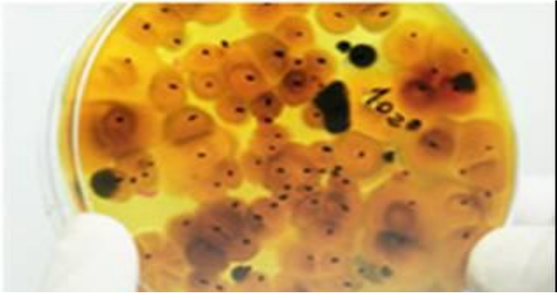

IN SILICO VERSUS IN VITRO ANALYSIS OF LDLR MUTATIONS

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Estudo Português de Hipercolesterolemia Familiar

BACKGROUND

The LDL receptor (LDLR) is a glycoprotein that mediates binding and internalization of cholesterol-rich lipoproteins from plasma. Mutations in the LDLR gene are the major cause of familial hypercholesterolaemia (FH), which results in impaired catabolism of circulating LDL. This common autosomal inherited metabolism disorder leads to premature atherosclerosis and increased risk of CHD. Many different mutations (currently more than 1300) have been identified in FH patients, but not all give rise to a defective LDLR.

Aim: The aim of this study was to compare results of *in silico* and *in vitro* functional analysis of 5 missense and 9 putative splicing mutations found in LDLR of patients of the Portuguese FH Study.

METHODS

Different LDLR mutants (p.V429L, p.W490R, p.S648P, p.P685S, p.V859M) were generated by site-directed mutagenesis and expressed in CHO–*ldl*A7 cells lacking endogenous expression of LDLR. To determine the effects of the different mutations on LDLR function we measured saturable uptake (binding plus internalization) and degradation of ^{125}I-labelled LDL at 37ºC and performed immunofluorescence microscopy of whole cells. *In silico* analysis was performed using different bioinformatics tools: PolyPhen, PolyPhen-2 and SIFT for missense mutations and Splice Site Score Calculation and Analyzer Splice Tool for splicing mutation.

RESULTS

- All mutant constructs were expressed as stable transfectants in CHO-*ldl*A7 cells lacking endogenous receptor protein expression (Figure 1), for variants p.V429L and p.W490R two bands were detected, though the mature form of the protein was greatly reduced and we could see an increased signal for the precursor form (apparent molecular weight ~90 kDa) of the receptor protein, more evident in variant p.W490R (Figure 1, lanes 2-3).
- Cells expressing constructs p.W490R and p.V429L were severely impaired in their ability to mediate uptake and degradation of ^{125}I-LDL (<15% of normal LDLR) (Figure 2 and 3).
- Variant p.S648P retained ~40% and variant p.P685S ~60% of normal LDLR activity (Figure 2 and 3).
- p.V859M cells showed essentially the same activity as cells expressing wild-type LDLR (Figure 2 and 3).
- *In silico* analysis failed to predict the correct effect on protein function of 4/5 missense mutations (table 1.). Only the functional assessment of variant p.P685L was correct for all software.
- *I n silico* splice site analysis fail to predict correctly the functional effect of 2/9 alterations (table 2.). In this case the bioinformatics tools is most similar results between them.

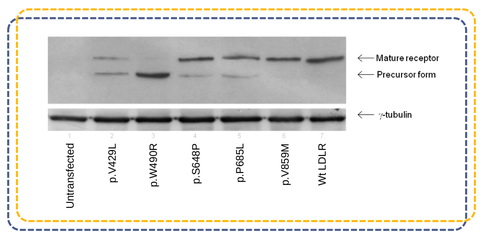

Figure 1. Western blot analysis of wild-type and mutant LDLR biosynthesis in CHO-ldlA7 cells. Whole cell extracts (30 µg) were fractioned in non-reducing 7% SDS-PAGE, transferred onto nitrocellulose membranes for incubation with a rabbit polyclonal anti-hLDLR antibody or rabbit monoclonal anti-γ-tubulin antibody and detected by chemiluminescence. In non-reducing conditions, mature LDLR runs at an apparent molecular weight of 130 kDa and the precursor form at 90 kDa.

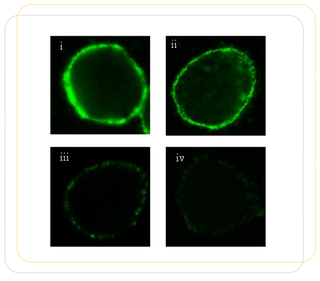

Figure 2. Mature LDLR protein present at cell visualised as membrane-bound fluorescence: confocal microscopy of immunostained cell surface LDLR for CHO-*ldl*A7 cells (A) expressing normal (i) and LDLR variants p.V859M (ii), p.S648P (iii), and p.V429L (iv).

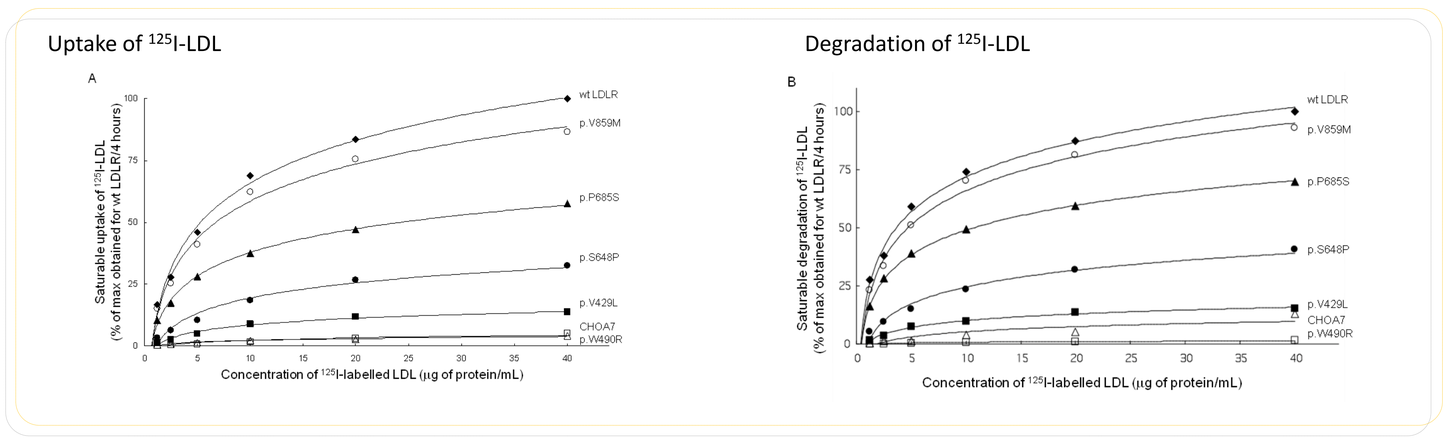

Figure 3. Uptake and degradation of 125I-LDL in CHO-ldlA7 cells. For analysis of 125I-LDL uptake (binding plus internalization) (A) and degradation (B), untransfected cells (CHOA7) and cells expressing the different variants of LDLR were incubated for 4 hours at 37ºC with increasing concentrations of 125I-LDL as indicated. Subsequently, cells were assayed for cell-associated radioactivity (uptake) and degradation of the protein component of LDL. Values were corrected for non-saturable association or degradation determined in the presence of an excess of unlabelled LDL (1 mg/mL).

Table 1. Comparison of results between *in silico* and *in vitro* analysis of missense alterations.

Putative mutation	Polyphen Prediction	Polyphen-2	SIFT Prediction
p.V429L	X	√	√
p.W490R	√	√	X
p.S648P	X	√	X
p.P685S	√	√	√
p.V859M	√	X	√

X – Failed to predict effect; √ - correctly predicted effect

Table 2. Comparison of results between *in silico* and *in vitro* analysis of splicing alterations.

Putative splicing mutation	SSSC	SSAT
c.190+4insTG	√	√
c.313+6T>C	√	√
c.818-2A>G	√	√
c.1060+1G>A	√	√
c.1061-8T>C	√	√
c.1359-5C>G	√	X
c.1845+1delG	√	√
c.2140+5 G>A	X	X
c.2389G>T (p.V797L)	√	√

X – Failed to predict effect; √ - correctly predicted effect

DISCUSSION & CONCLUSSION

Assessing the pathogenicity of the different variants found in patients with clinical diagnosis of FH is of great importance to distinguish pathogenic mutations from rare silent variants and has clinical implications for determining the associated cardiovascular risk. LDLR functional assays showed that variants p.V429L, p.W490R and p.S648P of the LDLR coding sequence severely impaired receptor function, while variant p.P685S had a milder effect and cells carrying p.V859M variant had LDL clearance rates comparable to cells expressing normal LDLR.

In silico analysis failed to predict correctly the effect of 4/5 alterations for missense alterations and 2/9 for splicing alterations. *In silico* analysis has improved during the last years but still needs to be further optimised to be used for this purpose.

Just finding an alteration in the LDLR gene sequence does not mean that it is the cause of a patients hypercholesterolaemia, as proven by the results for the p.V859M and other variants previously described (p.e. T726I, c.2140+5).

The correct assessment of mutation pathogenicity can only be done by in vitro studies and independent studies should be sought to guarantee the rightness of results.

Acknowledgements: Ana Catarina Alves was funded by FCT SFRH / BD / 27990 / 2006 ; project grant FCT_PTDC/SAU-GMG/101874/2008; S Silva was partially supported by a **Short Term Fellowship** from **Fundação Calouste Gulbenkian**, Portugal.