Homozygous Familial Hypercholesterolemia in Spain
Prevalence and Phenotype–Genotype Relationship

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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. 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 familial hypercholesterolemia in Spain between 1996 and 2015 (n=16,751). Clinical data included baseline lipid levels and atherosclerotic cardiovascular disease 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). At least 45 compound heterozygous for LDLR, 3 double heterozygous for LDLR and PCSK9, and 2 double heterozygous for LDLR and APOB. No PCSK9 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 with compound heterozygous cases, true homozygous cases showed more aggressive phenotypes with higher LDL-C and more atherosclerotic cardiovascular disease 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 familial hypercholesterolemia diagnosis accuracy. (Circ Cardiovasc Genet. 2016;9:504-510. DOI: 10.1161/CIRCGENETICS.116.001545.)

Key Words: alleles ■ hypercholesterolemia ■ lipids ■ mutation ■ registries

Familial hypercholesterolemia (FH) is a monogenic autosomal codominant disease characterized by low cell uptake of low-density lipoprotein cholesterol (LDL-C), resulting in high plasma LDL-C levels.1 Homozygous FH (HoFH) is caused by mutations in both copies of any of the 3 main genes involved in FH development: LDL receptor (LDLR; 95% of cases; OMIM #606945);1 apolipoprotein B (APOB; 2% to 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 for this reason, this form of FH has also been included in our studies. The classical HoFH prevalence is 1:100,000,1 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
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 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 4 research centers, with >16000 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 past 20 years.

Methods

We reanalyzed all diagnostic genetic studies for FH in Spain from 1996 to June 2015. All included studies were performed at 4 Spanish centers: Zaragoza University, Progenika Biopharma SA (A Grifols Company, Derio, Vizcaya), Hospital Clínico de Valencia, and Hospital Santa Creu i Sant Pau of Barcelona. These 4 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 who 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 LDLR exons and intron–exon boundaries and of the apolipoprotein (apo) B–binding domain of APOB to the LDL receptor. From 2004 to 2012, microarray analysis was performed to examine the most frequent LDLR and APOB mutations in Spanish populations. When no mutation was identified, complete sequencing was conducted of the LDLR promoter, exons, and intron–exon boundaries, as well as the APOB LDLR-binding domain. From 2012 to 2015, molecular diagnosis was performed using next-generation sequencing of the LDLR promoter, exons, and intron–exon boundaries; the APOB LDLR-binding domain; the PCSK9 promoter, exons, and intron–exon boundaries; and the LDLRAP1 promoter, exons, and intron–exon boundaries. From all analyzed studies, we selected the patients carrying at least 2 mutations within the 3 genes responsible for FH or in LDLRAP1, as previously described.

Patients were diagnosed with HoFH if they were homozygous for the same pathogenic mutation in LDLR, APOB, PCSK9, or LDLRAP1; double heterozygous for pathogenic mutations in 2 different genes (LDLR, APOB, or PCSK9); or compound heterozygous with 2 different pathogenic mutations in the same gene (LDLR, APOB, PCSK9, or LDLRAP1). To differentiate compound heterozygous patients carrying 2 mutations in trans from compound heterozygous patients in cis, mutation analysis was performed in first-degree relatives to verify familial segregation, haplotypes were examined using common LDLR variants (previously described by Tejedor et al.), and studies determined whether both mutations were present in >2 nonrelated patients. Null allele mutations were categorized as 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. NetGene2 and NNsplice were used to predict the effect of variants in potential splicing sites.

Clinical Diagnostic Features and Diagnostic Criteria

Genetic studies were performed using the clinical criteria recommended in the guidelines of the European Atherosclerosis Society, which are based on the Dutch Lipid Clinic Network criteria for HeFH diagnosis among adults. Clinical diagnosis of HoFH was based on an LDL-C level of >500 mg/dL without treatment or of >300 mg/dL while on high-intensive lipid-lowering therapy and the presence of tendon xanthomas before 10 years of age. All clinical information was obtained from the National Registry of Dyslipidemias of the Spanish Atherosclerosis Society, which includes data from most lipid clinics of Spain, or was obtained directly from the patients’ physicians. Clinical data for 3 cases were obtained from published data. For the genetic study, the following clinical features were recorded: current age; age at diagnosis; off-treatment levels of total cholesterol, LDL-C, high-density lipoprotein cholesterol (HDL-C), and triglycerides; presence and type of atherosclerotic cardiovascular disease (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 Estadístico). Statistical analyses were performed using SPSS 20.0 software (IBM, SPSS, Inc, Chicago, IL). Between-group comparisons were performed using the Student t or Mann–Whitney U tests. Data are expressed as means±SD for numeric variables that followed a normal distribution or as median and range for other numeric variables. Differences were considered significant when the 2-tailed P value was <0.05.

Results

A total of 16751 genetic studies for FH diagnosis were performed in Spain from 1996 to 2015. Of these studies, 11094 (66.2%) detected at least 1 pathogenic mutation, 531 (4.79% of the positive studies) showed multiple pathogenic mutations, and 97 (0.87%) carried 2 mutations corresponding to the HoFH criteria (Figure). All centers had similar detection rates of multiple pathogenic mutations: Zaragoza University, 6.7%; Progenika, 4.6%; Hospital Clínico de 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 LDLR). No cases were identified as homozygous for pathogenic mutations in the PCSK9 gene (Table I in the Data Supplement).

For the whole study period, the mean Spanish population was 43673 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 1:450000.

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. On the basis of 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 and their haplotypes with LDLR single-nucleotide variants. Four pairs of LDLR variants were classified as compound heterozygous in cis (Table II in the Data Supplement) 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 with receptor-defective HoFH and, also, in true homozygotes compared with 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% versus 43.8%). Moreover, ASCVD events started earlier in patients carrying null allele–related mutations (23±19 years versus 39±11 years; P=0.046). Among these reported ASCVD events, 87% were premature (before 55 years in men and 60 years in women) and 5 patients experienced ASCVD before 30 years of age. Compared with true LDLR null allele homozygotes, carriers of LDLRAP1 mutations showed higher LDL-C levels at diagnosis (806 versus 788 mg/dL) but fewer ASCVD events (20% versus 50%). Among all patients, 46.7% did not meet the classical HoFH criterion of a baseline LDL-C level >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, men were over-represented among most of the studied groups, except for double heterozygotes (Table 1).

**Discussion**

The present study results revealed an HoFH prevalence of 1:450,000. This is higher than that previously reported and almost twice the prevalence expected according to the classical clinical criterion of an off-treatment LDL-C level >500 mg/dL. This greater frequency is in agreement with the recently defined HeFH prevalence of ≈1:250 within unscreened representative populations from Denmark and United States. 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. 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 >6.5% of cases.

In fact, our presently obtained prevalence value is similar to the rates obtained using genetic criteria. This reinforces the idea that the classical clinical criteria identify only the most severe cases. 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. 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.15,20 

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, whereas 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 regard 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,8 but higher than that previously reported by Raal et al20 (38.3%). On the contrary, the presently determined mean age at first ASCVD among true homozygous patients (27.9 years) was similar to that in the cohort described by Raal et al20 (31.7 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 because of the availability of more efficient lipid-lowering treatments and the introduction of LDL apheresis.41,42 

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 >400 mg/dL.43 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 5 were genetically defined HoFH (1.4%). In Spain, ≈100000 patients could have FH according to the 1:500 reported prevalence.4 On the basis of this estimation, ≈20000

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 Homozygotes (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>LDLR &amp; APOB (3) LDLR &amp; PCSK9 (2)</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>Male sex, %</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>ASCVD, %</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 ASCVD</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>ASCVD type, %</td>
<td>100 CHD</td>
<td>100 CHD</td>
<td>100 CHD</td>
<td>71.4% CHD 28.6% stroke</td>
<td>100% CHD</td>
<td>NS</td>
</tr>
</tbody>
</table>

All true homozygous for LDLRAP1 were null alleles; the true homozygote for APOB presented defective alleles. ASCVD indicates atherosclerotic cardiovascular disease; CHD, coronary heart disease; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; N, number of patients; and TC, total cholesterol.

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>
</tr>
</thead>
<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>Male sex, %</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>ASCVD, %</td>
<td>50</td>
<td>43.8</td>
<td>0</td>
</tr>
<tr>
<td>Age ASCVD</td>
<td>23 (19)</td>
<td>39 (11)</td>
<td>...</td>
</tr>
</tbody>
</table>

ASCVD indicates atherosclerotic cardiovascular disease; CHD, coronary heart disease; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; N, number of patients; TC, total cholesterol; and y, years.

*Clinical data available only for 1 of 2 heterozygotes carriers of 2 null alleles in LDLR.
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, and the vast majority are heterozygotes.

In the present study, as well as in a previous Dutch study, patients with a genetic diagnosis of HoFH showed an LDL-C level of ≥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 other risk factors such as hypertension, smoking habits, diabetes mellitus, and 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.20 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 2 functional mutations within the same patient. In all of the included diagnostic centers, it was consistently observed that 5% of cases presented ≥2 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 because each parent carries 1 of the 2 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 (>10 kb) from a single molecule or single allele, which allow determination of the cis or trans status of 2 variants on the same gene. Although more severe phenotypes would be expected in compound heterozygous patients in trans, our present results demonstrate that this criterion is not sufficiently sensitive because of 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 cis in the LDLR, including c.(829G>A; c.12G>A) p.(Trp42*;Glu277Lys); c.(1061-8T>C;274C>G) p.(Glu354Asp); c.(313+1G>C; c.274C>G) p.(NA; Gln92Glu); and c.(2397-2405delCGTCTTCTCT1690A>C) p.(Lys799_Phe810del;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 inhibitor, lomitapide;26 PCSK9 inhibitors;46 and mipomersen, an antisense oligonucleotide that targets 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 before genetic analysis and showed acceptable metabolic control after treatment with statins and ezetimibe (article in preparation). This may indicate that the newer drugs should be used 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 at a priori deprived of newer drugs.

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. About 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 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 underdiagnosed. Nevertheless, the frequency of PCSK9 mutations causing FH in Spain is low.

Finally, it was difficult to identify compound heterozygous patients in trans when we had insufficient information available on 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 single-nucleotide variants. Thus, it is impossible to definitively determine that the 2 mutations were in different alleles.

Conclusions

The presently determined actual frequency of patients having 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
M. Stef is employee of Progenika Biopharma SA, a Grifols company, company dedicated to genetic diagnosis. The other authors report no conflicts.

Appendix
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References


**CLINICAL PERSPECTIVE**

The present study includes all diagnostic tests for familial hypercholesterolemia performed in Spain up to 2015. The study shows that genetically defined homozygous familial hypercholesterolemia (HoFH) prevalence is higher than the previously reported that was based mostly on low-density lipoprotein cholesterol (LDL-C) levels and that the classical clinical criteria identify only the most severe HoFH cases. Almost half of the patients who were genetically diagnosed with HoFH did not meet the classical clinical criteria for HoFH diagnosis. The study demonstrates that the lipid phenotype of HoFH is heterogeneous, and the causative genetic defect is the major driver of this heterogeneity. The highest LDL-C levels are found in true homozygous patients carrying null alleles, whereas the lowest blood LDL-C levels are present in compound heterozygous patients carrying defective alleles. The clinical phenotype of some cases of HoFH overlaps with the clinical phenotype of some severe cases of heterozygous familial hypercholesterolemia. Thus, the study suggests that the criteria used for the differential diagnosis of HoFH must be reconsidered. Our results also have therapeutic implications. LDL apheresis is the most used treatment in HoFH. However, new therapeutic options are available, and some of these agents have been specifically approved to treat only HoFH. Our findings support the application of these drugs based on LDL-C levels rather than a specific diagnosis.
Homozygous Familial Hypercholesterolemia in Spain: Prevalence and Phenotype–Genotype Relationship

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Supplemental Material
Supplementary tables

**Table 1. Mutations causing HoFH**

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<td>p[Asp307Valfs<em>3];pAsp307Valfs</em>3</td>
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**True Homozygotes: LDLRAP1**

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**True Homozygous: APOB**

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**Compound Heterozygotes: LDLR**

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c.[313+1G>C;274C>G];[2099A>G]
p.[NA;Gln92Glu];[Asp700Gly]
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c.[12G>A;829G>A];
[2397_2405delCGTCTT CCT;1690A>C]
p.[Trp4*; Glu277Lys];[Val800_Leu802del;Asn564His]
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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_2405delCGTCTT CCT;1690A>C];[39 4C>T]
p.[Val800_Leu802del;Asn564His];[Arg132 Trp]
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_2405delCGTCTT CCT;1690A>C];[26 7C>G]
p.[Val800_Leu802del;Asn564His];[Cys89Turp]
2

c.[313+1G>C;274C>G];[58G>A]
p.[NA;Gln92Glu];[Gly20Arg]
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c.[2086T>C];[1-?_67+?del]
p.[Cys696Arg];[NA]
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c.[2001T>A];[48C>A]
p.[Cys667*];[Leu16Leu]
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c.[1897C>T];[953G>T]
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c.[2375T>C];[1133A>C]
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p.[Ser177Leu];[NA]
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c.[451_453delGCC(;)1618G>A]  p.[Ala151del(;)Ala540Thr]  1


c.[2475C>A];[1195G>A]  p.[Asn825Lys];[Ala399Thr]  1

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c.[313+1G>C];[2140+1G>A]  p.[NA];[NA]  1

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c.[1816G>T(;)621C>T]  p.[Ala606Ser(;)Gly207Gly]  1

c.[1072T>C(;)2441G>A]  p.[Cys358Arg(;)Arg814Gln]  1

c.[460C>T];[1247G>A]  p.[Gln154*];[Arg416Gln]  1

c.[1133A>C];[1-?_67+?del]  p.[Gln378Pro];[NA]  1

c.[672_686del15(;)2390-?_2547+?del]  p.[Asp224_Glu228del(;)NA]  1

c.[97C>T(;)1093A>G]  p.[Gln33*];[Ser365Gly]  1

c.[916_919dupTCAG];[185C>T]  p.[Asp307Valfs*3];[Thr62Met]  1

c.[1618G>A];[451_453delGCC]  p.[Ala540Thr];[Ala151del]  1

c.[(-268)G>T(;)2093G>A]  p.[NA(;)Cys698Tyr]  1

c.[1246C>T];[1510A>G]  p.[Arg416Trp];[Lys504Glu]  1
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:** c.760C>T  **LDLR:** c.314-?_940+?del

**PCSK9:** c.60_65dupGCTGCT  **PCSK9:** c.60_65dupGCTGCT  **PCSK9:** c.[60_65dupGCTGCT]

**LDLR:** p.NA  **LDLR:** p.Gln254*  **LDLR:** NA

**PCSK9:** p.Leu22_Leu23dup  **PCSK9:** p.Leu22_Leu23dup  **PCSK9:** p.Leu22_Leu23dup

**Double Heterozygotes: LDLR and APOB**

**LDLR:** c.520G>A  **LDLR:** c.1?-_67+?del  **LDLR:** c.10588G>A

**APOB:** c.10580G>A  **APOB:** c.10588G>A

**LDLR:** p.Glu174Lys  **LDLR:** p.NA  **APOB:** p.Arg3527Gln  **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_2405delCGTCTTCT; 1690A>C]p.[Lys799_Phe801del;Asn564His]