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The Arg499His gain-of-function mutation in the C-terminal domain of *PCSK9*

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HIGHLIGHTS

- We identified a novel *PCSK9* GOF variant, p.(Arg499His), in two unrelated FH patients from Spain and Italy.
- p.(Arg499His) PCSK9 variant carriers show high LDL-C concentrations and FH phenotype.
- *In vitro* assays revealed reduced LDL receptor expression at membrane surface with p.(Arg499His).
- p.(Arg499His) is a GOF mutation that causes FH through an intracellular effect reducing LDLr availability.

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ABSTRACT

Background and aims: Familial hypercholesterolemia (FH) is a monogenic disease characterized by high levels of low-density lipoprotein cholesterol and premature atherosclerotic cardiovascular disease. FH is caused by loss of function mutations in genes encoding LDL receptor (*LDLR*), and Apolipoprotein B (*APOB*) or gain of function (GOF) mutations in proprotein convertase subtilisin/kexin type 9 (*PCSK9*). In this study, we identified a novel variant in *PCSK9*, p.(Arg499His), located in the C-terminal domain, in two unrelated FH patients from Spain and Italy.

Methods: We studied familial segregation and determined variant activity *in vitro*.

Results: We determined *PCSK9* expression, secretion and activity of the variant in transfected HEK293 cells; extracellular activity of the recombinant p.(Arg499His) *PCSK9* variant in HEK 293 and HepG2 cells; PCSK9 affinity to the LDL receptor at neutral and acidic pH; the mechanism of action of the p.(Arg499His) PCSK9 variant by co-transfection with a soluble construct of the LDL receptor and by determining total PCSK9 intracellular accumulation when endosomal acidification is impaired and when an excess of soluble LDLr is present in the culture medium. Our results show high LDL-C concentrations and FH phenotype in p.(Arg499His) carriers. *In vitro* functional characterization shows that p.(Arg499His) PCSK9 variant causes a reduction in LDLr expression and LDL uptake. An intracellular activity for this variant is also shown when blocking the activity of secreted PCSK9 and by inhibiting endosomal acidification.

Abbreviations: GOF, gain of function; MLPA, Multiplex Ligation-dependent Probe Amplification; LOF, loss of function; HGVS, Human Genome Variation Society; LOVD, Leiden Open Variation Database; ExAC, Exome Aggregation Consortium; gnomAD, Genome Aggregation Database; EVS, Exome Variant Server

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Conclusions: We demonstrated that p.(Arg499His) PCSK9 variant causes a direct intracellular degradation of LDLr therefore causing FH by reducing LDLr availability.

1. Introduction

Low-density lipoprotein cholesterol (LDL-C) is removed from circulation through the LDL receptor (LDLr). When an LDL particle binds its receptor, the LDL-LDLr complex is internalized and LDLr is mostly recycled to the surface or, less frequently, degraded in the lysosomes [1]. LDLr degradation is promoted by the proprotein convertase subtilisin-kexin type 9 (PCSK9) [2].

PCSK9 belongs to a family of 9 subtilisin-like serine proteases which are involved in the proteolytic maturation of different proteins like hormones and cytokines [3]. PCSK9 is a key player in plasma cholesterol metabolism that regulates LDLr levels by increasing LDLr degradation [2]. PCSK9 is a 692-amino acid glycoprotein, synthesized as a 72 kDa soluble zymogen (proPCSK9), which contains a signal peptide, an N-terminal peptide and a prodomain region, followed by a catalytic domain with the catalytic triad of serine proteases, aspartate (D), histidine (H) and serine (S), and a cysteine/histidine-rich-C-terminal domain (CTD). The proPCSK9 undergoes an autocatalytic process at the N-terminal domain, releasing a 14 kDa peptide which remains attached to the mature protein, inactivating the catalytic domain [4].

Genetic variation in *PCSK9* has an enormous impact on LDL-C concentration in humans and both gain-of-function (GOF) and loss-of-function (LOF) *PCSK9* mutations have been described [5,6]. While *PCSK9* LOF mutations cause hypocholesterolemia, GOF mutations are a rare cause of familial hypercholesterolemia (FH) [5], a monogenic disease characterized by very high levels of LDL-C and premature atherosclerotic cardiovascular disease (ASCVD) [1]. LOF mutations in the *LDLR* gene encoding LDLr or in *APOB*, are the most frequent causes of FH, with more than 1700 pathogenic variants reported [7]. GOF mutations in the *PCSK9* gene are a minor cause of FH, representing less than 1% of cases, with approximately 30 variants described so far [8]. *PCSK9* GOF mutations are causative of FH, because the enhancement in *PCSK9* function leads to increased LDLr degradation and reduced recycling to the cell surface. As a consequence, there is a reduction in LDL uptake and an increase in circulating LDL-C concentration [9].

PCSK9 GOF mutations are usually missense defects, located in any exon, except exon 3 [8]. Functional studies on human GOF variants show different mechanism to achieve the enhanced degradation of LDLr. Some mutations affecting the prodomain region result in an increase in internal and external LDLr degradation [10,11]. Variants that affect catalytic domain and prodomain share very similar mechanisms and effects [12]. The best characterized PCSK9 GOF mutation is p.(Asp374Tyr) which produces a ten-fold increase in LDLr degradation [13] by increasing the binding affinity of PCSK9 to the epidermal growth factor-like domain of LDLr [14,15]. This variant was demonstrated to inhibit LDL uptake still at a concentration 25 times lower than the wild-type PCSK9 [16]. The majority of GOF characterized variants located at catalytic domain present complete or partial resistance to furin processing (p.(Arg215His), p.(Arg218Ser), p.(Phe216Leu) and p.(Asp374Tyr)) resulting in higher amount of active PCSK9 molecules [17–20]. It has been shown that GOF activity of p.(Asp374His) is caused by a increased binding affinity to LDLr, but to a slightly lesser extent compared to p.(Asp374Tyr) [21]. There are other variants, such as p.(Arg237Trp), with similar processing and secretion rates compared to WT which has an unclear mechanism of action [4]. Among LOF variants within the catalytic domain, there have been several reported mechanisms of impaired function. Hence, p.(Gln152His), p.(Leu253Phe) and p.(Asn354Leu) show abolished or reduced autocatalytic cleavage [19,22,23], while LOF activities of p.(Asn157Lys) and p.(Gly236Ser) may be determined due to protein structure misfolding [14,19]. Although variants that affect the catalytic domain and prodomain have been studied, their effects are less known [24]. Furthermore, conflicting data has been published on how most GOF mutations can influence the biology of the LDLr, and the information about effects of variants in the CTD would be very useful to fully elucidate the pathophysiology of LDLr-PCSK9 interactions.

For new variants detected by genetic screening, it is important to assess their role in the disease. For this purpose, bioinformatic tools are a good approximation [25–27], but functional characterization by *in vitro* studies has proved to be the best method to provide evidence of the pathogenic role of the identified variants [28–30].

After identifying a novel p.(Arg499His) *PCSK9* variant in two unrelated FH patients from Spain and Italy, we characterized the GOF activity resulting in high LDL-C in mutation carriers, through familial studies and *in vitro* assays.

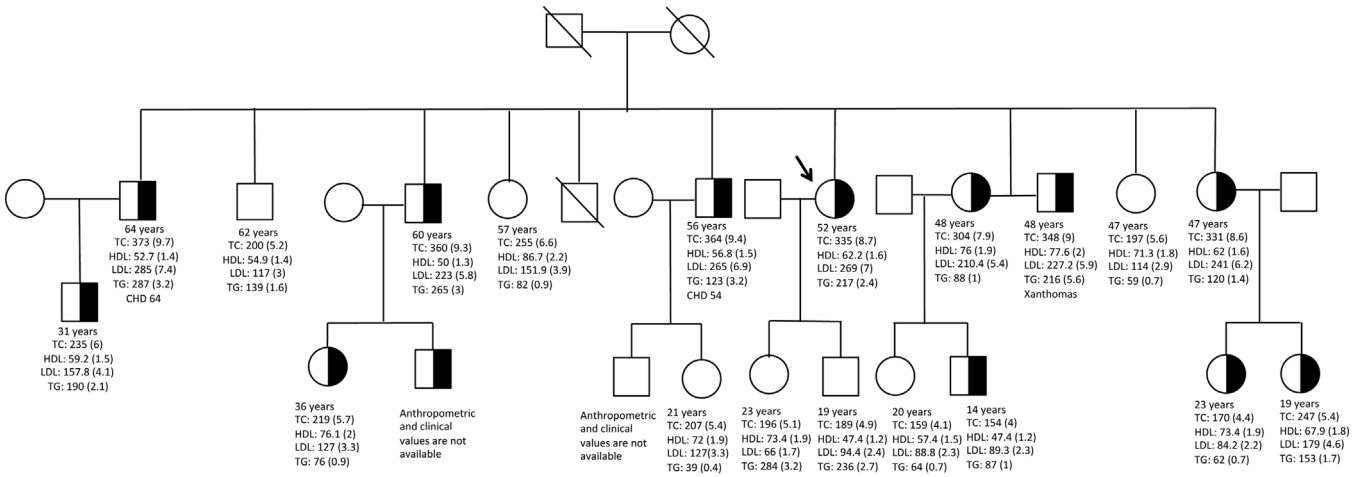

Fig. 1. Case 1 family tree showing the carriers of p.(Arg499His) *PCSK9* variant.
Half-blackened indicates carriers of the p.(Arg499His) variant. The crossed line indicates deceased patients. The arrow represents the proband. Age (in years) at lipid measurement, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG) in mg/dL and in (mmol/L) measured in the absence of lipid-lowering therapy, and history and age of onset of coronary heart disease (CHD) are given.

2. Materials and methods

2.1. Patients

Case 1. Fifty-one-year-old female native of Gran Canaria Island (Spain) with known hypercholesterolemia since the age of 36. Her untreated lipid levels were: total cholesterol 375 mg/dL (9.7 mmol/L), LDL-C 270 mg/dL (6.98 mmol/L), HDL-C 62 mg/dL (1.6 mmol/L), triglycerides 217 mg/dL (2.45 mmol/L). She had 11 siblings, one deceased in an accident and 8 with hypercholesterolemia (Fig. 1). Two brothers had suffered a myocardial infarction, at the age of 54 and 64, respectively. Her father had died of myocardial infarction at the age of 60. On physical examination, she had a body mass index (BMI) of 27.1 kg/m^2, corneal arcus, Achilles tendon xanthomas and she scored 14 points according to the Dutch Lipid Clinic Network diagnosis criteria for FH [31]. Hence, a clinical diagnosis of definite heterozygous FH was made.

Case 2. Nine year old female native from Naples with hypercholesterolemia with untreated total cholesterol 240 mg/dL (6.2 mmol/L), LDL-C 167 mg/dL (4.32 mmol/L), HDL-C 60 mg/dL (1.6 mmol/L), triglyceride 64 mg/dL (0.72 mmol/L), family history of hypercholesterolemia and premature cardiovascular disease, vertical transmission in the family of high LDL-C and a clinical diagnosis of probable heterozygous FH.

Family study. A total of 20 family members of Case 1 were studied, including their medical personal history, current treatments, cardiovascular risk factors, physical exam for the presence of xanthomata and corneal arcus, fasting blood sampling for lipid profile and DNA extraction. No family study was possible for Case 2.

Case 1 and Case 2's legal representatives and all family members signed written, informed consent for FH genetic analysis according to a protocol previously approved by the corresponding Ethical boards of our Institutions. This work has been carried out in accordance with the Declaration of Helsinki for experiments involving humans.

2.2. Genetic analysis

Genomic DNA was extracted from whole blood samples by using standard methods. Genetic screening for the presence of FH causative mutations in *LDLR* and *APOB* was carried out by Lipochip® platform (Progenika Biopharma SA A Grifols Company, Derio, Vizcaya, Spain) for Case 1 and by PCR amplification of the promoter, exons and exon-intron junctions, followed by direct sequencing, as previously described, for Case 2 [32,33]. Since no mutations were detected, Multiplex Ligation-dependent Probe Amplification (MLPA) was performed as previously reported [33] to search for large rearrangements in the *LDLR* gene. Molecular analysis of *PCSK9* included the amplification and direct sequencing of the promoter, exons and exon-intron junctions in both cases [32].

The coding region containing the mutation p.(Arg499His) in the *PCSK9* gene (NM_174936) was amplified by PCR and sequenced for all family members of Case 1.

DNA sequences were analysed using VariantReporter™ software (Applied Biosystems, Thermo Fisher Scientific, CA, USA) or CodonCode Aligner (CodonCode Corporation, MA, USA). The Human Genome Variation Society (HGVS) recommendations (http://varnomen.hgvs.org/) were used for variant nomenclature. The Human Gene Mutation Database (HGMD) and Leiden Open Variation Database (LOVD) 3.0 were consulted as mutation databases. To evaluate the Minor Allele Frequency (MAF), the following variant databases were consulted: dbSNP 149 (NCBI), Exome Aggregation Consortium (ExAC), genome Aggregation Database (gnomAD), Exome Variant Server (EVS) and 1000 genomes (1 kG).

2.3. Functional study

2.3.1. Site-directed mutagenesis and cloning

Plasmids carrying *PCSK9* variants were constructed by Innoprot (Derio, Spain). Briefly, variants were introduced into the human *PCSK9* cDNA (NM_174936.3), in the mammalian expression vector WT-PCSK9 plasmid (pCMV-PCSK9-FLAG) kindly provided by Prof. Horton [34], by oligonucleotide site-directed mutagenesis, using the QuickChange Lightning mutagenesis kit (Agilent Technologies Inc., CA, USA) according to the manufacturer's instructions. A 6x His tag was introduced to allow purification with no effects on PCSK9 activity. Restriction enzyme digestion of the appropriate fragments and the integrity of the remaining *PCSK9* cDNA sequences of all constructs were verified by direct sequence analysis.

2.3.2. PCSK9 expression in transfected HEK293 cells

A total of 5×10^5 HEK293 cells were transfected with 1 μg of a plasmid encoding WT-*PCSK9* and p.(Asp374Tyr) or p.(Arg499His) *PCSK9*-variants with Lipofectamine® LTX & Plus™ Reagent (Invitrogen, Thermo Fisher Scientific, CA, USA). Twenty-four hours post-transfection, cells were washed and then incubated with fresh DMEM medium containing 10% FBS, 2 mM L-glutamine and antibiotics (100 units/mL penicillin; 100 μg/mL streptomycin) (complete medium) for an additional 24 h. Next, cells were lysed to analyse intracellular PCSK9 by Western blot as described below.

2.3.3. PCSK9 quantification

PCSK9 concentration from patient's plasma and that secreted by HEK293 transfected cells to the extracellular media was determined by solid-phase immunoassay as described below. Additionally, binding of WT and p.(Arg499His) PCSK9 variant to LDL was determined following the same methodology, changing the ED-LDLr covering by LDL. For quantification, recombinant WT PCSK9 was used as standard, assayed in parallel in the same conditions, and the concentration was deduced from the calibration curve.

2.3.4. qRT-PCR PCSK9 quantification

RNA from HEK293 transfected with 1 μg of a plasmid encoding WT-*PCSK9* and p.(Asp374Tyr) or p.(Arg499His) *PCSK9*-variants was harvested from cells using TRIzol™ Reagent (Invitrogen, Thermo Fisher Scientific, CA, USA), and cDNA was synthesized from 40 ng of RNA using One Step SYBR® Primescript™ RT-PCR kit (Perfect Real Time) (Takara Bio Inc., Japan) according to the manufacturer's instructions. Reactions were performed in the BioRad C1000™ Cycling Platform. The real-time PCR program consisted of 40 cycles (95 °C for 15 s and 60 °C for 1 min) after initial 10 min incubation at 95 °C. In order to avoid differences in PCSK9 mRNA levels due to transfection efficiency among the independent experiments, the amount of secreted PCSK9 was adjusted using the ΔΔCT method. Therefore, PCSK9 mRNA levels were normalized in each independent assay to GAPDH as a reference gene. The primers used for PCSK9 were: forward 5′-AGGGGAGGACATCATT GGTG-3′ and reverse 5′-CAGGTTGGGGGTCAGTACC-3'. Those for GAPDH were: forward 5′-GGAGCGAGATCCCTCCAAAAT-3′ and reverse 5′-GGCTGTTGTCATACTTCTCATGG-3'.

2.3.5. PCSK9 purification from stably transfected HEK293 cells

HEK293 cells grown to sub-confluence were transfected with the different PCSK9 plasmids and selected with geneticin (G418 sulphate) (Gibco, Thermo Fisher Scientific, CA, USA) according to the manufacturer's instructions to obtain stably transfected cells. For PCSK9 purification, stably transfected HEK293 cells were grown at 80% confluence in complete DMEM medium. Then, the culture medium was replaced by Opti-MEM (Invitrogen, Thermo Fisher Scientific, CA, USA) without geneticin and cells were maintained under these conditions for 48 h. Finally, the medium was harvested and PCSK9 was purified using one-step nickel affinity chromatography. Purified PCSK9 variants were

stored at −80 °C in 50 mM Tris-HCl buffer supplemented with 150 mM NaCl and 10% glycerol, pH 8.0.

2.3.6. Lipoprotein labelling with fluorescein isothiocyanate

LDL was purified from blood plasma by centrifugation, at 12,000 × *g* at 4 °C for 19 h. LDL (1.019–1.050 g/mL) was isolated through isopicnic ultracentrifugation by adjusting plasma density to 1.21 g/mL by the addition of KBr.

LDL particles were labelled with fluorescein isothiocyanate (FITC) as previously described [35]. Briefly, 10 μL of FITC (2 mg/mL) were added to 1 mL LDL (1 mg/mL apoB) in 0.1 M $NaHCO_3$, pH 9.0, and mixed for 2 h by slow rocking at room temperature. The unreacted dye was removed by gel filtration on a Sephadex G-25 column equilibrated with PBS EDTA-free buffer. All fractions were assayed for protein content using bovine serum albumin as standard (Pierce BCA protein assay; Pierce, Thermo Fisher Scientific, CA, USA).

2.3.7. Quantification of LDL uptake by flow cytometry

LDLr expression and LDL uptake were assessed in HEK293 cells 48 h after transfection with the plasmids containing WT or p.(Arg499His) *PCSK9*-variant. To determine LDLr cell surface expression by fluorescence-activated cell sorter (FACS), cells were incubated with a mouse anti-LDLr primary antibody (clone IgG7; 1:100, 2.5 mg/L) (Cat. No.: 61087; Progen Biotechnik GmbH, Germany) for 1 h, at room temperature, then washed twice with PBS-1%BSA and incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (1:100) (Cat. No.: A11001; Molecular Probes, Thermo Fisher Scientific, CA, USA). For LDL uptake analysis, HEK293 cells were incubated for 4 h, at 37 °C with 20 μg/mL FITC-LDL and lipoprotein uptake was determined as previously described [35]. In addition, LDL uptake was determined by incubating purified WT or p.(Arg499His) *PCSK9* variant on HepG2 and HEK293 cells. Briefly, 2 μg/mL of each purified *PCSK9* variant were added to the cell culture medium and 2 h post-addition, 20 μg/mL FITC-LDL were added to the medium; thereafter the LDL uptake was determined 4 h later by FACS. In both experimental approaches, after incubation with FITC-LDL, cells were washed twice in PBS-1%BSA, fixed on 4% formaldehyde for 10 min and washed again twice with PBS-1%BSA. The amount of internalized LDL was determined as described before [35] by adding Trypan blue solution (Sigma-Aldrich, Merck, Germany) to a final concentration of 0.2%. Fluorescence intensities were measured in a FACSCalibur™ (BD Bioscience, NJ, USA) flow cytometer as previously described [35]. For each sample, fluorescence of 10,000 events was acquired for data analysis. All measurements have been performed at least in triplicate. In all assays, cells transfected with the known p.(Asp374Tyr) GOF *PCSK9* variant or treated with the recombinant purified p.(Asp374Tyr) PCSK9 were used as internal positive controls of the assay.

2.3.8. LDLr-ectodomain production and purification

The LDLr construct encoding the N-terminal extracellular ectodomain (ED-LDLr, corresponding to 1–789 amino acids) plus c-myc and His tags was purified by affinity chromatography from cells transfected with the pcDNA3.1-EC-*LDLR*-His plasmid, kindly provided by Prof. Leren [36]. Briefly, HEK293 cells at 70–80% confluence were transfected with the plasmid by calcium phosphate method for 24–48 h and selected in successive passages by geneticin (G-418 sulphate; Gibco, Thermo Fisher Scientific, CA, USA). For ED-LDLr expression and purification, the culture medium of positively transfected cells was changed to Opti-MEM (Invitrogen, Thermo Fisher Scientific, CA, USA) without geneticin and maintained under these conditions for three additional days. Then the medium was harvested, supplemented with protease inhibitors (cOmplete™ EDTA-free; Roche, Merck, Germany) and the ED-LDLr was affinity purified using one-step nickel affinity chromatography. For protein long-term maintenance, the buffer was changed to storage buffer (50 mM Tris-HCl, 50 mM NaCl, 10% glycerol, and 0.01% Brij-35, pH 7.5) [37] and frozen to −80 °C.

2.3.9. Solid-phase immunoassay for PCSK9-LDLr ectodomain binding

LDLr ectodomain fragments diluted in working buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$) were coated at a fixed concentration onto 96-well microtiter plates by incubation overnight at 4 °C. Plates were then blocked and incubated with a serial dilution of each of the different PCSK9 variants diluted in working buffer (for test at pH 7.4) or in buffer 10 mM Tris-Maleate, 50 mM NaCl, 2 mM $CaCl_2$, pH 5.2, during 2 h at room temperature, and then washed thoroughly with working buffer supplemented with 0.1% (w/v) Tween 20 (Sigma-Aldrich, Merck, Germany). For ligand detection, the antibodies, rat monoclonal anti-DYKDDDDK tag (clone L5) (Cat. No.: MA1-142; Thermo Fisher Scientific, CA, USA) and peroxidase-conjugated goat anti-rat (Cat. No.: 7077S; Cell Signalling Technology® Inc., MA, USA) were diluted in working buffer supplemented with 5% (w/v) BSA, applied directly to the plate and incubated for 1 h at room temperature, with an extensive washing between both incubations. After a final wash, antibody binding was determined using 50 μL per well of 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) substrate solution (Sigma-Aldrich, Merck, Germany) and measuring colour change at 405 nm. The time course for colour development was essentially linear and measurements were taken 30–60 min after the addition of substrate. For data processing, all absorbance values were corrected for unspecific binding, relativized to maximum absorbance and EC_{50} values were extracted from curves after fitting the data to 5-parameter logistic (5-PL) equation (SigmaPlot 13.0, Systat Software Inc., CA, USA).

2.3.10. Analysis of intracellular activity of PCSK9 by inhibiting endosomal acidification or blocking extracellular activity by ED-LDLr

To determine the mechanism of action of p.(Arg499His) *PCSK9* variant, total amount of LDLr was determined by flow cytometry in the presence of inhibitors of endosomal acidification (NH_4Cl and Bafilomycin A1, BFA1) or by addition of an excess of ED-LDLr into the culture media in order to block extracellular PCSK9 activity. Endosome acidification inhibition would prevent PCSK9-induced degradation of LDLr (both by its extracellular activity or by a intracellular activity that directly increases LDLr turnover by directing the complex to the lysosomes). This in turn, will lead to an accumulation of LDLr in early endosomes. On the other hand, any intracellular activity of PCSK9 can be studied by blocking extracellularly PCSK9 with ED-LDLr. Therefore and in order to determine total amount of LDLr (intracellular and plasma membrane), HEK293 cells were co-transfected with a plasmid containing enhanced green fluorescent protein (EGFP) tagged at the WT LDLr N-terminus and with the plasmids containing WT, p.(Asp374Tyr) or p.(Arg499His) PCSK9 variants. 36 h post-transfection, cells were washed and fresh culture media containing NH_4Cl (10 mM), BFA1 (50 nM) or ED-LDLr (5 μg/mL) (all final concentrations) was added and cells further incubated for 12 h. Then, fluorescence of EGFP-LDLr was determined by flow cytometry. Cell cultures, transfections and flow cytometry assays were performed as described above. For each sample, fluorescence of 10,000 events was acquired for data analysis. All measurements were performed at least in triplicate.

2.3.11. Analysis of p.(Arg499His) PCSK9 variant on the secretion of the ED-LDLr

pcDNA3.1-EC-*LDLR*-His plasmid was used to analyse the effect of p.(Arg499His) on intracellular LDLr trafficking. HEK293 cells stably transfected with the WT or p.(Arg499His) *PCSK9* variant were transiently co-transfected with pcDNA3.1-EC-*LDLR*-His using the calcium phosphate method. Forty-eight hours after transfection, HEK293 cells were washed with PBS and the medium was replaced with OptiMEM (Gibco, Thermo Fisher Scientific, CA, USA) supplemented with penicillin (50 units/mL) and streptomycin (50 μg/mL) for additional 24 h incubation. The medium was collected and cell debris was removed by centrifugation.

Western blot analysis of intracellular and secreted PCSK9 and ED-LDLr secretion in the presence of PCSK9.

PCSK9 expression and secretion analysis in HEK293 cells transfected with the different PCSK9 variants was performed in cell lysates and culture media by Western blotting. For that purpose, proteins from cell lysates or the supernatants were resolved by 8.5% Tris-Glycine SDS-PAGE. Gels were next blotted onto Nitrocellulose membranes (Protran BA 83, Whatman™, GE Healthcare, Germany), blocked for 1 h in TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk and immunoblotted with a rabbit polyclonal anti-human PCSK9 antibody (1:1000) (Cat. No: 10240; Cayman Chemical Company, MI, USA) for 16 h at 4 °C. Then, they were counterstained with a horseradish peroxidase-conjugated anti-rabbit antibody (Cat.No: 7074S; Cell Signalling Technology® Inc., MA, USA).

For ED-LDLr detection membranes were immunostained with a mouse monoclonal anti-c-Myc antibody (clone 9E10) (Cat. No.: MA1-980; Invitrogen, Thermo Fisher Scientific, CA, USA). A rabbit polyclonal IgG anti-GAPDH antibody (1:1000) (Cat. No: sc-26778; Santa Cruz Biotechnology Inc., CA, USA) was used to check protein loading and to normalize the extent of protein expression.

The signal was developed using SuperSignal West Dura Extended Substrate (Pierce, Thermo Fisher Scientific, CA, USA). ChemiDoc XRS (Bio-Rad, CA, USA) was used to detect the signals. The concentrations of the antibodies were optimized to achieve low background and a linear dose-dependent increase in signal intensity. Quantification in all cases was performed relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using NHI ImageJ software (https://rsbweb.nih.gob/ij/).

2.4. Statistical analysis

Data are presented as mean ± SD or means (interquartile range) for continuous variables. Experimental data presented in graphs are reported as percentage relative to the control. Flow cytometry and solid-phase immunoassays were performed independently 3 times and all measurements were performed in triplicate. Western blots from 3 independent experiments were quantified by densitometry to determine statistical significance. For comparisons between groups the T-test was used. Statistical significance was established at a p value < 0.05.

3. Results

The initial genetic screening of the probands found no pathogenic mutations in *LDLR* or *APOB*. *PCSK9* sequencing identified the c.1496G > A, p.(Arg499His) variant in both Cases 1 and 2, and in 12 of the 20 family members of Case 1.

To our knowledge, this variant has never been reported before as a cause of FH and was absent in the HGMD and LOVD mutation databases. It has been found at very low frequencies in GnomAD (MAF of 0.002%) and ExAC (MAF of 0.0047%), and was absent in EVS and 1000 genomes.

3.1. Familial segregation

Family lipid values, presence of ACSVD or xanthomas and *PCSK9* genotype are presented within the family tree (Fig. 1). There was high segregation of the hypercholesterolemia phenotype in p.(Arg499His) sibling carriers. The association of high LDL-C with the mutation was less clear in the second generation of young adults and children. Nevertheless, mean LDL-C in carrier family members was 199 (± 66.4) mg/dL (5.2 ± 1.7 mmol/L) *versus* 108 (± 28.1) mg/dL (2.79 ± 0.73 mmol/L), ($p = 0.003$) in non-carriers. Plasma PCSK9 levels were also determined both in control (n = 7) and cases (n = 13), but no statistically significant differences were observed (control: 0.17 ± 0.06 ng/mL *vs.* case: 0.17 ± 0.08 ng/mL). As the ELISA was performed by determining PCSK9 binding to LDLr, the similar detected amounts of PCSK9 among WT and p.(Arg499His) carriers indicates that the active (non-furin cleaved) PCSK9 levels are similar between control and cases. In addition, assessment of binding capacities of WT and p.(Arg499His) PCSK9 variant to LDL that could affect LDLr degradation as a previously proposed by Kosenko et al. [38] was also performed. Therefore, we determined the binding capacity of purified recombinant WT and p.(Arg499His) PCSK9 to LDL isolated from normolipidemic human plasma *in vitro*. The data obtained by ELISA showed no LDL binding statistical differences between WT and p.(Arg499His) PCSK9 variant (WT: 0.36 ± 0.02 μg/mg LDL *vs.* p.(Arg499His): 0.32 ± 0.03 μg/mg LDL).

3.2. Functional analyses

3.2.1. Extracellular secretion of p.(Arg499His) PCSK9 variant to the culture medium

HEK293 cells were transfected with DNA constructs encoding for WT, p.(Asp374Tyr), and p.(Arg499His) *PCSK9* variants and the effects of those mutations on secretion to the culture medium were analysed by ELISA, as described in the *Methods* section. As an internal control for the method, we used p.(Asp374Tyr) GOF variant because it has been demonstrated that this variant exerts its effect extracellularly by markedly increasing its affinity for LDLr [11,39]. Although similar WT and p.(Arg499His) plasma PCSK9 levels were determined both in control and cases, the amount of p.(Arg499His) PCSK9 variant secreted in the culture medium was lower compared to both WT and p.(Asp374Tyr) variant as determined by ELISA (0–24 h) (Fig. 2). To verify that the lower amount of PCSK9 variant in the culture medium was not dependent from different cellular production of PCSK9, a quantitative real time PCR of *PCSK9* mRNA was performed and revealed no differences among WT, p.(Asp374Tyr) and P.(Arg499His) within each experiment (n = 3, 3 replicate each) (Supplementary Figs. 1A and B). The amount of secreted PCSK9 was corrected using the ΔΔCT method and secreted PCSK9 values were normalized to *PCSK9* mRNA levels in each independent assay.

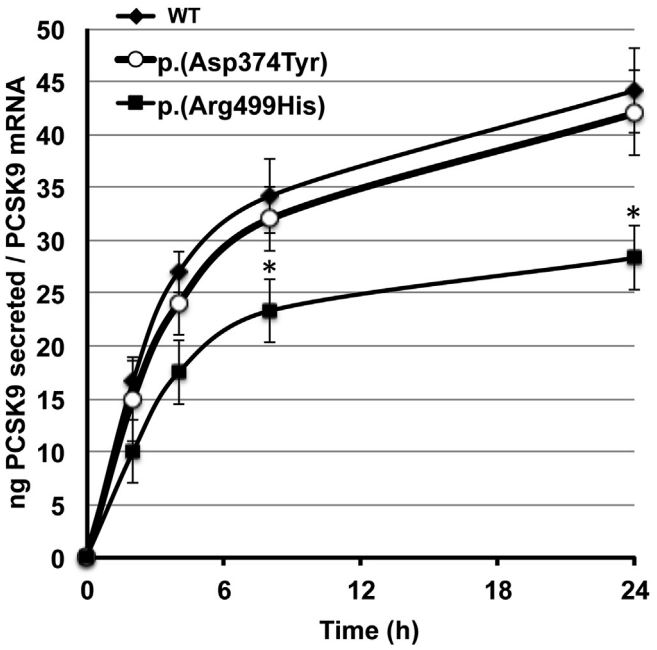

Fig. 2. PCSK9 secretion into the culture medium of WT, p.(Asp374Tyr) and p.(Arg499His) *PCSK9* variants.
HEK293 cells were transiently transfected with plasmid encoding the *PCSK9* variant. PCSK9 secretion to the culture medium was determined by ELISA and relativized to total *PCSK9* mRNA of transfected cells. The values represent the mean of triplicate determinations (n = 3); error bars represent ± SD. *$p < 0.01$ compared to WT PCSK9.

3.2.2. Transfection of HEK293 cells with p.(Arg499His) PCSK9 variant diminishes LDLr expression at the cellular surface and LDL uptake activity

In the first experimental approach to determine the activity of the p.(Arg499His) *PCSK9* variant, HEK293 cells were transiently transfected with WT, p.(Arg499His), or GOF p.(Asp374Tyr) *PCSK9* variants. The LDLr expression at cellular surface and the efficiency of fluorescent LDL uptake by the cells were measured by Western blot and Flow cytometry as described in the Materials and methods section. As shown in Fig. 3A (upper panel), expression of LDLr in the cells transfected with p.(Asp374Tyr) and p.(Arg499His) *PCSK9* variant is reduced when compared

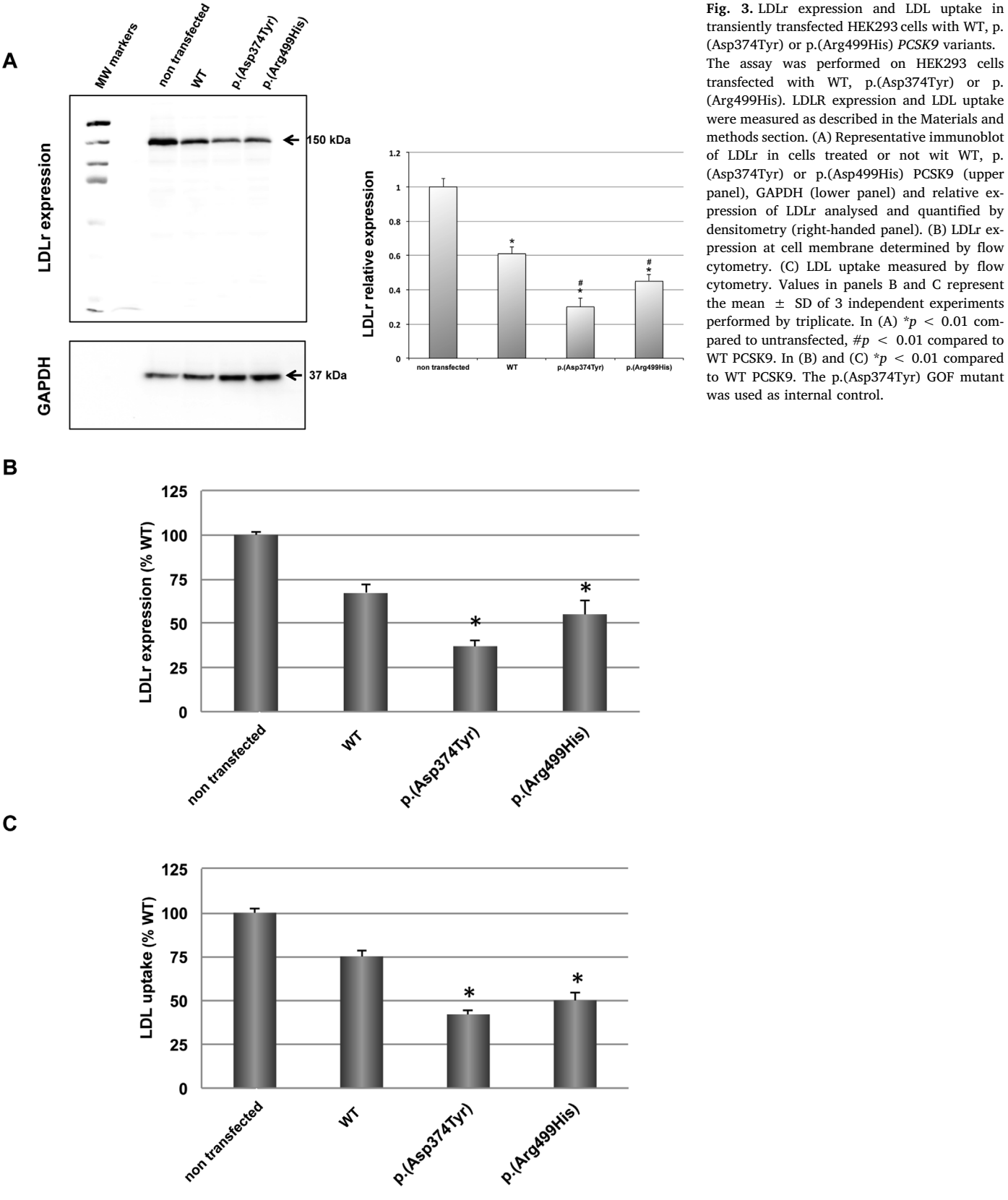

Fig. 3. LDLr expression and LDL uptake in transiently transfected HEK293 cells with WT, p.(Asp374Tyr) or p.(Arg499His) *PCSK9* variants. The assay was performed on HEK293 cells transfected with WT, p.(Asp374Tyr) or p.(Arg499His). LDLR expression and LDL uptake were measured as described in the Materials and methods section. (A) Representative immunoblot of LDLr in cells treated or not wit WT, p.(Asp374Tyr) or p.(Asp499His) PCSK9 (upper panel), GAPDH (lower panel) and relative expression of LDLr analysed and quantified by densitometry (right-handed panel). (B) LDLr expression at cell membrane determined by flow cytometry. (C) LDL uptake measured by flow cytometry. Values in panels B and C represent the mean ± SD of 3 independent experiments performed by triplicate. In (A) $*p < 0.01$ compared to untransfected, $\#p < 0.01$ compared to WT PCSK9. In (B) and (C) $*p < 0.01$ compared to WT PCSK9. The p.(Asp374Tyr) GOF mutant was used as internal control.

to WT PCSK9 as detected by Western blot. Equal loading of protein was confirmed in each blot by membrane stripping and further incubation with antibodies to visualise cytosolic GAPDH protein (Fig. 3A, lower panel). The relative expression of LDLr was analysed and quantified by densitometry (Fig. 3A, right-handed panel). Similar results were obtained when assessing LDLr expression by flow cytometry, as shown in Fig. 3B. LDLr expression was lower in cells transfected with the p.(Arg499His) *PCSK9* variant than in those transfected with WT *PCSK9*. In addition, and as shown in Fig. 3C, LDL uptake was significantly reduced (≈25%) in the presence of p.(Arg499His), compared to WT PCSK9. The p.(Asp374Tyr) variant (used as a GOF control) showed the expected reduction in LDL uptake (as previously reported to be at least 10-fold more active than wild-type PCSK9 and to reduce cell surface LDLr by 36% compared with WT-PCSK9) [11,40] (Fig. 3B). The use of p.(Asp374Tyr) *PCSK9* variant is suitable as a control to analyse the extracellular activity of PCSK9 variants because it has been shown that the GOF effect of p.(Asp374Tyr) variant is mainly due to an extracellular effect as a consequence of its markedly increased affinity for LDLr [11,39].

3.2.3. Recombinant purified p.(Arg499His) PCSK9 variant does not modify LDLr activity compared to WT PCSK9 when added exogenously to HEK293 and HepG2 cells

Extracellular activity of p.(Arg499His) PCSK9 variant was studied by determining FITC-LDL uptake in HEK293 and HepG2 cells treated with 2 μg/mL of purified PCSK9 variants. As shown in Fig. 4A, in HEK293 cells, activity of the p.(Arg499His) *PCSK9* variant was similar to WT PCSK9. The p.(Asp374Tyr) GOF variant caused the expected reduction of LDL uptake already described. Similar results were obtained in HepG2 cells when treated exogenously with purified PCSK9 variants (Fig. 4B).

3.2.4. Purified p.(Arg499His) PCSK9 variant shows a similar affinity for LDLr than WT PCSK9

Next, we tested binding affinity of p.(Arg499His) PCSK9 variant for the LDLr using a solid-phase binding immunoassay and compared them to those of WT PCSK9 and p.(Asp374Tyr) variants. The results at pH 7.4, shown in Table 1, indicate an EC_{50} for WT LDLr of 112.2 ± 16.9 nM, very similar to previously reported values [41]. The affinity value of p.(Arg499His) variant to LDLr was similar to that found for WT PCSK9 (110.6 ± 23.5 nM), whereas the EC_{50} value of p.(Asp374Tyr) GOF variant was lower (19.3 ± 9.5 nM), indicating a higher affinity to the LDLr compared to WT PCSK9 (19.3 nM *vs.* 112.2 nM, respectively) (Table 1). As show in Table 1, affinities of WT and p.(Asp499His) at pH 5.2 were also similar (23.2 ± 3.7 nM *vs* 33.2 ± 3.1 nM) while, as expected, affinity of p.(Asp374Tyr) GOF PCSK9 variant for LDLr was increased (7.4 ± 1.8 nM). These results indicate that the GOF effect of p.(Asp499His) variant is not due to increased affinity for LDLr.

Table 1
EC_{50} values for the binding of PCSK9 variants to LDLr, as determined by solid-phase immunoassay at pH 7.4 and pH 5.2.

	pH 7.4		pH 5.2	
	Mean	± SD	mean	± SD
WT	112.2	16.9	23.2	3.7
p.(Asp374Tyr)	19.3	9.5	7.4	1.8
p.(Arg499His)	110.6	23.5	33.2	3.1

3.2.5. p.(Arg499His) PCSK9 variant drives LDLr to intracellular degradation

In order to gain insight on the p.(Arg499His) PCSK9 mechanism of action, we determined the total amount of LDLr in cells transfected with the variant in the presence of endosomal acidification inhibitors which prevent intracellular LDLr degradation or in the presence of an excess of ED-LDLr in the culture medium thus preventing extracellular PCSK9 activity. Therefore, total amount of LDLr was determined in cells co-transfected with EGFP-LDLr with WT, p.(Asp374Tyr) or p.(Arg499His) in the presence or not of endosomal acidification inhibitors (NH_4Cl or BFA1). In addition, and in order to block extracellular activity of the secreted WT or *PCSK9* GOF variants, co-transfected cells were incubated with an excess of ED-LDLr (5 μg/mL). As control, we incubated cells with NH_4Cl (10 mM) or BFA1 (50 nM) to inhibit the endosomal acidification and we observed that EGFP-LDLr degradation is completely prevented by WT and both PCSK9 variants. However, blocking the extracellular activity of WT, p.(asp374Tyr) and p.(Arg499His) with ED-LDLr only prevented the extracellular activity of WT PCSK9. As shown in Fig. 5A, the presence of an excess of ED-LDLr in the culture medium did not prevent completely the activity of p.(Arg499His) which indicates an additional intracellular mechanism of LDLr driven by this variant.

To confirm this result, LDLr transport to the membrane was studied by co-transfecting stably WT, p.(Asp374Tyr) or p.(Arg499His) transfected HEK293 with ED-LDLr. This assay allows determining effective

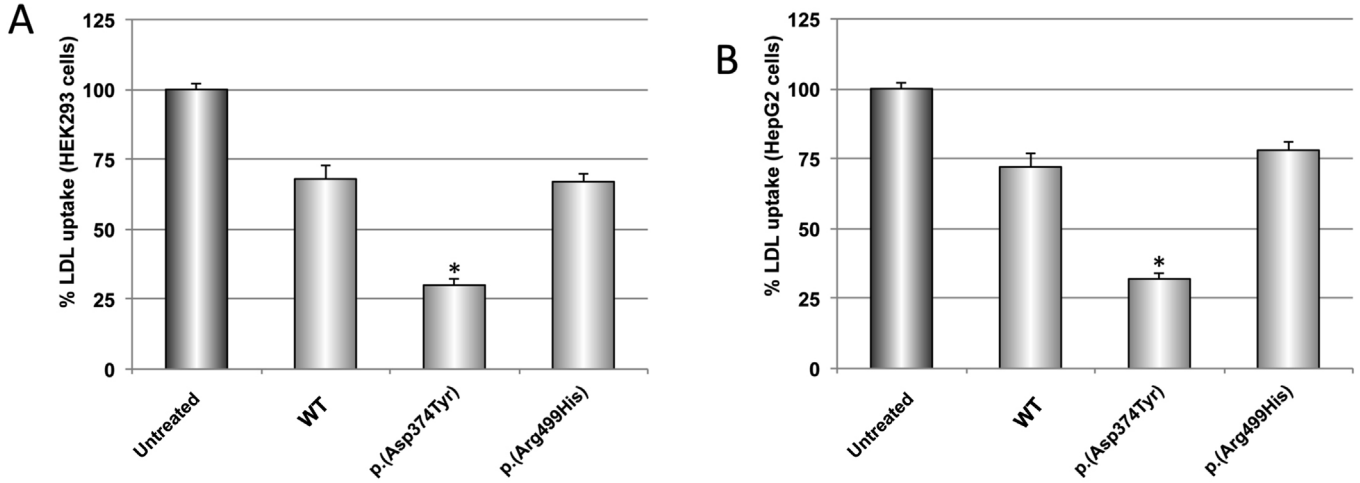

Fig. 4. Effect of purified WT, p.(Asp374Tyr) and p.(Arg499His) PCSK9 variants on LDL uptake.
(A) HEK293 or (B) HepG2 cells were incubated with the purified PCSK9 variants at 2 μg/mL for 2 h prior FITC-LDL addition. LDL internalization was determined after 4 h incubation at 37 °C as described in Materials and methods. Values represent the mean ± SD of 3 independent experiments performed by triplicate. *$p < 0.01$ *versus* WT PCSK9. The p.(Asp374Tyr) GOF mutant was used as internal control.

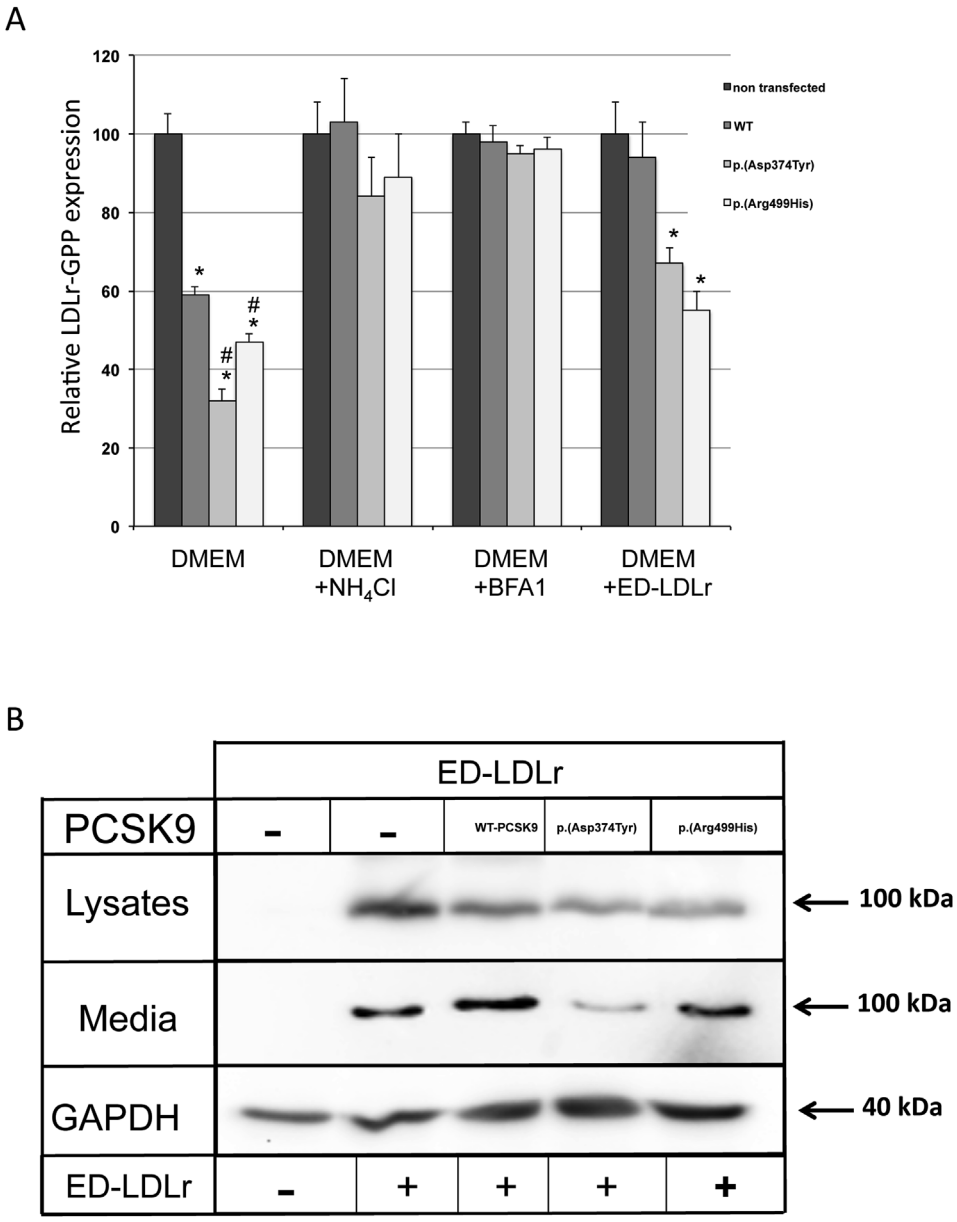

Fig. 5. Intracellular activity of p.(Arg499His). (A) Quantification of total LDLr expression by flow cytometry in the presence of endosomal acidification inhibitors, NH_4Cl (10 mM) or BFA1 (50 nM) or in the presence of an excess of ED-LDLr in the culture media. (B) Effect of p.(Arg499His) *PCSK9* variant on the secretion of LDLr ectodomain (ED-LDLr). Stably *PCSK9* transfected HEK293 cells were transiently co-transfected with ED-LDLr plasmid. The amount of the ED-LDLr was determined in lysates and in media by Western blot analysis. Values in (A) represent the mean ± SD of 3 independent experiments performed by triplicate. $^*p < 0.01$ *versus* untransfected; $\#p < 0.01$ *versus* WT PCSK9. A representative experiment from three independently performed assays is shown in (B).

intracellular anterograde trafficking of ED-LDLr by Western blot analysis of the secreted amount of ED-LDLr to the culture medium. As shown in Fig. 5B, the medium of cells co-transfected with ED-LDLr and p.(Arg499His) *PCSK9* variant contained lower amount of ED-LDLr than those transfected with WT PCSK9 (Fig. 5B) (uncropped blots are shown in Supplementary Fig. 2), corroborating the intracellular effect determined when extracellular activity is blocked by an excess of ED-LDLr (Fig. 5A). Surprisingly, we also detected a lower concentration of EGFP-LDLr when blocking the extracellular activity of p.(Asp374Tyr) and ED-LDLr in the culture media when cells were co-transfected with p.(Asp374Tyr) *PCSK9* variant (Fig. 5A and B). This data indicates that p.(Asp374Tyr) GOF PCSK9 variant could have and additional intracellular activity that needs to be further explored.

4. Discussion

We report the clinical phenotype and the pathogenic mechanism of a novel GOF mutation in the *PCSK9* gene in two unrelated probands from Spain and Italy. This new mutation changes an arginine into histidine in the CTD in the PCSK9 protein, c.1496G > A; p.(Arg499His). The GOF mutations altering the CTD of PCSK9 are very rare and the mechanisms inducing hypercholesterolemia are poorly understood [8]. Our work identifies the CTD of PCSK9 as a key determinant of the intracellular metabolism of the LDLr.

The phenotype of p.(Arg499His) GOF carriers is indistinguishable from FH caused by *LDLR* mutations with almost complete penetrance [42]. Most p.(Arg499His) GOF carriers have LDL-C concentrations in the range of FH secondary to *LDLR* mutations; and some p.(Arg499His) GOF carriers present with ASCVD, corneal arcus and tendon xanthomas in a frequency similar to the *LDLR*-caused FH [43]. The p.(Arg499His) mutation shows co-segregation with the hypercholesterolemic phenotype in the pedigree of Case 1 and the mutation was not found among normocholesterolemic subjects. Carriers of the p.(Arg499His) mutation showed LDL-C concentrations 2-fold higher than non-carriers (199 ± 66.4 mg/dL *versus* 108 ± 28.1 mg/dL, ($p = 0.003$)). The phenotype linked to *PCSK9* GOF mutations showed a large variability in severity depending on the mutation [17,44–47]. In particular, the p.(Asp374Tyr) mutation is associated with a very severe phenotype with very high LDL-C levels, premature coronary heart disease (CHD) and poor response to treatment [46].

Case 2 of our study showed that the mutation is expressed since childhood with high LDL-C concentrations. Some of the young affected patients in Case 1's family also showed elevated LDL-C, although the expression in the p.(Arg499His) carriers was variable, especially in the young members. This variability has also been described for other causes of FH [48]. Conversely, all the adult carriers showed elevated levels of LDL-C. Few reports about children with *PCSK9* GOF mutations have been published so far: they show a variable phenotype, usually less aggressive than *LDLR* LOF mutations [49,50]; an example is the PCSK9 GOF homozygous patient recently described with a mild phenotype [51].

Our study shows that p.(Arg499His) PCSK9 variant is efficiently produced but is secreted less efficiently than WT PCSK9. An extracellular mechanism of action can be excluded since similar affinities were obtained by solid-phase immunoassay and similar LDLr activities were obtained in experiments on cells incubated with purified WT and p.(Arg499His) PCSK9 variants. However, a GOF effect of p.(Arg499His) PCSK9 variant is appreciable in the experiments based on PCSK9 variant transfection. In fact, cells transfected with the p.(Arg499His) *PCSK9* variant showed a LDLr expression and activity significantly lower than those transfected with WT PCSK9. Additionally, when blocking the extracellular activity of PCSK9 a significant decrease of LDLr expression was observed for p.(Arg499His) but not for WT PCSK9. Conversely, the inhibition of endosomal acidification prevents LDLr degradation by both WT and mutant PCSK9. Taken together, these results suggest that an intracellular mechanism of action reducing LDLr availability is responsible of the GOF effect of the p.(Arg499His) PCSK9 variant.

PCSK9 GOF mutations have different mechanisms of action depending on the protein domain involved. The majority of them appear in the prodomain or in the catalytic domain, whereas the CTD GOF variants are less frequent [8,9]. The variants located in the prodomain have a broad mechanism of action; most frequently they increase LDLr degradation by both intracellular and extracellular effects [8,10,21]. Hence, some of them increase intracellular activity and LDLr degradation independently of autocatalytic activity [10], others show reduced autocatalytic cleavage but increased intracellular LDLr degradation [11,21,41] and finally, in some prodomain variants, the extracellular affinity of PCSK9 for LDLr is increased [10,21]. The catalytic domain is involved in the autocatalytic cleavage and the extracellular binding of PCSK9 to the LDLr and, consequently, the mutations located in this domain lead to partial or complete resistance to furin cleavage, increasing PCSK9 activity [46].

Finally, the CTD of PCSK9 has 3 repeat modules called M1, M2, M3 [52], which are necessary to induce PCSK9-mediated degradation of the PCSK9-LDLr complex. The best-characterized function of the PCSK9 CTD is to increase the affinity between PCSK9 and LDLr at the low pH of the endosomes, although this domain does not directly interact with the EGF-A domain of the LDLr [24]. Some variants in CTD increasing external degradation of LDLr are p.(Asn425Ser) and p.(Arg496Trp) [21]. Other variants in the CTD could lead to misfolding of PCSK9 and generate more stable extracellular unions [52]. Other intracellular mechanisms of the interaction between PCSK9 and LDLr have been described [53], and a possible role of the CTD for proper intracellular sorting of the PCSK9-LDLr complex has been suggested, although it still remains puzzling how *PCSK9* CTD governs LDLr degradation. This same intracellular sorting of LDLr to the lysosomes has been described for the GOF p.(Asp35Tyr) PCSK9 variant which shows and enhanced intracellular activity [10]. Additionally, some data suggest that once the PCSK9-LDLr complex reaches the trans-Golgi network (TGN), PCSK9 possibly interacts with a co-receptor through its CTD, which would lead the LDLr to the lysosomes [39]. These mechanism seems to explain the GOF activity of p.(Ser127Arg) and p.(Asp129Gly) *PCSK9* variants [39]. Our observation that the secretion of the ED-LDLr to the culture medium was markedly decreased when cells are co-transfected with the p.(Arg499His) *PCSK9* variant respect to the WT *PCSK9* supports the hypothesis of an intracellular action of the p.(Arg499His) *PCSK9* variant. Very interestingly and although our results confirm the extracellular GOF activity of p.(Asp374Tyr), we have also detected a intracellular activity of this variant, as determined when ED-LDLr is present in the culture media in excess. This intracellular activity can contribute to the pathogenic effect of this variant and needs to be further characterized in future works.

Our study has some limitations: in Case 2, no family member was available to study the segregation of hypercholesterolemia with the mutation. However, we had the chance to study the large family of Case 1, in which the association is clearly evident with strong penetrance in adults and less severe in young individuals. The functionality of the new mutation has been extensively studied "*in vitro*" with techniques that are considered to be a very close approximation to the "*in vivo*" situation, although they cannot fully reflect the real metabolism of *PCSK9*.

4.1. Conclusions

p.(Arg499His) is a new GOF *PCSK9* mutation located at the CTD with an intracellular GOF activity that drives LDLr to degradation. These data demonstrate the important role of this domain in the intracellular metabolism of the LDLr/PCSK9 complex.

Conflicts of interest

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

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Author contributions

R.M.S.H. and M.D.D.T. performed the genetic screening, recruited patients, collected samples and co-wrote the paper. F.J.N. recruited patients. A.B.-V., S.J. and U.G.-G. performed Western blot and FACS assays. K.B.U. and A.L.-S. performed solid-phase immunoassays. I.L.M. performed the genetic screening. M.B. and A.W. analysed data and co-wrote the paper. F.C, C.M and G.F. participated in the study design, conceived experiments, analysed data and co-wrote the paper. All authors have read and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.08.020.

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