# *In silico* Analysis of a Nonsense Mutation Linked to Autosomal Recessive Hypercholesterolemia Type 4

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**ABSTRACT**
Autosomal recessive familial hypercholesterolemia-4 (FHCL4) is a genetic disorder caused by mutations in *LDLRAP1*, a gene encoding a protein that allows the LDL receptor to be endocytosed and degraded in the liver. *In silico* tools were used to examine the rs121908325 variant linked to FHCL-4. The variant protein LDLRAP1$^{Q136X}$ affects the coding region of *LDLRAP1*, resulting in a glutamine amino acid being changed into a stop codon, truncating the protein at amino acid 136. Phylogenetic analysis of ten different animal species demonstrated that glutamine 136 is 100% conserved in the LDLRAP1 protein from the human protein to flies and frogs. An NCBI Conserved Domain Search for LDLRAP1 indicated that the LDLRAP1$^{Q136X}$ variant truncates the protein within a peptide binding domain, a phosphoinositol binding domain, as well as a domain that specifies the Pleckstrin protein superfamily of signaling proteins. IntFold 3D rendering of wild type (WT) and LDLRAP1$^{Q136X}$ proteins showed that the variant has a significant alteration in 3D structure, based on the loss of 172 amino acids. Further *in silico* analysis using amino acid interaction software showed that the LDLRAP1$^{Q136X}$ protein was missing its phosphotyrosine binding pocket. The rs121908325 mutation is rare, and none of the 70 genomes analyzed during a nutrition course at a large research I university carried this allele. These *in silico* studies demonstrate that the nonsense mutation at position glutamine 136 would impact specific domains of LDLRAP1, ultimately inhibiting the protein's ability to perform its function of facilitating removal of the LDL receptor from cell surfaces, leading to increased circulating cholesterol levels and potential cardiovascular health complications.

**KEYWORDS**
Cholesterol; Hypercholesterolemia; Low density lipoprotein; LDL; LDLR; LDLRAP1; Autosomal recessive hypercholesterolemia-4 (FHCL4); heart disease

## INTRODUCTION

Cholesterol is a waxy substance used to synthesize steroid hormones, make bile, insulate nerves, and form cell membranes. Its metabolism is regulated by exogenous and endogenous sources. In the endogenous pathway, the liver and extrahepatic tissues synthesize cholesterol, which enters circulation via lipoproteins secreted into bile. In the exogenous pathway, cholesterol from dietary and biliary sources is absorbed in the intestine and enters the bloodstream via chylomicrons. Humans consume about 300700 mg of cholesterol each day and about 1000 mg is secreted into bile, leaving a total of 1300-1700 mg of cholesterol to be metabolized in the intestines daily.[1]

Cholesterol is hydrophobic and is transferred throughout the body by two types of carriers. LDL carriers circulate cholesterol through the blood, and HDL carriers sequester cholesterol from the blood to the liver for recycling or degradation.[2] Elevated LDL cholesterol is tightly linked to atherosclerosis and cardiovascular disease. When cholesterol is stuck in the bloodstream arteries become more rigid, thicker, and harder.[3] Following an endothelium injury, an inflammatory response can result in a lesion. The lesion develops into a fatty streak with monocytes, platelets, and oxidized LDL accumulating in the injured area. Monocytes turn into macrophages and gorge the LDL using the LDL receptor (LDLR) on their cell surface. They then turn into foam cells. The fatty streak continues to grow and develops into plaque. A fibrous cap can form over the plaque, which occludes the blood vessel.[4] If a clot forms and blood flow stops completely, a myocardial infarction or stroke may occur. Although lifestyle factors contribute to these risks, hypercholesterolemia is often inherited, as some individuals simply make more cholesterol by *de novo* synthesis.[5]

Autosomal recessive familial hypercholesterolemia (FHCL) can be divided into four subtypes, each with variants within different genes along the cholesterol metabolism pathway. FHCL1 is associated with mutations of the *LDLR* gene, but also can have associated mutations in *APOA2*, *EPHX2* and *GHR*.[6] FCHL2 is associated with mutations in *APOB*, which acts as a ligand for

LDLR, while FHCL3 is caused by mutations in PCSK9 which binds to LDLR and inhibits LDL uptake.[6] The focus of this research is on the fourth type, FHCL4, which is linked with mutations in the gene encoding the low-density lipoprotein receptor adaptor protein 1 (*LDLRAP1*).[6] The main treatments for FHCL4 are lifestyle modifications: a diet low in saturated and trans fats, regular physical activity, weight control, and smoking cessation.[7] Statins can be used starting as early as age eight.[8] These lipid-lowering medications reduce the activity of hydroxymethyl glutaryl coenzyme A reductase, the enzyme responsible for cholesterol synthesis.[8] Statins are safe and effective for most patients of any age or gender, minimizing the risk of major CAD events by about 30 percent.[2,8]

LDLRAP1 interacts with the cytoplasmic tail of LDLR.[9-11] When this interaction occurs, LDLR is endocytosed into cells, resulting in removal of about 70% of LDL from circulation, decreasing plasma total cholesterol levels.[3,12] This process occurs in the liver and in macrophages, where the receptor with LDL can be degraded.[13] This process serves as a protective mechanism against hypercholesterolemia and injuries to the arterial wall. Deficiencies in *LDLRAP1* also cause macrophages to be unable to internalize and degrade LDL in the liver, leading to an increase in circulating cholesterol.[13,14] When circulating cholesterol is high, plaque is deposited in the coronary arteries and proximal aorta. The risks of an *LDLRAP1* defect are tendon xanthomas, atherosclerosis, and premature coronary artery disease (CAD). These risks are similar to those in individuals whose hypercholesterolemia is a consequence of their lifestyle.[14]

FHCL4 is caused by homozygous variants in the *LDLRAP1* gene, and as such is a recessively inherited condition.[9] The *LDLRAP1* gene is localized to chromosome 1, spanning 25 kb with 9 exons and 8 introns.[9,15,16] A single nucleotide variant (SNV) in *LDLRAP1*, rs121908325, has been found in a few families but is extremely rare, with an alternate allele prevalence of 0.003%.[17] The variant results in a premature stop codon, or nonsense mutation.[3,17] Only five studies describe patients with this variant, but none examined the functional consequences of this variant.[3,14,18-20] The first article describing this variant was from 1973[19], followed by an article from 2001 describing the location of *LDLRAP1* on chromosome 1p36.[3] In the 2001 article, rs121908325, along with five other variants, were identified as affecting the protein coding region of the gene.[3] It was not until 2016 when Farhed and colleagues described the Q136X variants as a founder mutation in Lebanese ancestry.[18]

The objective of this study was to evaluate the consequences of the rs121908325 nonsense mutation in the LDLRAP1$^{Q136X}$ variant protein. This work tests the hypothesis that *in silico* tools can be used to predict how the variant protein, LDLRAP1$^{Q136X}$ loses its intended purpose of removing LDL, leading to FHCL4.

## METHODS AND PROCEDURES

*In silico* methods were used to study the LDLRAP1 protein and the variant associated with FHCL4.

### *Initial identification of the variant*

A 2001 genome linkage study by Garcia and colleagues was initially used to identify the rs121908325 variant as the variant of interest for this study.[3] The study included four families with FHCL4: two Lebanese and two Sardinian. The individuals that inherited FHCL4 were the result of consanguineous mating. Studies by Sirinian and Nagai were also observed to further observe the interactions between LDLRAP1 variants and LDLR.[21,22]

### *Sequence analysis of LDLRAP1*

The NCBI protein sequence database was used to obtain protein sequence for LDLRAP1.[15] The "FASTA" tool was used to obtain the amino acid sequence of the reference protein sequence. Since the variant is a nonsense mutation, the variant amino acid sequence was constructed manually by deleting all amino acids following amino acid 136 (LDLRAP1$^{Q136X}$).[16,17] These two sequences were compared with the amino acid sequence from other animals using the NCBI orthologs database[15] and the COBALT Constraint-based multiple alignment/phylogenetic analysis tool.[23] *Homo sapiens* gene (NP_056442.2), *Mus musculus* (NP_663529.2), *Rattus norvegicus* (NP_001102741.1), *Danio rerio* (NP_001074104.1), *Xenopus tropicalis* (NP_001017114.1) *Macaca mulatta* XP_001107620.3), *Bos taurus* (NP_001077137.1), *Gallus gallus* (XP_015153218.2), *Canis lupus familiaris* (XP_855049.1), and *Sus scrofa* (XP_003127756.1) were selected. To observe conserved domains, the Conserved Domains database was used.[24] The gene name, *LDLRAP1*, was searched to find the cd13160 phosphotyrosine binding PH-like fold domain.

### *3D structural analysis of LDLRAP1 WT and variant sequences*

The FASTA sequences from the WT LDLRAP1 protein sequence (NP_056442.2) and the manually constructed variant sequence LDLRAP1$^{Q136X}$ were pasted into the IntFold feature of the Biomedics Web Servers Model.[25,26] The Protein Databank (PDB) files were downloaded, and PyMol[27] was used for molecular imagining. These images were compared visually to identify differences between the structure of the reference and LDLRAP1$^{Q136X}$ proteins.

*FunFold Analysis of Protein Domain Interactions*

The Biomedics Web Servers Model subprogram FunFold directly predicts possible ligand interactions.[28] The prediction was done using the amino acid sequences for both proteins and data for ligand interaction, as amino acids interacting with each ligand were collected. The PDB files were downloaded from FunFold and visualized using PyMol[27] software.

*ClinVar database analysis of SNP positions.*

The ClinVar database through NCBI[15,29,30] was used to link to Variation Viewer which shows the location of 52 pathogenic variants along the length of the *LDLRAP1* gene. This information was captured as a screenshot which was further annotated to produce the figure.

*GTEX analysis of gene expression*

GTEX multiple gene expression portal analysis[31] was used to compare the expression pattern of the LDL receptor mRNA (LDLR) and LDLRAP1. Both gene names were put into the search bar on the GTEX multi-gene query box with all tissues selected. The data showed the levels of the mRNA from each gene in tissues from the database.

*SNP analysis in DNA dataset*

Participants aged 20-23 enrolled in a Nutrition course at Research I university took part in a double-blind study that analyzed their genotypes and phenotypes (IRB protocol #24-150, approved as exempt). Each participant sent in a saliva sample via a 23andMe® kit and used numeric identifiers to upload their SNP data to a secure database site. The participants also completed surveys on health status. In the post-survey completed after receiving their 23andMe® kit results, participants were asked, "Do you know your cholesterol levels?" The results from the survey were compared with the raw genotype data received from the 23andMe® kit results.

**RESULTS**

The first step in analyzing the *LDLRAP1* gene was to compare the amino acid sequence of the protein across species and determine the homology. HomoloGene[15] showed that the glutamine at position 136 was well-conserved across species. As shown in **Figure 1**, comparison on the LDLRAP1 sequence from *Homo sapiens* (NP_056442.2) with *Mus musculus* (NP_663529.2), *Rattus norvegicus* (NP_001102741.1), *Danio rerio* (NP_001074104.1), *Xenopus tropicalis* (NP_001017114.1) *Macula mulatta* (XP_001107620.3), Bos taurus (NP_001077137.1), Gallus gallus (XP_015153218.2), Canis lupus familiaris (XP_855049.1), and Sus scrofa (XP_003127756.1) indicated a 100% conservation of glutamine 136 in these species, suggesting its importance in the structure and function of the LDLRAP1 protein. Based on the NCBI BLAST analysis and RefSNP report, the LDLRAP1$^{Q136X}$ protein generated by rs121908325 ended at amino acid 136 due to a nonsense mutation converting glutamine to a stop codon, truncating the protein.[16,17] Furthermore, 100% of the amino acids immediately following the position of the stop codon caused by rs121908325 were conserved. Overall, there were only 22 amino acids in the human sequence which differ or were absent compared to other species in our analysis. This yielded an overall conservation of the protein between species of 93%.

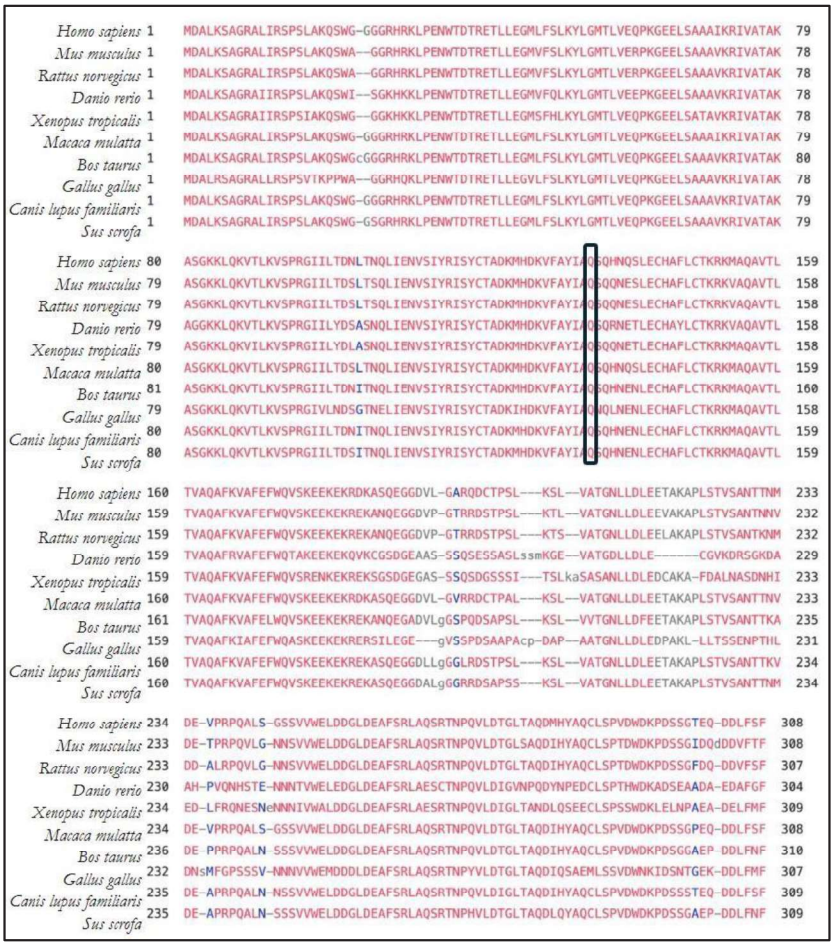


**Figure 1. Phylogenetic analysis of LDLRAP1 orthologs.** The amino acid sequences of *Homo sapiens* (NP_056442.2) *Mus musculus* (NP_663529.2), *Rattus norvegicus* (NP_001102741.1), *Danio rerio* (NP_001074104.1), *Xenopus tropicalis* (NP_001017114.1) *Macula mulatta* (XP_001107620.3), *Bos taurus* (NP_001077137.1), *Gallus gallus* (XP_015153218.2), *Canis lupus familiaris* (XP_855049.1), and *Sus scrofa* (XP_003127756.1) from top to bottom. The glutamine at position at 136 which is mutated by rs121908325 is indicated by the black box and is 100% conserved. Amino acids that differ from the reference sequence are colored in blue, while identical and homologous (charge, polarity, etc.) amino acids are in red. Grey amino acids are shown when that sequence is absent in one or more of the analyzed homologues. Dashes indicate spacing differences between sequences.

The 3D renderings for both the WT and the variant protein as predicted by the Biomedics Web Servers Model IntFOLD program[25,26] were compared in **Figure 2**. The LDLRAP1 reference protein model was quite large, whereas the variant protein, LDLRAP1$^{Q136X}$, was far less extensive due to the nonsense mutation that truncated it at position 136. In the reference protein, several domains are present past serine 135, which are missing in the mutant protein, including a large loop that connects to an alpha helix and to the end of the protein. The significance of these domains was not specifically tested using the modeling. However, the NCBI conserved domain database[15] was used to determine whether the nonsense mutation removed or altered any binding domains. The results of this analysis showed that the LDLRAP1 protein contains a phosphotyrosine binding/ phosphotyrosine-interaction domain (**Figure 3**). Notably, the nonsense mutation that truncates the protein at amino acid 136 for LDLRAP1$^{Q136X}$ is in the middle of that binding domain.

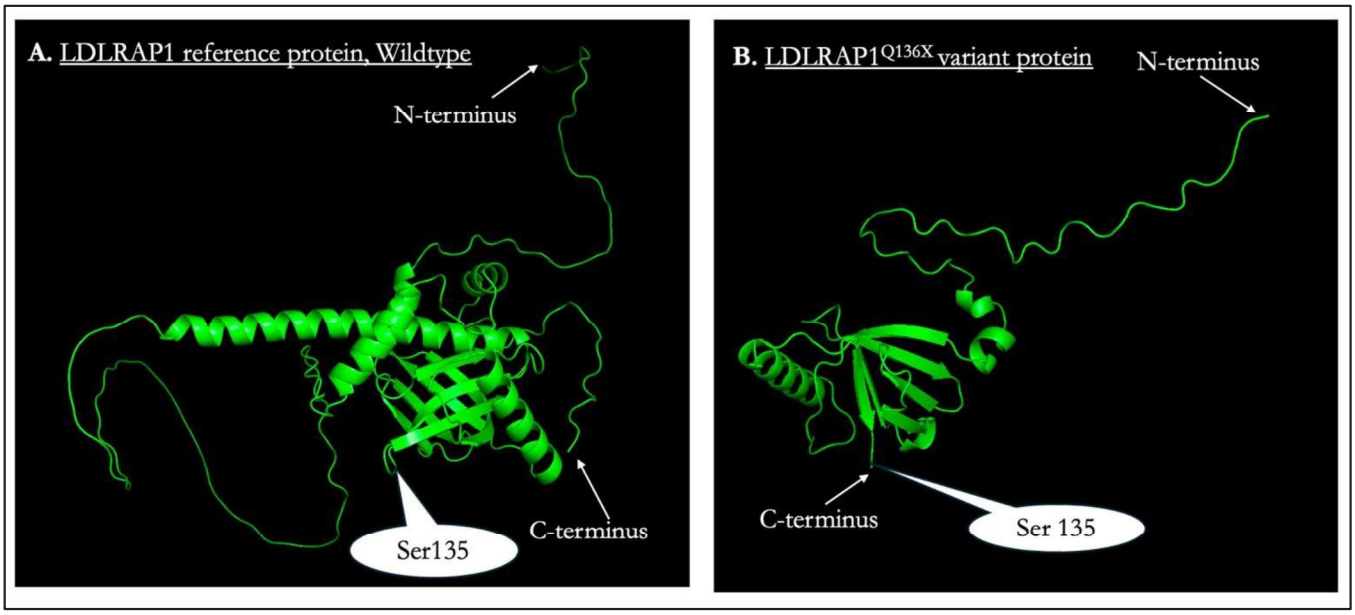


**Figure 2. IntFOLD 3D rendering of LDLRAP1. A.** The reference protein sequence[9] was input into the IntFOLD server.[25,26] **B.** The LDLRAP1$^{Q136X}$ sequence containing the deletion found in rs121908325 was generated by modification of the reference project sequence and input into the IntFOLD server. The N- and C- terminus, along with the position of serine 135 which is the last amino acid before the deletion is shown for both structures. The IntFOLD server was used with the datafiles displayed using PyMol.[27]

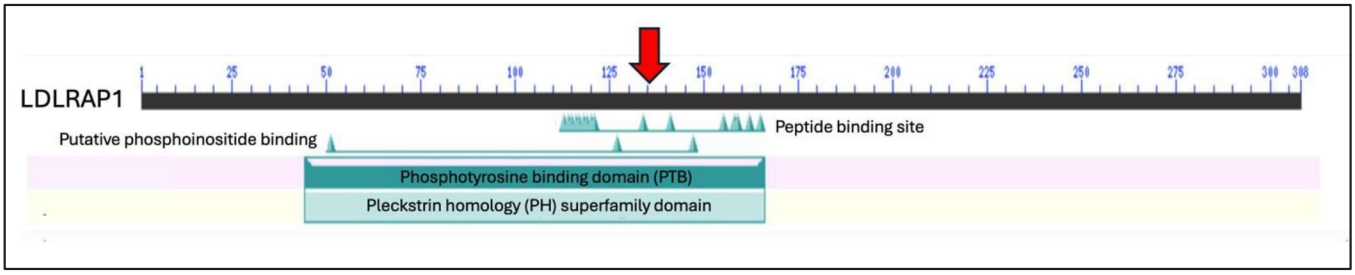


**Figure 3. NCBI Conserved Domain Search for LDLRAP1.** The LDLRAP1$^{Q136X}$ variant protein is truncated at amino acid 136, indicated by the red arrow. This region of the protein contains a peptide binding site, and a phosphinositol binding site, with interacting amino acids indicated by triangles. This region includes the mammalian phosphotyrosine binding domain, indicated by the dark green color (PTB), and is in a region that defines the Pleckstrin homology (PH)like superfamily of proteins, a large family of signaling proteins, indicated by the lighter green box.

FunFold database[28] was used to identify amino acid-ligand interactions in the reference and variant proteins. As shown in **Figure 4**, the reference protein contains a predicted tyrosine binding pocket that is missing in the variant protein LDLRAP1$^{Q136X}$, although the software highlights two tyrosine residues in the variant protein. These two residues are far upstream (amino acids 114 and 122) of the predicted interaction site with LDLR (amino acids 259 (Phe), 262 (Ala), 263 (Leu), 266 (Arg), 272 (Leu), 274 (Thr)) in the reference protein.

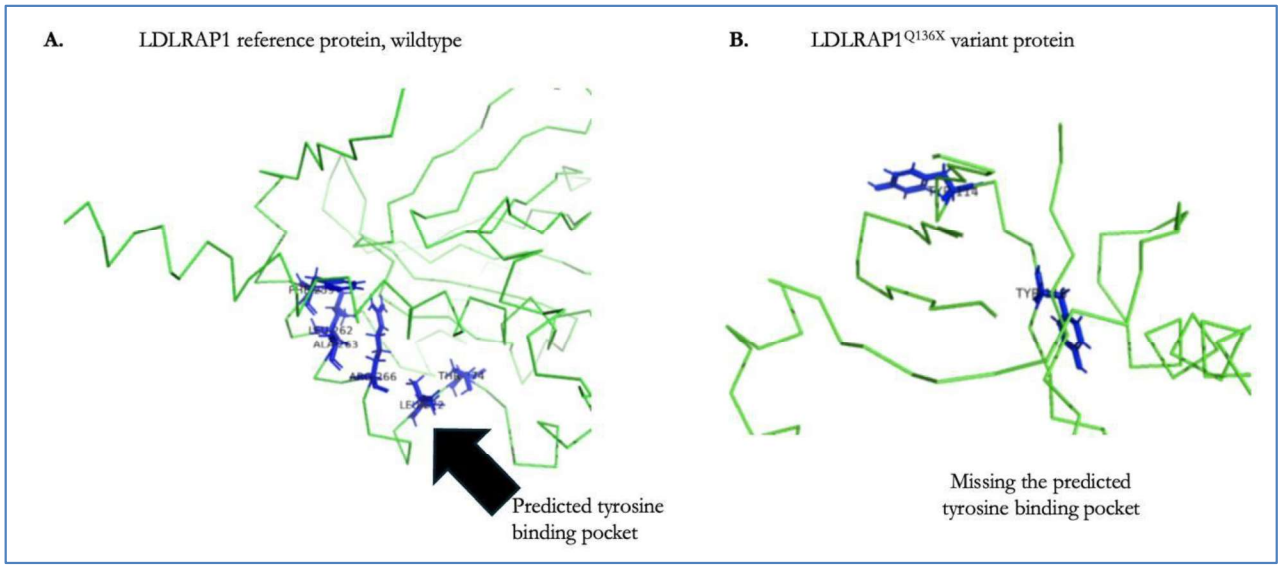


**Figure 4: Amino acid interaction predictions. A**. LDLR is phosphorylated following LDL binding, and LDLRAP1 binds to phosphotyrosine on LDLRAP1 at the PTB binding domain (arrow). The tyrosine interaction sites are at amino acids 259 (Phe), 262 (Ala), 263 (Leu), 266 (Arg), 272 (Leu), 274 (Thr) **B.** LDLRAP1$^{Q136X}$ is missing the predicted tyrosine binding pocket, with tyrosine amino acids indicated (Tyr 114 and Tyr 118) indicated in blue, but these amino acids further upstream than the predicted site in the reference protein, and not part of the predicted binding site for the reference protein. This structure is predicted not to bind to LDLR by the FunFold software.

Analysis of clinical pathogenic and likely pathogenic variants along the length of the *LDLRAP1* gene using ClinVar database[29,30,32] demonstrated that there are pathological variants located 3' to the position of rs121908325 (**Figure 5**). There are 29 pathogenic variants prior to rs121908325, and 22 following rs121908325. These data suggest that important functional domains are removed by the early termination that occurs for carriers of this variant.

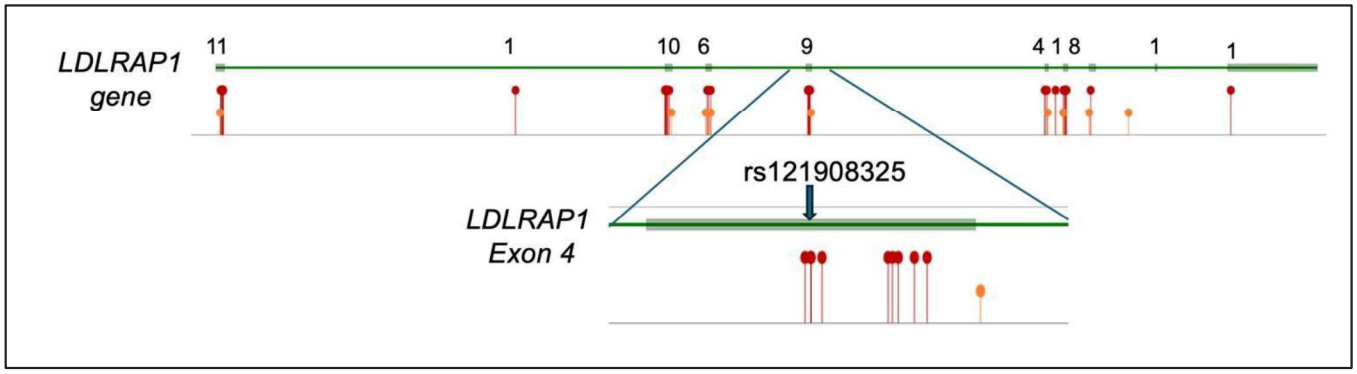


**Figure 5: Pathogenic variants in LDLRAP1.** The intron exon structure of the *LDLRAP1* gene is shown, with the locations of the pathogenic (red markers) and likely pathogenic (orange markers) variants shown, as mapped by the CLINVAR database.[29,30,32] The number of variants is shown above each exon/intron area for reference. Exon 4 is highlighted as is the position of rs131908325.

LDLRAP1 is an accessory protein for the LDL receptor, LDLR. GTEX multiple gene expression analysis[31] was used to examine co-expression of LDLR and LDLRAP1 to identify tissues that would be affected in rs121908325 carriers. As shown in **Figure 6**, LDLR and LDLRAP1 have similar mRNA expression levels in tissues with some notable differences. LDLR is highly expressed in the lung, adrenal gland, fallopian tube, esophagus, ovary, and cultured fibroblasts, while LDLRAP1 is highly expressed in the spleen, cervical region of the spinal cord, cerebellum, and cerebellar hemisphere. Both have moderate expression in the liver and blood cells.

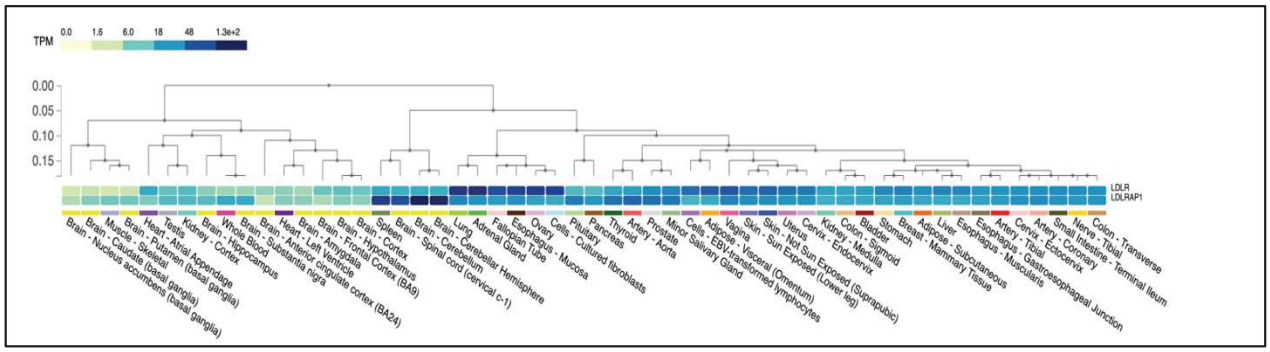


**Figure 6. GTEX multiple gene expression analysis** showing the co-expression level interaction between LDL and LDLRAP1. The Genotype Tissue Expression (GTEX)[31] online software was used to generate this figure.

Variant rs121908325 is rare, with NCBI reporting a worldwide alternate allele frequency of 0.003%.[17] Analysis of the raw data from 134 participants who took a 23andMe® test found only the reference genotype of CC for the rs121908325 variant, meaning that all participants had a normal genotype for this variant and would be expected to produce the full length LDLRAP1 protein. From the survey, 53 students did not know their cholesterol levels, six students reported high cholesterol levels, 68 reported normal cholesterol levels, and seven reported low cholesterol levels.

## DISCUSSION

The rs121908325 variant of *LDLRAP1* results in a nonsense mutation at amino acid 136 and early termination of the protein. While the variant has been previously reported, no additional *in silico* studies had been done to map the region lost by the early termination. Based on the findings from our *in silico* analysis, the hypothesis that this variant would disrupt protein function is supported. The results predict that LDLRAP1$^{Q136X}$ cannot serve its function of removing LDL, resulting in FHCL4.

In addition, the amino acid glutamine, located at the position of the termination event, was well-conserved across species, suggesting that its function within this area is also conserved across species. Because a few of the lower species were missing a few amino acids after glutamine, this area may be susceptible to changes in the amino acid sequence. However, no species tested was missing the entire domain of the protein past amino acid position 136.

The area of LDLRAP1 around amino acid 136 contains binding sites for signaling proteins. As a result, the mutated protein will likely not communicate with other cells and bind LDLR. The 3D rendered shape of the variant LDLRAP1 protein was utterly distinct from that of the full-length WT protein. The change in structure likely results in a change of function. In particular, the variant LDLRAP1 protein is missing the phosphorylated tyrosine binding pocket, so signaling proteins that use a tyrosine amino acid to interact with LDLRAP1 cannot bind to the tail of LDLR. With the loss of those domains, it is predicted that LDLR cannot serve its function of shuttling LDL out of the bloodstream and into hepatocytes or macrophages for degradation.

LDLRAP1 and LDL were expected to be most highly expressed in the liver. However, GTEX expression analysis revealed that LDLRAP1 was highly expressed in the adrenal gland, fallopian tube, esophagus, ovary, and cultured fibroblasts. This means that the protein may associate with other receptors and serve a different function in those tissues.[23] LDLRAP1 also have other binding partners whose cellular locations could be affected by this variant.[21] LDLR was most expressed in the lung, adrenal gland, fallopian tube, esophagus, ovary, and cultured fibroblasts. This supports the role of LDL in metabolism and synthesis of steroid hormones.

None of the participants included in the 23andMe™ study had the LDLRAP1$^{Q136X}$ variant, as was expected due to its worldwide population rarity. However, this variant has been shown to be a founder variant for individuals of Lebanese or Turkish descent.[18] In analyzing the 23andMe™ results, 19 students indicated Eastern European ancestry, which includes Turkish ancestry, and nine students indicated Northern African ancestry, which includes Lebanese ancestry. Our dataset did not further delineate ancestry data from these main groupings on the 23andMe report. Six participants in the study reported high cholesterol despite having the normal allele studied for the gene, meaning that other genetic variations and lifestyle factors played a role in serum cholesterol levels for these individuals. Given that the population in this study has an average age of 21.4 years, many participants had not yet had their cholesterol levels checked.

A previously published study found that in people who have two *LDLRAP1* variant alleles, hepatic LDLR function is impaired, resulting in clearance of LDL from the blood at a slower rate and reduction of HDL.[3] In our *in silico* analysis, LDLRAP1 expression was high in brain as well, and further studies should examine how brain function or neurons are impaired by variants

in LDLRAP1. These could include cognitive testing of carriers, or even cell-culture based studies to examine the role of LDLRAP1 protein in neurons. Furthermore, other work has found that LDLRAP1 is only recruited to the membrane after LDL binds, so a mutation would decrease its efficiency in binding to the receptor.[19] The data suggests that LDLRAP1 protein activity may be specific to LDLR function in polarized cells such as hepatocytes. This is consistent with the hypothesis that LDLRAP1 recycles LDLR from the lysosome to the basolateral cell surface.[3] Furthermore, another study found that patients with an *LDLRAP1* deficiency had over 20 times the amount of LDLR on the cell surface and 27 times the amount of LDLR outside of coated pits, upholding the information cited in the Garcia study.[3,12] The researchers concluded that *LDLRAP1* is required for LDL to bind to its receptor efficiently, and that function is impaired in patients with FHCL4.[12] Additional studies have shown that LDLRAP1 escorts megalin through endosomes and recycles endosomes in the Golgi region.[21] Megalin plays a role in the endocytosis of diverse proteins such as albumin, beta-2-microglobulin, Vitamin D binding protein, Vitamin A/retinol binding protein and even angiotensinogen in the renin-angiotensin system of blood pressure regulation.[33] Thus, it appears that interaction between LDLRAP1 and LDLR is essential for LDL clearance, but that LDLRAP1 may be involved in many other pathways.

## CONCLUSIONS

The findings from our *in silico* work confirmed that the *LDLRAP1*$^{Q136X}$ variant terminates the protein at position 136, resulting in loss of 172 amino acids. Given the loss of a significant portion of the protein, it is not surprising that this variant contributes to AHR. However, our new findings show that this deletion impacts the putative tyrosine amino acid binding domains of LDLRAP1, suggesting how and why the nonsense mutation impacts cell signaling in target tissues. We also provide new modeling that shows how deletion alters the predicted 3D structure of the remaining domains of the protein translated upstream to the deletion. Furthermore, our results allow us to predict that the presence of pathogenic variants in the DNA sequence downstream of rs121908325 impacts additional regions of the protein that are necessary to perform its function of removing LDL from circulation. Overall, these findings suggest that the truncation of LDLRAP1 due to the Q136X variant impairs its ability to efficiently remove LDL from circulation, potentially contributing to the development of cardiovascular diseases

Future research should focus on the mechanism by this variant in LDLRAP1 leads to FHCL4. For example, *in vitro* studies comparing the function of the LDLRAP1 and the variant in liver or brain cells, and in particular its interaction with LDLR would elucidate the variant's biological impact in cholesterol metabolism. Greater insight on this can lead to the discovery of treatments for hypercholesterolemia in general as the domains lost in rs121908325 variant are likely important signaling domains for the protein. Examination of the functions of LDLRAP1 and LDL in anatomical structures outside of the liver, and the effect of LDLRAP1 variants on megalin-associated pathway should be examined to determine if variants in this pathway are linked to the dysfunction in other tissues.

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Caroline Gardner graduated in May 2024 from Virginia Tech with a degree in Human Nutrition, Foods, and Exercise. She began her research in a Virginia Tech course called Eating For Your Genes and presented a poster at the Dennis Dean Undergraduate Research Conference. She plans to spend her first few years out of college as a medical scribe in Blacksburg, VA, then pursue a career as a physician assistant.

**PRESS SUMMARY**

Autosomal recessive familial hypercholesterolemia-4 (FHCL4) is a genetic condition caused by a defect in *LDLRAP1*. This gene makes a protein that helps the body get rid of LDL, or "bad" cholesterol. The researchers studied a variant called rs121908325 in this gene using online *in silico* databases and analyses, including those from the National Center for Biotechnology Information (NCBI). Their analysis showed that the rs121908325 polymorphism causes the protein to terminate early, likely rendering it incapable of removing LDL cholesterol. Using population analysis through NCBI, they found that this change is very rare. The rs121908325 variant has been associated with heart problems.