Atherosclerosis in the HIV and non-HIV setting: detecting and modifying cardiovascular risk
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High frequency of ApoB gene Mutations Causing Familial Hypobetalipoproteinemia in patients of Dutch and Spanish descent

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Sergio Castillo
Miguel Pocovi
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John J.P. Kastelein
and Joep C. Defesche

Abstract

**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 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.
Introduction

Familial hypobetalipoproteinemia (FHBL) is an autosomal co-dominant hereditary disorder of lipoprotein metabolism characterized by decreased low-density lipoprotein (LDL)-cholesterol and apolipoprotein B (apoB) plasma levels. ApoB is a key structural component of triglyceride and cholesterol-rich lipoproteins such as chylomicrons, very-low-density lipoproteins (VLDL) and LDL, and therefore plays a pivotal role in cholesterol metabolism. 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. In fact, heterozygous FHBL patients appear to be asymptomatic. However, the accompanying clinical phenotype is not well defined, since 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. 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 and most of these mutations prevent the translation of a full-length apoB protein. The frequency of apoB gene mutations causing truncated apoB and FHBL is considered rare and estimated to occur in 1.4% to 2.7% of individuals with persistent low levels of total and LDL-cholesterol.

Due 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 was to 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 frame-shift 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 truncation between B-30 and B-32 are only present in the density range of HDL. Therefore, analyzing only VLDL and LDL plasma fractions failed to identify certain apoB gene 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 localizations, encompassing 3p21.1-22 and 13q. In order to identify the cause of the FHBL phenotype in our probands, we chose to analyze the complete apoB gene by direct sequencing, rather than to analyze the apoB proteins in different lipoprotein fractions.

Material 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 dyslipidemia. Selection criterion was a LDL-cholesterol level below the 5th percentile for sex and age. Secondary causes for low
LDL-cholesterol levels, i.e. vegetarian, diet poor in fat 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 Ethical 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 below 4.5 mmol/L. ApoB and apoA1 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 the ApoB gene, fifty-four pairs of primers were designed. PCR amplification was carried out with 50 ng of genomic DNA in a 25 µl reaction volume containing 1x Taq DNA Polymerase buffer (Qiagen, Hilden, Germany), 50 µM of each dNTP, 0.4 µM of each primer, 5 µg BSA and 1 unit Taq DNA polymerase. The thermal cycling conditions were as followed: 96°C for 5 min once, then 35 cycles of 96°C for 20 sec, 55°C to 60°C (depending on primer CG content) for 20 sec, and 74°C for 30 sec in a PCR apparatus (T3 Biocycler, Biometra, Germany). The sequence reactions were performed using fluorescently labelled dideoxy chain terminations with the Big Dye Terminator ABI Prism Kit (Applied Biosystems, Foster City, CA, USA) according manufacturer’s protocol and analyzed on an Applied Biosystems Model 3730 automated DNA sequencer. Sequences were analyzed 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 ANOVA and by multiple linear regression analyses with adjustment for age and sex. A p-value of less than 0.05 was considered to be statistically significant.
Results

We identified 32 individuals meeting our inclusion criterion. After sequence analysis of the apoB gene, we identified 9 different mutations in 14 of our probands (table 1).

Table 1  Apolipoprotein B mutations identified.

<table>
<thead>
<tr>
<th>Exon</th>
<th>mutation</th>
<th>WT</th>
<th>MT</th>
<th>Bp position</th>
<th>Predicted size</th>
<th>Family</th>
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<td>CGA</td>
<td>TGA</td>
<td>1315</td>
<td>ApoB-9</td>
<td>SP809</td>
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<tr>
<td>11</td>
<td>R463W&lt;sup&gt;18&lt;/sup&gt;</td>
<td>CGG</td>
<td>TGG</td>
<td>1468</td>
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<td>SP810</td>
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<td>13</td>
<td>1718delAT</td>
<td>AT</td>
<td>delAT</td>
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<td>SP807</td>
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<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>
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<tr>
<td>25</td>
<td>Q1309X</td>
<td>CAA</td>
<td>TAA</td>
<td>4006</td>
<td>ApoB-29</td>
<td>NL808, NL822</td>
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<tr>
<td>26</td>
<td>R2507X</td>
<td>CGA</td>
<td>TGA</td>
<td>7600</td>
<td>ApoB-55</td>
<td>NL809, SP812</td>
</tr>
<tr>
<td>26</td>
<td>11548delTT</td>
<td>TT</td>
<td>delTT</td>
<td>11548</td>
<td>ApoB-84</td>
<td>NL825</td>
</tr>
<tr>
<td>26</td>
<td>11712delC&lt;sup&gt;19&lt;/sup&gt;</td>
<td>C</td>
<td>delC</td>
<td>11712</td>
<td>ApoB-86</td>
<td>NL801, NL802, NL806</td>
</tr>
</tbody>
</table>

The reference sequence used is NM_000384, with the A of the ATG-translation initiation codon numbered nucleotide +1 and the methionine numbered as amino acid –27.

The R412X<sup>17</sup> and 11712delC<sup>19</sup> mutations, resulting in truncated apoB-9 and apoB-86 proteins, respectively, and the missense mutation, R463W<sup>18</sup>, were described previously. Additionally, we identified six novel apoB gene mutations resulting in truncated apoB protein of different sizes. The frame shift 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 frame shift 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 to T transition of nucleotide 4006 in exon 25, thereby creating a Ddel 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 to 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 resulting in a stop codon at amino acid 3823 (11548delTT) leading to an predicted apoB-84 protein.

Screening for the frame-shift 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. Since 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 NlaIII restriction site when the R2507X mutation
was present. The six novel mutations found were screened in a group of 94 normolipemic con-
trols, in which none of the mutations were found. Moreover, each mutation showed complete
cosegregation 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 BMI or apoA1 levels after
adjustment for age and sex. Statistically significant differences between affected and unaf-
fected groups were found for plasma total cholesterol, LDL-cholesterol, HDL-cholesterol, trig-
glycerides and apoB levels (all \( p \)-values <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 appear 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 was suffering from occasional episodes of diarrhoea. The
proband of the NL808 family was a 59-year old male, who was referred to our Lipid Clinic be-
cause 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 the NL809 family, revealed DM type I at age 31 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 he-
dereditary 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 investiga-
tion in all. However, not enough relatives were available for linkage analysis in eight of these
kindreds. In five cases, family investigation showed no discernible pattern of the low choles-
terol trait. In another five cases it was evident that the low cholesterol trait was present due to
others causes than mutations in the apoB gene (figure 1).
Table 2  Clinical characteristics of FHBL carriers and their unaffected relatives.

<table>
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<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>
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<td>NL801</td>
<td>non-carriers</td>
<td>2</td>
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<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>
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<td>1.30±0.11</td>
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<td>4/1</td>
<td>31.2±19.6</td>
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<td>NL802</td>
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<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>
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<td>49.0±19.2</td>
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<td></td>
</tr>
<tr>
<td>carriers, hom</td>
<td>2</td>
<td>1/1</td>
<td>28.0±1.4</td>
<td>23.2±0.04</td>
<td>2.02±0.64</td>
<td>0.07±0.09</td>
<td>1.80±0.51</td>
<td>0.44±0.08</td>
<td>nd</td>
<td>nm</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>non-carriers</td>
<td>133</td>
<td>63/70</td>
<td>40.0±20.3</td>
<td>24.2±5.2</td>
<td>5.14±1.18</td>
<td>3.15±1.02</td>
<td>1.46±0.47</td>
<td>1.18±0.92</td>
<td>0.95±0.29</td>
<td>1.49±0.29</td>
</tr>
<tr>
<td>carriers</td>
<td>88</td>
<td>56/32</td>
<td>41.3±20.0</td>
<td>24.8±4.9</td>
<td>2.80±0.66</td>
<td>0.95±0.46</td>
<td>1.67±0.47</td>
<td>0.43±0.49</td>
<td>0.32±0.14</td>
<td>1.52±0.26</td>
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<tr>
<td>p-value</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.266</td>
<td></td>
<td></td>
</tr>
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</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. nm: not measured; nd: not detectable; htz: heterozygous; hom: homozygous; M: male; F: female.
Figure 1 Composition of the proband cohort studied after DNA analysis of the apoB gene.

Discussion

In 14 out 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. Statistically significant differences between carriers and non-carriers were found for plasma total cholesterol, LDL-cholesterol, 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. This leads to a reduced conversion from VLDL-particles 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 cholesteroles 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 cholesteroles 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, that 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 5 cases and our patients do not seem to differ clinically from patients examined by others.20-23
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-C 2.35 mmol/l; HDL-C 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. Since 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%. 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 hypocholesterolemia. Secondly, we applied very strict in- 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, since we choose to analyze the apoB gene by direct sequencing, rather than to analyze the apoB protein. The assessment of lipoprotein fractions would have failed to detect most of our mutations, since 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 did sequence at least 50 base pairs into each intron and analyzed up to 600 base pairs 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, yet unidentified genes could be the cause of the FHBL phenotype in these kindreds, since 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 (CVD), 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 cardiovascular disease. It can be hypothesized 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 (IMT) 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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