A Novel APOB Mutation Identified by Exome Sequencing Cosegregates With Steatosis, Liver Cancer, and Hypcholesterolemia

Angelo B. Cefalù,* James P. Pirruccello,* Davide Noto, Stacey Gabriel, Vincenza Valenti, Namrata Gupta, Rossella Spina, Patrizia Tarugi, Sekar Kathiresan, Maurizio R. Averna

Objective—In familial hypobetalipoproteinemia, fatty liver is a characteristic feature, and there are several reports of associated cirrhosis and hepatocarcinoma. We investigated a large kindred in which low-density lipoprotein cholesterol, fatty liver, and hepatocarcinoma displayed an autosomal dominant pattern of inheritance.

Approach and Results—The proband was a 25-year-old female with low plasma cholesterol and hepatic steatosis. Low plasma levels of total cholesterol and fatty liver were observed in 10 more family members; 1 member was affected by liver cirrhosis, and 4 more subjects died of either hepatocarcinoma or carcinoma on cirrhosis. To identify the causal mutation in this family, we performed exome sequencing in 2 participants with hypocholesterolemia and fatty liver. Approximately 22,400 single nucleotide variants were identified in each sample. After variant filtering, 300 novel shared variants remained. A nonsense variant, p.K2240X, attributable to an A>T mutation in exon 26 of APOB (c.6718A>T) was identified, and this variant was confirmed by Sanger sequencing. The genotypic analysis of 16 family members in total showed that this mutation segregated with the low cholesterol trait. In addition, genotyping of the PNPLA3 p.I148M did not show significant frequency differences between carriers and noncarriers of the c.6718A>T APOB gene mutation.

Conclusions—We used exome sequencing to discover a novel nonsense mutation in exon 26 of APOB (p.K2240X) responsible for low cholesterol and fatty liver in a large kindred. This mutation may also be responsible for cirrhosis and liver cancer in this family. (Arterioscler Thromb Vasc Biol. 2013;33:2021-2025.)

Key Words: carcinoma, hepatocellular ■ exome ■ fatty liver ■ hypobetalipoproteinemia, familial, 1

Hypobetalipoproteinemia (HBL) represents a heterogeneous group of disorders characterized by reduced plasma levels of total cholesterol, low-density lipoprotein-cholesterol (LDL-C), and apolipoprotein B (apoB) below the fifth percentile of the distribution in the population.1,2 Familial HBL (FHBL; OMIM 107730) is the most frequent monogenic form of HBL. It may be attributable to loss-of-function mutations in APOB or, less frequently, in genes of various sizes which, to a variable extent, lose the capacity to form plasma lipoproteins in liver and intestine and to export lipids from these organs.1,2 Missense nontruncating mutations of the APOB gene can also be the cause of FHBL.6,7 and are associated with a decreased secretion of the mutant apoBs because of an increased binding to microsomal triglyceride transfer protein.6,8 As a consequence of impaired hepatic export of lipoproteins (very-low-density lipoprotein), subjects with FHBL attributable either to truncating or nontruncating mutations of the APOB gene are prone to hepatic steatosis.5,9,10 In these subjects, the presence of fatty liver has been documented by abdominal ultrasound examination, magnetic resonance, or liver biopsy.9,11-14 Anecdotal reports have documented an association between fatty liver and steatohepatitis, liver cirrhosis, and hepatocarcinoma in patients with FHBL.15-17 We studied a large family in whom we observed an autosomal dominant pattern of low plasma cholesterol cosegregating with fatty liver and hepatocarcinoma. We hypothesized the presence in this family of a genetic susceptibility for cancer, which cosegregates with a causal mutation of FHBL. To identify the causal mutation in this family, we performed exome...
sequencing, an approach that allows the identification of all the coding variants present in affected family members.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Phenotype

DNA samples were available from 16 members across 2 generations. Lipid profiles, including apoB levels, and clinical characteristics of the family are presented in Table 1.

The proband (subject IV-5) showed low levels of total cholesterol, triglyceride, and LDL-C; low LDL-C levels were found in 9 more subjects of the family with a dominant transmission mode of inheritance (Table 1; Figure). Moreover 7 of 10 subjects with low cholesterol levels showed fatty liver as determined by liver ultrasonography (Table 1).

Exome Sequencing

The 2 samples that underwent exome sequencing each yielded 8.25 gigabases of sequence, with an average depth of coverage of ≈250 reads per targeted base. Approximately 22 400 single nucleotide variants were identified in each sample, of which 15 237 passed the previously described filters and were shared by both samples. After removing variants also identified in pilot 1 of the 1000 Genomes project, 1509 shared variants remained. After removing variants identified in 3 unrelated samples ascertained attributable to a non–LDL-C–related lipoprotein phenotype (hyperlalphalipoproteinemia), 300 novel shared variants remained. Of these, 112 were synonymous, 4 were nonsense, and 7 were at splice sites. Only 1 nonsense variant was within ±300 kilobases of lead single nucleotide polymorphisms in genomic regions associated with LDL-C in a recent large-scale genome-wide association study18: p. K2240X attributable to an A>T mutation in exon 26 of APOB (c.6718A>T), which encodes apoB 48.8.

The search for shared variants in the 2cM region encompassing the c.6718A>T mutation of the APOB gene also revealed an A>C mutation in exon 1 of the RHOB (ras homolog gene family, member B precursor) gene (c.244A>C, p.M82L).

Sanger Sequencing

The presence of the nonsense mutation in exon 26 (c.6718A>T, p.K2240X) was confirmed in 3 independent polymerase chain reaction amplifications and direct sequencing in the proband (subject IV-5). The mother of the proband (subject III-13) was found to carry the same mutation in the heterozygous state. Sanger sequencing of the same APOB gene region of exon 26 encompassing the mutation performed in the other 14 available family members confirmed that the nonsense mutation segregated with the low cholesterol trait (Figure).

Table 1. Clinical Characteristics, Plasma Lipids, and Apolipoprotein B

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age, y</th>
<th>TC, mg/dL</th>
<th>TG, mg/dL</th>
<th>HDL-C, mg/dL</th>
<th>ApoB, mg/L</th>
<th>LDL-C, mg/dL</th>
<th>BMI, kg/m²</th>
<th>Fatty Liver</th>
<th>Type of Severe Liver Disease</th>
<th>APOB Genotype</th>
<th>Other Clinical Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>II:1</td>
<td>*</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Hepatocarcinoma on cirrhosis</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II:5</td>
<td>*</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Hepatocarcinoma on cirrhosis</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III:1</td>
<td>63</td>
<td>103</td>
<td>48</td>
<td>43</td>
<td>42,2</td>
<td>28</td>
<td>Yes</td>
<td>Not present</td>
<td>K2240X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III:3</td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Hepatocarcinoma</td>
<td>NA</td>
<td>K2240X</td>
<td></td>
</tr>
<tr>
<td>III:5</td>
<td>60</td>
<td>234</td>
<td>45</td>
<td>NA</td>
<td>173,4</td>
<td>27</td>
<td>Yes</td>
<td>Not present</td>
<td>K2240X</td>
<td></td>
<td></td>
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<tr>
<td>III:7</td>
<td>58</td>
<td>136</td>
<td>55</td>
<td>55</td>
<td>68,2</td>
<td>28</td>
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<td>Not present</td>
<td>K2240X</td>
<td></td>
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<tr>
<td>III:9</td>
<td>50</td>
<td>140</td>
<td>76</td>
<td>NA</td>
<td>56</td>
<td>24,7</td>
<td>No</td>
<td>Not present</td>
<td>K2240X</td>
<td></td>
<td></td>
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<tr>
<td>III:11</td>
<td>*</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Yes</td>
<td>Hepatocarcinoma</td>
<td>NA</td>
<td></td>
<td>Referred hypcholesterolemia</td>
<td></td>
</tr>
<tr>
<td>III:13</td>
<td>50</td>
<td>121</td>
<td>52</td>
<td>37</td>
<td>50,2</td>
<td>22,5</td>
<td>Yes</td>
<td>Not present</td>
<td>K2240X</td>
<td></td>
<td></td>
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<tr>
<td>III:14</td>
<td>59</td>
<td>222</td>
<td>57</td>
<td>57</td>
<td>142</td>
<td>26,4</td>
<td>Yes</td>
<td>Not present</td>
<td>WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III:15</td>
<td>66</td>
<td>220</td>
<td>66</td>
<td>NA</td>
<td>135,8</td>
<td>28,6</td>
<td>Yes</td>
<td>Not present</td>
<td>WT</td>
<td>Type 2 diabetes mellitus</td>
<td></td>
</tr>
<tr>
<td>III:16</td>
<td>60</td>
<td>110</td>
<td>39</td>
<td>34</td>
<td>39,2</td>
<td>24</td>
<td>Yes</td>
<td>Liver cirrhosis</td>
<td>K2240X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV:1</td>
<td>30</td>
<td>107</td>
<td>53</td>
<td>53</td>
<td>52,4</td>
<td>25</td>
<td>No</td>
<td>Not present</td>
<td>K2240X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV:2</td>
<td>33</td>
<td>165</td>
<td>76</td>
<td>66</td>
<td>60</td>
<td>27,6</td>
<td>No</td>
<td>Not present</td>
<td>WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV:3</td>
<td>42</td>
<td>128</td>
<td>31</td>
<td>24</td>
<td>31,8</td>
<td>26,3</td>
<td>No</td>
<td>Not present</td>
<td>K2240X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV:4</td>
<td>31</td>
<td>174</td>
<td>58</td>
<td>23</td>
<td>102,2</td>
<td>NA</td>
<td>No</td>
<td>Not present</td>
<td>WT</td>
<td></td>
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<tr>
<td>IV:5</td>
<td>25</td>
<td>86</td>
<td>44</td>
<td>48</td>
<td>29,2</td>
<td>22</td>
<td>Yes</td>
<td>Not present</td>
<td>K2240X</td>
<td>Nonorganic seizures</td>
<td></td>
</tr>
<tr>
<td>IV:6</td>
<td>24</td>
<td>103</td>
<td>42</td>
<td>39</td>
<td>52,8</td>
<td>24,2</td>
<td>Yes</td>
<td>Not present</td>
<td>Hodgkin Lymphoma</td>
<td></td>
<td></td>
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<tr>
<td>IV:7</td>
<td>39</td>
<td>118</td>
<td>57</td>
<td>65</td>
<td>41,6</td>
<td>24,1</td>
<td>Yes</td>
<td>Not present</td>
<td>K2240X</td>
<td>Nonorganic seizures</td>
<td></td>
</tr>
<tr>
<td>IV:8</td>
<td>37</td>
<td>141</td>
<td>63</td>
<td>62</td>
<td>66,4</td>
<td>21,1</td>
<td>No</td>
<td>Not present</td>
<td>WT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ApoB indicates apolipoprotein B; BMI, body mass index; HDL-C, HDL-cholesterol; LDL-C, low-density lipoprotein-cholesterol; NA, not available; TC, total cholesterol; TG, triglycerides; and WT, wild type.

*Deceased.
There were 16 individuals in the family who had both plasma lipids phenotype and DNA available for genotyping. Of these 16 individuals, 10 were affected on clinical grounds (total cholesterol below the fifth percentile). All ten affected carried the **APOB** nonsense mutation. Of the 6 individuals who were unaffected (total cholesterol >fifth percentile), none carried the **APOB** nonsense mutation. The direct sequencing of the region of exon 1 of **RHOB** gene encompassing the c.244A>C variant showed that besides subject III-13 and IV-15, 2 other family members were heterozygous carriers of the variant p.M82L (II-16 and IV-7; Figure). To predict the effect of this amino acid change on protein function, we performed in silico analyses by using different algorithms: PolyPhen (www.bork.embl-heidelberg.de/PolyPhen/), SIFT (http://sift.jcvi.org/), and Mutationtaster (http://www.mutationtaster.org/). The PolyPhen and SIFT algorithms gave comparable results, indicating that the p.M82L (Polyphen score: 0.002, SIFT score: 0.11) amino acid substitution had a benign effect, whereas the Mutationtaster software predicted the p.M82L missense mutation to be damaging (Score: 15).

### Association of PNPLA3 Single Nucleotide Polymorphism rs738409 (I148M) With Hepatic Steatosis

As shown in Table 2, there were no significant differences in either PNPLA3 rs738409 minor allele (G) frequency allele or genotype frequencies between carriers and noncarriers of the c.6718A>T **APOB** gene mutation.

### Table 2. PNPLA3 Single Nucleotide Polymorphism rs738409 (I148M) in Carriers and Noncarriers of the c.6718A>T **APOB** Gene Mutation

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>C/C n (%)</th>
<th>C/G n (%)</th>
<th>G/G n (%)</th>
<th>C</th>
<th>G</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.6718A&gt;T <strong>APOB</strong> gene mutation carriers</td>
<td>10</td>
<td>2 (20)</td>
<td>8 (80)</td>
<td>0 (0)</td>
<td>0.6</td>
<td>0.4</td>
<td>NS</td>
</tr>
<tr>
<td>c.6718A&gt;T <strong>APOB</strong> gene mutation carriers with fatty liver</td>
<td>7</td>
<td>1 (14)</td>
<td>6 (86)</td>
<td>0 (0)</td>
<td>0.57</td>
<td>0.43</td>
<td>NS</td>
</tr>
<tr>
<td>Noncarriers of c.6718A&gt;T <strong>APOB</strong> gene mutation</td>
<td>6</td>
<td>2 (33)</td>
<td>3 (50)</td>
<td>1 (17)</td>
<td>0.58</td>
<td>0.42</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS indicates not significant.

Discussion

In the present study, we describe a large family in whom low plasma cholesterol, fatty liver, and hepatocarcinoma cosegregate in an autosomal dominant pattern. Using whole exome sequencing, we discovered that a novel nonsense mutation in exon 26 of the **APOB** gene (c.6718A>T, p.K2240X) segregates with low lipids and the liver phenotypes. A large number of **APOB** gene mutations truncating apoB have been reported to be the cause of FHBL, and novel mutations are continually being identified in subjects with FHBL.19 FHBL heterozygotes are generally asymptomatic but most of them develop fatty liver.19 In fact, individuals heterozygous for inactivating mutations in **APOB** show impaired very-low-density lipoprotein particle metabolism and have a 3-fold increase in hepatic triglyceride relative to healthy individuals.10 Earlier in vivo turnover studies have shown that effectiveness of lipid secretion from the liver depends on apoB length,20 implying that a variable amount of lipids might accumulate in the hepatocytes of FHBL carriers of different truncated apoBs. It was also suggested that fatty liver always develops in FHBL carriers of short and medium-size truncated apoBs (<apoB-48), whereas other additional environmental factors are needed in carriers of longer apoB forms.11 However, more recent data have shown that there is no evidence that the size of apoB truncation could be associated with a different degree of liver impairment. For instance, in the patients cohort studied by Sankatsing et al,21 hepatic steatosis was not more severe in patients carrying short truncated apoB.
apoBs not secreted into the plasma compared with carriers of longer truncations.

Even if fatty liver in FHBL has been considered, per se, a benign condition, a potential evolution to more severe forms of liver diseases, such as steatohepatitis, cirrhosis, or liver carcinoma, seems to be a relevant clinical issue. To date, only a few case reports on the association between FHBL and severe liver diseases have been published.

Lonardo et al. described a case of hepatocarcinoma without cirrhosis in a subject with FHBL attributable to a truncated form of apoB. The liver histology in this patient revealed a moderate degree of steatosis and fibrosis outside the hepatocarcinoma lesion, and the authors speculated that environmental factors (such as alcohol and smoking) could trigger the evolution of fatty liver attributable to FHBL. More recently, Bonnefont-Rousselot et al. have described a patient with FHBL and liver cirrhosis attributable to a truncated form of apoB. The liver biopsy revealed typical hepatic cirrhosis with irregular nodules and macronodular steatosis. In this case, classical causes of fatty liver and cirrhosis were excluded by a comprehensive clinical, biological, and histological work-up.

To our knowledge, our observation is the first description of the co-occurrence of FHBL, fatty liver, cirrhosis, and liver cancer. In particular, participants II:1, II:5, III:3, and III:11 died of hepatocarcinoma. Furthermore, in participant III:11, the histology of the liver tumor revealed a rare finding of fibrolamellar hepatocellular carcinoma. In fact, fibrolamellar carcinoma is a rare primary malignant liver tumor with distinctive histology that usually affects adolescents and young adults with a nearly even sex distribution, and most patients have no identifiable liver disease secondary to chronic infection with hepatitis B virus or hepatitis C virus.

An interesting question deals with the identification of factors that could elicit a progression of fatty liver attributable to FHBL to cirrhosis and liver cancer. Although environmental and lifestyle influences are well known and prevalent potential contributors of progression of fatty liver, other molecular processes may contribute to this condition. Recently, variants in genes affecting lipid metabolism, oxidative stress, insulin resistance, and immune regulation could act as predisposing factors to the development of hepatic steatosis and the development of progressive liver injury. Among these, 1 genetic variant that has consistently been associated in many independent studies with nonalcoholic fatty liver disease is a missense mutation (Ile148→Met148 [p.I148M]) in patatin-like phospholipase domain-containing (PNPLA3) gene (PNPLA3).

Moreover, this PNPLA3 variant is not only associated with hepatic steatosis but also with nonalcoholic steatohepatitis and cirrhosis, and these data provide strong molecular evidence of the importance of genetic factors on the progression of fatty liver to more severe forms of hepatic diseases. Among the APOB mutation carriers, individuals with the PNPLA3 p.I148M variant in this kindred did not show a higher susceptibility for fatty liver, suggesting that in this family the PNPLA3 gene does not act as a predisposing factor to the development of hepatic steatosis and the development of progressive liver injury.

In an attempt to identify other genetic determinants that could contribute to the progression of liver disease in this family, we searched for shared variants in the 2cM region encompassing the c.6718A>T mutation of the APOB gene. This analysis revealed an A>C mutation in exon 1 of the RHOB gene (c.244A>C, p.M82L).

Ras-homologous (Rho) small GTPases are involved in the regulation of a variety of cellular processes, and recent studies further confirmed the role of the Rho proteins in cancer by showing their involvement in cell transformation, invasion, metastasis, and angiogenesis.

In particular, RhoB has a tumor-suppressive role, including inhibition of cell proliferation and induction of apoptosis in several human cancer cells, and inhibition of tumor growth in a nude mouse xenograft model. Furthermore, RhoB is inducible by genotoxic stress, such as UV light, some growth factors (transforming growth factor-β), and chemotherapeutic drugs (cisplatin and 5-FU).

The results of genotyping of the p.M82L RhoB variant showed no cosegregation with the APOB gene mutation found to be responsible for the FHBL phenotype. However, it is interesting to note that the 4 carriers of the p.M82L variant are also carriers of the c.6718A>T mutation of the APOB gene and are all affected by fatty liver; in addition, 1 of them (subject III:16) developed cirrhosis.

The clinical and genetic findings from this large kindred suggest that the complex relationship between APOB mutations responsible for FHBL and the clinical and pathological sequelae of fatty liver accumulation requires further mechanistic studies.

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Disclosures

None.

References

Significance

Familial hypobetalipoproteinemia is a genetic disorder characterized by lower than fifth percentile plasma levels of total cholesterol, low-density lipoprotein-cholesterol, and apolipoprotein B (apoB). Most of the cases with familial hypobetalipoproteinemia are attributable to mutations in APOB gene, leading to defective hepatic secretion of apoB-containing lipoproteins (very-low-density lipoprotein). This results in an impaired export of triglycerides from the liver causing fat accumulation and hepatic steatosis. Few case reports have documented the association of familial hypobetalipoproteinemia with steatohepatitis, liver cirrhosis, and hepatocarcinoma. Here, we describe a large kindred in which a novel mutation of APOB gene (p.K2240X), identified by exome sequencing, cosegregates with hypobetalipoproteinemia, fatty liver, and hepatocarcinoma in an autosomal dominant pattern. We also found a variant in a tumor suppressor gene (RHOB), but no cosegregation was found with the lipid and hepatic phenotypes. In addition, genotyping of the PNPLA3 p.I148M does not show frequency differences between carriers and noncarriers of the APOB gene mutation.

A Novel APOB Mutation Identified by Exome Sequencing Cosegregates With Steatosis, Liver Cancer, and Hypocholesterolemia

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Material and Methods

FHBL patients (TC < 5th percentile) are routinely identified and studied in the Lipid Clinic of our Center for Genetic Dyslipidemias at the AOUP “P. Giaccone”, University of Palermo, Italy. Over the years we have collected a large number of patients who underwent a clinical, biochemical and genetic evaluation. Among these, one FHBL family with a high prevalence of associated hepatic diseases was selected for exome sequencing.

Study participants and pedigree

The proband (Figure 1 - subject IV:5) is a 25 year-old female of European ancestry who first came to our attention at the age of 18. Her clinical history was unremarkable except for low plasma TC and triglycerides (TG) detected in several occasions (86 mg/dl and 44 mg/dl respectively). Moreover, an ultrasound evaluation of the abdomen showed liver steatosis.

The analysis of plasma lipids of family members showed low plasma levels of TC and TG in several others. Noticeably in subjects who underwent to ultrasound evaluation, fatty liver was observed (subjects III:1, III:7, III:11, III:13, III:15, III:16 IV:5, IV:6 and IV:7) and in subject III:16 the diagnosis of liver cirrhosis was made after he suffered an acute gastroesophageal bleed.

It was also reported that four more subjects (II:1, II:5, III:3 and III:11) died of either hepatocarcinoma (III:3 and III:11) or carcinoma on cirrhosis (II:1 and II:5). In particular subject III-11 was referred to have a long-lasting unexplained history of hypocholesterolemia who died at the age of 58 from massive gastroesophageal bleeding after he was diagnosed with a hepatocarcinoma with a histologic finding of fibrolamellar hepatocellular carcinoma.

Chronic infection due to hepatitis B virus (HBV) or hepatitis C virus (HCV) and alcohol abuse (mean alcohol intake was < 15g/day) were excluded in all the studied subjects. For family members who had died of hepatocarcinoma, the exclusion of either chronic viral infection or alcohol abuse was made by analyzing the clinical records available and information provided by the relatives.

Moreover, as far as possible, other potential causes of chronic liver diseases, including hepatic disorders of iron and copper metabolism, were excluded by analyzing the clinical records.

Plasma Lipid analysis

Blood samples were collected after an overnight fast. Blood (10 mL) was collected into a plain tube and a tube containing EDTA (1 mg/mL) to obtain serum and plasma, respectively, and buffy coat by centrifugation at 3000 rpm for 15 min. Plasma TC, TG and high-density lipoprotein cholesterol (HDL-C) were measured using standard enzymatic–colorimetric procedures (Roche Diagnostics, Basel Switzerland) on a COBAS MIRA plus auto-analyzer (Roche Diagnostics, Basel Switzerland). LDL-C was calculated by the Friedewald formula. ApoB plasma levels were measured by immuno-nephelometry using a dedicated kit (Radim, Rome, Italy) on a DELTA (Radim, Rome, Italy) instrument.

Fatty liver

Ultrasound (US) examination of the liver was performed to assess for fatty liver. All US examinations were performed by a single operator with experience in liver disease ultrasonography for more than a decade.

The US of the liver was performed in the morning after a 10 h fasting using a realtime apparatus with a 2–5 MHz multi-frequency or 3.5 MHz convex probe. The fatty liver diagnosis was made in the presence of fine, packed high amplitude echoes that confer the brightness to the liver (bright liver) and hepatorenal echo contrast
Fatty liver severity was scored as follows: grade 1 = increased echogenicity or bright liver with normal visualization of diaphragm and intrahepatic vessel borders; grade 2 = increased echogenicity with posterior beam attenuation, but with slightly impaired visualization of the intrahepatic vessels and diaphragm; and 3 = marked increase in echogenicity and marked posterior beam attenuation resulting in failure to demonstrate the intrahepatic vessels, diaphragm, and posterior right lobe of the liver [1].

**Exome sequencing**

Two participants with hypocholesterolemia and liver steatosis (Subjects III:13 and IV:5) underwent exome sequencing. DNA from the two selected individuals was sent to the Broad Institute in accordance with protocols put in place by the institutional review boards of the AOUP “P. Giaccone” at University of Palermo and the Broad Institute. The DNA was subjected to solution hybrid selection using oligonucleotides synthesized on an Agilent array [2] in order to isolate the exonic genomic DNA, which was then sequenced using the Illumina HiSeq platform with 76-nucleotide paired-end reads. A total of 32,950,014 bases were targeted for sequencing. The sequence data was mapped to HG19 using BWA (which implements the Burrows-Wheeler transform) [3], and single nucleotide variants were called using the UnifiedGenotyper module of the Genome Analysis Toolkit (GATK) [4]. The variants were then filtered as follows: (1) Phred-scaled probability that a polymorphism exists at a site > 30; (2) ratio of variant quality score to number of reads > 5; (3) nonreference allele present in >25% of reads; (4) maximum contiguous homopolymer run of the variant allele in either direction on the reference < 5 bases; and (5) strand bias (as described in [30] less than -0.10. The variants were then annotated with a custom pipeline [5].

**Polymerase chain reaction (PCR) and DNA sequencing**

Genomic DNAs from all subjects were extracted from whole blood using the Wizard DNA Purification System (Promega Italia, Italy). A partial region of exon 26 of APOB gene was sequenced using the primer pairs for PCR amplification and the amplification conditions as previously described [6]. PCR fragments were purified with a commercial kit (Wizard PCR Prep–DNA Purification System; Promega Italia, Italy) then sequenced directly in both directions using BigDye Terminator Cycle sequencing kit 1.1 in a ABI 310 DNA sequencer (Applera Italia, Italy) and the results were analyzed with the Seqed software (Applied Biosystems, Warrington, UK). The direct sequencing of the region of exon 1 of RHOB gene variant was sequenced as described above by using the following primers: EX1F GGGCCAGGAGGACTACGA and EX1.R CAGTTGATGCAGCCGTTCT.

**Genotyping for rs738409 (I148M) in PNPLA3 gene**

Genotyping for PNPLA3 rs738409 was conducted in a StepOne Real time Apparatus (Life Technology) by using a commercial genotyping assay (cat. C_7241_10, Life Technology). The genotyping call was done with SDS software v.1.3.0 (ABI Prism 7500, Foster City, CA, USA). Genotyping was conducted in a blinded fashion relative to subject characteristics.

**References**