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RESEARCH ARTICLE

Functional analysis of new variants at the low-density lipoprotein receptor associated with familial hypercholesterolemia

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Abstract

Familial hypercholesterolemia is an autosomal dominant disease of lipid metabolism caused by defects in the genes *LDLR*, *APOB*, and *PCSK9*. The prevalence of heterozygous familial hypercholesterolemia (HeFH) is estimated between 1/200 and 1/250. Early detection of patients with FH allows initiation of treatment, thus reducing the risk of coronary heart disease. In this study, we performed in vitro characterization of new *LDLR* variants found in our patients. Genetic analysis was performed by Next Generation Sequencing using a customized panel of 198 genes in DNA samples of 516 subjects with a clinical diagnosis of probable or definitive FH. All new *LDLR* variants found in our patients were functionally validated in CHO-*ldlA7* cells. The LDLR activity was measured by flow cytometry and LDLR expression was detected by immunofluorescence. Seven new variants at *LDLR* were tested: *c.518 G>C*;p.(Cys173Ser),

c.[684 G>T;694 G>T];p.[Glu228Asp;Ala232Ser], *c.926C>A*;p.(Pro309His), *c.1261 A>G*;p.(Ser421Gly), *c.1594T>A*;p.(Tyr532Asn), and *c.2138delC*;p.(Thr713Lysfs* 17). We classified all variants as pathogenic except p.(Ser421Gly) and p.(Ala232Ser). The functional in vitro characterization of rare variants at the *LDLR* is a useful tool to classify the new variants. This approach allows us to confirm the genetic diagnosis of FH, avoiding the classification as "uncertain significant variants", and therefore, carry out cascade family screening.

KEYWORDS
familial hypercholesterolemia, functional study, genetic variants, LDLR gene

1 | INTRODUCTION

Familial hypercholesterolemia (FH; MIM# 143890) is one of the most common inherited metabolic diseases. The prevalence of the heterozygous form of FH (HeFH) is about 1/200–250 (Nordestgaard et al., 2013), and 1/160000–300000 in its homozygous form (HoFH; Authors/Task Force et al., 2016). It is clinically characterized by elevations in low-density lipoprotein cholesterol (LDL-c), the presence of tendon xanthomas, and a high risk of premature coronary heart disease (Goldstein, HH, Baudet, Sly, & Valle, 2001). The clinical phenotype of autosomal dominant FH is because of the defects in three genes: *LDLR* (MIM# 606945) affecting receptor-mediated endocytosis of LDL particles (Brown & Goldstein, 1986), *APOB* (MIM# 107730) showing decreased binding of LDL particles to the LDLR (Innerarity et al., 1990); and *PCSK9* (MIM# 607786) causing increased catabolism of the LDLR (Abifadel et al., 2003). Among the molecularly diagnosed cases, 86–88% are because of mutations in *LDLR*; 12% are caused by mutations in *APOB*; and <0.1–2% are caused by gain of function mutations in *PCSK9* (Motazacker et al., 2012). Later studies have shown that mutations in the *LDLRAP1* gene (MIM# 605747) result in an autosomal recessive form of FH (Garcia et al., 2001). In addition to these genes, hypercholesterolemia could be caused by pathogenic variants in other genes (Rios, Stein, Shendure, Hobbs, & Cohen, 2010).

The human *LDLR* gene is located on the distal short arm of chromosome 19 (p13.1–p.13.3; Lindgren, Luskey, Russell, & Francke, 1985), spans 45 kb, includes 18 exons (Sudhof, Goldstein, Brown, & Russell, 1985). The *LDLR* is ubiquitously expressed and is essential for cholesterol homeostasis. The correspondence between the six functional domains of the protein and the exons of the *LDLR* gene is well-established (Jeon & Blacklow, 2005). The signal peptide encoded by exon 1 is a sequence necessary for its transport to the cell membrane; it is cleaved during translocation into the endoplasmic reticulum (RE) leaving a mature protein of 839 amino-acids. The ligand binding domain, encoded by exons 2–6 participates in the interaction with lipoproteins. This domain is composed of seven modules named LDL receptor type A (**LR**). Each LR has approximately 40 residues with six conserved cysteine residues, which originate three disulfide bonds comprising a very stable domain. The COOH-terminal end of each LR has the sequence Ser-Asp-Glu (SDE), corresponding to negatively charged residues that interact with positive charges (Goldstein, Brown, Anderson, Russell, & Schneider, 1985) of regions rich in Arg and Lys present in ApoB and ApoE.

The epidermal grow factor (EGF) homology domain is encoded by exons 7–14. It is involved in the dissociation of the receptor and the lipoprotein in the endocytosis machinery and consists in three EGF-like repeats. The first two repeats are adjacent and separated from the third by a 280-amino-acid sequence that includes six copies of a conserved motif (YWTD). The first EGF-like repeat (EGF-A) interacts with a specific sequence of proprotein convertase subtilisin/kexin type 9 (PCSK9; Zhang et al., 2007). PCSK9 contributes to the posttranslational regulation of hepatic LDLR promoting its intracellular degradation. A sequence of 58 amino-acids enriched in serines and threonines is encoded by exon 15. This structure serves as an attachment site for O-linked sugar chains. Exon 16 and the 5´end of exon 17 encode 22 hydrophobic amino-acids, constituting the membrane anchoring domain, which is essential to the attachment of the receptor to the cell membrane. The remainder of exon 17 and the 5´end of exon 18 encode the 50 amino-acid cytoplasmic tails including the NPXY motif implicated in the endocytosis of the protein.

Currently, more than 1,700 different variants in the *LDLR* have been described in patients with a clinical diagnosis of FH (Leigh et al., 2017). However, a small number of variants have been functionally verified as causing of FH. In 2015, the American College of Medical Genetics and Genomics (ACMG) developed guidelines to classify the variants as a pathogenic, likely pathogenic, variant of unknown significance (VUS), likely benign and benign. Nevertheless, recent research has concluded the importance to validate functionally some variants (Chora, Medeiros, Alves, & Bourbon, 2018).

In this study, we report new genetic variants at the *LDLR* in patients with hypercholesterolemia, and their in vitro characterization.

2 | MATERIALS AND METHODS

2.1 | LDLR variants

All new variants found at the *LDLR* in DNA samples of 516 subjects with hypercholesterolemia sent to our reference center in Madrid from 2015 to 2016, were included in the study: *c.518 G>C*;p.(Cys173-Ser), c.[*684 G>T*;*694 G>T*];p.[Glu228Asp;Ala232Ser] *c.926C>A*;p.(Pro309His), *c.1261A>G*;p.(Ser421Gly), *c.1594T>A*;p.(Tyr532Asn), and *c.2138delC*;p.(Thr713Lysfs*17). All patients gave informed consent for the genetic analysis. Patients were clinically classified as having probable or definitive FE according to *Ducht Lipid Clinic Network Criteria*. In addition to the above-mentioned variants, we performed a functional analysis of the variant *c.800A>C*;p.(Glu267Ala), and the variant reported in dbSNP *c.1049 G>A*;p.(Arg350Gln) without the associated data. We analyzed the variant *c.829 G>A*;p.(Glu277Lys), which had been previously validated in the same cell model. The class 2 variant p.(Gly549Asp) was used as a control because it was functionally confirmed in fibroblasts of a homozygous patient, and the activity of the receptor was <2% (Hobbs, Brown, & Goldstein, 1992). We have considered new variants those that were not described in the databases ClinVar, LOVD, and Human Gene Mutation Database (HGMD).

2.2 | Genetic analysis

The genomic DNA from probands was extracted from EDTA treated whole blood samples using Chemagen (Chemagic DNA extraction special, Perkin Elmer Inc, Baesweiler, Germany). DNA quantification was performed using a NanoDrop ND-1000 Spectrophotometer.

The genetic analysis was performed by Next Generation Sequencing (NGS) using a customized panel of 198 genes. Library preparation and exome enrichment steps were performed according to the manufacturer's workflow (Nimblegen, Roche) and it was sequenced using MiSeq or NextSeq system Sequencing, Illumina. A subset of genes was chosen for analysis of hypercholesterolemia: *LDLR*, *APOB*, *PCSK9*, and *LDLRAP1*. NGS data was suitable for the analysis after passing the quality parameters established in our laboratory: Number of reads more than 30x in the 99% of the target bases.

Sanger sequencing was used to confirm the presence of the new variants. The *Multiplex ligation-dependent Probe Amplification* (MLPA, Salsa P062-D2 Kit, MRC-Holland, Amsterdam) was used for the detection of large rearrangements in *LDLR* according to manufacturer's instructions.

2.3 | In silico analysis

Bioinformatic analysis was performed using algorithms developed by our bioinformatic unit. Briefly, sequences were mapped to the *CRCh37/hg19* human reference sequence and databases used for analysis were HGMD® (http://www.hgmd.cf.ac.uk/ac/index.php) from BIOBASE Corporation; Online Mendelian Inheritance in Man (www.omim.org); Gene Tests (www.genetests.org). Variant annotation was carried out with Ensembl's variant Effect Predictor Tool and was on the basis of the transcripts ENST00000558518-*LDLR*, ENST0000023324-*APOB*, ENST00000302118-*PCSK9* and ENST00000374338-*LDLRAP1*. In silico predictors of pathogenicity used were Combined Annotation Dependent Depletion (CADD), Polymorphism Phenotyping (Polyphen), MutAssesor, Fasthmm, and Vest. Scores of conservation used: Gerp2, PhasCons, Phylop in relation to 13 species. MaxEntScan, NNSplice, GeneSplicer, and Human Splicing Finder were used as splicing predictors. The files were uploaded in BAM format for analysis using Alamut Visual V.2.8.0 (Interactive Biosoftware, France).

2.4 | Cell culture and transfection

Chinese hamster ovary (CHO) *ldlA7* cells (kindly provided by Dr. Monty Krieger, Massachusetts Institute of Technology, Cambridge, MA) were used as the LDLR-deficient cell line (Kingsley & Krieger, 1984; Krieger, Martin, Segal, & Kingsley, 1983). The cells were cultured in Ham's F-12 supplemented (Gibco, Life Technologies, Carlsbad, CA) with 5% heat-inactivated fetal bovine serum (Gibco, Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/L L-glutamine, Normocin 50 mg/ml (InvivoGen, Toulouse France). Adherent CHO-*ldlA7* cells were grown in monolayer culture into 75 cm^2 flasks and incubated at 37°C under 5% CO_2 atmosphere.

The constructs were generated into the expression vector *LDLR* NM_000527 Human cDNA ORF clone Human, C-GFPSpark®-tag (HG10231-ACG Sino-biological, Wayne). Site-directed mutagenesis was carried out with QuikChange lightning site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions. The plasmid sequences after mutagenesis were validated by Sanger sequencing.

CHO-*ldlA7* cells were seeded in 24-well plates at 15 × 104/well and when they reached about 70–90% confluent were transiently transfected. Briefly, Lipofectamine 3000 (Invitrogen™ Thermo Fisher Scientific, Waltham) was mixed with 2.5 μg of each of the LDLR expression plasmids previously mentioned, besides the cells were transfected with empty vector and wild type (WT) according to the manufacturer's instructions. The mix was added to the cells and they were incubated 48 hr.

2.5 | Quantification of LDL-receptor activity by flow cytometry

Human LDL was isolated from a single donor and labeled with 1,1′-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Life Technologies; Calvo, Gomez-Coronado, Suarez, Lasuncion, & Vega, 1998). The transfected cells were incubated for 4 hr at 4°C and 37°C for testing binding and uptake, respectively, with 20 μg of protein DiI-LDL/ml. Then, the cells were washed twice with phosphate-buffered saline-2% bovine serum albumin (PBS-2% BSA), and analyzed by flow cytometry (BD FACSCanto™). Forward scatter and side scatter gates were established to exclude dead cells and cell debris, also 4′,6-diamidino-2-phenylindole dihydrochloride

(DAPI; Invitrogen™, Thermo Fisher Scientific) solution was added directly to the samples to a final concentration of 0.1% to separate live cells of dead cells. The acquisition number of cells was set at 10^4. All assays were performed in triplicate.

2.6 | LDLR expression by immunofluorescence

CHO-*ldlA7* were seeded in coverslips at 3×10^4/well and transiently transfected with the constructs. After 48 hr the cells were incubated for 4 hr at 4°C or 37°C with 100 μg of protein Dil-LDL/ml. Then, the cells were washed three times with PBS-2%BSA and were fixed with 2% paraformaldehyde (PBS-2%PFA) for 10 min at room temperature, permeabilized with 0.1% TritonX-100 for 3 min at room temperature. The cells were incubated overnight at 4°C with primary antibody against GFP (Sino-Biological), fluorescent secondary antibody Alexa fluor-488 goat antimouse IgG (Invitrogen™, Thermo Fisher Scientific) for 1 hr and finally with DAPI during 20 min at room temperature; after each incubation period the samples were washed three times with PBS-2%BSA. The coverslips were mounted on glass slides with ProLong (Invitrogen™, Thermo Fisher Scientific). Images were obtained with a Leica DM5500 B fluorescence microscope (Leica Microsystems, Germany).

3 | RESULTS

A total of seven new variants at *LDLR* were tested: *c.518 G>C*;p.(Cys173Ser), c.[*684 G>T*;*694 G>T*];p.[Glu228Asp;Ala232Ser], *c.926C>A*;p.(Pro309His), *c.1261A>G*;p.(Ser421Gly), *c.1594T>A*;p.(Tyr532Asn), and *c.2138delC*; p.(Thr713Lysfs*17). All of these variants were functionally validated. Age, sex, origin of patients, and the localization of all the variants are shown in Table 1. All variants were found in index patients. Figure 1 shows the location of the *LDLR* variants analyzed in this study. The MLPA assay carried out in all patients did not identify any major rearrangements in the *LDLR* gene.

3.1 | In silico analysis

An in silico analysis of the conservation was performed for all variants showing conservation except for p.(Ala232Ser) and p.(Arg350Gln) with respect to Gerp2 (Table S1**)**. The predictors used showed damage or possible damage for all variants except p.(Ala232Ser). Because the variant p.(Ala232Ser) is located at the last nucleotide of exon 4, we performed an in silico analysis for splicing predictions. The splicing predictors SpliceSiteFinder and NNSPLICE revealed that this variant might disrupt the canonical splice donor site of exon 4. MaxEntScan, GeneSplicer, and Human Splicing Finder-like predicted a decreased splicing efficiency at the canonical donor site (Figure S1).

Ranges/cut-offs, websites, URL, software versions and parameters used in these results are reflected in Table S2.

Considering the ACMG criteria for variants classification, p.(Ala232Ser) was classified as likely benign; p.(Pro309His) and p.(Ser421Gly) as VUS and the rest of variants were classified as probably pathogenic or pathogenic.

3.2 | Effect of the variants in the LDLR expression

Immunofluorescence study revealed the expression of LDLR in all variants except for p.(Thr713fsLys*17). Colocalization of LDLR-Dil-LDL showed a lack of LDL uptake in the variants p.(Tyr532Asn) and p.(Thr713fsLys*17) as well as the control p.(Gly549Asp; Figure 2).

3.3 | Effect of the variants in the LDLR function in transfected cells

Dil-LDL uptake and binding capacity were measured by flow cytometry. The specific median intensity of fluorescence of each sample was estimated after subtracting the median intensity of florescence of the empty vector. We represent the data as the percentage of each variant in relation to the WT. The variants p.(Gly549Asp), p.(Tyr532Asn), and p.(Thr713fsLys*17) showed less than 1% of DIL-LDL binding and uptake with respect to WT. The variants p.(Glu277Lys), p.(Arg350Gln), and p.(Ser421Gly) showed >100%. The rest of the variants shown for LDL uptake and LDL binding: p.(Cys173Ser) 10% and 27%, c.684 G>T;p.(Glu228Asp) 12% and 13%, p.(Ala232Ser) 73% and 79%, p.(Glu267Ala) 38% and 69%, p.(Pro309His) 50% and 67%, (Figure 3**)**. The variants of less uptake p.(Cys173Ser), p.(Tyr532Asn), p.(Thr713fsLys*17) correlated with the highest LDL-c levels (250–329 mg/dL) in our patients and were classified as "damaging" according to the predictors of pathogenicity.

3.4 | Cosegregation studies

The cosegregation analysis was possible in four cases (Figure 4**)**. Proband 2, which carried the variants c.[684 G>T;694 G>T];p.[Glu228Asp;Ala232Ser] had LDL-c levels 250–329 mg/dL and this variant showed cosegregation (Figure 4a**)**. The p.(Ala232Ser) also showed nonsignificant differences on LDL uptake and binding capacity of LDL regarding WT. Using Alamut software, we visually confirmed that the variants p.(Glu228Asp) and p.(Ala232Ser) were in the same allele. Nevertheless, the impact of p.(Ala232Ser) as a splicing modifier was not tested because unfortunately, it was not possible to obtain the patient's RNA sample.

Two unrelated probands (3 and 4) carried **p.(Glu267Ala)**. Proband 4 had LDL-c 193 mg/dL. His mother carried the variant; she has hypercholesterolemia and is on statin treatment (Figure 4b**)**. Patient 5 was heterozygote for *LDLR:c.800A>C*:p.(Glu267Ala) plus *PCSK9:c.137 G>T*:p.(Arg46Leu), and showed raised LDL-c levels (250–329 mg/dL). This later variant in the *PCSK9* gene, has been associated with decrease LDL-c and a reduction in cardiovascular events (Abifadel et al., 2003); that indicates that the FH phenotype in this patient is because of the presence of p.(Glu267Ala).

The variant **p.(Glu277Lys)** was found in two individuals (patients 5 and 6). Patient 5 showed LDL-c ranging 155–189 mg/dL and had a family history of premature ischemic cardiopathy. The genetic analysis was

TABLE 1 Characteristics of patients and description of the *LDLR* variants found in the study

				Family history		Personal history	Physical examination							
ID	Sex	Age	Origin	LDL-c	CVD	CVD	Xanthomas	Arcus cornealis	LDL (mg/dl)	Exon	cDNA	Protein	LDLR domain	Reference
1	F	15	Spanish	Yes	No	No	No	No	250–329	4	*c.518G>C*	**p.(Cys173Ser)**	Ligand binding	New
2	M	47	Spanish	Yes	No	Yes	No	No	250–329	4	*c.[684G>T; 694G>T]*	**p.[Glu228Asp; Ala232Ser]**	Ligand binding	New
3	F	25	Spanish	Yes	No	No	No	No	190–249	6	*c.800A>C*	p.(Glu267Ala)	Ligand binding	Garcia-Garcia (et al. (2001)
4	F	62	Spanish	No	No	No	No	No	250–329	6	*c.800A>C*	p.(Glu267Ala)	Ligand binding	
5	F	59	Spanish	Yes	No	No	No	No	155–189	6	*c.829G>A*	p.(Glu277Lys)	EGF precursor homology	Ekstrom et al. (1995)
6	F	nd	nd	Yes	Yes	Yes	No	No	190–249	6	*c.829G>A*	p.(Glu277Lys)	EGF precursor homology	
7	M	14	Ecuadorian	Yes	No	No	No	No	190–249	6	*c.926C>A*	**p.(Pro309His)**	EGF precursor homology	New
8	F	41	Spanish	Yes	No	No	No	Yes	190–249	7	*c.1049G>A*	p.(Arg350Gln)	EGF precursor homology	dbSNP
9	F	45	Spanish	Yes	Yes	No	No	No	190–249	9	*c.1261A>G*	**p.(Ser421Gly)**	EGF precursor homology	New
10	F	74	Spanish	No	No	Yes	No	No	250–329	11	*c.1594T>A;*	**p.(Tyr532Asn)**	EGF precursor homology	New
11	F	29	German	No	Yes	No	No	No	250–329	14	*c.2138delC*	**p.(Thr713Lysfs*17)**	EGF precursor homology	New

Abbreviations: CVD, cardiovascular disease; ID, proband identification; LDL-c, low-density lipoprotein cholesterol.
Note. New variants at *LDLR* are marked in bold.

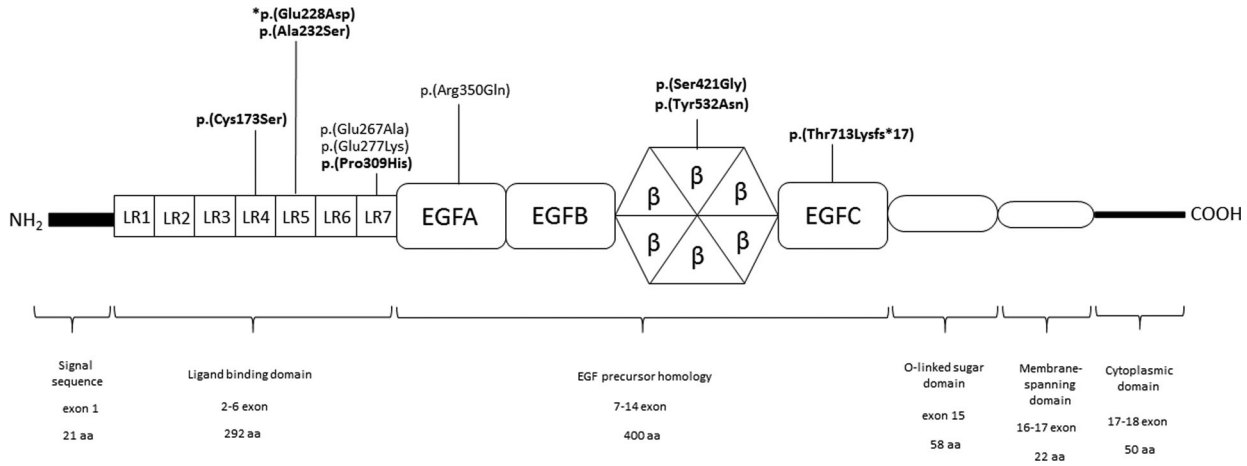

FIGURE 1 Diagram showing the location of the variants found in this study within the *LDLR* gene. New variants are represented in bold. *the variant p.(Glu228Asp) corresponds to the change c.684 G>T

possible in two members of the family: Sister with combined dyslipidemia and did not carry the variant and, the daughter who carried the variant and total cholesterol 241 mg/dL and LDL-c 141 mg/dL (Figure 4c). Patient 6, with the **p.(Glu277Lys),** had a history of cardiovascular disease (CVD) and showed LDL-c 190–249 mg/dL, with a family history of first-degree relatives with premature CVD and a first-degree relative with LDL-c 210 mg/dL. Clinical data of cosegregation was not available.

Patient 10, who carried the **p.(Tyr532Asn)** had two myocardial infarctions. We observed cosegregation of the variant with hypercholesterolemia (Figure 4d). Her affected son carried the variant, with raised LDL-c levels (250–329 mg/dL). Her granddaughter carried the variant as well, but not her daughter, who had normal levels of cholesterol. This variant showed very low LDL uptake and binding capacity of LDL compared with WT.

4 | DISCUSSION

Many genetic variants at the *LDLR* have been identified and reported to cause FH. We have characterized seven new variants plus three variants previously described. Among the studied variants, three of them are located at exon 4. The variant **p.(Cys173Ser)** affects a cysteine involved in one of the six disulfide bonds that constitute the LR4 module. Mutational studies of the seven LR modules of the LDLR, have revealed that modules three to seven participate in the binding of LDL particles (Russell, Brown, & Goldstein, 1989). The lack of cysteine in this position affects LDL binding, as confirmed in our assay that resulted in reduced LDL uptake and LDL binding 10% and 27%, respectively compared with WT. The variant *c.684 G>T*:**p.(Glu228Asp)** is located in module LR5. The structure LR5 has six cysteines that constitute three disulfide bonds leading to a scaffold that stabilizes the structure in connection with the calcium-binding site (Fass, Blacklow, Kim, & Berger, 1997). The Glu-228 constitutes the last amino-acid of the conserved sequence Ser-Asp-Glu (SDE) situated in the COOH-terminal of each LR (Hobbs, Russell, Brown, & Goldstein, 1990). These three amino-acids are negatively charged, allowing interaction with positively charged regions. The 228-Glu participates in Ca^{2+} coordination in concern with other residues so mutations in this residue destroy the Ca^{2+} binding site (Fass et al., 1997). Previous studies had revealed that mutations of the calcium-coordinating residues cause hypercholesterolemia because this structure is necessary

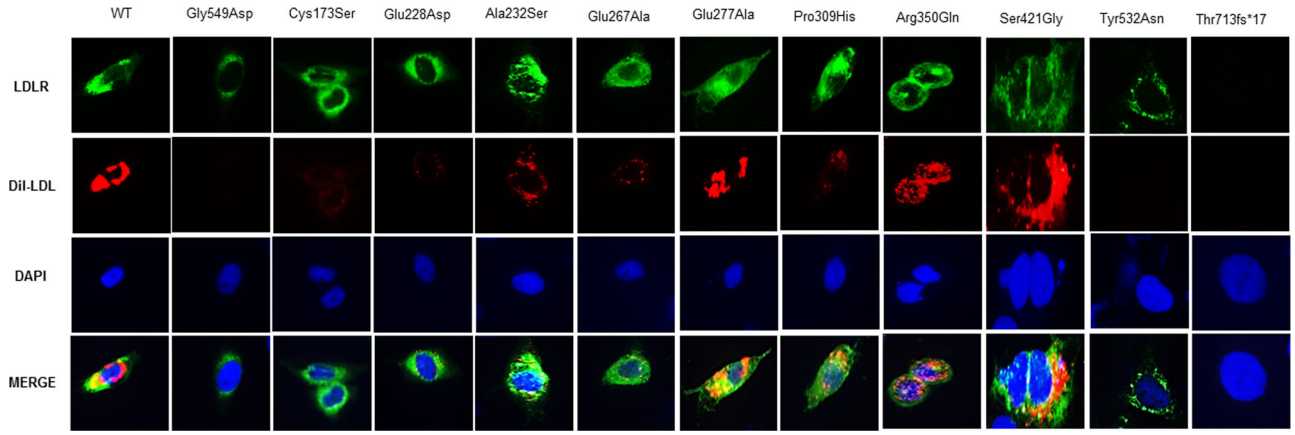

FIGURE 2 The expression of LDLR in the transiently transfected CHO-*ldlA7* cells, with WT, control (Gly549Asp), and LDLR variants. Images of immunofluorescence show LDLR expression in all variants except in p.(Thr713fsLys*17). Colocalization of LDLR-DiI-LDL are shown in the last row identifying lack of uptake LDL in the variants p.(Tyr532Asn) and p.(Thr713fsLys*17). LDL, low-density lipoprotein

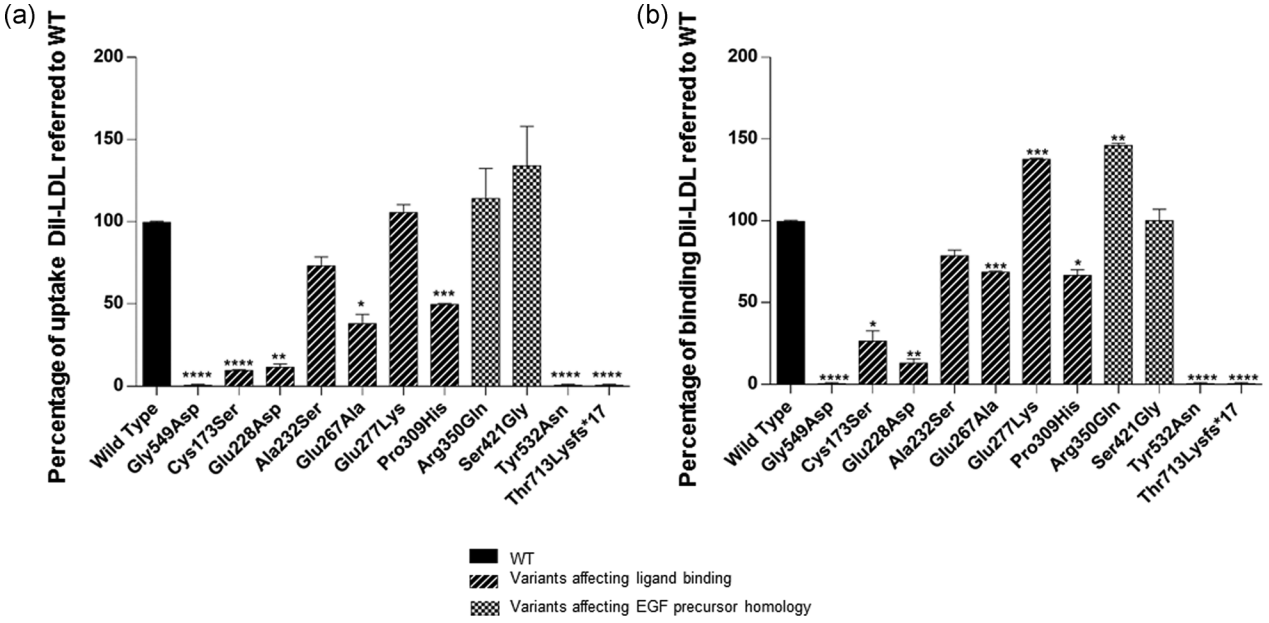

FIGURE 3 The LDLR activity in the transiently transfected CHO-*ldlA7* cells, with WT, control (Gly549Asp), and LDLR variants. (a) Uptake DiI-LDL. (b) Binding DiI-LDL. The analysis performed by flow cytometry as described in Material and Methods Each result corresponds to mean ± S.E.M. of the triplicate experiments compared with the WT. Statistical significance were determined by a two-tailed Student's *t* test and a 95% confidence interval. LDL, low-density lipoprotein; WT, wild type. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$ versus WT. The results were obtained in three independent experiments

for proper folding and function of the receptor (Atkins, Brereton, Kroon, Lee, & Smith, 1998; Goldstein & Brown, 1974). A different nucleotide change, *c.684 G>C* causing the same aminoacid alteration, has been described in a patient with clinical diagnosis of FH (Banares et al., 2017). The change of Glu-228 by other amino-acids, such as Lys, Gln or Ala have shown a reduced LDLR activity ranging from 2% to 8% (Hobbs et al., 1990, 1992; Leitersdorf, Tobin, Davignon, & Hobbs, 1990). Our results show a significant loss of LDL uptake and LDL binding in the variant c.684 G>T;p.(Glu228Asp) compared with WT. This variant occurs in *cis* with **p.(Ala232Ser),** and the variants showed cosegregation in the family. The functional analysis p.(Ala232Ser) revealed nonsignificant differences of LDL uptake and LDL binding compared to WT. Because this variant affects the last nucleotide of exon 4, the splicing could be affected, leading to an additive effect of this variant on cholesterol levels.

We report three variants affecting exon 6. The variant **p.(Glu267Ala)** was described for the first time in Spanish subjects (Garcia-Garcia et al., 2001). Glu267 is the last amino acid of the conserved sequence SDE, important for the interaction between LDLR and lipoproteins. We found significant differences in LDL binding and uptake with respect to WT. In our study, two index patients carried this variant, and we observed cosegregation in one of the families.

The variant **p.(Glu277Lys)** is seated in module LR7; the replacement of glutamic for lysine alters the negative charge. It is known that Asp and Glu side chains that coordinate calcium are essential for protein folding (Pena, Jansens, van Zadelhoff, & Braakman, 2010). This variant was reported for the first time in Swedish children with FH (Ekstrom, Abrahamson, Sveger, Lombardi, & Nilsson-Ehle, 1995) being widely distributed in patients with FH from different ethnicities. Pereira et al., (1995) revealed cosegregation of this variant in a Spanish family of Cuban descent. In our study, two index patients carried this variant and in one of them the variant showed cosegregation. However, we found affected neither the LDL-uptake nor the LDL binding regarding WT. Similar results were previously described. Ekstrom et al. (2000) described a patient who was carrier of p.[Glu277Lys;Ile423Thr] and tested separately in CHO-*ldlA7* cells both variants and they concluded that the deleterious effect on LDLR is because of the second one variant and not for the p.(Glu277Lys). In our case, we did not find any other variant that can explain the phenotype of hypercholesterolemia. It is possible that the variant p.(Glu277Lys) occurs in linkage disequilibrium with other undetected variant.

The new variant **p.(Pro309His)** is part of the LR7 (Russell et al., 1989) which consists in a change from a hydrophobic amino-acid to an amino acid with a positive charge. We observed significant differences in LDL uptake and binding in cells transfected with this variant compared with WT.

Four variants are situated on EGF precursor homology domain, p.(Arg350Gln), p.(Ser421Gly), p.(Tyr532Asn), and p.(Thr713Lysfs*17). The variant **p.(Arg350Gln)** had been reported in dbSNP without associated data (Database of Single Nucleotide Polymorphisms (dbSNP)Bethesda (MD)National Center for Biotechnology Information). However, change p.(Arg350Gln) was previously identified in an English patient who was compound heterozygote p.[Cys248Tyr];[Arg350Pro] and the LDLR activity measured in cultured skin fibroblasts or cultured Epstein-Barr virus-transformed lymphoblasts was 10–15% (Webb et al., 1996). Our patient carried p.(Arg350Gln) without other identified genetic variation, and we observed significant differences in LDL binding and uptake with regard to WT. The **new variant, p.(Ser421Gly)** lies within the β-propeller, a 280 amino-acid sequence that separate the

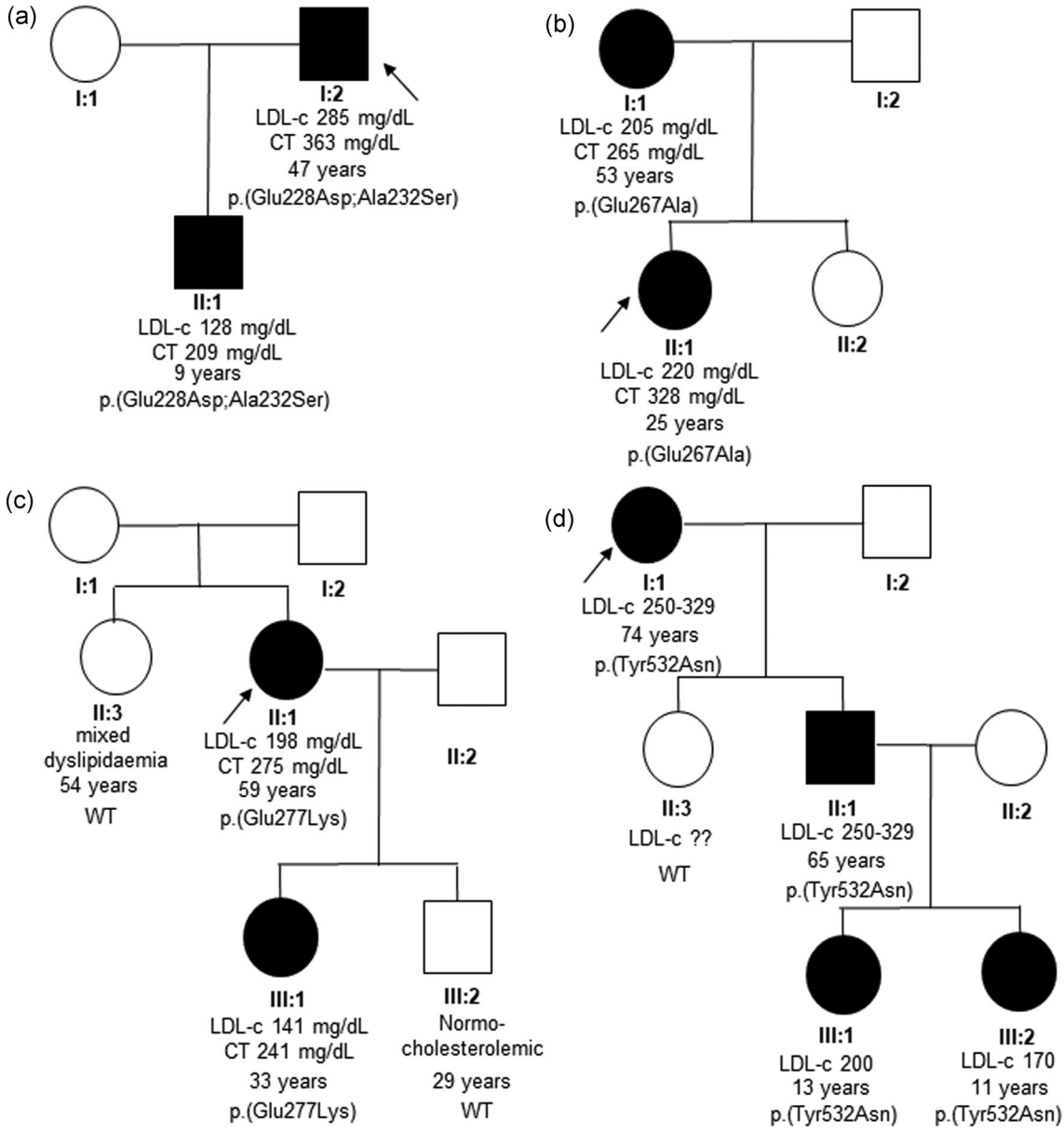

FIGURE 4 Pedigrees of families included in the study. The index cases are indicated with an arrow; circle and square symbols represent women and men respectively; shadow filled symbols indicate the affected members with FH. Line 1 below symbols correspond to the individual identification, line two indicates cholesterol total (CT) and levels without lipid-lowering therapy (LDL-c), line three indicates age and line 4 indicates the *LDLR* genotype: mutant or wild type (WT). (a) Pedigree of proband 3 p.[Glu228Asp;Ala232Ser]. (b) Pedigree of proband 4 p.(Glu267Ala). (c) Pedigree of proband 6 p.(Glu277Lys). (d) Pedigree of proband 11 p.(Tyr532Asn). FH, familial hypercholesterolemia

first two growth factor repeats, A and B, from the third, C. We did not observe differences in the LDL uptake and LDL binding when comparing p.(Ser421Gly) with the WT.

The variant **p.(Tyr532Asn)** affects one of the six copies of a conserved motif YWTD that spans from residues 396–664. The YWTD are folded into a compact structure known as a six-bladed β-propeller domain (Springer, 1998). Misfolded, misassembled and unassembled polypeptides are usually retained in the RE and degraded. These processes are quality control mechanisms that act in the RE to adjust the structural requirements for their expression (Hurtley & Helenius, 1989). The replacement of tyrosine by asparagine causes a change in the YWTD motif, impairing the correct folding of the structure previously mentioned. In this study, we have shown a lack of LDL uptake and LDL binding in CHO-*ldlA7* transfected with p.(Tyr532Asn). The variant showed cosegregation in the family supporting its pathogenicity.

The variant **p.(Thr713Lysfs*17)** is located at the third epidermal growth factor-like repeat. This variant is predicted to produce a premature stop codon and removing the last 130 amino. Previous studies have shown that mutations in the LDLR gene encoding residues at the cytoplasmic domain affect the interaction with COPII in the ER membrane, which plays an essential role for the membrane-spanning proteins (Dancourt & Barlowe, 2010; Hughes & Stephens, 2008). Also, the variant **p.(Thr713Lysfs*17)** could produce the loss of the LDLR cytoplasmic domain required for exiting the ER. At least 30

residues of the cytoplasmic domain are necessary to allow LDLR to exit from the ER (Strom et al., 2011). In our study, the variant p.(Thr713Lysfs*17) showed a lack of LDLR expression and activity.

In summary, we have reported seven new variants in *LDLR*. After the functional study, almost all variants were classified as likely pathogenic or pathogenic avoiding the classification as "variants of uncertain significance". This functional study allows us to confirm the FH in four out of eight patients with probable FH improving the efficacy of the FH screening. Moreover, the cascade screening lets us improve detection of relatives from a verified FH index case, which is more cost effective than any other screening method currently available (Marks et al., 2002). The capacity to achieve an early diagnosis of patients with FH, is economically and socially beneficial, as its impact on cardiovascular morbidity and mortality (Henderson, O'Kane, McGilligan, & Watterson, 2016). Recent studies have revealed that patients with FH and LDL-c levels above 190 mg/dL with mutations at the *LDLR*, exhibit a cardiovascular risk approximately three-folds higher than patients who show the same LDL-c levels but in which mutations at the *LDLR* cannot be demonstrated (Khera et al., 2016).

FH is associated with a very high risk of premature cardiovascular disease. The molecular diagnosis is necessary to achieve its definitive diagnosis and to allow for family cascade screening.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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