Novel functional APOB mutations outside LDL-binding region causing familial hypercholesterolaemia

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Familial hypercholesterolaemia (FH) is characterized by increased circulating low-density lipoprotein (LDL) cholesterol leading to premature atherosclerosis and coronary heart disease. Although FH is usually caused by mutations in LDLR, mutations in APOB and PCSK9 also cause FH but only a few mutations have been reported, APOB p.R3527Q being the most common. However, 30–80% of clinical FH patients do not present an identifiable mutation in any of the described genes. To identify the genetic cause of the hypercholesterolaemia in 65 patients without mutations in LDLR, PCSK9 or in fragments of exon 26 and 29 of APOB currently analysed, we performed whole sequencing of APOB by pyrosequencing. A total of 10 putative mutations in APOB were identified. Flow cytometry with fluorescently labelled LDL from patients and relatives showed that p.Arg1164Thr (exon 22) and p.Gln4494del (exon 29) presented a 40% decrease in internalization in lymphocytes and HepG2 cells, very similar to APOB3527. The proliferation assays with U937 cells showed reduced growth for both cases. The variant p.Tyr1247Cys was found to be neutral and other three alterations were considered polymorphisms. Our results emphasize the need to study the whole APOB in routine protocols to improve patient identification and cardiovascular risk assessment.

INTRODUCTION

Familial hypercholesterolaemia (FH) is one of the most common diseases of lipid metabolism. FH is inherited as an autosomal-dominant disorder and was the first genetic disorder of lipid metabolism for which the molecular mechanism was elucidated (1). FH usually results from inherited defects in the low-density lipoprotein receptor gene (LDLR) and is characterized by increased circulating low-density lipoprotein (LDL) cholesterol that leads to lipid accumulation in arteries and tendons (xanthomas), causing premature atherosclerosis and coronary heart disease (CHD) (1). Patients with mutations in other genes as the apolipoprotein B gene (APOB) and proprotein convertase subtilisin/kexin type 9 gene (PCSK9) present an indistinguishable phenotype and are now included in the definition of FH by many authors (2–4). These gene defects are however rare causes of FH (3). In contrast to the LDLR where more than 1300 mutations have been found (5) in APOB and PCSK9, only a few mutations have been found and proved to be mutations causing disease (6).

ApoB100 has two main functions: to maintain the integrity of the LDL particle and to enable binding of LDL to the LDL receptor, thereby promoting LDL clearance from the circulation by LDL-receptor-mediated internalization (1). ApoB100 is the ligand for the LDL receptor and the sole apolipoprotein on the surface of LDL; apoB100 is wrapped around the LDL particle as a belt and there are some critical residues that are crucial for the apoB–LDL-receptor affinity within this configuration (7).
The LDL-receptor-binding domain was first localized to the region between residues 3386 and 3396 (8); however, the most common mutation \textit{APOB3527} is outside this region (9). Later it was shown that alterations involving this amino acid destabilize important clusters for the apoB100 conformation, altering in this way its affinity for the LDL receptor (10,11); additionally, regions between amino acid 3174 and 3184 and also 4181 and 4540 were proved to be important for correct folding of the carboxyl terminus of apoB100 necessary for binding to the LDL receptor (11). In 2007, Krisko and Etchebest (12) proposed a model for the tertiary structure of apoB defining eight different domains and the domain for LDL-receptor binding was established to be between the residues 2820–3202 and 3243–3498. Nevertheless, this theoretical model was not proved experimentally since apoB, being one of the largest human proteins and only in its correct conformation when associated with lipids, is not easily crystallized (13).

\textit{APOB} mutations causing FH are estimated to occur in 1/300 to 1/700 people in several Caucasian populations in Europe (14). The first mutation to be identified and characterized in \textit{APOB}, \textit{APOB3500} now known as \textit{APOB3527}, was discovered in late 1980s (9,14). In the following years, other alterations were described as p.Asn1914Ser, p.His1923Arg, p.Arg3507Trp, p.Arg3527Trp, p.Arg3558Cys, p.Trp4396Tyr and p.Ala4481Thr (9,11,15–17); however, only p.Arg3507Trp, p.Arg3527Gln, p.Arg3558Cys, and p.Trp4396Tyr were proved to be pathogenic, although there are controversial data regarding p.Arg3558Cys that seems to be influenced by environmental factors (16). The remaining variants were classified as polymorphisms (15). The penetrance of \textit{APOB} mutations has been shown to be <100% and patients with \textit{APOB} mutations usually have a less-severe phenotype than FH patients due to LDLR mutations (1,14).

Recently, in 2012, two new alterations in \textit{APOB} have been published, found through a study of exome sequencing and linkage analyses, in two unrelated families. \textit{In vitro} assays in HepG2 showed 28–49% reduction in uptake assays with LDL from patients bearing heterozygous alterations in the residue p.Arg3059 and p.Lys3394 (18). These alterations are situated in the theoretical binding region postulated by Krisko and Etchebest (12). However, the co-segregation in these families is not completely showing in both cases low penetrance.

Due to the size of \textit{APOB}, this gene is not easily studied and routinely only a fragment of exon 26 and another of exon 29 are analysed, covering the regions where the four functional mutations have been described. The main aim of this study was to sequence the entire coding region of the \textit{APOB} in 65 index patients with a clinical diagnosis of FH but without detectable mutations in \textit{LDLR}, \textit{PCSK9} or in the two fragments of exon 26 and 29 of \textit{APOB} routinely screened, to identify and characterize the genetic cause of the hypercholesterolaemia in these patients.

**RESULTS**

**Subjects**

Sixty-five index patients (49 adults and 16 children) with clinical diagnosis of FH without a detectable mutation in routine diagnosis were sequenced by pyrosequencing. Clinical and biochemical characteristics of the 65 index cases included in the study are presented in Table 1.

**Table 1.** Clinical and biochemical characteristics of the 65 patients presented in the study

<table>
<thead>
<tr>
<th></th>
<th>Children (n = 16)</th>
<th>Adults (n = 49)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>9.47 ± 3.7</td>
<td>43 ± 12.3</td>
</tr>
<tr>
<td>Male (%)</td>
<td>50%</td>
<td>63%</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>281 ± 84</td>
<td>322 ± 49</td>
</tr>
<tr>
<td>LDL-c (mg/dl)</td>
<td>201 ± 80</td>
<td>232 ± 45</td>
</tr>
<tr>
<td>HDL-c (mg/dl)</td>
<td>59 ± 12</td>
<td>55 ± 16</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>88 ± 28</td>
<td>140 ± 30</td>
</tr>
<tr>
<td>apoA-I (mg/dl)</td>
<td>145 ± 22</td>
<td>144 ± 42</td>
</tr>
<tr>
<td>apoB (mg/dl)</td>
<td>127 ± 92</td>
<td>126 ± 44</td>
</tr>
<tr>
<td>Lp(a) (mg/dl)</td>
<td>93 ± 100</td>
<td>78 ± 41</td>
</tr>
<tr>
<td>Tendon Xant (%)</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>pCHD (%)</td>
<td>0%</td>
<td>13%</td>
</tr>
<tr>
<td>Medication (%)</td>
<td>12.5%</td>
<td>35%</td>
</tr>
</tbody>
</table>

Only pre-treatment values were considered.

**Pyrosequencing**

The coding region of \textit{APOB} in genomic DNA from 65 index patients (49 adults and 16 children) with clinical diagnosis of FH without a detectable mutation by routine diagnosis was sequenced. Pyrosequencing resulted in a total of 227 688 nucleotide reads, corresponding to a mean coverage of 35X/fragment/individual for \textit{APOB}. A total of 87 alterations were detected, of which more than half were previously described single-nucleotide polymorphisms (SNPs) while 32 were novel possible pathogenic variants (estimated alleles between 0.5 and 2.0). A total of 15 fragments containing these variants were resequenced by the Sanger method. These 32 variants were located in exons 5, 13, 14, 15, 16, 19, 22, 23, 24, 26 and 29, with the majority (25) in exons 26 and 29. From the 32 novel variations identified by pyrosequencing, only 7 were found by Sanger sequencing and three more alterations (2 novels and 1 described) were found by Sanger sequencing that had not been detected by pyrosequencing (Table 2 and Fig. 1). From these 10 putative alterations, two alterations did not co-segregate with hyperlipidaemia in the family of the proband, and for another 2 it was not possible to verify co-segregation because relatives were not available. The remaining 6 alterations were found to co-segregate with the phenotype in the proband’s family although presenting some cases of possible low penetrance. For three of these variants (two that co-segregate and one that did not co-segregate in the family), the ability of the patients’ LDL to bind and internalize the LDL receptor was determined (Table 3 and Fig. 1).

A panel of 96 normolipidaemic Portuguese control individuals was also screened for these putative alterations: alterations p.Asp1113His, p.Tyr1247Cys and p.Ser3274Gly were found in 2 normolipidaemic and p.Asp2213del alteration was found in 1 of them, being for this reason considered to be polymorphisms. The remaining alterations were not found in this normolipidaemic panel (Fig. 1). The 10 putative alterations found in \textit{APOB} were also screened in a panel of 96 FH mutation negative panel and only alterations p.Asp1113His and p.Ser3274Gly, described before here as polymorphisms, were found in these panels.
LDL binding and uptake assay

To assess the pathogenicity of p.Arg1164Thr (exon 22) and p.Gln4494del (exon 29) alterations, LDL particles from patients and relatives, carriers and non-carriers, as well as a normal control without alterations in APOB and a positive control with p.Arg3527Gln mutation were isolated. *In vitro* LDL assays were performed in lymphocytes and hepatocytes-like HepG2 cells. As it was possible to obtain a fresh serum sample from the patient with the alteration p.Tyr1247Cys and from her hypercholesterolaemic father who did not presented the alteration, LDL was also isolated from these subjects and analysed to confirm the polymorphism status. Functional assays did not show affected function (supplementary Fig. S1A and B).

The p.Arg1164Thr alteration has been found in Patient 3 (Family 3) and this variant co-segregated with hypercholesterolaemia in the family except in two young individuals (Fig. 2A). The *in silico* analysis predicts that this variant could be pathogenic but *in silico* predictions for a structural protein, such as apoB, may not be reliable so *in vitro* studies are necessary for pathogenicity validation. The alteration was not found in the 1000 genome project (19) or in the National Heart, Lung and Blood Institute (NHLBI) database (http://www.nhlbi.nih.gov/) (Table 3). Assays of LDL receptor function in lymphocytes showed that uptake (binding and internalization) of LDL from patient 3 was 40% less than uptake of LDL from control subjects (Fig. 2B and C). Her daughter presented a milder phenotype but showed a reduction similar to the well-known pathogenic p.Arg3527Gln (about 50%). The same results were observed in the uptake assay with HepG2 cells (Supplementary Material, Fig. S2A). All values presented by individuals carrying p.Arg1164Thr variant as well as the APOB3527 control sample were statistically different from the wild type (wt).

In family 10 in whom the p.Gln4494del variant was found, this alteration did not show complete co-segregate with hypercholesterolaemia in the family (Fig. 3A). The SIFT *in silico* analysis predicts that this variant could be pathogenic and the alteration was not found in the 1000 genome project (19) or in the NHLBI database (http://www.nhlbi.nih.gov/) (Table 3). Functional assays in control lymphocytes showed a decreased of ∼44% in the binding and internalization of LDL from index patient 10 when compared with normal control (Fig. 3B and C), while LDL from the severe hypercholesterolaemia patient’s mother presented a reduction more similar to p.Arg3527Gln. The same result was observed in the uptake assay with HepG2 cells (Supplementary Material, Fig. S2B). All individuals carrying p.Gln4494del variant as well as the APOB3527 control sample were statistically different from the wild type (wt).

In family 10 in whom the p.Gln4494del variant was found, this alteration did not show complete co-segregate with hypercholesterolaemia in the family (Fig. 3A). The SIFT *in silico* analysis predicts that this variant could be pathogenic and the alteration was not found in the 1000 genome project (19) or in the NHLBI database (http://www.nhlbi.nih.gov/) (Table 3). Functional assays in control lymphocytes showed a decreased of ∼44% in the binding and internalization of LDL from index patient 10 when compared with normal control (Fig. 3B and C), while LDL from the severe hypercholesterolaemia patient’s mother presented a reduction more similar to p.Arg3527Gln. The same result was observed in the uptake assay with HepG2 cells (Supplementary Material, Fig. S2B). All individuals carrying p.Gln4494del variant as well as the APOB3527 control sample were statistically different from the wild type (wt).

*U937 proliferation study*

To test the proliferation of U937 cells in the presence of altered apoB particles, LDL from patients and their relatives with and
without the putative variants was added to the medium. The results obtained were similar to the other assays with LDL from index patients and relatives with the alteration resulting in \( \approx 50–60\% \) growth compared with normal control, very similar to LDL from the APO3527 positive control (Fig. 2D and D). All LDL samples had significant lower growth rates than the wt sample.

**PCSK9, APOC3, APOE screening and exome study**

Although APOB mutations have been shown to have low penetrance (2,14), alterations in genes described to cause hypocholesterolaemia were investigated in order to seek further explanation of the mild phenotypes presented by 5/9 of the mutation carriers. Alterations in PCSK9 and the mutation p.Arg19X in APOC3 described to cause hypocholesterolaemia were screened but not found in the relatives with the two functional alterations but with normal/border line cholesterol values. The determination of the APOE genotype was also performed, but the results obtained (E3/E3 and E3/E4) did not justify the phenotype in these individuals.

Since the alteration found in family 10 did not show full co-segregation with the phenotype in this family, index patient 10 was also studied by exome sequencing. The preliminary results of this exome sequencing study found an alteration (p.Asp19His) in the ATP-binding cassette subfamily G member 8 gene (ABCG8) of the maternal grandmother (I:2, family 10) as well as in the index patient 10, his mother and uncle (Fig. 3A). This finding can present a justification for the apparent lack of co-segregation.

**DISCUSSION**

FH is one of the most widely studied monogenic disorders in the world and present well-defined clinical criteria (21). Mutations in LDLR, APOB and PCSK9 represents the dominant form and mutations in LDLR adaptor protein 1 gene (LDLRAP1) the recessive form of the disease (3). However, 30–80% of clinical FH patients do not present an identifiable mutation in any of these four genes (22,23). In our group, only 48% of clinical FH patients have an identifiable mutation (4). Other mutations in these genes or other gene defects must exist to explain the cause of hypercholesterolaemia in the remaining severe affected families. The fact that some index patients can have a polygenic dyslipidaemia instead of a monogenic dyslipidaemia as it has been described recently (24) cannot be discarded.

The molecular study of FH usually includes the study of promoter region, 18 exons and splice junctions of LDLR and 2 fragments of exons 26 and 29 of APOB (4,20,25). PCSK9 is not always routinely analysed and depending on the groups only exons where mutations have been found or all exons, promoter region and splice junctions are analysed (26–29).

Next-generation sequencing (NGS) has revolutionized research in genetics as well as having improved and widened genetic diagnosis of disease (18,23). To identify the genetic cause of the hypercholesterolaemia in patients with clinical
Table 3. *In silico* prediction, frequency and co-segregation data of alterations found in *APOB*

<table>
<thead>
<tr>
<th>ID</th>
<th>Exon</th>
<th>Alteration cDNA</th>
<th>Protein</th>
<th>Frequency Normolip. Panel</th>
<th>1000 Genome</th>
<th>NHLBI SIFT</th>
<th>PolyPhen2</th>
<th>Condel</th>
<th>Grantham Score</th>
<th>Mutation Taster</th>
<th>Co-segregation (C/TA;C/NA)</th>
<th>Functional characterization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19</td>
<td>c.2981C&gt;T</td>
<td>p.Pro994Leu</td>
<td>0/96</td>
<td>0.1%</td>
<td>0.1%</td>
<td>Damaging</td>
<td>Probably damaging</td>
<td>Deleterious</td>
<td>Disease causing</td>
<td>1/2;0/0</td>
<td>Unknown</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>c.3337G&gt;C</td>
<td>p.Asp1113His</td>
<td>2/96</td>
<td>0.3%</td>
<td>0.8%</td>
<td>Damaging</td>
<td>Possibly damaging</td>
<td>Deleterious</td>
<td>Moderately conservative</td>
<td>1/2;2/2</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>3</td>
<td>22</td>
<td>c.3491G&gt;C</td>
<td>p.Arg1164Thr</td>
<td>0/96</td>
<td>Nd</td>
<td>nd</td>
<td>Damaging</td>
<td>Possibly damaging</td>
<td>Deleterious</td>
<td>Moderately conservative</td>
<td>2/2;4/2</td>
<td>Causing disease</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>c.3740A&gt;G</td>
<td>p.Tyr1247Cys</td>
<td>0/96</td>
<td>0.1%</td>
<td>0.05%</td>
<td>Damaging</td>
<td>Possibly damaging</td>
<td>Deleterious</td>
<td>Moderately conservative</td>
<td>2/7;1/1</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>5</td>
<td>26</td>
<td>c.5599C&gt;T</td>
<td>p.Arg1867Trp</td>
<td>0/96</td>
<td>Nd</td>
<td>0.02%</td>
<td>Damaging</td>
<td>Possibly damaging</td>
<td>Deleterious</td>
<td>Moderately radical</td>
<td>2/2;0/0</td>
<td>Unknown</td>
</tr>
<tr>
<td>6</td>
<td>26</td>
<td>c.5690G&gt;A</td>
<td>p.Arg1897His</td>
<td>0/96</td>
<td>Nd</td>
<td>nd</td>
<td>Tolerated</td>
<td>Benign</td>
<td>Neutral</td>
<td>Polymorphism</td>
<td>3/4;0/1</td>
<td>Unknown</td>
</tr>
<tr>
<td>7</td>
<td>26</td>
<td>c.6639_6641 delTGAG</td>
<td>p.Asp2213del</td>
<td>1/96</td>
<td>Nd</td>
<td>0.32%</td>
<td>Tolerated</td>
<td>Benign</td>
<td>Neutral</td>
<td>Polymorphism</td>
<td>?</td>
<td>Unknown</td>
</tr>
<tr>
<td>8</td>
<td>26</td>
<td>c.7853T&gt;C</td>
<td>p.Ile2618Thr</td>
<td>0/96</td>
<td>Nd</td>
<td>nd</td>
<td>Tolerated</td>
<td>Benign</td>
<td>Neutral</td>
<td>Moderately conservative</td>
<td>2/2;0/1</td>
<td>Unknown</td>
</tr>
<tr>
<td>9</td>
<td>26</td>
<td>c.9835A&gt;G</td>
<td>p.Ser3279Gly</td>
<td>2/96</td>
<td>0.7%</td>
<td>0.8%</td>
<td>Damaging</td>
<td>Probably damaging</td>
<td>Neutral</td>
<td>Moderately conservative</td>
<td>2/3;3/4</td>
<td>Causing disease</td>
</tr>
<tr>
<td>10b</td>
<td>29</td>
<td>c.13480_13482delCAG</td>
<td>p.Gln4494del</td>
<td>0/96</td>
<td>Nd</td>
<td>nd</td>
<td>Damaging</td>
<td>np</td>
<td>np</td>
<td>Polymorphism</td>
<td>2/3;3/4</td>
<td>Causing disease</td>
</tr>
</tbody>
</table>

ID, family number; Normolip. Panel, Normolipidemic Portuguese Control Panel; NHLBI, exome sequencing project (ESP); Grantham Score: conservative (0–50), moderately conservative (51–100), moderately radical (101–150), or radical (≥151) according to the classification proposed by Grantham (20); co-segregation (C/TA;C/NA), alteration carriers/total affected; alteration carriers/total non-affected; aThese two individuals also presented the p.Ala431Thr in *LDLR*. 
bPatient with p.Asp19His alteration in *ABCG8*.
Figure 2. Results of functional assays for family 3. (A) Pedigree of the family 3 with p.Arg1164Thr alteration. Index case is indicated by an arrow. (B) LDL-binding capacity after 4 h incubation at 4°C in lymphocytes. (C) LDL internalization efficiency after 4 h incubation at 37°C in lymphocytes. (D) U937 cell proliferation assay after 48 h incubation with LDL, presented as fold induction with respect to control without added LDL. The values represents the mean from at least three determinations; wt represent the experiment with normal control; error bars represent ± SD. *P < 0.05 between wt and individuals with p.Arg1164Thr or APOB3527 (Student’s t-test, one-tailed).
Figure 3. Results on functional assays for family 10. (A) Pedigree of the family 10 with p.Gln4494del alteration. Index case is indicated by an arrow. In this family 2 alterations have been found, one in the APOB (grey ball symbol) and other in ABCG8 gene. (B) LDL-binding capacity after 4 h incubation at 4 °C in lymphocytes. (C) LDL internalization efficiency after 4 h incubation at 37 °C in lymphocytes. (D) U937 cell proliferation assay after 48 h incubation with LDL, presented as patient’s fold induction in respect to control without added LDL. The values represent the mean from at least three determinations; wt represent the experiment with normal control; error bars represent ± SD. *P < 0.05 between wt and individuals with p.Gln4494del or APOB3527 (Student’s t-test, one-tailed).
diagnosis of FH, we performed whole sequencing of \textit{APOB} by pyrosequencing, in 65 patients, without mutations in \textit{LDLR}, \textit{PCSK9} or in fragments of exons 26 and 29 of \textit{APOB} currently analysed. The results obtained by pyrosequencing revealed a large number of false positives and false negatives, but the software was recently updated and has demonstrated good results in diagnostics studies (30,31).

We identified 10 possible disease-causing alterations located in exons 19, 22, 24, 26 and 29. Functional studies for three of the alterations found in three unrelated families were performed using three different types of cells: lymphocytes, HepG2 cells and U937 cells. Alterations in exon 22 (p.Arg1164Thr) and 29 (p.Gln4494del) were proved to be mutations causing disease, while p.Tyr1247Cys, which did not co-segregate with the hypercholesterolaemia, was found not to affect apob function. All 10 putative variants were screened in a normolipidaemic panel and three alterations, p.Asp1113His, p.Asp2213del and Ser3279Gly, were shown to be a polymorphism due to the 1–2% prevalence found in the normolipidaemic control panel. Variants p.Asp1113His and p.Ser3274Gly were also present in 4/55 individuals from the FH mutation negative control panel. Four alterations are still waiting functional validation.

Functional studies in two families with alterations p.Arg1164Thr and p.Gln4494del showed a reduction of ~40–50% in the binding and uptake of patient’s LDL in lymphocytes and HepG2 as well as exhibiting a reduced ability to stimulated cell proliferation with patient’s LDL when compared with LDL of wt control. The U937 assays were found to be the best assays to study \textit{APOB} mutations since these cells are dependent on apoB-LDL receptor binding to grow since they do not synthesize cholesterol and require it in the extra cellular medium to be able to proliferate (32). Also these assays have less intermediate steps diminishing the associated errors of any complex experiment. The sensitivity and specificity of these assays have been shown to be 87.5% and 100%, respectively, demonstrating to be a reliable test (33).

Although the results of functional assays show that the alterations lead to reduced LDL receptor activity very similar to \textit{APOB}S3527 positive control, these two alterations did not show a 100% penetrance in any of the families studied. In a recently reported study of two novel functional alterations in \textit{APOB} (18), low penetrance was also seen in these families. However, they only performed functional studies for index patients and not for relatives with the alteration showing low penetrance, so it is not known if these individuals also presented affect function as the ones described here. It is known that patients with mutations in \textit{APOB} present a mildest and more variable phenotype and the penetrance is not 100% (2,14) probably due to the alternative pathway for LDL clearance through apoE in LDL cell surface that it is also a ligand for LDL receptor. Nevertheless, these patients can have an increased concentration of small dense LDL particles (sdLDL) and so patients with \textit{APOB} mutations also have an increased cardiovascular risk since these are the most atherogenic particles (8,34).

However, due to the lack of penetrance of these two alterations, further discussion is necessary. The alteration p.Arg1164Thr is not in the consensus region considered to be important for the binding of apoB to the LDL receptor and neither in the theoretical region postulated by Krisko and Etchebest (12). However, LDL from individuals with this alteration showed reduced affinity for the LDL receptor. It has been described that alterations in arginine residues resulting in a different amino acid substitution in apoB decreases the affinity of the LDL:LDL receptor complex (35–37). The lack of penetrance in the two youngest members of the family can be age related as in familial combined hyperlipidaemia (38).

In family 10, one member mildly hypercholesterolemic aged 74 years (I:1) and two normolipidemic aged 47 (II:1) and 12 (III:1) years presented the alteration p.Gln4494del in the \textit{APOB} tail important for the correct apoB conformation and stability of the protein in LDL cell surface. Functional assays performed for I:1 and II:1 showed reduced biding and internalization of the LDL:LDL receptor complex. This lack of penetrance cannot be explained totally by late onset and so other genetic or environmental factors must be playing a role. Also in this family individual II:2, grandmother of the index patient, from whom it was initially suspected that the hypercholesterolaemic trait was inherited, did not presented this alteration in \textit{APOB}. To understand better the genetics of this family, the exome sequencing of the index patient was performed. The index patient was also found to have an alteration in \textit{ABCG8}, p.Asp19His (rs11887534), described before to be associated with an increased susceptibility to form gallstones and increased LDL cholesterol, especially in women, since it increases the cholesterol transport from the liver to the bile leading to cholesterol saturation and accumulation as gallstones in the gall bladder (39–41). His hypercholesterolaemic mother and grandmother also presented the same alteration as well as his normolipidaemic uncle. Although this alteration in \textit{ABCG8} could explain the hypercholesterolaemia in the grandmother and the more severe phenotype in the index patient mother compared with other Portuguese heterozygous FH patients, the fact that his uncle was also found to be a carrier of this alteration and was normolipidaemic remains to be explained and only an extensive study of genetic and environmental causes could lead to a final conclusion. The clinical presentation that has been associated to the carrier status of this polymorphism (39–41) are in agreement with I:2 individual clinical history, since she has also made the removal of the gall bladder due to gall stone formation. In view of these findings, the index patient mother should be counselled regarding the possibility of gall stones formation.

To further investigate the low penetrance cases in these two families, all exons of \textit{PCSK9} and a fragment of \textit{APOC3} containing the p.Arg19X mutation were screened for mutations causing hypcholesterolaemia. APOE genotype was also performed; none of these results could explain the milder phenotype.

To conclude, we report here the finding of 10 novel possible causing mutations in \textit{APOB}. It was possible to characterize and proved to be functional mutations, the variants p.Arg1164Thr (exon 22) and p.Gln4494del (exon 29) by extensive studies supported by three different assays. Four variants were found to be a polymorphism and four alterations are still waiting functional validation. These results suggest that \textit{APOB} can carry more pathogenic mutations than initially described and that the whole analysis of \textit{APOB} should be performed, at least in patients without an identifiable mutation in routine protocol or in patients heterozygous for \textit{LDLR} mutations with a severe phenotype. This would be feasible with the development of the novel NGS and will be implemented in our laboratory. The genetic identification of FH patients is important for the
correct identification, assessment and management of these patients in order to decrease their elevated cardiovascular risk.

METHODS

Sixty-five index patients referred to our lab to the Portuguese FH Study following the Simon Broome criteria for FH as presented before (4,20) were analysed by pyrosequencing. These patients were previously analysed and found not to have detectable mutations in LDLR, PCSK9 or in fragments of exons 26 and 29 of APOB routinely studied (4).

Study approval

This research has been approved by the National Institute of Health Ethical Committee and the Portuguese FH Study has approval from the National Data Protection Commission.

Pyrosequencing

All exons of APOB were amplified in a pools of 20, 20 and 25 DNAs and sequenced by pyrosequencing (454 Life Science, Roche®) (service provided by BIOCANT, Cantanhede, PT, USA). Amplification of a total of 149 fragments with an average length of 230 bp was optimized in order to obtain maximum specificity and yield. Nucleotide reads obtained by pyrosequencing were aligned to the respective consensus sequences (APOB—NM_000384.2) using Amplicon Variant Analyzer software (AVA, Roche). The results were given by estimated alleles. An estimated allele of 1 for a variant is interpreted as at least 1 allele having that variant. All fragments containing alterations identified by pyrosequencing were resequenced by the Sanger method, for confirmation of the alteration found.

For sequence analysis, the references used for APOB, PCSK9, ABCG8 and APOC3 were respectively NM_000384.2, NM_174936.3, NM_002437.2 and NM_000040.1 and cDNA numbering was considered with nucleotide c.1 being A of the ATG initiation codon p.1 (42).

In silico analysis

In silico analysis was performed using different bioinformatics tools: polyPhen-2 (43), Sorting Intolerant From Tolerant (SIFT) (44), Consensus Deleteriousness score of missense SNVs (Condel) (45), Mutations taster (46) and Grantham Score (47).

Exome sequencing

The exome sequencing was performed by BaseClear B.V. (Netherlands), as follows: the DNA was fragmented, size selected and the ends of the sheared molecules blunted. Illumina adapters were added to both ends of the DNA fragments and a PCR amplification was performed on the molecules using the added adapter regions as priming sites. The resultant DNA fragments were then enriched by hybridization to the SeqCAP EZ probes provided by Nimblegen; this hybridization was performed over a 72 h period. The DNA was then washed and the DNA which is specifically bound to the SeqCAP EZ probes was eluted. The captured DNA was amplified using an LM-PCR. The enrichment was then measured using qPCR. The quantified and enriched DNA libraries were loaded on the Illumina sequencer and a run was performed as described by the manufacturer. The sequencing data produced were processed removing the sequence reads which were of too low quality; the sequence data were provided to us in FastQ format.

LDL isolation and labelling

Blood was drawn, after overnight fasting, into dry tubes and serum was obtained by means of low-speed centrifugation (2465 g). LDL was isolated from serum samples of individuals carrying APOB mutations in a two-step centrifugation as described before (32). Serum LDL (d. 1.019–1.050 g/ml) was isolated using sequential ultracentrifugation by adjusting density adding 3 M of potassium bromide to serum, then phosphate-buffered saline (PBS) buffer was added, resulting in two phases. The sample was centrifuged at 244,500g for 19.5 h at 4°C. The band corresponding to LDL was recovered and stored at 4°C and before the experiments LDL particles were labelled with FITC as previously described (48). Briefly, LDL samples (1 mg protein/ml) were dialysed in desalting columns (PD-10 Desalting Columns, GE Healthcare) in 0.1 M NaHCO3 (pH 9.0) and then added to 10 μl/ml FITC (2 mg/ml in dimethyl sulfoxide) and mixed gently by slow rocking at room temperature for 2 h in the dark. The unreacted dye was removed by gel filtration on a Sephadex G-25 column equilibrated with EDTA-free PBS buffer and the labelled LDL particles were stored at 4°C in the dark. All fractions were assayed for protein concentration by Bradford protein assay, with bovine serum albumin as standard.

Lymphocyte LDL uptake and binding assay

Isolation and culture of T-lymphocytes were performed as previously described before by Etxebarria (48). Lymphocytes (2 × 10^5 cells) were cultured for 72 h in lipoprotein-deficient serum LPDS and stimulated with anti-CD3/CD28 beads to obtain a uniform fraction of lymphoblast’s and maximum upregulation of LDLR. To determine LDL receptor activity, lymphocytes were incubated for 4 h with 20 μg/ml FITC–LDL at either 37°C for measuring LDL uptake activity, or at 4°C to determine LDL:LDL receptor binding. After incubation, lymphocytes were washed twice and fixed with paraformaldehyde for 10 min at room temperature, and washed again to remove completely the fixative. Fluorescence intensities were measured by fluorescence-activated cell sorting (FACS) in a FACSCalibur Flow cytometer according to the manufacturer’s instructions. For each sample, fluorescence of 10 000 events was acquired for data analysis and the results were expressed as the mean fluorescence of activated gated cells, selected in a forward versus side-scatter window. LDL receptor activity determining the amount of internalized LDL was assayed by adding Trypan blue solution (0.2% final concentration) to the samples, thus eliminating the extracellular signal due to the non-internalized LDL:LDL receptor complexes. This procedure allowed the removal of extracellular fluorescence by quenching and to determine the intensity of the remaining fluorescent particles inside the cells (which is unaffected by the external quencher). LDL:LDL receptor binding

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was determined in the washed cells by measuring the fluorescence intensity associated with the FITC-labelled LDL by FACS. All experiments were performed in triplicate. Statistical analysis to compare the different results in uptake and binding assays presented by patients and control subjects was performed by using Student’s t-test, one-tailed.

**Hepg2 cells LDL uptake assay**

FITC-labelled LDL uptake by HepG2 cells was also determined. Hepatoma cells were grown in monolayer at 37°C in DMEM with 10% (v/v) foetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin/streptomycin. 2 \times 10^5 cells were seeded in 24-well plates for 24 h prior the uptake assay. The FITC–LDL uptake assay was performed as described for lymphocytes assay. All experiments were performed in triplicate. Statistical analysis to compare the different results obtained for patients and control subjects was performed by using Student’s t-test, one-tailed.

**U937 cells proliferation assay**

U937 cells (ATCC® CRL-1593.2TM) were grown in RPMI with ampicillin/streptomycin and 10% FBS in a 75-cell culture flask, at 2 \times 10^5 cells/ml at 37°C in a humified atmosphere of 5% CO₂. Before proliferation assay, cells were seeded in 96-well culture plates for 24 h in RPMI containing 10% LPDS and the assay was started by adding 2 μg/ml LDL. The cells were further incubated for 48 h at 37°C allowing cell growth. U937 cell proliferation was determined by CellTiter96®AQ_ous Non-Radioactive Cell Proliferation Assay. This colorimetric method is based on the rate of reduction of the tetrazolium dye MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] in the presence of an electron-coupling reagent PMS [phenazine methosulfate, 5% (v/v)] by mitochondrial dehydrogenases of metabolically active cells, producing a soluble product called formazan which can be measured at 490 nm. The quantity of generated formazan product, as measured by the amount of 490 nm absorbance, is directly proportional to the number of viable cells in the culture. Cells were grown and incubated with LDL in triplicate for each index patient and relative. Measurements were performed once for each well and the mean of the triplicate assays for each subject was calculated. The proliferation rate in the presence of LDL:apoB100 from different patients and relatives has been expressed as fold increment of cellular growth in respect to basal growth of cells without added LDL.

For all experiments, a positive control with the APOB3527 and a negative control were analysed. Hypercholesterolaemic and normolipidaemic individuals were studied. Statistical analysis to compare the different results obtained for patients and control subjects was performed by using Student’s t-test, one-tailed.

**Normolipidaemic control panel**

A normolipidaemic control panel was constructed with 96 individuals from the Portuguese population with all lipid parameters [total cholesterol, LDL-c, HDL-c, triglycerides, ApoB, ApoAl and Lp(a)] within the values recommended by the European Cardiology Society (49). This panel was used to screen for the prevalence of the alterations found in APOB, by PCR and direct sequencing. Primers and PCR conditions are available upon request.

**FH mutation-negative panel**

An FH mutation-negative panel was constructed with 96 individuals from the Portuguese population with clinical diagnosis of FH but without mutations in one of the three genes studied in Portuguese FH Study. This panel was used to screen for the prevalence of the alterations found in APOB, by PCR and direct sequencing.

**PCSK9, APOE and APOC3 screening**

All exons of PCSK9, exon 4 of apolipoprotein E gene (APOE) and exon 2 of apolipoprotein C3 gene (APOC3) containing the mutation p.Arg19X (50,51), were amplified and sequenced as described before (4,20).

**Accession numbers**

The NCBI accession numbers for the APOB, PCSK9, ABCG8 and APOC3 sequences reported in this paper are, respectively, NM_000384.2, NM_174936.3, NM_022437.2 and NM_000040.1.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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**REFERENCES**


