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Mutational analysis of a cohort with clinical diagnosis of familial hypercholesterolemia: considerations for genetic diagnosis improvement

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Purpose: Familial hypercholesterolemia (FH) is a common autosomal dominant disorder of lipid metabolism caused by mutations in *LDLR*, *APOB*, and *PCSK9*. To fulfill the World Health Organization recommendation, the Portuguese FH Study was established. Here, we report the results of the past 15 years and present practical considerations concerning the genetic diagnosis of FH based on our experience.

Methods: Our approach comprises a biochemical and molecular study and is divided into five phases, including the study of whole *APOB* and functional assays.

Results: A total of 2,122 individuals were enrolled. A putative pathogenic variant was identified in 660 heterozygous patients: *LDLR* (623), *APOB* (33), and *PCSK9* (4); 8 patients presented with homozygous FH. A detection rate of 41.5% was observed. A stricter biochemical criteria was shown to improve patient identification. Overall, we have identified 3.4% and 80% of all heterozygous and homozygous patients, respectively, estimated to exist in our country.

Conclusion: The Portuguese FH Study has established the genetic diagnosis of FH in Portugal and is committed to continue the investigation of the genetic complexity of FH. Genetic diagnosis of FH should be expanded to include the study of all coding/flanking regions of *APOB* and functional in vitro studies, to improve the correct patient identification, and to avoid misdiagnosis.

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Key Words: *APOB*; familial hypercholesterolemia; functional assays; *LDLR*; *PCSK9*

INTRODUCTION

Familial hypercholesterolemia (FH) is a common autosomal dominant disorder of lipid metabolism, with a frequency between 1/200 and 1/500 for heterozygotes in most European countries.[1] Clinically, it is characterized by elevated concentrations of plasma cholesterol, which accumulates in arteries and tendons leading to premature coronary heart disease (pCHD).[2] FH most frequently results from loss-of-function mutations in the *LDLR* gene[3] representing >90% of the FH cases worldwide.[1] More than 1,600 mutations associated with FH have been described globally in *LDLR*.[4] Mutations in *APOB*[5–7] or *PCSK9* genes[8] are also associated with FH, but they are less frequent in FH patients (*APOB* <8% and *PCSK9* <3%),[9] although the number of functional *APOB* mutations has been increasing in the past 2 years.[6,7]

Because there is a genetic diagnosis and appropriate treatment to reduce the elevated cardiovascular risk of these patients, the World Health Organization has, since 1998, recommended universal screening for FH.[10] Based on this recommendation, the Portuguese FH Study was established in 1999 at the National Institute of Health, having implemented the molecular study of this pathology to promote early identification and characterization of FH patients and therefore to decrease their cardiovascular risk, through the implementation of early and correct counseling/treatment.

Here, we report the results of the past 15 years of the Portuguese FH Study, with identification and characterization of novel variants, and we also present practical considerations concerning the genetic diagnosis of FH based on our experience.

MATERIALS AND METHODS

Study design

The Portuguese FH Study is a research project supported mainly by external funds and is, for this reason, free of charge for all patients and health institutions. The study protocol and database have been approved by the National Institute of Health Ethics Committee and the National Data Protection Commission, respectively. Written informed consent was obtained from all participants before their inclusion in the study. During these past 15 years, patients with a clinical diagnosis of FH have been recruited all over the country by clinicians from several specialties. When a pathogenic variant is identified in a patient, the clinician is notified and asked to perform cascade screening in other relatives with and without a clinical diagnosis of FH for co-segregation analysis.

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For clinical criteria analysis, the study has been divided into three periods: implementation (period 1), exploratory (period 2), and consolidation (period 3). In periods 1 and 3, the majority of the patients fulfilled the Simon Broome (SB) criteria, as previously described[11]: total cholesterol >260 mg/dl or low-density lipoprotein cholesterol (LDL-C) >155 mg/dl for children, or total cholesterol >290 mg/dl or LDL-C >190 mg/dl for adults, as well as a family history of hypercholesterolemia or pCHD. In the exploratory period it was decided to include patients referred as having a clinical diagnosis of FH but with a milder phenotype than that indicated by the SB criteria.

The Portuguese FH study includes a biochemical and a molecular study and is divided into five phases (**Figure 1**).

Biochemical characterization of lipids and lipoproteins

Fasting blood samples were collected from all individuals at the time of their inclusion in the study. Total cholesterol (TC), direct LDL-C, high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), apolipoprotein A1 (apoA1), apolipoprotein B (apoB), and lipoprotein(a) (Lp(a)) were determined for all individuals in a Cobas Integra 400 plus (Roche, Risch-Rotkreuz, Switzerland) by enzymatic colorimetric and immunoturbidimetric methods.

Molecular analysis

Phase I includes DNA extraction, screening for the most common *APOB* mutations (fragments of exons 26 and 29), and molecular study of the promoter, splicing, and coding regions of the *LDLR* gene. Phase II includes the study of large rearrangements by multiplex ligation-dependent probe amplification (MLPA) technique.[12] Phase III includes the study of five exons (1, 2, 4, 7, 9) and flanking regions of *PCSK9*, where the putative *PCSK9* mutations have been described, and, in severely affected patients, the whole study of this gene. Phase IV comprises the study of promoter, all exons, and flanking regions of *APOB* in selected patients.[7] Phase V comprises the functional in vitro studies. Phases I and II are always executed for all patients, and phases III and IV are performed only if no putative mutation is detected in the previous phases. Phase V is pursued only for missense mutations, in-frame deletions/insertions (delins), and splicing variants when functional assays have not been performed and external funding is available.

The variants reported were considered "novel" when they were not listed in the University College London LDLR FH database (http://www.ucl.ac.uk/ldlr/LOVDv.1.1.0/, https://grenada.lumc.nl/LOVD2/UCL-Heart/home.php) or in the Human Gene Mutation Database accessed in March 2015.

For sequence analysis, the reference sequences used for *LDLR*, *APOB*, and *PCSK9* were, respectively, NM_000527.4, NM_000384.2, and NM_174936.3, and cDNA numbering was considered following the Human Genome Variation Society nomenclature, with nucleotide c.1 being A of the ATG initiation codon p.1. Variant nomenclature was revised using the program Mutalyzer (https://mutalyzer.nl). Variants were classified as pathogenic, likely pathogenic, benign, likely benign, or variant of unknown significance (VUS), according to the American College of Medical Genetics and Genomics Practice 2015 Guidelines.[13]

In silico analysis

The following software tools were applied to all variants, without functional studies, that were identified in our cohort for the first time since our last report: PolyPhen-2,[14] Sorting Tolerant From Intolerant (SIFT),[15] and Mutation Taster[16] for prediction of amino acid substitutions; and Splice-Site Predictor (Splice Port),[17] Neural Network Splice Site Prediction Tool,[18] and Neural Network Predictions of Splice Sites in Human (NetGen2)[19] for prediction of splicing defects. For variant classification, it was considered that a variant had a deleterious effect when all three software tools had a prediction of being pathogenic (probably damaging, deleterious, disease-causing, or <80% for splicing variants). If this was observed, then it was considered to be supportive evidence for variant classification following the American College of Medical Genetics and Genomics Guidelines.

Allele frequency analysis

If a variant has been described in more than 5% of the studied population (minor allele frequency >5%) in the 1000 Genomes database (1KG)[20] or the Exome Sequencing Project (http://evs.gs.washington.edu/EVS/) database,[21] then the variant was considered to be a common variant or a polymorphism and, therefore, a neutral variant.[22]

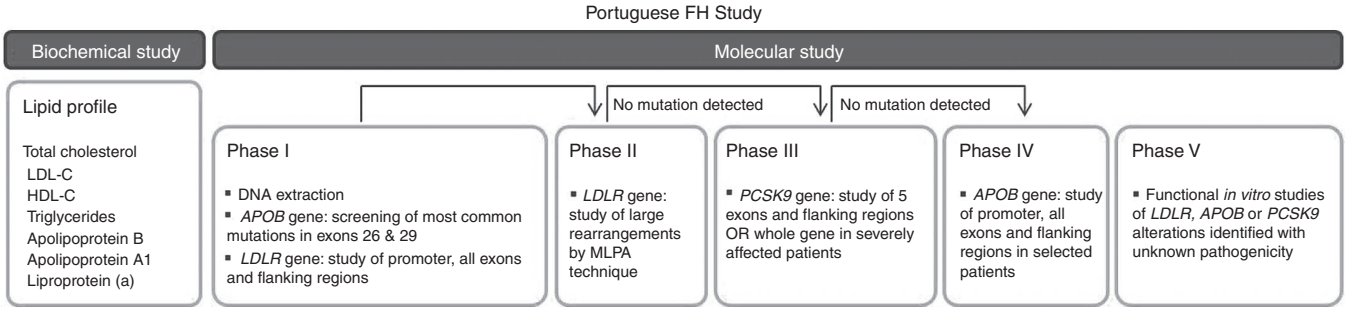

Figure 1 Portuguese familial hypercholesterolemia (FH) study workflow including the biochemical and molecular study of the genetic diagnosis of FH in Portugal.

For the majority of variants, screening of 95 normolipidemic individuals was also performed. When encountered in more than 1% of the normolipidemic population, the variant was considered to be a polymorphism.[23]

Statistical analysis

Statistical analysis was performed using SPSS software (version 22.0 for Windows; SPSS, Chicago, IL). Mean values of quantitative variables were compared with the Student's *t*-test for independent data, whereas median values were compared with the non-parametric Mann-Whitney tests. $P < 0.05$ was considered statistically significant.

Functional assays of LDLR synonymous variants

Total mRNA was obtained from freshly isolated blood mononuclear cells of the patients carrying these variants. The effect of the synonymous variants c.1911C>T, c.1920C>T, and c.1977C>A identified in the *LDLR* was investigated by direct sequencing of a reverse-transcription polymerase chain reaction product, as described previously.[12,24]

RESULTS

Portuguese FH study—15 years

A total of 2,122 individuals have been enrolled in the Portuguese FH Study, including 725 unrelated index patients (293 children and 432 adults) with a clinical diagnosis of FH. A total of 68 clinicians from 52 hospitals and clinics, public and private, from different clinical specialties, have referred clinical FH patients to the Portuguese FH Study (**Supplementary Figure S1** online) during these past 15 years. The majority of the patients are of Portuguese nationality but a small number are from other countries (5%), mostly African Portuguese-speaking countries (3%).

In our study, 88% (378/432) of the adults and 98% (288/293) of the children had undergone lipid screening before their inclusion in the study and a complete fasting lipid profile was performed at our institute for all patients referred to the study (**Table 1**).

In 301 of the 725 index patients a putative pathogenic variant was identified, giving a positive detection rate of 41.5%. A total of 367 relatives were also identified with one of these putative pathogenic variants; however, cascade screening has been successfully performed in only 66% of the families (198/301), giving an average rate of two affected relatives per index case. If cascade screening was performed by SB clinical criteria, then only 54% of the adult patients and 80% of the children would have been identified.

A total of 11 patients have tendon xanthomas and therefore have a clinical diagnosis of definite FH following SB criteria; however, only 82% (9/11) have a molecular diagnosis of FH.

Clinical criteria analysis

The Portuguese FH study has been divided into three periods for clinical criteria analysis.

Table 1 Demographic, biochemical, and clinical data of the clinical FH patients, as well as positive detection rate, during different periods of the Portuguese FH study

	Period 1 (1999–2005)	Period 2 (2006–2011)	Period 3 (2012–2014)
Pediatric patients	*N* = 37	*N* = 213	*N* = 45
Detection rate (%)	43.2	32.4	57.8
Age (years)	10.0 ± 3.7	10.3 ± 4.7	9.3 ± 4.5
Gender (% male)	43.2	43.9	28.9
Tendon xanthomas (%)	0	0	0
CHD (%)	0	0	0
Smokers (%)	0	0	0
Using medication (%)	59.5	28.2	28.9
Lipid profile (mg/dl)	*N* = 35	*N* = 180	*N* = 40
TC	292.1 ± 62.6	269.4 ± 52.6*,†	292.9 ± 55.2
LDL-C	222.3 ± 62.0	193.4 ± 49.6*,†	219.1 ± 52.2
HDL-C	55.2 ± 14.7	58.5 ± 17.8	58.8 ± 23.0
TG	93.5 ± 43.4	96.3 ± 53.7	89.0 ± 36.1
	N = 14	*N* = 142	*N* = 10
apoA1	145.3 ± 28.7	151.1 ± 29.2†	130.1 ± 18.5
apoB	140.2 ± 69.3	96.2 ± 22.5	128.4 ± 27.7
apoB/apoA1 ratio	1.0 ± 0.4	0.7 ± 0.3*,†	1.0 ± 0.3
Adult patients	*N* = 89	*N* = 294	*N* = 49
Detection rate (%)	74.7%	33.0%	46.9%
Age (years)	46.9 ± 14.1	43.6 ± 13.5	41.1 ± 11.3
Gender (% male)	33.3	47.3	56.0
Tendon xanthomas (%)	11.1	0.7	0
CHD (%)	27.8	21.6	33.3
Smokers (%)	23.5	17.0	4.9
Using medication (%)	92.2	80.6	65.3
Lipid profile (mg/dl)	*N* = 65	*N* = 200	*N* = 32
TC	381.7 ± 78.7	329.2 ± 81.0*	332.5 ± 62.3‡
LDL-C	289.0 ± 85.9	231.0 ± 62.4*	239.6 ± 52.9‡
HDL-C	53.6 ± 15.7	55.8 ± 16.6	54.3 ± 17.4
TG	158.8 ± 74.9	150.1 ± 66.1	148.8 ± 84.7
	N = 7	*N* = 51	*N* = 2
apoA1	152.6 ± 36.6	165.0 ± 48.4	168.5 ± 49.6
apoB	128.9 ± 77.4	116.1 ± 37.5	175.1 ± 43.2
apoB/apoA1 ratio	1.5 ± 1.0	1.0 ± 1.9*	1.2 ± 0.2

Data are expressed as mean ± standard deviation (SD) unless otherwise noted. Biochemical profile refers to pretreatment values.

CHD, coronary heart disease; FH, familial hypercholesterolemia; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride.

*$P < 0.05$ period 1 versus period 2. †$P < 0.05$ period 2 versus period 3.
‡$P < 0.05$ period 1 versus period 3.

The FH genetic/clinical identification rate has been determined for each period: overall; children; and adults. Period 1 had positive rates of 59.5%, 43.2%, and 74.7%; period 2 had positive rates of 35.3%, 32.4%, and 33.0%; and period 3 had positive rates of 52.1%, 57.8%, and 46.9%, respectively (**Table 1**; **Supplementary Figure S2** online).

The mean TC, LDL-C, HDL-C, TG, apoA1, apoB, and apoB/apoA1 ratio of each period as well as other demographic and clinical data are presented in **Table 1**.

Pediatric patients of periods 1 and 3 revealed a severe phenotype (no statistically significant differences between them). Pediatric patients of period 2 revealed a less severe phenotype with significantly lower TC, LCL-C, and apoB/apoA1 ratio mean values compared with pediatric patients of period 1 (TC, $P = 0.022$; LDL-C, $P = 0.006$; apoB/apoA1 ratio, $P = 0.002$) and period 3 (TC, $P = 0.003$; LDL-C, $P = 0.002$; apoA1, $P = 0.027$; apoB/apoA1 ratio, $P < 0.001$) (**Table 1**). Almost all mutation-positive pediatric patients fulfilled SB criteria (96.7%), but only approximately 80% of mutation-negative patients did so (**Supplementary Table S1** online).

Adult patients of period 1 revealed a more severe phenotype than adult patients of period 3 with significantly higher TC ($P = 0.001$) and LDL-C ($P = 0.002$) mean values. Adult patients of period 2 revealed a less severe phenotype with significantly lower TC, LDL-C, and apoB/apoA1 ratio mean values compared with adult patients of period 1 (TC, $P < 0.001$; LDL-C, $P < 0.001$; apoB/apoA1 ratio, $P = 0.006$), but no statistically significant differences were obtained when compared with adult patients of period 3 (**Table 1**).

The majority of mutation-positive adult patients fulfilled SB criteria (81.5%), but only approximately 58% of mutation-negative patients did so (**Supplementary Table S1** online).

Update on genetic variants

To date, a total of 140 different variants have been found in the Portuguese FH cohort. The majority were identified in the *LDLR* (123), followed by *APOB* (15) and *PCSK9* (2) genes. Since our last report in 2010,[12] 55 variants (22 novel; **Table 2**) have been identified for the first time in the Portuguese population: 41 in *LDLR* (19 missense, 1 nonsense, 5 splice site, 7 synonymous, 2 large rearrangement, and 7 insertions/deletions), 1 in *PCSK9*, and 13 in *APOB*. From these 55 variants, 23 are classified as pathogenic (1 nonsense, 6 frameshift, 2 large rearrangements, 4 splicing, 9 missense, and 1 in-frame deletion; 6 of these with functional assays proving their pathogenicity) and 6/55 missense variants are classified as likely pathogenic (**Supplementary Table S2a,b** online). A total of 19/55 variants are classified as benign or likely benign (**Supplementary Table S2c,d** online), including 10 proven to be non-pathogenic. In *LDLR,* one missense variant has already been described and has been proven to be benign,[25] and another has been described as non-disease-causing by other authors.[26] For three synonymous variants, functional studies are reported here for the first time and were proven to be non-pathogenic, and one splicing variant has been recently proven not to be a disease-causing mutation.[27] In *APOB*, four have been shown to be present in more than 1% of the normolipidemic subjects panel and, for this reason, are considered to be a polymorphism.[7] In *PCSK9*, one variant has been described as non-pathogenic.[28] The remaining 7/55 (**Supplementary Table S2e** online) are VUS, three in *LDLR* and four in *APOB*, that have never been reported in other populations and require further evidence, such as functional studies, to be classified as pathogenic variants. In silico and minor allele frequency analysis predict that four out of seven are neutral variants that do not affect the function of the protein involved, and one out of seven is classified as probably or possibly pathogenic by in silico analysis. The remaining two VUS are inframe deletions.

Heterozygous FH patients

A genetic defect was identified in a total of 660 patients (293 index patients and 367 relatives): 623 patients had a putative disease-causing variant in the *LDLR*, 33 had a putative disease-causing variant in *APOB*, and 4 had a putative disease-causing variant in *PCSK9*. These numbers include patients with a variant classification of pathogenic, likely pathogenic, likely benign, benign, and VUS, but exclude all variants so far proven to be benign by functional studies or minor allele frequency analysis.

Homozygous FH patients

Clinical characteristics of the eight homozygous FH patients found in our cohort are presented in **Table 3**: five are compound heterozygous and three are true homozygous. In general, Portuguese homozygous FH patients show a milder phenotype when compared with homozygous patients from other European populations.[29,30] Their LDL-C values were between 300 and 400 mg/dl, prevalence of pCHD was 30%, mean age of first event was 35 years, and there was absence of tendon xanthomas.

The most severely affected patient is a young man who had a myocardial infarction in the third decade of life (22 years old), although he is a heavy smoker. He has three different variants, two of which are in the same allele and have not been proven to be functional mutations, but based on in silico and minor allele frequency analysis these are predicted to be pathogenic. All homozygous patients are receiving lipid-lowering treatment; 75% use a combination of statins and ezetimibe and 25% are also on LDL apheresis (**Table 3**).

Mutation negative patients

A total of 424 index patients did not present an identifiable putative disease-causing variant in *LDLR*, in fragments of exons 26 and 29 of *APOB*, or in the *PCSK9* gene, including 20 patients with a proven benign variant (4.7%). The biochemical profile of mutation-negative patients was compared to mutation-positive patients and is presented in **Table 4**. The majority of these patients have a clinical FH phenotype, presenting mean LDL-C values above the 95th percentile, adjusted for age and sex, and a family history of hypercholesterolemia and/or pCHD, although

Table 2 *LDLR* and *APOB* variants identified in the Portuguese cohort during 2010–2014 and not reported previously in our population

Gene	Location	Nucleotide change	Protein	Number of carriers
Pathogenic variants				
LDLR	Exon 3	c.236dup	p.(Asn80Glnfs*50)	4
LDLR	Exon 3	c.310_313del	p.(Cys104Profs*101)	2
LDLR	Intron 10	c.1586+2T>A	p.(?)	1
LDLR	Exon 11	c.1587-?_1845+?del	p.(?)	1
LDLR	Exon 13	c.1846-?_2311+?dup	p.(?)	4
LDLR	Exon 17	c.2397_2412del	p.(Val800Glyfs*124)	2
APOB	Exon 29	c.13158del	p.(Glu4387Asnfs*7)	2
Likely pathogenic variants				
LDLR	Exon 4	c.666C>G	p.(Cys222Trp)	1
LDLR	Exon 5	c.799G>A	p.(Glu267Lys)	3
LDLR	Exon 8	c.1088C>A	p.(Thr363Asn)	1
LDLR	Exon 12	c.1802A>T	p.(Asp601Val)	4
Benign variants				
LDLR	Exon 13	c.1977C>A	p.(=)	6
Likely benign variants				
LDLR	Exon 9	c.1279A>C	p.(=)	7
LDLR	Exon 10	c.1383C>T	p.(=)	2
LDLR	Exon 10	c.1417A>G	p.(Ile473Val)	3
LDLR	Exon 13	c.1911C>T	p.(=)	1
LDLR	Exon 15	c.2291T>C	p.(Ile764Thr)	3
Variants of unknown significance (VUS)				
LDLR	Exon 11	c.1618_1620del	p.(Ala540del)	1
LDLR	Exon 12	c.1833_1841del	p.(Leu611_Val613del)	1
LDLR	Exon 13	c.1960C>T	p.(Leu654Phe)	1
APOB	Exon 26	c.11401T>A	p.(Ser3801Thr)	1
APOB	Exon 24	c.11477C>T	p.(Thr3826Met)	3

Variants were classified as pathogenic, likely pathogenic, benign, likely benign, and of unknown significance (VUS) according to American College of Medical Genetics and Genomics 2015 guidelines.[14]

27.5% also have apoB >120 mg/dl, which can indicate that these patients have familial combined hyperlipidemia[31] and could therefore have been misclassified as having FH.

DISCUSSION

A total of 668 Portuguese patients with a clinical diagnosis of FH have been identified with a putative disease-causing variant in one of the three genes associated with FH. However, only 503 patients have an established functional defect (null allele or defective allele proven by functional assays); in the remaining patients, a VUS or a likely benign variant has been identified. Less than one-third of the missense and splicing *LDLR* variants found in our cohort have functional studies (30%) proving their pathogenicity, although the majority of these variants have been described worldwide. Even so, this represents a better scenario than the worldwide reality, as can be confirmed in the FH database.[32] Analysis of the LDLR FH database (http://www.ucl.ac.uk/ldlr/LOVDv.1.1.0) has revealed that only 13% (83/651) of all reported missense, nonsense, and splicing variants have functional studies. The lack of in vitro analysis for the majority of the missense and splicing variants described internationally can lead to FH misdiagnosis[27] and therefore represents a serious problem for FH diagnosis.

As described, a higher prevalence of *LDLR* variants and a lower number of variants in *APOB* and *PCSK9* were observed.[9] However, the number of novel functional *APOB* mutations is increasing.[6,7] From 2010 until this report, a total of 13 novel *APOB* variants have been identified in our cohort: two have already been proven to be disease-causing variants, four have been shown to be polymorphisms,[7] and seven are rare variants that need to be characterized by in vitro studies, including the three novel variants reported here for the first time (p.(Ser3801Thr), p.(Thr3826Met) and p.(Glu4387Asnfs*7)). Although in silico analysis classifies three of the six reported rare missense variants in *APOB* as non-pathogenic, it is known that in silico analysis for complex proteins such as APOB is less

Table 3 Clinical and biochemical characterization of homozygote patients in the Portuguese familial hypercholesterolemia study

	Demographic		Clinical data			Basic lipid profile (mg/dl)				Alterations	
ID	Gender	Age (years)	CHD (age)	Xanthomas		TC	LDL-C	HDL-C	TG	c.DNA	Protein
20055	F	55	No	No	*pre*	—	—	—	—	c.[1285G>C];[1285G>C]	p.[Val429Leu);[(Val429Leu)]†
					on[a]	299	232	47	99		
21026	F	36	No	No	*pre*	490	435	42	64	c.[313+6C>T];[1291G>A]	p.[Leu184Serdel41]†;[(Ala431Thr)]†
					on[b]	355	280	51	219		
26048	F	25	No	No	*pre*	596	512	65	96	c.[1291G>A];[1291G>A]	p.[(Ala431Thr)];[(Ala431Thr)]†
					on[a]	370	299	54	84-		
26061	M	29	MI (23 years)	No	*pre*	561	515	—	—	c.[631C>G;1816G>T];[1178delA]	p.[(His211Asp)‡;(Ala606Ser)‡];[(Lys393Argfs*20)]†
					on[c]	346	298	29	96		
27023	M	55	MI (48 years)	Xanthelasma	*pre*	—	—	—	—	c.[670G>A];[2146G>A]	p.[(Asp224Asn)]‡;[(Glu716Lys)]‡
					on[b]	409	299	37	363		
29010	F	21	No	No	*pre*	345	210	51	—	c.[1216C>T];[c.1216C>T]	p.[(Arg406Trp)];[(Arg406Trp)]†
					on[b]	258	186	50	79		
11278	M	46	No	No	*pre*	600	—	—	—	c.[1633G>T];[c.1775G>A]	p.[(Gly545Trp)]‡;[(Gly592Glu)]†
					on[d]	185	108	63	36		
12040	F	61	Angina (50 years)	No	*pre*	587	401	69	67	c.[1060-?_1705+?del];[1216C>T]	p.[?];[(Arg406Trp)]†
					on[c]	389	232	62	112		

CHD, coronary heart disease; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; MI, myocardial infarction; pre, pretreatment values; TC, total cholesterol; TG, triglycerides; on, on treatment values with: [a]statin; [b]statin plus ezetimibe; [c]statin, ezetimibe and LDL apheresis; [d]statin, ezetimibe, and nicotinic acid. Variants classification: †pathogenic, assessment by functional assays; ‡probably pathogenic, assessment by in silico analysis.

likely to predict the correct functional effect.[33] Only in vitro functional studies will determine the pathogenicity of these recently found variants. To improve the genetic diagnosis of FH, our group is attempting to functionally characterize all variants found in our cohort; functional studies of novel variants in *LDLR*, *APOB*, and *PCSK9* are underway and will provide further characterization of the mutational complexity of FH patients.

Before the start of the Portuguese FH Study, not much was known about FH in Portugal. In the first years, using an adaptation of SB criteria, the study obtained a high positive rate of FH in adult patients (74.7%) but a lower rate for FH in children (43.2%). This means that the cause of the dyslipidemia in these children can be environmental instead of inherited, and therefore have been misclassified.[34] Afterwards, we conducted an exploratory period during which patients referred as having a clinical diagnosis of FH but with milder phenotypes than indicated by the SB criteria were included in the molecular study. We intended to investigate whether the phenotype of Portuguese FH patients was the same as reported in other European populations, or milder, because the homozygous patients identified, and presented here, seemed to have a milder phenotype than similar patients in other populations. During this phase it was observed that the positive rate declined between 10% (pediatric group) and 40% (adult group), leading us to conclude that Portuguese FH patients do not have a milder phenotype and, actually, that stricter biochemical clinical criteria would improve patient identification and would therefore be more cost-effective. In fact, for pediatric patients, we have already proposed to increase the LDL-C cut-off value to 190 mg/dl and to include an apoB/apoA1 ratio >0.68 in clinical criteria, because both biomarkers optimized the criteria's specificity and sensitivity and improved patient identification.[34] This can be seen during period 3, when these criteria have been used to select children for molecular study, resulting in an overall improvement of the positive rate (32.4–57.8%) in pediatric patients, reaching a positive rate of nearly 90% in 2014. In adult patients, we observed only a small improvement in the positive rate (13%), from period 2 to period 3, revealing that we have to be even stricter with the biochemical criteria applied to select adult patients for molecular study. For both pediatric and adult groups, the rate of mutation-positive patients that fulfilled SB criteria is more than 80% (mutation-positive children fulfilling SB criteria almost reaches 100%); however, the number of mutation-negative patients who fulfilled SB criteria is also high (pediatric group, 78.8%; adult group, 58.1%) and no putative disease-causing variant was found in these patients. Based on our previous studies,[7] we speculate that up to 10% of our clinical FH patients without an identifiable disease-causing variant might have an unknown functional mutation in *APOB*. In the past 2 years, five novel mutations outside the two fragments commonly studied in routine diagnosis were described and were proven to be pathogenic variants.[6,7,35] Analysis of all exons and flanking regions of *APOB* in our entire cohort is underway. Nevertheless, we believe that the

Table 4 Biochemical profile of mutation-positive and mutation-negative patients in the Portuguese familial hypercholesterolemia study

	Mutation-positive	Mutation-negative	*P* value
Pediatric patients			
Lipid profile (mg/dl)	*N* = 110	*N* = 140	
TC	304.0 ± 58.1	255.0 ± 41.9	**<0.001****
LDL-C	228.9 ± 54.5	178.7 ± 39.4	**<0.001****
HDL-C	53.7 ± 15.57	61.7 ± 19.5	**<0.001****
TG	90.1 ± 49.4	98.6 ± 50.6	0.186**
	N = 64	*N* = 102	
apoA1	139.0 ± 24.4	155.8 ± 29.7	**<0.001***
apoB	116.9 ± 28.4	92.4 ± 31.3	**<0.001****
apoB/apoA1 ratio	0.9 ± 0.3	0.6 ± 0.2	**<0.001****
Adult patients			
Lipid profile (mg/dl)	*N* = 120	*N* = 170	
TC	372.4 ± 82.5	317.0 ± 71.6	**<0.001****
LDL-C	283.4 ± 80.4	217.0 ± 46.3	**<0.001****
HDL-C	52.2 ± 15.2	57.5 ± 16.8	**0.008****
TG	145.2 ± 68.9	156.7 ± 70.6	0.197**
	N = 25	*N* = 40	
apoA1	157.8 ± 38.7	168.7 ± 49.8	0.408**
apoB	166.5 ± 59.7	121.4 ± 41.0	**<0.001****
apoB/apoA1 ratio	1.1 ± 0.7	1.0 ± 2.1	**0.001****

Data are expressed as mean ± standard deviation (SD) unless otherwise noted. Biochemical profile refers to pre-treatment values.

LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride.

*Student's *t*-test. **Mann-Whitney test. $P < 0.05$ was considered statistically significant.

majority of our mutation-negative patients will probably not have a monogenic disorder of lipid metabolism and possibly have a polygenic form of hypercholesterolemia, because they present significantly lower values for all lipid parameters when compared with mutation-positive patients. The gene score for polygenic dyslipidemia suggested by Talmud et al.[36] will be tested in our cohort. We also believe that a small fraction of our mutation-negative patients with a severe phenotype may have a defect in an unknown gene, although the majority of exome studies worldwide have not been able to identify these genes.[6,37] Another option is the existence of variants affecting known or unknown genes through an undescribed mechanism as an epigenetic phenomenon.

Based on the estimated frequency of heterozygous FH in Europe, Portugal should have at least 20,000 cases, but, despite all the efforts of our team, the present study identified only 3.4% of the heterozygous FH patients estimated to exist in our country, reflecting that FH, as in many other countries, is severely underdiagnosed.[1] However, 8 out of the 10 expected Portuguese FH homozygous patients, including true homozygotes and compound heterozygotes, have been identified during these past 15 years, which led us to conclude that the remaining must have died without diagnosis. In fact, through a child identified with heterozygous FH carrying a large rearrangement, it is suspected that her uncle, who died at the age of 8 years with a diagnosis of "blood problems," had homozygous FH, because both grandparents presented the same pathogenic variant. All of the identified homozygous patients are being treated with lipid-lowering drugs, but none has achieved the recommended LDL-C targets (<100 mg/dl or <70 mg/dl for patients with CHD).[30] Also, even though LDL apheresis is totally cost-free in our country, only two patients are on LDL apheresis, maybe due to the fact that this treatment is available at only two centers: one in Lisbon, the other in Oporto. Different therapeutic approaches are necessary to decrease the elevated cardiovascular risk of these patients.

The molecular analysis of FH patients needs to be expanded to include, at least, the study of all coding sequence, promoter, and splice site regions of *LDLR*, *APOB*, *PCSK9*, and probably *APOE*, due to the recent deletion described.[38] The inclusion of *LDLRAP1* in this panel could be an option as well, because it has not been established if heterozygous mutations can cause severe hypercholesterolemia.[9] With the advance of next-generation sequencing platforms, this can be easily performed by target exome sequencing. Because the inclusion of other lipid genes in a target exome sequencing panel does not greatly increase its cost, a broader panel with a selection of lipid genes,[39] as the several described to be associated with familial combined hyperlipidemia, and the polymorphisms used in the lipid gene score[36] could also be included in an FH genetic diagnosis panel to improve the clarification of the underlying cause of the dyslipidemia in patients with an FH phenotype. A basic panel with the five genes stated previously is being developed in our group, and an enlarged panel is being planned for the near future.

The Portuguese FH Study has established the genetic diagnosis of FH in Portugal and is committed to continue to investigate the genetic complexity of FH. For a more cost-effective FH identification, a case-finding program and a cascade-screening program need to be established. The collaboration of health authorities to tackle this important public health problem will be vital because the referral through the National Health Service is already possible (any National Health Service clinician can order this genetic test), but it is not routinely performed; this referral could improve patient identification. In 2013, the Directorate-General of Health has published a recommendation advising that a lipid screening should be performed for all children before the age of 10. This recommendation can improve FH detection, but it is too soon to evaluate its efficacy. It has been proven that FH patients, if identified early and treated, can have their life expectancy increased by many years.[40] Therefore, high priority should be given worldwide for early identification of FH patients to improve cardiovascular prevention.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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DISCLOSURE

The authors declare no conflict of interest.

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