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Functional analysis of six uncharacterised mutations in *LDLR* gene

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HIGHLIGHTS

- More than 2100 mutations have been already identified in *LDLR* gene.
  - •The relationship between the receptor activity and mutations have not been demonstrated in all cases.
  - •Functional studies of six uncharacterised variants in *LDLR* are reported here.
  - •The six variants could be categorised following ACMG guidelines as Benign, Pathogenic or Likely Pathogenic.
  - •Predictive *in silico* algorithms were not always consistent with the results of the functional studies on *LDLR*.

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ABSTRACT

Background and aims: Familial hypercholesterolemia (FH) is a primary hyperlipemia. It is an autosomal dominant genetic disorder of lipoproteins metabolism mainly caused by mutations in the low density lipoprotein receptor gene (*LDLR)*. We aimed to investigate the functional impact on the low density lipoprotein receptor (LDLR) activity of six uncharacterised variants located in the coding region of the *LDLR* gene, namely c.428G > T, c.640T > C, c.1708C > T, c.1736A > T, c.1981C > G and c.2114C > G (NM_000527.4) and to attempt to define their clinical status.
Methods: Functional studies were carried out using site-directed mutagenesis techniques and expression of LDLR protein *in vitro*. Results were correlated with clinical data and *in silico* analyses in order to assess the physiopathological role of these variants.
Results: This work provides functional information about 6 uncharacterised mutations in *LDLR*.
Conclusions: The six variants studied here appeared to affect the LDLR function *in vitro* to different degrees, ranging from receptors with normal to slightly reduced activity to receptors exhibiting less than 10% of the wild-type activity. According to these studies and The American College of Medical Genetics and Genomics (ACMG) Standards and Guidelines, two variants could be classified as "Likely Benign" (p.(Ala705Gly) and p.(Leu570Phe)), three variants as "Pathogenic" (p.(Asp579Val), p.(Cys143Phe) and p.(Trp214Arg)) and one variant as "Likely Pathogenic" (p.(Pro661Ala)).

1. Introduction

Familial hypercholesterolemia (FH, OMIM 143890) is an autosomal dominant hereditary disorder characterised by a reduction of the liver capacity in the clearance of the atherogenic low-density lipoproteins (LDL) resulting in a marked elevation of serum LDL cholesterol (LDL-c) levels. Consequently, FH causes early morbidity and mortality due to cardiovascular events [1]. FH is mainly caused by defects in the gene encoding the LDL receptor (LDLR). Pathogenic mutations on *LDLR* can affect different steps of the receptor cycle: synthesis, maturation, expression on the cell surface and correct insertion in the cell membrane, LDL binding, internalization and recycling. Most severe genetic defects

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Table 1
Clinical features of the patients carrying the mutations characterised here and summary of the results of functional and predictive analyses.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age (years)	42	75	54	34	39
Sex	Male	Female	Male	Male	Male
COL-T (mg/dl) (*)	500	426,4	429,4	409,4	393,4
LDL-c (mg/dl) (*)	441	349	350	356	331
Triglycerides (mg/dl) (*)	150	227	167	127	112
Lp(a) (mg/dl) (*)	74,8	167	180	17,2	55
HDL-c (mg/dL) (*)	29	32	46	28	40
DLCN	17	12	14	15	15
***LDLR* variant**	c.2114 C > G p.(Ala705Gly)	c.1708 C > T p.(Leu570Phe)	c.640 T > C p.(Trp214Arg)	c.1736 A > T p.(Asp579Val)	c.428 G > T p.Cys143Phe
Other variants	-	c.2029 T > C (p.(Cys677Arg))	-	-	-
Functional analyses					
***In vitro* activity of LDLR (% relative to wild-type)**	Near wild-type	50	75	50	20	< 10
Predictive analyses					
Mutation Taster	Disease causing-0,999	Disease causing-0,99999	Disease causing-0,99999999	Disease causing-0,999999999999	Disease causing-0,999999999999
Polyphen-2	Benign	Probably damaging	Probably damaging	Probably damaging	Probably damaging
SIFT	Tolerated	Tolerated	Tolerated	Deleterious	Tolerated
Mutation Assessor (Functional Impact)	Neutral	Medium	Low	Medium	High	High
Grantham score	Moderately conservative-60	Conservative-27	Conservative-22	Moderately radical-101	Radical-152	Radical-205

COL-T: total cholesterol, LDL-c: low density lipoprotein, Lp(a): lipoprotein a, HDL-c: high density lipoprotein; (*) before the initiation of lipid-lowering therapy, DLCN: Dutch Lipid Clinic Network Score.

SIFT: Scores from 0 to 1 (Score < 0,05 is predicted as "Not Tolerated"; Score > 0,05 is predicted as "Tolerated"). Polyphen: Scores from 0 to 1 (Score: 0 "Benign", Sore: 1 "Damaging"). Mutation Taster: Probability close to 1 indicates greater certainty in the prediction. Mutation Assessor: Functional Impact: Neutral-Low-Medium_High. Grantham Score: Conservative-Moderately Conservative-Radical (0.0–215).

are those that lead to a lack of protein production, for instance due to mutations in the promoter region or nonsense mutations [1]. Mutations in apolipoprotein B (*APOB*), and proprotein convertase subtilisin/kexin type 9 (*PCSK9*) genes, are recognised as less frequent causes of FH [2–5].

As demonstrated, the extent to which mutations affect LDLR activity can contribute to determine patient's phenotypes [6]. However, LDLR activity in an individual can be influenced by environmental factors and/or other genetic alterations involved in lipid metabolism [7]. Therefore, the relationship between receptor activity and phenotype may not be always direct.

At present, more than 2100 mutations in *LDLR* have been identified [2]. However, not all of them have proved to affect the receptor activity [8]. They have been assigned to 5 different classes depending on their effect [9–13].

Genetic testing is usually requested to assist in diagnosis, prognosis and treatment [8]. However, labelling missense changes as variants of uncertain significance (VUS) because of a lack of functional information does not help to solve diagnostic dilemmas. The ACMG and the Association for Molecular Pathology (AMP) have recognised the importance and utility of heterologous expression systems and have weighted the results of well-established functional testing [14]. In this study we aimed to evaluate the effect of 6 VUS in the *LDLR* gene previously reported by Gomez et al. [15] in a cohort of 38 FH patients by analysing the *in vitro* activity of the LDLR. Secondly, the correlation between genotype, functional analysis results and clinical features of the patients was investigated.

2. Materials and methods

2.1. Patients selection

This study included six VUS detected in five patients with FH phenotype defined by Dutch Lipid Clinic Network score ≥8 points (DLCN) [16] previously reported [15]. All patients gave their informed consent before blood sample collection. Data was retrieved from their clinical records or was obtained by direct interrogation at the moment the samples were taken. Anonymity of personal data and confidentiality of the results were maintained throughout all the phases of the study. The study was approved by the Institutional Bioethics Committee (DDI (1282) 5014).

2.2. Variants analysed

The six following VUS in *LDLR* were included in this study: chr19: 11105334c.428G > T p.(Cys143Phe), chr19:11105546 c.640T > C p.(Trp214Arg), chr19:11116861 c.1708C > T p.(Leu570Phe), chr19:11116889 c.1736A > T p.(Asp579Val), chr19:11120227 c.1981C > G p.(Pro661Ala), and chr19: 11120496 c.2114C > G p.(Ala705Gly) (GRCh38, NM_000527.4).

2.3. In silico analysis

The following bioinformatic tools were used to predict the effect of the variants on the receptor activity: Mutation Taster (http://www.mutationtaster.org) [17], Polyphen-2 (http://genetics.bwh.harvard.edu/pph2) [18], Mutation Assessor (http://mutationassessor.org/r3/) [19], and Sorting Intolerant From Tolerant (SIFT) (http://sift.jcvi.org) [20]. Mutation Taster is a free online application to evaluate DNA sequence variants for their disease-causing potential. The software estimates the impact of the change at the DNA and protein level, and the alteration is predicted as one of four possible types: disease causing, disease causing automatic, polymorphism and automatic polymorphism. PolyPhen-2 predicts the possible impact of amino acid substitutions on the stability and function of human proteins using structural and comparative evolutionary considerations. Mutation

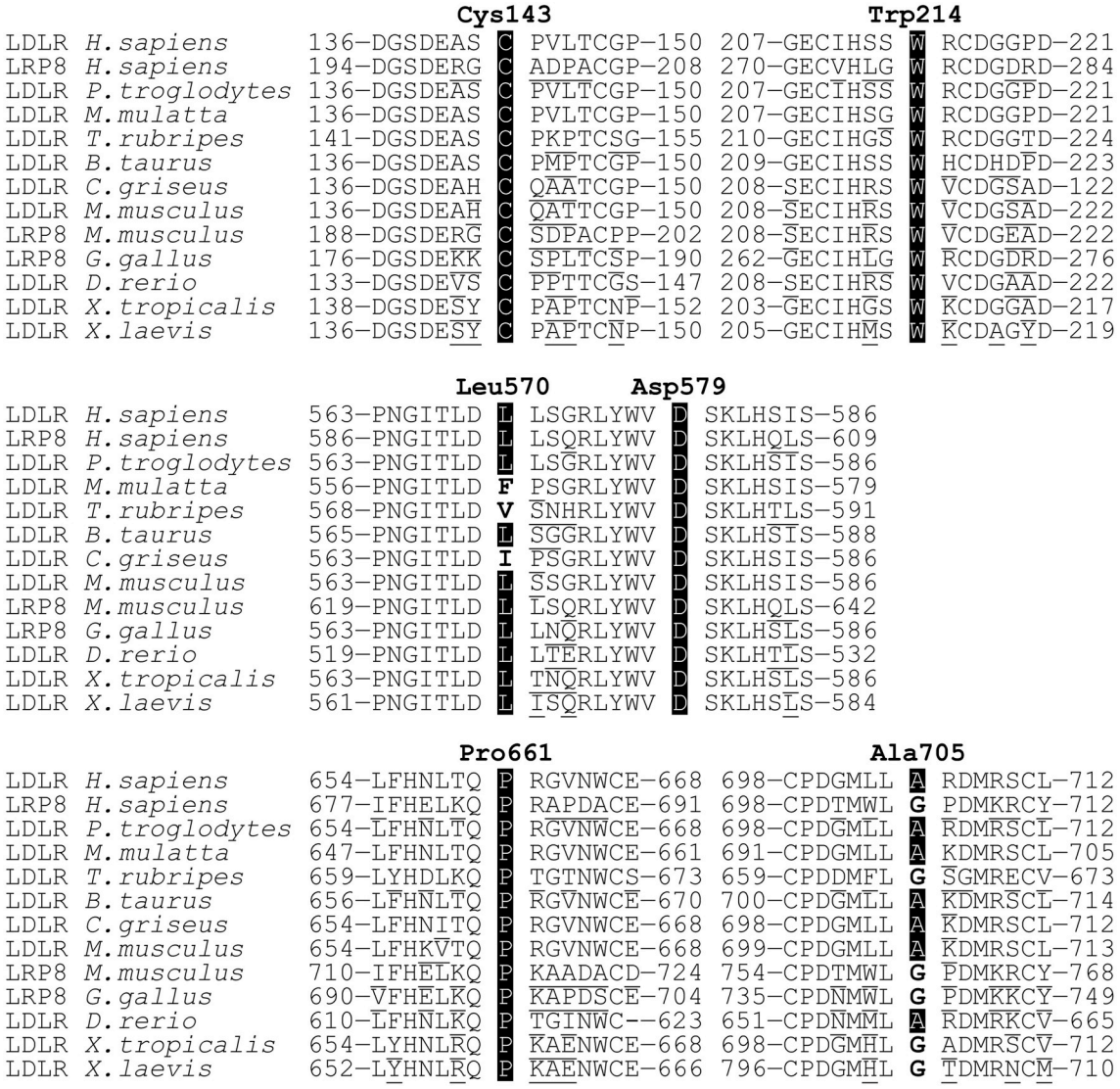

Fig. 1. Comparative analysis of *LDLR* sequences in multiple species.

Assessor predicts the functional impact of amino acid substitutions in proteins based on the evolutionary conservation of the amino acid sites. The SIFT server sorts intolerant from tolerant substitutions and classifies substitutions as tolerated or deleterious.

The multi-species comparison analysis has been done using The Clustal Omega version of Clustal programme. This server allows aligning multiple sequences and looking at the amino acid conservation in different species [21].

The Grantham's Score has been assigned to the six investigated variants. The Grantham's score depends on 3 properties: composition, polarity and molecular volume [22]. This score was taken from an amino acid substitution matrix which takes into account the physico-chemical characteristics of amino acids and scores substitutions according to the degree of difference between the original and the new amino acid.

2.4. Functional studies

2.4.1. Site-directed mutagenesis

Individual *LDLR* point mutations were introduced into the human *LDLR* cDNA in the expression vector pLDLR-2 (American Type Culture Collection, Manassas, VA, USA) under control of a SV40 promoter. The vector contains a portion of pBR322 and SV40 sequences including SV40 ori, 16S and 19S donor and acceptor splicing sites, and poly-adenylation signals. Oligonucleotide site-directed mutagenesis was performed using the QuikChange XL Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. The presence of the desired nucleotide alteration was confirmed by PCR and restriction enzyme digestion of the appropriate fragments using HpyAV, MspI, MboI, *Hin*FI, BstNI, and HaeIII (New England BioLabs, Ipswich, MA, USA) for c.428G > T p.(Cys143Phe), c.640T > C p.(Trp214Arg), c.1708C > T p.(Leu570Phe), c.1736A > T p.(Asp579Val), c.1981C > G p.(Pro661Ala), and c.2114C > G p.(Ala705Gly), respectively. The integrity of the remaining *LDLR* cDNA sequence of all constructs was checked by Sanger sequencing.

2.4.2. Expressions of LDLR in vitro

Chinese Hamster ovary (CHO) cells are an epithelial cell line derived from the ovary of the Chinese hamster. LDLR-deficient CHO-*ldlA7* cells do not express the LDLR [23].

CHO-*ldlA7* cells were cultured in Ham's F12 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Lonza, Basel, CH). Cells were plated into 6-well dishes and transfected with wild-type and mutated pLDLR-2 plasmids using Lipofectamine LTX with Plus reagent

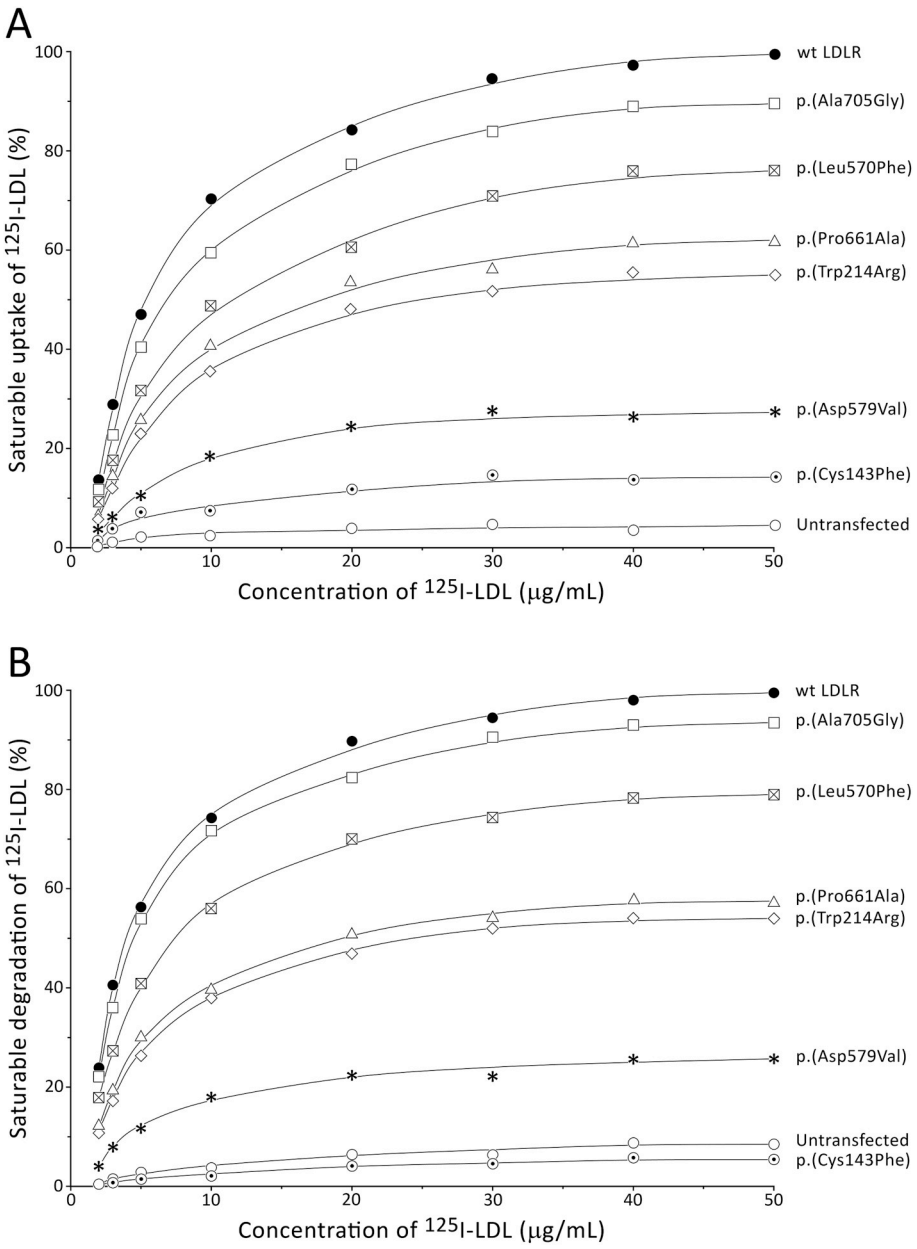

Fig. 2. ^{125}I-LDL metabolism: uptake and degradation of ^{125}I-LDL in CHO-*ldlA7* cells.

(ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Transfected cells were kept at 37 °C in 95% air and 5% CO_2 for 48 h to allow for maximal LDLR expression.

2.4.3. Real-time quantitative PCR

CHO-*ldlA7* cells (0.5×10^6) were seeded in 6-well tissue-culture plates overnight and transfected as described in Section 2.4.1. Two days after transfection, total RNA was harvested from cells using the TRIzol reagent (ThermoFisher) according to the manufacturer's directions and cDNA synthesized from ca. 1 μg of RNA using the Maxima First Strand cDNA Synthesis kit (ThermoFisher) according to the manufacturer's instructions. The qRT-PCR was performed in triplicates using the SuperScrip III Platinum SYBR Green One-Step qPCR kit (ThermoFisher). Primers used to amplify human *LDLR* and hamster *GAPDH* (housekeeping control gene) specific fragments were as reported previously [24,25]. Reactions were performed in triplicate on an ABI 7500 Real-Time PCR system (ThermoFisher). Expression of all LDLR transcripts was determined relative to GAPDH levels by the comparative C_T method [26].

2.4.4. Uptake and catabolism of ^{125}I-LDL

^{125}I-labelled LDL (^{125}I-LDL) was prepared by a modification [27] of the iodine monochloride method [28]. One mCi of $Na^{125}I$ (GE Amersham, Piscataway, NJ, USA) was used to iodinate 2.5 mg of LDL in the presence of 30 nmol of iodine monochloride in 0.5 M glycine/NaOH (pH 10). Free iodine was removed by gel filtration (Sephadex G-25) followed by overnight dialysis against Tris-phosphate buffered saline. The specific radioactivity ranged from 1 to 3×10^5 cpm/μg protein. LDL uptake and catabolism in CHO-*ldlA7* cells expressing wild-type or mutant LDLR was determined by using ^{125}I-LDL as previously described [25,29,30]. Briefly, LDLR-expressing CHO-*ldlA7* cells were seeded in triplicates in 12-well plates (ca. 2×10^5 cells/dish). After 30 h cells were cells were harvested, washed, and pre-incubated (12 h) in Ham's F12 medium containing 10% (v/v) fetal calf lipoprotein-deficient serum, cholesterol (90 μg/mL) and 25-hydroxycholesterol (9 μg/mL) (Sigma-Aldrich, St. Louis, MA, USA). Cells were then washed and

Table 2
Classification of variants according to ACMG criteria.

Variant	Evidence of pathogenicity	Category of pathogenicity	Evidence of benign impact	Category of benign impact	ACMG classification
p.Ala705Gly (c.2114C > G)	Moderate Supporting	PM2	Strong	BS3	Likely benign
		PP4	Supporting	BP1	
			Supporting	BP2	
			Supporting	BP4	
p.Leu570Phe (c.1708C > T)	Moderate Supporting	PM2	Strong	BS3	Likely benign
		PP4	Supporting	BP1	
			Supporting	BP2	
			Supporting	BP4	
p.Pro661Ala (c.1981C > G)	Strong	PS3	-	-	Likely pathogenic
	Moderate	PM2			
	Moderate	PM5			
	Supporting	PP4			
p.Trp214Arg (c.640T > C)	Strong	PS3	-	-	Pathogenic
	Moderate	PM2			
	Moderate	PM5			
	Supporting	PP3			
	Supporting	PP4			
p.Asp579Val (c.1736A > T)	Strong	PS3	-	-	Pathogenic
	Moderate	PM2			
	Moderate	PM5			
	Supporting	PP3			
	Supporting	PP4			
p.Cys143Phe (c.428G > T)	Strong	PS3	-	-	Pathogenic
	Moderate	PM2			
	Moderate	PM5			
	Supporting	PP3			
	Supporting	PP4			

ACMG: American College of Medical Genetics and Genomics. Evidence of pathogenicity PS3: Strong: Well-established *in vitro* or *in vivo* functional studies supporting of damaging effect on the gene or gene product.PM2: Moderate: Absent from controls in Exome Sequencing Project, 1000 Genome Project and or Exome Aggregation Consortium. PM5: Moderate: Novel missense change at amino acid residue where at different missense change determined to be pathogenic has been seen before. PP3: Supporting: Multiple lines of computational evidence support a deleterious effect on the gene product. PP4: Supporting: Patient's phenotype or family history is highly specific with the disease with a single genetic aetiology. Evidence of benign impact (BS3: Strong: Well-established *in vitro* or *in vivo* functional studies show no damaging effect on protein function or splicing. BP1: Supporting: Missense variant in a gene for which primary truncating variants are known to cause disease. BP2: Supporting: Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern. BP4: Supporting: Multiple lines of computational evidence suggest no impact on gene or gene product.

incubated at 37 °C (4 h) in Ham's F12 medium containing 5% (v/v) fetal calf lipoprotein-deficient serum and increasing concentrations of ^{125}I-LDL. LDL uptake, determined at 37 °C, accounts for both binding and internalization of LDL particles. Saturable uptake and degradation of ^{125}I-LDL were determined by cell incubation in the presence or absence of an excess (1 mg/mL) of unlabeled LDL, as described before [25,31,32]. Each experiment was repeated with triplicate samples for each transfected cell line. Saturation and degradation data are reported as the fraction of the value obtained for wild-type LDLR after 4 h of incubation.

2.5. Variant classification according to ACMG criteria

The ACMG Standards and Guidelines provide an approach to evaluating evidence for variants observed in patients with suspected inherited (primarily Mendelian) disorders. These guidelines provide two sets of criteria, one for classification of pathogenic or likely pathogenic variants and one for classification of benign or likely benign ones. Each pathogenic criterion is weighted as very strong (PVS1), strong (PS1-4), moderate (PM1-6) or supporting (PP1-5); and each benign criterion is weighted as stand-alone (BA1), strong (BS1-4), or supporting (BP1-6). These criteria are then combined according to scoring rules [33].

2.6. Correlation analysis

The Spearman's rank correlation test was applied to the association between clinical parameters and LDLR activity, considering significant a p value < 0.05. The statistical analysis was performed with SPSS 20.0 [34].

3. Results

3.1. Clinical features and in silico analyses

The main clinical features of the patients carrying the variants characterised here are summarized in Table 1, together with the results of functional and predictive analyses. All patients had total cholesterol levels above of 190 mg/dl and LDL-c above 150 mg/dl. All values were taken before the initiation of lipid-lowering therapy. The DLCN scores were higher than 8 points in all of patients.

Regarding the results of the predictive analyses, it can be seen that they were not always consistent. The Mutation Taster server classified all variants as “Disease Causing” with slightly differences in the prediction certainty. Polyphen-2 classified p.Ala705Gly and p.Pro661Ala as “Benign” variants and p.Leu570Phe, Trp214Arg, Asp579Val and Cys143Phe as “Probably Damaging” variants. The SIFT system assigned the p.Asp579Val as “Deleterious” while the other five variants were classified as “Tolerated”. The Mutation Assessor software classified p.Ala705Gly variant as “Neutral Functional Impact”, p.Pro661Ala and p.Trp214Arg variants as “Medium Functional Impact”, p.Leu570Phe as “Low Functional Impact” variant and the p.Asp579Val and Cys143Phe as “High Functional Impact” variants. The Grantham's Score was highest for p.Asp579Val and p.Cys143Phe changes (Scores: 152 and 205 respectively).

The multi-species comparison analysis results are shown in Fig. 1. It can be seen that c. 428 C > T p.(Cys143Phe), c.640 T > C p.(Trp214Arg), c.1736 A > T p.(Asp579Val) and c.1981 C > G p.(Pro661Ala) variants introduced changes in highly conserved positions. On the contrary, p.Ala705Gly was present in 6 out 13 of the sequences tested, while p.Leu570Phe was also observed in *M. mulatta*;

furthermore, two other amino acids substitutions for this codon were observed in two other species.

3.2. Functional analyses

3.2.1. Expression of LDLR variants

The mRNA levels in the CHO-*ldlA7* cells transfected with both the wild type (*wt*) and mutated constructs were determined by qRT-PCR after RNA extraction as describe in Material and Methods.

The relative mRNA expression (normalized to GAPDH expression) was similar in the *wt* and in the mutated transfected cells meaning that the gene expression was not affected by each of the introduced variants. mRNA expression was null in CHO-*ldlA7* untransfected cells or cells transfected with the null-plasmid.

3.2.2. Uptake and catabolism of ^{125}I-LDL

Cell lines expressing the *wt* LDLR or the LDLR with c.428 G > T p.(Cys143Phe), c.640 T > C p.(Trp214Arg), c.1708 C > T p.(Leu570Phe), c.1736A > T p.(Asp579Val), c.1981C > G p.(Pro661Ala) and c.2114 C > G p.(Ala705Gly) variants were assayed for their ability to saturable uptake and degradation of the ^{125}I-LDL. As shown in Fig. 2a, the uptake of LDL was highly decreased in c.428 G > T p.(Cys143Phe) variant as well as in non-transfected cells. We found that c.1736 A > T p.(Pro579Val) variant showed a little higher level of uptaking (20% of the wild-type *LDLR*) than non-transfected cells and c.428 G > T p.(Cys143Phe9) variant. It was observed a superior level in the uptake of ^{125}I-LDL in c.640 T > C p.(Trp214Arg) and c.1981 C > G p.(Pro661Ala) variants (almost 50% of wild-type *LDLR*). On the other hand, we found that c.1708 C > T p.(Leu570Phe) and c.2114 C > G p.(Ala705Gly) variants almost kept their function in uptaking ^{125}I-LDL. Fig. 2b shows a similar pattern for the degradation of ^{125}I-LDL. Thus, both processes seemed to be affected in the same way for each of the variants.

3.2.3. ACMG categories

The ACMG variant classification is shown in Table 2. One benign strong criterion (BS3) and 3 benign supporting criteria (BP1, BP2 and BP4) were observed for variants c.2114C > G (p.Ala705Gly) and c.1708C > T (p.Leu570Phe) (Evidence of pathogenicity: (i) PM2: Absent from controls in Exome Sequencing Project, 1000 Genome Project and or Exome Aggregation Consortium; (ii) PP4: Patient's phenotype or family history is highly specific with the disease with a single genetic aetiology. Evidence of benign impact: (i) BS3: well-established *in vitro* or *in vivo* functional studies show no damaging effect on protein function or splicing; (ii) BP1: missense variant in a gene for which primary truncating variants are known to cause disease; (iii) BP2: observed in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in *cis* with a pathogenic variant in any inheritance pattern; (iv) BP4: multiple lines of computational evidence suggest no impact on gene or gene product). Therefore, according to this scoring rules, variants c.2114C > G (p.Ala705Gly) and c.1708C > T (p.Leu570Phe) should be classified as “Likely Benign”.

Next, one pathogenicity strong criterion (PS3) and one pathogenicity moderate criterion (PM2, PM4 and PM5) were present for variant c.1981C > G (p.Pro661Ala) (Evidence of pathogenicity: (i) PS3: Strong: Well-established *in vitro* or *in vivo* functional studies supporting of damaging effect on the gene or gene product; (ii) PM2: Moderate: Absent from controls in Exome Sequencing Project, 1000 Genome Project and or Exome Aggregation Consortium; (iii) PM5: Moderate: Novel missense change at amino acid residue where at different missense change determined to be pathogenic has been seen before; (iv) PP4: Supporting: Patient's phenotype or family history is highly specific with the disease with a single genetic aetiology). Thus, variant c.1981C > G (p.Pro661Ala) should be classified as “Likely Pathogenic”.

Finally, one pathogenicity strong criterion (PS3), 2 pathogenicity moderate criteria (PM2 and PM5) and 2 pathogenicity supporting criteria (PP3 and PP4) were present in variants c.640T > C (p.Trp214Arg), c.1736A > T (Asp579Val) and c.428G > T (Cys143Phe) (Evidence of pathogenicity: (i) PS3:Strong: Strong: well-established *in vitro* or *in vivo* functional studies supporting of damaging effect on the gene or gene product; (ii) PM2: Moderate: absent from controls in Exome Sequencing Project, 1000 Genome Project and or Exome Aggregation Consortium; (iii) PM5: Moderate: novel missense change at amino acid residue where at different missense change determined to be pathogenic has been seen before; (iv) PP3: Supporting: multiple lines of computational evidence support a deleterious effect on the gene product; (v) PP4: Supporting: patient's phenotype or family history is highly specific with the disease with a single genetic aetiology). Thus, variants c.640T > C (p.Trp214Arg), c.1736A > T (Asp579Val) and c.428G > T (Cys143Phe) should be classified as “Pathogenic”.

3.2.4. Comparison of functional, predictive and multi-species analyses, and ACMG classification

In an attempt to summarize our findings we allocated the six variants into three *ad hoc* groups: high, medium and low activity.

First, in the “high activity” group we included variants c.2114C > G p.(Ala705Gly) and c.1708C > T p.(Leu570Phe), which displayed normal to slightly reduced (≈75%) activity *in vitro*. Both of them appeared to be tolerated positions according to the multi-species comparison, while the results of the *in silico* analyses were somehow unclear. Based on the results of the functional studies performed, it appeared that the clinical features of the patients cannot be attributed to an impaired LDLR function caused by the solely presence of these variants. Furthermore, Patient 2 who carries the c.1708C > T p.(Leu570Phe) also carries another variant that has been previously associated with hypercholesterolemia (c.2029 T > C variant p.(Cys677Arg), HGMD (CM920466) [11], (Table 1), and which might probably be responsible for her clinical phenotype.

Next, variants c.1981 C > G p.(Pro661Ala) and c.640 T > C p.(Trp214Arg) showed about 50% of the wild-type receptor activity and fall in the “medium activity” group. The *in silico* predicted function for these variants were again inconsistent among the different algorithms while the multi-species analyses showed a conserved site in both cases. Regarding c.1981 C > G, a variant involving the same codon but introducing a different amino acidic change (c.1982 C > T p.(Pro661Leu)) had been reported in HGMD (CM973629) in association with hypercholesterolemia [12]. Similarly, the c.641G > C and c.642G > A variants affecting the same codon as in c.640 T > C p.(Trp214Arg) but introducing the p.(Trp214Ser) and p.(Trp214*) amino acidic changes, have also been reported in HGMD (CM107985 and CM993942) [35,36].

Lastly, In the “low activity” group we allocated variants c.1736 A > T p.(Asp579Val) and c.428G > T p.(Cys143Phe), which exhibited less than 20% and 10% of the normal LDLR activity respectively. These variants were also predicted as pathogenic by the *in silico* analysis and the phylogenetic analysis showed them as two highly conserved positions. Furthermore, despite none of them have been found in the searched databases, different substitutions affecting the same codons were previously associated with hypercholesterolemia: c.1735G > A (p.Asp579Asn), 1735G > T (p.Asp579Tyr), c.1736A > C (p.Asp579Ala), c.1736A > G (p.Asp579Gly) (CM920463, CM108008, CM066112, and CM002353) [11,35,37,38]; c.427C > T (p.Cys143Arg), c.427C > G (p.Cys143Gly), c.428G > A (p.Cys142Tyr), c.428G > C (p.Cys143Ser) and c.429C > A (p.Cys143*) (CM012390, CM087511, CM012777, CM005340, CM970876) [39–42]. Thus, we found these two variants as strong evidence supporting the development of the clinical features of the two patients who carry them.

The five patients carrying the six variants analysed, presented different levels of hypercholesterolemia (Table 1) as well as partially

overlapping clinical conditions and familial history of cardiovascular disease. Within this framework, it was not possible to establish a direct correlation between the severity of a particular phenotype and the effect on LDLR function. Nevertheless, it could be hypothesized that the solely presence of variants with low and medium effect on the LDLR activity, might not be enough to cause their pathological condition. Other genetic or epigenetic factors should be present to explain the phenotype. On the other hand, since it was demonstrated here, variants c.1736 A > T p.(Asp579Val) and c.428G > T p.(Cys143Phe) strongly affected the LDLR activity *in vitro*, revealing a relevant role in the development of the hypercholesterolemia.

3.2.5. Correlation analysis

The correlation analysis for the LDLR activity did not show a significant association with LDL cholesterol ($R = 0.48$, $p > 0.05$), HDL cholesterol ($R = -0.1$, $p > 0.05$), total cholesterol ($R = 0.75$, $p > 0.05$), triglycerides ($R = 0.72$, $p > 0.05$), lipoprotein (a) ($R = 0.57$, $p > 0.05$) or DLCN score ($R = 0.07$, $p > 0.05$).

4. Discussion

We carried out an *in vitro* analysis on mutated *LDLR* through the evaluation of expression of six *LDLR* variants and their performance for the uptake and degradation of ^{125}I-LDL in CHO-*ldlA7* cells in order to explore the effect on the protein receptor function. We also performed *in silico* analyses to assess the correlation between functional and bioinformatics studies.

Based on the functional studies performed in this work we provided evidence on the effect of six uncharacterised variants in the *LDLR* that can be used to set their clinical relevance. According to ACMG standards and the available information, variants p.(Ala705Gly) and p.(Leu570Phe) could be classified as “Likely Benign”, p.(Asp579Val), p.(Cys143Phe) and p.(Trp214Arg) as “Pathogenic” variants, and variant p.(Pro661Ala) as “Likely Pathogenic”.

We observed a connection between the *in vitro* activities of LDLR carrying these variants and multi-species comparison results. However, concordance with the results of *in silico* predictive algorithms was not always consistent, mainly due to ambiguous expected effects.

Future familial genetic analyses in order to investigate the co-segregation of these variants with the disease will help us to confirm their actual status.

Our results reinforce the clinical importance of functional studies in the confirmation of mutations on *LDLR* to explain the phenotype of patients with FH.

Author contributions

C.R and P.A perfomed the functional studies. All of the authors analysed the results and contributed in writing the manuscript.

Declaration of competing interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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