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Update of the Portuguese Familial Hypercholesterolaemia Study

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ABSTRACT

The main aim of the Portuguese Familial Hypercholesterolaemia Study is to identify the genetic cause of hypercholesterolaemia in individuals with a clinical diagnosis of Familial Hypercholesterolaemia (FH). A total of 1340 blood samples were collected from 482 index patients and 858 relatives with the collaboration of clinicians from several hospitals all over the country. The genetic diagnosis of FH in this study is based on the analyses of three genes: *LDLR*, *APOB* and *PCSK9*. In the last 10 years, the Portuguese FH Study identified a genetic defect in a total of 171 index patients, corresponding to an overall of 48% of the total received cases with clinical diagnosis of FH. Although the Simon Broome FH register criteria have been adapted to our study, 59 patients that did not fulfil all criteria were included in the study and a mutation causing disease was identified in 8 of these patients. In the *LDLR* gene were found 80 different mutations in 165 unrelated index patients: 159 heterozygous, 3 compounds heterozygous and 3 true homozygous. The *APOB* p.Arg3527Gln and the *PCSK9* p.Asp374His mutation were not found in any of our patients since our last report, but a novel mutation in the *APOB* gene, predicted to cause a single amino acid substitution p.Tyr3560Cys, was found in one patient. The cascade screening in relatives of these 171 index patients allowed the identification and genetic characterization of a total of 404 FH patients in Portugal.

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1. Introduction

Familial Hypercholesterolaemia (FH) is an autosomal dominant disorder clinically characterized by high levels of LDL-associated cholesterol in plasma. This accumulation causes its deposition on arteries and tendons leading to accelerated atherosclerosis and increased risk of premature coronary heart disease (CHD) [1].

FH usually results from mutations in three different genes involved in lipid metabolism [2]. These include, most commonly, mutations in the low density lipoprotein receptor gene (*LDLR*), less commonly in the apolipoprotein B (*APOB*) gene and rarely in the proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene [3]. At present, more than 1000 mutations have been described worldwide in the *LDLR* gene (<http://www.ucl.ac.uk/fh>) and the residual LDL receptor activity varies considerably between those [4]. Mutations in exons 26 and 29 of the *APOB* gene have been identified in FH patients, the most common of which is the nucleotide change c.10708G>A, predicted to lead to the amino acid substitution p.Arg3527Gln [5]. From the *PCSK9* mutations reported worldwide

[6] none was found in the Portuguese population except the mutation, p.Asp374His, located at the same codon as the p.Asp374Tyr described previously [6], but leading to a different amino acid substitution.

In 1999, it was first established by the Portuguese FH Study, a DNA diagnosis for patients with clinical diagnosis of FH and cascade screening in relatives of the affected index patients. This study has been based at the National Institute of Health, Lisbon, where the biochemical characterization and DNA diagnosis have been performed with no costs for the patient/medical institution partly due to a collaboration established in 2006 with the Portuguese Society of Cardiology. The number of index patients has expanded significantly since then due an increasing network of 24 clinicians located throughout several hospitals in Portugal. The genetic diagnosis of FH is performed in three phases and an optional fourth phase. Phase I comprises screening for the common mutations in *APOB* gene and analysis of *LDLR* gene. Phase II includes identification of large rearrangements in the *LDLR* gene using MLPA technique. Phase III consists in screening of *PCSK9* gene and is only performed if no mutation was found in phases I and II. Phase IV is only performed when putative splicing mutations, not described before or without functional studies, are found. RNA from peripheral blood mononuclear cells (PBMC) is extracted and the cDNA is analysed to identify the effect of the alteration on the splicing mechanism.

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Table 1
New LDLR mutations identified in the Portuguese FH patients since last mutation report in 2008.

Location	Nucleotide change	Protein	Predicted effect	References
Promoter	c.–135C>G		LDLR not produced	[9]
Promoter	c.–13A>G		LDLR not produced	Novel
Exon 2	[EX2_3del]		Large deletion of 2 exons	Novel^a
Intron 2	c.190+4insTG		Splicing error	Novel
Exon 3	c.274C>G	p.Gln92Glu		[10]
Exon 4	c.427T>C	p.Cys143Arg		[11]
Exon 4	c.473C>G	p.Ser158Cys		Novel
Exon 4	c.502G>C	p.Asp168His		[12]
Exon 4	c.551G>A	p.Cys184Tyr		[13]
Exon 4	c.590G>T	p.Cys197Phe		[14]
Exon 8	c.1176C>A	p.Cys392X	Stop codon	[15]
Exon 9	c.1322T>C	p.Ile441Thr		[16]
Intron 9	c.1359–5C>G		Splicing error	[9]
Exon 10	c.1325A>G	p.Tyr442Cys		[17]
Exon 10	c.1403T>A	p.Val468Asp		Novel
Exon 11	c.1599G>A	p.Trp533X	Stop codon	[18]
Exon 12	c.1756delT	p.Ser586GlnfsX78	Truncated peptide	Novel
Intron 12	c.1846–1G>A		Splicing error	[12]
Exon 13	c.1876G>A	p.Glu626Lys		[19]
Exon 13	c.1936delC	p.Leu646TyrfsX18	Truncated peptide	Novel
Exon 13	c.1966C>A	p.His656Asn		[20]
Exon 14	c.2054C>T	p.Pro685Leu		[21]
Intron 14	c.2140+1G>A		Splicing error	[22]
Exon 15	c.2146G>A	p.Glu716Lys		Novel
Exon 16	[EX16_18del]		Large deletion of 3 exons	Novel^a
Exon 16	c.2385delC	p.Pro795ProX20	Truncated peptide	Novel
Exon 16	c.2389G>T	p.Val797Leu	Splicing error	[9]

Mutations not described before are in bold.

^a Similar large rearrangements were already described but since the deletions breakpoints are unknown it is possible that the Portuguese rearrangements are different from the previously described.

2. Methods

2.1. Patients

A total of 1340 blood samples were collected from 482 index patients and 858 affected and unaffected relatives. The 482 index patients (318 adults and 164 children) were referred to our lab following the Simon Broome criteria for FH, as presented before [7,8].

2.2. Biochemical characterization

The biochemical parameters, including total cholesterol, LDL-cholesterol, HDL cholesterol, triglycerides, apolipoprotein AI, apolipoprotein B and lipoprotein (a), were determined for all individuals in an Hitachi 911 (Boehringer, Mannheim, Roche) until 2007 and in Cobas Integra 400 plus (Roche) since then, by an enzymatic colorimetric method.

2.3. DNA isolation

Genomic DNA was isolated from whole blood EDTA samples, using the Wizard[®] Genomic DNA Purification kit (Promega) according to the manufacturer's instruction.

2.4. DNA analysis

The promoter region and the 18 exons of the LDLR gene were amplified from genomic DNA by polymerase chain reaction (PCR) and screened for sequence alterations using denaturing high pressure liquid chromatography (DHPLC) and automated sequencing. Large rearrangements in the LDLR gene were identified by multiplex ligation-dependent probe amplification (MLPA) technique using the SALSA[®] MLPA[®] kit P062 LDLR (MRC-Holland). Screening for the common mutations in the APOB gene was performed by PCR amplification and direct sequencing of exons 26 and 29. The

12 exons of PCSK9 gene were amplified by PCR and analysed by direct sequencing. All the described techniques were performed as reported before [8].

2.5. RNA extraction and reverse transcriptase (RT) reaction

PBMC were isolated from 8 ml of fresh blood collected in CPT tube (cell preparation tube with sodium citrate, BD Vacutainer[®]) by centrifugation for 30 min at 2800 rpm, 4 °C (separation of PBMC must occur during the first 2 h after collection). The upper layer containing plasma and PBMC was transferred to a 15 ml falcon tube. The PBMC were obtained by centrifugation for 10 min at 1400 rpm, 4 °C. The mononuclear cells (pellet) were then washed with PBS by centrifugation as above. Total RNA was extracted with the RNeasy[®] Mini Kit (Qiagen), including the optional DNase I incubation as described in the kit protocol. This procedure yielded 6.5–20 µg of total RNA (200–600 ng/µl). To prepare cDNA, 1 µg of RNA was reverse transcribed with TaqMan Reverse Transcription (Applied Biosystems) as described in the kit protocol [9].

2.6. RT-PCR and analysis of PCR fragments

Amplification of the cDNA fragments of interest was performed in a T3000 thermocycler (Biometra) as reported before [9].

3. Results

3.1. Clinical analysis

Biochemical data referring to total cholesterol and LDL-cholesterol, for the FH patients genetically identified in Portugal (before treatment values were only available for 351 Portuguese FH patients) is shown in Supplementary Fig. S1A.

Coronary heart disease is present in 49 of the total Portuguese FH patients genetically identified (17% of the adult FH patients). Premature CHD analysis of the 49 FH patients distributed according

to age group is shown in [Supplementary Fig. S1B](#). From the analyses of clinical questionnaires 64 premature deaths have occurred in the reported families (55.0 ± 14.0 years).

3.2. Molecular analysis

From the 482 index patients received only 359 patients have their molecular study completed, so the results presented are relative to these patients.

3.2.1. LDLR gene

A total of 80 mutations were identified in 165 index patients participating in the Portuguese FH Study, 34 being exclusive to the Portuguese population.

Since our last report [8] 27 different mutations have been found, 10 of which have not been reported previously ([Table 1](#)). These 27 mutations found in the Portuguese FH patients include 2 mutations in the promoter region, 13 missense mutations, 2 nonsense mutations, 5 splice site mutations, 3 small deletions and 2 large deletions.

Six index patients were found to carry two defective LDLR alleles. Three presented the same mutation in both alleles and three presented different mutations in the two defective alleles, four of these patients had been previously reported and one is a true homozygous for the p.Arg406Trp, an already described mutation [8]. The new patient who was found to be compound heterozygous for p.Asp224Asn and p.Glu716Lys presented a severe phenotype. This patient and his daughter carried the same allele with p.Asp224Asn mutation but his daughter did not inherit the allele carrying p.Glu716Lys ([Fig. 1](#)) confirming that the two mutations are not on the same allele and that the index patient is a compound heterozygous. The p.Glu716Lys variation has not been reported before and requires *in vitro* confirmation of its pathogenic effect.

The screening of 443 individuals in these 165 families identified in the Portuguese FH Study, lead to the additional identification of 226 genetically diagnosed FH patients.

Index patients in whom no mutation was found in the APOB and LDLR genes were analysed by MLPA in order to identify possible large rearrangements in the LDLR gene. Two large deletions were identified in two unrelated patients. One of the large deletions was found in one family with a severe phenotype ([Supplementary Fig. S2](#)); a 5-year-old girl and her father with premature CHD carried the same allele with a deletion of the 3'-end of the LDLR gene comprising exons 16–18. The other large deletion, of exons 2 and 3, was found in a child, his brother and father with a severe phenotype and a family history of premature CHD. Both large deletions are novel in the Portuguese population.

3.2.2. APOB gene

The APOB p.Arg3527Gln mutation was found in three unrelated index patients as reported previously [8]. Screening relatives in two of these families lead to the identification of six more patients carrying this APOB mutation.

One novel mutation in the APOB gene, predicted to cause a single amino acid substitution p.Tyr3560Cys, was found in one child with ancestors in Cape Verde Islands, an ex-Portuguese colony. This mutation has been reported before [22]. Unfortunately this patient had no relationship with both parents and so it was not possible to identify other relatives.

3.2.3. PCSK9 gene

The PCSK9 gene analysis was performed for all patients without LDLR or APOB mutations, 48 patients have been fully studied and the remaining were studied for alterations in exons 2, 3, 4, 5 and 7. The only mutation identified in this gene, p.Asp374His, has

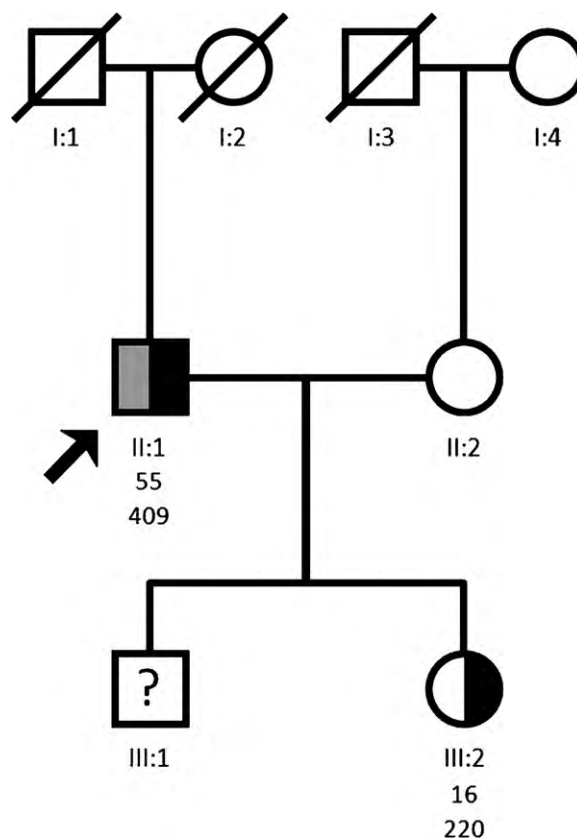


Fig. 1. Pedigree of the compound heterozygous patient carrying p.Asp224Asn and p.Glu716Lys mutations. The arrow indicates the index patient. Age in years and total cholesterol values in mg/dl are shown below each symbol. Half shaded symbol black correspond to p.Asp224Asn mutation. Half shaded symbol grey corresponds to p.Glu716Lys mutation. Open symbols represent individuals not available for the study. Open symbol with question mark represent individual with clinical FH but that was not available for the study.

been reported previously by our group [8] and was only present in three patients of the Portuguese FH Study. Five novel variants were found in possible FH patients without mutations in LDLR or APOB gene: c.–73A>G (1 patient), c.–67C>A (1 patient), c.400–22C>A (9 patients), c.1181–53T>C (6 patients) and c.1180+22T>C (1 patient). The unique variants need further characterization to analyse the possible effect in cholesterol metabolism.

3.2.4. cDNA analysis of putative splicing mutations

Blood samples for mRNA extraction and cDNA analysis were obtained from four index patients. Two of these variants, c.1060+1G>A and c.2547+1G>A, caused total exon skipping, one caused retention of 10 nucleotides of intron 5 (c.818–2A>G) and variant c.190+4insTG caused retention of 2 nucleotides of intron 2 ([Fig. 2](#)). All putative splicing mutations analysed are predicted to create a premature stop codon. Alteration c.1060+1G>A causes skipping of exon 7 generating a complete different protein from exon 6 forward and a premature stop codon at aa 779. Alteration c.2547+1G>A causes skipping of exon 17 and a premature stop codon at aa 805. Alteration c.818–2A>G causes the retention of 10 bp of intron 5 generating a premature stop codon at aa 303. Alteration c.190+4insTG involved activation of a novel splicing-donor site (GT) at IVS2+3+4 (instead of IVS2+1+2), resulting in the defective splice out of intron 2, leading to the reading-through of exon 2 plus the following intronic 2 bp sequence, generating a complete different protein from exon 2 forward and a premature stop codon at aa 206 ([Fig. 2](#)).

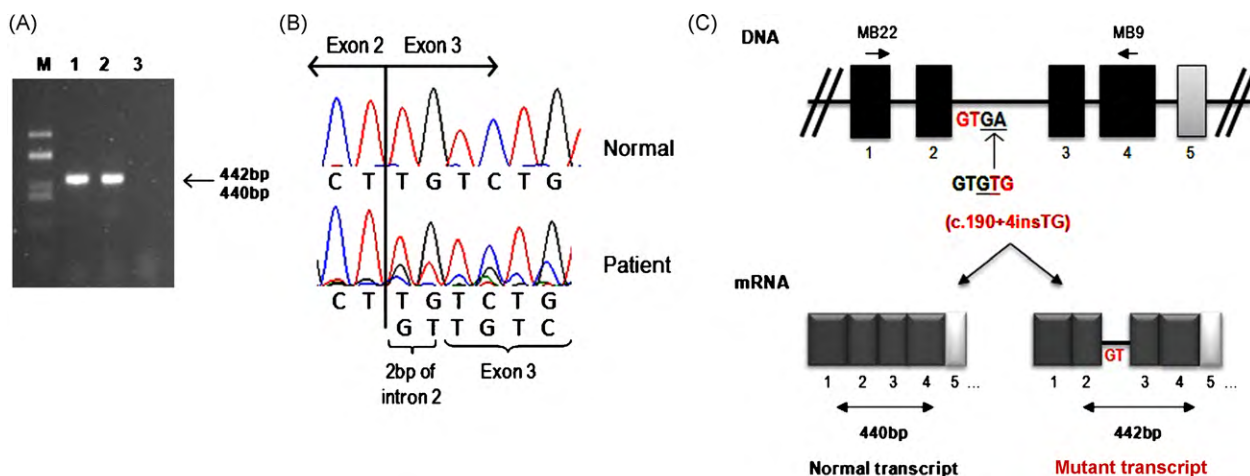


Fig. 2. Effect of the c.190+4insTG mutation on LDLR cDNA of the reported patient. (A) Agarose gel electrophoresis of a fragment containing exons 1–4 amplified from the patient’s cDNA. Total mRNA was isolated from fresh blood mononuclear cells of the patient; M, marker; lane 1, patient; lane 2, control cDNA; lane 3, water blank. (B) Nucleotide sequence of the single fragment from a normal subject (upper panel), and the fragment amplified from the patient’s cDNA (lower panel), with the double sequence indicated below. (C) Diagram showing the location of primers used and the two fragments obtained (MB22: GGCTGGAAATTGCGCTGGAC and MB9: CTGGCGGACCACAGGTGAGC).

4. Discussion

In 1999 a lipid network was established for the Portuguese FH Study and since then DNA based diagnosis has been set up to study patients with a clinical diagnosis of FH. The screening protocol used in this study has lead to the genetic identification of a total of 171 index patients, corresponding to an overall identification of 48% of the received cases with clinical diagnosis of FH. The low percentage of index cases genetically identified in our population can be due to several reasons but, the one reason is the definition of the criteria for inclusion in the study that is not always followed by the rule. Following the Simon Broome Registry criteria, based in a family history of hypercholesterolaemia and a cut off value for total cholesterol of 260 mg/dl or LDLc of 155 mg/dl for children under 16, and a cut off value of 290 mg/dl or LDLc of 190 mg/dl for adults, in 45% of the children and in 51% of adults studied, a mutation causing disease was identified. Since patient referral to the Portuguese FH Study has been very weak (it is estimated that 80% of FH patients are not even clinically identified), it was decided to study all the patients referred by the clinicians as long as they fulfilled the criteria of having a total cholesterol above the 95th percentile for age and sex and a family history of hypercholesterolaemia. In fact, of the 8 children studied with cholesterol between 200 and 260 mg/dl (or LDLc between 120 and 155 mg/dl), none had a disease-causing mutation, while in the adult group, of the 34 patients with total cholesterol values between 240 and 290 mg/dl (or LDLc of 144–190 mg/dl), a mutation was identified in 5 patients, none of these patients had premature CHD. Also 3/17 patients with triglycerides above 250 mg/dl and premature CHD, had a mutation causing disease; 6 of these patients had also premature CHD but a mutation causing disease was not found. This means that a mutation was found in 14% (8/59) of the patients that did not fulfill the Simon Broome criteria. Even so, since the genetic diagnosis of FH is still time consuming and expensive it was decided that Portugal will continue to use Simon Broome criteria since it is more cost effective. When other less expensive and less time consuming technologies are available this decision will be revised.

For 52% of the patients it was not possible to identify a mutation in any of the three genes analysed. One possible explanation is that the underlying gene defect causing FH in these patients is in a non-coding region of the LDLR gene that may affect its expression or RNA processing, not detectable by the methodologies used in this study. The other explanation is that the defect lies in another

gene involved in LDLR function, regulation or LDL metabolism. Some patients can also be misdiagnosed and instead of FH can have familial combined hyperlipidaemia (FCHL) which is thought to be a dominant condition associated with hyperlipidaemia and premature CHD, with very few candidate genes identified. Since 51/188 did not fulfil the Simon Broome clinical criteria for FH it is also possible that these patients are being clinically misdiagnosed with FH. Our group intends to examine these options in the future in the genetically unidentified families of the Portuguese FH Study.

The large rearrangements detected by the MLPA methodology are being confirmed by long PCR but when deletions occur at the 5’-end or 3’-end of the gene confirmation is more difficult. MLPA is a simple and rapid method for detecting large rearrangements in the LDLR gene and our results support the reliability of MLPA for genetic diagnostic and is our opinion that should be included in the methodology for genetic testing of FH as suggested by other authors [23]. In Portugal large rearrangements correspond to 6% of the LDLR mutations, very similar as described in other populations [23].

APOB mutations are a rare cause of FH in Portugal compared to other countries [24]. An APOB mutation was only found in about 2% of all FH cases identified.

Our lab has developed a quick and simple method to analyse putative splicing mutations [9]. Whenever it is possible these alterations are studied to increase the scientific knowledge of the effect of these alterations and, most important, to determine if they are mutations causing disease or not. In this paper we report the functional effect of 3 mutations previously described [8] and 1 novel mutation is fully characterized. For one of the alterations reported in this paper (1846–1G>A), it was not possible to obtain a fresh blood sample for RNA extraction and cDNA analysis.

The cascade screening of relatives allowed a total of 404 FH patients to be genetically identified in Portugal. This corresponds only to 2.02% of the estimated total number of Portuguese FH patients. Cascade screening in families proved to be an efficient method for the identification of FH patients. This approach allows a more time and cost effective diagnosis of FH diagnosis. A second phase of the project funded by the Portuguese Cardiology Society will start this year and will be dedicated to the cascade screening of the patients already identified as having FH. Until now the relatives screening has been low due to the inexistence of a research nurse able to collect blood samples from relatives living all over the country but, this difficulty will be overcome by the new grant. Also

it will be contemplated the option of sample collection of epithelial cells by swabs or mouth wash solutions to screen for mutations in children or relatives specially those living abroad.

Clinician adhesion to the study has been increasing but it is still far from the expected, especially since major efforts to find external funding have been made to maintain the study free of cost for the patients and medical institutions.

The genetic identification of a mutation causing disease in FH patients, confirms the clinical diagnosis based on the plasma cholesterol levels determinations and provides an unequivocal diagnosis of the disease and allows the early identification of affected relatives. This is especially important for young patients since they can receive appropriate dietary and lifestyle advice and adequate therapeutic measures when necessary in order to reduce their risk of CHD. If adequate measures (counselling and therapeutic) are taken early enough the cardiovascular risk of young FH patients can decrease to equal to the general population.

As reported by the clinicians, in our population, the genetic diagnosis increases the patient adherence to treatment although majority of the patients are not receiving adequate treatment as described elsewhere [25]. This is due to mainly three reasons: patients do not take the medication prescribed because, (1) they are not aware of their increased cardiovascular risk; (2) they have social-economics problems or (3) the clinicians are not prescribing the adequate treatment for patient with genetic condition. The overall result is that the cardiovascular risk of the majority of the Portuguese FH patient is not reduced. Awareness strategies to alert the patients and the health authorities have to be implemented at larger scale so cardiovascular prevention can be achieved.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2010.07.012.

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