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Familial hypercholesterolaemia in Portugal

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on behalf of the investigators of the Portuguese FH study [1]

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Abstract

Familial hypercholesterolaemia (FH) is characterised clinically by an increased level of circulating LDL cholesterol that leads to lipid accumulation in tendons and arteries, premature atherosclerosis and increased risk of coronary heart disease (CHD). Although Portugal should have about 20,000 cases, this disease is severely under-diagnosed in our country, this being the first presentation of Portuguese data on FH. A total of 602 blood samples were collected from 184 index patients and 418 relatives from several centres throughout Portugal. Fifty-three different mutations were found in 83 index patients, 79 heterozygous and 4 with two defective LDLR alleles. Additionally, 4 putative alterations were found in 8 patients but were not considered mutations causing disease, mainly because they did not co-segregate with hypercholesterolaemia in the families. Three unrelated patients were found to be heterozygous for the $APOB_{3500}$ mutation and two unrelated patients were found to be heterozygous for a novel mutation in *PCSK9*, predicted to cause a single amino acid substitution, D374H. Cascade screening increased the number of FH patients identified genetically to 204. The newly identified FH patients are now receiving counselling and treatment based on the genetic diagnosis. The early identification of FH patients can increase their life expectancy and quality of life by preventing the development of premature CHD if patients receive appropriate pharmacological treatment.
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Keywords: Familial hypercholesterolaemia; Low density lipoprotein receptor; Mutations; Coronary heart disease; Index patient; Cholesterol

1. Introduction

Familial hypercholesterolaemia (FH) is an autosomal dominant disorder with a frequency of about 1/500 in most European countries [1]. FH usually results from inherited

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defects in the low density lipoprotein receptor gene (*LDLR*) but mutations in the gene for apolipoprotein B (*APOB*) or, more recently described, mutations in the proprotein convertase subtilisin/Kexin type 9 (*PCSK9*) gene also cause the same phenotype. FH is clinically characterised by an increased level of circulating LDL cholesterol that leads to lipid accumulation in tendons (xanthomas) and arteries, premature atherosclerosis and increased risk of coronary heart disease (CHD). Although Portugal should have about 20,000 cases, this disease is severely under-diagnosed in our country where no prior clinical or genetic studies have been performed. Men and women with FH aged between 20 and 39 years old have an 100-fold increased risk to develop premature CHD compared to the normal population [2]. It is important to identify as early as possible individuals with FH to improve their prognosis by administration of appropriate therapeutic interventions and also to allow provision of genetic counselling and to ensure access to specialised medical services. The aim of this project was to set up a network of lipid clinics and an analytical laboratory for the "Portuguese FH study" [3] and perform the molecular characterization of patients with a clinical diagnosis of Familial hypercholesterolaemia (FH) to allow cascade screening of the relatives of index patients. It was also expected that comparison of the clinical and molecular characteristics of Portuguese FH patients with FH patients from other countries may provide some insight about the underlying reason for the different severity of the phenotypes presented by FH patients.

2. Methods

2.1. Patient recruitment

A total of 602 blood samples were collected from 184 index patients and 418 relatives of 124 of these families. The 184 index patients, 51 children (aged 18-year old or under) and 133 adults, were referred to our institute by 21 clinicians working in public hospitals, mainly in cardiology, internal medicine, paediatric and genetic services, throughout the country and islands (Fig. 1). A clinical questionnaire adapted from the "Simon Broome Heart Research Study" [4] was completed by clinicians for each index patient and informed consent was signed by all index patients and relatives. For children under 18 the declaration was signed by the parents.

2.2. Biochemical characterization

The biochemical parameters including total cholesterol, HDL cholesterol, triglycerides, apolipoprotein AI, apolipoprotein B and lipoprotein (a), were determined for all subjects in an Hitachi 911 (Boehringer Mannheim, Roche) by an enzymatic colorimetric method, following the manufacturer's instructions. LDL cholesterol values were calculated using the Friedewald formula.

2.3. DNA analysis

Amplified fragments from genomic DNA comprising all exons of the LDLR gene, were analysed for mutations by highly sensitive denaturing high pressure liquid chromatography (DHPLC) (Transgenomics, USA) and automated sequencing. Large rearrangements in the LDLR were identified by multiplex ligation-dependent probe amplification (MLPA) with the kit for the LDLR gene (SALSA P062B kit) from MRC-Holland (Amsterdam, The Netherlands), following the manufacturer's instructions. MLPA amplification products were analysed with the GeneScan Analysis Software Version 3.1.2 and peak heights were measured with Genotyper Software Version 2.5 (Applied Biosystems) and exported to excell sheets for further processing. The peak

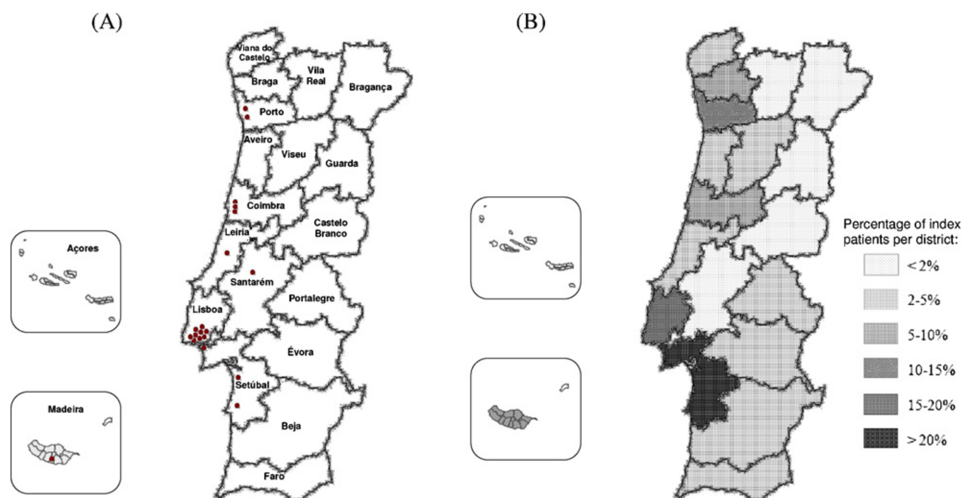

Fig. 1. Demographic distribution of Clinicians and index patients participating in the Portuguese FH study. (A) Portuguese network of clinicians currently collaborating with the Portuguese FH study. (B) Origin of 184 unrelated index patients registered in the Portuguese FH study, by district.

heights were normalised and then divided by average normalized peaks from four normal control subjects. The results are given as relative allele copy number as compared to normal controls, so a ratio of ~1 is obtained if both alleles are present, a ratio of ~0.5 if an allele is absent and ~1.5 if an allele is duplicated.

APOB gene was analysed by amplification and sequencing of a fragment of exon 26 containing the four most common *APOB* mutations and a fragment of exon 29 as described before [5].

All 12 exons of PCSK9 gene were amplified and sequenced as described before [6]

2.4. LDLR and APOB gene haplotyping

LDLR haplotypes were analysed by PCR amplification and sequencing of fragments containing known common variants in this gene namely, 81T > C (SfaNI), 1060+10G > C (SmaI), 1171G > A (StuI), 1413G > A (BsmAI), 1617C > T (BstNI), 1773 C > T (HincII), 1959 T > C (AvaII), 2232G > A (MspI), 2389 +46, 2547 −80, 2547 −42 and 2583 +52, corresponding to exon 2, intron 7, exons 8, 9, 11–13, 15, intron 16, 17 and 3′UTR region.

APOB haplotypes of patients with the $APOB_{3500}$ mutation were determined by PCR amplification and restriction analysis and/or sequencing of four polymorphic sites in the APOB gene, as described previously [7,8]

2.5. Long-range PCR

For PCR amplification of large fragments of the LDLR gene, Expand Long Template PCR System was used according to manufacturer's instructions (Roche Applied Science). The reactions were carried out in a Biometra T3000 thermocycler (Germany). The fragments obtained were analysed by direct sequencing.

2.6. Analysis of human single tandem repeats

Single tandem repeats (STR) analysis was performed as a test for paternity using the AmpF*l*STR® Profiler Plus™ following manufacturer's instructions (Applied Biosystems, USA). The products obtained were analysed with the Genemapper Software Version 3.7 (Applied Biosystems)

All sequencing reactions, MLPA reactions and STR analysis described here were analysed in a 3100 Genetic Analyser with 16 capillaries (Applied Biosystems, USA) using POP6 polymer according to the manufacturer's instructions. PCR conditions and primers are available on request.

2.7. Statistical analysis

All data were analysed with SPSS software (version 13.0). To test for association between variables, the Pearson Chi square test and the Fisher exact test were used. When these could not be applied, non-parametric tests were used: Mann–Whitney (for two independent samples) and Kruskall–Wallis (for more than two independent samples). For all tests used $p < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical data analysis

The clinical and biochemical characteristics and relevant life habits of the 184 Portuguese FH patients are summarized in Table 1.

3.2. Molecular analysis

3.2.1. LDLR

Fifty-three different mutations were found in 83 index patients, 23 of which have not been described previously (Table 2a). These include 31 point mutation, 3 nonsense mutations, 6 splice site mutations, 11 small deletions and insertions and 2 large deletions. Additionally, 2 alterations, 2 missense and 2 potential splicing alterations, were found in 8 index patients (Table 2b) where in all but one, other mutations have not been found in *APOB*, *LDLR* or *PCSK9*. The pathogenicity of these alterations is not clear, mainly due to lack of co-segregation of the alteration with hypercholesterolaemia in the family. One of these alterations, G248D, has been described before as a mutation causing disease (online FH database: www.ucl.ac.uk/fh) but it did not co-segregate with the hypercholesterolaemia in one of the three different families where this alteration was found. In fact in this family this alteration was not present in two hypercholesterolaemic relatives and it was found in two normolipidaemic relatives. This suggests that this variant has no deleterious effect on LDL receptor function. Another alteration, 818 −2A/G, was identified in this family that co-segregates with the hypercholesterolaemia in this family (Fig. 2A) and was for this reason considered the mutation causing disease.

Four patients were found to carry two defective LDLR alleles, even though three of these patients did not present a typical "homozygous" phenotype (Table 3): one homozygous for V408L, one homozygous for A410T and one compound heterozygous for A410T and a potential splicing mutation 313+6T/C. The compound heterozygous patient inherited the 313+6T/C mutation from her father but the A410T mutation has appeared *de novo* in her maternal allele and she transmitted it to her two sons. This result was confirmed by the analysis of human STRs (data not shown) that proved that the mother was indeed the biological mother.

Patients in whom no mutation in the LDLR or APOB genes could be identified by dHPLC and/or sequencing were analysed by MLPA to search for large rearrangements in the LDLR gene. Two large deletions were found by MLPA in three unrelated patients with a severe FH phenotype; one index patient was found to carry an allele with deletion of

Table 1
Patient distribution in the paediatric and adult groups. Clinical and biochemical characteristics, smoking and alcohol consumption habits percentages

	Paediatric (under 18)	*n*	Adults	*n*
Age (years)	9.98 ± 3.89	51	46.63 ± 13.82	130
BMI (kg/m^2)	19.92 ± 3.45	51	26.11 ± 4.95	119
Female (%)	54.9	28	60.9	81
Male (%)	45.1	23	39.1	52
Total cholesterol (mg/dl)	297.95 ± 7.84[a]	46	361.72 ± 84.55	89
	248 ± 53.19[b]	46	279 ± 81.5	115
LDL cholesterol (mg/dl)	221.53 ± 66.69[a]	40	276.01 ± 85.82	75
	168 ± 48.74[b]	46	209 ± 191.36	115
HDL cholesterol (mg/dl)	56.05 ± 17.90[a]	40	52.47 ± 15.08	79
	58 ± 15.56[b]	46	57 ± 15.35	115
Triglycerides (mg/dl)	110.29 ± 76.69[a]	42	162.61 ± 114.59	81
	111 ± 67.54[b]	46	153 ± 110.14	115
ApoB (mg/dl)	109.23 ± 55.58[b]	46	147.32 ± 67.22	115
ApoAI (mg/dl)	136.08 ± 34.95[b]	46	138.74 ± 35.11	115
Lp(a) (mg/dl)	73.45 ± 80.01[b]	45	65.41 ± 65.03	111
Alcohol consumption (%)	2.0	1	36.8	46
Smokers (%)	2.0	1	15.0	19
Hypertension (%)	2.0	1	28.7	37
Diabetes (%)	0.0	0	1.6	2
Thyroid (%)	0.0	0	4.7	6
Tendon xanthomas (%)	0.0	0	8.5	11
CHD (%)[c]	0.0	0	25.4	33
On medication (%)[d]	34	17	88.8	111
Exercise (%)	56.0	28	39.5	49

[a] Values before medication. Not available for all individuals.
[b] Values at present time, most under medication; only obtained for 161/184 index patients.
[c] CHD is considered angina, coronary artery bypass graft (CABG) and miocardial infarction.
[d] Medication is considered statins, fibrates, eztimibe and resins in the paediatric group.

exons 8–12 and, the other two patients, carried a deletion of the 5′-end of the LDLR gene, comprising the promoter, exons 1 and 2, plus the deletion of exons 8–12, as showed in Fig. 3 for one of the patients. These mutations were also found in relatives of these index patients. In individuals that showed half the normal copy number of exons 8–12, the deletion of these five exons was confirmed by long-range PCR (Fig. 3C). The pattern of inheritance in the family indicated that both deletions occurred on the same allele. The haplotype analysis of these three patients revealed that they share the same haplotype for the mutant allele: C (1060 +1), G (2232), C (2389 +46), G (2547 −80), G (2547 −42).

The most common mutation, A410T, represents 13% of all genetically identified FH cases in this study and only two different haplotypes have been found: C (81, SfaNI), C (1060 +10, SmaI), G (1171, StuI), G (1413, BsmAI), C (1617, BstNI), T (1773 HincII), C (1959, AvaII), G (MspI) and C (81, SfaNI), G (1060 +10, SmaI), G (1171, StuI), A (1413, BsmAI), C (1617, BstNI), C(1773 HincII), T (1959, AvaII), G (MspI).

3.2.2. *APOB*

Three unrelated patients with clinical diagnosis of FH were found to be heterozygous for the R3500Q mutation in the APOB gene. The mutation was also found in three hypercholesterolaemic relatives of these patients. The genotypes for four *APOB* polymorphic sites were determined in these individuals. The R3500Q allele was associated with the haplotype PvuII−/XbaI−/MspI+/EcoRI− in the three families although we did not have relatives of the third family and so this haplotype is only estimated. These are the first $APOB_{3500}$ patients to be described in the Portuguese population and carry the same Caucasian haplotype as described before [7]

3.2.3. *PCSK9*

Two unrelated patients and one relative were found to be heterozygous for a novel mutation in *PCSK9*, predicted to cause a single amino acid substitution, D374H. These patients presented a very severe phenotype with extremely high total cholesterol levels, mean value of 505.40 ± 86.82 mg/dl versus other FH patients with mean value of 353.93 ± 83.0 mg/dl (LDLR) and 279.54 ± 52.97 mg/dl (APOB), and several relatives with premature CHD (Fig. 4 in supplement). Total cholesterol values in PCSK9 patients were significantly different from those in LDLR and APOB patients ($p = 0.034$; $p = 0.022$). This mutation has not been reported before, but a different nucleotide substitution in the same codon has been described in four English patients (D374Y) who also had a very severe phenotype [9].

3.3. *Cascade screening*

Screening of 206 relatives in families of 88 index patients, 19 children (under 18) and 69 adults, lead to the identification of 116 additional genetically diagnosed FH patients, 27

Table 2a
Mutations found in the LDLR gene

Nucleotide change	Predicted effect	No of index patients	No of relatives
c.-21A > C	M-21L in signal peptide	1	0
[Pr_EX2del + EX8_12del]	**Large deletion of 7 exons**	**1**	**8**
c.226G > T	**G55W in ligand binding domain**	**1**	**3**
c.241C > T	R60C in ligand binding domain	1	0
c.265T > C	C68R in ligand binding domain	1	2
c.292G > C	**G77R in ligand binding domain**	**1**	**0**
c.313+1G > A	Splicing error	1	0
c.313+6T > C	**Splicing error**	**1**	**1**
c.326G > T	C88F in ligand binding domain	1	3
c.369_393del25	**S102fsX176**	**1**	**1**
c.530C > T	S156L in ligand binding domain	3	7
c.551G > A	C163Y in ligand binding domain	1	3
c.619_639del21	**In frame. G186_S192del**	**1**	**2**
c.631C > G	**H190D in ligand binding domain**	**1**	**0**
c.661G > T	D200Y in ligand binding domain	1	1
c.662A > G	D200G in ligand binding domain	1	1
c.682G > A	D203N in ligand binding domain	7	14
c.670G > A	E207K in ligand binding domain	1	0
c.818 −2A > G	**Splicing error**	**2**	**4**
c.862G > A	E267K in ligand binding domain	1	0
c.1016_1017insG	**L318fsX336**	**1**	**3**
c.1027G > A	G322S in EGF precursor homology domain	1	1
c.1031C > T	Stop codon. Q324X	1	3
c.1048C > T	Stop codon. R329X	1	0
c.1060+1G > A	Splicing error	2	0
c.1085delA	D341fsX348	1	1
c.1178delA	**K372fsX392**	**1**	**0**
EX8_12del	**Large deletion of 5 exons**	**1**	**4**
c.1216C > T	R385W in EGF precursor homology domain	4	9
c.1222G > A	E387K in EGF precursos homology domain	1	0
c.1285G > C	**V408L in EGF precursor homology domain**	**1**	**0**
c.1291G > A	A410T in EGF precursor homology domain	11	14
c.1432G > A	G457R in EGF precursor homology domain	2	4
c.1455C > G	**H464Q in EGF precursos homology domain**	**1**	**1**
c.1468T > C	**W469R in EGF precursor homology domain**	**1**	**3**
c.1633G > T	**G524W in EGF precursor homology domain**	**1**	**1**
c.1659_1661delCTCinsATACTTTCA	**Stop codon. Y532X**	**1**	**1**
c.1690A > C	N543H in EGF precursor homology domain	1	0
c.1775G > A	G571E in EGF precursor homology domain	3	4
c.1816G > T	A585S in EGF precursor homology domain	3	0
c.1840T > A	**F593I in EGF precursor homology domain**	**1**	**1**
c.1845+1delG	**Splicing error**	**1**	**0**
c.1886delT	**F608fsX642**	**1**	**0**
c.1935_1936delA	**N624fsX646**	**1**	**1**
c.1942T > C	**S627P in EGF precursor homology domain**	**1**	**0**
c.1999T > C	C646R in EGF precursor homology domain	1	0
c.2053C > T	P664S in EGF precursor homology domain	1	0
c.2056C > T	Stop codon. Q665X	1	3
c.2077_2078delAA	**K672fsX694**	**1**	**1**
c.2389G > A	V776M in membrane spanning domain	1	5
c.2399_2403delTCTTCinsGGGT	V779fsX910	1	0
c.2417_2418insG	V785fsX795	1	0
c.2547+1G > A	**Splicing error**	**2**	**2**

Novel mutations are in bold.

Table 2b
Putative mutations causing disease found in the LDLR gene

Nucleotide change	Predicted effect	No index patients	No relatives
c.806G > A	G248D in ligand binding domain	4	6
c.1061 −8T > C	Splicing error?	1	7
c.1359 −5C > G	**Splicing error?**	**2**	**3**
c.2575G > A	**V838M in internalisation signal domain**	**1**	**1**

Novel alterations are in bold.

children and 89 adults, making a total of 46 children and 158 adults genetically identified as having FH.

3.4. Patients with no mutations in LDLR, APOB and PCSK9 genes

No alteration could be found in the LDLR, APOB gene or PCSK9 gene of 88 patients, 16 of whom presented a severe phenotype with extremely high total cholesterol (mean value of 321.6 ± 66.0 mg/dl, mean age 48.6 ± 12.9) and premature heart disease as well as strong family history and will therefore be candidates for mutations in other genes. The remaining 72 patients, 28 children and 44 adults, did not present with premature CHD but had severe hypercholesterolaemia (children mean total cholesterol value of 255.9 ± 29.9, mean age 10.0 ± 4; adults, mean total cholesterol value of

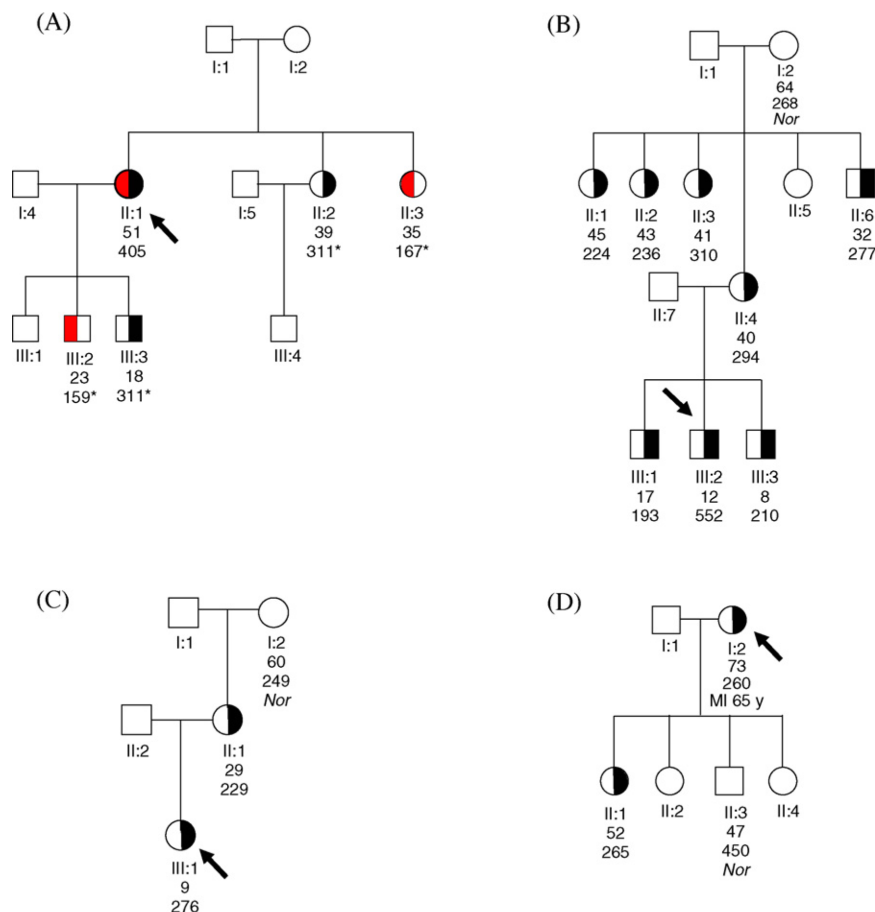

Fig. 2. Pedigrees of families where a putative alteration in the LDLR was found but does not co-segregate with the hypercholesterolaemia in the family. The arrow indicates the index patient. Age in years and total cholesterol values in mg/dL (*correspond to values on treatment) are shown below each symbol. Half shaded symbols represent heterozygous individuals for the alteration found. Open symbols represent individuals where the alteration was not found (*Nor*) or individuals who were not screened. (A) Pedigree of the familiy of the index patient with the G248D (half shaded red) and 818 −2 A/G mutation (half shaded black) G248D is found in two normolipidaemic individuals and on none of the hypercholesterolaemic individuals. (B) Pedigree of the familiy of the index patient with the 1061 −8 T/C (half shaded black) alteration. The hypercholesterolaemic grandmother (I:2) does not have the alteration and the grandfather is normolipidaemic as far as we know. (C) Pedigree of the family of the index patient with the V838M (half shaded black) alteration. The hypercholesterolaemic grandmother (I:2) does not have the alteration and the grandfather is normolipidaemic as far as we know. (D) Pedigree of the family of the index patient with the 1359 −5 C/G (half shaded black) alteration. The hypercholesterolaemic son (II:3) does not have the alteration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 3
Clinical and molecular characteristics of Portuguese FH homozygous

Subject	Mutations	Age	Sex	BMI	Lipid values	TC	LDL-c	HDL-c	TG	Apo A–I	Apo B	Clinical data
1	[V408L] + [V408L]	55	F	23.2	w/o treat	–	–	–	–	–	–	Stenosis
					On treat	299	232	47	99	100.0	139.0	
2	[A410T] + [313+6C > T]	36	F	30.4	w/o treat	490	435	42	64	117.0	245.7	–
					On treat	377	298	51	142	132	187.3	
3	[A410T] + [A410T]	25	F	18.5	w/o treat	596	512	65	96	–	–	–
					On treat	370	299	54	84	119.0	156.9	
4	H190D + K372fsX392 + A585S[a]	29	M	25.1	w/o treat	561	515	–	–	–	–	MI, 23 years CABG,
					On treat	346	298	29	96	86.0	189.3	CA, 24 years

Treat, treatment; TC, total cholesterol; TG, triglycerides; all lipids values are given in mg/dl. MI, myocardial infarction; CABG, coronary artery bypass graft; CA, coronary angioplasty.

[a] Parents were not available for testing so it could not be determined which mutations were in the same allele.

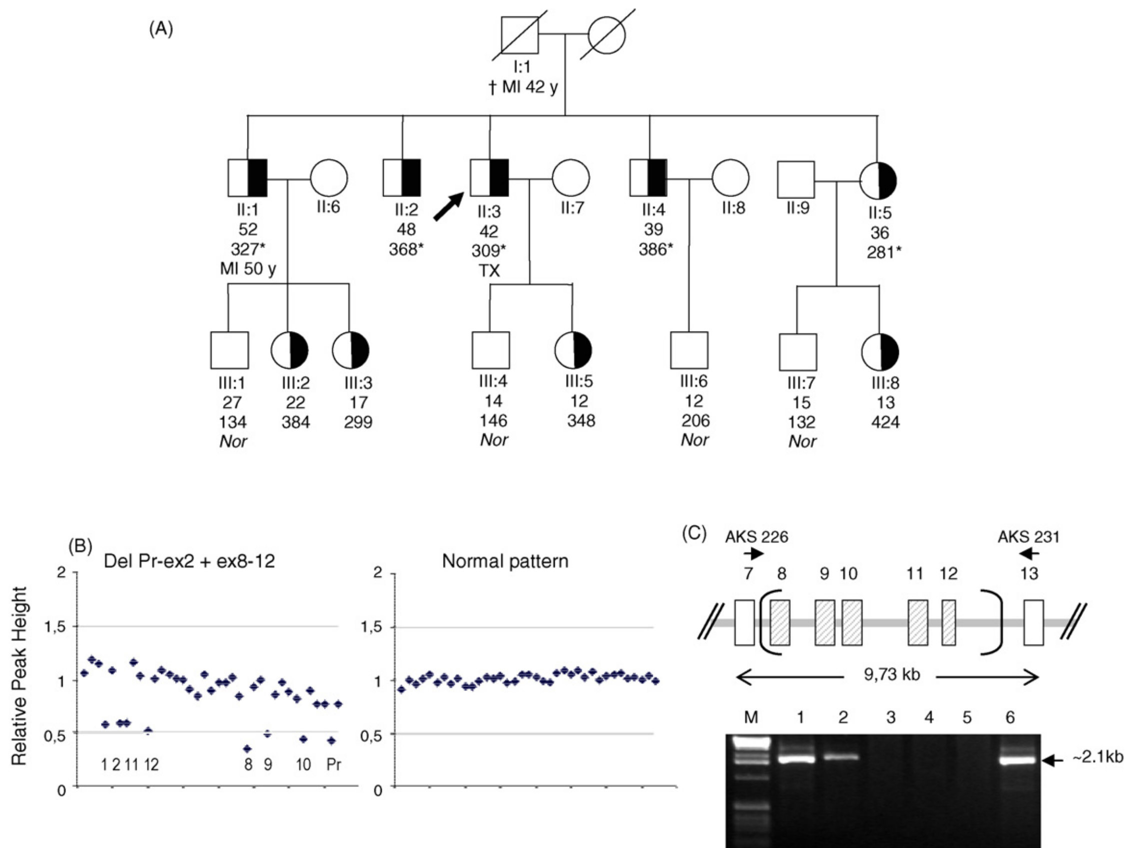

Fig. 3. Proband pedigree, mutation detection by MLPA and confirmation by PCR amplification. (A) Proband pedigree. The arrow indicates the index patient. Age in years and total cholesterol values in mg/dL (*correspond to values on treatment) are shown below each symbol. Half shaded black symbols represent heterozygous individuals for the deletion of promoter + exons 1–2 and 8–12. Open symbols represent normolipidaemic individuals (*Nor*) where the mutation was not found or individuals who were not screened. (B) After normalization of all the peak heights relative to control samples, the normal height of each peak, corresponding to the amplification product of all the exons of the LDLR gene and several control sequences, is ∼1. If the gene has a large rearrangement, such as the deletions found in this patient, the relative height of the peak drops to ∼0.5. (C) Long PCR amplification of a fragment comprising exon 7–exon 13. The fragment obtained for index patient and affected relatives with the deletion of exons 8–12 was ∼2.1 kb and the normal allele was not amplified (9.73 kb). M, 1 kb ladder; lane 1, index patient (II:3, 20042); lane 2, relative III:5, lane 3, relative III:4; lane 4, relative III:6; lane 5, relative III:7; lane 6, relative III:8. *Abbreviations*: †, deceased; MI, myocardial infarction; TX, tendon xanthomas.

343.6 ± 64.4, mean age 47.2 ± 11.2) and a family history of increased cholesterol values, consistent with a genetic disorder of lipid metabolism.

3.5. Statistical analysis

Only patients with an LDLR mutation were considered for this analysis.

3.5.1. Comparison by mutation, age, sex and CHD occurrence

The statistical analysis revealed that patients with mutations that lead to mRNA instability (stop codons, frameshift mutations, large deletions and splicing mutations) have higher total cholesterol values ($p = 0.025$) than patients with missense mutations, but there was no significant effect of mutation type or (age-adjusted) plasma cholesterol levels on incidence of CHD.

When the adult population was compared between sexes, it was observed that a higher percentage of males were smokers ($p = 0.006$) and a higher percentage of males presented with CHD ($p = 0.042$). Female FH patients with CHD were older ($p = 0.004$) and had higher Lp(a) values ($p = 0.039$) than FH females without CHD and the same was seen in the male group ($p = 0.022$; $p = 0.048$).

There were no significant differences in biochemical characteristics between sexes in the paediatric group.

3.5.2. Comparison between countries

Age, BMI, total cholesterol values, LDL cholesterol values, % of patients with premature CHD and % of patients with tendon xanthomas were compared between Portugal and Spain and between Portugal and the UK. Spanish values were obtained from the literature [10] and UK data was kindly provided by Dr R. Naoumova, Hammersmith Hospital Lipid Clinic. There were no significant biochemical differences between FH patients from Portugal and Spain except for total cholesterol values which are higher in Spanish women than in Portuguese women. Perhaps more surprising, FH patients in the UK do not exhibit higher values of total or LDL cholesterol than Portuguese patients and do not have a higher incidence of CHD than Portuguese or Spanish FH patients. This is despite a higher occurrence of tendon xanthomas in the UK, present in only 8.5% of Portuguese FH patients, 22.5% of Spanish FH patients and 78.8% of English FH patients, although the mean age of the UK patients was significantly greater than the Portuguese (56.8 ± 17.1 versus 48.7 ± 14.2).

4. Discussion

A network of lipid clinics was established for the Portuguese FH study and we regularly receive samples for this study. A genetic defect was identified in 88 patients with FH, of whom 79 were heterozygous for a mutation in the *LDLR*, two were true homozygous and two were compound heterozygous for two different alleles, three were heterozygous for the $APOB_{3500}$ mutation and two for a novel mutation in the *PCSK9*. These patients represent approximately 50% of the clinical FH patients referred to our study. Twenty-three mutations in the LDLR gene and the mutation in the PCSK9 gene have not been reported before, being until date, exclusive to the Portuguese population as far as we know. Family studies lead to the additional identification of 116 affected relatives, increasing the number of FH patients identified genetically to 204.

The majority of the described alterations, specially the point mutations, fulfilled all the accepted criteria for a functional mutation [11] except the functional studies that are still being performed for the few exceptions. Additionally, four alterations were found in eight patients but were not considered mutations causing disease, since they did not co-segregate in the families with the hypercholesterolaemia or were alterations in non-coding regions close to the intron exon junction that potentially affect splicing. These four putative functional alterations are under investigation. Co-segregation of the mutation with the hypercholesterolaemia in the families studied was always a strong criteria that has been followed by our group and a report has only been sent to the assistant clinician when relatives were available to confirm this. Other mutations have been described as fulfilling almost all Cotton criteria and subsequently proven not to be a functional mutation [12] similarly to one of our index cases (Fig. 2A), so all attention has to be given to mutations that do not fulfil all these criteria and functional studies should be performed. Unfortunately only a few, for example A410T [13] or 313 +1 [14] have been proved by independent functional studies to be the causal mutation.

The importance of always studying the whole gene was also supported by one of the cases presented (Fig. 2A) where the first mutation found did not co-segregate with hypercholesterolaemia in the family, but when the whole gene was studied a splice site mutation was found that co-segregated with the phenotype in the family. However, the first alteration found in this family (G248D) that was not considered to be the mutation causing disease, was also found in other three unrelated FH families without any other detectable defect in the three genes studied. Thus it is possible that this mutation could have a mild effect and functional studies are being performed.

Based on the estimated frequency of homozygous FH in Europe (about one in a million) [1], Portugal should have about 10 homozygous patients, but to our knowledge, none have been described. Four patients have been identified in our study with two defective alleles of the LDLR but only one presented with a typical homozygous phenotype. For one of the compound heterozygous patients, there is an explanation, since it was observed that the mutation in the donor splice site appears to result in mis-splicing of only a small portion of the mRNA from that allele (data to be reported elsewhere). For the patient homozygous for V408L, the only explanation

is that some modulation by environmental and genetic factors produces the less severe phenotype and for the patient homozygous for the A410T perhaps CHD has not yet developed due the young age of this patient. In fact the only patient with a typical homozygous phenotype only developed CHD at age 22 and smoked 20 cigars per day.

Several distinct mutations were found in our population, as seen in most European countries, but few alterations occurred more than once. All these more frequent mutations were found in apparently unrelated index patients but several haplotype analysis revealed evidence for a common ancestor. The haplotype analysis of the 11 patients carrying the A410T, the most frequent mutation accounting for 13% of all mutations found, showed that this mutation could have occurred twice since only two different haplotypes were found. Also the majority of these patients come from the same region in the south, which could imply that these patients must have a common ancestor. Since all patients with del exon 8–12 share the same haplotype it could be possible that they share a common ancestor and that the promoter region plus exons 1 and 2 deletion occurred subsequently on the allele already carrying the exon 8–12 deletion. The patients carrying the D203N probably have a common ancestor since the patients or their parents come from the same village in the Algarve and a family connection was subsequently revealed between three of these patients. The existence of a few common mutations would help the establishment of a rapid genetic diagnosis for FH in Portugal, but insufficient patients have yet been studied to determine correctly the frequency of these most common mutations.

Interestingly only four mutations were found in common with the Spanish FH population, namely M-21L [15], S156L [16], D200G [17], and G322S [18] and this last one also with the Brazilian population [19]. From the available literature it seems that not enough FH patients have been studied in these three countries for results in the populations to be compared. Castillo and co-workers [20] described 13 Spanish patients with the $APOB_{3500}$ mutation, 11 coming from a region just north of Portugal. Surprisingly our three patients are from a central region of Portugal and share the same haplotype.

In two unrelated patients a mutation was identified in the PCSK9 gene, as well as in one relative. All these patients presented a very severe phenotype with premature CHD, as other patients with similar PCSK9 mutations [9]. The mutation described, D374H has not been described before although, another nucleotide change has been described in the same codon (D374Y) [21] that is accepted as a functional mutation [22,23].

It was not possible to identify a mutation in any of the three genes analysed in about 50% of the clinical FH patients studied, although 16 presented a severe phenotype with very high total cholesterol values and premature CHD. Other genetic causes for hypercholesterolaemia have to be sought and therefore these patients will be good candidates for other gene defects yet to be known to be involved in FH. The most interesting patients belong to the paediatric group since in these patients, hypercholesterolaemia due to environmental factors can be more easily ruled out. The possibility that a mutation in the studied genes could have been missed must be considered since no method is 100% effective.

To determine if the phenotype of Portuguese FH patients resembles that of a group of FH patients from Spain, a neighbouring country with shared ancestors and culture, first of all the lipid profile of these two general populations was compared. Surprisingly, this revealed that total mean cholesterol in the Spanish population is significantly lower than in the Portuguese, that is 4.9 mmol/l versus 5.5 mmol/l for men and 5.0 mmol/l versus 5.5 mmol/l for women [24,25]. In contrast, the values for the Portuguese population are very similar to the ones found in the English population, 5.5 mmol/l for male and 5.6 mmol/l for women [26] despite it being a north European country with a distinct geography and culture from Portugal. Thus it seemed less certain that Portuguese and Spanish FH patients would be similar. However, when the clinical characteristics of Portuguese and Spanish FH patients were compared there were no statistically significant differences in age, BMI, pre-treatment total cholesterol (except for women), LDLc and percentage of patients with CHD. The Portuguese FH patients were also compared to a group of English FH patients from the Hammersmith Hospital Lipid Clinic and the only significant difference observed was a higher percentage of patients with tendon xanthomas in English FH patients. However the similarity of the clinical characteristics of FH patients of these three countries with different backgrounds could be limited in this analysis because the numbers of these three groups of patients are very different. Since there are no differences in mean lipid levels in the FH population between these three countries, two alternative explanations could be advanced to explain the fact that UK FH patients have such a high percentage of tendon xanthomas; either tendon xanthomas are not being well diagnosed in Portugal or Portuguese FH patients are less susceptible to the development of tendon xanthomas for some environmental reason. The first explanation seems to be more plausible since a more recent publication [27] showed that 40% of Spanish FH patients had tendon xanthomatosis when tendon ultrasonography was used to analyse Achilles tendon xanthomas.

FH fulfils the World Health Organization criteria for screening programs [28]. Since the introduction of statins (HMG-CoA reductase inhibitors), in the last decade, the prognosis of FH patients has improved substantially and therefore it is considered appropriate to do a systematic screening for FH [29] and there are no negative ethical implications in the study of this disorder. Nevertheless FH is severely under-diagnosed in Portugal, this being the first clinical and molecular study of FH in this country. The number of FH patients studied is still very small but steps are now being undertaken to increase this number, namely a collaboration with the Portuguese Cardiology Society that will allow, in the next 3 years, the complete study of 300 new families with FH. After the national publication of this study [3], our NHS

has become aware of the importance of early identification of this disorder as part of a programme for the prevention of premature CHD and has given its scientific support to the Portuguese FH Study.

The newly identified FH patients are now receiving counselling and treatment based on the genetic diagnosis and it is expected that some of the younger patients may not develop premature CHD due to the early identification of their disease through this study. The present study also helped to establish the appropriate therapeutic regime for each patient. None of the patients ceased their medication because a mutation was not found in the LDL receptor gene, but several patients in whom a mutation was found were treated subsequently with a more aggressive therapeutic regime.

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