

Original article

Diagnostic yield of sequencing familial hypercholesterolemia genes in individuals with primary hypercholesterolemia



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ABSTRACT

Introduction and objectives: Our objective was to approximate the prevalence of mutations in candidate genes for familial hypercholesterolemia (FH) in a middle-aged Spanish population and to establish the predictive value of criteria for clinical suspicion in the detection of causative mutations.

Methods: Unrelated individuals aged ≥ 18 years from the Aragon Workers' Health Study (AWHS) with high low-density lipoprotein cholesterol (LDL-C) and clinical suspicion of FH (participants with LDL-C concentrations above the 95th percentile, participants with premature cardiovascular disease and/or participants with high LDL-C [130 mg/dL] under statin therapy), assuming that any participant with FH exhibits at least 1 trait, were selected and the *LDLR*, *APOB*, *PCSK9*, *APOE*, *STAP1* and *LDLRAP1* genes were sequenced by next generation sequencing technology.

Results: Of 5400 individuals from the AWHS, 4514 had complete data on lipid levels and lipid-lowering drugs, 255 participants (5.65%) met the criteria for suspicion of FH, 24 of them (9.41%) were diagnosed with hyperlipoproteinemia(a), and 16 (6.27% of those sequenced) were found to carry causative mutations in candidate genes: 12 participants carried 11 different pathogenic *LDLR* alleles and 4 participants carried 1 pathogenic mutation in *PCSK9*. LDL-C concentrations > 220 mg/dL and LDL-C > 130 mg/dL despite statin therapy showed the strongest association with the presence of mutations ($P = .011$).

Conclusions: Our results show that the prevalence of FH in Spain is 1:282 and suggest that the combination of high untreated LDL-C and high levels of LDL-C despite statin therapy are the best predictors of a positive FH genetic test.

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Rendimiento diagnóstico de la secuenciación de genes de hipercolesterolemia familiar en sujetos con hipercolesterolemia primaria

RESUMEN

Introducción y objetivos: Nuestro objetivo fue aproximar la prevalencia de mutaciones en los genes candidatos de hipercolesterolemia familiar (HF) en una población española de mediana edad, y determinar el valor predictivo de los criterios clínicos de sospecha de HF en la detección de mutaciones causales.

Métodos: Se seleccionaron individuos mayores de 18 años no relacionados de la cohorte Estudio de Salud de los Trabajadores de Aragón (AWHS) con colesterol unido a lipoproteínas de baja densidad (cLDL) $>$ percentil 95, con enfermedad cardiovascular prematura o con cLDL $>$ 130 mg/dl con tratamiento hipolipemiente, asumiendo que al menos una de las características estará presente en todos los individuos con HF. En estos participantes se secuenciaron los genes *LDLR*, *APOB*, *PCSK9*, *APOE*, *STAP1* y *LDLRAP1* mediante secuenciación masiva.

Palabras clave:

Prevalencia HF

Mutación en genes candidatos

LDLR

PCSK9

STAP1

Lipoproteína (a)

Criterios de sospecha de HF

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Resultados: De 5.400 individuos del AWHs, 4.514 tenían datos lipídicos y registro farmacológico hipolipemiante completo, 255 participantes (5,65%) cumplían los criterios de sospecha de HF, 24 de ellos (9,41%) fueron diagnosticados de hiperlipoproteinemia(a) y 16 (6,27% de los secuenciados) presentaron alguna mutación causal en genes candidatos: 12 participantes portaban 11 alelos patogénicos diferentes en *LDLR* y 4 participantes portaban una mutación patogénica en *PCSK9*. Las concentraciones de cLDL > 220 mg/dl y el cLDL > 130 mg/dl a pesar del tratamiento con estatinas mostraron la mayor asociación con la presencia de mutación ($p = 0,011$).

Conclusiones: Nuestros resultados muestran que la prevalencia española de HF es 1:282 y sugieren que una concentración de cLDL elevado, y niveles altos de cLDL a pesar de la terapia con estatinas son los mejores predictores para un diagnóstico genético positivo de HF.

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Abbreviations

AWHS: Aragon Workers' Health Study
 CHD: coronary heart disease
 FH: familial hypercholesterolemia
 LDL-C: low-density lipoprotein cholesterol

INTRODUCTION

Familial hypercholesterolemia (FH) is a genetic disorder characterized by very high plasma total cholesterol concentrations, due to increased low-density lipoprotein cholesterol (LDL-C), with a high risk of premature coronary heart disease (CHD).¹ Traditionally, FH has been described as a monogenic disease, with autosomal codominant transmission and an estimated prevalence of around 1:500 in the general population.² However, there are some discrepancies in the prevalence of FH. Recent studies have revealed that clinically defined FH is probably more common than previously reported, with a prevalence of 1:217 in the Copenhagen General Population study.³ Early diagnosis is crucial in FH because lipid-lowering treatment has been demonstrated to drastically reduce CHD,⁴ especially if treatment begins early in life.⁵ However, FH is underdiagnosed and undertreated in the general population.⁶

The clinical diagnosis of FH relies on high plasma LDL-C, family history of hypercholesterolemia, personal and family history of premature CHD, and signs of cholesterol deposition such as tendon xanthomas and premature arcus cornealis. These variables are often clinically scored by applying the Make Early Diagnosis to Prevent Early Death (MEDPED) criteria, the Dutch Lipid Clinic Network (DLCN) MEDPED modification, or the Simon Broome Register Group (SBRG) criteria.⁷ These clinical criteria have been demonstrated to be strongly associated with genetic diagnosis.^{8,9} However, these criteria include information that is not always available or requires some expertise, and they are mostly used in specialized units.^{6,10} For this reason and to improve diagnosis, the European Atherosclerosis Society Consensus Panel recommends the criteria for suspicion prior to clinical diagnosis based on the following: a) for adults, plasma total cholesterol above 310 mg/dL or the 95th percentile by age and sex for country; b) for children, plasma total cholesterol level above 230 mg/dL or the 95th percentile by age and sex for country; c) premature CHD (< 55 years in men and < 60 years in women); d) tendon xanthomas; e) sudden premature CHD death in a family member (< 55 years in men and < 60 years in women).⁶ However, the clinical value of these criteria for suspicion has not been validated.

Molecular genetic diagnosis is the gold-standard in a monogenic disease and is strongly recommended in FH.¹¹ FH is caused by mutations in several genes: *LDLR*, coding for the low-density

lipoproteins (LDL) receptor; *APOB*, coding for apolipoprotein B,¹² and *PCSK9*, coding for the enzyme proprotein convertase subtilisin/kexin type 9.¹³ One new *locus* causing FH has been identified: the p.(Leu167del) mutation in *APOE*.¹⁴ In addition, several mutations in the signal transducing adaptor family member *STAP1* have been associated with FH,¹⁵ although the role of this gene has recently been questioned.^{16,17} Furthermore, plasma levels of lipoprotein(a) (Lp(a)) is an inheritable trait associated with increased CHD risk, and it has been reported that hyperlipoproteinemia(a) (hyperLp(a)) is a common cause of autosomal dominant hypercholesterolemia.¹⁸ Knowledge of the causative mutation confirms diagnosis, confers prognosis, facilitates family screening, and improves patient adherence to treatment.¹⁹ The presence of pathogenic mutations in FH causative genes in patients with a clinical diagnosis of FH confers a substantially increased risk for CHD,²⁰ and therefore genetic tests allow identification of persons with the highest CHD risk among people with hypercholesterolemia. However, this genetic testing also has some difficulties, including the genetic complexity of the disease with several genes involved, thousands of different mutations, some of them of uncertain pathogenic significance, and the poor availability of genetic analysis in some health systems.

The objective of the present study was to approximate the prevalence of mutations in candidate genes in a middle-aged Spanish population based on criteria for clinical suspicion and to analyze their predictive value for the presence of causative mutations.

METHODS

Participants

Individuals were selected from the Aragon Workers' Health Study (AWHS). The AWHs is a longitudinal cohort study of cardiovascular risk factors and subclinical atherosclerosis performed among 5400 workers at the Opel Spain factory in Aragon, Spain, who have been followed up since 2009.²¹ Here, we studied unrelated individuals ≥ 18 years old from the AWHs, with annual or biannual determinations since 2009. The following criteria were used to select those with clinical suspicion of FH: a) participants with untreated LDL-C concentrations > 95th percentile of the Spanish population stratified by age and sex²² in at least 2 measurements; b) participants with premature CHD (< 55 years in men and < 60 years in women) at baseline; c) participants under statin therapy and LDL-C > 130 mg/dL in at least 2 measurements; d) participants with untreated LDL-C concentrations > 95th percentile of the Spanish population in just 1 measurement but who also had LDL-C > 130 mg/dL under statin therapy. The exclusion criteria were the presence of secondary causes of hypercholesterolemia at baseline or during follow-up,

which include severe obesity (body mass index [BMI] > 35 kg/m²), poorly controlled type 2 diabetes (HbA1c > 8%), renal disease with glomerular filtration rate < 30 mL/min and/or macroalbuminuria, liver disease (alanine transaminase > 3 times the upper normal limit), hypothyroidism (thyroid stimulating hormone > 6 mIU/L), pregnancy, autoimmune diseases, and treatment with protease inhibitors. We defined hyperLp(a) participants as those participants with suspicion of FH who had Lp(a) > 50 mg/dL, but normal LDL-C concentrations adjusted by Lp(a) (< 95th percentile of the Spanish population)²².

All participants gave written informed consent, and the study was approved by the Aragón Ethics Committee for Clinical Research (CEICA).

Assessment of cardiovascular risk factors, personal and family history of cardiovascular disease, intake of drugs affecting lipid metabolism, and anthropometric measurements were performed in all participants at baseline and annually or biannually during the follow-up.

Lipid analysis

Lipid and lipoprotein analyses were performed on EDTA plasma samples collected after an overnight fast of at least 10 hours annually or biannually since 2009. Measurements up to 2015 were included. Total cholesterol and triglyceride levels were determined by standard enzyme methods. High-density lipoprotein cholesterol (HDL-C) was measured directly by an enzyme reaction using cholesterol oxidase (UniCel Dx C 800; Beckman Coulter, Inc, USA). LDL-C was calculated by Friedewald's formula. Lipid values were adjusted according to statin therapy in those participants taking lipid-lowering drugs.²³ None of these participants received PCSK9 inhibitors. Lp(a) was determined by IMMAGE kinetic nephelometry (Beckman Coulter, Inc, USA). LDL-C adjusted by Lp(a) was obtained by subtracting the concentrations of Lp(a) divided by 3 to the LDL-C concentration calculated by the Friedewald formula.^{24,25}

Genetic analysis

Whole blood genomic DNA was isolated using standard methods. *LDLR* (NM_000527.4), *PCSK9* (NM_174936.3), *APOE* (NM_000041.3), *STAP1* (NM_0121108.3), and *LDLRAP1* (NM_015627.2) genes and exons 26 and 29 of *APOB* (NM_000384.2), which encodes the *LDLR* binding site, were sequenced by next generation sequencing with the SeqProLipo Platform (Progenika Biopharma Grifols, Spain). This platform includes point mutations, large rearrangements, and copy number variations.

To evaluate the pathogenicity of identified variants we used SIFT,²⁶ PolyPhen-2,²⁷ Mutation Taster,²⁸ and PredictSNP.²⁹ The effect of variants in potential splicing sites was predicted with FruitFly.³⁰ To compare the frequency of identified variants in the study participants with that of the general population, we compiled the allele frequencies of the identified variants from the 1000 Genomes Project³¹ and ExAc Browser Data.³² Rare variants were defined as genetic variants with a frequency < 1% in the general population. A rare variant was defined as a causative variant when it had been previously associated with FH or possibly pathogenic when the bioinformatic analysis prediction classified it as pathogenic.

Statistical analysis

Analyses were performed using statistical computing software R version 3.5.0.³³ The distribution of the variables was analyzed

with the Shapiro test. Quantitative variables with a normal distribution are expressed as mean ± standard deviation and were analyzed with the ANOVA test. Variables with a skewed distribution are expressed as median and interquartile range and were analyzed with the Kruskal-Wallis test. Qualitative variables are expressed as percentages and were analyzed with the chi-square test. The level of significance was set at $P < .05$. Logistic binary regression was used to determine the best European Atherosclerosis Society criteria diagnosis with the presence of a mutation in candidate genes as the dependent variable.

RESULTS

Study participants meeting FH clinical criteria

Of the 5400 individuals from the AWHs, 4514 participants had the information required by the inclusion and exclusion criteria. A total of 886 individuals were excluded because they had incomplete recorded lipid data, less than 2 complete lipid panel and/or incomplete information about lipid-lowering drug use. Of the 4514 participants, 255 (5.65%) met the following inclusion criteria (not mutually exclusive): *a*) 127 participants had untreated LDL-C concentrations > 95th percentile of the Spanish population in at least 2 measurements; *b*) 21 participants had premature CHD at baseline (< 55 years in men and < 60 years in women); *c*) 39 participants had LDL-C > 130 mg/dL in at least 2 measurements while taking statin therapy and; *d*) 113 participants had untreated LDL-C concentrations > 95th percentile of the Spanish population in 1 measurement and also had LDL-C > 130 mg/dL on a least another occasion with statin therapy (figure 1).

Participants with high levels of LDL-C despite statin therapy and those with CHD at baseline were older and had higher BMI than the rest of the participants. Participants with statin therapy and high levels of LDL-C despite drug treatment, together with participants with high LDL-C concentrations in 1 measurement and also high levels of LDL-C with statin therapy, had higher concentrations of total cholesterol, LDL-C and triglycerides than the remaining participants. Participants with premature CHD had higher levels of Lp(a). The percentage of participants with high levels of LDL-C in at least 2 measurements who did not take any lipid-lowering therapy was 93.7% (table 1).

Genetic analysis

Sequencing analysis reported 5280 variants in candidate genes among the 255 sequenced participants (figure 1): *a*) 1792 variants in *LDLR*, 11 of which have been described as pathogenic and previously associated with FH; *b*) 249 variants in *APOB*, although none of them has been described as pathogenic or previously associated with FH; *c*) 1697 variants in *PCSK9*, only 1 of which has been described as pathogenic and previously associated with FH; *d*) 72 variants in *APOE*, 2 of which have been previously associated with type III hyperlipoproteinemia; *e*) 869 variants in *STAP1*, 3 of which have been described as possibly pathogenic by bioinformatic analysis; *f*) 601 variants in *LDLRAP1* gene, 2 of which have been classified as possibly pathogenic by bioinformatic analysis. However, neither of these 2 possibly pathogenic variants in *LDLRAP1* were present in homozygosity, a condition that has been described as necessary to cause hypercholesterolemia.

The percentage of mutations in candidate genes was similar among groups; however, participants with high levels of LDL-C in at least 2 measurements showed the highest percentage of causative mutations, exceeding 8% of cases (table 1). A total of 12 patients were carriers of 11 distinct pathogenic alleles in

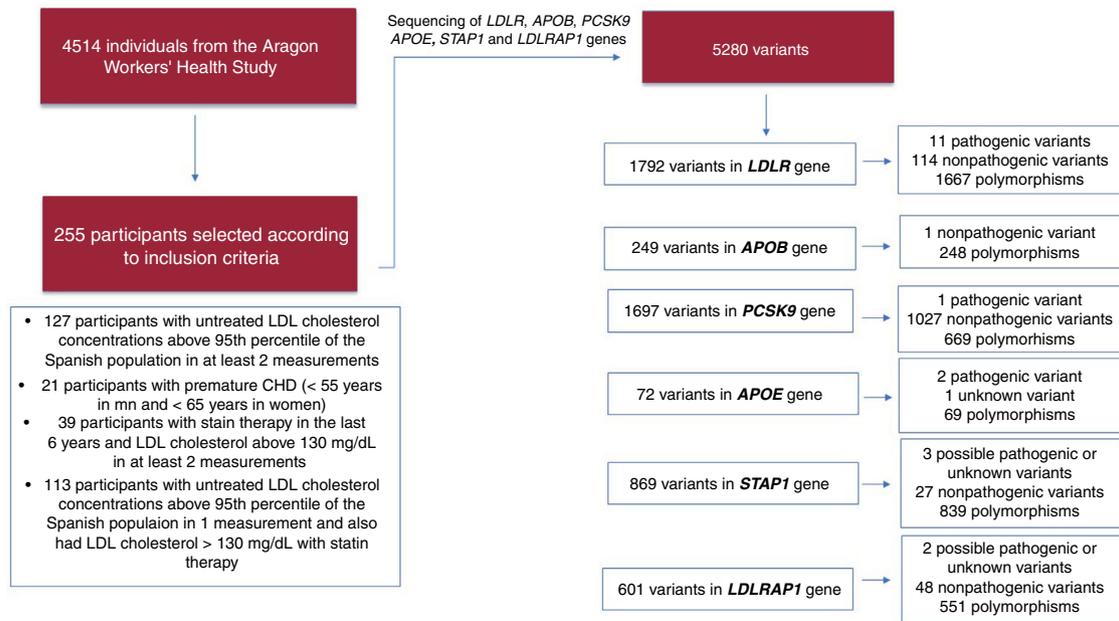


Figure 1. Selection criteria and identified variants in candidate familial hypercholesterolemia genes. CHD, coronary heart disease; LDL, low-density lipoprotein.

heterozygosity in the *LDLR*: 1 of them with a causative variant located in the promoter region (c.-135C>G); 7 of them had a causative variant, located in coding regions, producing an amino acid change (c.530C>T, c.826T>G, c.862G>A, c.1247G>A,

c.1529C>T, c.1775G>A, and c.1816G>A); 1 of them with a causative variant, located in an intronic region, producing an alternative splicing change (c.1586+5G>A); 1 allele had 2 causative variants located in *cis*: (c.274C>G) and (c.313+1G>C) and 1 of

Table 1

Baseline biochemical and clinical characteristics, and number of mutation carriers according to selection criteria.

	Participants with untreated LDL-C concentrations above 95th percentile of the Spanish population in at least 2 measurements (n = 127)	Participants with premature CHD (< 55 years in men and < 60 years in women) (n = 21)	Participants with statin therapy in the last 6 years and LDL-C above 130 mg/dL in at least 2 measurements (n = 39)	Participants with untreated LDL-C concentrations above 95th percentile of the Spanish population in 1 measurement and also LDL-C > 130 mg/dL with statin therapy (n = 113)
Age, years	40.2 ± 8.47	48.0 ± 4.59	47.7 ± 4.45	46.4 ± 5.23
BMI, kg/m ²	26.8 ± 3.11	26.4 ± 2.38	28.2 ± 2.89	27.4 ± 2.73
Men	119 (93.7)	21 (100)	39 (100)	108 (95.6)
Total cholesterol, mg/dL	318 ± 57.4	271 ± 61.4	352 ± 48.6	351 ± 61.0
LDL-C, mg/dL	223 ± 47.3	177 ± 45.7	235 ± 38.9	239 ± 48.6
HDL-C, mg/dL	51.7 ± 8.92	49.0 ± 8.34	52.8 ± 11.3	53.6 ± 9.57
Triglycerides, mg/dL	143 [113-201]	99.5 [82.9-163]	178 [141-247]	150 [116-217]
Apolipoprotein B, mg/dL	135 ± 29.1	101 ± 51.2	120 ± 22.1	139 ± 30.00
Lipoprotein(a), mg/dL	41.1 [8.48-75.4]	72.3 [42.4-80.7]	25.5 [15.2-34.6]	24.4 [9.53-60.3]
Glucose, mg/dL	89.1 ± 11.4	94.6 ± 27.3	95.1 ± 12.3	90.5 ± 12.4
Hb1Ac, %	5.30 [5.20-5.30]	5.30 [5.30-5.30]	5.30 [5.30-5.30]	5.40 [5.35-5.50]
Insulin, U/ml	5.50 [3.58-7.60]	4.10 [3.60-7.10]	5.30 [4.10-7.13]	5.70 [3.95-9.20]
Statin therapy				
None	119 (93.7)	10 (47.6)	0 (0)	84 (74.3)
Low intensity	0 (0)	0 (0)	6 (15.4)	5 (5.95)
Moderate intensity	7 (5.52)	10 (47.6)	31 (79.5)	22 (19.5)
High intensity	1 (0.78)	1 (4.76)	2 (5.13)	2 (1.77)
Causative mutation in candidate genes				
None	114 (89.8)	20 (95.2)	36 (92.3)	103 (91.2)
<i>LDLR</i> mutation carriers	8 (6.30)	0 (0)	2 (5.55)	9 (7.96)
<i>PCSK9</i> mutation carriers	3 (2.36)	1 (4.76)	0 (0)	0 (0)
<i>STAP1</i> mutation carriers	2 (1.57)	0 (0)	1 (2.56)	1 (0.88)

BMI, body mass index; CHD, coronary heart disease; HbA1c, glycosylated hemoglobin; HDL-C, High-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. Total, LDL-C and HDL-C and triglyceride levels have been adjusted according to statin therapy.²³ Selected participants may belong to more than one group. Data are expressed as No. (%), mean ± standard deviation or median [interquartile range].

Table 2
Causative variants in candidate genes identified in patients selected in this study

Gene	SNV	Nucleotide	Amino acid change	Number of carriers	Bioinformatic analysis				Clin Var	Frequency ExAc ³²	Frequency 1000 G ³¹
					SIFT ²⁶	POLYPHEN-2 ²⁷	Mutation Taster ²⁸	PredictSNP ²⁹			
<i>LDLR</i>	rs879254375	c.-135C>G	NA	1 participant	NA	NA	NA	NA	Pathogenic	-	-
<i>LDLR</i>	rs774467219 rs112029328	c.[274C>G; 313+1G>C]	p.[Gln92Glu;NA]	1 participant	Tolerated (0.15)	Possibly damaging (0.736)	Deleterious (0.510)	Neutral (0.252)	Pathogenic	0.0009715	0.001
<i>LDLR</i>	rs121908026	c.530C>T	p.(Ser177Leu)	1 participant	Deleterious (0.01)	Probably damaging (0.999)	Deleterious (0.896)	Deleterious (0.000090)	Pathogenic	8.958e-06	-
<i>LDLR</i>	rs879254692	c.826T>G	p.(Cys276Gly)	1 participant	Deleterious (0)	Probably damaging (0.969)	Deleterious (0.856)	Deleterious (0.000005)	Pathogenic	-	-
<i>LDLR</i>	rs368657165	c.862G>A	p.(Glu288Lys)	1 participant	Deleterious (0.05)	Probably damaging (0.918)	Deleterious (0.714)	Deleterious (0.000018)	Pathogenic	0	-
<i>LDLR</i>	-	c.941-?_1845+?del	Deletion from exon 7 to exon 12	1 participant					Pathogenic		
<i>LDLR</i>	rs773658037	c.1247G>A	p.(Arg416Gln)	1 participant	Deleterious (0.01)	Probably damaging (0.957)	Deleterious (0.806)	Deleterious (0.000045)	Pathogenic	1.793e-05	0
<i>LDLR</i>	rs755154048	c.1529C>T	p.(Thr510Met)	1 participant	Deleterious (0.02)	Possibly damaging (0.791)	Deleterious (0.679)	Deleterious (0.000013)	Pathogenic/ Likely pathogenic	8.952e-06	-
<i>LDLR</i>	rs781362878	c.1586+5G>A	NA	1 participant	NA	NA	NA	NA	Uncertain/ Pathogenic	1.796e-05	
<i>LDLR</i>	rs137929307	c.1775G>A	p.(Gly592Glu)	2 participants	Deleterious (0.01)	Probably damaging (0.925)	Deleterious (0.779)	Deleterious (0.000015)	Pathogenic	8.951e-05	-
<i>LDLR</i>	rs72658865	c.1816G>A	p.(Ala606Thr)	1 participant	Deleterious (0.02)	Possibly damaging (0.5)	Deleterious (0.550)	Deleterious (0.000034)	Likely benign/ Uncertain	8.952e-06	-
<i>PCSK9</i>	rs371488778	c.60_65dup GCTGCT	p.(Leu22_Leu23dup)	4 participants	NA	NA	NA	Deleterious (0.000002)	Uncertain/ Pathogenic	0.002144	-

NA, not applicable.

Table 3

Clinical characteristics and lipid profile according to the primary cause of hypercholesterolemia

	Participants without mutation in candidate genes and without hyperLp(a) (n = 211)	Participants carrying <i>LDLR</i> mutations (n = 12)	Participants carrying <i>PCSK9</i> mutations (n = 4)	Participants carrying <i>STAP1</i> mutations (n = 4)	Participants with hyperLp(a) (n = 24)	P
Age, years	44.3 ± 7.53	44.3 ± 6.89	43.0 ± 9.09	37.8 ± 13.7	41.7 ± 9.43	.242
BMI, kg/m ²	27.3 ± 2.83	26.5 ± 3.38	28.9 ± 3.41	26.0 ± 1.36	25.7 ± 3.46	.061
Men, %	207 (96.3)	11 (91.7)	4 (100)	4 (100)	21 (87.5)	.356
Total cholesterol, mg/dL	323 ± 53.8	406 ± 97.8	321 ± 13.2	304 ± 24.7	298 ± 41.9	<.001
HDL-C, mg/dL	52.1 ± 9.96	56.2 ± 6.77	53.8 ± 11.8	53.5 ± 9.00	55.2 ± 10.2	.440
LDL-C, mg/dL	220 ± 38.6	297 ± 91.8	221 ± 18.8	208 ± 26.0	221 ± 28.8	<.001
LDL-C adjusted by Lp(a), mg/dL	218 ± 37.3	296 ± 92.6	220 ± 19.6	207 ± 25.9	164 ± 26.9	<.001
Triglycerides, mg/dL	172 ± 80.3	132 ± 37.3	126 ± 27.3	111 ± 23.2	119 ± 52.1	.004
Lipoprotein(a), mg/dL	20.3 [8.25-41.0]	17.2 [13.9- 20.6]	4.50 [3.20-6.00]	4.50 [3.13-7.90]	80.0 [68.8-114]	<.001

BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a).

The P value was calculated by ANOVA test or Kruskal-Wallis and chi-square, as appropriate.

Data are expressed as No. (%), mean ± standard deviation or median [interquartile range].

them had a large rearrangement (c.941-?_1845+?del). All of them have been described as pathogenic by bioinformatic analysis and have been previously reported as a cause of FH.^{34–40} Only 1 patient carried a rare variant in exon 26 of the *APOB* gene, (c.10621A >G). However, this variant has not been previously associated as a cause of FH, has been classified as benign by bioinformatic analysis, and seems not to affect the binding region structure. A total of 5 patients carried 2 rare variants in the *PCSK9* gene (c.60_65dup GCTGCT and c.743G >A), and only the first one has been previously associated with FH. Four of these patients were carriers of the c.60_65dupGCTGCT in-frame indel, which has been described as uncertain or likely pathogenic by Clin Var⁴¹ and Garcia et al.⁴² A total of 5 patients carried 3 rare variants in the *APOE* gene, none of them previously described as a cause of FH. One of them has not been previously associated with any hyperlipidemia and has been classified as unknown by bioinformatic analysis (c.335C >A). The other 2 variants, (c.460C >A, c.487C >T), were present in 3 and 1 participants, respectively. Both variants have been previously associated with type III hyperlipoproteinemia.^{43,44} A total of 4 patients carried 3 unknown rare variants in the *STAP1* gene; 2 of them carried 1 variant located at the 5' region (c.-60A >G) and the other 2 were carriers of 2 different variants, located in coding regions, which produce amino acid substitution (c.619G >A and c.803T >C). None of these variants have been previously associated with FH. Two patients carried 2 unknown rare variants in heterozygous state in the *LDLRAP1* gene, 1 of them producing an amino acid change (c.605C >G) and the other could affect splicing (c.748-7C >G). However, mutations in *LDLRAP1* only produce hypercholesterolemia when they are found in homozygosity (table 2 and table 1 of the supplementary data).

HyperLp(a)

In 24 (9.41%) participants with criteria for suspicion of FH due to LDL-C > 95th percentile, when the cholesterol transported in Lp(a) was analyzed, their LDL-C were no longer > 95th percentile. None of these hyperLp(a) participants was a carrier of a pathogenic mutation in FH candidate genes.

Characteristics according to genetic analysis

Table 3 shows the clinical characteristics and lipid profile according to the genetic classification including hyperLp(a)

participants. Participants carrying *LDLR* mutations had the highest levels of total and LDL-C. In addition, participants carrying *PCSK9* mutations had significantly higher levels of total and LDL-C than those carrying *STAP1* mutations and participants classified with hyperLp(a) ($P < .001$ and $P < .001$, respectively). Moreover, participants carrying *STAP1* mutations had the lowest levels of total and LDL-C, and had lower levels of total and LDL-C than participants without mutation in candidate genes.

Mutations in the *LDLR* gene were responsible for 75% of some mutations in candidate genes, vs 25% of those in participants who carried some mutation in the *PCSK9* gene. Figure 2 shows the percentage of mutations according to groups by baseline LDL-C levels. The higher the percentage, the greater the increase in LDL-C: 0.40% of participants with baseline LDL-C < 190 mg/dL, 1.20% of participants with baseline LDL-C from 190-220 mg/dL, and 4.80% of participants with baseline LDL-C > 220 mg/dL had a mutation in candidate genes. Considering those patients with a mutation in candidate FH genes plus those with hyperLp(a), almost 16% of the group with criteria for suspicion had monogenic disease.

Logistic binary regression was used to study the association of clinical data with 2 response variables: mutation in any gene and mutation in the *LDLR* gene. The analysis showed that baseline

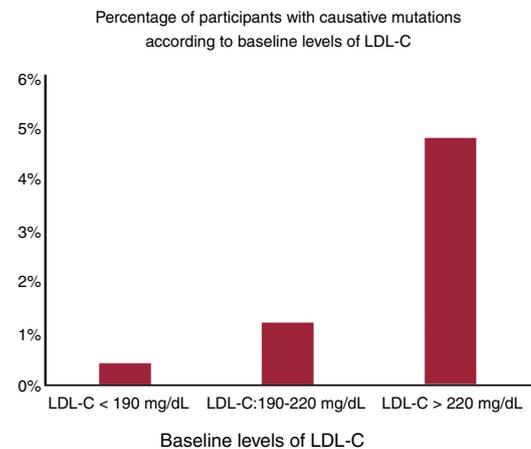


Figure 2. Percentage of participants with causative mutations according to baseline levels of low-density lipoprotein cholesterol. LDL-C, low-density lipoprotein cholesterol.

Table 4
Logistic binary regression with presence of mutation in candidate genes as dependent variable

A	OR	95%CI	P	R ² Nagelkerke
Baseline age, years	0.962	0.879-1.065	.243	0.192
Baseline BMI, kg/m ²	1.018	0.838-1.234	.852	
Baseline LDL-C, mg/dL	1.023	1.012-1.036	<.001	
B	OR	95%CI	P	R ² Nagelkerke
Baseline age, years	0.958	0.873-1.061	.379	0.213
Baseline BMI, kg/m ²	1.026	0.839-1.25	.797	
Baseline LDL adjusted by Lp(a), mg/dL	1.024	1.013-1.037	<.001	
C	OR	95%CI	P	R ² Nagelkerke
Baseline age, years	0.961	0.880-1.059	.389	0.157
Baseline BMI, kg/m ²	0.995	0.821-1.204	.959	
Baseline total cholesterol, mg/dL	1.017	1.008-1.025	<.001	
D	OR	95%CI	P	R ² Nagelkerke
CRITERION 1 ^a	2.332	0.822-7.591	.127	0.099
CRITERION 2 ^b	0.735	0.039-4.153	.775	
CRITERION 3 ^c	0.324	0.026-7.607	.389	
CRITERION 4	4.01	1.163-18.40	.011	

95%CI, 95% confidence interval; BMI, body mass index; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); OR, odds ratio. Participants with untreated LDL-C concentrations above 95th percentile of the Spanish population in one measurement and also LDL-C > 130 mg/dL with statin therapy. Table 4A-C show the logistic binary regression using the presence of any mutation (yes/no) in any candidate gene. Table 4D shows the logistic binary regression using the presence of any mutation (yes/no) in the *LDLR* gene.

^a Participants with untreated LDL cholesterol concentrations above 95th percentile of the Spanish population in at least 2 measurements.

^b Participants with premature CHD (<55 years in men and <60 years in women).

^c Participants with statin therapy in the last 6 years and LDL cholesterol above 130 mg/dL in at least 2 measurements.

Table 5
Number of participants carrying a mutation based on the criteria they meet

CRITERION 1	Participants who meet criteria				PPV	NPV*	Sensibility*	Specificity*
	Yes (n=127)		No (n=128)					
Mutation	Yes	No	Yes	No				
Participants with mutation	13	114	7	121	0.102	0.945	0.650	0.514
Participants with <i>LDLR</i> mutation	8	119	4	124	0.063	0.969	0.667	0.510
Participants with <i>PCSK9</i> mutation	3	124	1	127	0.024	0.992	0.750	0.506
Participants with <i>STAP1</i> mutation	2	125	2	126	0.016	0.984	0.500	0.498
CRITERION 2	Yes (n=21)		No (n=234)		PPV	NPV*	Sensibility*	Specificity*
Mutation	Yes	No	Yes	No				
Participants with mutation	1	20	19	215	0.048	0.919	0.050	0.915
Participants with <i>LDLR</i> mutation	0	21	12	222	0.000	0.949	0.000	0.914
Participants with <i>PCSK9</i> mutation	1	20	3	231	0.048	0.987	0.750	0.920
Participants with <i>STAP1</i> mutation	0	21	4	230	0.000	0.983	0.000	0.916
CRITERION 3	Yes (n=39)		No (n=216)		PPV	NPV*	Sensibility*	Specificity*
Mutation	Yes	No	Yes	No				
Participants with mutation	3	36	17	199	0.077	0.921	0.150	0.847
Participants with <i>LDLR</i> mutation	2	37	10	206	0.051	0.954	0.166	0.848
Participants with <i>PCSK9</i> mutation	0	39	4	212	0.000	0.981	0.000	0.845
Participants with <i>STAP1</i> mutation	1	38	3	213	0.026	0.986	0.250	0.849
CRITERION 4	Yes (n=113)		No (n=142)		PPV	NPV*	Sensibility*	Specificity*
Mutation	Yes	No	Yes	No				
Participants with mutation	10	103	10	132	0.088	0.930	0.500	0.562
Participants with <i>LDLR</i> mutation	9	104	3	139	0.080	0.979	0.750	0.572
Participants with <i>PCSK9</i> mutation	0	113	4	138	0.000	0.972	0.000	0.550
Participants with <i>STAP1</i> mutation	1	112	3	139	0.009	0.979	0.250	0.553

NPV, negative predictive value; PPV, positive predictive value.

* Regarding the sample selected due to the presence of at least one criterion.

LDL-C, LDL-C adjusted by Lp(a) and total cholesterol levels were significantly associated with the presence (yes/no) of any mutation in candidate genes (OR, 1.023; $P < .001$; OR, 1.024; $P < .001$ and OR, 1.017; $P < .001$ respectively, table 4A–C). Nevertheless, no criterion showed a significant association with the presence of any mutation in candidate genes. The presence of mutation in the *LDLR* (yes/no) was significantly associated with baseline total and LDL-C (OR, 1.009; $P = .010$ and OR, 1.016; $P = .0488$, respectively) and the criteria for suspicion of FH: untreated LDL-C concentrations above the 95th percentile in 1 measurement and also LDL-C > 130 mg/dL while taking statins ($P = .011$, table 4D).

Table 5 shows the number of participants with a mutation based on the criteria they met, positive and negative predictive values and sensitivity and specificity regarding the sample selected due to the presence of at least 1 criterion. The first criterion had the highest positive predictive value (10.2%) to detect the presence of functional mutations in candidate genes (*LDLR*, *PCSK9* and *STAP1* genes), especially to detect mutations in the *LDLR* and *PCSK9* genes (6.3% and 2.4%). Nevertheless, the fourth criterion showed the highest positive predictive value to detect mutations in the *LDLR* gene (8.0%), showing in this selected sample higher specificity than the first criterion (0.510 and 0.572, respectively).

DISCUSSION

This study analyses the frequency of pathogenic mutations in candidate genes for monogenic FH in a population with clinical suspicion of FH. Three major conclusions can be drawn from our results. First, high LDL-C is the main factor associated with a positive genetic diagnosis; second, a high LDL-C alone is not specific enough to be used for FH identification, requiring genetic analysis, and third, Lp(a) concentration should be included in the diagnostic algorithm for FH. Our study analyses more than 4500 individuals from the AWHs, a healthy middle-aged population, showing that approximately 5% (255 participants) fulfilled the criteria for diagnostic suspicion of FH, but only 16 participants (0.4%) had a pathogenic mutation in the *LDLR* or *PCSK9* genes.

FH identification is an important issue because FH mutation carriers have a substantially increased risk for CHD,²⁰ and therefore genetic study allows identification of the highest risk within hypercholesterolemic participants.⁵ The analysis of candidate genes together with Lp(a) quantification allows identification of almost 16% of the causes of these severe hypercholesterolemia groups. Our data are in agreement with previously published reports on the prevalence of hyperLp(a) as a cause of primary hypercholesterolemia^{18,45} and reinforces the idea that genetic evaluation of patient with suspected FH should include candidate genes and Lp(a) concentration quantification.⁹

The concept of FH is evolving and, possibly with the current clinical criteria, the disease is a genetic severe hypercholesterolemia syndrome, which is sometimes monogenic, and sometimes polygenic or with a complex background.⁴⁶ This article refers to heterozygous FH, which is the most common form within FH with a definite clinical diagnosis and the form most closely associated with CVD, and which therefore requires an earlier presumptive diagnosis.

Traditionally, the prevalence of FH has been estimated at 1:500.² However, subsequent studies have revealed that clinically defined FH is probably more common than previously reported, with a prevalence of 1:217 in the Copenhagen General Population study.³ In our study, we found that 255 participants, from a total of 4514 individuals studied, met the criteria for suspicion of FH, which indicates that 1:18 met the criteria for suspicion of FH. Of 255 participants, 20 participants had a rare variant in the *LDLR*, *PCSK9*, or *STAP1* genes, which would suggest a prevalence of FH of

1:226. Of 20 participants with rare variants in candidate genes, 12 of them had a mutation in *LDLR*, 4 of them were carriers of a mutation in *PCSK9*, and 4 of them had a variant in *STAP1*.

According to recent studies,^{16,17,47} the role of *STAP1* has not been clearly associated with FH phenotype and, for that reason, we recalculated the prevalence of FH using only carriers of mutations in *LDLR* and *PCSK9* genes. In this way, the prevalence would decrease from 1:226 to 1:282. In the present study, we have demonstrated that *LDLR* FH carriers have the extreme FH phenotype, as reported previously.^{48,49} Therefore, if we take into account only *LDLR* carriers, the prevalence of FH decreases from 1:226 to 1:376.

Sequencing analysis of *LDLR*, *APOB*, *PCSK9*, *APOE*, *STAP1* and *LDLRAP1* reported 5280 variants, but only 16 of them were possibly pathogenic or pathogenic according to *in silico* analysis: a) eleven pathogenic variants in the *LDLR* gene previously associated with FH,^{34–40} b) 1 pathogenic variant in the *PCSK9* gene (c.60_65dupGCTGCT), which has a frequency less than 0.5% in the general population and has been classified as pathogenic by bioinformatic analysis. However, further studies are needed to investigate the functionality of this variant; c) 1 rare variant in *APOB* (c.10621A >G). This missense variant, which produces an amino acid change, p.(Ile3515Val), in the mature protein, is located in the beta 2 domain of apolipoprotein B and computational prediction tools and conservation analyses suggest that this variant would not impact protein function. In addition, computational splicing tools suggest that this variant would not impact the RNA splicing⁵⁰; d) 3 rare variants in the *STAP1* gene; 1 of them located in the 5'UTR (c.-60A >G), and 2 of them located in coding regions (c.619G >A and c.803T >C), which produce amino acid change, p.(Ile268Thr) and p.(Asp207Asn), respectively. All these variants have been classified as pathogenic by bioinformatic analysis; however, none of them has previously been associated with FH. Further research is needed to investigate the role of *STAP1* in FH phenotype, as previous studies have reported participants carrying *STAP1* mutations with normal levels of total and LDL-C and incomplete association with FH;^{16,17,51} e) 2 rare variants in *LDLRAP1* gene, 1 of them located in intronic region (c.748-7C >G), which could produce an alternative splicing, and another one located in coding region (c.605C >G), producing an amino acid change, p.(Ser202Cys). This missense variant has been classified as pathogenic by bioinformatic analysis. However, the individual carried this variant in heterozygosity, which would not explain the FH phenotype; f) 3 missense rare variants in *APOE* gene, 2 of them (c.460C >A and c.487C >T) described as pathogenic by Clin Var⁵² and associated with type III hyperlipoproteinemia,^{43,44} but not with FH.

Our results showed that LDL-C levels were significantly associated with the presence of a causative mutation in candidate genes. While in our sample from a working population CHD was small and it had the smallest positive predict value for mutations, participants with untreated LDL-C concentrations above > 220 mg/dL and those with LDL-C above 130 mg/dL despite statin therapy showed a significant association with the presence of a pathogenic mutation. Among them, the probability of finding a causative mutation in candidate gene was 4 times higher than that in patients meeting the remaining criteria for suspicion of FH. In the future, the progressive decline in the costs of DNA analysis will probably facilitate universal screening programs in the population. However, because of their current costs, candidates must be selected for genetic testing. Our results suggest that not only high levels of untreated LDL-C are a good marker of FH in the general population, but the combination of these levels with high levels of LDL-C despite statin therapy could be a better predictor of FH. Furthermore, participants with high levels of untreated LDL-C and high levels of LDL-C with statin therapy showed the highest

percentage of mutations in LDLR (7.96%), and these participants also had a more extreme FH phenotype.

The percentage of mutations in candidate genes varies widely, from 55.6% when a history of family monogenic pattern of hypercholesterolemia information is available,⁸ to less than 2% in the case of exclusive use of LDL levels > 190 mg/dL.²⁰ Our study fully agrees with the concept that an isolated high LDL-C without information of family history of severe hypercholesterolemia allows identification of a small percentage of FH.

Limitations

Our study has some limitations. First, AWHs is a cohort with a high prevalence of men, obesity, hypertension, and hypercholesterolemia, which may not be representative of the general population. However, because it is a relatively young population there, will be no bias in the prevalence of FH mutations. Second, we report some mutations, c.743G >A in the PCSK9 gene and c.10621A >G in APOB, with uncertain significance. However, both are described as neutral or benign by bioinformatic analysis and the ClinVar database, so there are not enough data to classify them as pathogenic variants. Sequencing analysis of the APOB gene included only exon 26 and exon 29, while sequencing the entire APOB gene could report other variants as a cause of FH phenotype. Nevertheless, hypercholesterolemia due to APOB defects would require that the protein would have defective binding to the LDL receptor, since mutations causing only lower expression or improper folding of ApoB would produce hypocholesterolemia. Therefore, the variants causing FH are expected to be located in the coding region of the ApoB binding domain (in exon 26 and 29). In fact, most APOB mutations described as a cause of FH have been identified in exon 26.⁴⁹ Finally, possibly pathogenic mutations in the PCSK9 gene (c.60_65dupGCTGCT) and in the STAP1 gene (c.-60A >G, c.619G >A and c.803T >C) need further studies to gain greater insight into their role in the FH phenotype.

CONCLUSIONS

The present study analyses the positive predictive value of clinical diagnostic criteria for FH. Our study included 4514 individuals from the AWHs, a healthy middle-aged population. A total of 255 individuals met the diagnostic criteria for clinical suspicion of FH, of whom 16 (6.27%) had mutations in the LDLR and PCSK9 genes, which corresponds to a prevalence of FH of 1:282 in this population. Furthermore, 24 participants (9.41%) were diagnosed with hyperlipoproteinemia(a), which let us to identify almost 16% of the etiology of these severe hypercholesterolemia groups. Untreated LDL-C concentrations and high levels of LDL-C despite statin therapy showed a significant association with the presence of mutations in candidate genes. These results suggest that the combination of high untreated LDL-C with high levels of LDL-C despite statin therapy could be a first step, but not the only one, for FH screening in the population.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

WHAT IS KNOWN ABOUT THE TOPIC?

- FH is a genetic disorder characterized by high plasma total and LDL cholesterol concentrations and high risk of CVD.
- Clinical criteria have been demonstrated to be highly associated with genetic diagnosis.
- However, 20% to 40% of patients with the FH phenotype do not have a mutation in candidate genes.
- The European Atherosclerosis Society has recommended new diagnostic criteria for suspicion of FH, which have not yet been validated.

WHAT DOES THIS STUDY ADD?

- The prevalence of FH in Spain is 1:282.
- Only 6.27% of participants with suspected FH had functional mutations in the LDLR and PCSK9 genes.
- A total of 9.41% of participants with suspected FH were diagnosed with hyperlipoproteinemia(a).
- The combination of high untreated LDL-C with high levels of LDL-C despite statin therapy is the best predictor for a positive finding of FH mutation.

APPENDIX. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version available at <https://doi.org/10.1016/j.rec.2020.06.003>

REFERENCES

1. Civeira F. International Panel on Management of Familial Hypercholesterolemia. Guidelines for the diagnosis and management of heterozygous familial hypercholesterolemia. *Atherosclerosis*. 2004;173:55–68.
2. Goldstein JL, Brown MS. The LDL receptor. *Arterioscler Thromb Vasc Biol*. 2009;29:431–438.
3. Benn M, Watts GF, Tybjaerg-Hansen A, Nordestgaard BG. Mutations causative of familial hypercholesterolemia: screening of 98 098 individuals from the Copenhagen General Population Study estimated a prevalence of 1 in 217. *Eur Heart J*. 2016;37:1384–1394.
4. Besseling J, Hovingh GK, Huijgen R, Kastelein JJP, Hutten BA. Statins in Familial Hypercholesterolemia: Consequences for Coronary Artery Disease and All-Cause Mortality. *J Am Coll Cardiol*. 2016;68:252–260.
5. Perez-Calahorra S, Laclaustra M, Marco-Benedí V, et al. Effect of lipid-lowering treatment in cardiovascular disease prevalence in familial hypercholesterolemia. *Atherosclerosis*. 2019;284:245–252.
6. Nordestgaard BG, Chapman MJ, Humphries SE, et al. Familial hypercholesterolemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: consensus statement of the European Atherosclerosis Society. *Eur Heart J*. 2013;34:3478–3490.
7. Austin MA, Hutter CM, Zimmern RL, Humphries SE. Genetic causes of monogenic heterozygous familial hypercholesterolemia: a HuGE prevalence review. *Am J Epidemiol*. 2004;160:407–420.
8. Civeira F, Ros E, Jarauta E, et al. Comparison of genetic versus clinical diagnosis in familial hypercholesterolemia. *Am J Cardiol*. 2008;102:1187–1193.193.e1.
9. Berberich AJ, Hegele RA. The complex molecular genetics of familial hypercholesterolemia. *Nat Rev Cardiol*. 2019;16:9–20.
10. Chan DC, Pang J, Hooper AJ, et al. A Comparative Analysis of Phenotypic Predictors of Mutations in Familial Hypercholesterolemia. *J Clin Endocrinol Metab*. 2018;103:1704–1714.
11. Mach F, Baigent C, Catapano AL, et al. 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk. *Eur Heart J*. 2020;41:111–188.

12. Soria LF, Ludwig EH, Clarke HR, Vega GL, Grundy SM, McCarthy BJ. Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100. *Proc Natl Acad Sci USA*. 1989;86:587–591.
13. Abifadel M, Varret M, Rabès J-P, et al. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat Genet*. 2003;34:154–156.
14. Cenarro A, Etxebarria A, de Castro-Orós I, et al. The p.Leu167del Mutation in APOE Gene Causes Autosomal Dominant Hypercholesterolemia by Down-regulation of LDL Receptor Expression in Hepatocytes. *J Clin Endocrinol Metab*. 2016;101:2113–2121.
15. Fouchier SW, Dallinga-Thie GM, Meijers JCM, et al. Mutations in STAP1 are associated with autosomal dominant hypercholesterolemia. *Circ Res*. 2014;115:552–555.
16. Blanco-Vaca F, Martín-Campos JM, Pérez A, Fuentes-Prior P. A rare STAP1 mutation incompletely associated with familial hypercholesterolemia. *Clin Chim Acta*. 2018;487:270–274.
17. Lamiquiz-Moneo I, Restrepo-Córdoba MA, Mateo-Gallego R, et al. Predicted pathogenic mutations in STAP1 are not associated with clinically defined familial hypercholesterolemia. *Atherosclerosis*. 2019;292:143–151.
18. Meriño-Ibarra E, Puzo J, Jarauta E, et al. Hyperlipoproteinaemia(a) is a common cause of autosomal dominant hypercholesterolaemia. *J Inherit Metab Dis*. 2007;30:970–977.
19. Farnier M, Civeira F, Descamps O; FH expert working group. How to implement clinical guidelines to optimise familial hypercholesterolaemia diagnosis and treatment. *Atheroscler Suppl*. 2017;26:25–35.
20. Khera AV, Won H-H, Peloso GM, et al. Diagnostic Yield and Clinical Utility of Sequencing Familial Hypercholesterolemia Genes in Patients With Severe Hypercholesterolemia. *J Am Coll Cardiol*. 2016;67:2578–2589.
21. Casasnovas JA, Alcaide V, Civeira F, et al. Aragon Workers' Health Study—Design and Cohort Description. *BMC Cardiovasc Disord*. 2012;12:45.
22. Gómez-Gerique JA, Gutiérrez-Fuentes JA, Montoya MT, et al. [Lipid profile of the Spanish population: the DRECE (diet and risk of cardiovascular disease in Spain) study. DRECE study group]. *Med Clin (Barc)*. 1999;113:730–735.
23. Grundy SM, Stone NJ, Bailey AL, et al. 2018 AHA/ACC/AACVPR/AAPA/ABC/ACPM/ADA/AGS/APHA/ASPC/NLA/PCNA Guideline on the Management of Blood Cholesterol: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *J Am Coll Cardiol*. 2019;73:e285–e350.
24. Rhoads GG, Dahlen G, Berg K, Morton NE, Dannenberg AL. Lp(a) lipoprotein as a risk factor for myocardial infarction. *JAMA*. 1986;256:2540–2544.
25. Dahlén GH, Weinehall L, Stenlund H, et al. Lipoprotein(a) and cholesterol levels act synergistically and apolipoprotein A-I is protective for the incidence of primary acute myocardial infarction in middle-aged males. An incident case-control study from Sweden. *J Intern Med*. 1998;244:425–430.
26. Vaser R, Adusumalli S, Leng SN, Sikk M, Ng PC. SIFT missense predictions for genomes. *Nat Protoc*. 2016;11:1–9.
27. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. *Nat Methods*. 2010;7:248–249.
28. Schwarz JM, Rödelsperger C, Schuelke M, Seelow D. MutationTaster evaluates disease-causing potential of sequence alterations. *Nat Methods*. 2010;7:575–576.
29. Bendl J, Musil M, Štourač J, Zందుకా J, Damborský J, Brezovský J. PredictSNP2: A Unified Platform for Accurately Evaluating SNP Effects by Exploiting the Different Characteristics of Variants in Distinct Genomic Regions. *PLoS Comput Biol*. 2016;12:e1004962.
30. Choi Y, Sims GE, Murphy S, Miller JR, Chan AP. Predicting the functional effect of amino acid substitutions and indels. *PLoS ONE*. 2012;7:e46688.
31. 1000 Genomes Project Consortium, Abecasis GR, Auton A, et al. An integrated map of genetic variation from 1,092 human genomes. *Nature*. 2012;491:56–65.
32. Lek M, Karczewski KJ, Minikel EV, et al. Analysis of protein-coding genetic variation in 60,706 humans. *Nature*. 2016;536:285–291.
33. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2018. Available at: <https://www.r-project.org/>. Accessed 29 Apr 2019.
34. Cenarro A, Jensen HK, Casao E, et al. Identification of recurrent and novel mutations in the LDL receptor gene in Spanish patients with familial hypercholesterolemia. *Mutations in brief no 135 Online Hum Mutat*. 1998;11:413.
35. Mozas P, Cenarro A, Civeira F, Castillo S, Ros E, Pocovi M. Mutation analysis in 36 unrelated Spanish subjects with familial hypercholesterolemia: identification of 3 novel mutations in the LDL receptor gene. *Hum Mutat*. 2000;15:483–484.
36. Tichý L, Freiberger T, Zapletalová P, Soška V, Ravčuková B, Fajkusová L. The molecular basis of familial hypercholesterolemia in the Czech Republic: spectrum of LDLR mutations and genotype-phenotype correlations. *Atherosclerosis*. 2012;223:401–408.
37. Etxebarria A, Palacios L, Stef M, et al. Functional characterization of splicing and ligand-binding domain variants in the LDL receptor. *Hum Mutat*. 2012;33:232–243.
38. Bertolini S, Pisciotto L, Rabacchi C, et al. Spectrum of mutations and phenotypic expression in patients with autosomal dominant hypercholesterolemia identified in Italy. *Atherosclerosis*. 2013;227:342–348.
39. Etxebarria A, Benito-Vicente A, Palacios L, et al. Functional characterization and classification of frequent low-density lipoprotein receptor variants. *Hum Mutat*. 2015;36:129–141.
40. Brønne I, Kleineck M, Reiz B, et al. Systematic analysis of variants related to familial hypercholesterolemia in families with premature myocardial infarction. *Eur J Hum Genet*. 2016;24:191–197.
41. VCV000265916.5 - ClinVar - NCBI. Available at: <https://www.ncbi.nlm.nih.gov/clinvar/variation/265916/>. Accessed 13 May 2020.
42. Garcia UG, Vicente AB, Etxebarria A, et al. The leucine stretch length of PCSK9 signal peptide and its role in development of autosomal dominant hypercholesterolaemia: Unravelling the activities of P.LEU23DEL and P.LEU22_LEU23DUP variants. *Atherosclerosis*. 2017;263:e37. <http://dx.doi.org/10.1016/j.atherosclerosis.2017.06.138>.
43. Pocovi M, Cenarro A, Civeira F, et al. Incomplete dominance of type III hyperlipoproteinemia is associated with the rare apolipoprotein E2 (Arg136->Ser) variant in multigenerational pedigree studies. *Atherosclerosis*. 1996;122:33–46.
44. Hsia SH, Connolly PW, Hegele RA. Restriction isotyping of apolipoprotein E R145C in type III hyperlipoproteinemia. *J Invest Med*. 1995;43:187–194.
45. Langsted A, Kamstrup PR, Benn M, Tybjaerg-Hansen A, Nordestgaard BG. High lipoprotein(a) as a possible cause of clinical familial hypercholesterolaemia: a prospective cohort study. *Lancet Diabetes Endocrinol*. 2016;4:577–587.
46. Masana L, Ibarretxe D, Rodríguez-Borjabad C, et al. Toward a new clinical classification of patients with familial hypercholesterolemia: One perspective from Spain. *Atherosclerosis*. 2019;287:89–92.
47. Loaiza N, Hartgers ML, Reeskamp LF, et al. Taking One Step Back in Familial Hypercholesterolemia: STAP1 Does Not Alter Plasma LDL (Low-Density Lipoprotein) Cholesterol in Mice and Humans. *Arterioscler Thromb Vasc Biol*. 2020;40:973–985.
48. García-Alvarez I, Castillo S, Mozas P, et al. Differences in clinical presentation between subjects with a phenotype of familial hypercholesterolemia determined by defects in the LDL-receptor and defects in Apo B-100. *Rev Esp Cardiol*. 2003;56:769–774.
49. De Castro-Orós I, Pocovi M, Civeira F. The genetic basis of familial hypercholesterolemia: inheritance, linkage, and mutations. *Appl Clin Genet*. 2010;3:53–64.
50. VCV000630672.1 - ClinVar - NCBI. Available at: <https://www.ncbi.nlm.nih.gov/clinvar/variation/630672/>. Accessed 9 Aug 2019.
51. Amor-Salamanca A, Castillo S, Gonzalez-Vioque E, et al. Genetically Confirmed Familial Hypercholesterolemia in Patients With Acute Coronary Syndrome. *J Am Coll Cardiol*. 2017;70:1732–1740.
52. VCV000017850.1 - ClinVar - NCBI. Available at: <https://www.ncbi.nlm.nih.gov/clinvar/variation/17850/>. Accessed 9 Aug 2019.