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# Molecular analysis of *APOB*, *SAR1B*, *ANGPTL3*, and *MTTP* in patients with primary hypocholesterolemia in a clinical laboratory setting: Evidence supporting polygenicity in mutation-negative patients

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## HIGHLIGHTS

- Primary hypobetalipoproteinemia is a syndrome with heterogenic nature and presentation.
- Primary hypobetalipoproteinemia has been considered monogenic. Our findings suggest that it may also have a polygenic origin.
- Polygenicity should be examined in patients with clinical and biochemical data of primary hypobetaliproteinemia.

## ARTICLE INFO

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## ABSTRACT

*Background and aims:* Primary hypobetalipoproteinemia is generally considered a heterogenic group of monogenic, inherited lipoprotein disorders characterized by low concentrations of LDL cholesterol and apolipoprotein B in plasma. Lipoprotein disorders include abetalipoproteinemia, familial hypobetalipoproteinemia, chylomicron retention disease, and familial combined hypolipidemia. Our aim was to review and analyze the results of the molecular analysis of hypolipidemic patients studied in our laboratory over the last 15 years.
*Methods:* The study included 44 patients with clinical and biochemical data. Genomic studies were performed and genetic variants were characterized by bioinformatics analysis. A weighted LDL cholesterol gene score was calculated to evaluate common variants associated with impaired lipid concentrations and their distribution among patients.
*Results:* Twenty-three patients were genetically confirmed as affected by primary hypobetalipoproteinemia. In this group of patients, the most prevalent mutated genes were *APOB* (in 17 patients, with eight novel mutations identified), *SAR1B* (in 3 patients, with one novel mutation identified), *ANGPTL3* (in 2 patients), and *MTTP* (in 1 patient). The other 21 patients could not be genetically diagnosed with hypobetalipoproteinemia despite presenting suggestive clinical and biochemical features. In these patients, two *APOB* genetic variants associated with lower LDL cholesterol were more frequent than in controls. Moreover, the LDL cholesterol gene score, calculated with 11 SNPs, was significantly lower in mutation-negative patients.
*Conclusions:* Around half of the patients could be genetically diagnosed. The results suggest that, in at least some of the patients without an identified mutation, primary hypobetalipoproteinemia may have a polygenic origin.

*Abbreviations:* HBL, hypobetalipoproteinemia; LDLc, LDL cholesterol; ApoB, apolipoprotein B; MTTP, microsomal triglyceride transfer protein; PCSK9, proprotein convertase subtilisin/kexin type 9; SAR1B, secretion associated Ras related GTPase; ANGPTL3, angiopoietin-like 3; MYLIP, myosin regulatory light chain interacting protein; DGAT1, diacylglycerol O-acyltransferase; NPC1L1, Niemann-Pick C1-like 1 cholesterol transporter 1; FHBL, familial hypobetalipoproteinemia; TG, triglyceride; CMRD, chylomicron retention disease; CM, chylomicron; VLDL, very low density lipoprotein; ABL, abetalipoproteinemia; FHBL1, familial hypobetalipoproteinemia type 1; FHBL2, familial hypobetalipoproteinemia type 2; HDLc, HDL cholesterol; TC, total cholesterol; SNP, single nucleotide polymorphism; HBL/M-, hypobetalipoproteinemia mutation-negative patients; HBL/M+, hypobetalipoproteinemia mutation-positive patients

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## 1. Introduction

Primary hypobetalipoproteinemia (HBL) is a heterogenic group of inherited lipoprotein disorders characterized by low concentrations of low-density lipoprotein (LDL) cholesterol (LDLc) and apolipoprotein B (ApoB) in plasma [1]. HBL may manifest as a fat malabsorption syndrome commonly accompanied by growth failure, fat-soluble vitamin deficiency, hepatic steatosis, abdominal distension, diarrhea, vomiting, and neurological and ophtalmological disorders in the context of hypolipidemia [1]. Primary HBL can be classified according to various criteria, including clinical and biochemical phenotype, the gene involved, and the mode of inheritance of the condition.

Familial hypobetalipoproteinemia (FHBL) type 1 (FHBL1) (OMIM 615558) is a codominant disorder caused by mutations in the *APOB* gene frequently giving rise to a truncated ApoB protein [2]. Affected heterozygotes are asymptomatic or present with hepatic steatosis, and low levels of LDLc and ApoB [3]. Homozygotes may be clinically and biochemically indistinguishable from abetalipoproteinemic patients [2,3]. FHBL1 can also be caused by loss-of-function mutations in the proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene which encodes a protein that targets the LDL receptor for lysosomal degradation. *APOB* and *PCSK9* genes are also associated with familial hypercholesterolemia.

Familial combined hypolipidemia (OMIM 605019), also known as familial hypobetalipoproteinemia type 2 (FHBL2), is a rare recessive disorder caused by mutations in the angiopoietin-like 3 (*ANGPTL3*) gene [4]. Loss-of-function mutation increases lipoprotein lipase activity and may decrease high-density lipoprotein (HDL) cholesterol (HDLc) by increasing endothelial lipase, two enzymes that in physiological conditions are catabolized by the action of ANGPTL3. Patients typically present with no clear phenotype other than hypolipidemia [2].

Chylomicron retention disease (CMRD) or Anderson's disease (OMIM 246700) is a rare recessive disorder caused by mutations in the secretion associated Ras Related GTPase (*SAR1B*) gene characterized by the accumulation of lipid droplets within the enterocytes and the selective absence of ApoB-48-containing particles from plasma [5]. Biochemical manifestations include reduced LDLc levels and absence of chylomicrons (CM) [5].

Abetalipoproteinemia (ABL) (OMIM 200100) is a rare recessive disorder caused by mutations in the microsomal triglyceride transfer protein (*MTTP*) gene. The gene encodes a molecular chaperone critical for the formation of triglyceride-rich lipoproteins such as very low-density lipoproteins (VLDL) and CM. Patients typically present with an absence of LDLc and CM, low TG levels, and very low vitamin E levels [3].

Most of these disorders are very rare. Estimated prevalence for familial hypobetalipoproteinemia is 1 in 1000–3000 individuals and less than 1 in 1 million individuals for the rest of types of primary HBL [3,6]. Some studies have reported variants in other genes very infrequently associated with primary HBL, such as the *APOC3* gene [7], the miosin regulatory light chain interacting protein (*MYLIP*) gene [8], and the diacylglycerol O-acyltransferase 1 (*DGAT1*) gene [9] [10].

It is noteworthy that extreme cholesterol phenotypes can result from the accumulation of common small-effect LDLc affecting alleles [11,12]. Therefore, the objective of this study was to review, analyze, and evaluate genetic variants in 44 hypolipidemic patients recruited to our laboratory over the last 15 years. This included the search for a potentially polygenic basis for the disorder in patients who were negative for mutations in the well-established genes causing primary HBL.

## 2. Materials and methods

### 2.1. Subjects

Subjects were recruited over a 15-year period and were referred to our Metabolism Unit for genetic analysis based on biochemical and/or clinical suspicion of primary HBL, according to the requesting practitioners. Only two patients were followed at our hospital; thus, clinical and/or biochemical data were provided by the medical centers of origin (mainly from Spain) in summarized form, and therefore, the clinical and/or biochemical information obtained was sometimes heterogeneous or incomplete. Primary HBL is usually defined as persistent low total cholesterol with plasma LDLc and ApoB concentrations below the fifth percentile, 130 mg/dL, 62 mg/dL and 63 mg/dL respectively for the Spanish population [13,14]. In order to avoid molecular analysis of secondary HBL, the exclusion criteria included severe chronic liver disease, chronic pancreatitis, cystic fibrosis, end-stage renal disease, hyperthyroidism, cachexia, and malabsorption. In some cases, relatives were also submitted for study. Genetic studies were performed to confirm the diagnosis. All blood samples were obtained in accordance with the Helsinki Declaration of 1964, as revised in 2000. The Ethics Committee of the Hospital de la Santa Creu i Sant Pau reviewed and approved the study protocol and all individuals provided written informed consent. To study the polygenic basis, a sample of 503 European subjects from the 1000 Genomes Project (www.1000genomes.org) was used as the control group and gene score distribution compared.

### 2.2. Gene amplification and resequencing

Genomic DNA was isolated from whole blood (usually EDTA or citrate blood) using the QIAamp DNA blood minikit (Qiagen, Hilden, Germany). All the exons and exon-flanking intronic regions of the *MTTP*, *APOB*, *SAR1B*, *PCSK9*, *ANGPTL3*, *APOC3*, *MYLIP*, and *DGAT1* genes were amplified by PCR. The resulting products were purified using GFX PCR DNA and a Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) and sequenced using a Big Dye Terminator cycle sequencing kit v.3.1 (Applied Biosystems, Foster, CA, USA) on an ABI3130XL automated analyzer (Applied Biosystems). The resulting chromatograms were analyzed with the Staden package program [15]. The primers used for gene amplification and Sanger sequencing are available upon request. A sequential analysis was performed to optimize the diagnostic process. The *APOB* gene was sequenced first, and if it was negative, the *SAR1B*, *ANGPTL3*, and *MTTP* genes were sequenced. Finally, if no putative pathogenic variants were detected, the rest of the genes were also sequenced.

### 2.3. Characterization of variants and bioinformatics analysis

The nomenclature of the allelic variants follows the recommendations of the Human Molecular Genome Variation Society (http://www.hgvs.org). All variants were checked against the Human Gene Mutation Database (HGMD, www.hgmd.cf.ac.uk), 1000 Genome Project (phase3browser.1000genomes.org), and ClinVar (www.ncbi.nlm.nih.gov/clinvar) databases in order to characterize them. No functional studies were performed hence bioinformatics functional analysis was used. The impact of point mutations on the structure and function of the protein was assessed with the following software: SIFT (sift.bii.a-star.edu.sg) [16], PolyPhen2 (genetics.bwh.harvard.edu/pph2/index.shtml) [17], Panther (www.pantherdb.org) [18], Provean (provean.jcvi.org) [19], i-Mutant (gpcr.biocomp.unibo.it/cgi/predictors/I-Mutant3.0) [20], SNPs3D (www.snps3d.org) [21], PMut (mmb.pcb.ub.es/pmut2017) [22], Mutation Taster (www.mutationtaster.org) [23], and Mutation Assessor (mutationassessor.org) [24]. Variants affecting introns were analyzed using Human Splicing Finder v.3.0 software (www.umd.be/HSF3/HSF.html) [25]. According to the American College of Medical Genetics and Genomics guidelines [26], point mutations causing premature stop codons, small insertions, or deletions causing a frameshift and a premature stop codon, large rearrangements, and mutations affecting intron donor or acceptor splice sites (positions +1, +2, −2, or −1) were considered directly pathogenic. The remaining variants (synonymous mutations, missense mutations, small in-frame insertions and deletions, mutations affecting a promoter, 5′UTR or

3′UTR, and intronic variants) were considered pathogenic depending on the existence of functional analysis previously reported in the literature, identification as pathogenic or likely pathogenic in databases such as ClinVar, or in the absence of previous information, when the programs used in the bioinformatics analysis gave as a result a probable alteration in protein function, protein expression, or gene regulation.

### 2.4. Frequency distribution of variants and LDLc gene score

We used contingency tables to evaluate the frequency distribution of the observed nonpathogenic variants in patients that tested negative for a pathogenic mutation versus 503 European controls from the 1000 Genomes Project. We also calculated a weighted LDLc gene score [11] (the weighted sum of the estimated per-allele beta coefficients reported by the Global Lipid Genetics Consortium [27] Supplementary Table 1) and compared its distribution among patients with HBL with no confirmed pathogenic variant, patients with an identified pathogenic mutation, and 503 European controls from the 1000 Genomes Project. Using TaqMan probes (Thermo Fisher Scientific, USA), 11 SNPs influencing LDLc were genotyped: *PCSK9* (rs2479409), *CELSR2-SORT1* (rs629301), *APOB* (rs1367117), *ABCG8* (rs4299376), *SLC22A1* (rs1564348), *HFE* (rs1800562), *MYLIP* (rs3757354), *NYNRIN* (rs8017377), *LDLR* (rs6511720), and *APOE* (rs429358 and rs7412) [11]. This LDLc-specific gene score was calculated for each individual following the method described in Talmud et al. (2013) [11]: when the effect is LDL-lowering (negative), the major allele is considered the risk allele and the absolute effect-size is used as the weight. When the effect for the minor allele is LDL-rising (positive), it is considered directly the risk allele. The weighted score is then calculated as the sum of the product of the number of copies of the risk allele by its corresponding effect-size.

### 2.5. Statistical analysis

A non-parametric sample *t*-test and Fisher's exact test were performed with SPSS v13.0 (IBM).

## 3. Results

The study included 44 patients, 27 males and 17 females aged 0.5–76 years (mean age 15.4 ± 14.0 years). All the patients were Spanish, except for two Italians and one German. All available demographic, clinical, and biochemical information is presented in Supplementary Table 2. Among the 44 patients studied, 23 (52.3%) were considered genetically diagnosed, 17 presented with mutations in the *APOB* gene (73.9%), three in the *SAR1B* gene (13.0%), two in the *ANGPTL3* gene (8.7%), and one in the *MTTP* gene (4.4%). The results for all genetically diagnosed patients are presented in Table 1. Twenty-one patients (47.7%) did not present with a pathogenic mutation; however, most of them presented with genetic variants potentially associated with changes in lipid metabolism.

*APOB mutations*: Of the 17 patients with *APOB* mutation, total cholesterol (TC) was provided in 12 (25–111 mg/dL, average ± standard deviation (SD), 72.8 ± 30.9). LDLc was provided in 10 patients (1–46 mg/dl, average ± SD, 19.9 ± 13.7). ApoB values were referred in nine patients (4–35 mg/dl, average ± SD, 24.1 ± 9.3). HDLc was provided in eight patients (21–89 mg/dl, average ± SD, 44.8 ± 18.5). TG was provided in 10 patients, nine of whom presented with decreased values and one of whom presented with values that fell within the reference interval. TG-concentration values (7–96 mg/dL, average ± SD, 29.2 ± 25.5).

Notably, patient 4 was homozygote for the novel *APOB* mutation c.2726C > A (p.Ser909*). All the *in silico* prediction programs classified this mutation as damaging. The mutation generates an estimated 80% size reduction of the ApoB protein. The parents of the proband were studied and both presented with the mutation c.2726C > A (p.Ser909*) in heterozygosity. No clinical data were provided. The mother presented with decreased TC (93 mg/dL), LDLc (25 mg/dL), apoB (< 22 mg/dL), TG (23.7 mg/dL), and vitamin A (0.23 mg/L) levels and a normal HDLc (63.3 mg/dL). The father also presented with decreased TC (87 mg/dL), LDLc (34 mg/dL), ApoB (30.9 mg/dL), and HDLc (25.9 mg/dL) levels but with a normal TG concentration

**Table 1**
Genetic data from patients with one or two pathogenic variants potentially causing primary HBL.

| Patient | Gene | DNA (pathogenic variants)[a] | Protein[b] | ApoB % | ID[c] | Proband[d] | LDLc score |
|---|---|---|---|---|---|---|---|
| 8 | *APOB* | c.3G > C | p.Met1Ile | | new | Hz | 0.935 |
| | | c.6630_6631delAA | p.Leu2212* | ApoB-48 | new | Hz | |
| 7 | *APOB* | c.133C > T | p.Arg45* | ApoB-0 | new | Hz | 1.035 |
| 13 | *APOB* | c.1124G > A | p.Ser375Lysfs*93 | ApoB-10 | CS072142 [40] | Hz | 0.928 |
| 12 | *APOB* | c.1315C > T | p.Arg439* | ApoB-9 | rs142066904/CM021487 [41] | Hz | 0.811 |
| 1 | *APOB* | c.1407C > A | p.Tyr469* | ApoB-10 | new | Hz | 0.841 |
| 9 | *APOB* | c.1407C > A | p.Tyr469* | ApoB-10 | new | Hz | 1.008 |
| 2 | *APOB* | c.2726C > A | p.Ser909* | ApoB-19 | new | Hz | 0.604 |
| 4 | *APOB* | c.2726C > A | p.Ser909* | ApoB-19 | new | Hm | 0.734 |
| 14 | *APOB* | c.2726C > A | p.Ser909* | ApoB-19 | new | Hz | 0.158 |
| 11 | *APOB* | c.2781delT | p.Ser928Profs*25 | ApoB-20 | new | Hz | 0.845 |
| 5 | *APOB* | c.3843-2A > G | NA | | CS072139 [42] | Hz | 0.986 |
| 15 | *APOB* | c.3997C > T | p.Arg1333* | ApoB-29 | rs121918383/CM880006 [43] | Hz | 0.974 |
| 10 | *APOB* | c.6034C > T | p.Arg2012* | ApoB-44 | rs147863759/CM962634 [44] | Hz | 0.682 |
| 3 | *APOB* | c.7600C > T | p.Arg2534* | ApoB-55 | rs145143533/CM051005 [45] | Hz | 0.942 |
| 17 | *APOB* | c.7600C > T | P.Arg2534* | ApoB-55 | rs145143533/CM051005 [45] | Hz | 0.682 |
| 16 | *APOB* | c.10430_10440delTTGACCACAAG | p.val3477Alafs*2 | ApoB-76 | new | Hz | 0.863 |
| 6 | *APOB* | c.11728G > T | p.Glu3910* | ApoB-86 | new | Hz | 0.573 |
| 20 | *SAR1B* | c.1-4482_58 + 1406del5946InsGCATGATGGCGGGT | NA | - | CG081079 [30] | Hm | 0.993 |
| 18 | *SAR1B* | c.83_84delTG | p.Leu28Argfs*7 | - | new | Hm | 0.593 |
| 19 | *SAR1B* | c.409G > A | p.Asp137Asn | - | rs28942109/CM035597 [29] | Hm | 0.931 |
| 21 | *ANGPTL3* | c.363_367delCTCAA | p.Asn121Leufs*3 | - | rs398122988/CD1110133 [31] | Hm | 1.098 |
| 22 | *ANGPTL3* | c.363_367delCTCAA | p.Asn121Leufs*3 | - | rs398122988/CD1110133 [31] | Hm | 1.046 |
| 23 | *MTTP* | c.1691T > C | p.Ile564Thr | - | CM068327 [46] | Hm | 0.863 |

HBL, hypobetalipoproteinemia. NA: not applicable.
[a] , positions are given according to the reference sequences: NM_000384 (*APOB*), NM_016103 (*SAR1B*), NM_014495 (*ANGPTL3*), and NM_000253 (*MTTP*).
[b] , positions are given according to the UniProt sequences: P04114 (*APOB*), Q9Y6B6 (*SAR1B*), Q9Y5C1 (*ANGPTL3*), and P55157 (*MTTP*).
[c] , in dbSNP (rs) and/or Human Gene Mutation Database (HGMD).
[d] , Hm, homozigous; Hz, heterozigous.

(133.3 mg/dL).

Patient 8 was a compound heterozygote for the variants c.3G > C (p.Met1Ile) and c.6627_6628delAA (p.Leu2212*). We described both mutations for the first time, and their pathogenicity was predicted by all the *in silico* prediction programs. We estimated that the second mutation generates a 50% size reduction of the ApoB protein. Data and DNA only from the father were sent, testing positive for the c.3G > C mutation (p.Met1Ile) and negative for c.6627_6628delAA (p.Leu2212*) and presenting with decreased LDLc (39 mg/dl) and ApoB (21 mg/dL). One case reported in the literature exhibited an interaction between a heterozygous ApoB mutation and an ApoE2/E2 genotype [28]. In this case, both father and proband presented with an ApoE3/E3 genotype.

Other novel mutations described in the *APOB* gene in this study were c.133C > T (p.Arg45*); c.1407C > A (p.Tyr469*); c.11728G > T (p.Glu3910*); c.2781delT (p.Ser928Profs*25); and c.10430_10440delTTGACCACAAG (p.Val3477Alafs*2), all of which cause a truncated protein. All the consulted *in silico* programs predicted their pathogenicity. Some previously reported mutations were also found in our cohort and have been included in Table 1 together with a designation of the ApoB truncated forms. Of the submitted relatives, five presented with the familial mutation in the *APOB* gene and therefore were genetically diagnosed with FHBL1.

In addition, some variants previously reported to be associated with more subtle changes in lipid or lipoprotein plasma levels were also identified, see Table 2. These reported variants were found in both genetically diagnosed and non-genetically-confirmed patients; of these variants, only three were found exclusively in negative patients: c.2188G > A (p.Val730Ile), c.3383G > A (p.Arg1128His), and c.3427C > T (p.Pro1143Ser).

*SAR1B mutations*: Three patients were genetically diagnosed for *SAR1B* pathogenic variants, all in homozygous form. We found one novel variant, c.83_84delTG (p.Leu28Argfs*7), and two previously reported. Only one of the patients, a 26-year-old male, was referred with biochemical data and was found to be homozygous for p. Asp137Asn. Two relatives were also diagnosed, one presented the variant c.409G > A (p.Asp137Asn) [29], and the other one presented with c.1-4482_58 + 1406del5946insGCATGATGGCGGGT [30], both of them in homozygosity.

*ANGPTL3 mutations*: As previously reported by our group, two patients were genetically confirmed as homozygous [31].

*MTTP mutations*: Only one patient was found to be homozygous for a previously described pathogenic variant.

Moreover, variants associated with subtle changes in the lipid profile were found, see Table 2.

*Results of PCSK9, MYLIP, APOC3 and DGAT1 sequencing*: Some variants potentially associated with plasma lipid level variability were found for *PCSK9, MYLIP* and *APOC3* genes, see Table 2. No variants previously associated with primary HBL were detected for the *DGAT1* gene.

*Contingency tables to evaluate the frequency distribution of three common genetic variants of the APOB gene:* Several common single nucleotide polymorphisms (SNP) associated with impaired lipid concentrations [27] were identified in those patients that tested negative for primary HBL. After performing a Fisher's exact test, three SNP presented with a statistically significantly different frequency of distribution when compared to 503 European subjects from the 1000 Genomes Project (Table 3). The *APOB* variants with statistical significance were the following: rs1367117, allele T, associated with high LDLc ($p = 0.00052$); rs1042031, allele A, associated with low LDLc ($p = 0.021$); and rs1042034, allele A, associated with high LDLc ($p = 0.0014$).

Concerning the 21 patients who could not be genetically diagnosed with primary HBL, TC was provided in 12 of them (57–114 mg/dL, average ± SD, 85.7 ± 19.9). LDLc was provided in 12 patients, all of whom had decreased values except for one patient (13–52 mg/dl, average ± SD, 29.9 ± 12.9). ApoB values were referred in 11 patients (16–50 mg/dl, average ± SD, 30.3 ± 10.0). HDLc was provided in six patients, two of whom presented with decreased values and four of whom presented with values that fell within the reference interval (17–64 mg/dl, average ± SD, 42.5 ± 16.0). TG was provided in nine patients, six of whom presented with decreased values and three of whom were normal (12–75 mg/dL, average ± SD, 38.8 ± 21.1.

### 3.1. Analysis of the weighted LDLc gene score

Regarding a possible polygenic basis of the hypolipidemia of these patients, LDLc weighted scores expressed as mean (SD) were as follows: 0.8937 (0.213) for the control population (503 subjects from the European population in the 1000 Genome Project); 0.8315 (0.212) for 23 primary HBL patients with identified mutation (HBL/M+); 0.7357 (0.325) for 20 primary HBL mutation-negative patients (HBL/M-). The gene score using the 11 SNPs was significantly different between HBL/M- and controls ($p = 0.0436$), whereas no significant differences were found between HBL/M+ and controls ($p = 0.182$) or between HBL/M- and HBL/M+ ($p = 0.268$) (Fig. 1). Six of the HBL/M- subjects (29%) presented a weighted LDLc gene score below the 10th percentile (0.623) of the control population. The results for the LDLc scores of all patients are presented in Tables 1 and 2.

## 4. Discussion

The present study presents the outcomes of our molecular diagnosis of hypolipidemic patients in a clinical laboratory setting. Although the study actually resulted from an unprogrammed clinical practice setting, it constitutes the largest experience published regarding genetic diagnosis of primary HBL in Spain. The most recent genetic studies in Spanish patients presenting with low LDLc described the clinical and genetic heterogeneity of a total of five patients with FHBL1 [32,33]. The benefits of knowing the patient genotype include identification of additional family members as well as appropriate clinical follow-up and treatment, which includes adherence to a low-fat diet and supplementation with essential fatty acids and fat soluble vitamins. Out of 44 referred patients, 23 were genetically confirmed for the studied genes with 9 new mutations described. Among them, the most prevalent mutated genes found in our study were *APOB, SAR1B*, *ANGPTL3*, and *MTTP*, with substantially fewer mutations found in these other genes. Despite their important role in lipid metabolism, Sanger sequencing did not allow identifying pathogenic variants in *PCSK9*, *APOC3*, *MYLIP*, or *DGAT1*. The inclusion of relatives in the study revealed seven further cases that were genetically confirmed. Interestingly, two of the relatives with *APOB* mutations did not present any sign of disease, demonstrating that screening for mutations in relatives should be strongly recommended because some cases might be underdiagnosed. Thus, early diagnosis is important for preventing or minimizing clinical sequelae.

There was a clear overlap between clinical and biochemical data and the affected genes involved in primary HBL. The latter further revealed that it may not be a feasible strategy to predict whether or not a patient will present with a monogenic detectable mutation by means of clinical and biochemical phenotype. However, steatosis or liposoluble vitamin deficiency would rule out mutations in *ANGPTL3* as a monogenic cause of HBL [2]. Additionally, the pattern of Mendelian inheritance in a family may direct the analysis towards *APOB* (co-dominant) or *ANGPTL3, SAR1B* and *MTTP* (recessive).

Because most of our HBL/M+ patients presented with *APOB* mutations, it is worth recalling that the clinical symptoms included hepatic steatosis, liposoluble vitamin deficiency, diarrhea, growth failure, cognitive impairment, and multineuritis. Previously published data described *APOB* heterozygous mutation carriers as asymptomatic or with increased susceptibility to diseases that may be underdiagnosed for producing few symptoms, such as liver steatosis [1]. The symptomatology observed in some of our patients may be a consequence of a

**Table 2**
Variants of unknown significance associated to genetically and non-genetically diagnosed patients.

| Patient | Gene | DNA[a] | Protein[b] | ID[c] | Associated with[d] | Proband[e] | LDLc score |
|---|---|---|---|---|---|---|---|
| 1 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 0.841 |
| | | c.11248C > T | p.His3750Tyr | new | | Hz | |
| 2 | *APOB* | c.293C > T | p.Thr98Ile | rs1367117/CM020623 | Cholesterol [48,49] | Hz | 0.604 |
| 3 | *APOB* | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hm | 0.942 |
| 5 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 0.986 |
| | | c.8216C > T | p.Pro2739Leu | rs676210/CM980093 | Hypocholesterolemia [51,52] | Hz | |
| 6 | *APOB* | c.8216C > T | p.Pro2739Leu | rs676210/CM980093 | Hypocholesterolemia [51,52] | Hz | 0.573 |
| | | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hz | |
| 7 | *APOB* | c.293C > T | p.Thr98Ile | rs1367117/CM020623 | Cholesterol [48,49] | Hz | 1.035 |
| | | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | |
| | | c.8216C > T | p.Pro2739Leu | rs676210/CM980093 | Hypocholesterolemia [51,52] | Hz | |
| | | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hz | |
| 8 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hm | 0.935 |
| | | c.1254G > A | p.Glu4181Lys | rs1042031/CM068651 | Ischemia and stroke [53,54] | Hz | |
| | | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hm | |
| 9 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 1.088 |
| | | c.3740A > G | p.Tyr1247Cys | rs61741164 | Possibly damaging | Hz | |
| | | c.11245C > T | p.His3750Tyr | new | | Hz | |
| | | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hm | |
| | | c.13451C > T | p.Thr4484Met | rs12713450/CM104784 | Hypertrigliceridemia [55] | Hz | |
| 15 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 0.974 |
| 16 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47]**7** | Hz | 0.863 |
| | | c.11248C > T | p.His3750Tyr | new | | Hz | |
| 17 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 0.682 |
| | | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hz | |
| 19 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 0.931 |
| 21 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 1.098 |
| | | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hm | |
| 22 | *APOB* | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hm | 1.046 |
| 23 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 0.863 |
| 24 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 1.102 |
| | | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hm | |
| | *MYLIP* | c.1025A > G | p.Asn342Ser | rs9370867/CM116474 | High degrad. of LDLR [56]<br>No effect [57,58] | Hz | |
| | *APOC3* | c.*71G > T | NA | rs4225/CR1611071 | ↓TG concentration [59] | Hm | |
| 25 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 0.413 |
| | | c.8216C > T | p.Pro2739Leu | rs676210/CM980093 | Hypocholesterolemia [51,52] | Hz | |
| | | c.13013G > A | p.Ser4338 Asn | rs1042034/CM133911 | Total cholesterol [50] | Hz | |
| | *PCSK9* | c.43_44insCTG | p.Leu23_Gly24insLeu | rs35574083/CI102605/CE062370 | Low LDLc [60,61] | Hz | |
| | | c.137G > T | p.Arg46Leu | rs11591147/CM061168 | Low LDLc, ↓risk CHD [62] | Hz | |
| | | c.158C > T | p.Ala53Val | rs115836807CM102603 | Hypocholesterolemia [60] | Hz | |
| | | c.1420G > A | p.Val474Ile | rs562556/CM045990 | LDLc [63,64] | Hz | |
| | *MTTP* | c-101-478G > T | Not applicable | rs1800591/CR982417 | Low LDLc, SNAFLD [65,66] | Hz | |
| | | c.891G > C | p.Gln297His | rs2306985/CM1510847 | Low LDLc [65] | Hz | |
| | | c.383T > C | p.Ile128Thr | rs3816873 | | Hz | |
| | *APOC3* | c.*71G > T | NA | rs4225/CR1611071 | ↓TG concentration [59] | Hm | |
| 26 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 0.847 |
| | | c.8216C > T | p.Pro2739Leu | rs676210/CM980093 | Hypocholesterolemia [51,52] | Hz | |
| | | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hz | |
| | *APOC3* | c.*71G > T | NA | rs4225/CR1611071 | ↓TG concentration [59] | Hm | |
| 27 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM 984191 | ApoB [47] | Hz | 0.864 |
| | | c.8216C > T | p.Pro2739Leu | rs676210/CM980093 | Hypocholesterolemia [51,52] | Hz | |
| | | c.13013G > A | p.Ser4338 Asn | rs1042034/CM133911 | Total cholesterol [50] | Hz | |
| | *PCSK9* | c.43_44insCTG | p.Leu23_Gly24insLeu | rs35574083/CI102605/CE062370 | Low LDLc [60,61] | Hz | |
| | | c.158C > T | p.Ala53Val | rs11583680/CM102603 | Hypocholesterolemia [60] | Hz | |
| | | c.1420G > A | p.Val474Ile | rs562556/CM045990 | LDLc [63,64] | Hz | |
| 28 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 0.181 |
| | | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hm | |
| | *MTTP* | c.891G > C | p.Gln297His | rs2306985/CM1510847 | Low LDLc [65] | Hz | |
| | | c.1151A > C | p.Asp384Ala | rs17029215/CM057721 | | Hz | |
| 29 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 0.896 |
| | | c.8216C > T | p.Pro2739Leu | rs676210/CM980093 | Hypocholesterolemia [51,52] | Hz | |
| | | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hz | |
| | *APOC3* | c.102T > C | p.Gly34 = | rs4520/CM942309 | ↓ plasma TG [67] | Hz | |
| | | c.*40G > C | NA | rs5128/041556 | ↑TG concentrations59 | Hz | |
| | | c.*71G > T | NA | rs4225/CR1611071 | ↓TG concentration [59] | Hz | |
| 30 | *APOB* | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hz | - |
| | *PCSK9* | c.1420G > A | p.Val474Ile | rs562556/CM045990 | LDLc [63,64] | Hz | |
| 31 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 0.963 |
| | | c.8216C > T | p.Pro2739Leu | rs676210/CM980093 | Hypocholesterolemia [51,52] | Hz | |
| | | c.13013G > A | p.Ser4338 Asn | rs1042034/CM133911 | Total cholesterol [50] | Hz | |
| | *MTTP* | c.891G > C | p.Gln297His | rs2306985/CM1510847 | Low LDLc [65] | Hm | |

(*continued on next page*)

**Table 2** (*continued*)

| Patient | Gene | DNA[a] | Protein[b] | ID[c] | Associated with[d] | Proband[e] | LDLc score |
|---|---|---|---|---|---|---|---|
| 32 | *APOB* | c.3383G > A | p.Arg1128His | rs12713843/CM040371 | "Recessive" HBL [68] | Hz | 1.100 |
| | | c.8216C > T | p.Pro2739Leu | rs676210/CM980093 | Hypocholesterolemia [51,52] | Hz | |
| | | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hz | |
| | *PCSK9* | c.43_44insCTG | p.Leu23_Gly24insLeu | rs35574083/CI102605/CE062370 | Low LDLc [60,61] | Hz | |
| | | c.137G > T | p.Arg46Leu | rs11591147/CM061168 | Low LDLc, ↓risk CHD [62] | Hz | |
| | | c.158C > T | p.Ala53Val | rs11583680/CM102603 | Hypocholesterolemia [60] | Hz | |
| | | c.1420G > A | p.Val474Ile | rs562556/CM045990 | LDLc [63,64] | Hz | |
| | *APOC3* | c.*71G > T | NA | rs4225/CR1611071 | ↓TG concentration [59] | Hm | |
| | *MTTP* | c.891G > C | p.Gln297His | rs2306985/CM1510847 | Low LDLc [65] | Hz | |
| | | c.1151A > C | p.Asp384Ala | rs17029215/CM057721 | | Hz | |
| 33 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 1.046 |
| | | c.8216C > T | p.Pro2739Leu | rs676210/CM980093 | Hypocholesterolemia [51,52] | Hz | |
| | | c.13013G > A | p.Ser4338 Asn | rs1042034/CM133911 | Total cholesterol [50] | Hz | |
| | *PCSK9* | c.43_44insCTG | p.Leu23_Gly24insLeu | rs35574083/CI102605/CE062370 | Low LDLc [60,61] | Hz | |
| | | c.158C > T | p.Ala53Val | rs11583680/CM102603 | Hypocholesterolemia [60] | Hz | |
| | *APOC3* | c.102T > C | p.Gly34 = | rs4520/CM942309 | ↓ plasma TG [67] | Hz | |
| | | c.*40G > C | NA | rs5128/041556 | ↑TG concentrations [69] | Hz | |
| | | c.*71G > T | NA | rs4225/CR1611071 | ↓TG concentration [59] | Hz | |
| 34 | *APOC3* | c.*71G > T | NA | rs4225/CR1611071 | ↓TG concentration [59] | Hz | 0.927 |
| 35 | *APOB* | c.2188G > A | p.Val730Ile | rs12691202/CM981492 | Impairment of HBL [70] | Hz | 0.601 |
| | *APOC3* | c.102T > C | p.Gly34 = | rs4520/CM942309 | ↓ plasma TG [67] | Hm | |
| 36 | *APOC3* | c.102T > C | p.Gly34 = | rs4520/CM942309 | ↓ plasma TG [67] | Hz | 0.987 |
| | | c.*71G > T | NA | rs4225/CR1611071 | ↓TG concentration [59] | Hz | |
| 37 | *APOB* | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hz | 0.740 |
| | *APOC3* | c.*71G > T | NA | rs4225/R1611071 | ↓TG concentration [59] | Hz | |
| 38 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | −0.159 |
| | | c.8216C > T | p.Pro2739Leu | rs676210/CM980093 | Hypocholesterolemia [51,52] | Hz | |
| | | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hz | |
| | *APOC3* | c.*71G > T | NA | rs4225/CR1611071 | ↓TG concentration [59] | Hz | |
| 39 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hz | 0.679 |
| | *APOC3* | c.102T > C | p.Gly34 = | rs4520/CM942309 | ↓ plasma TG [67] | Hm | |
| 40 | *APOB* | c.3427C > T | p.Pro1143Ser | rs72653077/CM087422 | Hypocholesterolemia [35] | Hz | 0.534 |
| 41 | *APOB* | c.1853C > T | p.Ala618Val | rs679899/CM984191 | ApoB [47] | Hm | 0.860 |
| | | c.8216C > T | p.Pro2739Leu | rs676210/CM980093 | Hypocholesterolemia [51,52] | Hz | |
| | | c.1254G > A | p.Glu4181Lys | rs1042031/CM068651 | Ischemia and stroke [53,54] | Hz | |
| | | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hz | |
| 42 | *APOB* | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hm | 0.991 |
| | | c.293C > T | p.Thr98Ile | rs1367117/CM020623 | Cholesterol [48,49] | Hz | |
| 43 | *APOB* | c.293C > T | p.Thr98Ile | rs1367117/CM020623 | Cholesterol [48,49] | Hz | 0.687 |
| | | c.13013G > A | p.Ser4338Asn | rs1042034/CM133911 | Total cholesterol [50] | Hm | |
| | | c.13451C > T | p.Thr4484Met | rs12713450/CM104784 | Hypertriglyceridemia [55] | Hz | |
| 44 | *APOB* | c.293C > T | p.Thr98Ile | rs1367117/CM020623 | Cholesterol [48,49] | Hz | 0.455 |

[a] Positions are given according to the reference sequences: NM_000384 (APOB), NM_016103 (SAR1B), NM_014495 (ANGPTL3), NM_000253 (MTTP), NM_174936 (PCSK9), NM_013262 (MYLIP), and NM_000040 (APOC3).

[b] Positions are given according to the UniProt sequences: P04114 (APOB), Q9Y6B6 (SAR1B), Q9Y5C1 (ANGPTL3), P55157 (MTTP), Q8NBP7 (PCSK9), Q8WY64 (MYLIP), and P02656 (APOC3).

[c] In dbSNP (rs) and/or Human Gene Mutation Database (HGMD).

[d] CHD: coronary heart disease; SNAFLD: susceptibility to non alcoholic fatty liver disease.

[e] Hm, homozigous; Hz, heterozigous.

**Table 3**
APOB common variants associated with plasma LDLc concentrations.

| SNP ID | HGMD[a] | Location[b] | DNA[b] | Protein[c] | *p*[d] | MA[e] | Freq[f] | Freq[g] | Impact[h] | Teslovich et al. (2010)[i] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | EUR | HBL/M- | | |
| rs1367117 | CM020623 | exon 4 | c.293C > T | p.Thr98Ile | 0.00052 | T | 0.298 | 0.028 | moderate | Allele T associated with high LDLc ($p$ = 4.5E-18) |
| rs1042031 | CM068651 | exon 29 | c.12541G > A | p.Glu4181Lys | 0.021 | A | 0.213 | 0.028 | moderate | Allele A associated with low LDLc ($p$ = 1.83E-20) |
| rs1042034 | CM133911 | exon 29 | c.13013G > A | p.Ser4338Asn | 0.0014 | G | 0.218 | 0.417 | moderate | Allele A associated with high LDLc ($p$ = 8.32E-22) |

[a] Identification in Human Gene Mutation Database.

[b] According to reference sequence NM_000384.

[c] According to UniProt sequence P04114.

[d] Signification in Fisher's exact test.

[e] Minor allele (MA).

[f] Frequency in the European population of the 1000 Genomes database.

[g] Frequency in hypobetalipoproteinemia mutation negative patients (HBL/M - ).

[h] Result of the *in silico* analysis regarding the impact on the functionality of the protein.

[i] Association in the GWAS of Teslovich et al. [27].

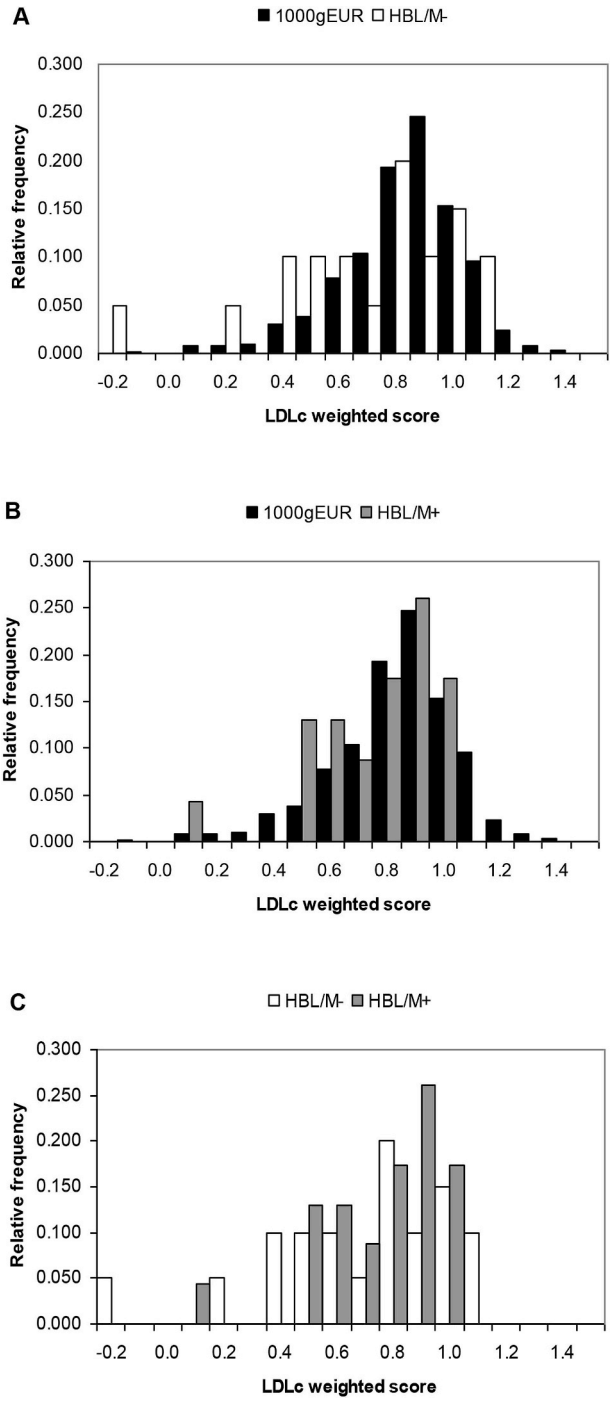


**Fig. 1.** Weighted LDLc score as mean (SD) was 0.8937 (0.213) for EUR controls, 0.8315 (0.212) for HBL/M+ patients, and 0.7357 (0.325) for HBL/M-patients. The significance of gene score between HBL/M - and controls (A), HBL/M+ and controls (B), and HBL/M - and HBL/M+ (C) was $p = 0.044$, $p = 0.182$, and $p = 0.268$, respectively.

decreased intestinal capacity for chylomicron synthesis/secretion, which is largely dependent on the conservation of the intestinal ApoB-48 sequence [34]. Protein truncation will always affect the complete liver-specific ApoB-100, indicating that the *APOB* gene will not form the full protein needed for VLDL, intermediate-density lipoprotein (IDL), LDL and Lipoprotein (a) structure and function [35].

There were 21 index patients who could not be genetically diagnosed. It is well known that up to 50% of interindividual variation in plasma LDLc is due to genetic variation by an accumulation of small-effect LDLc-impairing alleles [11,12]. In that sense, we observed a statistically significantly different frequency distribution of three *APOB* common genetic variants associated with LDLc concentration when comparing 503 subjects from the 1000 Genome Project with negative patients (HBL/M - ) (Table 3). Two of the *APOB* genetic variants could be contributing to the observed phenotype, because the allele associated with decreased levels of LDLc was more frequent in HBL/M-than in the control population. These findings led us to test the recently proposed LDLc gene score to distinguish polygenic and monogenic forms of familial hypercholesterolemia [11,12] and primary HBL [36] through a panel of SNPs that are known to influence LDLc levels. The primary HBL group without known mutation (HBL/M - ) had a significantly lower mean weighted LDLc gene score when compared to the European 1000 Genome Project participants, suggesting that some HBL/M-patients may present a polygenic inheritance pattern. It is noteworthy that in a recent work performed with 121 women with LDLc below the first percentile, around 50% of the cases could be explained by polygenic predisposition [36]. As in recent studies that have shown a polygenic basis for some cases previously identified as monogenic familial hypercholesterolemia [11,12], we did not find differences in the weighted LDLc gene of HBL/M+ and HBL/M - , suggesting that polygenicity influences phenotype expression in both forms of the disease.

This study has several limitations mainly due to the retrospective manner in which it was conducted. One is the heterogeneous degree of clinical information available according to that supplied by the clinician who requested the molecular analysis. The requests for molecular analyses were accepted although the information received was sometimes incomplete. Despite this drawback, the results allowed identifying pathogenic variants in 52% of the cases, representing a higher success rate compared to the one obtained by our group and collaborators in the case of familial hypercholesterolemia [37]. This fact argues in favor of a solid suspicion of primary HBL by the practitioners that requested the genetic study. Another limitation is that large rearrangements in the studied genes were not evaluated. According to HGMD, large rearrangements in these genes are scarce. Lastly, the idea of finding negative patients harboring other pathogenic and low-frequency variants only detectable through whole exome or genome sequencing should not be ruled out.

In conclusion, our report expands the existing molecular information relating to primary HBL, confirming the heterogenic nature and presentation of this syndrome. Although this condition has been considered a monogenic disorder, our findings support the idea that some cases may also have a polygenic origin as recently suggested [36]. The concept of polygenicity, demonstrated previously in other dyslipidemias [11,12,38,39], should be further examined in patients with clinical and biochemical data of primary HBL without monogenic disorders.

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## Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

## Author contributions

FBV, JMMC, JJ and MT devised the study. JMMC and MT did the data analysis. ABV, MC, SM, RR, and NF contributed to the genotyping.

FBV, JMMC and MT interpreted the data and wrote the manuscript. JJ also discussed the results and contributed to the final version of the manuscript.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.01.036.

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