ONLINE MUTATION REPORT

High frequency of APOB gene mutations causing familial hypobetalipoproteinaemia in patients of Dutch and Spanish descent

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J Med Genet 2005;**42**:e23 (http://www.jmedgenet.com/cgi/content/full/42/4/e23). doi: 10.1136/jmg.2004.029454

Background: Familial hypobetalipoproteinaemia (FHBL) is an autosomal co-dominant hereditary disorder of lipoprotein metabolism characterised by decreased low density lipoprotein (LDL) cholesterol and apolipoprotein B (APOB) plasma levels. High levels of plasma APOB and LDL cholesterol are strong predictors for risk of cardiovascular disease (CVD), while individuals with low APOB and LDL cholesterol levels are thought to have lower than average risk for CVD, and in fact, heterozygous FHBL patients appear to be asymptomatic.
Methods: Rather than identifying truncated APOB proteins in plasma fractions separated by gel electrophoresis, which will miss any mutations in proteins smaller than 30 kb, we analysed the *APOB* gene directly, using PCR.
Results: We identified nine different mutations, six of which are novel. Each mutation showed complete co-segregation with the FHBL phenotype in the families, and statistically significant differences between carriers and non-carriers were found for plasma total, LDL, and HDL cholesteroll, triglycerides, and APOB levels, but not for APOA1 levels. All carriers of an APOB mutation were completely free from CVD.
Conclusions: Prolonged low levels of LDL cholesterol and elevated levels of HDL cholesterol may reduce the progression of atherosclerotic disease, but this has not been unequivocally shown that this is indeed the case in individuals with FHBL, and is the subject of a current study.

INTRODUCTION

Familial hypobetalipoproteinaemia (FHBL) is an autosomal co-dominant hereditary disorder of lipoprotein metabolism characterised by decreased low density lipoprotein (LDL) cholesterol and apolipoprotein B (APOB) plasma levels.[1 2] APOB is a key structural component of triglyceride and cholesterol rich lipoproteins such as chylomicrons, LDL, and very low density lipoproteins (VLDL), and therefore plays a pivotal role in cholesterol metabolism.[3] High levels of plasma APOB and LDL cholesterol are strong predictors for risk of cardiovascular disease (CVD), while individuals with low APOB and LDL cholesterol levels are thought to have lower than average risk for CVD.[4] In fact, heterozygous FHBL patients appear to be asymptomatic. However, the accompanying clinical phenotype is not well defined, as only a few kindreds with a definite molecular diagnosis have been investigated in detail. The few reported symptomatic FHBL subjects suffered from diarrhoea, neurological manifestations, fatty liver, retarded growth, weight loss, and vitamin A and E deficiency.[5–8] In FHBL kindreds assessed at the molecular level, low LDL cholesterol and APOB levels are caused by mutations in the gene encoding APOB-100. To date, over 40 different molecular defects have been reported, most of which prevent the translation of a full length APOB protein. The frequency of *APOB* gene mutations causing truncated APOB and FHBL is considered rare, and is estimated to occur in 1.4% to 2.7% of individuals with persistent low levels of total and LDL cholesterol.[2 9–11]

Owing to the size of the *APOB* gene (spanning 43 kb, of which 14 kb is translated into the APOB-100 protein), the commonly used approach is identify truncated forms of the APOB protein by sodium dodecyl sulphate–polyacrylamide gel electrophoresis of delipidated VLDL and LDL plasma fractions. This strategy will only detect point mutations and frameshift mutations that lead to truncated APOB proteins that are larger than APOB-30. Truncated proteins smaller than APOB-30 are not incorporated in lipoproteins but are rapidly degraded or retained in the endoplasmic reticulum. Additionally, APOB truncations between B-30 and B-32 are only present in the density range of HDL.[12] Therefore, analysing only VLDL and LDL plasma fractions fails to identify certain *APOB* mutations. Furthermore, evidence exists that other loci might also contribute to a low cholesterol trait. Linkage to these putative loci was found in two genetic localisations, encompassing 3p21.1–22[13] and 13q.[14] In order to identify the cause of the FHBL phenotype in our probands, we chose to analyse the complete *APOB* gene by direct sequencing, rather than to analyse the APOB proteins in different lipoprotein fractions.

MATERIALS AND METHODS

Study subjects were selected by analysis of cholesterol levels collected during the course of a number of studies addressing several forms of genetic dyslipidaemia. Selection criterion was an LDL cholesterol level below the fifth percentile for sex and age.[15] Secondary causes for low LDL cholesterol levels, such as vegetarian diet, low fat diet, or cancer, were excluded. The probands were of Dutch or Spanish descent and provided information on their own health status and the structure of their kindreds. Blood samples were obtained from probands and their relatives after an overnight fast of at least 12 hours. All study subjects provided written informed consent and the study protocol was approved by the institute's ethics review board.

Plasma concentrations of total cholesterol, HDL cholesterol, and triglycerides were measured by commercially available kits (Boehringer Mannheim, Mannheim, Germany). LDL cholesterol concentrations were calculated by the Friedewald formula only when the triglyceride concentration was <4.5 mmol/L.[16] APOB and APOAI were determined on a Behring nephelometer BN100 using standard and references supplied by the manufacturer (Behring, Marburg, Germany). Genomic DNA was prepared from 10 ml whole blood on an AutopureLS apparatus according to manufactory's protocol (Gentra Systems, Minneapolis, MI, USA).

To analyse the promoter region, all 29 exons and the intronic boundaries of *APOB*, 54 pairs of primers were designed. PCR amplification was carried out with 50 ng of genomic DNA in a 25 µl reaction volume containing 1× *Taq* DNA polymerase buffer (Qiagen, Hilden, Germany), 50 µmol/l of each dNTP, 0.4 µmol/l of each primer, 5 µg bovine serum albumin, and 1 U *Taq* DNA polymerase. The thermal cycling conditions were as follows: 96°C for 5 minutes, then 35 cycles of 20 seconds at 96°C, 20 seconds at 55°C to 60°C (depending on primer CG content), and 30 seconds at 74°C in a PCR apparatus (T3 Biocycler, Biometra, Germany). The sequence reactions were performed using fluorescently labelled dideoxy chain terminations with a Big Dye Terminator ABI Prism kit (Applied Biosystems, Foster City, CA, USA) according manufactory's protocol and analysed on an Applied Biosystems automated DNA sequencer (model 3730). Sequences were analysed with the Sequencher package (GeneCodes Co, Ann Arbor, MI, USA).

All data were analysed using SPSS software (version 10.1; SPSS, Chicago, IL, USA) by one way analysis of variance and by multiple linear regression analyses with adjustment for age and sex. A p value <0.05 was considered to be statistically significant.

RESULTS

We identified 32 individuals meeting our inclusion criterion. After sequence analysis of *APOB*, we identified nine different mutations in 14 of our probands (table 1).

The R412X[17] and 11712delC[19] mutations, resulting in truncated APOB-9 and APOB-86 proteins, respectively, and the missense mutation, R463W,[18] have been described previously. Additionally, we identified six novel *APOB* mutations resulting in truncated APOB of different sizes. The frameshift causal deletion of nucleotides AT at base pair 1718 (1718delAT) in exon 13 resulted in a stop codon at amino acid position 547, which leads to a predicted APOB-12 protein. Deletion of an adenosine at nucleotide 2534 (2534delA) in exon 17 causes a frameshift resulting in amino acid changes running from amino acid 818 to 834, and finally a stop codon at amino acid 835, leading to an APOB-18 protein. The deletion of a cystidine at base pair 2783 (2783delC), resulted in amino acid changes running from amino acids 902 to 924 and finally a stop codon at 925, which leads to a truncated APOB-20 protein. A nonsense mutation comprising a single C→T transition of nucleotide 4006 in exon 25, thereby creating a *Dde*I restriction site, changes the codon for glutamine at amino acid 1309 into a stop codon (Q1309X), leading to a predicted APOB-29 protein. The C→T substitution at position 6700 in exon 26, converting an arginine at amino acid 2507 into a stop codon (R2507X). The predicted protein contains 2506 amino acid residues, and is designated as APOB-55. Finally, a deletion of TT at base pair position 11548 in exon 26 results in a stop codon at amino acid 3823 (11548delTT), leading to an predicted APOB-84 protein.

Table 1 Apolipoprotein B mutations identified

Exon	Mutation	WT	MT	Pos (bp)	Predicted size	Family
10	R412X[17]	CGA	TGA	1315	APOB-9	SP809
11	R463W[18]	CGG	TGG	1468	NA	SP810
13	1718delAT	AT	delAT	1718	APOB-12	SP807
17	2534delA	A	delA	2534	APOB-18	NL804
18	2783delC	C	delC	2783	APOB-20	NL826, NL827
25	Q1309X	CAA	TAA	4006	APOB-29	NL808, NL822
26	R2507X	CGA	TGA	7600	APOB-55	NL809, SP812
26	11548delTT	TT	delTT	11548	APOB-84	NL825
26	11712delC[19]	C	delC	11712	APOB-86	NL801, NL802, NL806

Pos, position. The reference sequence used was NM_000384, with the A of the ATG translation initiation codon numbered nucleotide +1 and the methionine numbered as amino acid −27.

Screening for the frameshift mutations was performed by direct sequencing of the relevant region of the *APOB* gene, and both nonsense mutations were screened by PCR followed by digestion with the appropriate restriction enzyme. As the R2507X mutation did not introduce or delete a restriction site, a mutagenic forward primer was designed that substituted an A at nucleotide position 7598 with a C, creating an *Nla*III restriction site when the R2507X mutation was present. The six novel mutations found were screened in a group of 94 normolipaemic controls, in which none of the mutations were found. Moreover, each mutation showed complete co-segregation with the FHBL phenotype in the families.

Clinical information on each FHBL family is listed in table 2. Individuals with and without the FHBL trait did not differ significantly from each other with regard to body mass index (BMI) or APOA1 levels after adjustment for age and sex. Statistically significant differences between affected and unaffected groups were found for plasma total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, and APOB levels (all $p<0.001$). Within the group of patients with FHBL we could not establish a relation between LDL cholesterol and APOB levels and the size of the truncated APOB protein.

Although most heterozygous FHBL patients appeared to be asymptomatic, some individuals did have complaints that may be associated with low LDL cholesterol and APOB levels. The proband of the NL806 family indicated that he experienced occasional episodes of diarrhoea. The proband of the NL808 family was a 59 year old man, who was referred to our lipid clinic because of high glucose levels and was diagnosed with diabetes mellitus (DM) type 2. Medical examination revealed extremely low cholesterol levels and severe obesity, with a BMI of 39.2 kg/m^2. Glucose levels remained high after medication. Medical examination of the 33 year old male proband of family NL809 revealed DM type I at 31 years of age, and neurological complaints of anaesthesia in his feet and paraesthesia in his hands. Vitamin A levels were slightly elevated (>3.9 µmol/l) and vitamin E levels were low (12 µmol/l). The diabetes was well managed by diet and insulin. Vitamin E levels returned to normal after oral administration of 400 mg vitamin E daily, after which his neurological complaints diminished.

Of the 27 individuals with persistent low levels of total and LDL cholesterol and a proven hereditary trait in their families, 14 were identified with a functional *APOB* mutation, representing a disease frequency of 52%. In 18 probands, we were not able to identify a causal *APOB* gene mutation to explain the low cholesterol levels. To demonstrate linkage or exclusion of linkage of the *APOB* gene to the low cholesterol phenotype we attempted to perform family investigation in all these probands; however, insufficient relatives were available for linkage analysis in eight of these kindreds. In five cases, family investigation showed no discernible pattern of the low cholesterol trait. In another five cases, it was evident that the low cholesterol trait was due to causes other than mutations in the *APOB* gene (fig 1).

DISCUSSION

In 14 of 32 probands with low cholesterol levels, we were able to identify an *APOB* gene mutation, resulting mainly in truncated forms of APOB. Although the functionality of these mutations was not validated in a strictest sense, it is well established that truncated APOB proteins are the cause of FHBL. Moreover, all mutations co-segregated with the FHBL phenotype, and therefore it seems likely that these variants are the cause of the FHBL phenotype in our families.

Table 2 Clinical characteristics of FHBL carriers and their unaffected relatives

Family	Status	n	M/F	Age	BMI	TC	LDL	HDL	TG	APOB	APOA1
NL801	Non-carriers	2	0/2	41.5 (38.9)	21.8 (5.6)	3.81 (0.71)	2.26 (0.76)	1.17 (0.41)	0.86 (0.81)	0.76 (0.23	1.30 (0.11)
	Carriers	5	4/1	31.2 (19.6)	22.8 (5.3)	3.00 (0.33)	0.75 (0.38)	2.15 (0.38)	0.22 (0.17)	0.47 (0.44)	1.74 (0.24)
NL802	Non-carriers	56	31/25	39.7 (18.1)	25.3 (4.7)	5.23 (1.22)	3.35 (1.02)	1.33 (0.35)	1.27 (1.03)	1.01 (0.28)	1.41 (0.25)
	Carriers	4	3/1	49.0 (19.2)	26.0 (1.7)	2.68 (0.62)	1.07 (0.52)	1.23 (0.25)	0.86 (0.92)	0.43 (0.14)	1.43 (0.06)
NL804	Non-carriers	35	18/17	34.9 (21.6)	22.8 (6.6)	4.97 (1.08)	2.84 (0.85)	1.68 (0.59)	1.01 (0.65)	0.85 (0.25)	1.55 (0.34)
	Carriers	23	14/9	39.2 (19.1)	23.0 (4.0)	3.01 (0.75)	1.02 (0.51)	1.84 (0.43)	0.33 (0.34)	0.33 (0.12)	1.51 (0.24)
NL806	Non-carriers	17	5/12	46.1 (17.5)	25.0 (3.6)	5.73 (1.37)	3.69 (1.17)	1.44 (0.49)	1.29 (1.08)	1.10 (0.35)	1.62 (0.30)
	Carriers	19	10/9	36.7 (18.4)	26.7 (5.2)	2.78 (0.61)	1.04 (0.47)	1.59 (0.32)	0.28 (0.15)	0.30 (0.08)	1.56 (0.21)
NL808	Carriers	3	3/0	52.3 (5.9)	39.2	2.80 (0.75)	1.03 (0.31)	1.61 (0.70)	0.38 (0.40)	0.29	1.00
NL809	Non-carriers	2	1/1	47.5 (24.7)	NM	4.56 (0.16)	2.26 (0.58)	1.69 (1.16)	1.35 (0.95)	0.83 (0.33)	1.66 (0.61)
	Carrier	1	1/0	33.0	NM	1.98	0.29	1.62	0.16	0.27	1.56
NL822	Non-carrier	1	0/1	53.0	25.7	7.11	5.18	1.23	1.55	NM	NM
	Carriers	2	2/0	42.5 (16.3)	28.1 (0.9)	2.63 (0.41)	1.11 (0.32)	1.42 (0.04)	0.24 (0.13)	NM	NM
NL825	Non-carriers	6	3/3	35.8 (22.0)	21.8 (3.8)	4.50 (0.94)	2.70 (0.75)	1.45 (0.27)	0.72 (0.28)	0.81 (0.21)	1.45 (0.21)
	Carriers	7	3/4	38.8 (23.5)	21.4 (3.4)	3.57 (1.15)	1.64 (1.15)	1.70 (0.35)	0.49 (0.33)	0.52 (0.32)	1.57 (0.23)
NL826	Non-carriers	4	2/2	28.3 (18.5)	22.7 (5.9)	4.81 (0.85)	2.08 (0.94)	1.50 (0.50)	1.88 (1.96)	0.88 (0.27)	1.57 (0.21)
	Carriers	5	3/2	40.2 (25.6)	24.0 (6.2)	2.30 (0.83)	0.77 (0.76)	1.19 (0.47)	1.02 (1.22)	0.31 (0.13)	1.28 (0.29)
NL827	Non-carriers	8	2/6	60.5 (22.5)	24.7 (3.7)	5.14 (0.83)	3.10 (0.56)	1.61 (0.22)	0.96 (0.25)	0.96 (0.18)	1.61 (0.18)
	Carriers	9	6/3	54.3 (21.3)	26.3 (5.2)	3.00 (0.60)	1.00 (0.26)	1.71 (0.59)	0.66 (0.50)	0.33 (0.07)	1.56 (0.35)
SP807	Carriers	2	2/0	45.0 (33.9)	26.7 (2.9)	2.37 (1.27)	1.12 (0.77)	0.97 (0.23)	0.92 (0.95)	0.23 (0.09)	NM
SP809	Non-carrier	1	0/1	12.0	19.5	4.74	3.21	0.95	1.23	0.93	0.97
	Carriers	2	0/2	40.5 (10.6)	22.4 (0.71)	2.42 (0.49)	0.69 (0.12)	1.40 (0.54)	0.73 (0.37)	0.25 (0.92)	1.30 (0.11)
SP810	Carrier	1	1/0	15	23.5	1.71	0.49	1.06	0.49	0.14	NM
SP812	Non-carrier	1	1/0	36.0	24.7	3.81	2.24	1.06	1.11	0.31	NM
	Carriers, htz	2	1/1	60.5 (3.5)	26.3 (1.27)	2.85 (0.38)	0.85 (0.33)	1.78 (0.18)	0.48 (0.25)	0.29 (0.02)	NM
	Carriers, hom	2	1/1	28.0 (1.4)	23.2 (3.04)	2.02 (0.64)	0.07 (0.09)	1.80 (0.51)	0.44 (0.08)	ND	NM
All	Non-carriers	133	63/70	40.0 (20.3)	24.2 (5.2)	5.14 (1.18)	3.15 (1.02)	1.46 (0.47)	1.18 (0.92)	0.95 (0.29)	1.49 (0.29)
	Carriers, htz	88	56/32	41.3 (20.0)	24.8 (4.9)	2.80 (0.66)	0.95 (0.46)	1.67 (0.47)	0.43 (0.49)	0.32 (0.14)	1.52 (0.26)
p value					0.737	<0.001	<0.001	<0.001	<0.001	<0.001	0.266

Age is shown in years. Lipids: (TC, total cholesterol; LDL, LDL cholesterol; HDL, HDL cholesterol and TG, triglyceride are in mmol/l; Apolipoprotein B (APOB) and apolipoprotein AI (APOA1) are in mg/dl. BMI, body mass index; NM, not measured; ND, not detectable; htz, heterozygous; hom, homozygous; M, male; F, female.

Statistically significant differences between carriers and non-carriers were found for plasma total, LDL, and HDL cholesterol, triglycerides, and APOB levels, but not for APOA1 levels. The low levels of LDL cholesterol are the result of failure to produce normal amounts of VLDL from truncated APOB proteins, leading to a reduced conversion from VLDL to LDL particles, as normally occurs through the action of lipoprotein lipase. Additionally, a reduction of the activity of cholesterylester transfer protein (CETP) through low numbers of VLDL particles results in a reduced transfer rate of cholesterolesters from HDL to VLDL and of triglycerides from VLDL to HDL, thus leading to elevated HDL cholesterol and reduced triglyceride levels. The normal level of APOA1 in both carriers and non-carriers is in line with similar HDL particle numbers in both groups, albeit with a different composition in term of cholesterolesters and triglycerides.

Some of the FHBL patients presented with a mild clinical phenotype not definitely linked to their lipoprotein disorder. In one case, occasional episodes of diarrhoea were noted, consistent with FHBL, and in another case mild neurological symptoms were found, which diminished after supplementation with vitamin E. The two cases of diabetes were in all likelihood not related to FHBL. The combination of DM and FHBL, with or without neurological complaints, has been described before in five cases and our patients do not seem to differ clinically from those examined by others.[20–23]

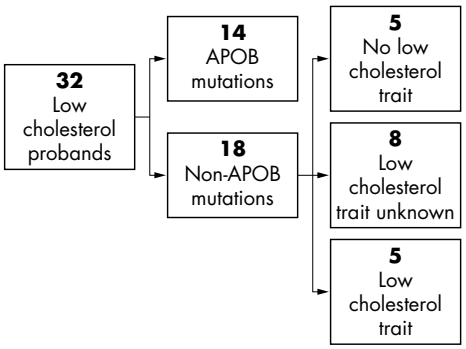

Figure 1 Composition of the proband cohort studied after DNA analysis of the *APOB* gene.

One interesting case was an 8 year old girl, initially diagnosed with familial defective apolipoprotein B (FDB) caused by the R3500Q mutation that she inherited from her father. However, her lipid profile (TC 3.71 mmol/l; LDL 2.35 mmol/l; HDL 1.25 mmol/l; TG 0.23 mmol/l; APOB 0.54 g/l) did not match the phenotypic characteristic of FDB. The subsequent identification of the 11712delC mutation, inherited from her mother, explained her normal cholesterol level. As the girl had no complaints associated with neither FDB nor FHBL, we could assume that the resulting phenotypic expression is a consequence of the compensation of one disorder by the other.

The precise prevalence of *APOB* gene mutations causing truncated APOB and FHBL is not known. However, several larger studies in individuals with persistent low levels of total and LDL cholesterol show an estimated frequency between 1.4% and 2.7%.[2 9–11] From these studies, it appears that truncated APOB is rare in healthy subjects with low LDL cholesterol levels. However, these data are very different from the frequency of 52% for the mutations we identified in our study population. This discrepancy might be explained by a number of different factors. Firstly, we only included individuals free from any secondary causes of hypocholesterolaemia, secondly, we applied very strict inclusion and exclusion criteria for enrolment, and lastly, our cohort was substantially larger than any other previously studied. However, additionally, this difference could be explained by the approach used, as we choose to analyse the *APOB* gene by direct sequencing, rather than through analysing the APOB protein. The assessment of lipoprotein fractions would have

failed to detect most of our mutations, as they represent truncated APOB proteins smaller than APOB-30.

Interestingly, we were not able to identify a causal *APOB* gene mutation in all FHBL patients. Although we sequenced at least 50 bp into each intron and analysed up to 600 bp upstream of the promoter region, the presence of mutations outside these regions could not be ruled out, nor the presence of a large deletion or insertion. Additionally, as yet unidentified genes could be the cause of the FHBL phenotype in these kindreds, as evidence is accumulating that other genetic factors besides the *APOB* gene may lead to a FHBL trait, such as loci identified on chromosome 3p21.1–22[13] and chromosome 13q.[14] Identification of these putative genes would provide novel insights into the mechanisms operating in APOB metabolism.

It is well established that high levels of plasma APOB are strong predictors for risk of cardiovascular disease, but less is known about this risk in individuals with a FHBL phenotype. In our study, all carriers of an APOB mutation were completely free from CVD. It can be hypothesised that prolonged low levels of LDL cholesterol and elevated levels of HDL cholesterol will reduce the progression of atherosclerotic disease. Nevertheless, it has not been unequivocally shown that this is indeed the case in individuals with FHBL. Assessment of the thickness of the intima–media complex in individuals with FHBL, compared with non-affected siblings could be used to test this hypothesis. Such a study is currently under way in our centre and is the subject of a future report.

ACKNOWLEDGEMENTS

We gratefully acknowledge the contribution of physicians K Hovingh, E Stroes, and W van Dorp of the Dutch Lipid Clinics for submitting blood samples of FHBL patients. We also wish to thank our genetic field worker, Mrs Holtkamp, for collection of blood samples of family members. This work was supported by a grant 2000B138 of the Netherlands Heart Foundation.

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Competing interest: none declared

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Received 22 November 2004
Revised version received 7 January 2005
Accepted for publication 10 January 2005

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