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A new variant (c.*1A > G*) in *LDLRAP1* causing autosomal recessive hypercholesterolemia: Characterization of the defect and response to PCSK9 inhibition

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HIGHLIGHTS

- A new variant c.*1A > G* in *LDLRAP1* causing autosomal recessive hypercholesterolemia was found.
- c.*1A > G* in *LDLRAP1* produces an N-terminal truncated autosomal recessive hypercholesterolemia (ARH) protein.
- Lymphocyte LDLR activity is significantly reduced but far from abolished.
- Evolocumab biweekly substantially lowered LDLc levels in ARH patients.

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ABSTRACT

Background and aims: Autosomal recessive hypercholesterolemia (ARH) is a rare disorder caused by mutations in *LDLRAP1*, which impairs internalization of hepatic LDL receptor (LDLR). ARH patients respond relatively well to statins or the combination of statins and Ezetimibe, but scarce and variable data on treatment with PCSK9 inhibitors is available. We aimed to identify and characterize the defect in a hypercholesterolemic patient with premature cardiovascular disease and determine the response to lipid-lowering treatment.

Methods and results: Gene sequencing revealed a homozygous *c.1A > G*:p.? variant in *LDLRAP1*. Primary lymphocytes were isolated from the ARH patient, one control and two LDLR-defective subjects, one LDLR:p.(Cys352Ser) heterozygote and one LDLR:p.(Asn825Lys) homozygote. The patient had undetectable full-length ARH protein by Western blotting, but expressed a lower-than-normal molecular weight peptide. LDLR activity was measured by flow cytometry, which showed that LDL binding and uptake were reduced in lymphocytes from the ARH patient as compared to control lymphocytes, but were slightly higher than in those from the LDLR:p.(Cys352Ser) heterozygote. Despite the analogous internalization defect predicted in ARH and homozygous LDLR:p.(Asn825Lys) lymphocytes, LDL uptake was higher in the former than in the latter. LDL-cholesterol levels were markedly reduced by the successive therapy with Atorvastatin and Atorvastatin plus Ezetimibe, and the addition of Evolocumab biweekly decreased LDL-cholesterol by a further 39%.

Conclusions: The *LDLRAP1:c.1A > G* variant is associated with the appearance of an N-terminal truncated ARH protein and to reduced, although still significant, LDLR activity in lymphocytes. Residual LDLR activity may be relevant for the substantial response of the patient to Evolocumab.

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1. Introduction

Around 70% of human plasma cholesterol circulates as a component of low-density lipoproteins (LDL). Most LDL is cleared from the circulation through the hepatic LDL receptor (LDLR) by endocytosis of the LDL/LDLR complex [1]. Reduction in activity or number of receptors leads to an increase in cholesterol plasma levels.

Familial hypercholesterolemia (FH) is a genetic disorder clinically characterized by increased LDL-cholesterol (LDLc) levels and a high risk of premature coronary heart disease [1]. Besides mutations in the *LDLR* gene (*LDLR*, MIM 606945) [1], defects in *APOB* (MIM 107730) [2] and *PCSK9* (MIM 607786) [3] also cause FH with an autosomal dominant inheritance pattern. Moreover, mutations in *LDLRAP1* (MIM 605747) result in an autosomal recessive form of FH (ARH) [4] which has low prevalence (1–9/1,000,000).

ARH patients show lower plasma LDLc levels and a retarded onset of cardiovascular disease compared to homozygotes for *LDLR* mutations [5,6], and marked reductions of LDL catabolism and hepatic LDL uptake [7]. The function of LDLR is also defective in cultured lymphocytes from these patients [8–10], but, contrary to homozygotes for *LDLR* mutations [1], it is preserved in skin fibroblasts [7].

The human *LDLRAP1* gene is located on the short arm of chromosome 1 (1p36.11) and encodes an LDLR adaptor protein known as ARH protein. This protein binds to LDLR and mediates its internalization by coupling the receptor to the clathrin machinery at the plasma membrane. The ARH protein accomplishes this by simultaneously engaging the FDNPxY motif within the cytoplasmic tail of LDLR via its N-terminal phosphotyrosine-binding (PTB) domain, and clathrin and the β_2-adaptin subunit of AP-2 via motifs within its C-terminus [4,11]. The requirement of ARH is tissue-specific. In fibroblasts, Disabled 2 (Dab-2) has been shown to compensate for the absence of the ARH protein [12], thus explaining the normal LDLR activity in fibroblasts from ARH patients.

ARH is generally more responsive to statins [5,13,14] or statins plus Ezetimibe [14,15]. Some ARH patients, however, fail to attain LDLc goals with these treatments. Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors have recently emerged as valuable lipid-lowering drugs [16]. PCSK9 regulates LDLR levels by triggering its degradation [17,18]. There are very few data regarding the treatment of ARH patients with PCSK9 inhibitors. Available results show widely variable responses to this treatment added to conventional therapy, LDLc reduction ranging, for example, from none to ~40% after up to 12 weeks [19–22]. The pathophysiological mechanism for this variability is unclear.

Currently, about 15 different variants in *LDLRAP1* have been associated with ARH according to The Human Gene Mutation Database (HMGD; http://www.hgmd.cf.ac.uk/ac/gene.php?gene=LDLRAP1). In this paper, we describe a novel variant in *LDLRAP1* affecting the start codon in a subject with severe hypercholesterolemia that eventually developed cardiovascular disease. Moreover, we also analyze the response to the treatment and the impact of that mutation on the ARH protein and LDLR activity in the patient's lymphocytes.

2. Materials and methods

2.1. Patient

The patient is a 48-year-old male from Dominican Republic. He came to our primary care center (PCC) in Madrid in January 2016 for the study of his hypercholesterolemia. His parents are cousins and had eight children (six males and two females), three of them deceased for unknown reasons. The patient reported that his parents and one alive sister, all of them living in Dominican Republic, have normal cholesterol levels. The lipid status of his brothers was unknown. The patient referred a sedentary lifestyle, with alcohol intake on weekends, and a diet rich in carbohydrates and saturated fats. Among his past medical history, the following features may be highlighted: altered basal glycemia (January 2011) and a renal colic (January 2016), with no subsequent episodes. He was aware of his hypercholesterolemia from 1996, with a total cholesterol (TC) concentration of 400–600 mg/dL. When he came for the first time to the PCC, the TC levels were 621 mg/dL, LDLc 535 mg/dL, HDLc 60 mg/dL and triglycerides 130 mg/dL. He was not taking the prescribed medication, statins, because of economic reasons. Physical examination revealed a BMI of 29 kg/m^2, abdominal circumference of 94 cm and blood pressure of 130/80 mmHg. No xanthelasmas or xanthomas were present. Bilateral corneal arch was present since before he was 45 years old. The electrocardiogram did not show abnormalities. The rest of the physical examination was normal. Liver enzymes, bilirubin and thyroid stimulating hormone were normal. An ultrasound exam was performed to detect the presence of plaques in the carotid and femoral arteries and to measure the carotid intima-media thickness, detecting an increase in the average carotid intima-media thickness (1.17 mm), but not showing plaques in any of the explored arteries. The treatment history from January 2016 and the corresponding lipid responses are shown in Results.

The patient gave informed consent for genetic testing and biochemical analysis.

2.2. Serum lipid analysis

Blood samples were collected in thrombin tubes (BD Vacutainer®) after an overnight 12h fast. After clot retraction, tubes were centrifuged at 1500 × *g* for seven minutes, serum total cholesterol, triglyceride and HDLc concentrations were measured in an automated analyzer (Advia 2400, Siemens). LDLc was calculated using the Friedewald formula.

2.3. Genetic analysis

The genomic DNA (gDNA) was extracted from blood using the Chemagen system. DNA was analysed by Next-Generation Sequencing (NGS) using a customized panel. We chose a specific subset of genes for analysis: *LDLR*, *APOB*, *PCSK9* and *LDLRAP1*. Library preparation and exome enrichment steps were performed according to manufacturer's workflow and sequencing was performed using the MiSeq system (Illumina, San Diego, California, USA). Sanger sequencing was used to confirm the presence of the new variant identified by NGS. The multiplex ligation-dependent probe amplification (MLPA, MRC-Holland) was also carried out to detect gross deletion/insertion at the *LDLR*.

2.4. Bioinformatic analysis

Bioinformatic analysis was performed using algorithms developed by our Bioinformatic Unit. Briefly, sequences were mapped to the CRCh37/hg19 human reference sequence and data bases used for analysis were Human Gene Mutation Database (HGMD® Public) (www.biobaseinternational.com/hgmd) 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 based on the transcripts *LDLR*-ENST00000558518, *APOB*-ENST00000233242, *PCSK9*-ENST00000302118, *LDLRAP1*-ENST00000374338. The *in silico* predictors of pathogenicity used were CADD (Combined Annotation Dependent Depletion), Polyphen (Polymorphism Phenotyping), MutAssesor, Fasthmm and Vest. The scores of conservation used were Gerp2, PhasCons, Phylop. 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).

2.5. Isolation and culture of human peripheral blood lymphocytes

Blood was drawn from fasting subjects in tubes containing lithium

heparin, diluted 1 in 2 with phosphate-buffered saline (PBS), pH 7.4, and layered on Lymphoprep (Nycomed Pharma AS, Zürich, Switzerland). After centrifugation at 800 × *g* for 40 min, mononuclear cells were collected from the interphase and washed three times with PBS. Monocytes were removed by adherence to plastic for 2 h in RPMI 1640 (Lonza, Verviers, Belgium) containing 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 10 μg gentamicin and 1% heat-inactivated foetal bovine serum (FBS; Gibco, Life Technologies, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere of 5% CO_2. Then, lymphocytes were cultured in RPMI 1640 containing 2 mM glutamine, antibiotics and 10% heat-inactivated lipoprotein-deficient FBS (LPDS, d > 1.21 g/mL).

2.6. Western blot

Cultured lymphocytes were centrifuged at 480 × *g* for 5 min. The pellets were lysed with RIPA assay buffer supplemented with protease and phosphatase inhibitors (Thermo Scientific, Waltham, Massachusetts, USA) for the extraction of total cellular proteins. The cell lysates were incubated on ice for 5 min and afterwards were centrifuged at 18000 × *g* for 15 min at 4 °C, and the supernatants were collected.

Total protein was measured by the Bradford assay, separated by 10% sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk at room temperature for 2 h. The blots were incubated overnight at 4 °C with primary antibodies against ARH-N-terminal (1/1500), ARH-C-terminal (1/5000) (Abcam, Cambridge, UK), LDL receptor (1/1000) (Thermo Scientific, Waltham, Massachusetts, USA) and β-actin (1/5000) (Sigma-Aldrich, Saint Louis, Missouri, USA) followed by species-specific horse radish peroxidase-labelled secondary antibodies (1/5000) (Thermo Scientific, Waltham, Massachusetts, USA) for 1 h. The proteins were revealed by chemiluminescence (ECL; Amersham). β-Actin was used as a loading control.

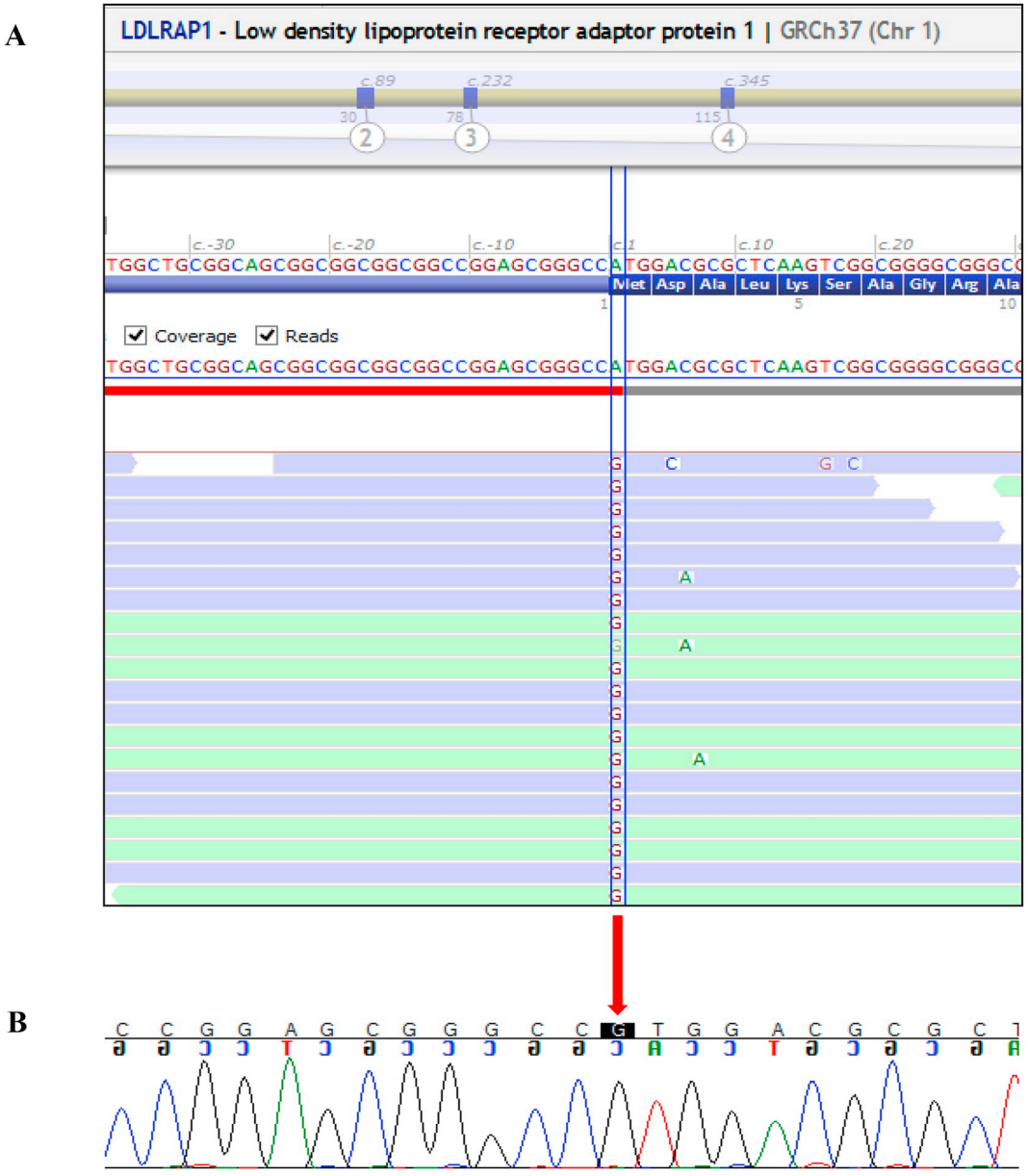

Fig. 1. Sequencing results of *LDLRAP1*.
(A) NGS analysis. BAM file visualized with Alamut software shows the presence of the *c.1A > G* variant in all reads. The transition from *A* to *G* at nucleotide position 1 predicts the change of Met to Val at amino acid position 1. (B) Chromatograms of Sanger sequencing confirming the presence of the *c.1A > G* variant in homozygosity.

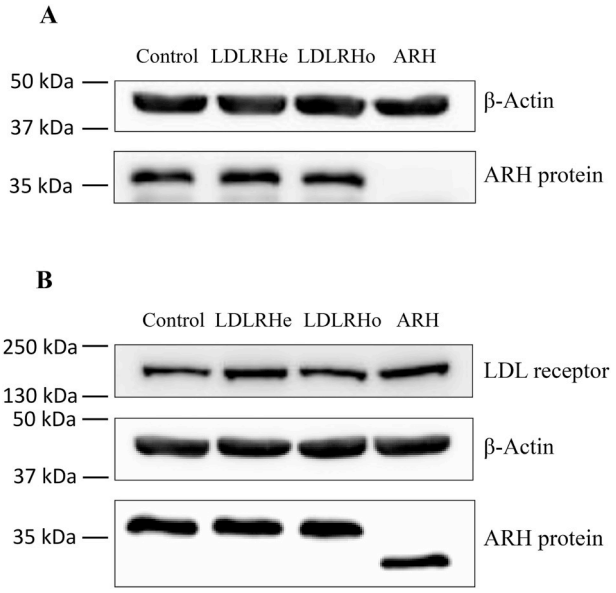

Fig. 2. Expression of the ARH protein and LDLR in lymphocytes from the ARH patient and heterozygous LDLR:p.(Cys352Ser), homozygous LDLR:p.(Asn825Lys) and control subjects.
(A) 50 μg of protein was loaded per lane and probed with an antibody to ARH-N-terminal. (B) 50 μg of protein was loaded per lane and probed with an antibody to ARH-C-terminal or an antibody to LDLR as indicated. ARH, ARH patient; LDLRHe, LDLR:p.(Cys352Ser) heterozygote; LDLRHo, LDLR:p.(Asn825Lys) homozygote.

2.7. LDL-receptor activity

Human LDL was isolated from a single donor and labelled with 1,1′-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Life Technologies) as reported [23]. LDLR activity was determined in lymphocytes as previously described [24]. LDLR was up-regulated by incubation in RPMI 1640 containing 2 mM glutamine, antibiotics and 10% heat-inactivated LPDS for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Lymphocytes (5×10^5 cells/mL) were subsequently incubated for 2 h at 4 °C (binding) or 37 °C (uptake), respectively, with up to 35 μg DiI-LDL/ml. A 30-fold excess of unlabeled LDL was added to extra tubes to determine non-specific binding and uptake. Then, cells were washed, resuspended in PBS and analysed by flow cytometry (FACScalibur, Becton Dickinson, Franklin Lakes, New Jersey, USA). Forward scatter and side scatter gates were established to exclude dead cells and cell debris. The acquisition number of cells was set at 10^4. The specific median intensity of fluorescence (MIF) was estimated after subtracting cell autofluorescence and, moreover, non-specific binding or uptake, respectively, from the total value obtained with the corresponding DiI-LDL concentration. Saturation binding and uptake curves were fitted to a one-site binding model and Bmax values were calculated by using Prism 4.00 (GraphPad Software).

2.8. Quantification of cell surface LDLR expression

After the incubation in RPMI 1640 containing LPDS for 72 h, 5×10^5 lymphocytes were incubated with an allophycocyanin-conjugated antibody against the human LDLR (clone 472413, R&D Systems) at 0.625 μg/mL or an IgG1 isotype control (Life Technologies) for 20 min at room temperature in the dark. Then the cells were washed twice with 1% bovine serum albumin in PBS at 4 °C and immediately analysed by flow cytometry. For each sample, 10^4 lymphocytes were acquired for analysis and the results were expressed as the MIF after subtracting the fluorescence of the isotype control. All measurements were performed in duplicate.

3. Results

3.1. Genetic analysis

The results from NGS analysis showed no pathogenic changes in *LDLR* and *APOB*. Moreover, no gain-of-function or loss-of-function mutations were found in *PCSK9*. However, a previously undescribed variant *c.1A > G:*p.? at the *LDLRAP1* was found in homozygosity, thus affecting the translation start codon (*AUG > GUG*). This was confirmed by Sanger sequencing (Fig. 1). *In silico* analysis showed that three out of five predictors of pathogenicity classified this variant as damaging: CADD 15.83 (S ≥ 14), Polyphen 0.437 (S ≥ 0.3) and VEST 0.947 (S ≥ 0.65); the other two predictors classified the variant as possibly damaging: Sift 0.133 (range 0.06–0.23) and Fathmm 0.02 (range -1-(0.08)). All conservation predictors proved conservation: Gerp.2 3.95 (> 2.45), PhastCons (placenta) 0.99 (range 0–1), PhastCons-(vertebrate) 1 (range 0–1), PhyloP-(placenta) 0.9650 (S ≤ 2) and PhyloP-(vertebrate) 1.0390 (S ≤ 2). Samples from the patient's parents were not available.

3.2. Expression of the ARH protein

The Western blotting analysis using an anti-ARH N-terminal antibody showed that the patient's lymphocytes had undetectable ARH protein expression, whereas lymphocytes from an age-matched, normolipidemic, healthy male (control), and two FH male subjects bearing a heterozygous *c.1054T > A*:p.(Cys352Ser) [25] or a homozygous *c.2475C > A:*p.(Asn825Lys) mutation [26], respectively, showed the band corresponding to the ARH protein at ~35 kDa (Fig. 2A). Additionally, to confirm the complete absence of protein the blots were incubated with a C-terminal antibody. This antibody showed a band at ~35 kDa in lymphocytes from the control and the two LDLR-defective subjects. Remarkably, it revealed a band at ~32 kDa in the patient's lymphocytes (Fig. 2B). On the other hand, the four individuals showed expression of mature LDLR (Fig. 2B).

3.3. Expression of cell surface LDL receptor

Cell surface LDLR expression was also determined in the ARH, control and two LDLR-defective subjects. As shown in Fig. 3, cell surface LDLR expression in lymphocytes from the ARH patient was the highest among the four subjects studied. On the other hand, the expression levels were higher in lymphocytes from the LDLR:p.(Asn825Lys) homozygote and lower in lymphocytes from the LDLR:p.(Cys352Ser) heterozygote than in control cells.

3.4. Lymphocyte LDL-receptor activity

The saturation curves of LDL binding and uptake in lymphocytes from the ARH patient and the control subject were determined. ARH-deficient lymphocytes showed much lower LDL binding (B_{max} = 4.6 ± 0.2 *vs.* 11.1 ± 0.7, arbitrary units of fluorescence ± S.E.) and uptake (B_{max} = 15.8 ± 0.5 *vs.* 29.9 ± 1.2) than control lymphocytes. Given that it has been described that immortalized lymphocytes from ARH patients display decreased LDL uptake and degradation [8–10], but increased LDL binding [9,10,27], an additional LDLR activity analysis was performed in new blood lymphocyte isolates. In this case, besides the lymphocytes from the patient and control subjects, the lymphocytes from the two LDLR-defective male patients were also analysed for comparison. As expected, the lymphocytes from the LDLR:p.(Cys352Ser)-heterozygous patient displayed decreased LDL binding and uptake, their B_{max} values being approximately 40–50% of those in control lymphocytes (Fig. 4). LDL binding and uptake in the patient's lymphocytes were slightly higher than those in lymphocytes from the LDLR:p.(Cys352Ser) heterozygote (Fig. 4), thus confirming that both parameters were reduced to about

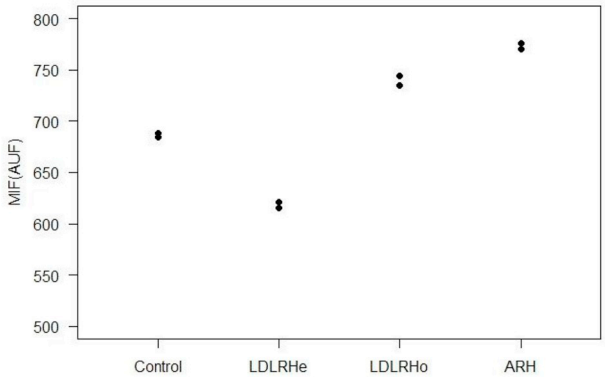

Fig. 3. Cell surface LDLR expression in lymphocytes from the ARH patient and heterozygous LDLR:p.(Cys352Ser), homozygous LDLR:p.(Asn825Lys) and control subjects.
The analysis was performed by flow cytometry. The individual values of each duplicate determination are shown. ARH, ARH patient; LDLRHe, LDLR:p.(Cys352Ser) heterozygote; LDLRHo, LDLR:p.(Asn825Lys) homozygote; MIF, median intensity of fluorescence; AUF, arbitrary units of fluorescence.

half of those found in control lymphocytes.

As mentioned above, the other LDLR-defective patient was homozygous for the p.(Asn825Lys) variant [26], which precludes the interaction of the LDLR with the ARH protein. This causes a functional defect analogous to that expected when the ARH protein is defective. As shown in Fig. 4, LDL binding to lymphocytes from the LDLR:p.(Asn825Lys) homozygote was very similar to that of the patient's lymphocytes. However, LDL uptake in lymphocytes from the LDLR:p.(Asn825Lys) homozygote was 23% (B_{max}) of that in control lymphocytes and, hence, clearly lower than the uptake in the patient's lymphocytes. Therefore, lymphocytes from the ARH patient have defective LDL binding and uptake, but retain a higher capacity to take up LDL than lymphocytes from the LDLR:p.(Asn825Lys) homozygote.

3.5. Response to cholesterol-lowering treatment

Table 1 shows the changes in lipid levels along cholesterol-lowering treatment. From January 2016, the patient was prescribed 40 mg/day of Atorvastatin and LDLc levels decreased from 535 mg/dL to 258 mg/dL. Subsequently, Atorvastatin 80 mg plus Ezetimibe 10 mg daily reduced LDLc levels to 146 mg/dL. Triglyceride levels were progressively lowered. In March 2017, the patient started with effort angina. An angiography was performed and he was diagnosed with multivessel atheromatous coronary artery disease and subjected to stent revascularization. Then, Evolocumab, a PCSK9 inhibitor, was started at a dose of 420 mg per month, on top of Atorvastatin plus Ezetimibe. The patient was also prescribed acetylsalicylic acid (100 mg/day), Losartan (12.5 mg/day) and Ticagrelor (90 mg/12 h). In May 2017 LDLc levels had barely decreased, thus failing to attain the LDLc goals. Therefore, it was decided to administer 420 mg Evolocumab biweekly. After initiation of this dosage, contact with the patient was lost for several months, so that the next lipid analysis was performed at approximately eight months on this treatment. It was found that LDLc levels were lowered to 87 mg/dL, a 39% reduction as compared with the previous determination, and triglyceride levels were decreased to 51 mg/dL (54% reduction). Evolocumab treatment produced no significant adverse effects.

4. Discussion

The clinical features of the patient studied herein, namely raised LDLc levels and bilateral corneal arch, together with his reportedly normolipidemic and consanguineous parents are consistent with the diagnosis of ARH. Genetic variants in *LDLRAP1* have been shown to cause ARH [4,5,28]. We found a new variant, *LDLRAP1:c.1A > G,* associated with this disorder. We also found that *LDLRAP1:c.1A > G* lymphocytes have an N-terminal truncated ARH protein and defective LDLR activity. Moreover, the patient responded well to Evolocumab administered biweekly.

The *LDLRAP1* variant found in the present study affects the *AUG* start codon, thereby presumably impairing the initiation of protein translation. It is known that codons other than *AUG* can act as initiation codons in eukaryotes, although less efficiently [29,30]. In most cases, the near-cognate codons *CUG*, *GUG* and *UUG*, are used as start codons, *CUG* being the most efficient [31]. In mouse and human cells, the protein DAP5 uses exclusively *GUG* as the start codon, which encodes Val [32,33]. In our study, Met is predicted to be substituted by Val, which arises the question as to whether the latter serves as the start amino acid in ARH protein synthesis. Interestingly, immunoblot analysis of the patient's lymphocytes using anti-ARH N-terminal and C-terminal antibodies revealed a protein with lower-than-normal molecular weight (~32 vs. 35 kDa) that is compatible with an N-terminal truncated form of the ARH protein. This suggests that translation is initiated from one of the two downstream *AUG* codons at positions 46 and 55, which are in an appropriate sequence context for translation initiation [9]. A similar ~32 kDa protein was previously detected in lymphocytes from an ARH patient homozygous for a nonsense mutation at codon 22 [9].

In the ARH patient, hypercholesterolemia is associated with

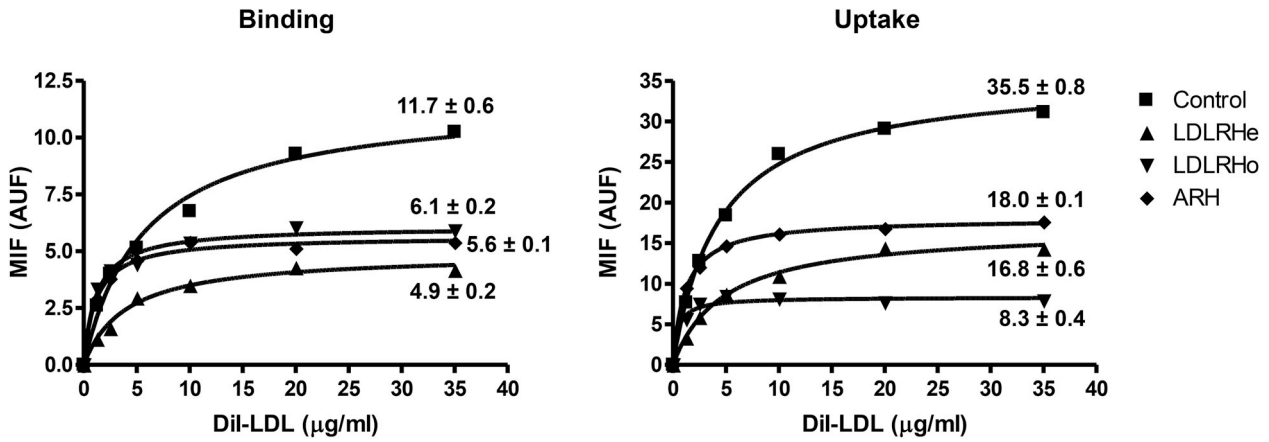

Fig. 4. LDLR activity in lymphocytes from the ARH patient and heterozygous LDLR:p.(Cys352Ser), homozygous LDLR:p.(Asn825Lys) and control subjects.
Saturation curves of DiI-LDL binding and uptake. The numerical value next to each curve is the corresponding estimated B_{max} ± S.E. ARH, ARH patient; LDLRHe, LDLR:p.(Cys352Ser) heterozygote; LDLRHo, LDLR:p.(Asn825Lys) homozygote; MIF, median intensity of fluorescence; AUF, arbitrary units of fluorescence.

Table 1
Evolution of lipid profile of the patient following different lipid-lowering therapies.

Date of measurement	Treatment (initiation date)					
	No treatment	Atorvastatin 40 mg (January 15, 2016)	Atorvastatin 80 mg + Ezetimibe 10 mg/day (February 10, 2016)		Atorvastatin 80 mg + Ezetimibe 10 mg/day	
					Evolocumab 420 mg/month (March 31, 2017)	Evolocumab 420 mg/2 weeks (June 6, 2017)
	January 14, 2016	February 10, 2016	June 6, 2016	December 5, 2016	May 17, 17	February 20, 2018
Total cholesterol (mg/dL)	621	340	251	241	238	166
Triglycerides (mg/dL)	130	83	74	62	110	51
LDL cholesterol (mg/dL)	535	258	179	146	142	87
HDL cholesterol (mg/dL)	60	66	58	82	74	69

defective lymphocyte LDLR activity, both LDL binding and uptake being significantly reduced. Given that the liver, the major site for LDL catabolism, critically depends on the ARH protein for LDLR internalization [7,34], hypercholesterolemia in the patient can be attributed to defective hepatic uptake of LDL. Nevertheless, LDLR activity was slightly higher than in lymphocytes heterozygous for the LDLR:p.(Cys352Ser) mutation, which contain half the normal amount of functional LDLR. The lower-than-normal LDL binding in *LDLRAP1:c.1A > G* lymphocytes contrasts with their higher expression of cell surface LDLR compared with control lymphocytes. Similarly, other authors have reported a poor correspondence between cell surface LDLR expression and LDL binding in immortalized *LDLRAP1* mutant lymphocytes [27]. Previous reports using these cells showed increased LDL binding [9,10,27]. However, Michaely et al. found that the majority of LDLR on the surface of ARH-deficient lymphocytes was unable to bind LDL and concluded that ARH is not only required for internalization of the LDL/LDLR complex, but also for efficient binding of LDL, perhaps by stabilizing the interaction of the receptor with the lipoprotein particle [27]. Interestingly, lymphocytes from the LDLR:p.(Asn825Lys) homozygote also had higher surface LDLR expression and lower LDL binding than control lymphocytes. This mutation alters the highly conserved FDNPxY sequence motif (positions 823–828) located in the cytoplasmic domain of LDLR, so that point mutations in that sequence alter the binding of the ARH protein to LDLR [11]. Therefore, a defective interaction between these two proteins may impair LDL binding.

Despite reduced LDL uptake, *LDLRAP1:c.1A > G* lymphocytes preserve a higher uptake activity than those with internalization-defective LDLR, as observed by comparing the former with LDLR:p.(Asn825Lys) lymphocytes. The defective binding of the ARH protein to LDLR impairs subsequent receptor uptake, thus causing FH [11,26]. Therefore, analogous LDL uptake defects in both mutant lymphocytes could be expected, but, instead, they showed differential uptake activity. Consistently with these results, a previous study [9] found that LDL degradation is reduced but not abolished in lymphocytes from patients with ARH, and that these lymphocytes preserved the ability to down-regulate the LDLR in response to LDL addition, thus suggesting that ARH is not absolutely required for LDL internalization in lymphocytes. Moreover, a significant LDL uptake by lymphocytes has been previously found in a study including 28 ARH subjects and five *LDLRAP1* variants [35]. In this same study, it was also reported that exogenous PCSK9, which binds LDLR and triggers its degradation in lysosomes [17,18], reduces LDLR expression and LDL uptake in lymphocytes from ARH patients, although to a lower extent than in control lymphocytes [35]. All together, these findings are consistent with the presence in lymphocytes of an additional mechanism for LDLR internalization, which partially compensates for the absence of normal ARH protein.

Dab-2 is an alternative PTB domain-containing protein that, like ARH, binds the FDNPxY motif within the cytoplasmic tail of the LDLR. However, Dab-2 is an unlikely candidate to mediate LDL/LDLR internalization in *LDLRAP1:c.1A > G:*p.? lymphocytes, because this cell type does not express detectable Dab-2 [36]. However, it is possible that a different, alternative mechanism exists in lymphocytes.

On the other hand, it cannot be ruled out that the N-terminal truncated ARH protein found in the patient's lymphocytes preserves some functional activity. The PTB domain of the ARH protein interacts with the FDNPxY motif on the LDLR tail and simultaneously with cell membrane phosphoinositides, whereas specific sequences within the C-terminal region of ARH bind clathrin and its adaptor AP-2 [4,11]. The N-terminal truncated ARH protein would only lack, at the most, the first few amino acids of the N-terminal end of the PTB domain (residues 45–175). This may be compatible with residual activity of the N-terminal truncated ARH protein, thus contributing to the remaining LDL uptake by the patient's lymphocytes. Whether or not this truncated protein is present in the patient's hepatocytes is unknown.

Although there was a marked impressive reduction in LDLc concentration while on Atorvastatin and this drug plus Ezetimbe, the subsequent diagnosis of cardiovascular disease led to treat the patient with 420 mg Evolocumab monthly, which unfortunately resulted in no additional reduction in cholesterol concentration. This resistance to Evolocumab treatment could be due to high circulating levels of PCSK9. However, the unavailability of serum previous to the treatment with the PCSK9 inhibitor impeded to address this question. The possibility of a gain-of-function mutation on *PCSK9* can be discarded since, as mentioned above, the patient does not carry any pathogenic variant of *PCSK9*. This lack of response moved us to further increase the Evolocumab dosage to 420 mg every other week, which produced a substantial reduction in LDLc levels. Among the very few ARH patients previously reported to be treated with PCSK9 inhibitors, LDLc reduction has showed wide variability, ranging from none to very significant responses [20–22]. The mechanism for the reduction of LDLc by PCSK9 inhibitors in ARH patients is unclear. Residual function of the ARH protein may underlie this effect, allowing an increase in LDLR activity when PCSK9-induced degradation of the receptor is blocked. Supporting this hypothesis, in vitro inhibition of PCSK9 in ARH lymphocytes with Alirocumab restored LDL uptake [35]. In keeping with this, it has been suggested that the extent of residual LDLR function is important in determining the response to PCSK9 inhibition in homozygotes for LDLR-defective FH [21,22,37–39].

In summary, we characterized ARH protein expression and LDLR activity in lymphocytes from an ARH patient carrying a missense mutation in the first nucleotide of the coding sequence of *LDLRAP1*. This variant is associated to undetectable full-length and the presence of N-terminal truncated ARH protein. Lymphocyte LDLR activity is significantly reduced but far from abolished. On the other hand, treatment of the patient with Evolocumab biweekly substantially lowered LDLc levels. Further studies are required to ascertain whether the N-terminal truncated ARH protein preserves some functional activity and whether,

if present in hepatocytes, this determines the response to PCSK9 inhibition.

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

CRJ, DGC and FC performed the laboratory work; CRJ, DGC and SRN conceived the study and wrote the manuscript; MFV, CL, JSR, DGN and JMM provided subject's data; MAL and JMM discussed the data and contributed to their interpretation.

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