previously unreported entries, which have not yet been assigned dbSNP identifiers.

mM/l) with similar evolution despite wt stability and an efficient glycemic control (HbA1c = 6.5 %).

Family history was associated with type 2 diabetes on the maternal side, but the exact severity of HTG in subjects BI1 and BII2 was unknown. Additionally, all the proband's 4 sisters had abdominal obese phenotypes (BIII1, 2, 4, 5); 2 had mild (BIII1 and BIII5) and 1 severe (BIII2) HTG. BIII2, a 56-year-old female, displayed HTG ranging between 7 and 10 mM/l under various unsuccessful treatments (fibrates, long-chain omega-3 fatty acid). Her highest recorded triglyceridemia was 24 mM/l without history of pancreatitis. She has been obese (BMI = 40.1; waist circumference = 113 cm) and hypertensive for more than 30 years and had recently been diagnosed with type 2 diabetes (HbA1c = 7.1%). PAGE of plasma lipoproteins from BIII2 and BIII3 revealed type 5 hyperlipoproteinemia. Their *APOE* genotypes were respectively *E3E3* and *E3E4*.

As in family A, the overall presentation was a familial severe hyperchylomicronemia highly resistant to treatment with WT *LPL* genes and elevated plasma apoCII concentrations. Additionally, in family B this dyslipemia was closely associated with overt abdominal obesity and mild type 2 diabetes.

Patient CI1. Hyperchylomicronemia was discovered at 34 in this white male upon the occurrence of acute pancreatitis. He displayed a permanent unmanageable HTG during 20-year follow-up with 3 additional episodes of acute pancreatitis. At 49, he presented a silent myocardial infraction. At 60, he died from a cause unrelated to his dyslipidemia.

Most of the time, his plasma TG concentrations ranged between 15 and 30 M/l with several peaks above 40 mM/l (maximal range 6.5–65 mM/l) despite strict dietary compliance and a combination of fibrate and omega-3 fatty acids. He had type 5 hyperlipidemia, as determined by electrophoresis of plasma lipoprotein and ultracentrifugation, with normal plasma apoB concentration (1.1 g/l), low LDLc and HDLc (0.58 and 1.1 mM/l, respectively), and 3 times more TGs in chylomicrons than in VLDL fraction. *APOE* genotype was *E3/E3*; the presence of *LPL* gene mutation was excluded, and plasma apoCII/apoCIII ratio was normal.

He was not diabetic, of normal wt (70 kg; for 1.71 m height; waist circumference = 90 cm) and only presented transient fasting hyperglycemia upon acute peak of HTG. Except for beta blocker introduced after myocardial infarction, he had no additional cause of secondary dyslipidemia. His brother had a mild mixed hyperlipidemia and his sister was normolipidemic. Overall, the presentation was a late onset fasting hyperchylomicronemia over a period of 30 years, resistant to treatment.

APOA5-Q139X and haplotype study in pedigrees

Members of both families were screened for the presence of the Q139X allele; 6 additional heterozygote carriers were identified, 1 from family A and 5 from family B (Figure 1). Fasting hyperchylomicronemia had occurred in 5 out of 9 carriers (AII1, AIII2, BIII2, BIII3, and CI1) but not in any of the 10 adult noncarriers tested from both families (Figure 1, A and B).

All 5 Q139X carriers with severe HTG were either homozygote (CI1) or heterozygote with minor haplotypes on the second *APOA5* allele (Table 1). In family A (Figure 1A), the proband (AII1) transmitted Q139X to his son (AIII2). AII1 also had the S19W signal peptide variant (*APOA5*3* haplotype) whereas his son (AIII2) had the maternal *c.A-3G* allele with the minor alleles for *single nucleotide polymorphism 2* (*SNP2*) and *SNP3* (*APOA5*2* haplotype). In family B (Figure 1B), the 2 severely dyslipidemic Q139X carriers (proband BIII3 and BIII2) had the S19W polymorphism on the second allele (*APOA5*3* haplotype). In contrast, the 4 carriers identified without history of severe HTG had the most common *APOA5*1* haplotype on the second chromosome. Although in their fifties, BIII4 remained normolipidemic, and BIII5 had only moderate HTG. The 2 normolipidemic carriers from the younger generation, BIV1 and BIV6, are only in their thirties.

Apoa5 distribution in plasma lipoproteins

In both families, murine apolipoprotein A-V (Apoa5) plasma concentrations were not related to the presence of Q139X (Figure 1). Q139X is predicted to determine a truncation of Apoa5 at residue 116 of the mature protein, generating a 15-kDa peptide. In order to assess the presence of the putative truncated peptide in the plasma of heterozygotes, we analyzed by Western blotting the Apoa5 distribution in lipoprotein subfractions obtained following ultracentrifugation. A 15-kDa peptide containing the N terminal epitopes of Apoa5 was found in the 6 Q139X heterozygotes tested but not in 3 noncarriers or 10 unrelated normolipidemic controls (Figure 2). This 15-kDa peptide was exclusively located in the unbound fraction ($d > 1.21$). The 40-kDa full-length Apoa5 was detected in plasma-derived VLDL and HDL fractions from the noncarriers and unrelated normolipidemic controls. In contrast, in the 6 Q139X heterozygotes tested, both the 40-kDa and the 15-kDa truncated Apoa5 were recovered exclusively from the unbound fraction (Figure 2). As shown in Figure 2, this altered association of WT Apoa5 to lipoproteins was present in all the carriers whether they were hyperlipidemic (AII1, AIII2, BIII2, and BIII3) or not (BIII4 and BIII5).

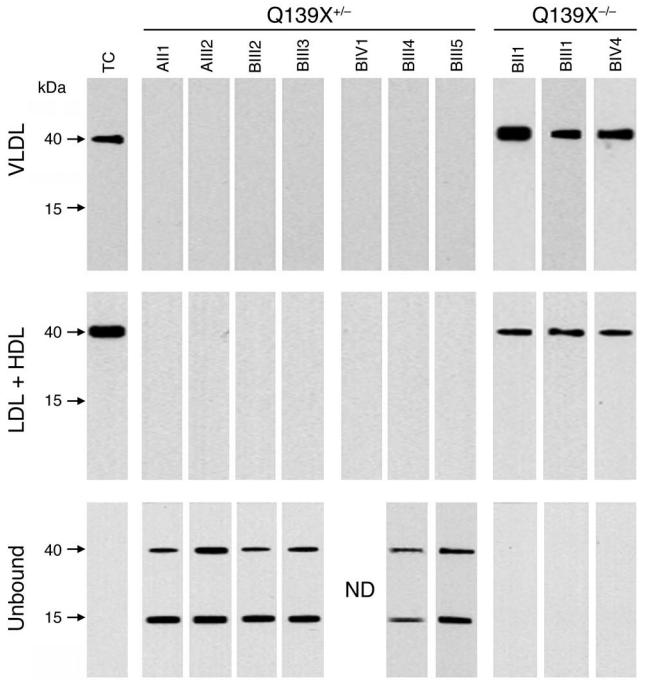


Figure 2
Western blot analysis of Apoa5 distribution in plasma lipoproteins. Ultracentrifugation fractions were as follows: VLDL fraction, $d < 1.006$ g/ml; LDL + HDL, $1.019 < d < 1.210$; unbound to lipoproteins, $d > 1.21$. TC is representative of the analysis of plasma lipoprotein fraction of 10 unrelated normolipidemic controls.

Plasma APOB100 kinetics

Plasma APOB100 kinetics were studied in 3 patients with Q139X mutation (AII1, AIII2 and BIII3). Kinetic curves (Figure 3) clearly show a much slower isotopic enrichment of VLDL-apoB compared with the normolipidemic control subjects, indicating a decreased VLDL-apoB catabolic rate. As shown in Table 2, severely dyslipidemic patients with Q139X had a 20- to 44-fold increase of VLDL-apoB pool due to a 22- to 26-fold decrease in VLDL-apoB fractional catabolic rate (FCR) (transfer to IDL/LDL). VLDL-apoB production rates (PRs) were identical to controls in 2 patients (AII1 and AIII2) and only slightly increased in 1 patient (BII3).

The 3 patients had a significant increase of IDL-apoB due to a 2.6- to 15-fold reduction of apoB-IDL FCR. This reduction was entirely attributable to the low apoB-IDL indirect FCR (transfer to LDL) whereas apoB-IDL direct FCR was not reduced in the patients (Table 2). As a consequence, LDL-apoB PRs were reduced in the 3 patients with conserved LDL-apoB FCRs, resulting in a decrease of LDL-apoB pool (Table 2). These results indicate a major alteration in the lipolysis of TG-rich lipoproteins in 3 heterozygote Q139X carriers.

LPL defect

To further assess the lipolysis defect associated with dyslipidemia in Q139X carriers, LPL activity was measured in postheparin plasma from all the severely dyslipidemic patients with Q139X (CI1, AII1, AIII2, BIII2, and BIII3) and from 2 carriers without severe HTG (BIII4 and BIII5). Postheparin LPL activities were dramatically reduced in all 5 severely hyperlipidemic Q139X carriers as compared with unrelated controls (Figure 4). The residual activities were similar to those found in homozygous *LPL* deficiency. By contrast, BIII4 and BIII5 had normal LPL activities (Figure 4). Hepatic lipase activity in postheparin plasma from 4 severely dyslipidemic patients (AII1, AIII2, BIII2, and BIII3) was similar to that found in controls (7.6 ± 2.7 vs. 7.7 ± 2.3 μM FFA/ml/h, P = not significant). We subsequently analyzed LPL protein in postheparin plasma by Western blotting to determine whether functionally inactive LPL was released or LPL was not released. LPL protein was present in postheparin plasma from 2 normolipidemic Q139X carriers (BIII4 and BIII5) as in the controls but remained undetectable in the postheparin plasma from the 5 severely hypertriglyceridemic carriers: AII1, AIII2, BIII2, BIII3, and CI1 (Figure 4).

Discussion

We report what we believe to be a new form of familial hyperchylomicronemia with vertical transmission, late onset, incomplete penetrance, and an unusual resistance to conventional treatment. Our results strongly suggest that the newly identified APOA5-Q139X private nonsense mutation is a determinant of this new dyslipidemia based on the following: (a) observation in the 2 pedigrees that hyperchylomicronemia occurs only in the Q139X carriers and cosegregates with the Q139X mutant allele in pedigree A; (b) presence in the carriers of a 15-kDa peptide corresponding to the N terminal end of Apoa5, as expected for a truncation at residue 139; and (c) altered association of WT Apoa5 to plasma lipoproteins in all the carriers from both families. Additionally (d), we demonstrate that severe HTG in Q139X mutation carriers results from an LPL defect leading to lipolysis impairment.

In family A, the fasting hyperchylomicronemia was expressed in at least 3 consecutive generations. Moreover, in both pedigrees this dyslipidemia occurred exclusively among the Q139X carriers, none of which had an identifiable deleterious mutation of the second *APOA5* allele. This, together with similar dyslipidemia in the homozygote CI1 and 3 heterozygote carriers (AII1, AIII2, and BIII3), initially suggested that Q139X caused a dominant hyperchylomicronemia. But subsequent study of family B indicated the *Q139X*$^{+/-}$ genotype was incompletely penetrant with only 2 out of 6 carriers affected in family B. A role for compound heterozygocity with either the *APOA5*3* (with S19W) or the *APOA5*2* haplotype (with *c.A-3G, SNP2,* and *SNP3* minor alleles) (Table 1) was indicated by the complete association of hyperchylomicronemia with these compound heterozygote genotypes. Two normolipidemic carriers

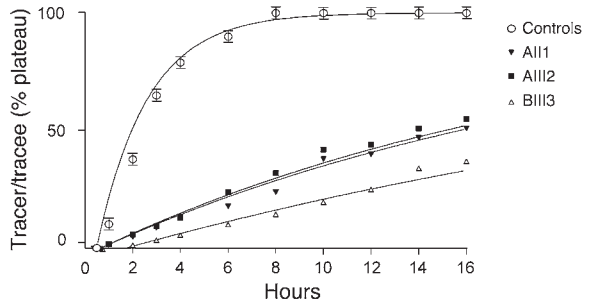


Figure 3
Kinetic curves of VLDL-apoB in plasma. ^{13}C-leucine enrichment curves of human APOB100 in patients AII1 (filled triangle), AIII2 (filled squares), and BIII3 (open triangles) and in 10 controls (open circles). Results are expressed as tracer/tracee values (mean of triplicates). For controls, data are shown as mean ± SEM.

Table 2
Kinetic parameters of VLDL, IDL, and LDL apoB in 3 Q139X mutated patients and in control subjects

	VLDL apoB				IDL apoB				LDL apoB		
	apoB (mg/l)	Production (mg/kg/d)	FCR (pool/d)		apoB (mg/l)	Production (mg/kg/d)	FCR (pool/d)		apoB (mg/l)	Production (mg/kg/d)	FCR (pool/d)
			Direct catab.	Transfer to IDL/LDL			Direct catab.	Transfer to LDL			
Patient AII1	743	17.0	0	0.51	112	17.1	2.1	1.28	164	6.6	0.90
Patient AIII2	658	14.2	0	0.48	197	14.0	0.6	0.98	239	8.9	0.83
Control men[A] (n = 5)	33 ± 4	14.8 ± 4.1	0	11.3 ± 4.0	39 ± 9	17.5 ± 7.0	1.4 ± 0.9	7.4 ± 4.1	771 ± 180	26.9 ± 8.2	0.82 ± 0.24
Patient BIII3	1562	30.2	0	0.43	597	33.8	0.98	0.28	407	8.2	0.45
Control women[A] (n = 5)	35 ± 3	15.6 ± 2.3	1.6 ± 1.8	9.4 ± 3.3	18.0 ± 2.0	15.6 ± 4.6	2.1 ± 1.6	17.6 ± 6.3	608 ± 66	12.0 ± 4.3	0.47 ± 0.15

[A]Values in mean ± SD. Catab.; catabolism.

are only in their thirties (BIV1 and BIV6), and we cannot exclude the possibility that they will later display severe HTG. However, 2 carriers are in their fifties and were still either normolipidemic (BIII4) or showed moderate HTG, likely due to abdominal obese phenotype (BIII5). Moreover, a unique other *APOA5* deleterious mutation, *Q148X*, was reported recently in a pedigree with severe hyperchylomicronemia in a homozygote patient (12). None of the reported 10 heterozygotes had severe HTG, and they all had the frequent *APOA5*1* haplotype on the second chromosome (12). Therefore, as in our study, heterozygote nonsense *APOA5* mutation alone did not result in severe chylomicronemia. This is consistent with our observation that the 2 variant haplotypes were crucial determinants of dyslipidemia in heterozygote Q139X carriers.

The effect of *APOA5*2* and *APOA5*3* minor haplotypes on Apoa5 function is unknown. Previous studies demonstrated that *APOA5*2* and *APOA5*3* are both strong, independent determinants of plasma TG concentration variability across human populations (6, 9–11, 13–16). Furthermore, several studies suggested that S19W and/or *c.A-3G* enhances HTG in various physiological and pathological conditions such as pregnancy (17), familial combined hyperlipidemia (16, 18, 19), and mixed hyperlipidemia (20). Moreover, the S19W variant was a crucial determinant of HTG in *APOE2* homozygotes (21). Our results highly strengthen these studies and further highlight the TG-raising properties of the 2 *APOA5* minor haplotypes. On the other hand, population studies indicate that homozygocity for these minor haplotypes only results in a mild increase in plasma TG levels (9, 16), suggesting only a mild Apoa5 impairment. This may indicate a complex association of genetic and environmental factors in line with the observed late onset of dislipidemia in this study.

We show that severe chylomicronemia in homozygote and heterozygote Q139X carriers was caused by a profound LPL defect, and we provide what we believe to be the first clear evidence in humans of a functional interplay between Apoa5 and LPL. The apoB kinetic data in 3 dyslipidemic *APOA5* mutated patients clearly indicate a major alteration in the lipolysis of TG-rich lipoproteins with a striking reduction of VLDL and IDL catabolism. Unfortunately, kinetic study could not be performed in the homozygote CI1 due to his death. The reduction of catabolism was of greater magnitude for VLDL than for IDL, in line with the LPL defect observed in the 5 severely hyperlipemic carriers. A slight increase of VLDL-apoB production likely contributes to the hyperlipidemia in 1 patient (BIII3). It is most likely the consequence of her diabetes mellitus rather than of Apoa5 truncation because her VLDL-apoB PR is as usually observed in type 2 diabetic patients (22, 23). Accordingly, recent experimental studies indicated that Apoa5 does not modulate hepatic VLDL production (24) but induces an LPL-dependent acceleration of the catabolism of TG-rich lipoproteins (24–26).The increase in IDL pool despite the reduced VLDL catabolism is likely due to the 22-fold increase in VLDL pool leading to a residual conversion into LDL.

The defective VLDL catabolism resulted from strikingly decreased LPL activity in all severely dyslipidemic Q139X carriers. Moreover, Q139X homozygocity (CI1) was associated with the lowest postheparin LPL activity (Figure 4), similar to those in homozygotes for nonsense LPL mutations, such as *R192X*, *IVS1–1G→A*, or *Y288X* (data not shown). Q139X nonsense mutation is predicted to encode a truncated Apoa5 variant, missing the entire hydrophobic region with the lipid-binding domain (27) and a putative heparin-binding domain between aa 186 and 227 (28). In our study, the Q139X truncated peptide was poorly bound to VLDL unlike Apoa5 in control subjects. Furthermore, in heterozygote carriers, the binding of Apoa5 expressed from the second allele to lipoproteins was also impaired. This might be critical to Apoa5 dysfunction because recent experimental studies suggested that upregulation of lipolysis by Apoa5 involves its binding both to lipid and to heparan sulfate-proteoglycan (HSPG). It was proposed that Apoa5 accelerates lipolysis by facilitating the interaction of TG-rich lipoproteins to HSPG-bound LPL (24). First, experimental data suggest that Apoa5 upregulates lipolysis by LPL-HSPG complexes but not by free LPL (24). Second, a recent study indicates that Apoa5 binds to HSPG and enhances the binding of VLDL and

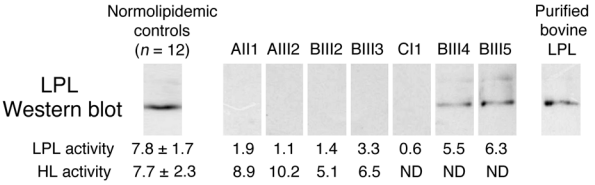


Figure 4
LPL activity (μmol FFA/ml/h) and mass in postheparin plasma. LPL activity was as follows in patients with LPL deficiency: 2.3 ± 0.8 μmol FFA/ml/h in homozygote LPL deficiency (n = 6) and 3.6 ± 0.7 in heterozygote *LPL* deficiency (n = 8). HL, hepatic lipase.

chylomicrons to LPL-HSPG complexes (28). This feature is likely to be defective in the Q139X truncated peptide, resulting in an inability to upregulate lipolysis.

However, LPL protein expression was dramatically decreased on HSPG only in severely dyslipidemic carriers. In contrast, their hepatic lipase activity is normally released by heparin. Hence, rather than simply a defective stimulation of normally expressed LPL, heterozygote carriers who fail to express and/or target LPL to HSPG developed severe HTG whereas those who maintained a normal releasable pool of LPL did not. It remains to be established how LPL expression was altered in the heterozygous carriers as they became hyperchylomicronemic. One possibility is a dominant-negative effect of the 15-kDa truncated Apoa5 expressed in the plasma of all carriers. We speculate that the truncated product could interfere with the normal association of Apoa5 to lipoproteins and affect either indirectly or directly the LPL expression on the endothelium. Such a dominant-negative effect would also account for the severity of lipolysis defect in compound heterozygotes with only mild impairment of the second allele.

The full expression of the syndrome, as observed in the Q139X homozygote CI1 and in all affected heterozygotes, is characterized by type 5 hyperlipidemia, in the thirties, switching from intermittent to permanent severe HTG, unusually resistant to dietary and hypolipidemic drug therapy. Previously, chylomicronemia was diagnosed at 9 years old in the only reported homozygote for a deleterious *APOA5* mutation (*Q148X*) (12). Although we cannot exclude early onset in the Q139X homozygote (CI1), late onset in our study was suggested by late diagnosis in all patients; patient AIII2 was normolipidemic at 24. This suggests in our pedigrees the crucial involvement of environmental factors to modulate the expressivity and timing of dyslipidemia Therefore, this raises the question of how LPL expression was progressively impaired, leading to severe HTG. Studies in *apoa5* KO mice suggested that LPL overexpression can compensate for *apoa5* defect and normalize plasma TG concentration (24). Accordingly, we propose that, in our patients, LPL upregulation might have initially compensated for *APOA5* deficiency. Later on, age-related factors could have limited LPL availability, which became unable to uphold lipolysis. In family B, there was some association between obesity and severe HTG, but this was not the case in family A, in which wt was normal for both patients. The absence of dietary errors in family A and the lack of response to dietary intervention in all patients suggest that nutritional factors may not be a major determinant of the disease. Type 2 diabetes was present in 3 of 5 patients and has likely favored severe HTG through hepatic VLDL-TG overproduction, as in patient BII3 (Table 2) (23). Furthermore, type 2 diabetes is often associated with mildly reduced LPL activity and mass in fasting and postprandial state (29–31) This could have further impaired lipolysis in addition to Apoa5 deficiency itself. Even though type 2 diabetes has likely contributed to severe HTG in 3 patients (AII1, BIII2, and BIII3), this is not the case for patient AIII2, who remained normoglycemic and normoinsulinemic.

We demonstrate that homozygote *APOA5* deficiency due to Q139X nonsense mutation causes familial chylomicronemia through severe impairment of LPL expression. Furthermore, severe heterozygote phenotype can occur, depending on the complex interplay between the rare heterozygous truncation, common susceptibility *APOA5* haplotypes, and age-related factors. Overall, our results strongly support a role for the *APOA5* gene in the regulation of the lipolysis of TG-rich lipoproteins in humans.

Methods

Patient assessment

Probands and pedigrees are detailed in Results. A total of 140 unrelated patients with hyperchylomicronemia were selected when referred to the Hôpital Louis Pradel lipid clinics on the basis of current or history of documented transient episodes of severe fasting HTG. The presence of hyperchylomicronemia was assumed when plasma TG concentration was above 15 mM/l with a TG/TC ratio above 2.5 (in g/l) (32). The study was performed according to the requirements and approval of the Comité Consultatif d'Éthique (Dijon, France), and written informed consent was obtained from all the patients included in the study.

APOA5 genomic sequence analysis

APOA5 gene mutation analysis. Genomic DNA was extracted as described (5). Prior to direct sequencing of the *APOA5* (nt –25 to +1820), genomic DNA (0.2 μg) was subjected to PCR with the 2 primers APOA5gF1 (5′-CAGGTGGGCAGGGGAGAGGTGGTA-3′) and APOA5gR1 (5′-ATGGCAGCCCTGGGGAGACAAGTG-3′) generating a 2526-bp product. PCR was performed with Taq-polymerase (40 mU/μl) as indicated (Qbiogene Inc.), primers (0.4 μM/l each), DMSO 8% (Sigma-Aldrich), MgCl2 (4.5 mM/l), and dNTP (0.2 μm/l). A total of 35 cycles were performed at a denaturating temperature of 95°C for 40 seconds followed by annealing temperature of 68°C (1 min) and extension temperature of 72°C (1 min 30 sec). PCR products were directly sequenced on both strands with a CEQ2000 DNA analysis system using the CEQ-DTCS QuickStart kit (Beckman Coulter) The sequencing primers were the following: APOA5sF1 (5′-CCTTCGTCTCCTTCTTCCCCTAACC-3′), APOA5sR1 (5′-TGTGGAGAGGGACTAGGTAATCAGG-3′), APOA5sF2 (5′-TGGCTCTTCTTTCAGGTGGGTCTCC-3′), APOA5sR2 (5′-CCAGCAGCGGCCACAGAGGTTGAG-3′), APOA5sF3 (5′-TGGGGACAAAGGAGATGAT-3′), APOA5-seqR3 (5′-GCCTTCACCTCCTCCAACTC-3′), APOA5sF4 (5′-GCAGCTGCAGGAGGAGTTGG-3′), APOA5sR4 (5′-AACTGGGCCTTGGTGTC), APOA5sF5 (5′-GGGGGAAGACACCAA-3′), APOA5sR5 (5′-TGCGGAGCCACACTG-3′), APOA5sF6 (5′-TCCACCCATACGCCGAGAGC-3′), APOA5sR6 (5′-GCGGAAAGCCTGAAGTCG-3′), APOA5sF7 (5′-CCCGGACCCCCAGATGCT-3′), APOA5sR7 (5′-AGGCTGTGAGTGATGTCTT-3′), APOA5sF8 (5′-AGTGGCAAGGTTCTGAG-3′), and APOA5sR8 (5′-AGACAGCAGCCCCTTTGGTG-3′).

PCR-RFLP screening of the APOA5-Q139X mutation. Genomic DNA was PCR amplified with 0.1 μM/l of primers AV139XF (5′-TGCAGGAGGAGTTGGAGGAGGTGA-3′) and AV139XR (5′-TGCACGCGCAGGGCCAG-3′) as indicated earlier, except for an annealing temperature of 60°C and without DMSO. PCR products were subsequently digested with Pvu-II restriction enzyme (Roche Diagnostics Corp.) before gel electrophoresis analysis.

APOA5 haplotype analysis. To unambiguously assess haplotypes, 7040 bp *APOA5* PCR products were generated and subcloned in the PGEM-T vector system (Promega) prior to sequencing. The PCR primers were APOA5F9 (5′-GAGCTCAGGCCCATTCAAAACAA-3′) and APOA5R9 (5′-GCTCACCAGGCTCTCGGCGTATG-3′). PCRs (35 cycles) were performed using the expanded high-fidelity PCR system as described by the manufacturer (Roche Diagnostics Corp.). Q139X-positive and -negative clones were sequenced with the following primers: APOA5F9, APOA5F10 (5′-CTAACTGTATGGCCCCAATCTAAT-3′), APOA5F11 (5′-TCTGCTGATGACTCCCAAAACTC-3′), APOA5F12 (5′-AAGAAAAAGCCCCTGCACTCAAAG-3′), APOA5F13 (5′-GATTGATTCAAGATGCATTTAGGAC-3′) and APOA5R13 (5′-CCCCAGGAACTGGAGCGAAAGT-3′), APOA5sF1, APOA5sR1, APOA5sF2, and APOA5sR2.

Circulating apoB kinetic study

Experimental protocol. A kinetic study of APOB100 was performed in the 3 patients with the *APOA5* mutation (AII1, AIII2, and BIII3). The results were compared with those of control normolipidemic men and women. The kinetic study was performed in fed state. Food intake, with a leucine-poor diet (1700 kcal/d; 55% carbohydrates, 39% fats, and 7% proteins), was fractionated in small equal portions that were provided every 2 hours, starting 6 hours prior to the tracer infusion up to the end of the study, in order to avoid important variations in apolipoprotein plasma concentration as previously performed by other groups (33, 34). To determine the kinetic of APOB100, the subjects received an intravenous injection of a 0.7 mg/kg bolus of L-[1-^{13}C]leucine (99% ^{13}C; Eurisotop) immediately followed by a 16-hour constant infusion at 0.7 mg/kg/h. Blood samples were collected at hours 0, 0.25, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 15, and 16. Serum was separated by centrifugation and stored at 4°C. Inhibitors of protease (aprotinin, 17 mg/l) and bacterial growth (sodium azide, 500 mg/l) were added to each sample.

Analytical procedures. Analytical procedures were performed as previously described in detail (22, 23, 35).

Isolation of apolipoproteins. VLDL (density [*d*] < 1.006 g/ml), IDL (1.006 < *d* < 1.019) and LDL (1.019 < *d* < 1.063) were isolated from plasma by sequential flotation ultracentrifugation, using a 50.4 rotor in an L7 apparatus (Beckman Coulter). IDL and LDL fractions were then dialyzed against a 10 mM/l ammonium bicarbonate buffer pH 8.2 containing 0.01% EDTA and 0.013% sodium azide. VLDL, IDL, and LDL fractions were delipidated with diethylether-ethanol, and APOB100 from each lipoprotein fraction was isolated by preparative SDS-PAGE (3%). After staining with Coomassie blue R-250, APOB100 bands were excised from the polyacrylamide gels and hydrolyzed in 6 M HCl at 110°C for 16 hours under nitrogen vacuum. Samples were then lyophilized in a SpeedVac (Savant Instruments). Lyophilized samples were dissolved in 50% acetic acid and applied to an AG-50W-X8 200-400 mesh cation exchange resin (Bio-Rad Laboratories); aa were recovered by elution with 4N NH_4OH (34).

Determination of leucine enrichment by gas chromatograph/combustion/isotope ratio mass spectrometry (GC/C/IRMS). aa were converted to N-acetyl O-propyl (NAP) aa esters and analyzed with a Finnigan Mat Delta C isotope ratio mass spectrometer (Finnigan Mat) coupled to an HP 5890 series II gas chromatograph (Hewlett-Packard) (36), as previously described (37).

Modeling. Apolipoprotein kinetics data were expressed as tracer-to-trace mass ratios (37, 38) $z(t)$, calculated as follows: $z(t) = e(t) / e_i - e(t)$, where e_i is the tracer enrichment, $e(t) = a(t) - aN$, and $a(t)$ and aN are the isotope abundance of the labeled and the unlabeled species, respectively.

The data were analyzed with the Simulation, Analysis, and Modeling (SAAM) II program (SAAM Institute Inc.) using a multicompartmental model (39). In the model chosen, VLDL apoB kinetic data are represented by compartments 1 and 2, plasma IDL apoB kinetic data by compartments 11 and 12, and LDL apoB kinetic data by compartment 21. As the experiment was performed in the steady state, fractional synthetic rate equaled FCR (38).

Direct FCR of VLDL apoB and FCR from VLDL to IDL or LDL, expressed in pool/d, were calculated as follows: direct $FCR_{VLDL} = M_2\, k(0,2)/(M_1 + M_2)$; $FCR_{VLDL\rightarrow IDL\rightarrow LDL} = M_1\,[k(11,1) + k(21,1)]/(M_1 + M_2)$, where $k(i,j)$ is the fractional transfer coefficient from compartment j to i, and Mj represents the apoB mass (expressed as concentration/l plasma) of compartment j. Total apoB VLDL FCR is the sum of direct FCR_{VLDL} and $FCR_{VLDL\rightarrow IDL\rightarrow LDL}$ (transfer to IDL/LDL). Direct FCR of IDL apoB and FCR from IDL to LDL were calculated as follows: direct $FCR_{IDL} = M_{12}\, k(0,12)/(M_{11} + M_{12})$ and $FCR_{IDL\rightarrow LDL} = M_{11}k(21,11)/(M_{11} + M_{12})$. Total apoB IDL FCR is the sum of direct FCR_{IDL} and $FCR_{IDL\rightarrow LDL}$ (transfer to LDL). The FCR of LDL apoB was calculated as follows: $FCR_{LDL} = k(0,21)$. PRs of the APOB100 in each lipoprotein fraction were calculated as follows: Pr = apoB FCR (for each lipoprotein fraction) × apoB pool size/body wt, where apoB pool size is calculated by multiplying the apoB concentration in the lipoprotein fraction (VLDL, IDL, or LDL) by the estimated plasma volume (4.5% of body wt).

LPL activity and Western blotting. Postheparin plasma was obtained 10 minutes after intravenous injection of heparin (50 IU/kg) and assay of lipase activity using a radio-labeled 14C-triolein emulsion as previously described (40, 41). Human heat-inactivated serum (10%) was used as a source of apoCII. To measure LPL activity, hepatic lipase was inhibited by preincubation with a specific goat polyclonal anti-serum (a gift from S. Griglio, U551 INSERM, Paris, France). LPL Western blotting was performed in postheparin plasma as previously described (40). In brief, postheparin plasma (200 μl) was mixed with heparin-sepharose CL-6B (Pharmacia), incubated for 1 hour at 4°C in 0.1 M/l phosphate buffer, pH 7.2, containing 0.15 M/l NaCl, 1 mM/l EDTA, 10% glycerol, and 10% diethyl p-nitrophenylphosphate. The slurry was washed twice prior to elution with 0.8 and 1.3 M/l NaCl buffer. Eluted fractions were TCA precipitated, and the pellet was analyzed on 10% SDS-PAGE electrophoresis and transferred to nitrocellulose. Blot was exposed to anti-LPL 5D2 mAb (1:500) and revealed with ECL (Amersham Biosciences).

Plasma Apoa5 assay and Western blot analysis. Apoa5 plasma concentrations were measured as previously described (25). For Western blot analysis, proteins were extracted from ultracentrifugation fractions as indicated in the kinetic study (22). Equal amounts of total proteins (100 μg) were subjected to a 15% SDS-PAGE and then transferred onto a nitrocellulose membrane. The blots were first incubated with a goat anti-human Apoa5 (1:300) directed to the N terminal region, then with an anti-goat IgG horseradish peroxidase conjugate (1:1000; Sigma-Aldrich). Detection was performed by a chemiluminescent method (ECL, Amersham Biosciences). Normal control plasma was analyzed from normolipidemic subjects (n = 10).

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