Phenotypic expression of familial hypobetalipoproteinemia in three kindreds with mutations of apolipoprotein B gene

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Abstract **We report the clinical phenotype in three kindreds with familial heterozygous hypobetalipoproteinemia (FHBL) carrying novel truncated apolipoprotein Bs (apoBs) of different sizes (apoB-8.15, apoB-33.4 and apoB-75.7). In D.A. kindred, we found three carriers of a C-deletion in exon 10 leading to the synthesis of apoB-8.15 not detectable in plasma. They showed steatorrhea and fatty liver. In N.L. kindred, the proband is heterozygous for a nonsense mutation in exon 26, leading to the formation of apoB-33.4. He had premature cerebrovascular disease and fatty liver; two apoB-33.4 carriers in this kindred showed only fatty liver. In B.E. kindred, the proband is heterozygous for a T-deletion in exon 26, which converts tyrosine at codon 3435 into a stop codon, resulting in apoB-75.7. The proband, a heavy alcohol drinker, had steatohepatitis, whereas his teetotaller daughter, an apoB-75.7 carrier, had no detectable fatty liver. This study suggests that: *i*) fatty liver invariably develops in FHBL carriers of short and medium-size truncated apoBs (< apoB-48), but its occurrence needs additional environmental factors in carriers of longer apoB forms; *ii*) intestinal lipid malabsorption develops only in carriers of short truncated apoBs, which are not secreted into the plasma; and *iii*) cerebrovascular disease due to premature atherosclerosis may occur even in FHBL subjects.**—Tarugi, P., A. Lonardo, C. Gabelli, F. Sala, G. Ballarini, I. Cortella, L. Previato, S. Bertolini, R. Cordera, and S. Calandra. **Phenotypic expression of familial hypobetalipoproteinemia in three kindreds with mutations of apolipoprotein B gene.** ***J. Lipid Res.*** **2001.** 42: **1552–1561.**

Supplementary key words truncated apoBs • fatty liver • lipid malabsorption • carotid atherosclerosis

Familial hypobetalipoproteinemia (FHBL) is an autosomal codominant disorder characterized by reduced plasma levels of cholesterol and apolipoprotein B (apoB) containing lipoproteins (1, 2). In most of the FHBL kindreds investigated so far at the molecular level, hypobetalipoproteinemia is caused by mutations in the gene encoding apoB-100. These mutations interfere with the translation of a full-length apoB-100 mRNA and cause the production of truncated apoBs of various size (1, 2), which may or may not be detectable in plasma. Truncated forms of apoB are detectable in plasma only when their size is larger than apoB-25/B-27 (i.e., apoBs with a size corresponding to 25–27% of apoB-100, designated according to the centile nomenclature) (3). The size of detectable truncated apoBs, as estimated by PAGE, gives a clue for the localization of the mutation in the apoB gene, thus facilitating sequence analysis. This is not the case in those FHBL kindreds with no truncated apoBs detectable in plasma; in these situations, the complete sequence of the apoB gene appears to be the only reliable way to define whether mutations of this gene are the cause of the lipid disorder. Because the sequence of the whole apoB gene is an arduous task, most of these FHBL kindreds (representing approximately two-thirds of all FHBL kindreds collected in the largest series) (4) have not been characterized at the molecular level.

The clinical phenotype of heterozygous FHBL is not well defined because only few genotyped kindreds have been investigated in detail. Most FHBL subjects are apparently asymptomatic and have been discovered during large cholesterol screening programs (4). A few symptomatic cases of heterozygous FHBL have been reported. These subjects have gastrointestinal and, less frequently, neurological manifestations of variable severity (2, 5). Other FHBL cases are brought to medical attention by the presence of an otherwise unexplained fatty liver (6–11). However, there are few reports of liver disease in FHBL sub-

Abbreviations: apo, apolipoprotein; BMI, body mass index; FHBL, familial hypobetalipoproteinemia; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; MTP, microsomal triglyceride transfer protein; SPECT, single photon emission computed tomography; TC, total cholesterol.

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jects in whom the mutations of the apoB gene have been fully characterized (12, 13).

In view of observational studies suggesting that hypocholesterolemic individuals have a lower risk of developing cardiovascular disease (14–16), it has been assumed that FHBL may protect against atherosclerosis (17). However, there are no epidemiological or clinical studies that have specifically addressed this question in heterozygous FHBL subjects with known mutations of the apoB gene.

In this study, we report three FHBL kindreds with novel mutations of the apoB gene leading to the formation of truncated apoBs of various sizes, either detectable (apoB-33.4 and apoB-75.7) or undetectable (apoB-8.15) in plasma. The aim of this study was the characterization of the phenotype associated with these different truncated apoBs.

PATIENTS AND METHODS

Clinical data

D.A. kindred. The proband D.A. (subject II-3 in **Fig. 1**) is a 39-year-old female. She was referred to the hospital in 1997 for a long-standing history of diarrhea that was exacerbated after a lipid-rich meal. At the age of 12 months, she had been admitted to hospital for the presence of diarrhea, weight loss, and retarded growth. Common causes of intestinal malabsorption (notably celiac disease and lactose intolerance) were ruled out. The presence of a mild steatorrhea associated with low plasma total cholesterol (TC) levels (80–85 mg/dl) suggested, at that time, the diagnosis of hypobetalipoproteinemia. After treatment with a low-fat diet supplemented with fat-soluble vitamins, her condition improved and she developed normally. However, she has always complained of occasional episodes of diarrhea following fat-rich meals. At the time of the observation (1997), she was overweight [body mass index (BMI) = 29.0 kg/m^2] and had a mild hepatomegaly. Neurological examination was negative. Ultrasound scanning revealed fatty liver but a normal morphology of the gallbladder and biliary system. Routine laboratory tests, including an oral glucose tolerance test, were within the normal values. Plasma lipid values are given in Results. Plasma levels of vitamins A, E, and D_3 were within the normal range. Stool analysis, performed when she was on a standard diet, revealed the presence of steatorrhea (fecal fat =15–20 g/24 h).

The proband's pedigree spans three generations and includes nine living members (Fig. 1). The proband's mother (I-2 in Fig. 1), who was HCV-Ab positive, died from liver cirrhosis at the age of 68; at the age of 30 she had been found to have hypocholesterolemia (TC = 105 mg/dl). The proband's father (I-1 in Fig. 1) died from stroke at the age of 71; his plasma cholesterol was reported to be within the normal values (TC = 185 mg/dl).

The proband's daughter (III-3 in Fig. 1) had been found to have hypocholesterolemia (TC = 82 mg/dl) associated with very low levels of TGs (26 mg/dl) and apoB (24 mg/dl) when she was 7 years old. She had a history of chronic diarrhea which subsequently improved after the administration of a low-fat diet supplemented with lipid-soluble vitamins. In 1997 she was found to be obese (BMI = 34.6 kg/m^2). Neurological examination was negative. All laboratory tests were normal apart from plasma lipids (see Results). Stool analysis showed a mild steatorrhea (fecal fat=10–15 g/24 h). Ultrasound scanning indicated the presence of fatty liver.

The proband's son (III-4 in Fig. 1) was found to have hypocholesterolemia (TC = 77 mg/dl) at the age of 8 months during a routine laboratory investigation. At the time of observation he was found to be in good health.

Although liver and intestinal biopsies were proposed to all of the proband's family members found to have fatty liver and steatorrhea, no consent was given for these procedures.

N.L. kindred. Proband N.L. (I-1 in **Fig. 2**), a 77-year-old male, was identified during a cholesterol screening program aimed at selecting individuals with low plasma cholesterol (below the 5th percentile). He had smoked 15–20 cigarettes a day for 40 years, quitting at the age of 65. He had had an ischemic stroke with left hemiparesis at the age of 41.

At the time of the study, there were no symptoms or signs of coronary artery disease. Routine laboratory tests, including an oral glucose tolerance test, were within the normal values. An exercise stress test and Doppler ultrasound examination of the lower limbs were normal. Carotid Doppler ultrasonography revealed a fibrocalcific atherosclerotic plaque at the origin of the right internal carotid artery, causing a luminal narrowing of 30% and a diffuse medial-intimal thickness. A single photon emission computed tomography (SPECT) evaluation of the cerebral perfusion with Xenon-133 showed the presence of ischemic areas in the right temporal-parietal and left temporal-rolandic regions.

The proband's sister (I-2 in Fig. 2) is a 73-year-old asymptomatic subject. Although obese (BMI = 30.1 kg/m^2) and hypertensive (treated with ACE inhibitors, calcium antagonists, and diuretics for several years), she had no history of atherosclerotic disease.

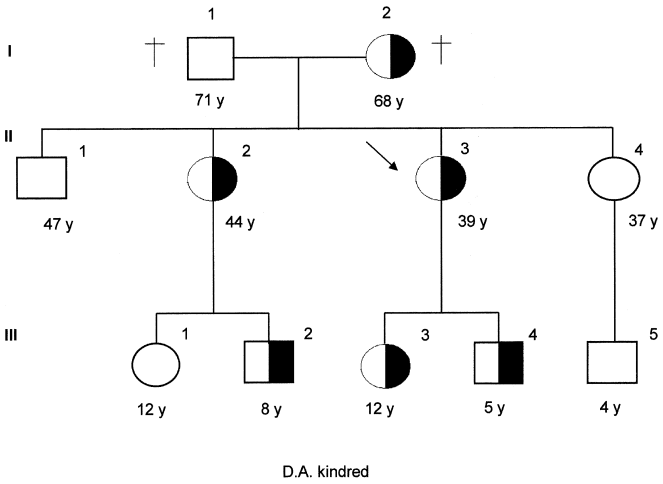

Fig. 1. The D.A. kindred. The proband is indicated by an arrow. Subjects with hypobetalipoproteinemia are indicated by half-shaded symbols. Subject II-1 was not available for the study.

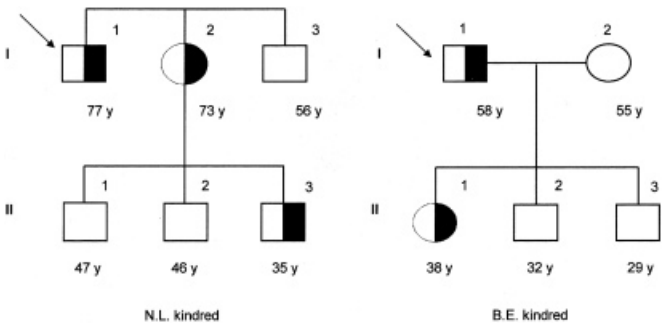

Fig. 2. The N.L. and B.E. kindreds. The probands are indicated by arrows. Subjects with hypobetalipoproteinemia are indicated by half-shaded symbols. Subject I-3 in the N.L. kindred and subject I-2 in B.E. kindred were not available for the study.

Fundus oculi examination revealed a grade II hypertensive retinopathy. Laboratory tests disclosed the presence of hypocholesterolemia (see Results). Carotid Doppler ultrasonography was normal. The son of the proband' s sister (II-3 in Fig. 2), age 35, was found to be hypocholesterolemic (see Results). He was asymptomatic and had normal blood pressure but was heavily overweight (BMI = 29.7 kg/m^2). Liver ultrasound examination revealed the presence of fatty liver in both these subjects. However, no consent was given for liver biopsy.

B.E. kindred. The proband (I-1 in Fig. 2), a 58-year-old male with a long-standing history of high alcohol intake (>100 g/day), was admitted to hospital on account of abdominal pain and altered bowel habit. At age 42, he had been medically treated for pulmonary tuberculosis for ~6 months. The patient alleged his liver function tests to have been normal before the antitubercular treatment. At age 43, his liver function tests were found to be altered, and hypocholesterolemia was first documented. The physical examination was unremarkable apart from overweight (BMI = 28 kg/m^2) and hepatomegaly. Laboratory tests disclosed a mild elevation of serum transaminases. Routine blood chemistry (with the exception of lipid values), fasting insulin, total urine porphyrins, serum copper, α_1-antitrypsin, ceruloplasmin, HBsAg, HBcAb, HCV, antibodies against smooth muscle, anti-mitochondria, anti-nuclei, anti-liver-kidney microsome, and oral glucose tolerance test values were all negative or in the normal range. The plasma level of ferritin was 1,321 ng/ml (n.v. 300–400 ng/ml), and serum iron and transferrin were within the normal range. The search for the common mutations (C282Y and H63D) of the hemochromatosis gene was negative. Plasma lipids are given in Results.

Ultrasound scanning was consistent with fatty liver. Esophagogastroduodenoscopy was negative. Colonscopy disclosed a few sigmoid diverticula without signs of inflammatory changes. Chest X-rays showed bi-apical scarring. Percutaneous liver biopsy revealed sparse chronic inflammatory changes in the portal areas with mild perisinusoidal and periportal fibrosis. Steatosis was present in 80% of the hepatocytes; occasional areas of liver cell necrosis and lipogranulomata were also observed. Iron accumulation was present in hepatocytes and, to a much larger extent, in Kuppffer cells. The score of iron overload was 8/20 according to Sciot et al. (18). The patient was dismissed with the diagnosis of "alcoholic steatohepatitis with fibrosis. Familial heterozygous hypobetalipoproteinemia. Diverticular disease of the sigmoid colon." The proband's daughter (II-1 in Fig. 2), an asymptomatic 38-year-old female, was found to have hypocholesterolemia (see Results). She had a history of repeated extra-uterine pregnancies, for which she had been operated on twice. She had normal liver function tests and no ultrasonographic evidence of fatty liver or gallstones. The proband's sons (II-2 and II-3 in Fig. 2) were normolipidemic.

All members of the three kindreds available for the study were subjected to the evaluation of the lipid profile and to the screening for the mutation of the apoB gene found in the corresponding index cases. Healthy subjects were selected from among the laboratory staff and medical students. All gave their informed consent for the study.

The study protocol was approved by the Institutional Human Investigation Committee of each participating institution.

Ultrasonographic examination of the liver

Subjects were allowed a free diet (according to their dietary habits) in the days preceding the ultrasonographic scanning of the liver. The consumption of alcohol (if any) was not specifically restricted before the liver ultrasound examination.

The technique of ultrasonographic scanning of the liver was detailed elsewhere (19). The liver was scanned after an overnight fast. Contiguous longitudinal and transverse sections with and without holding of the breath were performed. The scanning was performed and the images examined by two independent observers. The interobserver variation for the presence or absence of steatosis, investigated in 60 consecutive cases, was found to be fairly good (Spearman's $\rho = 0.987$, $P < 0.01$).

In a previous study, we found that the absence of fatty liver at ultrasound examination was confirmed by computed tomography (liver to spleen ratio >0.95) (13). It is generally accepted that for "bright" liver to be present, at least 25–30% of hepatocytes must have undergone fatty changes (20). However, in our ongoing study on nonalcoholic fatty liver disease (21), we found an ultrasonographic "bright" liver in subjects with fatty changes in as few as 10–20% of the hepatocytes (unpublished observation).

Analysis of plasma lipids and lipoproteins

Blood was collected after an overnight fast, unless otherwise specified. Plasma TC and TG, apoA-I, and apoB were measured as previously specified (12). Plasma lipoproteins were separated either by continuous density gradient ultracentrifugation (12) or sequential ultracentrifugation (22).

Apolipoprotein analysis

Aliquots (15–50 μg of protein) of lipoprotein fractions were precipitated in 10% TCA and extracted with ethanol–diethyl ether 3:2 (v/v). For the analysis of apoB, the samples were separated by a linear (3.5/5–10%) gradient SDS-PAGE (12). For analysis of the other apolipoproteins, the samples were separated by a linear 5–20% gradient SDS-PAGE (12).

Immunoblot analysis

Aliquots of total plasma and plasma lipoproteins (50–60 μg of protein) were delipidated and separated by linear 3.5/5–10% or 5–20% gradient SDS-PAGE (12). For immunoblotting, apolipoproteins were electrotransferred from the gel to a Zeta Probe membrane (BioRad Laboratories, Richmond, CA). The membranes were incubated with anti-human apoB-100 sheep polyclonal antiserum, as previously described (12). In the case of N.L. kindred, immunoblotting of apoB was also performed by using monoclonal antibodies BSol12, 1D1, 2D8, and 4G3 kindly provided by Dr. Y. Marcel (University of Ottawa, Ontario, Canada). These antibodies recognize the amino-terminal end of apoB (BSol 12) and the epitopes in the amino acid regions 401–582 (1D1), 1,297–1,480 (2D8), and 2,980–3,080 (4G3) of apoB (23).

Analysis of the apoB gene

Genomic DNA was extracted from peripheral blood leukocytes by a standard procedure (24). In the case of the D.A. kindred, in which no truncated apoB was detected in plasma lipoproteins, the whole apoB gene was analyzed. The primer pairs for PCR amplification are listed in **Table 1** (25, 26). For all PCR amplifications, 1 μg of genomic DNA was added to a 100-μl mixture containing 0.2 mmol/l of each deoxynucleoside triphosphate, 100 pmol of each primer, and 3.5 U Taq DNA polymerase (Expand High Fidelity; Roche Diagnostics GmbH, Germany) in PCR buffer (50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 9, and 1.5 mmol/l $MgCl_2$). The amplification conditions were: 95°C for 5 min followed by 30 cycles at *a*) 94°C for 1 min/60°C for 1 min/72°C for 3 min (exons 1, 2, 4, 5–6, 7–8, 19–20, 21, 22, 23, 27–28); *b*) 95°C for 1.5 min/67°C for 1.5 min (exon 3); *c*) 94°C for 1 min/55°C for 1 min/72°C for 3 min (exons 9, 13, 14, 17, 18, 24); *d*) 92°C for 1 min/55°C for 2 min/72°C for 2 min (exon 10); *e*) 94°C for 1 min/59°C for 1 min/72°C for 3 min (exons 11–12, 15, 16, 25, and 29, fragments a and b); *f*) 94°C for 1 min/63°C for 1 min/72°C for 3 min (exon 26, fragments a and d); *g*) 94°C

TABLE 1. Oligonucleotide primers for PCR amplification of apoB gene

Exons	Forward Primers (5′→3′)	Reverse Primers (5′→3′)	Size of PCR Products (*nt*)
1	TGTAGAAAAGCAAACAGGTCAGGC	CCGCCAGCTGGTCCAATGCC	453
2	GGAAGCTCACAGAATTTCTTTCTC	GTAGAAGAGAGTTGGCATCCCTT	~275
3	CCAGAATTGGCTGTCCTTGGGAG	TACACAACTCCGGGAAGGTCGCG	280
4	TTGGTGCTCTGATTAGAGATTAAGC	CACAAGTTCATACCTCAGCGGAC	301
5–6	AGTGCCACCCAGCTTACTTCCA	TCAAAGGTGCCCACTAGCTCAA	1,200
7–8	TCTGAGTTTATCTAGTGGTACAG	TGGCTAAGCCATGATAGGCACAT	1,083
9	AGCAGATCTAGCAGGCATTGAA	TTGAAAGTTCAGTCAGTTACCAT	~427
10	TTCTGAGCTCCAAGTTGGGTT	AAGAATTCAATTTGTGTTTGCTGA	~560
11–12	ATCTTTCCCAGCACCACTTATTGA	GATGAAATCTAGAGTCTCATTCC	703
13	AGAAGATTCAATACCAGCCATTT	GGTCTAGATCTGCTACACATTT	486
14	AGATCTAGACCCAAAGACTTAGG	CTTAGTTTTCCTCTGGGTAGCT	472
15	ACATCAAGAGTGGGACTACTAGG	GAATTGTTTTTGCATTGAGACCC	315
16	TGGGAACTGAAAGGTGTTTGACA	CAGTGAATTCAAGGCAAACCTCC	400
17	ATAAAGAGTAATTACTCTCCAATG	CATTCTGGTGGAAGCTTGAAGTT	432
18	AACTCTAGAGAACTGAGAACTCG	TGATCTAGATCAACTGTTTAGCC	~450
19–20	CCCTGAGAATTTGTGATGTCCATT	GAATTCTGAACCTGAGACTGCG	816
21	AAACATAGCTTCTTACCACACATC	AGAACATGGCTTGGTCAGGTATGA	358
22	CTCTGAACCATCCTTGTATCT	CACCTGCATTACTTTGGAAGT	339
23	CTGTGGTTACAGGCTGAACTA	TGCACCTAGCTCAGAGTTGAG	318
24	GTCCAGCTTAATAATTAACTTGTC	ACAGGTTGTACTGAATAAAATATC	508
25	TTTGAATGACTGATGTCTGACTG	AAGACTTCCAAGTAGCAAGGAAG	554
26a	CAGATGGAGGAGTCTATTGCACA	TGAACCTTAGCAACAGTGTCTGCTT	1,492
26b	ACATCTATGCCATCTCTTCTG	ATCAATAGCCTCAATGTGTTG	1,367
26c	AAGAGACACATACAGAATATAG	ACAAAGTCAATTGTAAAGGAAG	1,190
26d	GGTTTTCCACACCAGAATTTACCAT	TACTTATACTGATTGAACCTAGCAC	1,363
26e	TAACTATGCACTGTTTCTGAG	GAGTACAGCATTGAAGAATTG	1,197
26f	AGTCAAAACCTACTGTCTCTTCCTC	ACGTGTAGGGTATACATGTATCTCTTTTCT	1,595
27–28	CGTCCTACTGTTATGAATCTAATAAAATAC	CTCGCTCTTGGGGGCGTGTCACTCATTAGG	494
29a	ACACATGAACTGACATATGAAAGAT	TGAAGATTACGTAGCACCTCTG	963
29b	ATAGATGTAATCTCGATGTATAGG	TGCAAGGCTGGCTCACTGTATG	1,018

For some exons, the indicative size (~) of the PCR products is given. In view of their size, exons 26 and 29 were separated into fragments (indicated by lowercase letters). The nucleotide positions of the 5′ and 3′ end of each fragment (fr.) in cDNA are as follows: fr. 26a, from nt −129 (in intron 25 with respect to the first nucleotide of exon 26) to nt 5,707; fr. 26b, nt 5,641–7,007; fr. 26c, nt 6,936–8,125; fr. 26d, nt 8,059–9,421; fr. 26e, nt 9,344–10,540; fr. 26f, from nt 10,474 to nt 152 of intron 26; fr. 29a, from nt −151 (in intron 28 with respect to the first nucleotide of exon 29) to nt 13,027; fr. 29b, nt 12,915–13,932. Nucleotide numbering according to Knott et al. (25).

for 1 min/53°C for 1 min/72°C for 3 min (exon 26, fragments b, c, and e); and *h*) 94°C for 1 min/67°C for 1 min/72°C for 3 min (exon 26, fragment f). The amplification products were purified with PCR Clean up Kit (Roche Diagnostics GmbH), analyzed by 2% agarose gel electrophoresis, and sequenced with a Big Dye terminator cycle sequencing kit (PE; Applied Biosystems, Warrington, UK) on an Applied Biosystems 377 DNA sequencer by using appropriate primers. Sequence variations found in automated sequencing were always checked by manual sequencing (12) using appropriate primers.

In the case of the N.L. kindred, the presence in SDS-PAGE of a truncated form of apoB with an estimated molecular mass of 160–190 kDa (see Results) suggested that the putative mutation might be located at the 5′ end of exon 26 of the apoB gene. Therefore, we amplified a 551-bp fragment spanning from the intron 25-exon 26 junction to the first 510 nucleotides of exon 26. The fragment was amplified using the following primers: 5′-TACTGCTTAATTTAACCAATGTCT-3′ (forward primer in intron 25 from nt −41 to nt −8 with respect to nt 4,345 of the cDNA) and 5′-CATTGGTGTCAGATTTTAAAGTCA-3′ (reverse primer in exon 26 from nt 4,854 to nt 4,831 of the cDNA). The conditions were 95°C for 5 min followed by 30 cycles at 95°C for 1 min, 52°C for 1 min, 72°C for 1 min, and final extension at 72°C for 10 min. The amplification product was purified with High Pure PCR Product Purification Kit (Roche Diagnostics). Both strands of the 551-bp fragment were sequenced directly by using the same primers and the Ampli Cycle sequencing kit (PE; Applied Biosystems).

In the case of the B.E. kindred, the presence in SDS-PAGE of a truncated form of apoB with an estimated molecular mass of ~400–450 kDa (see Results) suggested that the putative mutation might be located at the 3′ end of exon 26 (fragments e and f, Table 1). These regions were amplified and sequenced as specified above in the case of the D.A. kindred.

Screening of the apoB gene mutations found in the kindreds

The screening of D.A. family members for the C-deletion in exon 10 found in the proband (see Results) was performed by automated sequencing of PCR-amplified exon 10 (see above). For the screening of the C→T transition in exon 26 (at nucleotide 11,605 of the cDNA) that introduces an *Nla*III restriction

TABLE 2. Characteristics of the members of the D.A. kindred

Subjects	BMI	TC	TG	LDL-C	HDL-C	ApoB	ApoA-I
	kg/m²	*mg/dl*					
Proband (II-3)	29.0	125	24	52	69	34	141
Daughter (III-3)	34.6	77	35	22	49	22	115
Son (III-4)	23.5	65	21	14	48	17	110
Sister (II-2)	28.3	113	40	32	75	29	164
Sister (II-4)	24.7	176	86	108	58	68	153
Niece (III-1)	20.0	167	55	98	60	58	143
Nephew (III-2)	21.5	89	24	23	62	20	131
Nephew (III-5)	13.6	113	99	54	43	52	111

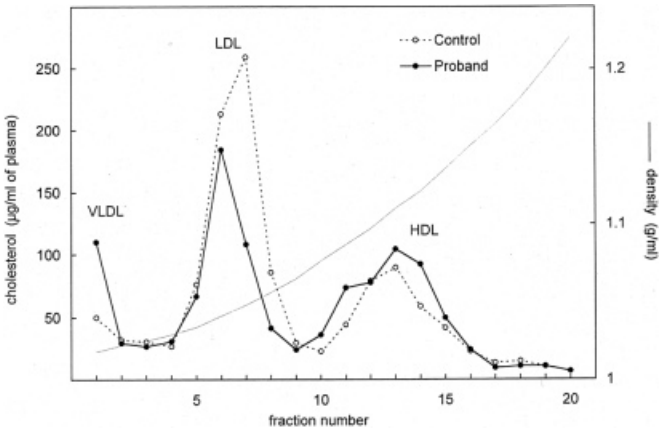

Fig. 3. Density profile of plasma lipoproteins. Plasma lipoproteins were separated by density gradient ultracentrifugation from proband D.A. and an age- and sex-matched control subject. VLDL + IDL (intermediate density lipoproteins; fractions 1–3); LDL (fractions 4–9); HDL (fractions 10–18).

site, the 3′ end of exon 26 (nt 11,359–11,912 of the cDNA) was amplified by PCR and digested by the restriction enzyme *Nla*III (New England Biolabs, Inc.). The primers were: 5′-TTGTCATGC CTACGTTCCATGTCCC-3′ (forward primer) and 5′-TAGTTCAT ATTCTAGGAACTGTACG-3′ (reverse primer). The amplification conditions were 95°C for 5 min followed by 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min. The digestion products were separated on a 3% agarose gel (Metaphor; FMC BioProducts, Rockland, ME). The digestion of the 553-bp PCR-amplified product generated three fragments (499, 34, and 20 bp) from the normal allele and four fragments (272, 227, 34, and 20 bp) from the mutant allele.

The screening for the mutations found in the probands of the N.L. and B.E. kindreds was performed by direct sequencing of the apoB gene regions harboring the mutations.

RESULTS

D.A. kindred

Plasma TC, LDL cholesterol (LDL-C), and apoB of proband D.A. (**Table 2**) were found to be greatly reduced compared with the values found in age- and sex-matched subjects from the general population (TC = 195.9 ± 36.0 mg/dl; LDL-C = 125.8 ± 33.2 mg/dl; apoB = 86.7 ± 20.0 mg/dl). The density profile of plasma lipoproteins isolated from the proband in nonfasting conditions (**Fig. 3**) was characterized by a marked reduction of the LDL peak (fractions 4–9, d = 1.035–1.060 g/ml) as compared to the density profile of a normolipidemic age- and sex-matched family member. The same profile was observed in the proband' s children (data not shown). The apoB in all lipoprotein density fractions was analyzed by SDS-PAGE (5–10% and 5–20% polyacrylamide gradient gels). In the proband as well as in the other hypocholesterolemic family members, the only detectable forms of apoB were apoB-48 and apoB-100 (data not shown). Immunoblot of 5–10% and 5–20% polyacrylamide gradient gels of lipoprotein fractions and lipoprotein infranate failed to show truncated forms of apoB (data not shown).

Analysis of apoB gene. The analysis of the apoB gene showed that the proband was heterozygous for a C-deletion (either at position nt 1,310 or 1,311) in exon 10 (**Fig. 4**). This deletion (regardless of its position) causes a shift in the reading-frame that results in a stretch of three novel amino acids preceding a premature stop codon at position 371. The predicted translation product of this mutant allele is a truncated protein of 370 amino acids, corresponding to 8.15% of mature apoB-100 (designated apoB-8.15). The nucleotide sequence of the exon 10 region involved in this deletion does not allow the unequivocal identification of the precise position of the deleted C-nucleotide (nt 1,310 or 1,311 of the cDNA). However, the deletion of either of these nucleotides has the same effect because it leads to an identical translation product (Fig. 4). This deletion occurs in a stretch of short direct repeats that are known to be hot spots for minute deletions or insertions occurring during DNA replication (slipped mispairing) (27).

The analysis of apoB gene extended to all family members available for study demonstrated that four of them were carriers of the mutation, which cosegregated with the FHBL plasma lipid phenotype in the pedigree. The

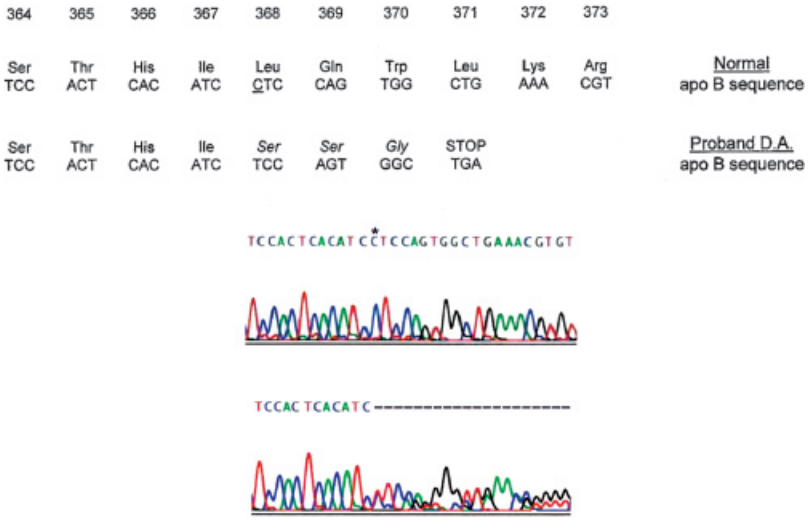

Fig. 4. Sequence analysis of exon 10 of the apoB gene in proband D.A. The upper panel illustrates the location of the C-deletion (underlined) in exon 10, which results in a premature stop codon at amino acid position 371. The lower panel shows the corresponding sequence electropherograms from a normal control (top) and the proband (bottom). The putative deleted nucleotide is indicated by a star.

search for the C-deletion in exon 10 was also performed in 12 unrelated FHBL probands of our series in whose plasma no truncated apoBs had been detected. None of them was found to carry this mutation.

During the apoB gene sequencing, we found that the proband was heterozygous for a novel C→T transition in exon 26 (at nucleotide 11,605 of the cDNA), which converts the ACG codon (threonine) into ATG (methionine) (T3799M) (data not shown). Because this mutation introduces a *Nla*III restriction site, a rapid screening method was developed based on PCR amplification of the 3′ end of exon 26 (see Patients and Methods) followed by *Nla*III digestion. We screened family members as well as unrelated normolipidemic subjects (data not shown). This analysis showed that this C→T transition *a*) cosegregated with the FHBL lipid phenotype in the kindred and *b*) was not found in 50 normolipidemic subjects.

N.L. kindred

Plasma lipids of the proband and all available family members are shown in **Table 3**. The plasma levels of TC, LDL-C, and apoB in the proband, his sister, and one of the sister's sons were consistent with heterozygous FHBL. Of the other family members, subject II-1 (Fig. 2) had normal lipid levels whereas subject II-2 had mixed hyperlipidemia. The analysis of apoB separated in SDS-PAGE revealed not only apoB-100 but also an abnormal band of lower molecular weight (migrating in the 160- to 190-kDa region of the gel), which was present predominantly in the HDL density range (data not shown). Immunoblot analysis of plasma apoB (**Fig. 5**) confirmed the presence of this abnormal band in proband N.L. and his sister, suggesting the presence of a truncated apoB with a size ranging from 30% to 35% of that of apoB-100. Immunoblotting with a battery of monoclonal antibodies showed that the abnormal band reacted with antibodies BSol12, 1D1, and 2D8 but not with antibody 4G3 (data not shown). In view of the epitope assignments of these antibodies (23), these findings indicated that the truncated apoB corresponded to the NH_2-terminal portion of apoB-100 (upstream from residue 1,480). These combined results suggested that the putative mutation leading to the formation of the truncated apoB was located in the 5′ end of exon 26 of the apoB gene.

Analysis of apoB gene. The sequence analysis of a 551-bp fragment spanning from the intron 25-exon 26 junction to the first 510 nt of exon 26 showed that the proband and his sister were heterozygous for a T→A transversion at nucleotide 4,739 of apoB cDNA (**Fig. 6**). This substitution converts the TAT codon (tyrosine) at position 1,510 into a stop codon (TAA). The predicted truncated apoB encoded by this mutant allele is a protein of 1,509 amino acids that corresponds to the 33.4% of the mature apoB-100 (apoB-33.4). This mutation was also found in subject III-3 of this kindred (Fig. 2).

TABLE 3. Characteristics of the members of the N.L. kindred

Subjects	BMI	TC	TG	LDL-C	HDL-C	ApoB	ApoA-I
	kg/m²			*mg/dl*			
Proband (I-1)	28.4	99	35	38	54	32	113
Sister (I-2)	30.1	101	80	32	53	38	156
Nephew (II-1)	nd	187	105	122	44	94	126
Nephew (II-2)	nd	138	512	118	38	128	134
Nephew (II-3)	29.7	96	136	32	37	36	110

nd, not determined.

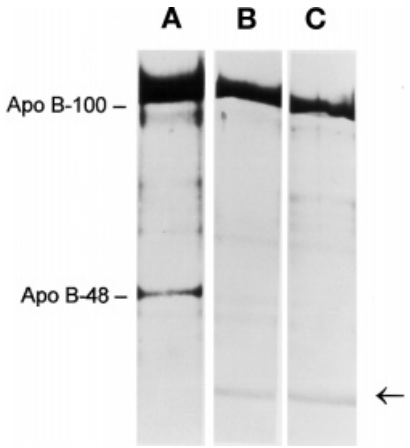

Fig. 5. Western blot of SDS-PAGE (3.5–10% linear gradient gel) of plasma apoB in the N.L. kindred. Lane A, VLDL from a normal subject; lane B, plasma of proband N.L.; lane C, plasma of proband's sister. The putative truncated apoB form is indicated by an arrow.

B.E. kindred

The plasma lipid levels in the proband and his daughter (**Table 4**) as well as the density gradient profile of plasma lipoproteins (data not shown) were consistent with heterozygous FHBL. The analysis of apoB separated by SDS-PAGE revealed, besides apoB-100, an abnormal band of lower molecular weight (migrating in the 400- to 450-kDa region of the gel), which was clearly detectable in lipoproteins isolated in the density range of 1.050–1.070 g/ml (**Fig. 7**). Immunoblot analysis of apoB confirmed this abnormal band in the proband and his daughter, suggesting the presence of a truncated apoB with a size ranging from 70% to 80% of that of apoB-100 (data not shown). This result indicated that the putative mutation leading to the formation of the truncated apoB might be located at the 3′ end of exon 26 of the apoB gene.

Analysis of apoB gene. The sequence of the candidate region of exon 26 showed that the proband and his daughter were heterozygous for a T-deletion at nucleotide 10,514 of apoB cDNA (**Fig. 8**). This deletion causes a single-nucleotide shift in the reading frame that converts the tyrosine codon at position 3,435 into a stop codon. The predicted truncated apoB encoded by this mutant allele is a protein of 3,434 amino acids that corresponds to 75.7% of the mature apoB-100 (apoB-75.7).

DISCUSSION

In this work, we have analyzed the FHBL phenotype in three kindreds carrying novel mutations of the apoB gene leading to truncated apoB forms of various lengths.

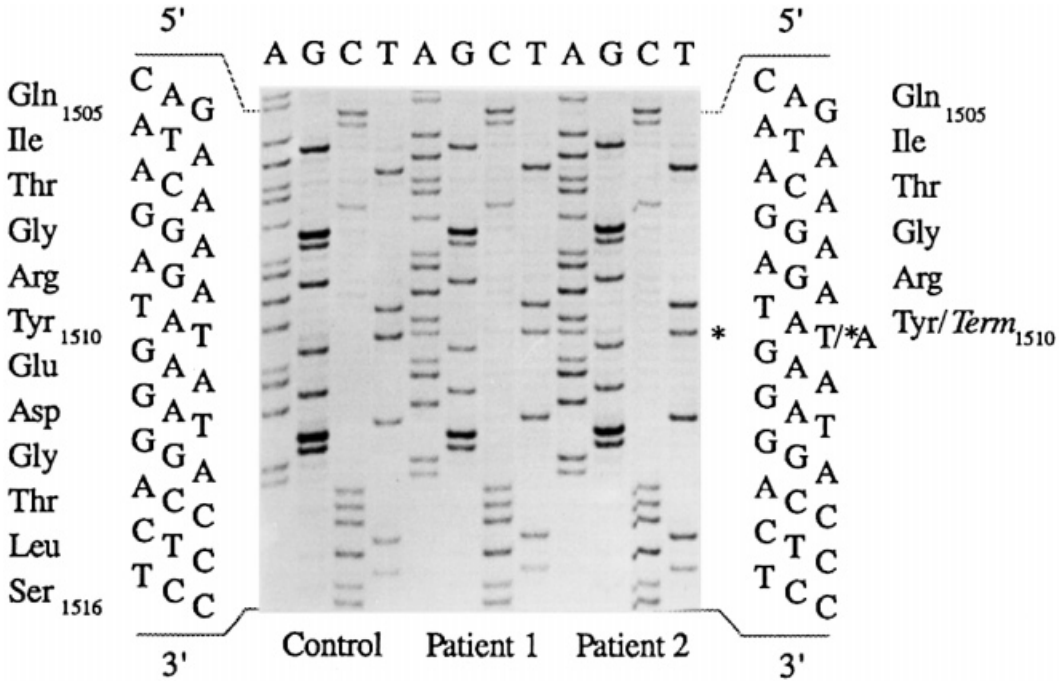

Fig. 6. Sequence analysis of the 5′ end of exon 26 of the apoB gene. Proband N.L. (patient 1) and his sister (patient 2) were found to be heterozygous for a T→A transversion (indicated by a star) that converts the tyrosine codon at position 1,510 into a termination (*Term*) codon.

Proband D.A. belongs to our series of "symptomatic" heterozygous FHBL subjects (12, 13) with no truncated apoBs detectable in plasma. In our series of 22 "symptomatic" FHBL subjects, approximately two-thirds have no truncated apoBs detectable in plasma. This finding raises the question as to whether, in a specific FHBL patient/pedigree, hypobetalipoproteinemia is due to mutations in the apoB gene or in other genes (yet to be identified) affecting apoB metabolism. In the presence of a large family, the cosegregation analysis of apoB gene markers may be a suitable tool to answer this question (4, 28). In small families (the most common situation), the only feasible approach is to sequence the whole apoB gene. This strategy, adopted in the study of the D.A. kindred, demonstrated that the proband was heterozygous for a single-nucleotide deletion in exon 10, which leads to the production of a truncated apoB of 370 amino acids (apoB-8.15). This novel mutation is the third reported so far as the cause of very short truncated apoBs (with a size <10% of that of apoB-100) (28–30), which are not secreted in plasma as lipoprotein constituents or in lipid-free form.

TABLE 4. Characteristics of the members of the B.E. kindred

Subjects	BMI	TC	TG	LDL-C	HDL-C	ApoB	ApoA-I
	kg/m²			*mg/dl*			
Proband (I-1)	28.0	103	57	45	49	26	134
Daughter (II-1)	27.2	110	34	52	53	27	139
Son (II-2)	nd	191	51	121	62	65	157
Son (II-3)	nd	182	124	126	36	71	124

nd, not determined.

ApoB-8.15 is completely devoid of the lipid-associating domains present in the NH_2-terminal region of apoB (31) and of the region of the α1 globular domain (residues 430–570) involved in the binding of the microsomal TG transfer protein (MTP) (32, 33). It is most likely that apoB-8.15 does not associate with lipids and, for this reason, is rapidly degraded intracellularly in both liver and intestine. Thus, in proband D.A., the only apoB synthesized in both tissues is the one encoded by the normal allele. However the plasma level of apoB-100 in apoB-8.15 carriers is much lower than the 50% expected value (i.e., ~50% of the level found in controls) (Table 2). A recent study of apoB-100 and TG turnover in FHBL heterozygotes for very short truncated apoBs (apoB-2 and apoB-9) gives an explanation for this finding (34). This study showed that the production rates of VLDL apoB-100 and TG were ~25% and 40% of normal, respectively, whereas the catabolism of both apoB-100 and TGs was not altered (34).

The presence of steatorrhea suggested that proband D.A. might have lipid malabsorption possibly due to a reduced availability of apoB-48 (haplo-insufficiency) for the formation of chylomicrons. This explanation seems in contrast with the results obtained by Averna et al. (35), who compared fat absorption in heterozygotes with apoB truncations longer than apoB-48 or shorter than apoB-48 and found no effect of the apoB length on postprandial lipemia. However, in that study, heterozygotes for truncations shorter than apoB-48 were carriers of apoBs with a size ranging from apoB-31 to apoB-46. Because these peptides contain the MTP binding domain as well as part of lipid-associating domains of apoB-48, it is reasonable to

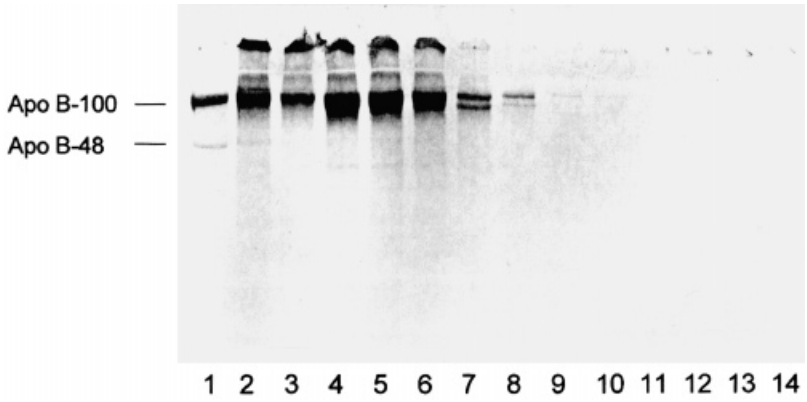

Fig. 7. SDS-PAGE (5–10% linear gradient gel) of apoB in plasma lipoproteins of proband B.E. An aliquot of lipoproteins (15 μg of protein in lanes 2–7 and 50 μg of protein in lanes 8–14) isolated by density gradient ultracentrifugation was applied to each lane. Lane 1, VLDL from a normal subject; lane 2, VLDL + IDL (d < 1.025 g/ml); lanes 3–7, LDL (d = 1.025–1.070 g/ml); lanes 8–14, HDL (d = 1.070 – 1.210g/ml) isolated from proband B.E.

assume that they can bind lipids and form lipoprotein particles that may contribute to postprandial lipemia. As a matter of fact, we have never observed steatorrhea in 21 carriers of truncated apoBs with a size ranging from apoB-33.4 to apoB-75.7. This may not be the case with very short truncated apoBs not detectable in plasma.

Proband N.L., identified during a population screening of subjects with hypocholesterolemia, was found to be heterozygous for a novel nonsense mutation leading to the formation of a truncated protein of 1,509 amino acids (apoB-33.4). This subject, who had suffered from a major cerebrovascular accident (with no angiographic evidence of congenital vascular malformation) at the age of 41, had signs of carotid atherosclerosis later in life. This was an unexpected finding because individuals with low levels of LDL-C are considered to be protected from atherosclerosis, and FHBL is usually perceived as a condition predisposing to longevity (17). These assumptions are challenged by some anecdotal observations of atherosclerotic disease in heterozygous FHBL subjects carrying detectable truncated apoBs. Severe coronary and aortic atherosclerotic lesions were reported in a 71-year-old male suspected of being a carrier of apoB-55 (36). Coronary atherosclerotic plaques were reported in a 59-year-old female carrier of apoB-46 (37), and myocardial infarction occurred in two carriers of the apoB-40/89 kindred (38). Finally, an extensive arterial calcification was reported in a patient with homozygous FHBL associated with diabetes mellitus (39). These sporadic observations suggest that, in some cases, an LDL-C level below the 5th percentile may not prevent cardiovascular disease in the presence of other cardiovascular risk factors (i.e., smoking, as in the case of proband N.L.).

Proband B.E. was found to be heterozygous for a single nucleotide deletion leading to the formation of apoB-75.7. Liver histology revealed the presence of steatohepatitis which, on the basis of historical data, was related to high alcohol intake (alcoholic steatohepatitis). The same mutation was present in his teetotaller asymptomatic daughter, who had no ultrasonographic evidence of fatty liver. A truncated form of apoB similar in size (apoB-75) to the one described in the B.E. kindred was reported by Krul et al (40) in an apparently asymptomatic FHBL subject carrying a C-deletion at position 10,366 resulting in a premature stop codon at amino acid residue 3,387.

In vivo turnover studies have shown that all truncated apoBs detectable in plasma are secreted at reduced rates

Fig. 8. Sequence analysis of the 3′ end of exon 26 of the apoB gene in proband B.E. The upper panel illustrates the location of the T-deletion (underlined) in exon 26 (nt 10,514 of apoB cDNA). This mutation causes a single-nucleotide shift in the reading frame that converts the tyrosine codon at position 3,435 into a stop codon. The lower panel shows the corresponding sequence electropherograms from a normal control (top) and the proband (bottom). The deleted nucleotide is indicated by a star.

(compared with apoB-100) and the rate of secretion is dependent on the length of the truncation. It has been calculated that the secretion is reduced by 1.4% for 1% of apoB truncated (41). These findings imply that the lipid secretion from the liver may be impaired in heterozygous FHBL, and a variable amount of lipids might accumulate in the hepatocytes. The presence of fatty liver was a constant clinical feature in carriers of apoB-8.15 and apoB-33.4 (D.A. and N.L. kindreds). This finding, together with previously reported observations in four other FHBL kindreds carrying apoB-38.95 (12, 13) and one kindred carrying apoB-38.15 (P. Tarugi and S. Lancelloti, unpublished observations), lends further support to the concept that heterozygous FHBL should not be overlooked in the etiological differential diagnosis of otherwise unexplained fatty liver.

The finding of a severe fatty liver in a carrier of apoB-75.7 was somewhat unexpected. This truncated apoB, which contains the bulk of the lipid-associating domains of apoB-100, is predicted to form a sufficient amount of lipoprotein particles to allow the secretion of TGs from the liver and prevent their intracellular accumulation, at least under physiological conditions. It is possible, however, that in the presence of environmental factors that increase hepatic TG synthesis or reduce apoB production, the amount of apoB-75.7 and apoB-100 synthesized by the liver is not sufficient to prevent lipid accumulation. In proband B.E., the presence of steatohepatitis is most likely the result of a combination of environmental factors such as high alcohol intake, overweight, and previous treatment with antitubercular drugs. This interpretation is supported by the study of the proband's daughter. In the absence of steatogenic environmental factors, she has no ultrasonografic evidence of fatty liver, indicating that the carrier status of apoB-75.7 is not sufficient per se to induce hepatic lipid accumulation to such an extent as to be detectable by liver ultrasound scanning.

The relationship between FHBL and fatty liver is supported by two recently developed animal models. Chen et al. (42) generated genetically engineered mice carrying a truncated apoB (apoB-38.9/apoB-100 mice) that display the FHBL lipoprotein phenotype and show a fatty liver due to the reduced ability of apoB-38.9 to transport TGs. This model supports our finding of the presence of fatty liver in carriers of truncated apoBs ranging from apoB-33.4 to apoB-38.95. Leung et al. (43) reported that hepatic TG stores are 4-fold higher in apoB$^{+/-}$ mice than in control animals. These mice can mimic the situation found in carriers of very short truncated apoBs not detectable in plasma such as apoB-2 (28, 30), apoB-8.15, and apoB-9 (29, 30). These animal models represent useful tools to investigate the genotype-phenotype correlations and clarify the role of environmental factors on the pathogenesis of liver disease in FHBL.

In conclusion, this study and previous observations (12, 13, 42, 43) suggest that carriers of truncated apoBs shorter than a certain size (presumably shorter than apoB-48) invariably develop fatty liver because of an impaired capacity of these truncated apoBs to export lipids from the hepatocytes. In contrast, truncated apoBs longer than apoB-48 may not affect this capacity to such an extent as to induce fatty liver unless additional steatogenic factor(s) are present.

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