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Fouchier, S.W.; Sankatsing, R.R.; Peter, J.; Castillo, S.; Pocovi, M.; Alonso, R.; Kastelein, J.J.P.; Defesche, J.C.

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

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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. 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 cholesterol, 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.

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. 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. 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 manufacturer'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 x 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 fluorescein labelled dideoxy chain terminations with a Big Dye Terminator ABI Prism kit (Applied Biosystems, Foster City, CA, USA) according manufacturer’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 and 11712delC mutations, resulting in truncated APOB-9 and APOB-86 proteins, respectively, and the missense mutation, R463W, 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 576, leading 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 position 1309 into a stop codon at 1315, 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 C→T transition of nucleotide 4006 in exon 25, thereby creating a DdeI 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 (11548delITT), leading to an predicted APOB-84 protein.

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 MlaI restriction site when the R2507X mutation was present. The six novel mutations found were screened in a group of 94 normolipaeic 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/m². 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 1 Apolipoprotein B mutations identified

<table>
<thead>
<tr>
<th>Exon</th>
<th>Mutation</th>
<th>WT</th>
<th>MT</th>
<th>Pos (bp)</th>
<th>Predicted size</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>R412X</td>
<td>CGA</td>
<td>TGA</td>
<td>1315</td>
<td>APOB-9</td>
<td>SP809</td>
</tr>
<tr>
<td>11</td>
<td>R463W</td>
<td>CGG</td>
<td>TGG</td>
<td>1468</td>
<td>APOB-12</td>
<td>SP810</td>
</tr>
<tr>
<td>13</td>
<td>1718delAT</td>
<td>AT</td>
<td>delAT</td>
<td>1718</td>
<td>APOB-12</td>
<td>SP807</td>
</tr>
<tr>
<td>17</td>
<td>2534delA</td>
<td>A</td>
<td>delA</td>
<td>2534</td>
<td>APOB-18</td>
<td>NL804</td>
</tr>
<tr>
<td>18</td>
<td>2783delC</td>
<td>C</td>
<td>delC</td>
<td>2783</td>
<td>APOB-20</td>
<td>NL826, NL827</td>
</tr>
<tr>
<td>25</td>
<td>Q1309X</td>
<td>CAA</td>
<td>TAA</td>
<td>4006</td>
<td>APOB-29</td>
<td>NL808, NL822</td>
</tr>
<tr>
<td>26</td>
<td>R2507X</td>
<td>CGA</td>
<td>TGA</td>
<td>6700</td>
<td>APOB-55</td>
<td>NL809, NL827</td>
</tr>
<tr>
<td>26</td>
<td>11548delTT</td>
<td>delTT</td>
<td>11548</td>
<td>APOB-84</td>
<td>NL825</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>11712delC</td>
<td>C</td>
<td>delC</td>
<td>11712</td>
<td>APOB-86</td>
<td>NL801, NL802</td>
</tr>
</tbody>
</table>

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.
In one case, occasional episodes of diarrhoea were noted, phenotype not definitely linked to their lipoprotein disorder.

Different composition in terms of cholesterolesters and triglycerides. The rate of cholesterolesters from HDL to VLDL and of triglycerides from VLDL to HDL, thus leading to elevated HDL cholesterol levels. Additionally, a reduction of the activity of lipoprotein lipase. 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.

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 mother. However, her lipid profile (TC 3.71 mmol/l; LDL 2.35 mmol/l; HDL 1.25 mmol/l; TG 0.23 mmol/l) is 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.

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

<table>
<thead>
<tr>
<th>Family</th>
<th>Status</th>
<th>n</th>
<th>M/F</th>
<th>Age</th>
<th>BMI</th>
<th>TC</th>
<th>LDL</th>
<th>HDL</th>
<th>TG</th>
<th>APOB</th>
<th>APOA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL801</td>
<td>Non-carriers</td>
<td>2</td>
<td>0/2</td>
<td>41.5 (38.9)</td>
<td>21.8 (5.6)</td>
<td>3.81 (0.71)</td>
<td>2.26 (0.76)</td>
<td>1.17 (0.41)</td>
<td>0.86 (0.81)</td>
<td>0.76 (0.23)</td>
<td>1.30 (0.11)</td>
</tr>
<tr>
<td>Carriers</td>
<td>5</td>
<td>4/1</td>
<td>31.2 (19.6)</td>
<td>22.8 (5.3)</td>
<td>3.00 (0.33)</td>
<td>0.75 (0.38)</td>
<td>2.15 (0.38)</td>
<td>0.22 (0.17)</td>
<td>0.47 (0.44)</td>
<td>1.74 (0.24)</td>
<td></td>
</tr>
<tr>
<td>NL802</td>
<td>Non-carriers</td>
<td>56</td>
<td>31/25</td>
<td>39.7 (18.1)</td>
<td>25.3 (4.7)</td>
<td>5.23 (1.22)</td>
<td>3.35 (1.02)</td>
<td>1.33 (0.35)</td>
<td>1.27 (1.03)</td>
<td>1.01 (0.28)</td>
<td>1.41 (0.25)</td>
</tr>
<tr>
<td>Carriers</td>
<td>4</td>
<td>3/1</td>
<td>49.0 (19.2)</td>
<td>26.0 (1.7)</td>
<td>2.68 (0.62)</td>
<td>1.07 (0.52)</td>
<td>1.23 (0.25)</td>
<td>0.86 (0.92)</td>
<td>0.43 (0.14)</td>
<td>1.43 (0.06)</td>
<td></td>
</tr>
<tr>
<td>NL804</td>
<td>Non-carriers</td>
<td>35</td>
<td>18/17</td>
<td>34.9 (21.6)</td>
<td>22.8 (6.6)</td>
<td>4.97 (0.75)</td>
<td>2.84 (0.85)</td>
<td>1.68 (0.59)</td>
<td>0.85 (0.65)</td>
<td>0.85 (0.25)</td>
<td>1.34 (0.34)</td>
</tr>
<tr>
<td>Carriers</td>
<td>23</td>
<td>14/9</td>
<td>39.2 (19.1)</td>
<td>23.0 (4.0)</td>
<td>3.01 (0.75)</td>
<td>1.02 (0.51)</td>
<td>1.84 (0.43)</td>
<td>0.33 (0.34)</td>
<td>0.33 (0.12)</td>
<td>1.51 (0.24)</td>
<td></td>
</tr>
<tr>
<td>NL806</td>
<td>Non-carriers</td>
<td>17</td>
<td>5/12</td>
<td>46.1 (17.5)</td>
<td>25.0 (3.6)</td>
<td>5.73 (1.57)</td>
<td>3.69 (1.17)</td>
<td>1.44 (0.49)</td>
<td>1.29 (0.18)</td>
<td>1.62 (0.30)</td>
<td></td>
</tr>
<tr>
<td>Carriers</td>
<td>19</td>
<td>10/9</td>
<td>36.7 (18.4)</td>
<td>26.5 (7.2)</td>
<td>2.78 (0.61)</td>
<td>1.04 (0.49)</td>
<td>1.50 (0.32)</td>
<td>0.28 (0.15)</td>
<td>0.30 (0.08)</td>
<td>1.56 (0.21)</td>
<td></td>
</tr>
<tr>
<td>NL808</td>
<td>Carriers</td>
<td>3</td>
<td>3/0</td>
<td>52.3 (5.9)</td>
<td>39.2</td>
<td>2.80 (0.75)</td>
<td>1.03 (0.31)</td>
<td>1.61 (0.70)</td>
<td>0.38 (0.40)</td>
<td>0.29 (1.00)</td>
<td></td>
</tr>
<tr>
<td>NL809</td>
<td>Carriers</td>
<td>2</td>
<td>1/1</td>
<td>47.5 (24.7)</td>
<td>21.7 (5.9)</td>
<td>5.16 (1.6)</td>
<td>2.26 (0.58)</td>
<td>1.69 (1.16)</td>
<td>1.35 (0.95)</td>
<td>0.83 (0.33)</td>
<td>1.66 (0.61)</td>
</tr>
</tbody>
</table>

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 A1 (APOA1) are in mg/dl. BMI, body mass index; NM, not measured; ND, not detectable; htz, heterozygous; hom, homozygous; M, male; F, female.
failed to detect most of our mutations, as they represent truncated \textit{APOB} proteins smaller than \textit{APOB-30}.

Interestingly, we were not able to identify a causal \textit{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 \textit{APOB} gene may lead to a FHBL trait, such as loci identified on chromosome 3p21.1–22\textsuperscript{13} and chromosome 13q.\textsuperscript{14} Identification of these putative genes would provide novel insights into the mechanisms operating in \textit{APOB} metabolism.

It is well established that high levels of plasma \textit{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 \textit{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**

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**Authors’ affiliations**

S W Fouchier, R R Sankatsing, J Peter, J J P Kastelein, J C Defesche, Department of Vascular Medicine, Academic Medical Center at the University of Amsterdam, Amsterdam, The Netherlands

S Castillo, M Pocovi, Department of Biochemistry, Cellular Biology Universidad de Zaragoza, Zaragoza, Spain

R Alonso, Lipid Unit Research, Fundacion Jimenez Diaz, Madrid, Spain

Competing interest: none declared

Correspondence to: Dr J C Defesche, Department of Vascular Medicine G1–113, Academic Medical Center, University of Amsterdam, P.O. Box 22660, 1100 DD Amsterdam, The Netherlands; j.defesche@amc.uva.nl

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