

The importance of an integrated analysis of clinical, molecular, and functional data for the genetic diagnosis of familial hypercholesterolemia

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Abstract

Purpose

Familial hypercholesterolemia (FH) is one of the most common monogenic disorders, and the high concentrations of low-density lipoprotein (LDL) cholesterol presented since birth confers on these patients an increased cardiovascular risk. More than 1,600 alterations have been described in the LDL receptor gene (*LDLR*), but a large number need to be validated as mutations causing disease to establish a diagnosis of FH. This study aims to characterize, both at the phenotypic and genotypic levels, families with a clinical diagnosis of FH and present evidence for the importance of the integration of clinical, molecular, and functional data for the correct diagnosis of patients with FH.

Methods

A detailed analysis of the phenotype and genotype presented by 55 families with 13 different alterations in the *LDLR* was conducted. For eight of these, an extensive functional characterization was performed by flow cytometry, confocal microscopy, and reverse transcriptase polymerase chain reaction.

Results

Carriers of neutral alterations presented a significantly lower incidence of premature cardiovascular disease, lower levels of atherogenic lipoproteins and a large number of these individuals had LDL-cholesterol values below the 75th percentile. presented a significantly lower incidence of premature cardiovascular disease, lower levels of atherogenic lipoproteins and a large number of these individuals had LDL-cholesterol values below the 75th percentile. However, the functional study was essential to determine the pathogenicity of variants.

Conclusion

The data collected illustrate the importance of this integrated analysis for the correct assessment of patients with FH who can otherwise be misdiagnosed.

Keywords

functional studies; genetic diagnosis; integrated analysis; neutral alteration; pathogenic mutation

Introduction

Familial hypercholesterolemia (FH) is one of the most common and well-known monogenic disorders; mutations in the low-density lipoprotein (LDL) receptor gene (*LDLR*) are the underlying cause in more than 90% of cases.^{1,2,3} Patients with FH have an

increased cardiovascular risk as a result of the high concentrations of LDL cholesterol (LDL-C) present from birth.⁴ The World Health Organization recommends large-scale screening to identify patients who can most benefit from the early implementation of lipid-lowering treatment. The early identification and treatment of these patients can reduce or even eradicate their elevated cardiovascular risk if LDL-C concentrations are reduced for their lifetime.⁵ Clinical identification is possible, but in most cases it is not sufficient to identify these patients⁶; therefore, recent dyslipidemia guidelines recommend DNA testing whenever possible.⁷ In the 30 years since mutations in *LDLR* were identified as the prime defect in patients with FH, the laboratory techniques to identify these mutations have improved greatly. New variants are found every day in different populations,^{8,9,10} but the functional effect of these variants is usually not assessed, which can lead to misdiagnosis. With the novel sequencing technologies being applied for genetic diagnosis of FH, it is expected that an increasing number of variants of unknown clinical significance will be found in *LDLR*. Therefore, understanding whether these alterations disturb the function of the protein becomes important. In fact, the last update of the FH database⁸ recognizes that, based on *in silico* analysis, only about 80% of all reported alterations are pathogenic, and more than half of the missense mutations reported so far do not have functional studies. Cosegregation studies have been very useful in assessing variant pathogenicity^{11,12,13}; however, all these data are not usually analyzed as a whole when considering the genetic diagnosis of FH.

A detailed description and analysis of the phenotype and genotype presented by 55 families with a clinical diagnosis of FH carrying five functional and eight neutral alterations are discussed here. For this, an extensive functional characterization was performed for eight of these alterations without functional studies. An integrated discussion of clinical, molecular, and functional data is presented, highlighting the importance of this analysis for the correct assessment of patients with FH.

Materials and Methods

Study population

Data on phenotype/genotype of 135 participants of the Portuguese FH Study are presented. Written informed consent was obtained from all participants before their inclusion in the study. The study protocol and database were previously approved by the National Institute of Health Ethical Committee and National Data Protection Committee.

Lipid profile

Fasting blood samples were collected from individuals at the time of their inclusion in the study. Total cholesterol, direct LDL-C, high-density lipoprotein cholesterol (HDL), triglycerides, apolipoprotein A1 (apoA1), apolipoprotein B (apoB), and lipoprotein(a) were determined for all individuals using Cobas Integra 400 plus (Roche, Basel, Switzerland) and enzymatic colorimetric and immunoturbidimetric methods.

For all individuals, LDL-C percentiles were calculated by age and sex, according to the reference values for the Spanish population,¹⁴ because of the absence of percentile distributions for fasting serum lipids in the Portuguese population. In some individuals there were only LDL-C values while receiving medication; in that case, those values were corrected for the type of medication. For statins, the reduction factor used was 30% (LDL-C under treatment $\times 1.3$); for the combination of a statin and ezetimibe, the factor used was 50% (LDL-C under treatment $\times 1.5$).^{15,16}

Segregation analysis

The cosegregation of the alterations with the hypercholesterolemia in each family was evaluated and expressed as the number of “alteration carriers/total affected” or “alteration

carriers/total nonaffected.” “Total affected” referred to all relatives with hypercholesterolemia (>75th percentile, adjusted for sex and age) and “total nonaffected” was related to all relatives with normal lipid values (<75th percentile, adjusted for sex and age).

Molecular analysis

A genetic diagnosis of FH was made by the molecular study of the *APOB* (fragments of exons 26 and 29), *LDLR* (including the study of large rearrangements), and *PCSK9* genes, as reported previously.¹¹

In silico analysis

The predicted effects of *LDLR* nonsynonymous missense alterations were assessed using the following open-access software: PolyPhen-2,¹⁷ Sorting Tolerant From Intolerant (SIFT),¹⁸ and Mutation taster.¹⁹

The effect on splicing of *LDLR* putative splice site variants was assessed using Splice-Site Predictor (Splice Port),²⁰ Neural Network Splice Site Prediction Tool (NNSSP),²¹ and Neural Network Predictions of Splice Sites in Humans (NetGen2).²²

Functional assays of *LDLR* variants: c.-13A>G, c.818-3C>G, and c.1706-10G>A

Total messenger RNA was obtained from blood mononuclear cells freshly isolated from the patients carrying these variants. Functional assays were performed as described before.^{9,23}

Site-directed mutagenesis, transfection, and Western blot analysis

The oligonucleotides used to generate the different plasmids carrying the LDL receptor variants under study are presented in the **Supplementary Methods** online as well as the mutagenesis protocol. Cell transfection and semiquantitative immunoblotting were performed as described before.²⁴

LDL isolation and lipoprotein labeling

LDL was isolated from 3 ml serum samples from healthy individuals using two-step centrifugation (**Supplementary Methods** online). LDL was labeled with fluorescein isothiocyanate as previously described.²⁵

Quantification of LDLR expression and activity by flow cytometry

LDLR cell surface expression and LDL binding and uptake were determined by flow cytometry, specifically fluorescence-activated cell sorting, as previously described.²⁴

Confocal laser scanning microscopy

Confocal laser scanning microscopy was used to analyze LDLR expression and intracellular colocalization of LDLR, as described before²⁴ (**Supplementary Methods** online).

Kinetics of LDLR variants

Expression at different incubation times in the presence of LDL was measured, and studies of LDL–LDLR binding at different pH values were performed, as previously described.²⁶

Statistical analysis

Statistical analysis of the lipid profile was performed using SPSS software (version 17.0 for Windows; SPSS, Chicago, IL). Frequencies of qualitative variables were compared using the χ^2 test. Mean values of quantitative variables were compared using the Student *t* test or analysis of variance for independent data, whereas median values were compared with the nonparametric Mann–Whitney or Kruskal Wallis median tests.

For functional assay studies, all measurements were performed at least three times, with $n = 3$ unless otherwise stated. Results are presented as mean \pm SD. Levels of

significance were determined using a two-tailed Student *t* test. A *P*-value <0.05 was considered statistically significant.

Results

In the Portuguese FH study cohort, about 35 variants (30%) have an uncertain pathogenic effect. In an effort to continue the characterization of all these variants, eight *LDLR* alterations were chosen for this study based on the following criteria: (i) the prevalence in our cohort (**Supplementary Figure S1** online), (ii) lack of cosegregation, (iii) the severity of the phenotype, and (iv) sample availability for functional studies.

In silico analysis

The results obtained by different software packages are presented in **Tables 1** and **2**.

Table 1. *In silico* and *in vitro* analyses of missense alterations found in the *LDLR* gene

Alteration		Frequency	In silico analysis				Reference				
Complementary DNA	Protein		1KG (%)	ESP (%)	Normolipidemic panel ^b	Polyphen2	SIFT	Mutation Taster	Functional study	First described	Functional study
c.226G>T	p.Gly76Trp	3/3; 5/6	NF	NF	0/95	Probably	Deleterious	Disease causing	Benign	¹¹	Reported here
c.806G>A	p.Gly269Asp	9/14; 2/2	0.1	0.008	0/95	Benign	Tolerated	Disease causing	Benign	¹⁴	¹¹
c.829G>A	p.Glu277Iys	2/2; 1/1	0.001	0.023	0/95	Benign	Tolerated	Disease causing	Benign	¹⁶	¹⁸
c.1216C>T	p.Arg406Trp	35/36; 0/2	NF	NF	0/95	Probably	Deleterious	Disease causing	Pathogenic	¹⁶	Reported here
c.1322T>C	p.Ile441Thr	11/12; 0/1	NF	NF	0/95	Probably	Deleterious	Disease causing	Pathogenic	⁷	Reported here
c.1633C>T	p.Gly545Trp	32/33; 0/4	NF	NF	0/95	Probably	Deleterious	Disease causing	Pathogenic	¹¹	Reported here
c.2093G>T	p.Cys698Phe	2/0; 0/0	NF	NF	0/95	Probably	Deleterious	Disease causing	Pathogenic	⁷	Reported here
c.2575G>A	p.Val859Met	2/3; 1/1	0.001	0.008	0/95	Benign	Deleterious	Polymorphism	Benign	¹¹	¹⁹

Bold indicates the alterations correctly assessed by the three *in silico* software packages.

^aCosegregation values show alteration carriers/total affected; alteration carriers/total nonaffected. ^bPanel of 95 Portuguese normolipidemic individuals. 1KG, 1000 Genomes Project; ESP, National Heart, Lung, and Blood Institute GO Exome Sequencing Project; NF, not found.

Table 1. *In silico* and *in vitro* analyses of splicing and promoter alterations in the *LDLR* gene

Alteration		Frequency	In silico analysis				Reference				
Intron	Complementary DNA		1KG (%)	ESP (%)	Normolipidemic panel ^b	Splice Port	NN5SP	NetGen2	Functional study	First described	Functional study
Promoter	c.-13A>G	3/5; 0/0	NF	0.008	0/95	60 (exon 1A)	0 (exon 1A)	100 (exon 1A)	Benign	⁸	¹¹
5	c.818-3C>G	3/3; 0/1	NF	NF	0/95	0 (exon 6A)	0 (exon 6A)	0 (exon 6A)	Pathogenic	Novel	Reported here
7	c.1061-8T>C	12/13; 3/4	0.520	0.799	2/75	100 (exon 8A)	100 (exon 8A)	100 (exon 8A)	Benign	¹⁰	¹³
11	c.1706-10G>A	3/4; 1/1	0.080	0.146	0/95	100 (exon 12A)	100 (exon 12A)	100 (exon 12A)	Benign	Novel	Reported here
14	c.2140+5G>A	4/4; 0/0	0.280	0.899	1/75	86 (exon 14D)	33 (exon 14D)	86 (exon 14D)	Benign	¹⁰	¹³

Bold indicates the alterations correctly assessed by the three *in silico* software packages.

^aCosegregation values show alteration carriers/total affected; alteration carriers/total nonaffected. ^bPanel of 95 Portuguese normolipidemic individuals. D, splice donor; A, splice acceptor; 1KG, 1000 Genomes Project; ESP, National Heart, Lung, and Blood Institute GO Exome Sequencing Project; NF, not found.

Splicing and promoter alterations assays

Functional studies of alterations c.-13A>G and c.1706-10G>A revealed that both are nonfunctional, whereas c.818-3C>G is a functional alteration leading to the retention of two nucleotides in intron 5 (**Supplementary Figure S2** online). The promoter sequence variant was classified as nonpathogenic because both alleles (T/C) of the *LDLR* single nucleotide polymorphism rs2228671 were present in patients' messenger RNA, indicating that the variant does not affect messenger RNA expression. We also verified that both alleles of the same single nucleotide polymorphism (SNP) were present in a patient with the c.1706-10G>A alteration, and there was no intron retention or exon skipping.

Expression of *LDLR* variants in CHO-*IdIA7* cells

Only one band was detected for wild-type (wt) *LDLR* and the p.Gly76Trp variant, corresponding to the mature form (apparent molecular weight 130 kDa) (**Supplementary Figure S3** online, lanes 1 and 2). Two bands were detected for the p.Arg406Trp variant, though the precursor form of the protein is reduced (**Supplementary Figure S3** online, lane 3). Finally, p.Ile441Thr, p.Gly545Trp, and p.Cys698Phe *LDLR* variant expression was detected only as the precursor form (**Supplementary Figure S3** online, lanes 4, 5 and 6). Equal loading of protein was confirmed in each blot by membrane stripping and

further incubation with antibodies to visualize cytosolic GAPDH protein (**Supplementary Figure S3a** online). The extent of protein expression was determined by quantitative densitometric analysis (**Supplementary Figure S3b** online). No statistical differences in expression were observed among the LDLR constructs, as shown in **Supplementary Figure S3b** online.

Functional study of the LDLR variants by fluorescence-activated cell sorting

As shown in **Figure 1a**, only p.Gly76Trp shows LDLR expression at cell surface, similar to the wt (wt: 100 ± 5 ; p.Gly76Trp: 102 ± 2 (arbitrary units)). Expression of p.Arg406Trp, p.Ile441Thr, p.Gly545Trp, and p.Cys698Phe was significantly reduced compared with the wt LDLR (p.Arg406Trp: 65 ± 5 ; p.Ile441Thr: 7 ± 5 ; p.Gly545Trp: 7 ± 5 ; p.Cys698Phe: 8 ± 6 ; $P < 0.01$). Binding of p.Gly76Trp was similar to wt binding (wt: 100 ± 3 ; p.Gly76Trp: 90 ± 3); however, the binding activities of the other four variants analyzed were diminished when compared with wt (p.Arg406Trp: 60 ± 4 ; p.Ile441Thr: 5 ± 3 ; p.Gly545Trp: 6 ± 4 ; p.Cys698Phe: 3 ± 1 ; $P < 0.01$) (**Figure 1b**). As shown in **Figure 1c**, and in agreement with LDLR expression and binding results, LDL internalization by p.Gly76Trp is similar to wt (wt: 100 ± 4 ; p.Gly76Trp: 95 ± 5), and the LDL uptake determined for the other four variants is significantly diminished compared with wt (p.Arg406Trp: 62 ± 4 ; p.Ile441Thr: 10 ± 3 ; p.Gly545Trp: 11 ± 4 ; p.Cys698Phe: 8 ± 5 ; $P < 0.01$). According to the results obtained, it can be concluded that p.Gly76Trp is a nonpathogenic LDLR variant, whereas p.Arg406Trp is a pathogenic variant with diminished activity ($\approx 40\%$), and p.Ile441Thr, p.Gly545Trp, and p.Cys698Phe are pathogenic with a near complete loss of activity ($\approx 10\%$).

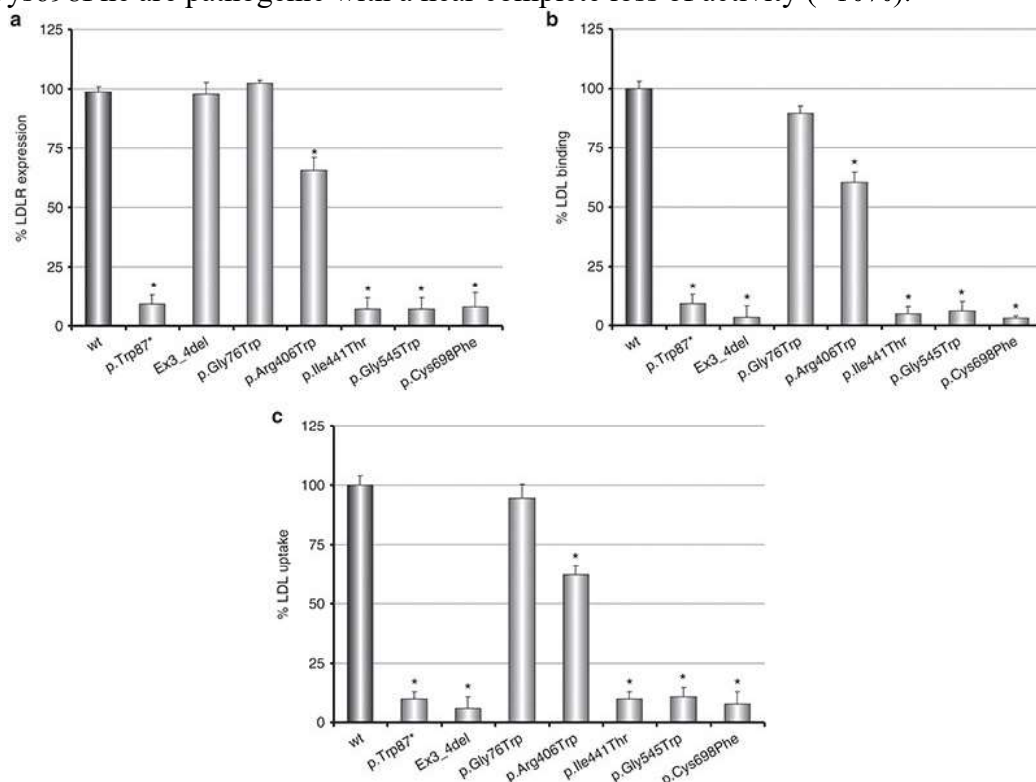


Figure 1. Functional characterization of LDLR variants in CHO-dla7 transfected cells. (a) LDLR expression at the cellular membrane. (b) Low-density lipoprotein (LDL)–LDLR binding after 4 h incubation at 4 °C. (c) LDL internalization efficiency after 4 h incubation at 37 °C. Cells ($n = 10,000$) were acquired in a Facsclibur cytometer, and values of LDL uptake and binding and LDLR expression were calculated as described in the Methods. The values represent the mean of triplicate determinations ($n = 3$); error bars represent \pm SD. * $P < 0.001$ compared with wild type using the Student t test.

Determination of LDLR class mutation by confocal microscopy

According to the results obtained by fluorescence-activated cell sorting, p.Arg406Trp is most probably a class 2b (LDLR partially retained in the endoplasmic reticulum) or class 5 (impaired recycling of LDLR), and p.Ile441Thr, p.Gly545Trp, and p.Cys698Phe are probably class 2a (total retention of LDLR in the endoplasmic reticulum). As shown in **Figure 2**, wt does not colocalize with calregulin, residing mostly at the cellular membrane. However, p.Arg406Trp LDLR showed partial colocalization with the endoplasmic reticulum, indicating that it belongs to class 2b. p.Ile441Thr, p.Gly545Trp, and p.Cys698Phe LDLR variants colocalize almost completely with calregulin and thus are classified as class 2a variants.

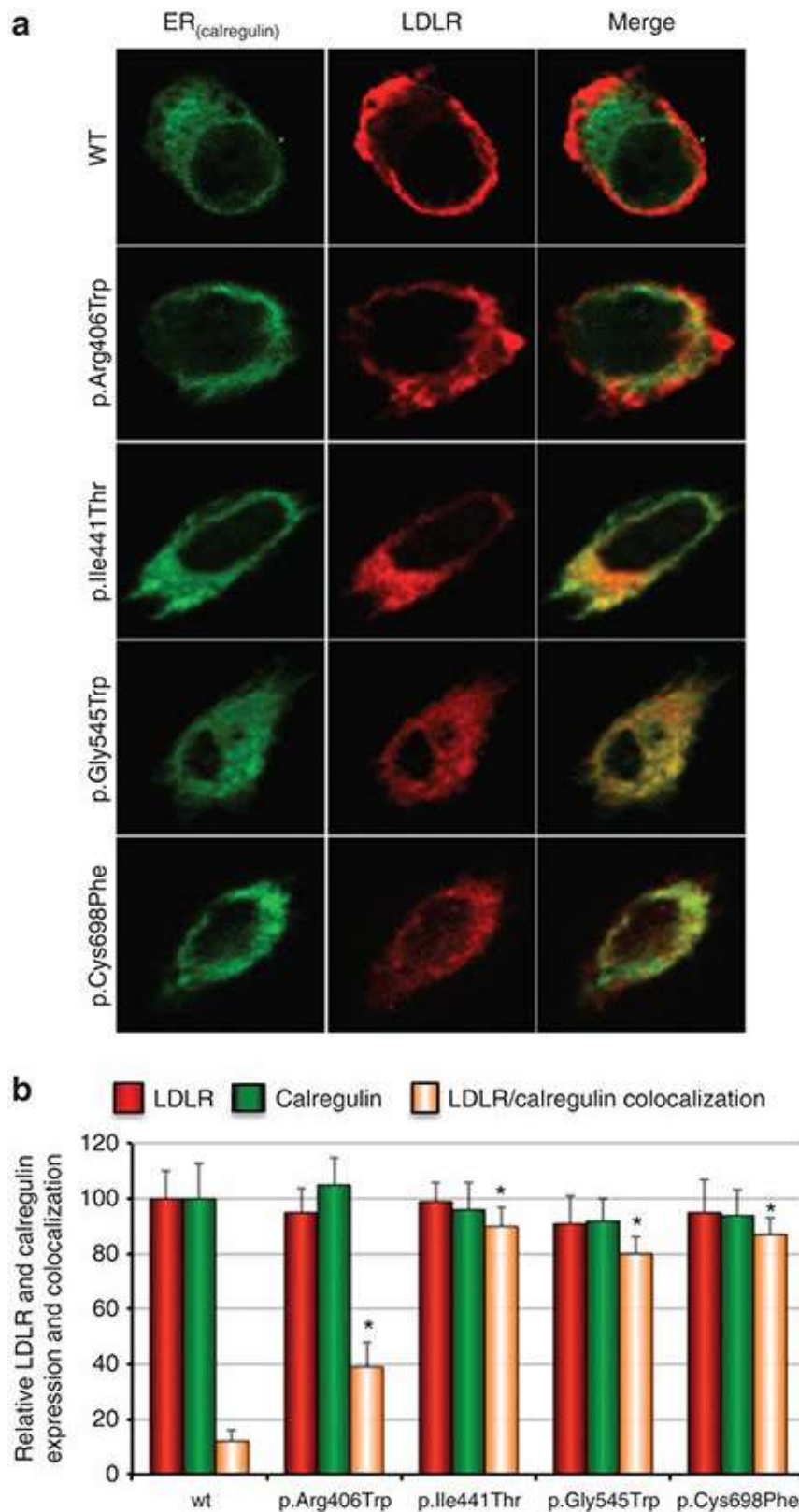


Figure 2. Analysis of wild type, p.Arg406Trp, p.Ile441Thr, p.Gly545Trp, and p.Cys698Phe LDLR by confocal microscopy. (a) LDLR expression and colocalization with calregulin, a specific endoplasmic reticulum marker, was determined 48 h after cell transfection. Immunostaining was performed with anti-hLDLR and anti-calregulin antibodies. Texas Red- and Alexa Fluor 488-labeled secondary antibodies were used to visualize LDLR and

calregulin, respectively. The images show a representative individual cell ($n = 30$). (b) The relative amount of LDLR (red) and calregulin (green) and the percentage of LDLR to calregulin colocalization (orange). The histograms represent the mean \pm SD ($n = 30$ cells). * $P < 0.001$ compared with wild type using the Student t test.

Kinetics of p.Arg406Trp LDLR expression

Kinetic studies were performed to exclude any possibility of an impaired defect in the recycling of p.Arg406Trp LDLR. Expression of LDLR at the cell surface was determined by fluorescence-activated cell sorting upon the addition of LDL, and the percentage of LDLR relative to time 0 (without LDL) was determined at each incubation time. As shown in **Supplementary Figure S4a** online, expression of wt LDLR does not diminish after the addition of LDL during incubation. Expression of p.Arg406Trp mutant is also not decreased over time during incubation with LDL (wt: 100 ± 4 ; p.Arg406Trp: 65 ± 4 at time 0). As an internal control, the previously characterized class 5 variant p.Arg416Trp²⁶ was used to illustrate the decrease of LDLR expression at the cell membrane occurring as a result of impaired recycling. We also mimicked the acid-dependent mechanism of lipoprotein release that occurs in the endosomal compartment upon acidification. As shown in **Supplementary Figure S4b** online, LDL-LDLR binding for wt was dependent on pH, being 75% less efficient at pH 5.5 compared with pH 7.5. Similar results were determined for the p.Arg406Trp mutant, indicating that there is no impaired release of LDL/LDLR upon endosomal acidification, confirming that this variant is not a class 5 LDLR mutation. By contrast, for the positive control p.Arg416Trp LDLR, LDL release at an acidic pH was not as efficient as in wt, resulting in binding 37% lower at pH 5.5 than at pH 7.5 (**Supplementary Figure S4b** online).

Lipid profile of functional and nonfunctional alterations

To increase the accuracy of our analysis, we choose to include the results of five nonpathogenic (neutral) variants previously reported (p.Gly269Asp, p.Glu277Lys, c-1061-8T>C, c.2140+5G>A, and p.Val859Met).^{23,27,28,29}

Biochemical characteristics of each mutation carrier are shown in **Supplementary Table S1** online. Only data for adults without treatment were included (except lipoprotein(a)) because no values were registered for the majority of pediatric patients. When the phenotype of functional alteration carriers and neutral alteration carriers were compared, carriers of functional alterations presented a severe phenotype; the differences were statistically significant for all parameters except high-density lipoprotein cholesterol and apoA1, as well as age (**Supplementary Table S1** online). Also, the mean values of LDL-C percentile were significantly higher in the carriers of functional alterations than in those identified with neutral alterations (83.6 ± 22.4 vs. 61.2 ± 34.2 mg/dl; $P = 0.002$) (**Supplementary Table S1** online).

A family history of premature cardiovascular disease was more common in carriers of functional alterations (74.2%) than in carriers of neutral alterations (29.2%) (**Supplementary Table S2** online). The same was observed in patients with LDL-C values above the 75th percentile (92.5% vs. 61.1%; $P < 0.001$). For index patients, however, there were no statistically significant differences concerning LDL-C values above the 95th percentile (75.9% vs. 79.2%; $P = 0.520$) (**Supplementary Table S2** online).

Because the p.Arg406Trp alteration presented a decrease in protein activity (close to borderline) of only 40%, the phenotype of individuals with this alteration was compared with that of carriers of functional alterations and neutral alterations (**Table 3**). Individuals with p.Arg406Trp presented a more severe phenotype when compared with carriers of

neutral alterations for total cholesterol, LDL-C, apoB, apoB/apoA1 ratio, and LDL-C percentile mean values (333.9 ± 78.5 vs. 278.2 ± 59.23 mg/dl ($P = 0.02$); 237.7 ± 112.4 vs. 168.2 ± 53.0 mg/dl ($P = 0.002$); 144.5 ± 44.3 vs. 103.0 ± 28.2 mg/dl ($P = 0.002$); 0.87 ± 0.29 vs. 0.63 ± 0.28 mg/dl ($P = 0.027$); and 82.4 ± 22.8 vs. 61.2 ± 34.2 ($P = 0.03$), respectively). When compared with carriers of functional alterations, however, a statistically significant difference was not found, although the mean levels of total cholesterol, LDL-C, apoB, and apoB-to-apoA1 ratio were higher for p.Arg406Thr (333.9 ± 78.5 vs. 322.2 ± 68.2 mg/dl ($P = 0.82$); 237.7 ± 112.4 vs. 230.7 ± 94.1 mg/dl ($P = 0.96$); 144.5 ± 44.3 vs. 117.4 ± 35.4 mg/dl ($P = 0.19$); 0.87 ± 0.29 vs. 0.79 ± 0.21 mg/dl ($P = 0.492$); 82.4 ± 22.8 vs. 84.4 ± 22.4 ($P = 0.73$)) (Table 3). Triglycerides, high-density lipoprotein cholesterol, and ApoA1 values were similar between these three groups. The cosegregation data are very similar between p.Arg406Thr and functional carriers, and carriers of neutral variants present lower cosegregation rates, showing a high percentage of nonaffected individuals with neutral variants. The analysis of LDL-C percentiles, however, presented contradictory results: p.Arg406Thr carriers showed the lowest rates for index cases, with LDL ≥ 75 th percentile and LDL ≥ 95 th percentile.

Table 3. Biochemical characterization of adult individuals with p.Arg406Trp versus functional alterations versus neutral alterations

	p.Arg406Trp ^a	n	Functional alterations ^a	n	Neutral alterations ^a	n	P value ^b	P value ^c
Age (years)	44.1 ± 12.0	20	43.9 ± 14.0	30	46.5 ± 14.0	40	0.970	0.512
Total cholesterol	333.9 ± 78.5	19	322.2 ± 68.2	26	278.2 ± 59.23	31	0.818	0.022
LDL-C	247.8 ± 71.8	11	239.2 ± 69.5	18	185.3 ± 52.7	29	0.877	0.004
LDL-C cor ^d	237.7 ± 112.4	21	230.7 ± 94.1	31	168.2 ± 53.0	42	0.963	0.002
High-density lipoprotein cholesterol	56.9 ± 12.4	12	57.0 ± 16.5	19	61.7 ± 26.2	29	0.734	0.944
Triglycerides	132.6 ± 54.9	12	108.4 ± 38.9	19	155.6 ± 66.8	29	0.220	0.300
Lipoprotein(a) ^e	43.5 ± 72.1	21	88.6 ± 61.6	31	40.4 ± 50.6	41	0.001	0.732
Apolipoprotein B	144.5 ± 44.3	11	117.4 ± 35.4	7	103.0 ± 28.8	23	0.193	0.002
Apolipoprotein A1	172.7 ± 42.9	11	147.9 ± 13.3	7	188.9 ± 69.3	22	0.161	0.836
Apolipoprotein B-to-apolipoprotein A1 ratio	0.87 ± 0.29	11	0.79 ± 0.21	7	0.63 ± 0.28	22	0.533	0.027
sdLDL	52.9 ± 10.1	3	45.74 ± 14.2	4	30.4 ± 12.4	7	0.492	0.067
LDL percentile	82.4 ± 22.8	21	84.4 ± 22.4	31	61.2 ± 34.2	42	0.728	0.029
≥75th (%) index	75%	12	100%	17	91.7%	24	0.060	0.530
≥95th (%) index	66.7%	12	82.4%	17	79.2%	24	0.295	0.069
≥75th (%) relatives ^f	95.7%	21	90.0%	31	61.5%	28	0.601	0.001
≥95th (%) relatives ^f	52.2%	21	63.3%	31	44.2%	28	0.296	0.350
Cosegregation	35/36 (97.2%); 0/2 (0%)		87/91 (95.6%); 0/9 (0%)		38/48 (79.2%); 12/16 (75%)		NA	NA

Statistically significant values are bold.

NA, not applicable

^aValues are expressed as mean ± SD. ^bP value based on comparison of p.Arg406Trp carriers versus functional alterations carriers. ^cP value based on comparison of p.Arg406Trp carriers versus neutral alterations carriers. ^dValues of LDL-C are corrected for medication (with statin ×1.3 and statin plus ezetimibe ×1.5). ^eTreatment and pretreatment values. ^fAmong alteration carriers.

Discussion

A detailed analysis of the phenotype and genotype presented by 55 families with clinical diagnosis of FH and 13 different alterations in the *LDLR* was conducted. The aim of this investigation was to highlight the importance of the integrated analysis of clinical, molecular, and functional data for the correct diagnosis of FH. Of these 13 alterations, 7 have been described in other populations as well as in a Portuguese FH cohort. All variants, except two that are novel, are described in the FH database as putative mutations causing disease, and eight did not have functional studies. For this reason, an extensive functional characterization of these eight *LDLR* alterations was performed, allowing the functional assessment of these variants. Pathogenicity status was attributed to five alterations, and three had a neutral effect on protein activity. To increase accuracy, data on five neutral alterations with functional characterization, and found before in different

populations, were included in the analysis. Carriers of neutral alterations presented a significantly lower incidence of premature cardiovascular disease, lower concentrations of atherogenic lipoproteins, and also a lower LDL-C percentile than functional alterations carriers. The cosegregation of the variants with the hypercholesterolemia phenotype was less well established for neutral variants; only 78.7% of the hypercholesterolemic subjects were alteration carriers, and 87.5% of normolipidemic individuals presented these alterations compared with 96.3% and 0%, respectively, for functional alteration carriers. Deciding which alterations are pathogenic based on the lipid profile is not straightforward, however, and if there are no data of hypercholesterolemic or normolipidemic individuals within the same family, performing cosegregation studies is also not possible. Only when data of at least 50 alteration carriers are known is it possible to use cosegregation to assess a variant's pathogenicity,¹³ and usually this is not an easy requirement to fulfill in the majority of FH cohorts. In silico analysis was also not conclusive for the majority of the alterations, having assessed correctly only 7/13 alterations. So, functional study is essential to determine a variant's pathogenicity, and these studies can be performed by any research laboratory with access to a flow cytometer and a confocal microscopy since the protocol has been published,²⁴ or collaborations can be established. Nevertheless, when the lipid and molecular profiles of affected and nonaffected relatives are known, this information is useful for the pathogenicity assessment of a variant, and it should always be taken into consideration.

The importance of this integrated analysis of clinical, molecular, and functional data is demonstrated by the assessment of p.Arg406Thr, a mutation described worldwide but for which functional assays have not been performed. The lipid profile and cosegregation analysis of p.Arg406Thr carriers and the other functional mutations carriers under study are similar and a statistically significant exists between these and carriers of neutral alterations for all atherogenic particles except high-density lipoprotein cholesterol, apoA1, and triglycerides. This indicates that this alteration is probably a disease-causing mutation. When the LDL-C percentiles were analyzed, however, the results were not as straightforward; the prevalence of index cases with the alterations and with LDL-C \geq 75th percentile and LDL-C \geq 95th percentile was lower for p.Arg406Thr (although without statistical significance) than for index carriers of the neutral or functional alterations. For relatives, the lower prevalence was seen for neutral alterations carriers but without significant differences. In conclusion, this phenotype/genotype analysis was not able to produce a valid pathogenicity assessment for p.Arg406Thr. When the functional study was performed, the LDLR activity was determined and the reason for the oscillation in the lipid profile was understood; this variant retained 60% of LDLR activity concerning expression, binding, and internalization. This allows a total activity of the LDLR receptor of 80% (assuming that the nonmutated allele produces 50% of the active protein) compared with 50–55% that is seen for a null mutation or for the mutations characterized in this study (p.Ile442Thr, p.Gly545Trp, and p.Cys698Phe). By contrast, p.Gly76Trp—classified here as neutral—presented a total activity of about 95% for binding and internalization. Because the p.Arg406Thr variant retains 60% activity, it can be considered a mild mutation, and the variation in the phenotype of carriers can be attributed to environmental factors that are known to affect the phenotype, even in patients with FH.³⁰ The cutoff value for determining whether an LDLR variant is considered a functional mutant by in vitro studies has not been established, but, based on several published studies,^{1,27,29,31,32,33} in vitro LDLR activity less than 70–80% (either in expression, binding, or internalization), corresponding to 85–90% total LDLR activity, could classify a variant as pathogenic.

The functional classification of a variant is also important for patient management so patients can be advised according to their condition. Because carriers of these neutral alterations present a milder phenotype, most probably do not have FH and therefore need different counseling and treatment approaches to tackle their dyslipidemia. The results obtained for p.Arg406Thr can also have a clinical implication; these patients probably need a less aggressive medication to control their LDL-C concentrations because their mutant LDLR still retains some activity (60% for the mutated allele, 80% in total). This way the determination of the variant functionality can be a step forward for the personalized treatment of patients with FH.

The integrated analysis presented here is important for the correct assessment of patients with FH who might otherwise be misdiagnosed. A detailed analysis of the protein at the molecular level, adding to the clinical and molecular data already obtained routinely, provides information relevant to understanding the phenotype observed in these patients and that can be translated into clinical management improvements.

Disclosure

The authors declare no conflict of interest.

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Author Contributions

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