

# 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*), 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.<sup>1</sup> 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);<sup>1</sup> apolipoprotein B (*APOB*; 2% to 5% of cases; OMIM #107730);<sup>2</sup> and proprotein convertase subtilisin/kexin type 9 (*PCSK9*; <1% of cases; OMIM #607786).<sup>3,4</sup> Additionally, recessive autosomal FH has been described, involving mutations on both *LDLRAP1* alleles (<1% of cases; OMIM #695747).<sup>5</sup> The recessive form of FH is clinically indistinguishable from HoFH,<sup>6-8</sup> although less aggressive phenotypes have also been described,<sup>9</sup> for this reason, this form of FH has also been included in our studies. The classical HoFH prevalence is 1:1 000 000,<sup>1</sup> with

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higher rates in genetically isolated populations, such as French Canadians, Afrikaners from South Africa, or Christian Lebanese people.<sup>10-13</sup> However, recent publications showed that the true prevalence may be higher, with estimated HoFH prevalences of 1:160 000 in Denmark,<sup>14</sup> 1:300 000 in the Netherlands,<sup>15</sup> and 1:800 000 in Germany.<sup>16</sup> Additionally, a recent document from the Spanish Atherosclerosis Society refers to 44 genetically confirmed cases in Spain.<sup>17</sup>

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.<sup>15,18-20</sup> These findings can be explained based on the variations in the number of genes involved, the specific pathogenic mutations, and

Received May 10, 2016; accepted September 28, 2016.

The Data Supplement is available at <http://circgenetics.ahajournals.org/lookup/suppl/doi:10.1161/CIRCGENETICS.116.001545/-/DC1>.

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*Circ Cardiovasc Genet* is available at <http://circgenetics.ahajournals.org>

DOI: 10.1161/CIRCGENETICS.116.001545

the resulting decrease of function.<sup>18,21</sup> Moreover, genetic diagnosis of HeFH or HoFH can be challenging because of the frequent coexistence of 2 functional mutations within a patient and the potential difficulty of identifying whether they are in the same allele (compound heterozygous in *cis*) or in 2 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 severe clinical criteria.<sup>22</sup> Consequently, many genetically diagnosed patients do not meet the classical clinical criteria for HoFH,<sup>15,20</sup> 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.<sup>23,24</sup> 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.<sup>23</sup> 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.<sup>4,25</sup> 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.<sup>24</sup> 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*).<sup>21</sup> 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),<sup>26</sup> and studies

determined whether both mutations were present in >2 nonrelated patients. Null allele mutations were categorized as nonsense, frame-shift, 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.<sup>27–29</sup> NetGene2 and NNSplice were used to predict the effect of variants in potential splicing sites.<sup>30,31</sup>

## 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.<sup>32</sup> 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.<sup>22</sup> 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.<sup>33,34</sup> 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 Estadística).<sup>35</sup> 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 mean±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, 11 094 (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 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. 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.

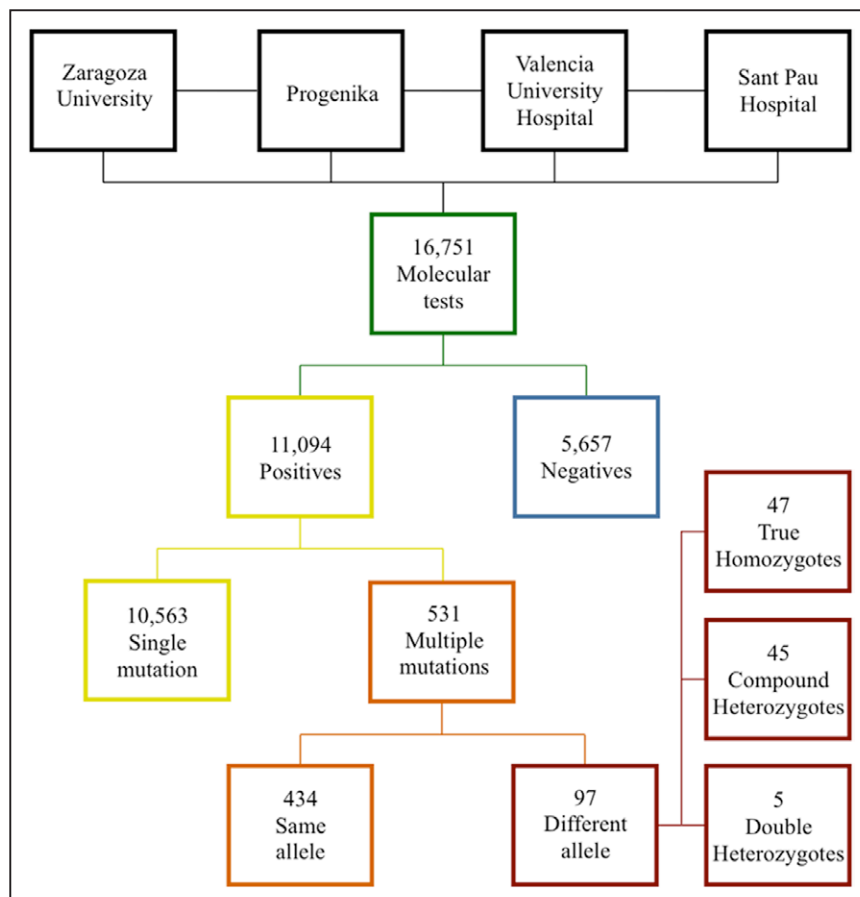
Tables 1 and 2 present the clinical characteristics of HoFH. Baseline LDL-C levels were correlated with genotype severity, being higher in receptor-negative HoFH compared with receptor-defective HoFH and, also, in true homozygotes compared with compound or double heterozygotes (Table 1). Genotype was 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 ( $23\pm 19$  years versus  $39\pm 11$  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 1:450000. 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.<sup>1</sup> This greater frequency is in agreement with the recently defined HeFH prevalence of  $\approx 1:250$  within unselected representative populations from Denmark<sup>36</sup> and United States.<sup>37</sup> 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.<sup>38</sup> 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.<sup>4</sup>

In fact, our presently obtained prevalence value is similar to the rates obtained using genetic criteria.<sup>21</sup> This reinforces the idea that the classical clinical criteria identify only the most severe cases.<sup>20</sup> 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.<sup>22</sup> In our study, to estimate the molecular prevalence of this disease, we included patients with autosomal recessive



**Figure.** Genetic familial hypercholesterolemia diagnosis performed in Spain between 1996 and 2015.

**Table 1. Clinical Characteristics of True Homozygotes, Compound Heterozygotes, and Double Heterozygotes for *LDLR*, *APOB*, *PCSK9*, and *LDLRAP1***

	True Homozygotes ( <i>LDLR</i> )	True Homozygotes ( <i>LDLRAP1</i> )	True Homozygous ( <i>APOB</i> )	Compound Heterozygotes ( <i>LDLR</i> )	Double Heterozygotes	<i>P</i>
N	41	5	1	45	<i>LDLR</i> & <i>APOB</i> (3) <i>LDLR</i> & <i>PCSK9</i> (2)	
Age, y	36.8 (18.4)	32.4 (18.7)	74	37.9 (18.56)	52.5 (28.2)	NS
Age of diagnosis, y	6.5 (0.6–57)	1.2 (0.2–12)	50	21.8 (16.2)	43 (10–63)	0.034
Male sex, %	73.3	80	100	51.7	25	0.065
TC, mg/dL	692 (262)	886 (298)	391	465 (279–950)	370 (25.54)	0.005
LDL-C, mg/dL	625 (271.5)	806.3 (287)	329	397 (197–890)	304 (37)	0.008
HDL-C, mg/dL	43 (14)	39 (9)	33	48.7 (15)	48 (7)	NS
Triglycerides, mg/dL	100 (42)	115 (84–522)	144	105 (26–514)	65 (17)	NS
ASCVD, %	46.4	20	100	25	25	NS
Age of ASCVD	31.7 (17)	55	56	34.3 (17.4)	43	NS
ASCVD type, %	100 CHD	100 CHD	100 CHD	71.4% CHD 28.6% stroke	100% CHD	NS

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.

hypercholesterolemia and double heterozygotes—subjects who have been excluded from previous studies.<sup>15,20</sup>

HoFH is a heterogeneous disease that does not always exhibit the classical phenotype.<sup>19,39</sup> This heterogeneity seems to depend in part on the causative genetic defect.<sup>40</sup> 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.<sup>8,18,21,39</sup> 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,<sup>8</sup> but higher than that previously reported by Raal et al<sup>20</sup> (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 al<sup>20</sup> (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.<sup>41,42</sup>

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.<sup>43</sup> 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, ≈100 000 patients could have FH according to the 1:500 reported prevalence.<sup>4</sup> On the basis of this estimation, ≈20 000

**Table 2. Clinical Characteristics Depending on the Type of Mutation in *LDLR***

	True Homozygotes		Compound Homozygotes			<i>P</i>
	Null Alleles	Defective Alleles	Null/Null Alleles*	Null/Defective Alleles	Defective/Defective Alleles	
N	15	26	2	24	19	
Current age, y	24.2 (18)	43.8 (15)	51	35.7 (21)	40.1 (16)	NS
Diagnosis age, y	5 (2.5–35)	8 (0.6–57)	40	10.5 (7–29)	21 (7–49)	NS
Male sex, %	64.3	81.3	0	64.7	36.4	NS
TC, mg/dL	845 (208)	559 (233)	525	527 (154)	423 (279–950)	0.006
LDL-D, mg/dL	788 (208)	488 (248.5)	428.2	452 (288–760)	340 (197–890)	<0.001
HDL-C, mg/dL	35.6 (10)	48 (15)	84	47 (14)	48 (13.6)	0.011
Triglycerides, mg/dL	121 (50)	85 (28)	64	109 (58–514)	104.4 (43.2)	NS
ASCVD, %	50	43.8	0	25	27.3	NS
Age ASCVD	23 (19)	39 (11)	...	32 (23.9)	37 (5.19)	NS

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,<sup>15</sup> 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 others 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,<sup>20</sup> 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.*(Trp4\*;Glu277Lys); *c.*(1061-8T>C;274C>G)<sup>26</sup>; *c.*(313+1G>C; *c.*274C>G) *p.*(NA;Gln92Glu); and *c.*(2397\_2405delCGTCTTCCT;1690A>C) *p.*(Lys799\_Phe801del;Asn564His),<sup>44</sup> 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;<sup>45</sup> PCSK9 inhibitors;<sup>46</sup> and mipomersen, an antisense oligonucleotide that targets APOB mRNA.<sup>47</sup> 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 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.<sup>4</sup>

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.

### Acknowledgments

We thank Amelia Oleaga, Leire Pérez, Rosaura Figueras, Miguel Ángel Lasunción, Margarita Martínez de Atienza, Nuria Espinosa, José Antonio Gimeno, Luis Irigoyen, Maria Gema Grau, Ángel Brea, Antonio Pérez, and Pablo Prieto for providing clinical data. We also thank Ana Wägner and Mauro Boronat for their critical reading of the article.

### Sources of Funding

This study was supported by grants from the Spanish Ministry of Economy and Competitiveness PI12/01087, PI12/01703, IPT-2011-0817-010000, ISCIII -PI14/01648, Marató TV3 foundation grant 201524-31 and Red de Investigación Cardiovascular (RIC) RD12/0042/0055.

### 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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## 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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*Circ Cardiovasc Genet.* 2016;9:504-510; originally published online October 26, 2016;  
doi: 10.1161/CIRCGENETICS.116.001545

*Circulation: Cardiovascular Genetics* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1942-325X. Online ISSN: 1942-3268

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## **Supplemental Material**

## Supplementary tables

**Table 1.** Mutations causing HoFH

Nucleotide substitution (c-DNA)	Allele name (Protein)	N
<b>True Homozygous: <i>LDLR</i></b>		
c.[1162_1173del12];[1162_1173del12]	p.[His388_Ala391del];[His388_Ala391del]	1
c.[1199_1207delACCTCTTCT]; [1199_1207delACCTCTTCT]	p.[Tyr400_Phe402del];[Tyr400_Phe402del]	1
c.[1965C>G];[1965C>G]	p.[Phe655Leu];[Phe655Leu]	1
c.[1048C>T];[1048C>T]	p.[Arg350*];[Arg350*]	1
c.[1045delC];[1045delC]	p.[Gln349Serfs*21];[Gln349Serfs*21]	2
c.[1301C>G];[1301C>G]	p.[Thr434Arg];[Thr434Arg]	2
c.[97C>T];[97C>T]	p.[Gln33*];[Gln33*]	1
c.[2397_2405delCGTCTTCCT;1690A>C];[23 97_2405delCGTCTTCCT;1690A>C]	p.[Val800_Leu802del;Asn564His];[Val800 _Leu802del;Asn564His]	1
c.[800A>C];[800A>C]	p.[Glu267Ala];[Glu267Ala]	1
c.[682G>T];[682G>T]	p.[Glu228*];[Glu228*]	1
c.[953G>T];[953G>T]	p.[Cys318Phe];[Cys318Phe]	1

c.[1916T>A];[1916T>A]	p.[Val639Asp];[Val639Asp]	1
c.[1775G>A];[1775G>A]	p.[Gly592Glu];[Gly592Glu]	1
c.[2397_2405delCGTCTTCCT;1690A>C];[2397_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_Pro105delinsSer]	2
c.[1775G>A];[1775G>A]	p.[Gly592Glu];[Gly592Glu]	2

c.[313+5G>A];[313+5G>A]	NA	1
c.[313+2dupT];[313+2dupT]	p.[Leu64_Pro105delinsSer];[Leu64_Pro105delinsSer]	1
c.[2475C>G];[2475C>G]	p.[Asn825Lys];p.[Asn825Lys]	1
[c.2054delC]; [c.2054delC]	p.[Pro685ArgFs*24];[ p.[Pro685ArgFs*24 ]]	1
[c.916_919dupTCAG] [c.916_919dupTCAG]	[pAsp307Valfs*3];[pAsp307Valfs*3]	2

**True Homozygotes: *LDLRAP1***

c.[431dupA];[431dupA]	p.[His144Glnfs*27];[His144Glnfs*27]	1
c.[207delC];[207delC]	p.[Ala70ProfsX19 ];[Ala70ProfsX19]	1
c.[603dupC];[c.603dupC]	p.[Ser202LeuFs*19];[Ser202LeuFs*19]	1
c. [345-?_459+?del]; [345-?_459+?del]	NA	2

**True Homozygous: *APOB***

c.[10580G>A];[10580G>A]	p.[Arg3527Gln];[Arg3527Gln]	1
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**Compound Heterozygotes: *LDLR***

c.[346T>C];[2100C>G]	p.[Cys116Arg];[Asp700Glu]	1
c.[1246C>T];[737G>A]	p.[Arg416Trp];[Gly246Glu]	1
c.[97C>T(;);584G>A]	p.[Gln33*(;);Ser195Asn]	1

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];[Cys89Trp]	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

c.[2390-?_2547+?del];[1775G>A]	p.[NA];[Gly592Glu]	1
c.[451_453delGCC(;)]1618G>A]	p.[Ala151del(;)]Ala540Thr]	1
c.[9delC];[1567G>A]	p.[Trp4Glyfs*202];[Val523Met]	1
c.[2475C>A];[1195G>A]	p.[Asn825Lys];[Ala399Thr]	1
c.[1816G>A(;)]556G>C]	p.[Ala606Thr(;)]Gly186Arg]	1
c.[313+1G>C];[2140+1G>A]	p.[NA];[NA]	1
c.[1816G>T(;)]631C>G]	p.[Ala606Ser(;)]His211Asp]	3
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.(-140)C>T];[858C>A]	p.[NA];[Ser286Arg]	1
[c.902A>G ];[c.1646G>T]	p.[Asp301Gly];[Gly549Val]	1

**Double Heterozygotes: *LDLR* and *PCSK9***

<i>LDLR</i> : c.(-228)G>C	<i>LDLR</i> : p.NA	1
<i>PCSK9</i> :c.60_65dupGCTGCT	<i>PCSK9</i> : p.Leu22_Leu23dup	
<i>LDLR</i> : c.760C>T	<i>LDLR</i> : p.Gln254*	1
<i>PCSK9</i> :c.60_65dupGCTGCT	<i>PCSK9</i> : p.Leu22_Leu23dup	
<i>LDLR</i> : c.[314-?_940+?del]	<i>LDLR</i> : NA	1
<i>PCSK9</i> :c.[60_65dupGCTGCT]	<i>PCSK9</i> : p.Leu22_Leu23dup	

**Double Heterozygotes: *LDLR* and *APOB***

<i>LDLR</i> : c.520G>A	<i>LDLR</i> : p.Glu174Lys	1
<i>APOB</i> : c.10580G>A	<i>APOB</i> : p.Arg3527Gln	
<i>LDLR</i> : c.1-?_67+?del	<i>LDLR</i> : p.NA	1
<i>APOB</i> : c.10588G>A	<i>APOB</i> : 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]