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## Familial hypercholesterolemia: Molecular characterization of possible cases from the Azores Islands (Portugal)

Teresa Cymbron<sup>a,b,\*</sup>, Patrícia Mendes<sup>c</sup>, Amanda Ramos<sup>a,b</sup>, Mafalda Raposo<sup>a,b</sup>, Nadiya Kazachkova<sup>a,b</sup>, Ana Margarida Medeiros<sup>e,f</sup>, Jácome Bruges-Armas<sup>b,d</sup>, Mafalda Bourbon<sup>e,f</sup>, Manuela Lima<sup>a,b</sup>

<sup>a</sup> Centre of Research in Natural Resources (CIRN), Department of Biology, University of the Azores, 9501-801 Ponta Delgada, Azores, Portugal

<sup>b</sup> Institute for Molecular and Cell Biology (IBMC), University of Porto, 4150-180 Porto, Portugal

<sup>c</sup> The Azores School of New Technologies (ENTA), 9504-540 Ponta Delgada, Azores, Portugal

<sup>d</sup> Specialized Service of Epidemiology and Molecular Biology (SEEBMO), Hospital of Santo Espírito, 9700-049 Angra do Heroísmo, Azores, Portugal

<sup>e</sup> Grupo de Investigação Cardiovascular, Unidade I&D, Departamento de Promoção da Saúde e Prevenção de Doenças Não Transmissíveis, Instituto Nacional de Saúde Dr. Ricardo Jorge, 1649-040 Lisboa, Portugal

<sup>f</sup> Centre for Biodiversity, Functional and Integrative Genomics (BioFIG), Faculty of Sciences, University of Lisboa, 1749-016 Lisboa, Portugal

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### ABSTRACT

Familial hypercholesterolemia (FH) is an autosomal dominant disorder of the cholesterol metabolism, which constitutes a risk factor for coronary arterial disease (CAD). In the Azores Islands (Portugal), where mortality from CAD doubles its rate comparatively to the rest of the country and where a high frequency of dyslipidemia has been reported, the prevalence and distribution of FH remain unknown. The molecular characterization of a group of 33 possible cases of FH of Azorean background was undertaken in this study. A DNA array was initially used to search mutations in the *LDLR*, *APOB* and *PCSK9* loci in 10 unrelated possible cases of FH. No mutations were detected in the array; after sequencing the full *LDLR* gene, 18 variants were identified, corresponding to two missense (c.806G > A; c.1171G > A) and sixteen synonymous alterations. Six of the synonymous variants which are consistently described in the literature as associated with altered cholesterol levels were used to build haplotypes. The most frequent

*Abbreviations:* APOB, apolipoproteína B-100; CAD, coronary arterial disease; CI, conservation index; FH, familial hypercholesterolemia; LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin 9; TC, total cholesterol.

\* Corresponding author at: CIRN/Department of Biology, University of the Azores, Rua da Mãe de Deus, Apartado 1422, 9501-801 Ponta Delgada, Azores, Portugal. Tel.: +351 296650477; fax: +351 296650100.

E-mail address: [tcymbron@uac.pt](mailto:tcymbron@uac.pt) (T. Cymbron)

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haplotype corresponded to TTCGCC (45%), a “risk” haplotype, formed exclusively by alleles that were reported to increase cholesterol levels. Some of the variants detected in the full sequencing of the *LDLR* gene fell within the ligand-binding domain of this gene, defined by exons 2 to 6. To add information as to the role of such variants, these exons were sequenced in the remaining 23 possible FH cases. Two missense alterations (c.185C > T; c.806G > A) were found in this subset of possible FH cases. The missense alteration c.185C > T, identified in one individual, is novel for the Portuguese population. *In silico* analysis was not conclusive for this alteration, whose role will have to be further investigated. This study represents the first approach to the establishment of the mutational profile of FH in the Azores Islands.

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## Introduction

Familial hypercholesterolemia (FH; MIM# 143890) is an autosomal dominantly inherited disorder of cholesterol metabolism that affects approximately 1 in 500 individuals worldwide, thus corresponding to one of the most common mendelian disorders (Goldstein et al., 1995). FH is characterized by an excess of low-density lipoprotein cholesterol (LDL-c) in circulation, leading to deposits in peripheral tissues (xanthomas), and, more importantly, in the arterial wall, causing premature and accelerated atherosclerosis and an increased risk of coronary artery disease (CAD) at a young age (Chater et al., 2006). Defects in at least three different genes encoding for proteins involved in hepatic clearance of LDL-c from the plasma can cause FH. Mutations in the apolipoproteina B-100 (*APOB*) and in the proprotein convertase subtilisin/kexin 9 (*PCSK9*) genes can produce a phenotype of FH. The most frequently implicated gene, however, is the one encoding for the low-density lipoprotein receptor (*LDLR*). Mutations can occur all over the *LDLR* gene, but the majority of variants fall within the ligand-binding domain, which is encoded by exons 2 to 6 (Al-Khateeb et al., 2011). Although the diagnostic algorithm of FH currently involves the screening of the *LDLR*, *APOB* and *PCSK9* genes, molecular studies have been showing that in a high number of clinically defined index-cases no pathogenic mutations can be identified at these three loci. In the study of Motazacker et al. (2012), for example, 41% of the index-cases could not be molecularly diagnosed; this percentage was around 50% in the study of Bourbon et al. (2008). On the other hand, the role of some genetic variants described in the previously referred loci (*LDLR*, *APOB* and *PCSK9*), remains to be established (Rafiq et al., 2011). Synonymous variants, for example, although previously dismissed as “non-pathogenic”, are increasingly recognized as being associated with clinically relevant phenotypes. In FH their potential to modulate splicing efficiency through the alteration of exon splicing enhancers has been demonstrated (Bourbon et al., 2008; Defesche et al., 2008; Zhu et al., 2007). Portugal, in contrast to most of the other Southern European countries, has a high mortality rate due to CAD, which represents the second cause of death in the country (WHO, 2011). In the Portuguese Azores Islands the death rate from CAD is two-fold higher than in mainland Portugal (Eurostat, 2009). A previous study using apparently healthy Azorean subjects showed a high prevalence of dyslipidemia in this population (Borges, 2012). Because FH is one of the risk factors of CAD, the molecular characterization of 10 Azorean index-cases with possible FH was performed by analyzing the *LDLR*, *APOB* and *PCSK9* loci, using a DNA array (LIPOchip) and subsequently sequencing the full *LDLR* gene. An adult possible FH case characterized according to the Simon Broome Heart Research Trust criteria is an individual with total cholesterol (TC) over 290 mg/dl and/or LDL cholesterol (LDL-c) over 190 mg/dl, and with family history of myocardial infarction or high level cholesterol (adapted from the Simon Broome Heart Research Trust (1991)). To further clarify the role of some genetic variants detected in the *LDLR* gene, we additionally sequenced exons 2 to 6 and the corresponding exon/intron boundaries in a series of 23 index-cases of possible FH.

## Subjects and methods

At two regional health centers (Vila Franca and Nordeste), blood samples were collected after informed consent from 33 unrelated index-cases, 23 women (69.7%) and 10 men (30.3%) born in the Azores and of

Azorean ancestry. The individuals selected fulfilled the criteria of “possible FH”, according to the [Simon Broome Heart Research Trust \(1991\)](#). Secondary causes of hypercholesterolemia, such as diabetes, hypothyroidism and nephrotic syndrome, were considered exclusion criteria. TC was determined for all subjects in collaborating laboratories, using standardized protocols. LDL-c concentration was calculated by the Friedewald formula ([Friedewald et al., 1972](#)). DNA was extracted using the salting-out method ([Miller et al., 1988](#)). From the 33 individuals collected, 10 (7 women and 3 men) who presented high levels of TC and whose samples simultaneously fulfilled the necessary DNA purity and concentration requirements were selected for microarray analysis. Genotyping was performed using the LIPOchip® Array version 7 (Progenika, Derio, Spain) ([Stef et al., 2013](#)). This platform includes a microarray for the detection of point mutations and small insertions/deletions (indels) as well as copy number variations in the *LDLR* gene. All exons of the *LDLR* gene were analyzed; the array included the analysis of 207 point mutations in the gene *LDLR*, 4 mutations in the gene *APOB* and seven in the gene *PCSK9* (information provided by Progenika). The chip further detects point mutations in exon 26 of the *APOB* gene as well as a few mutations in the *PCSK9* gene ([Palacios et al., 2012](#)). Whenever the LIPOchip® Array fails to identify mutations a second step is carried out by sequencing the promoter, the translated exon sequences, and the exon–intron boundaries of the 18 exons of the *LDLR* gene ([Palacios et al., 2012](#)). For the remaining 23 index-cases, exons 2 to 6, which define the ligand-binding domain of the *LDLR* gene, were sequenced. Primers were designed to flank the entire coding region and the intron–exon junctions of each exon. All primer sequences, annealing temperatures, as well as expected PCR products are available upon request. Sequencing of purified PCR products was performed using the BigDye Terminator v3.1 sequencing kit (Applied Biosystems) following the manufacturer's protocols and sequences were run in an ABI 310 sequencer (Applied Biosystems).

Haplotypic analyses for the six synonymous variants associated with cholesterol levels were performed using the Arlequin3.11 software package ([Excoffier et al., 2005](#)). Allelic frequencies at population level were obtained for comparative purposes from dbSNP (NCBI) using the 1000 Genomes as a source.

**Table 1**

Genetic variants obtained after *LDLR* gene sequencing in a total of 33 possible HF cases, grouped by type of variation and set of individuals used in this study.

<i>LDLR</i> gene sequencing							
18 exons, <i>N</i> = 10				Exons 2 to 6, <i>N</i> = 23			
Age (Years)	Sex (M F)	TC (mg/dl)	LDL-C (mg/dl)	Age (Years)	Sex (M F)	TC (mg/dl)	LDL-C (mg/dl)
54 ± 13	3 7	296 ± 18	194 ± 20	50 ± 11	7 16	293 ± 22	204 ± 23
Variant name <sup>a</sup>	Location	SNP ID <sup>b</sup>		Variant name	Location	SNP ID	
<i>Missense variants</i>							
c.806G > A; p.Gly269Asp	Exon 5	rs143992984		c.185C > T; p.Thr62Met	Exon 2		No reference
c.1171G > A; p.Ala391Thr	Exon 8	rs11669576		c.806G > A; p.Gly269Asp	Exon 5		rs143992984
<i>Synonymous variants</i>							
c.81C > T; p.Cys27Cys	Exon 2	rs2228671		c.81C > T; p.Cys27Cys	Exon 2		rs2228671
c.1060 + 7 T > C	Intron 7	rs2738442		c.90C > T; p.Asn30Asn	Exon 2		No reference
c.1060 + 10G > C	Intron 7	rs12710260					
c.1359-30C > T	Intron 9	rs1003723					
c.1413A > G; p.Arg471Arg	Exon 10	rs5930					
c.1617C > T; p.Pro539Pro	Exon 11	rs5929					
c.1706-55A > C	Intron 11	rs2738447					
c.1706-69G > T	Intron 11	rs7259278					
c.1725C > T; p.Leu575Leu	Exon 12	rs1799898					
c.1773C > T; p.Asn591Asn	Exon 12	rs688					
c.1959 T > C; p.Val653Val	Exon 13	rs5925					
c.2232A > G; p.Arg744Arg	Exon 15	rs5927					
c.2389 + 46C > T	Intron 16	rs2738460					
c.2548-80G > A	Intron 18	rs2116897					
c.2548-42A > G	Intron 18	rs6413504					
c.* + 52G > A	Intron 18	rs14158					

<sup>a</sup> Official name following rules of human genome variation society (HGVS).

<sup>b</sup> SNP identification in NCBI-SNP database.

To infer the putative effects of a missense alteration identified (c.185C > T; p.Thr62Met) several analyses were performed. The amino acid conservation index (CI), defined as the percentage of species from the NCBI list that have the wild-type amino acid in a given position, was estimated for position 62 of the LDLR protein. A total of 39 amino acid reference sequences of different mammal species were used for amino acid CI calculation. Sequences were aligned using Muscle v3.6 (Edgar, 2004). The pathogenicity of the c.185C > T variant was further studied using the software packages “Mutation Taster” (Schwarz et al., 2010) and “MutPred” (Li et al., 2009).

## Results and discussion

### Mutation screening (LDLR, APOB and PCSK9) of 10 index-cases of possible FH

In the 10 unrelated “possible FH” cases analyzed using the LIPOchip® Array, no mutations were detected. The sequencing of the promoter and the 18 exons of the *LDLR* gene in these individuals revealed the presence of eighteen variants, corresponding to two missense alterations and sixteen synonymous variants (Table 1). The sample analyzed therefore contains a high number of variants when compared with samples used in similar studies, such as the one by Rafiq et al. (2011); this latter study, which screened the whole *LDLR* gene in 17 samples of Pakistani origin, described only two synonymous variants (also detected in the present sample). This indication of high genetic diversity of the Azores is not all together surprising, since previous research conducted in the Azorean populations, using different genetic systems, has shown considerable levels of diversity (Montiel et al., 2005; Santos et al., 2003). In one of the 10 individuals analyzed two missense alterations, both in the heterozygous state, were detected (Table 1). On what concerns the missense alteration identified at exon 5 (c.806G > A, rs143992984), in silico analysis of this variant predicts that given the high levels of nucleotide and amino acid conservation, it should be pathogenic (Etxebarria et al., 2012). However, segregation analysis of this variant in a Portuguese family failed to prove its association with the FH phenotype (Bourbon et al., 2008). Furthermore, functional validation indicates that this alteration does not impair lipoprotein-binding capacity, and therefore should be classified as non-pathogenic (Etxebarria et al., 2012). The other missense alteration identified at exon 8 (c.1171G > A, rs11669576) has been classified as non-pathogenic ([www.ucl.ac.uk/ldlr/LOVDv.1.1.0](http://www.ucl.ac.uk/ldlr/LOVDv.1.1.0)) and is rare in the Portuguese mainland population (data not shown).

**Table 2**

Demographic, lipid profile and genetic variants in *LDLR* gene previously associated with LDL-c levels for each of ten individuals with possible HF analysed in LipoCHIP array. Data in bold are the alleles which are associated with altered cholesterol levels.

Sample ID	Age	Gender	TC	LDL-c	Variants previously associated with LDL-c levels					
					High levels				Low levels	
					c.1359-30C > T	c.1773C > T	c.1959 T > C	c.2548-42A > G	c.81C > T	c.1617C > T
					rs1003723	rs688	rs5925	rs6413504	rs2228671	rs5929
<b>1</b>	62	F	293	204	<b>C/T</b>	C/C	T/T	A/A	<b>C/T</b>	C/C
<b>2</b>	48	F	333	189	<b>T/T</b>	<b>T/T</b>	<b>C/C</b>	<b>G/G</b>	C/C	C/C
<b>3</b>	52	F	281	208	<b>T/T</b>	C/T	T/C	A/G	<b>C/T</b>	C/C
<b>4</b>	55	M	298	219	C/C	C/C	T/T	A/A	C/C	<b>C/T</b>
<b>5</b>	27	M	268	198	<b>T/T</b>	<b>T/T</b>	<b>C/C</b>	<b>G/G</b>	C/C	C/C
<b>6</b>	54	F	317	170	C/C	C/C	T/T	A/A	<b>C/T</b>	C/C
<b>7</b>	50	F	299	155	<b>T/T</b>	C/T	T/C	<b>G/G</b>	C/C	C/C
<b>8</b>	43	F	295	175	<b>T/T</b>	<b>T/T</b>	<b>C/C</b>	<b>G/G</b>	C/C	C/C
<b>9</b>	75	F	292	214	<b>C/T</b>	C/T	T/C	A/G	<b>C/T</b>	C/C
<b>10</b>	69	M	280	205	C/C	C/C	T/T	A/G	C/C	C/C
Minor allele frequency (MAF)			1000	genomes	28%	28%	33%	36%	6%	12%
			<b>Present study</b>		<b>60%</b>	<b>45%</b>	<b>45%</b>	<b>55%</b>	<b>20%</b>	<b>5%</b>
			Ancestral allele		C	C	T	A	T	C

Total cholesterol (TC), low density lipoprotein-cholesterol (LDL-c).

Six out of the sixteen synonymous variants identified in the present study (Table 1), which have been consistently confirmed in several studies as being associated with alterations in cholesterol levels (Linsel-Nitschke et al., 2008; Martinelli et al., 2010; Miljkovic et al., 2010), are described in detail in Table 2. When compared with the normal population, a higher frequency of the risk alleles was observed in the 10 index-cases of possible FH (Table 2). Concerning 2 synonymous variants related to low LDL-c levels the T variant at c.81C > T, rs2228671 was found in the heterozygous state in 4 subjects (40%). This allele reaches a frequency of approximately 6% in Europe and appears to have the strongest association with LDL-c levels across multiple European populations, each copy being related to a decrease of LDL-c levels and to a significantly lower risk of coronary artery disease (Linsel-Nitschke et al., 2008). By the other hand, the T allele of the synonymous variant c.1617C > T, rs5929 is very rare in populations of European ancestry (Miljkovic et al., 2010). Noteworthy, the T allele is present in one of the 10 index-cases analyzed (10%); this variant displays its higher percentage in African populations such as the Yoruba in Nigeria (12.3%) and the Masai in Kenya (13.5%); a strong association of this allele with a protective role concerning LDL-c levels in multi generational families of African descent has been described (Miljkovic et al., 2010). The presence of an allele typical of African populations in the analyzed sample could be justified by the Azorean population genetic background. In fact, both historical and genetic data support a sub-Saharan African contribution to the peopling of the Azores (Matos, 1989; Mendonça, 1996).

In the total of 10 index-cases analyzed seven different haplotypes for the six synonymous variants associated with cholesterol levels (Table 2) were present (data not shown). The most frequent haplotype is TTCGCC (45%), which is a “risk” haplotype, being formed exclusively by risk alleles. This haplotype is present in 3 subjects (2, 5 and 8).

#### *Analysis of exons 2 to 6 of the LDLR gene in an additional series of 23 possible FH index-cases*

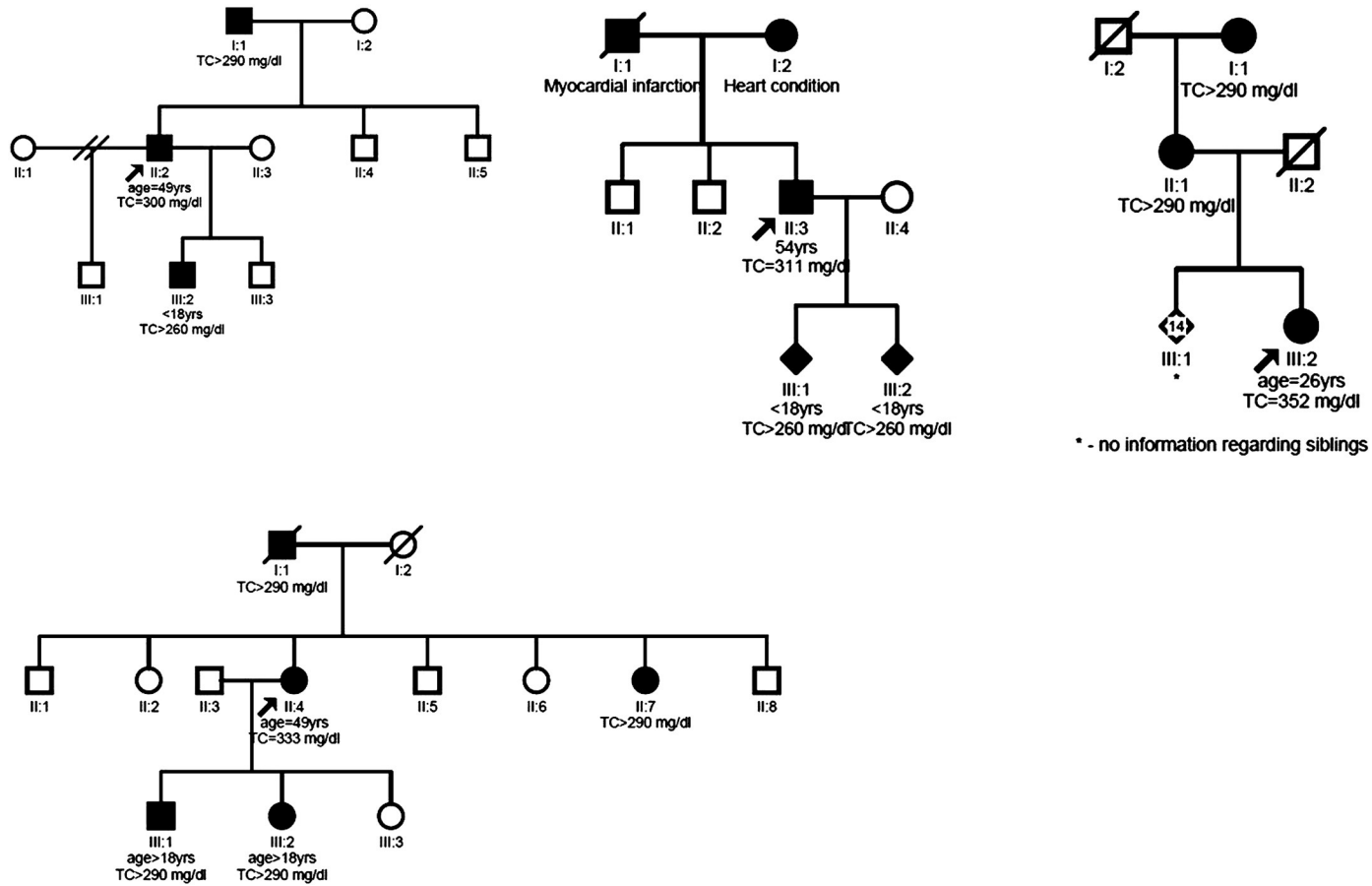
To add information as to the role of the variants detected in the initial 10 index-cases, 23 additional cases of possible FH were also studied. For these samples, demographic and lipid profile data are presented in Table 3. The sequencing of exons 2 to 6 of the *LDLR* gene of these 23 cases allowed the identification of

**Table 3**

Demographic and lipid profile data for each of 23 individuals selected for the analysis of exons 2 to 6 of the *LDLR* gene.

Sample ID	Age	Gender	TC	LDL-c
11	57	F	293	201
12	58	F	329	238
13	26	F	352	266
14	52	F	290	214
15	56	M	266	192
16	71	M	297	186
17	54	M	269	194
18	41	F	280	207
19	60	F	269	194
20	63	F	278	207
21	58	F	305	211
22	34	F	300	209
23	54	M	311	225
24	49	M	300	150
25	44	M	265	191
26	53	M	287	193
27	51	F	322	233
28	46	F	285	193
29	53	F	320	221
30	47	F	265	195
31	56	F	277	195
32	21	F	307	176
33	44	F	280	205

Total cholesterol (TC) and low density lipoprotein-cholesterol (LDL-c) levels are represented as mg/dl. M = male and F = female.



**Fig. 1.** Shows 4 pedigrees of cases included in our sample, as illustrative of the types of dyslipidemia present in these “possible cases”. These pedigrees show an autosomal dominant pattern of inheritance, characteristic of FH.

four variants (Table 1): two of these were missense alterations, both transitions and in a heterozygous state; the 2 remaining alterations correspond to synonymous variants. The missense alteration c.806G > A, rs143992984 was already found in the mutation screening of the 10 index-cases of possible FH (Table 1). Concerning the missense alteration c.185C > T, identified in one individual, is reported in the British Heart Foundation database ([www.ucl.ac.uk/ldlr/LOVDv.1.1.0](http://www.ucl.ac.uk/ldlr/LOVDv.1.1.0)) for Caucasians populations, but is novel for the Portuguese population (data not shown). No functional studies have been performed for this alteration (Fouchier et al., 2005), whose in silico analysis predicts not to be pathogenic. The amino acid CI estimated for position 62 of the LDLR protein across the protein reference sequences of the class Mammals that is 89.7%, suggesting it to be a highly stable position. Because in silico analysis of this variant failed to produce conclusive results, and since segregation studies were impossible to perform, the association of this variant with the phenotype remains to be established.

In exon 2 two synonymous variations were also detected (Table 1). The variant c.81C > T, rs2228671 was detected in 10 individuals, and is frequent in the Portuguese mainland population (data not shown); on the other hand the c.90C > T variant, identified in one individual of our sample is also being described for the first time for the Portuguese population (data not shown). Fig. 1 shows 4 pedigrees of cases included in our sample, as illustrative of the types of dyslipidemia present in these “possible cases”. These pedigrees show an autosomal dominant pattern of inheritance, characteristic of FH.

Although no pathogenic mutations were found in the studied population, several genetic alterations were detected, allowing the establishment of a FH variant profile in the Azores. This profile deserves further investigation, since data derived will promote the early detection of this disease and the minimization of its impact.

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