Homozygous Familial Hypercholesterolemia in Spain: Prevalence and Phenotype-Genotype Relationship

Running title: Sánchez-Hernández et al.; Homozygous Familial Hypercholesterolemia in Spain

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Abstract:

**Background** - Homozygous familial hypercholesterolemia (HoFH) is a rare disease characterized by elevated plasma levels of low-density lipoprotein cholesterol (LDL-C) and extremely high risk of premature atherosclerotic cardiovascular disease (ASCVD). HoFH is caused by mutations in several genes, including LDL receptor \((LDLR)\), apolipoprotein B \((APOB)\), proprotein convertase subtilisin/kexin type 9 \((PCSK9)\), and LDL protein receptor adaptor 1 \((LDLRAP1)\). No epidemiological studies have assessed HoFH prevalence, or the clinical and molecular characteristics of this condition. Here we aimed to characterize HoFH in Spain.

**Methods and Results** - Data were collected from the Spanish Dyslipidemia Registry of the Spanish Atherosclerosis Society and from all molecular diagnoses performed for FH in Spain between 1996 and 2015 \((n=16,751)\). Clinical data included baseline lipid levels and ASCVD events. A total of 97 subjects were identified as having HoFH—of whom, 47 were true homozygous (1 for \(APOB\), 5 for \(LDLRAP1\), and 41 for \(LDLR\)), 45 compound heterozygous for \(LDLR\), 3 double heterozygous for \(LDLR\) and \(PCSK9\), and 2 double heterozygous for \(LDLR\) and \(APOB\). No \(PSCK9\) homozygous cases were identified. Two variants in \(LDLR\) were identified in 4.8% of the molecular studies. Over 50% of patients did not meet the classical HoFH diagnosis criteria. The estimated HoFH prevalence was 1:450,000. Compared to compound heterozygous cases, true homozygous cases showed more aggressive phenotypes with higher LDL-C and more ASCVD events.

**Conclusions** - HoFH frequency in Spain was higher than expected. Clinical criteria would underestimate the actual prevalence of individuals with genetic HoFH, highlighting the importance of genetic analysis to improve FH diagnosis accuracy.

**Key words:** hypercholesterolemia, lipids, genetics, receptors, registries
Introduction

Familial hypercholesterolemia (FH) is a monogenic autosomal co-dominant disease characterized by low cell uptake of LDL-C, resulting in very high plasma LDL-C levels.\(^1\) Homozygous FH (HoFH) is caused by mutations in both copies of any of the three main genes involved in FH development: LDL receptor (\(LDLR\); 95% of cases; OMIM #606945);\(^1\) apolipoprotein B (\(APOB\); 2–5% of cases; OMIM#107730);\(^2\) and proprotein convertase subtilisin/kexin type 9 (\(PCSK9\); <1% of cases; OMIM #607786).\(^3,4\) Additionally, recessive autosomal FH has been described, involving mutations on both \(LDLRAP1\) alleles (<1% of cases; OMIM# 695747).\(^5\) The recessive form of FH is clinically indistinguishable from HoFH,\(^6-8\) although less aggressive phenotypes have also been described,\(^9\) and for this reason was also included in our studies. The classical HoFH prevalence is 1:1,000,000,\(^1\) with higher rates in genetically isolated populations, such as French Canadians, Afrikaners from South Africa, or Christian Lebanese people.\(^10-13\) However, recent publications showed that the true prevalence may be higher, with estimated HoFH prevalences of 1:160,000 in Denmark,\(^14\) 1:300,000 in the Netherlands,\(^15\) and 1:800,000 in Germany.\(^16\) Additionally, a recent document from the Spanish Atherosclerosis Society refers to 44 genetically confirmed cases in Spain.\(^17\)

Recent genetic studies of FH highlight the great variability of the clinical phenotypes in HoFH and heterozygous FH (HeFH), the poor genotype-phenotype correlation, and the large clinical overlap between HoFH and HeFH.\(^15,18-20\) These findings can be explained based on the variations in the number of genes involved, the specific pathogenic mutations, and the resulting decrease of activity/function.\(^18,21\) Moreover, genetic diagnosis of HeFH or HoFH can be challenging due to the frequent co-existence of two functional mutations within a patient, and the potential difficulty of identifying whether they are in the same allele (compound heterozygous in
cis) or in two different alleles (compound heterozygous in trans).

Molecular diagnosis of HoFH is currently based on cases showing the most aggressive phenotype. This leads to a selection bias in which the HoFH definition typically includes very severe clinical criteria. Consequently, many genetically diagnosed patients do not meet the classical clinical criteria for HoFH, and the actual prevalence of this disease remains unknown.

In Spain, the genetic basis of FH has been investigated for the past 20 years, and our country pioneered the inclusion of biochips and next generation sequencing in FH diagnostic procedures. These genetic-based studies have been conducted almost exclusively in four research centers, with over 16,000 genetic studies performed in patients with clinical suspicion of FH.

Our present study aimed to determine the actual prevalence of HoFH in Spain, to describe the clinical impact of the different genotypes, and to facilitate the diagnosis of patients carrying double mutations. We analyzed all cases with a clinical diagnosis of HoFH or HeFH within all FH genetic studies performed in Spain over the last 20 years.

Methods

We re-analyzed all diagnostic genetic studies for FH in Spain from 1996 to June of 2015. All included studies were performed at four Spanish centers: Zaragoza University, Progenika Biopharma SA (A Grifols Company, Derio, Vizcaya), Hospital Clínico of Valencia, and Hospital Santa Creu i Sant Pau of Barcelona. These four centers are the reference genetic laboratories for the lipid units in Spain. The Spanish public healthcare system includes the whole Spanish population and comprises lipid units around the country where all severe FH cases are referred to perform genetic diagnosis. All patients that underwent molecular diagnosis were informed and
signed an informed consent, in each center the study was approved by all the ethics committees.

**Molecular Diagnosis**

The methods of molecular diagnosis changed over time. Until 2004, molecular diagnosis involved complete sequencing of \textit{LDLR} exons and intron-exon boundaries, and of the apolipoprotein (apo) B-binding domain of \textit{APOB} to the LDL receptor.\textsuperscript{23} From 2004 to 2012, microarray analysis was performed to examine the most frequent \textit{LDLR} and \textit{APOB} mutations in Spanish populations. When no mutation was identified, complete sequencing was conducted of the \textit{LDLR} promoter, exons, and intron-exon boundaries as well as the \textit{APOB} LDLR-binding domain.\textsuperscript{4, 25} From 2012 to 2015, molecular diagnosis was performed using next generation sequencing of the \textit{LDLR} promoter, exons, and intron-exon boundaries; the \textit{APOB} LDLR-binding domain; the \textit{PCSK9} promoter, exons, and intron-exon boundaries; and the \textit{LDLRAP1} promoter, exons, and intron-exon boundaries (from 2012 to 2015).\textsuperscript{24} From all analyzed studies, we selected the patients carrying at least two mutations within the four genes responsible for FH or in \textit{LDLRAP1}, as previously described.

Patients were diagnosed with HoFH if they were homozygous for the same pathogenic mutation in \textit{LDLR}, \textit{APOB}, \textit{PCSK9}, or \textit{LDLRAP1}; double heterozygous for pathogenic mutations in two different genes (\textit{LDLR}, \textit{APOB}, or \textit{PCSK9}); or compound heterozygous with two different pathogenic mutations in the same gene (\textit{LDLR}, \textit{APOB}, \textit{PCSK9}, or \textit{LDLRAP1}).\textsuperscript{21} To differentiate compound heterozygous patients carrying two mutations in \textit{trans} from compound heterozygous patients in \textit{cis}, mutation analysis was performed in first-degree relatives to verify familial segregation, haplotypes were examined using common \textit{LDLR} variants (previously described by Tejedor et al),\textsuperscript{26} and studies determined whether both mutations were present in more than two non-related patients. Null allele mutations were categorized as either nonsense, frameshift,
splicing, or large rearrangements.

Pathogenicity of LDLR variants had been previously assessed by LDL uptake by cultured fibroblast for all the mutations identified in true homozygous. In compound heterozygotes when the assessment of LDL uptake by fibroblast was not available, in silico predictions were used to evaluate the pathogenicity of these genetic variant: PolyPhen-2, SIFT and Mutation Taster.²⁷-²⁹ NetGene2 and NNSplice were utilized to predict the effect of variants in potential splicing sites.³⁰,³¹

Clinical Diagnostic Features and Diagnostic Criteria

Genetic studies were performed using the clinical criteria recommended in the guidelines of the European Atherosclerosis Society (EAS), which are based on the Dutch Lipid Clinic Network (DCLN) criteria for HeFH diagnosis among adults.³² Clinical diagnosis of HoFH was based on an LDL-C level of over 500 mg/dL without treatment, or of over 300 mg/dL while on high-intensive lipid-lowering therapy, as well as the presence of tendon xanthomas before 10 years of age.²² All clinical information was obtained from the National Registry of Dyslipidemias of the Spanish Atherosclerosis Society (SEA), which includes data from most lipid clinics of Spain, or was obtained directly from the patients’ physicians. Clinical data for three cases were obtained from published data.³³,³⁴ For the genetic study, the following clinical features were recorded: current age; age at diagnosis; off-treatment levels of total cholesterol, LDL-C, HDL-C, and triglycerides; presence and type of ASCVD; and age at first ASCVD event.

Statistical Analysis

Prevalence was calculated as the number of HoFH cases divided by the mean total number of population for the whole period. Mean population was estimated using demographic data provided by the Spanish National Institute for Statistics (Instituto Nacional Estadística, INE).³⁵
Statistical analyses were performed using SPSS 20.0 software (IBM, SPSS, Inc., Chicago, IL, USA). Between-group comparisons were performed using the Student’s t or Mann-Whitney U tests. Data are expressed as mean ± standard deviation (SD) for numeric variables that followed a normal distribution, or as median and range for other numeric variables. Differences were considered significant when the two-tailed P value was below .05.

Results

A total of 16,751 genetic studies for FH diagnosis were performed in Spain from 1996 to 2015. Of these studies, 11,094 (66.2%) detected at least one pathogenic mutation, 531 (4.79% of the positive studies) showed multiple pathogenic mutations, and 97 (0.87%) carried two mutations corresponding to the HoFH criteria (Fig 1). All centers had similar detection rates of multiple pathogenic mutations: Zaragoza University, 6.7%; Progenika, 4.6%; Hospital Clínico of Valencia, 3.1%; and Hospital Santa Creu i Sant Pau of Barcelona, 6%. Cases of HoFH showed the following distribution: 47 true homozygous (41 for LDLR, 5 for LDLRAP1, and 1 for APOB); 45 compound heterozygous for LDLR; and 5 double heterozygous (3 for PCSK9 and LDLR, and 2 for APOB and LDLRL). No cases were identified as homozygous for pathogenic mutations in the PCSK9 gene (supplementary table 1). For the whole studied period, the mean Spanish population was 43,673,187 people and 97 genetic HoFH were identified. Hence, for the same period, the estimated prevalence of genetically diagnosed HoFH in Spain was found to be 1:450,000.

In compound-heterozygous cases, to elucidate whether the LDLR variants were in cis or trans positions, we reviewed familial genetic studies that were also included in the database. Based on allelic segregation, 33 HoFH cases were unequivocally in trans. However, no familial details were available for 12 subjects. We therefore examined their LDLR variants throughout the
entire database, and found that all 12 were distributed in heterozygosis. Thus, these 12 patients were considered compound-heterozygous in *trans*. This diagnosis was supported by the HoFH-like phenotype observed in these cases as well as their haplotypes with *LDLR* SNVs. Four pairs of *LDLR* variants were classified as compound-heterozygous in *cis* (supplementary table 2) because they were frequently associated with each other throughout the database, and because familial genetic studies confirmed their presence in the same allele.

Tables 1 and 2 present the clinical characteristics of HoFH. Baseline LDL-C levels were correlated with genotype severity, being higher in receptor-negative HoFH compared to receptor-defective HoFH and, also, in true homozygotes compared to compound or double heterozygotes (table 1). Genotype was also strongly associated with ASCVD events (table 1), with more ASCVD events in true homozygous carrying null alleles than in those carrying defective alleles (50% vs 43.8%). Moreover, ASCVD events started earlier in patients carrying null allele-related mutations 23±19 years old vs 39±11 years old; *P*=.046. Among these reported ASCVD events, 87% were premature (before 55 years in men and 60 in women), and 5 patients experienced ASCVD before 30 years of age. Compared to true *LDLR* null allele homozygotes, carriers of *LDLRAP1* mutations showed higher LDL-C levels at diagnosis (806 mg/dL vs 788 mg/dL) but fewer ASCVD events (20% vs 50%). Among all patients, 46.7% did not meet the classical HoFH criterion of a baseline LDL-C level above 500 mg/dL. This criterion was not met by 32.4% of true homozygotes, 64% of compound heterozygotes, and 100% of double heterozygotes. Regarding sex distribution, males were overrepresented among most of the studied groups, except for double heterozygotes (table 1).

**Discussion**

The present study results revealed an HoFH prevalence of 1:478,000. This is higher than
previously reported, and almost twice the prevalence expected according to the classical clinical
criterion of an off-treatment LDL-C level above 500 mg/dL.¹ This greater frequency is in
agreement with the recently defined HeFH prevalence of approximately 1:250 within unselected
representative populations from Denmark³⁶ and USA.³⁷ Spain is home to a genetically
heterogeneous population that lacks any relevant genetically isolated groups, which explains the
absence of recurrent mutations responsible for FH or other monogenic diseases.³⁸ Over 400
different FH-causing mutations have been described in the Spanish population, almost all of
which have been described in other populations, and none accounting for more than 6.5% of
cases.⁴

In fact, our presently obtained prevalence value is similar to the rates obtained using
genetic criteria.²¹ This reinforces the idea that the classical clinical criteria identify only the most
severe cases.²⁰ Further supporting this notion, almost half of our patients who were genetically
diagnosed with HoFH did not meet the classical clinical criteria for HoFH diagnosis.²² In our
study, to estimate the molecular prevalence of this disease, we included patients with autosomal
recessive hypercholesterolemia and double heterozygotes—subjects who have been excluded
from previous studies.²⁶

HoFH is a heterogeneous disease that does not always exhibit the classical phenotype.¹⁹,
³⁹ This heterogeneity seems to depend in part on the causative genetic defect.⁴⁰ The highest LDL-
C levels are found in true homozygous patients carrying null alleles, while the lowest blood
LDL-C levels are measured in compound heterozygous patients carrying defective alleles and in
double heterozygous patients.⁸, ¹⁸, ²¹, ³⁹ This continuum is also observed with regards to ASCVD
occurrence, which is more frequent among patients with severe mutations, and less common with
decreasing mutation malignancy. The presently noted percentage of ASCVD patients (~50%)
among true homozygous subjects was similar to that described by Pisciotta et al, but higher than previously reported by Raal et al (38.3%). On the other hand, the presently determined mean age at first ASCVD among null-allele patients (27.9 years) was similar to in the cohort described by Raal et al (26.2 years). Overall, our results are compatible with previous reports in patients with HoFH. Additionally, our present findings suggest that the clinical prognosis of these patients has gradually improved in recent years due to the availability of more efficient lipid-lowering treatments and the introduction of LDL apheresis.

The clinical phenotype of some cases of HoFH overlaps with the clinical phenotype of some severe cases of HeFH. Thus, we must reconsider the criteria used for the differential diagnosis of HoFH. Along these lines, the American Heart Association recently suggested that HoFH be suspected in cases with an untreated LDL-C level above 400 mg/dL. In this sense, 21.23% of the patients from the Spanish Atherosclerosis Society Registry presented untreated LDL-C levels between 300 and 500 mg/dL and only five were genetically defined HoFH (1.4%).

In Spain around 100,000 patients could suffer from FH according to the 1:500 reported prevalence. Based on this estimation approximately 20,000 FH patients could have untreated LDL-C levels between 300 and 500 mg/dL, but only a minority (0.25%) will have genetically defined HoFH. Hence, a high percentage of HoFH patients have LDL-C between 400 and 500 mg/dL, but a low percentage of those with genetically confirmed FH in that range are homozygotes, the vast majority are heterozygotes.

In the present study, as well as in a prior Dutch study, patients with a genetic diagnosis of HoFH showed an LDL cholesterol level of over 290 mg/dL (7.5 mmol/L). The complexity of the diagnosis of HoFH should be considered, and it seems necessary a better definition of this disease, that should include genetic information, phenotypic characterization and cardiovascular
risk. It must be taken into account that a confirmed genetic diagnosis of HoFH does not completely reflect cardiovascular risk, and risk stratification to identify severe FH is needed considering LDL-C levels and others risk factors such as hypertension, smoking habits, diabetes, high levels of lipoprotein(a). We think that this complexity should be considered in the diagnosis of this disease. Further refinement of these diagnostic criteria will require additional comparisons among molecularly diagnosed cases from different research studies. Improved diagnostic criteria could be used in familial screenings to make new diagnoses among patients’ relatives, as well as for testing of new, specifically-designed drugs. Our present conclusions are similar to those of Raal et al,\(^3\) highlighting that HoFH is a heterogeneous disease resulting in a wide range of elevated LDL-C levels, such that diagnosis should not be limited to selected patients with a more severe phenotype.

Another major finding of our study is the frequent presence of two functional mutations within the same patient. In all of the included diagnostic centers, it was consistently observed that about 5% of cases presented two or more pathogenic variants. To our knowledge, no previous evidence demonstrates the prevalence of multiple mutations among patients with clinical suspicion of FH. Additionally, it is difficult to elucidate whether mutations affect the same allele (cis position) or different alleles (trans position), complicating the classification of patients as HeFH or HoFH, respectively. To this end, family segregation is the best practical identification method—as the analysis of first-degree relatives (particularly parents) confirms whether mutations are in different alleles since each parent carries one of the two mutations. However, other procedures are necessary when DNA from first-degree relatives is unavailable.

Newly developed techniques enable the sequencing of long stretches of DNA (over 10 kb) from a single molecule or single allele, which allow determination of the cis or trans status
of two variants on the same gene. While more severe phenotypes would be expected in compound heterozygous patients in \textit{trans}, our present results demonstrate that this criterion is not sufficiently sensitive due to the frequently overlapping phenotypes between homozygous and heterozygous patients. Moreover, variants can be associated in linkage disequilibrium within the same allele, and they must be separately identified in different populations. For instance, in Spain, several variants have been described in \textit{cis} in the \textit{LDLR}, including \(c. (829G>A; 12G>A)\) \(p. (Trp4*; Glu277Lys)\); \(c. (1061-8T>C; 274C>G)\),\(^{26}\) \(c. (313+1G>C; c.274C>G)\) \(p. (NA; Gln92Glu)\); \(c. (2397_2405delCGTCTTCCT; 1690A>C)\) \(p. (Lys799_Phe801del; Asn564His)\),\(^{44}\) but may have different segregation patterns in other populations.

Our results also have therapeutic implications. LDL apheresis is currently the most used treatment for metabolic control of the most severe cases of HoFH. However, new therapeutic options are available, including the microsomal triglyceride transfer protein (MTP) inhibitor lomitapide;\(^{45}\) PCSK9 inhibitors;\(^ {46}\) and mipomersen, an antisense oligonucleotide that targets \textit{APOB} mRNA.\(^ {47}\) Of these agents, lomitapide and mipomersen have been specifically approved to treat only HoFH. Our present findings support the application of these drugs based on LDL-C levels. Many of our patients had been diagnosed with HeFH prior to genetic analysis, and showed acceptable metabolic control following treatment with statins and ezetimibe (article in preparation). This may indicate that the newer drugs should be employed not only in cases with a specific clinical diagnosis but also in response to the LDL-C level achieved after treatment with traditional lipid-lowering drugs. Such a change would prevent situations in which severe HeFH patients with poor control of LDL-C might be \textit{a priori} deprived of newer drugs.

\textbf{Study Limitations}

The present study includes all diagnostic centers that performed molecular diagnosis of FH in
Spain up to 2015; however, there is insufficient evidence to guarantee that genetic analysis was performed in all cases with clinical suspicion of HoFH. Consequently, the present prevalence was calculated based on the cases that were seen in specialized units that performed molecular analyses. Considering the structure of our public healthcare system, in which the most severe cases are referred to these units, it can be presumed that our study included all severe FH cases. However, some HoFH cases with less aggressive phenotypes, showing LDL-C levels similar to HeFH phenotypes, could not have been subjected to molecular analysis. Regarding prevalence estimation, the follow-up information in this cohort was not available in all HoFH cases, and the clinical status was referred at the time of genetic analysis. However, most of the diagnoses have been performed in recent years, and the mortality rate in this cohort is <5%, therefore we think that the prevalence estimation is quite accurate.

Although all genetic analyses included complete LDLR sequencing and APOB-3500 detection, few studies included analysis of the PCSK9 gene. Therefore, the frequency of mutations at this locus could be under-diagnosed. Nevertheless, the frequency of PCSK9 mutations causing FH in Spain is very low.4

Finally, it was difficult to identify compound heterozygous patients in trans when we had insufficient information available regarding their relatives. The results of thousands of previous genetic studies indicate that some mutations are frequently present in cis. However, this status was only discerned in a few cases by constructing haplotypes with LDLR SNVs. Thus, it is impossible to definitively determine that the two mutations were in different alleles.

Conclusions

The presently determined actual frequency of patients suffering from HoFH is higher than previously reported prevalence rates that have largely included only the most aggressive cases. A
high percentage of patients genetically diagnosed with HoFH do not meet the clinical diagnostic criteria. This highlights the relevance of molecular diagnosis, and the need for improved procedures for HoFH diagnosis. The aggressiveness of the HoFH phenotype ranges from severe in homozygous patients, to milder in double or compound heterozygous patients. High suspicion is required to diagnose this disease in patients who show LDL-C levels lower than those previously considered as diagnostic.

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Disclosures: Marianne Stef is employee of Progenika Biopharma SA- A Grifols Company, company dedicated to genetic diagnosis. The rest of authors declare no conflicts of interest.

References:


Table 1: Clinical characteristics of true homozygotes, compound heterozygotes and double heterozygotes for LDLR, APOB, PCSK9 and LDLRAP1.

<table>
<thead>
<tr>
<th></th>
<th>True Homozygotes (LDLR)</th>
<th>True Homozygous (LDLRAP1)</th>
<th>True Homozygous (APOB)</th>
<th>Compound Heterozygotes (LDLR)</th>
<th>Double Heterozygotes</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>41</td>
<td>5</td>
<td>1</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>36.8 (18.4)</td>
<td>32.4 (18.7)</td>
<td>74</td>
<td>37.9 (18.56)</td>
<td>52.5 (28.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Age of diagnosis (y)</td>
<td>6.5 [0.6-57]</td>
<td>1.2 [0.2-12]</td>
<td>50</td>
<td>21.8 (16.2)</td>
<td>43 [10-63]</td>
<td>0.034</td>
</tr>
<tr>
<td>Sex male (%)</td>
<td>73.3</td>
<td>80</td>
<td>100</td>
<td>51.7</td>
<td>25</td>
<td>0.065</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>692 (262)</td>
<td>886 (298)</td>
<td>391</td>
<td>465 [279-950]</td>
<td>370 (25.54)</td>
<td>0.005</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>625 (271.5)</td>
<td>806.3 (287)</td>
<td>329</td>
<td>397 [197-890]</td>
<td>304 (37)</td>
<td>0.008</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>43 (14)</td>
<td>39 (9)</td>
<td>33</td>
<td>48.7 (15)</td>
<td>48 (7)</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>100 (42)</td>
<td>115 [84-522]</td>
<td>144</td>
<td>105 [26-514]</td>
<td>65 (17)</td>
<td>NS</td>
</tr>
<tr>
<td>CVD (%)</td>
<td>46.4</td>
<td>20</td>
<td>100</td>
<td>25</td>
<td>25</td>
<td>NS</td>
</tr>
<tr>
<td>Age of CVD</td>
<td>31.7 (17)</td>
<td>55</td>
<td>56</td>
<td>34.3 (17.4)</td>
<td>43</td>
<td>NS</td>
</tr>
<tr>
<td>CVD Type (%)</td>
<td>100 CHD</td>
<td>100 CHD</td>
<td>100 CHD</td>
<td>71.4% CHD</td>
<td>100% CHD</td>
<td>NS</td>
</tr>
</tbody>
</table>

N: number of patients; Y: years; TC: total cholesterol; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; CVD: cardiovascular disease; CHD: Coronary heart disease.

All true homozygous for LDLRAP1 were null alleles; the true homozygote for APOB presented defective alleles.
Table 2: Clinical characteristics depending on the type of mutation in *LDLR*

<table>
<thead>
<tr>
<th></th>
<th>True homozygotes</th>
<th>Compound homozygotes</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>Null Alleles</td>
<td>Defective Alleles</td>
<td>Null/Null Alleles</td>
</tr>
<tr>
<td>N</td>
<td>15</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td>Current age (y)</td>
<td>24.2 (18)</td>
<td>43.8 (15)</td>
<td>51</td>
</tr>
<tr>
<td>Diagnosis age (y)</td>
<td>5 [2.5-35]</td>
<td>8 [0.6-57]</td>
<td>40</td>
</tr>
<tr>
<td>Sex, male (%)</td>
<td>64.3</td>
<td>81.3</td>
<td>0</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>845 (208)</td>
<td>559 (233)</td>
<td>525</td>
</tr>
<tr>
<td>LDL-D (mg/dL)</td>
<td>788 (208)</td>
<td>488 (248.5)</td>
<td>428.2</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>35.6 (10)</td>
<td>48 (15)</td>
<td>84</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>121 (50)</td>
<td>85 (28)</td>
<td>64</td>
</tr>
<tr>
<td>CVD (%)</td>
<td>50</td>
<td>43.8</td>
<td>0</td>
</tr>
<tr>
<td>Age CVD</td>
<td>23 (19)</td>
<td>39 (11)</td>
<td>32 (23.9)</td>
</tr>
</tbody>
</table>

N, number of patients; Y, years; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; CVD, cardiovascular disease.

*Clinical data available only for one of two heterozygotes carriers of two null alleles in *LDLR*.
Figure Legend:

Figure 1: Genetic FH diagnosis performed in Spain between 1996 and 2015.
Homozygous Familial Hypercholesterolemia in Spain: Prevalence and Phenotype-Genotype Relationship


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Supplemental Material
Supplementary tables

Table 1. Mutations causing HoFH

<table>
<thead>
<tr>
<th>Nucleotide substitution (c-DNA)</th>
<th>Allele name (Protein)</th>
<th>N</th>
</tr>
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<tbody>
<tr>
<td><strong>True Homozygous: LDLR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.[1162_1173del12];[1162_1173del12]</td>
<td>p.[His388_Ala391del];[His388_Ala391del]</td>
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<tr>
<td>c.[1199_1207delACCTCTTCT];[1199_1207delACCTCTTCT]</td>
<td>p.[Tyr400_Phe402del];[Tyr400_Phe402del]</td>
<td>1</td>
</tr>
<tr>
<td>c.[1965C&gt;G];[1965C&gt;G]</td>
<td>p.[Phe655Leu];[Phe655Leu]</td>
<td>1</td>
</tr>
<tr>
<td>c.[1048C&gt;T];[1048C&gt;T]</td>
<td>p.[Arg350*];[Arg350*]</td>
<td>1</td>
</tr>
<tr>
<td>c.[1045delC];[1045delC]</td>
<td>p.[Gln349Serfs<em>21];[Gln349Serfs</em>21]</td>
<td>2</td>
</tr>
<tr>
<td>c.[1301C&gt;G];[1301C&gt;G]</td>
<td>p.[Thr434Arg];[Thr434Arg]</td>
<td>2</td>
</tr>
<tr>
<td>c.[97C&gt;T];[97C&gt;T]</td>
<td>p.[Gln33*];[Gln33*]</td>
<td>1</td>
</tr>
<tr>
<td>c.[2397_2405delCGTCTTCTCT;1690A&gt;C];[2397_2405delCGTCTTCTCT;1690A&gt;C]</td>
<td>p.[Val800_Leu802del;Asn564His];[Val800_Leu802del;Asn564His]</td>
<td>1</td>
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<tr>
<td>c.[800A&gt;C];[800A&gt;C]</td>
<td>p.[Glu267Ala];[Glu267Ala]</td>
<td>1</td>
</tr>
<tr>
<td>c.[682G&gt;T];[682G&gt;T]</td>
<td>p.[Glu228*];[Glu228*]</td>
<td>1</td>
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<tr>
<td>c.[953G&gt;T];[953G&gt;T]</td>
<td>p.[Cys318Phe];[Cys318Phe]</td>
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c.[1916T>A];[1916T>A]
  p.[Val639Asp];[Val639Asp]  1

c.[1775G>A];[1775G>A]
  p.[Gly592Glu];[Gly592Glu]  1

c.[2397_2405delCGTCTTCCT;1690A>C];[23
  97_2405delCGTCTTCCT;1690A>C]
  p.[Val800_Leu802del;Asn564His];[Val800
  _Leu802del;Asn564His]  1

c.[1897C>T];[1897C>T]
  p.[Arg633Cys];[Arg633Cys]  2

c.[621C>T];[621C>T]
  p.[Gly207Gly];[Gly207Gly]  1

c.[1706-?_1845+?del];[1706-?_1845+?del]
  NA  1

c.[898A>G];[898A>G]
  p.[Arg300Gly];[Arg300Gly]  1

c.[1965C>G];[1965C>G]
  p.[Phe655Leu];[Phe655Leu]  1

c.[1783C>T];[1783C>T]
  p.[Arg595Trp];[Arg595Trp]  3

c.[1027G>A];[1027G>A]
  p.[Gly343Ser];[Gly343Ser]  1

c.[502G>A];[502G>A]
  p.[Asp168Asn];[Asp168Asn]  1

c.[97C>T];[97C>T]
  p.[Gln33*];[Gln33*]  1

c.[1342C>T];[1342C>T]
  p.[Gln448*];[Gln448*]  2

c.[1775G>A];[1775G>A]
  p.[Gly592Glu];[Gly592Glu]  1

c.[313+2dupT];[313+2dupT]
  p.[Leu64_Pro105delinsSer];[Leu64_Pro105
delinsSer]  2

c.[1775G>A];[1775G>A]
  p.[Gly592Glu];[Gly592Glu]  2
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<th>Allele</th>
<th>Phenotype</th>
<th>Count</th>
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<tr>
<td>c.[313+5G&gt;A];[313+5G&gt;A]</td>
<td>NA</td>
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<tr>
<td>c.[313+2dupT];[313+2dupT]</td>
<td>p.[Leu64_Pro105delinsSer];[Leu64_Pro105delinsSer]</td>
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<td>c.[2475C&gt;G];[2475C&gt;G]</td>
<td>p.[Asn825Lys];p.[Asn825Lys]</td>
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<tr>
<td>[c.2054delC]; [c.2054delC]</td>
<td>p.[Pro685ArgFs<em>24];[Pro685ArgFs</em>24]</td>
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<tr>
<td>[c.916_919dupTCAG] [c.916_919dupTCAG]</td>
<td>p[Asp307Valfs<em>3];p[Asp307Valfs</em>3]</td>
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**True Homozygotes: LDLRAP1**

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<th>Count</th>
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<td>p.[His144Glnfs<em>27];[His144Glnfs</em>27]</td>
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<tr>
<td>c.[207delC];[207delC]</td>
<td>p.[Ala70ProfsX19 ];[Ala70ProfsX19]</td>
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<tr>
<td>c.[603dupC];[c.603dupC]</td>
<td>p.[Ser202LeuFs<em>19];[Ser202LeuFs</em>19]</td>
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</table>

**True Homozygous: APOB**

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<th>Allele</th>
<th>Phenotype</th>
<th>Count</th>
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<tbody>
<tr>
<td>c.[10580G&gt;A];[10580G&gt;A]</td>
<td>p.[Arg3527Gln];[Arg3527Gln]</td>
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**Compound Heterozygotes: LDLR**

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<th>Allele</th>
<th>Phenotype</th>
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<tr>
<td>c.[346T&gt;C];[2100C&gt;G]</td>
<td>p.[Cys116Arg];[Asp700Glu]</td>
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<tr>
<td>c.[1246C&gt;T];[737G&gt;A]</td>
<td>p.[Arg416Trp];[Gly246Glu]</td>
<td>1</td>
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<tr>
<td>c.[97C&gt;T(;)584G&gt;A]</td>
<td>p.[Gln33*(;),Ser195Asn]</td>
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c.[313+1G>C;274C>G];[2099A>G] p.[NA;Gln92Glu];[Asp700Gly] 1

c.[12G>A;829G>A];
[2397_2405delCGTCTTCCT;1690A>C] p.[Trp4*; Glu277Lys];[Val800_Leu802del;Asn564His] 1

c.[313+1G>C;274C>G];[240C>A] p.[NA;Gln92Glu];[Asn80Lys] 2

c.[346T>C];[12G>A ; 829G>A] p.[Cys116Arg];[Trp4*; Glu277Lys] 1

c.[12G>A;829G>A ];[1358+1G>A] p.[Trp4*, Glu277Lys];[NA] 1

c.[2397_2405delCGTCTTCCT;1690A>C];[394C>T] p.[Val800_Leu802del;Asn564His];[Arg132Trp] 1

c.[313+1G>C;274C>G];[1027G>A] p.[NA;Gln92Glu];[Gly343Ser] 1

c.[514G>A];[1103G>A] p.[Asp172Asn];[Cys368Tyr] 1

c.[2397_2405delCGTCTTCCT;1690A>C];[267C>G] p.[Val800_Leu802del;Asn564His];[Cys89Thr] 2

c.[313+1G>C;274C>G];[58G>A] p.[NA;Gln92Glu];[Gly20Arg] 1

c.[2086T>C];[1-?_67+?del] p.[Cys696Arg];[NA] 1

c.[2001T>A];[48C>A] p.[Cys667*];[Leu16Leu] 1

c.[1897C>T];[953G>T] p.[Arg633Cys];[Cys318Phe] 1

c.[2375T>C];[1133A>C] p.[Ile792Thr];[Gln378Pro] 1

c.[530C>T];[1-?_67+?del] p.[Ser177Leu];[NA] 1
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<th>Mutation Details</th>
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<th>Count</th>
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<td>p.[NA];[Gly592Glu]</td>
<td>1</td>
</tr>
<tr>
<td>c.[451_453delGCC(;1618G&gt;A)</td>
<td>p.[Ala151del(;Ala540Thr]</td>
<td>1</td>
</tr>
<tr>
<td>c.[2475C&gt;A];[1195G&gt;A]</td>
<td>p.[Asn825Lys];[Ala399Thr]</td>
<td>1</td>
</tr>
<tr>
<td>c.[1816G&gt;A(;556G&gt;C]</td>
<td>p.[Ala606Thr(;Gly186Arg]</td>
<td>1</td>
</tr>
<tr>
<td>c.[313+1G&gt;C];[2140+1G&gt;A]</td>
<td>p.[NA];[NA]</td>
<td>1</td>
</tr>
<tr>
<td>c.[1816G&gt;T(;631C&gt;G]</td>
<td>p.[Ala606Ser(;His211Asp]</td>
<td>3</td>
</tr>
<tr>
<td>c.[1816G&gt;T(;621C&gt;T]</td>
<td>p.[Ala606Ser(;Gly207Gly]</td>
<td>1</td>
</tr>
<tr>
<td>c.[1072T&gt;C(;2441G&gt;A]</td>
<td>p.[Cys358Arg(;Arg814Gln]</td>
<td>1</td>
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<td>c.[460C&gt;T];[1247G&gt;A]</td>
<td>p.[Gln154*];[Arg416Gln]</td>
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<tr>
<td>c.[1133A&gt;C];[1-?_67+?del]</td>
<td>p.[Gln378Pro];[NA]</td>
<td>1</td>
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<tr>
<td>c.[672_686del15(;2390-?_2547+?del]</td>
<td>p.[Asp224_Glu228del(;NA]</td>
<td>1</td>
</tr>
<tr>
<td>c.[97C&gt;T(;1093A&gt;G]</td>
<td>p.[Gln33*(;Ser365Gly]</td>
<td>1</td>
</tr>
<tr>
<td>c.[916_919dupTCAG];[185C&gt;T]</td>
<td>p.[Asp307Valfs*3];[Thr62Met]</td>
<td>1</td>
</tr>
<tr>
<td>c.[1618G&gt;A];[451_453delGCC]</td>
<td>p.[Ala540Thr];[Ala151del]</td>
<td>1</td>
</tr>
<tr>
<td>c.[(-268)G&gt;T(;2093G&gt;A]</td>
<td>p.[NA(;Cys698Tyr]</td>
<td>1</td>
</tr>
<tr>
<td>c.[1246C&gt;T];[1510A&gt;G]</td>
<td>p.[Arg416Trp];[Lys504Glu]</td>
<td>1</td>
</tr>
</tbody>
</table>
c.[1816G>T];[631C>G]  p.[Ala606Ser];[His211Asp]  1

c.[2390-?_2583+?del];[1898G>A]  p.[NA];[Arg633His]  1

c.[1898G>A];[2390-?_2583+?del]  p.[Arg633His];[NA]  1

c.[58G>A];[1697T>C]  p.[Gly20Arg];[Ile566Thr]  1

c.[11G>A(;)1694G>A]  p.[Trp4*(;)Gly565Asp]  1

c.[902A>G ];[c.1646G>T]  p.[Asp301Gly];[Gly549Val]  1

Double Heterozygotes: LDLR and PCSK9

LDLR: c.(-228)G>C  LDLR: p.NA  1

PCSK9:c.60_65dupGCTGCT  PCSK9: p.Leu22_Leu23dup

LDLR: c.760C>T  LDLR: p.Gln254*

PCSK9:c.60_65dupGCTGCT  PCSK9: p.Leu22_Leu23dup

LDLR: c.[314-?_940+?del]  LDLR: NA  1


Double Heterozygotes: LDLR and APOB

LDLR: c.520G>A  LDLR: p.Glu174Lys  1

APOB: c.10580G>A  APOB: p.Arg3527Gln

LDLR: c.1-?_67+?del  LDLR: p.NA  1

APOB: c.10588G>A  APOB: p.Val3530Met
Table 2. Frequent LDLR variants associated in cis position

- c.[313+1G>C; 274C>G] p.(Gln92Glu)
- c.[829G>A; c.12G>A] p.[Glu277Lys; Trp4*]
- c.[1061-8T>C; 274C>G]
- c.[2397_2405delCGTCTTCCT; 1690A>C] p.[Lys799_Phe801del; Asn564His]