Title: The Clinical Genome Resource (ClinGen) Familial Hypercholesterolemia Variant Curation Expert Panel consensus guidelines for *LDLR* variant classification

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Conflict of interest notifications:

R.A.H. reports consulting fees from Acasti, Aegerion, Akcea/Ionis, Amgen, HLS Therapeutics, Novartis, Pfizer, Regeneron and Sanofi.

ABSTRACT

Purpose: In 2015, the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) published consensus standardized guidelines for sequence-level variant classification in Mendelian disorders. To increase accuracy and consistency, the Clinical Genome Resource (ClinGen) Familial Hypercholesterolemia (FH) Variant Curation Expert Panel (VCEP) was tasked with optimizing the existing ACMG/AMP framework for disease-specific classification in FH. Here, we provide consensus recommendations for the most common FH-associated gene, *LDLR*, where >2,300 unique FH-associated variants have been identified.

Methods: The multidisciplinary FH VCEP met in person and through frequent emails and conference calls to develop *LDLR*-specific modifications of ACMG/AMP guidelines. Through iteration, pilot testing, debate and commentary, consensus among experts was reached.

Results: The consensus *LDLR* variant modifications to existing ACMG/AMP guidelines include: 1) alteration of population frequency thresholds; 2) delineation of loss-of-function variant types; 3) functional study criteria specifications; 4) co-segregation criteria specifications; and 5) specific use and thresholds for *in silico* prediction tools, among others.

Conclusion: Establishment of these guidelines as the new standard in the clinical laboratory setting will result in a more evidence-based, harmonized method for *LDLR* variant classification worldwide, thereby improving the care of FH patients.

Key words: familial hypercholesterolemia, *LDLR*, ClinGen, ACMG/AMP, variant classification

INTRODUCTION

Familial hypercholesterolemia (FH) (OMIM: #143890) is a common (~1:250 individuals affected)[1] genetic dyslipidemia characterized by lifelong exposure to elevated low-density lipoprotein cholesterol (LDL-C) levels. Early identification and appropriate treatment are imperative for prevention of premature atherosclerotic cardiovascular disease; however, less than 10% of individuals with FH worldwide have been diagnosed.[2,3]

FH is predominantly caused by heterozygous variants in one of three genes: the LDL receptor gene (*LDLR;* >90% of molecularly defined cases), the apolipoprotein B gene (*APOB;* ~5-8% of cases), or the proprotein convertase subtilisin/kexin type 9 gene (*PCSK9;* ~1% of cases).[4] A single variant in the apolipoprotein E gene (*APOE;* p.Leu167del) can cause autosomal dominant hypercholesterolemia, and this variant may explain the cause of hypercholesterolemia in 1-2% of patients with FH phenotype in some countries.[5] Identification of a pathogenic variant in an FH-associated gene can strongly affirm a diagnosis, motivates and simplifies family-based 'cascade screening', has potential to direct therapeutic strategy and/or promote adherence, and may impact insurance coverage of certain medications. Genetic testing has increasingly become a central part of diagnosing FH in many countries. Genetic testing in FH is recommended by the United Kingdom National Institutes for Health and Clinical Excellence,[6] both the European and International Atherosclerosis Societies,[2] and an international expert panel convened by the FH Foundation and American College of Cardiology,[7] among others. The US Centers for Disease Control and Prevention Office of Public Health Genomics also recommends the use of genetic information in the care of FH.[8] Moreover, the American College of Medical Genetics and Genomics (ACMG) list *LDLR*, *APOB*, and *PCSK9* among the 59 'medically actionable'

genes,[9] which has, in part, led to frequent inclusion of these genes on commercially available clinical panels,[10] as well as direct-to-consumer tests.

With the increasingly widespread implementation of genetic testing for FH, it is becoming ever more essential to establish a consensus, standardized method for the clinical classification of identified variants. In a 2018 study of >6,500 FH-associated variants submitted to the ClinVar database, there were at least 12 different variant classification criteria (including internal laboratory-specific ACMG/AMP criteria or other classification methods) being used among 30 submitters from 14 different countries.[11] This heterogeneity leads to discordance in variant classification. For instance, 379 unique FH variants had conflicting classifications in ClinVar.

Application of the American College of Medical Genetics and Genomics / Association for Molecular Pathology (ACMG/AMP) guidelines[12] has been a major advancement toward achieving a more critical and consistent approach to variant classification for many disorders, including FH.[11] However, because these guidelines are meant to be generalizable to all Mendelian disorders, they include inherent ambiguities that may lead to differences in their classification and application among users. Indeed, 114 FH-associated variants in ClinVar have conflicting classifications despite each laboratory having cited the same ACMG/AMP guidelines as their applied criteria. Gene-specific modifications to these guidelines are essential to provide the clarity required for standardized variant classification.

In 2013, the Clinical Genome Resource (ClinGen) Consortium was established as a centralized collaborative resource that aims to define the clinical relevance of genes and variants.[13] Among their major initiatives is the commission of disease/gene expert panels to provide consensus specifications of ACMG/AMP variant classification criteria. The ClinGen FH Variant Curation Expert Panel (VCEP) has been tasked with providing gene-specific

recommendations for *LDLR*, *APOB* and *PCSK9*. Here, we describe consensus ACMG/AMP specifications for the *LDLR* gene, where more than 2,300 unique variants have been identified in patients with a clinical association of FH.[11]

MATERIALS AND METHODS

ClinGen FH Variant Curation Expert Panel

FH VCEP membership includes clinicians, laboratory diagnosticians, research scientists, genomic medicine specialists, and genetic counsellors, who share expertise knowledge in FH. To achieve international harmonization of variant classification practice, additional emphasis was placed on global representation with members coming from 12 countries (United States, Canada, Brazil, United Kingdom, Portugal, Spain, France, Netherlands, Czech Republic, Japan, Australia, and Israel). The FH VCEP is part of the larger ClinGen Cardiovascular Domain Working Group.

Specification of ACMG/AMP criteria

A core group of eleven FH VCEP members reviewed all criteria in the original ACMG/AMP guidelines and began to propose initial *LDLR*-specific modifications based on expert opinion and prior publications.[14] Proposed modifications were discussed frequently through conference calls, emails, and several in-person meetings at international conferences until consensus was reached. Proposed guidelines, in various iterations, were consistently evaluated in analyses using well-known variants ranging from pathogenic to benign. ClinGen's Sequence Variant Interpretation (SVI) committee provided feedback and suggestions, which were incorporated in multiple rounds of revisions. Finalized criteria were

ultimately voted upon and approved by all members of FH VCEP. Note that given differences in mechanisms of disease, prevalence, and penetrance, it was decided that *APOB*- and *PCSK9*-specific guidelines will be completed separately.

Validation and pilot testing

Following guideline approval from the SVI committee, a formalized pilot study of 54 *LDLR* variants was performed in the ClinGen Variant Curation Interface (VCI; https://curation.clinicalgenome.org/). The VCI is a publicly available, comprehensive resource that systematically facilitates individual and group-level curation activities in accordance with the ACMG/AMP guidelines.

Pilot study curations in the VCI were performed independently by two trained VCEP biocurators, followed by a review from two VCEP leadership members. Publicly available data used for curation were supplemented with internal case-level data from VCEP member laboratories. When applicable, internal laboratory data used in the classifications were uploaded and saved into the VCI. Following independent curation of the 54 *LDLR* pilot-variants, biocurators extracted the data from the VCI and sent it to the reviewers. Any discordance in the application of criteria codes or in the final classification for each variant was recorded. Discordances were resolved in discussion among the biocurators and reviewers. Final classifications were approved by the reviewers and were submitted to ClinVar under the FH VCEP affiliation. The ontology used for FH due to *LDLR* variation was 'hypercholesterolemia, familial' (MONDO:0007750), with semi-dominant inheritance (HP:0032113) and the reference sequence used for *LDLR* was NM_000527.5.

Rules for combining pathogenic and benign criteria follow the original ACMG/AMP scoring algorithm (Richards et al., 2015)[12] (**Table S1**).

RESULTS AND DISCUSSION

Summary of specifications

FH VCEP specifications for *LDLR* variant classification in FH are summarized in **Table 1**. The type of *LDLR*-specific alterations we made to the original ACMG/AMP criteria codes can be categorized into the following: 14 disease-specific/strength-level changes, 13 disease-specific changes, one strength-level change, and four clarification changes (based on recent ClinGen recommendations). Additionally, we found six criteria codes not applicable to *LDLR*. Key *LDLR* modifications include alteration of population data frequency thresholds, delineation of loss-of-function (LoF) variant types, functional study criteria specifications, co-segregation criteria specifications, and specific use and thresholds for *in silico* prediction tools.

Population data (PM2, BA1, BS1)

The FH VCEP recommends using the gnomAD PopMax Filtering Allele Frequency (FAF) in evaluation of BA1 and BS1 codes[15], while evaluation of PM2 should be performed using the PopMax Minor Allele Frequency (MAF). Frequency thresholds specific for *LDLR* variants in FH are displayed in **Table 2** and were calculated using the CardioDB metrics allele frequency web tool (https://www.cardiodb.org/allelefrequencyapp/) based on prevalence, penetrance, and allelic/genetic heterogeneity. Allele frequency thresholds were equal to FAF ≥0.005 (0.5%) for BA1, FAF ≥0.002 (0.2%) and <0.005 (0.5%) for BS1, and MAF ≤0.0002 (0.02%) for PM2. Note that if both exomes and genomes have a FAF/MAF value presented in gnomAD, consider the value corresponding to the higher number of alleles tested (i.e., higher total allele number). When evaluating whole-exon deletions and duplications, which are a

relatively common pathogenic variant type in *LDLR*,[16] the gnomAD Structural Variant (SV) dataset should be queried, applying the same thresholds as defined above.

It is important to keep in mind that both case and control gnomAD cohorts are expected to contain many individuals with FH, given FH is relatively common in the general population (1 in 250 individuals; or an estimated ~34 million affected worldwide), and >90% of individuals are thought to be undiagnosed.[2,3] Further, there are multiple cardiac case cohorts included in gnomAD, such as those from the Framingham Heart Study, Jackson Heart Study, Multi-Ethnic Study of Atherosclerosis, and Myocardial Infarction Genetics Consortium studies.

Loss of function (PVS1) and in-frame indels (PM4)

LDLR satisfies ClinGen's three requirements for applicability of PVS1.[17] That is 1) it is a definitive gene for FH; 2) three or more LoF variants reach an ACMG/AMP classification of "Pathogenic" without PVS1 (**Table S2**); and 3) >10% of variants associated with the phenotype are LoF (across more than one exon). In fact, frameshift variants alone represent ~20% of all unique FH-associated variants in ClinVar and are distributed throughout the gene.[11]

In accordance with the PVS1 flowchart outlined in Abou Tayoun et al., 2018,[17] we have specified PVS1 (**Figure 1**) based on well-established evidence in *LDLR*. Notably, any stop codon amino-terminal of amino acid 830 (NM_000527.5; located in exon 17) has been shown to remove a region known to be critical to protein function (i.e., the NPXY sequence of the cytoplasmic tail, required for LDLR internalization).[18] Note that alternative splicing of exons #1-18 from *LDLR*'s biologically relevant transcript is not known to occur. Therefore, PVS1 (Very Strong) includes the following variants: 1) deletion of full gene; 2) deletion of single or

multiple exons (exons 1-17) that lead to an out-of-frame consequence; 3) nonsense or frameshift variants causing a premature stop codon amino-terminal of amino acid 830 (NM_000527.5:p.Lys830); 4) variants in canonical +/- 1,2 GT/AG splice sites that predict a frameshift in exons 1-17; and 5) intragenic exon duplications proven to occur in tandem, that predict a frameshift in exons 1-17. PVS1_Strong includes: 1) deletion of single or multiple exons (exons 1-17) that do not predict a frameshift; 2) variants in canonical +/-1,2 GT/AG splice sites that predict in-frame deletions in exons 1-17; and 3) intragenic exon duplications presumed to occur in tandem that predict frameshifts in exons 1-17. PVS1_Moderate includes: 1) variants in the initiation codon; 2) whole-exon deletion of exon 18; and 3) nonsense/frameshift variants carboxy-terminal of amino acid 830 (NM_000527.5:p.Lys830). Further, **Table S3** provides information on the phase of *LDLR* exons for determining in- or out-of-frame consequences for applicable variants.

In addition, in-frame deletions or insertions smaller than a whole exon, or in-frame whole-exon duplications not considered in the PVS1 criteria are applicable to PM4, if they also meet PM2.

Experimental studies (PS3, BS3)

Following the SVI recommendations for application of functional studies codes PS3/BS3,[19] we have defined the mechanism of disease, evaluated the applicability of classes of assays used in the field, and evaluated individual instances of assays, in determining appropriate strength levels.

In summary, LDLR is expressed at the cell surface, where it binds circulating plasma LDL particles. The LDLR-LDL (receptor-ligand) complex is internalized at clathrin-coated pits via receptor-mediated endocytosis. Once internalized (as part of the endosome), acidic conditions

mediate release of the LDL ligand from its receptor, and the receptor is recycled back to the cell surface where it can repeat this process; a single LDLR protein can be recycled ≥100 times.[20] Pathogenic variants may induce a loss of function at any part of the LDLR cycle,[21] disrupting LDLR activity, and leading to FH due to an inability to effectively clear LDL-C from the bloodstream. The most reliable functional assays are adapted from the Nobel Prize winning work of Drs. Michael Brown and Joseph Goldstein[22] and allow the characterization of the whole LDLR cycle, which can be evaluated sufficiently at three key steps: 1) LDLR expression/biosynthesis, 2) LDL particle binding, and 3) LDL internalization. Such assays compare LDLR activity in wild-type cells against cells harboring a specific variant and are currently performed by flow cytometry with fluorescently-labeled LDL (commercially available or isolated from a wild-type individual) in 1) heterologous cells (with no endogenous LDLR) transfected with a mutant plasmid or 2) patient cells (fibroblasts, lymphocytes or lymphoblasts). Older studies using radioactively-labelled LDL (^{125}I-LDL) (e.g., Hobbs et al., 1992)[23] are also valid if they use these same cell types. A more in-depth analysis of the rationale and methodologies of LDLR functional assays are presented in Bourbon, Alves, & Sijbrands, 2017.[24]

We have determined three different strength "levels" of LDLR functional assays (**Table 3**), based on the appropriateness of the methodology. Level 1 studies (set at PS3/BS3) are the most reliable; these include flow cytometry assays which evaluate the whole LDLR cycle (i.e., LDLR expression/biosynthesis, LDL binding and LDLR-LDL internalization) performed in heterologous cells (with no endogenous LDLR) transfected with a mutant plasmid. Using heterologous cells with site-directed mutagenesis ensures that the assay is variant-specific. **Figure S1** demonstrates thresholds and controls used to validate flow cytometry assays in heterologous cells. Level 2 and Level 3 studies (set at PS3_Moderate, PS3_Supporting/BS3_Supporting) represent additional techniques that allow for evaluation

of only part of the LDLR cycle, or which use less robust cells/materials. It is important to note that when using patient cells, DNA sequence analysis of *LDLR* should indicate the assay is variant-specific (i.e., no other candidate variants identified in *LDLR*, including whole-exon deletions/duplications). Although the historical Brown and Goldstein LDLR activity assays using patient cells were thoughtfully designed to be gene-specific (for e.g., APOB-containing LDL particles used are always wild-type and LDLR is overexpressed in the cultured cells), patient-specific genetic factors may still modify outcomes. Lastly, studies in compound heterozygous patient cells are not considered as valid functional assays since it is difficult to delineate the individual effect of each variant.

Hotspot/well-established functional domains (PM1)

LDLR exon 4 is considered a mutational hot spot for missense variants in a well-established functional domain critical to protein function, since it encodes LDLR type A repeats 3, 4 and 5, which compose the well-established ligand (LDL) binding domain;[25] exon 4 also has the highest number of FH-associated variants per nucleotide with no variants proven benign by functional studies.[14] In addition, *LDLR* contains 60 highly conserved cysteine residues (located throughout exons 2-8 and 14) critical to protein function; these 60 cysteine residues are involved in disulfide bond formation, essential for proper protein folding.[26,27] Thus, PM1 is applicable to any missense change in the amino acids of exon 4 (NM_000527.5:c.314-694 or p.105-232) that are also rare (i.e., PM2 is met), or to any missense change in the 60 highly conserved cysteines, which we have listed in **Table S4**.

Observed in healthy adults (BS2)

Pathogenic variants in *LDLR* are known to be highly penetrant, where "affected" status is typically identifiable as early as childhood[28] through a simple and routine laboratory measure

of plasma LDL-C level. Therefore, we have determined BS2 is applicable for *LDLR* variants identified in ≥3 heterozygous or ≥1 true homozygous well-phenotyped, normolipidemic, untreated, unrelated adults. At a minimum, "well-phenotyped" refers to LDL-C measurements taken over multiple time points (≥2), with consistent results. Individuals considered in BS2 should not be taking any lipid-lowering therapy near the time of measurement and should have an LDL-C level below the ethnic and country-specific 50th centile (adjusted for age and sex) (e.g., Starr et al. 2008).[29] Because lipid-lowering therapies (e.g., statins) are among the most widely prescribed medications in the general population, and neither medication status nor LDL-C level are typically available in commonly used, publicly available resources such as gnomAD or ExAC, such resources must not be used for evaluation of BS2 in FH. Rather, we recommend evaluation of BS2 in well-phenotyped normolipidemic cohorts only, which are likely to be more available in internal laboratory settings. It is important to follow these caveats closely, given BS2 is a strong-level criterion.

Specificity of phenotype (PP4) and case-control data (PS4)

There are a variety of validated clinical diagnostic criteria used for FH, which include the Dutch Lipid Clinic Network (DLCN) criteria,[30] Simon Broome criteria,[31] the United States MEDPED criteria,[32] and other country-specific criteria. We have determined that PP4 is applicable to any rare (i.e., PM2 is met) *LDLR* variant identified in a patient with a diagnosis of FH based on any validated clinical criteria; examples include a DLCN score ≥6, Simon Broome score of "possible" or "definite" FH, or a MEDPED diagnostic score of "FH". Because 1) all validated clinical criteria require extreme LDL-C levels to be present in the patient together with a family history positive for high LDL-C and/or premature coronary heart disease, and 2) the *LDLR* gene is specific for FH (>90% of cases), we believe strongly

in the appropriateness of PP4 for *LDLR*. However, in any case, PP4 is applicable only after alternative causes of high LDL-C are excluded. Alternative causes for high LDL-C are reviewed in Sturm et al., 2018[7] and include polygenic dyslipidemia, elevated lipoprotein(a) [Lp(a)], nephrotic syndrome, obstructive liver disease, hypothyroidism, diabetes, FH due to *PCSK9, APOB,* or *APOE* variants, or FH phenocopies due to bi-allelic variants in *LDLRAP1, LIPA,* or *ABCG5/8*.

For the case-control criterion PS4, different strength levels may be applied depending on the number of unrelated FH cases with the rare variant. PS4 is applicable if the variant is found in ≥10 unrelated FH cases (FH diagnosis met using validated clinical criteria); PS4_Moderate is applicable if found in 6-9 unrelated FH cases; and PS4_Supporting is applicable if found in 2-5 unrelated FH cases. Note that in applying PS4-level criteria, the variant must also meet PM2.

Segregation data (PP1, BS4)

We have determined three strength levels for application of PP1 depending on the number of families/individuals studied. PP1_Strong is applicable when there is co-segregation of the variant with affected status in ≥6 informative meioses; PP1_Moderate when in 4-5 informative meioses; and PP1_Supporting when in 2-3 informative meioses. Index cases should not be counted as positive cases for co-segregation results. When the same variant is identified in more than one family, data can be added to reach stronger evidence levels. **Figure S2** shows a typical example of co-segregation in a pedigree, with an explanation on informative meiosis for a FH-associated variant. Note that when an index case presents with a heterozygous FH phenotype and the hypercholesterolemia is associated with one branch of the family, individuals from the other branch should not be considered for co-segregation

analysis. BS4 is applicable when there is lack of co-segregation in ≥2 index case families (unrelated) and there is data on ≥2 informative meioses in each family. When applying BS4 there should be at least one instance where an unaffected family member carries the variant (i.e., genotype-positive, phenotype-negative).

For co-segregation analysis we consider an affected individual as one with an untreated total cholesterol (TC) or LDL-C level above the 75th centile adjusted for age and sex. Each country/region should preferably use their TC and LDL-C centile charts. Given the widespread use of lipid-lowering therapies in the general population, untreated TC or LDL-C measurements may not be obtainable for some individuals under consideration for PP1/BS4. For those with only known *treated* TC or LDL-C levels, several imputation factors may be applied for an estimation of untreated measurements; namely by specific medication and dose (preferred)[33], or by the more general 0.8 and 0.7 correction factors corresponding to an estimated 20% TC and 30% LDL-C reduction on treatment, respectively.[34] Unaffected family members should have 'untreated' TC and LDL-C below the 50th centile adjusted for age and sex.

It is important to consider both affected and unaffected individuals when evaluating co-segregation. Alternative causes of high TC or LDL-C values, such as those described above, should be considered carefully given their ability to explain instances of hypercholesterolemia in genotype-negative family members. It is important to note that cholesterol concentrations are influenced by the co-inheritance of common variants of small effect; Trinder et al. have recently demonstrated that individuals who have a LDL-C polygenic risk score in the lowest decile have LDL-C concentrations considerably lower than those in the highest decile (3.61 mmol/L versus 4.37 mmol/L, respectively)[35]. Lastly, be aware that although rare, FH patients with a pathogenic *LDLR* variant could also be positive

for a rare monogenic cholesterol-lowering variant (possible in *APOB* or *PCSK9* genes for example), as has been described in Emi et al., 1991[36] and Motazacker et al., 2012.[37] If identifiable, these individuals should not be considered for co-segregation analysis.

In silico **prediction (PP3, BP4)**

For *in silico* classification of missense variants in *LDLR* we suggest the use of REVEL, an ensemble method for pathogenicity prediction that combines predictions from 13 individual commonly used computational tools.[38] Use of a single meta-predictor such as REVEL will eliminate discrepancies in which programs are used by curators and what specificities to account for when manually performing *in silico* analysis. REVEL was also selected because of its accessibility; REVEL scores are pre-computed and automatically displayed in the ClinGen VCI (under the 'Variant Type' tab), or are available for download (LDLR: https://rothsj06.u.hpc.mssm.edu/revel/revel_segments/revel_chrom_19_009082971-013246689.csv.zip). To determine *LDLR*-specific score thresholds for PP3/BP4 we evaluated REVEL scores for *LDLR* missense variants with 1) Pathogenic/Likely pathogenic or Benign/Likely Benign classifications in ClinVar, 2) damaging or neutral results according to *LDLR*-specific PS3/BS3 functional study evidence, and 3) concordant *in silico* results for Poly-Phen, SIFT, PROVEAN and MutationTaster (**Figure S3**). Using the PP3 threshold of ≥0.75 (as defined in Ioannidis et al., 2016)[38], the vast majority of variants with an association of 'pathogenic' using any comparison (as above) have REVEL scores above this threshold. Using the BP4 threshold of ≤0.5, ~half of variants with an association of 'benign' from ClinVar classifications or other *in silico* predictors have REVEL scores below this threshold. Therefore, we recommend a REVEL score ≥0.75 as supportive evidence of pathogenicity (PP3), and a REVEL score ≤0.5 as supportive evidence of benign (BP4).

For *in silico* prediction of splicing effects, we recommend evaluation only if no functional data is available; furthermore, variants already considered in PVS1 (or modified strength) should not be further considered in PP3/BP4. We suggest the use of MaxEntScan (MES)[39] which is highly reputable and publicly available. We have defined distinct thresholds for MES depending on the variant location, as described in **Figure 2.**

Lastly, if both missense and splicing prediction are applicable, only one prediction of a damaging effect is sufficient in applying PP3; however, both need to predict a neutral effect in applying BP4.

Other variants in the same codon (PS1, PM5)

When there are other described variants in the same codon as a missense variant being classified, PS1 is applicable if at least one missense variant has a classification of pathogenic (classified using these *LDLR*-specific guidelines), and the variant predicts the same amino acid change. PM5 (Moderate) is applicable if there is one pathogenic missense variant that predicts a different amino acid in the same codon. Lastly, PM5_Strong is applicable in the same context if there are ≥2 pathogenic missense variants which predict different amino acids in the same codon. Note that for these codes to be applied, the curated variant(s) should 1) not already be considered in PM1 (hotspot/well-established functional domain), and 2) have an *in silico* predicted splicing impact of benign. Combining PS1/PM5 with PM1 can be considered "double-counting", i.e., evaluating a variant under a similar premise twice, while investigating potential splicing impact provides greater confidence that pathogenicity is related to a predicted altered amino acid rather than creation of a *de novo* splice site or activation of cryptic splice site.

Allele data (*cis/trans*) (PM3, BP2)

LDLR variants show a semi-dominant pattern of inheritance on plasma cholesterol concentration, such that the phenotypes in homozygous or compound heterozygous patients are significantly more severe than in heterozygotes. Because of this, both PM3 and BP2 criteria (observed in *trans* with a pathogenic variant) can be used when case-level data are available for individuals with more than one FH-associated variant. PM3 is applicable when a candidate *LDLR* variant is identified in a patient with a clear homozygous or compound heterozygous FH phenotype (defined here as untreated LDL-C ≥13 mmol/L or ≥500 mg/dl), who has an additional known pathogenic variant in *LDLR* (in *trans*), *APOB,* or *PCSK9*. The candidate variant must also meet PM2. PM3 must not be used if *cis/trans* status in *LDLR* has not been established. BP2 is applicable to any additional *LDLR* variants identified in a patient with a clear heterozygous FH phenotype (defined here as untreated, elevated LDL-C that is <8 mmol/L or <310 mg/dl, in adults) who already has a known pathogenic variant in *LDLR (*in *trans)*, *APOB,* or *PCSK9.*

For both PM3 and BP2, known pathogenic variants in *LDLR* must have been classified as pathogenic according to these guidelines, while known pathogenic variants in *APOB* or *PCSK9* should have been formally assessed by general ACMG/AMP guidelines until these gene-specific guidelines have been established, at which time both variant classifications should be re-evaluated. Although to the best of our knowledge no formal studies evaluating the prevalence of double-heterozygotes in these FH genes have been completed, they are uncommon in our experience.

De novo occurrence (PS2, PM6)

The FH VCEP recommends following the SVI recommendations for PM6 and PS2, which can be found at https://clinicalgenome.org/working-groups/sequence-variant-interpretation. These recommendations evaluate PM6/PS2 based on a points system centered around three parameters: confirmed versus assumed status, phenotypic consistency, and number of *de novo* observations. Although data to address *de novo* occurrence in *LDLR* directly are lacking, we have no evidence to suggest that this a common feature in FH, given that to date, only one member of FH VCEP has anecdotally observed a *de novo* occurrence in their clinical practice, and to the best of our knowledge, such cases have only been reported once in the literature.[40] However, they are of course possible, and should be considered.

Criteria not applicable (BP1, PP2, BP3, BP6, PP5, BP5)

BP1 (missense variant in a gene for which primarily truncating variants are known to cause disease) is not applicable, since the majority of FH-associated *LDLR* variants are missense variants. Following SVI counsel regarding PP2 (missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease), PP2 is not applicable, on the basis of a low z-score = 0.12 for *LDLR* in the gnomAD missense constraint table. BP3 (in-frame deletions/insertions in a repetitive region without a known function) is not applicable, given that there are no regions in *LDLR* without a known function. BP6 and PP5 (variant previously classified by a reputable source) have been advised by ClinGen not to be used, so are not applicable. Lastly, the FH VCEP has decided to remove BP5 (alternative mechanism for disease), given that this premise is already evaluated in our specifications for BP2.

Pilot study

The pilot study of the final specifications was done on 54 *LDLR* variants in the ClinGen VCI. Variants, listed in **Table S5**, were chosen to reflect *LDLR* variant variability and included one multi-exon deletion, 40 missense, seven intronic/splicing, four nonsense and two synonymous variants. A total of nine institutions, which included six clinical and three research laboratories, provided internal case-level data (via standardized template), supplementing classifications for 42 of 54 variants. For the remaining 12 variants, and whenever necessary, data from published literature was used to count number of cases, and to evaluate co-segregation and functional evidence. **Figure S4** shows the impact of internally shared or literature only case-level data in pilot variant classifications. Without internal case-level data sharing, 30/42 variants were classified as VUS; however, when considering shared case-level data, half of these variants were able to be upgraded/downgraded. Namely, 14 variants were upgraded to Likely pathogenic/Pathogenic, while one was downgraded to Benign.

Preliminary results had complete agreement (in both classification and individual criteria used) for 16 variants, agreement in classifications but not in each criterion used for 27 variants, and discrepancies in both classification and criteria used for the remaining 11 variants. Differences in classification were eight counts of Likely pathogenic versus VUS, two of Likely pathogenic versus Pathogenic, and one of Benign versus VUS. A careful review of the discrepancies determined that most resulted from extracting different gnomAD MAF/FAF data from the VCI, or from differences in applying PS4_moderate versus PS4_supporting due to slight differences in case counts. Minor refinements were added to the guidelines to address these discrepancies: we clarified the use of MAF/FAF (exomes versus genomes feature) in gnomAD and designed a more efficient template for tracking case-level

data. Consequently, finalized pilot results had complete agreement in both classification and criteria used for all 54 variants and represented six Benign, two Likely benign, 18 VUS, 15 Likely pathogenic and 13 Pathogenic variants. The number of times each criterion was used is represented in **Figure S5**. Reviewers approved the final classifications which are now published in the ClinGen Evidence Repository and submitted to ClinVar under the FH VCEP affiliation.

LIMITATIONS

There are multiple criteria specifications that require diagnostic information and case-level data, which if not readily available, may limit the classification of variants. For instance, PS4 and PP4 criteria require that cases considered are clinically diagnosed with FH based on validated clinical criteria. PP1-level, PS2/PM6, PM3, BS2, BS4, and BP2 criteria require that further case-level data are available, including LDL-C measurements, genetic results, family history, and medication status. However, although this information may be difficult to ascertain in some settings, it is necessary to apply these criteria correctly. Whenever possible, we encourage curators to actively seek this information if it is not initially available. As the FH VCEP works toward classifying all ~2,300 *LDLR* variants currently in the ClinVar database using the guidelines presented here, we are hopeful we can overcome some of these limitations through internal data sharing efforts. We encourage any laboratory with internal data on *LDLR* variants in FH patients to upload these data in ClinVar, as these data can have a major impact on the proper classification of variants.

CONCLUSIONS AND FUTURE DIRECTIONS

Here, the FH VCEP presents consensus recommendations for *LDLR* variant classification. Application of these guidelines will provide evidence-based, standardized classification of *LDLR* variants for use in clinical diagnostics and research. Future directions include sustained variant curation, with the aim of classifying all ~2,300 unique *LDLR* variants at the '3-star' status in ClinVar. 3-star status indicates variants reviewed by an expert panel, according to a set of ClinGen approved VCEP-adapted ACMG/AMP criteria. It is noteworthy that in 2018 the ClinGen Variant Curation Expert Panel protocol was recognized by the U.S. Food and Drug Administration (FDA), whereby variant classifications with 3-star status in ClinVar are now associated with an FDA-recognized tag, and can be used to support clinical validity of genetic tests. In the future, we expect this may have implications for obtaining insurance coverage for certain medications, for enrolment in certain clinical trials or research studies, or in the feedback of incidental findings from whole-exome or whole-genome sequencing. Given these possible implications, we will prioritize the classification of *LDLR* variants with the greatest potential impact, such as those that are LoF variant types, those with many and/or conflicting submissions currently in ClinVar, or those known to be on clinically available arrays/panels. We are hopeful that the FH VCEP classification of all ~2,300 *LDLR* variants is completed within the next four years; we also plan to review all classifications on a two-year basis to ensure that recently emerging data are considered.

Finally, please be aware that the *LDLR*-specific guidelines presented here are subject to change in response to emerging data and newly available resources, which will continually influence the evolving nature of variant classification methodology, both specific to *LDLR* and also more broadly throughout the clinical genetics community. For this reason, please

refer to the FH VCEP page in the ClinGen website (https://clinicalgenome.org/affiliation/50004) for the most currently accepted version.

DATA AVAILABILITY

All data generated or analysed during this study are included in this manuscript (and its supplementary information files).

ACKNOWLEDGEMENTS

ClinGen is primarily funded by the National Human Genome Research Institute (NHGRI), through the following three grants: U41HG006834, U41HG009649, U41HG009650. ClinGen also receives support for content curation from the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), through the following three grants: U24HD093483, U24HD093486, U24HD093487. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. JRC acknowledges her PhD fellowship funded by the Science and Technology Foundation (SFRH/BD/108503/2015). LT and TF are supported by the Ministry of Health of the Czech Republic, grant NU20-02-00261. SEH is an Emeritus British Heart Foundation Professor and is funded by PG08/008, and by the National Institute for Health Research University College London Hospitals Biomedical Research Centre. MT is supported by a Vanier Canada Graduate Scholarship. LRB is a Michael Smith Foundation for Health Research Scholar and a Canada Research Chair in Precision Cardiovascular Disease Prevention. RAH is supported by the Jacob J. Wolfe Distinguished Medical Research Chair, the Edith Schulich Vinet Canada Research Chair in Human Genetics, the Martha G.

Blackburn Chair in Cardiovascular Research, and operating grants from the Canadian Institutes of Health Research (Foundation Grant) and the Heart and Stroke Foundation of Ontario (G-18-0022147). JWK is supported by the NIH through grants P30DK116074 (to the Stanford Diabetes Research Center), R01 DK116750, R01 DK120565, R01 DK106236; and by the American Diabetes Association (grant #1-19-JDF-108). Lastly, the FH VCEP would like to thank Drs. Steven Harrison, Leslie Biesecker, Heidi Rehm and the additional members from the ClinGen SVI for their feedback and guidance in establishing these guidelines.

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ETHICS DECLARATION

Our study contains only the curation of already de-identified publicly available data, namely, the curation of de-identified genetic variants already present in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/). As per ClinVar policy, variant submitters are

expected to have obtained appropriate consent for data submission and sharing. Geisinger Health System provided IRB approval for ClinGen submission of genetic variants to ClinVar. We have obtained ethics approval for our study by the ClinGen Data Access, Protection, and Confidentiality Committee, and adhere to their ethics policies.

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Figure 1. *LDLR*-specific recommendations for application of PVS1.

Abbreviations: NMD, nonsense-mediated decay

Figure 2. FH VCEP suggestions for evaluating splicing effects using MaxEntScan (MES) dependent on variant location A, B, or C. (**A**) Variant is located at -20 to +3 bases related to the authentic acceptor splice site or at -3 to +6 related to the authentic donor splice site: A result of authentic splice site strength variant/wild-type score <0.8 is supportive evidence of pathogenicity (PP3), while a score ≥1.0 is supportive evidence of benign (BP4). (**B**) Variant creates *de novo* acceptor splice site, which is at least 50 bases upstream of the authentic donor splice site, or *de novo* donor splice site, which is at least 50 bases downstream of the authentic acceptor splice site: A result of *de novo* splice site strength variant/authentic wild-type score in >0.9 is applicable to PP3, while a score <0.8 is applicable to BP4. (**C**) Variant is located at -20 to +3 bases relative to an intra-exonic AG dinucleotide, which is at least 50 bases upstream of the authentic donor splice site, or at -3 to +6 bases relative to an intra-exonic GT dinucleotide, which is at least 50 bases downstream of the authentic acceptor splice site: Results of both variant cryptic/wild-type cryptic score in >1.1 and cryptic acceptor/authentic acceptor score or cryptic donor/authentic donor score in >0.9 is applicable to PP3. Note: BP4 is applicable to exonic variants outside of the 50 base limits detailed above, given the unlikelihood of such variants to impact splicing in *LDLR*.

Abbreviations: Var, variant; Wt, wild-type.

Conflict of interest notifications:

R.A.H. reports consulting fees from Acasti, Aegerion, Akcea/Ionis, Amgen, HLS Therapeutics, Novartis, Pfizer, Regeneron and Sanofi.

Figure 1 (revised)

Figure 2

Table 1. Summary of ACMG/AMP guideline specifications for *LDLR*.

Gene	**Disease**	**Transcript**
LDLR	***hypercholesterolemia, familial (MONDO:0007750)***	***NM_000527.5***
PATHOGENIC CRITERIA		
Criteria	**Criteria Description**	***LDLR*** **Specification**
VERY STRONG CRITERIA		
PVS1	See PVS1 flow diagram (**Figure 1**).	Disease specific / strength
STRONG CRITERIA		
PS1	Missense variant at the same codon as a variant classified pathogenic (by these guidelines), and predicts the same amino acid change. **Caveat**: there is no *in silico* predicted splicing impact for either variant.	Clarification
PS2	Variant is *de novo* in a patient with the disease and no family history. Follow SVI guidance for *de novo* occurrences: https://clinicalgenome.org/working-groups/sequence-variant-interpretation/	Clarification
PS3	Variant meets Level 1 pathogenic functional study criteria. See **Table 3**.	Disease specific / strength
PS4	Variant is found in ≥10 unrelated FH cases (FH diagnosis met by validated clinical criteria).	Disease specific / strength

	Caveat: variant must also meet PM2.	
PVS1_Strong	See PVS1 flow diagram (**Figure 1**).	Disease specific / strength
PM5_Strong	Missense variant at a codon with ≥2 missense variants classified pathogenic (by these guidelines), and predicts a different amino acid change.	Strength
PP1_Strong	Variant segregates with phenotype in ≥6 informative meioses in ≥1 family. Must include ≥2 affected relatives (LDL-C >75th centile) with the variant.	Disease specific / strength
MODERATE CRITERIA		
PM1	Missense variant located in exon 4, or a missense change in one of 60 highly conserved cysteine residues (listed in **Table S4**). **Caveat**: variant must also meet PM2.	Disease specific
PM2	Variant has a PopMax MAF ≤0.0002 (0.02%) in gnomAD. Consider exceptions for known founder variants.	Disease specific
PM3	This criterion can be used for a candidate *LDLR* variant observed in an individual with a homozygous FH phenotype when there is only one other pathogenic variant in *LDLR* (in *trans*), *APOB* or *PCSK9*. **Caveat**: variant must also meet PM2.	Disease specific

PM4	In-frame deletion/insertions smaller than one whole exon, or in-frame whole-exon duplications not considered in any PVS1 criteria. **Caveat**: variant must also meet PM2.	Disease specific
PM5	Missense variant at the same codon as a variant classified pathogenic (by these guidelines), and predicts a different amino acid change.	Clarification
PM6	See PS2 above.	Clarification
PS3_Moderate	Variant meets Level 2 pathogenic functional study criteria. See **Table 3**.	Disease specific / strength
PS4_Moderate	Variant is found in 6-9 unrelated FH cases (FH diagnosis made by validated clinical criteria). **Caveat**: variant must also meet PM2.	Disease specific / strength
PP1_Moderate	Variant segregates with phenotype in 4-5 informative meioses in ≥1 family. Must include ≥2 affected relatives (LDL-C >75th centile) with the variant.	Disease specific / strength
PVS1_Moderate	See PVS1 flow diagram (**Figure 1**).	Disease specific / strength
SUPPORTING CRITERIA		
PP1	Variant segregates with phenotype in 2-3 informative meioses in ≥1 family. Must include ≥1 affected relative (LDL-C >75th centile) with the variant.	Disease specific / strength

PP2	*Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease.*	*N/A*
PP3	REVEL score ≥0.75 (missense variants), or predicted impact to splicing using MaxEntScan (see **Fig. 2** for suggested thresholds).	Disease specific
PP4	Any *LDLR* variant identified in an FH patient [diagnosis based on validated clinical criteria, e.g., Dutch Lipid Clinic Network (≥6), Simon Broome (possible/definite), MEDPED], **after alternative causes of high cholesterol are excluded**. **Caveat**: variant must also meet PM2.	Disease specific
PP5	*Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation.*	*N/A*
PS3_Supporting	Variant meets Level 3 pathogenic functional study criteria. See **Table 3**.	Disease specific / strength
PS4_Supporting	Variant is found in 2-5 unrelated FH cases (FH diagnosis made by validated clinical criteria). **Caveat**: variant must also meet PM2.	Disease specific / strength
BENIGN CRITERIA		
STAND ALONE CRITERIA		
BA1	Variant has a PopMax FAF ≥0.005 (0.5%) in gnomAD.	Disease specific

STRONG CRITERIA		
BS1	Variant has a PopMax FAF ≥0.002 (0.2%) in gnomAD.	Disease specific
BS2	Variant is identified in ≥3 heterozygous or ≥1 homozygous **well-phenotyped, untreated, normolipidemic** adults (unrelated).	Disease specific
BS3	Variant meets Level 1 benign functional study criteria. See **Table 3**.	Disease specific / strength
BS4	Lack of segregation in ≥2 index case families (unrelated), when data is available for ≥2 informative meioses in each family. **Caveat**: must be ≥1 unaffected relative (LDL-C <50th centile) who is positive for the variant.	Disease specific
SUPPORTING CRITERIA		
BP1	*Missense variant in gene where only LoF causes disease.*	*N/A*
BP2	If a FH patient with a heterozygous phenotype has a proven pathogenic variant in *LDLR (*in *trans), APOB* or *PCSK9*, BP2 is applicable to any additional *LDLR* variants.	Disease specific
BP3	*In-frame deletions/insertions in a repetitive region without a known function.*	*N/A*
BP4	REVEL score ≤0.5 (missense variants), or no predicted impact to splicing using MaxEntScan (see **Fig. 2** for suggested thresholds).	Disease specific

BP5	*Variant found in a case with an alternate molecular basis for disease.*	*N/A*
BP6	*Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation.*	*N/A*
BP7	Variant is synonymous. **Caveat**: variant must also meet BP4 (i.e., no predicted impact on splicing).	Disease specific
BS3_Supporting	Variant meets Level 3 benign functional study criteria. See **Table 3**.	Disease specific / strength

Abbreviations: FH, familial hypercholesterolemia; MAF, minor allele frequency; FAF, filtering allele frequency; LoF, loss of function. Note: PopMax refers to the gnomAD subpopulation with the highest allele frequency.

Table 2. *LDLR*-specific population data frequency thresholds.

	gnomAD Frequency	Prevalence	Penetrance	Allelic Het.	Genetic Het.
BA1	PopMax FAF ≥0.005 (0.5%)[a]	1/250	50%	1.0	1.0
BS1	PopMax FAF ≥0.002 (0.2%) and <0.005 (0.5%)	1/250	95%	1.0	0.9
PM2	PopMax MAF ≤0.0002 (0.02%)	1/250	95%	0.1	0.9

Note: PopMax refers to the gnomAD subpopulation with the highest allele frequency. [a] BA1 metrics were equal to 0.4%; however, we conservatively increased the BA1 threshold to 0.5%. Abbreviations: FAF, filtering allele frequency; MAF, minor allele frequency; Het., heterogeneity.

Table 3. PS3/BS3 functional study criteria specifications for *LDLR*.

Pathogenic	
PS3 (Level 1)	**(1)** Study of the *whole* LDLR cycle (LDLR expression/biosynthesis, LDL binding, and LDL internalization) performed in heterologous cells (with no endogenous LDLR) transfected with a mutant plasmid. Assay result of <70% of wild-type activity in either expression/biosynthesis, binding OR internalization.
PS3_Moderate (Level 2)	**(1)** Study of a) only *part* of the LDLR cycle following Level 1 methodology, or b) *whole or part* of the LDLR cycle in true homozygous patient cells. A variant with assay results of <70% of wild type activity in either LDLR expression/biosynthesis, LDL binding OR internalization. **(2)** RNA studies, using RNA extracted from heterozygous or true homozygous patient cells, where aberrant transcript is confirmed by sequencing and is quantified as >25% of total transcript from heterozygous cells or 50% of total transcript from homozygous cells. **(3)** Variants with two or more Level 3 functional studies (must be different assays); or any Level 3 functional study #1-4 performed by two or more independent labs with concordant results.
PS3_Supporting (Level 3)	**(1)** Study of LDLR cycle (*whole or part*) in heterozygous patient cells, with assay results of <85% of wild-type activity in either LDLR expression/biosynthesis, LDL binding OR internalization.

	(2) Luciferase studies with transcription levels of <50% compared to wild-type (applicable to 5'UTR/promoter variants). **(3)** Minigene splicing assays with <10% wild-type transcript present where an aberrant transcript from the candidate variant is confirmed by sequencing. **(4)** High-throughput assays, which include alternative microscopy assays (e.g., Thormaehlen et al., 2015), Multiplex Assays of Variant Effect (MAVE) (e.g., Weile & Roth, 2018) and deep mutational scanning assays, can be considered here, only if assay has been validated with a minimum of four pathogenic and four benign variant controls in LDLR. *Note: % activity thresholds will be defined by the FH VCEP as more data becomes available. **(5)** RNA studies, using RNA extracted from heterozygous or homozygous patient cells, with aberrant transcript confirmed by sequencing (but without transcript quantification).
Benign	
BS3 (Level 1)	**(1)** Study of the *whole* LDLR cycle (LDLR expression/biosynthesis, LDL binding, and LDL internalization) performed in heterologous cells (with no endogenous LDLR) transfected with a mutant plasmid. Assay result of >90% of wild-type activity in expression/biosynthesis, binding AND internalization.

	Note: studies of only *part* of the LDLR cycle are not eligible for BS3 or BS3_Supporting.
BS3_Supporting (Level 3)	**(1)** Study of the *whole* LDLR cycle in a) true homozygous patient cells, with assay result of >90% of wild-type activity in biosynthesis, binding AND internalization; or in b) heterozygous patient cells with assay result of >95% of wild-type activity in biosynthesis, binding AND internalization. **(2)** Luciferase studies with transcription levels of >90% when compared to wild-type (applicable to 5'UTR/promoter variants). **(3)** RNA studies, using RNA extracted from heterozygous or true homozygous patient cells, with a) aberrant transcripts quantification, where aberrant transcript is <10% of total transcript OR b) without transcript quantification where no aberrant transcript is confirmed by sequencing. **(4)** Minigene splicing assay where only wild-type transcript is present and confirmed by sequencing. **(5)** High-throughput assays as defined above; only applicable when assay can indicate the *whole* LDLR cycle (LDLR expression/biosynthesis, LDL binding AND internalization) is unaffected.

Note: functional assays performed in compound heterozygous patient cells are not considered applicable in PS3/BS3 criteria since it is difficult to delineate the individual effect of each variant.

SUPPLEMENTARY TABLES AND FIGURES

Table S1. Rules for combining pathogenic and benign criteria in ACMG/AMP guideline specifications for *LDLR*.

PATHOGENIC		
1 Very Strong AND	1 or more Strong	
	2 or more Moderate	
	1 Moderate AND	1 Supporting
	2 or more Supporting	
≥2 Strong		
1 Strong AND	3 or more Moderate	
	2 Moderate AND	2 or more Supporting
	1 Moderate AND	4 or more Supporting
LIKELY PATHOGENIC		
1 Very Strong AND	1 Moderate	
1 Strong AND	1- 2 Moderate	
	2 or more Supporting	
3 or more Moderate		
2 Moderate AND	2 or more Supporting	
1 Moderate AND	4 or more Supporting	
BENIGN		
1 Stand Alone		
2 or more Strong		
LIKELY BENIGN		
1 Strong AND	1 Supporting	
2 or more Supporting		
Variant of Uncertain Significance (VUS)		
Criteria shown above are not met OR the criteria for pathogenic and benign are contradictory		

Adapted from Richards et al., 2015[1]; no changes to original scoring algorithm.

Table S2. *LDLR* loss-of-function variants that reach an ACMG/AMP classification of "Pathogenic" without the application of PVS1.

***LDLR* variant**	**Applicable criteria***	**Sum of criteria**
c.313+1G>A	(PVS1_Strong); PS4; PP1_Strong; PM2; PS3_Moderate; PP4	2 Strong, 2 Moderate and 1 Supporting
c.564C>G (p.Tyr188Ter)	(PVS1); PP1_Strong; PS3_Moderate; PM2; PS4_Supporting; PP4	1 Strong, 2 Moderate and 2 Supporting
c.2140+1G>A	(PVS1); PP1_Strong; PM2; PS4_Moderate; PS3_Supporting; PP4	1 Strong, 2 Moderate and 2 Supporting

*The criteria in parentheses in this column were not applied.

Table S3. *LDLR* exon information.

Exon No.	Start (g.)	Stop (g.)	Start (c.)	Stop (c.)	Length	Start Phase	End Phase
1	11089463	11089615	-86	67	153	-	1
2	11100223	11100345	68	190	123	1	1
3	11102664	11102786	191	313	123	1	1
4	11105220	11105600	314	694	381	1	1
5	11106565	11106687	695	817	123	1	1
6	11107392	11107514	818	940	123	1	1
7	11110652	11110771	941	1060	120	1	1
8	11111514	11111639	1061	1186	126	1	1
9	11113278	11113449	1187	1358	172	1	2
10	11113535	11113762	1359	1586	228	2	2
11	11116094	11116212	1587	1705	119	2	1
12	11116859	11116998	1706	1845	140	1	0
13	11120092	11120233	1846	1987	142	0	1
14	11120370	11120522	1988	2140	153	1	1
15	11123174	11123344	2141	2311	171	1	1
16	11128008	11128085	2312	2389	78	1	1
17	11129513	11129670	2390	2547	158	1	0
18	11131281	11133820	2548	2583	35	0	-

Phase: the position of an exon/intron boundary within a codon. A phase of zero means the boundary falls between codons, one means between the first and second base and two means between the second and third base. Genomic (g.) coordinates correspond to reference sequence NC_000019.9, and coding (c.) coordinates correspond to *LDLR* transcript NM_000527.5.

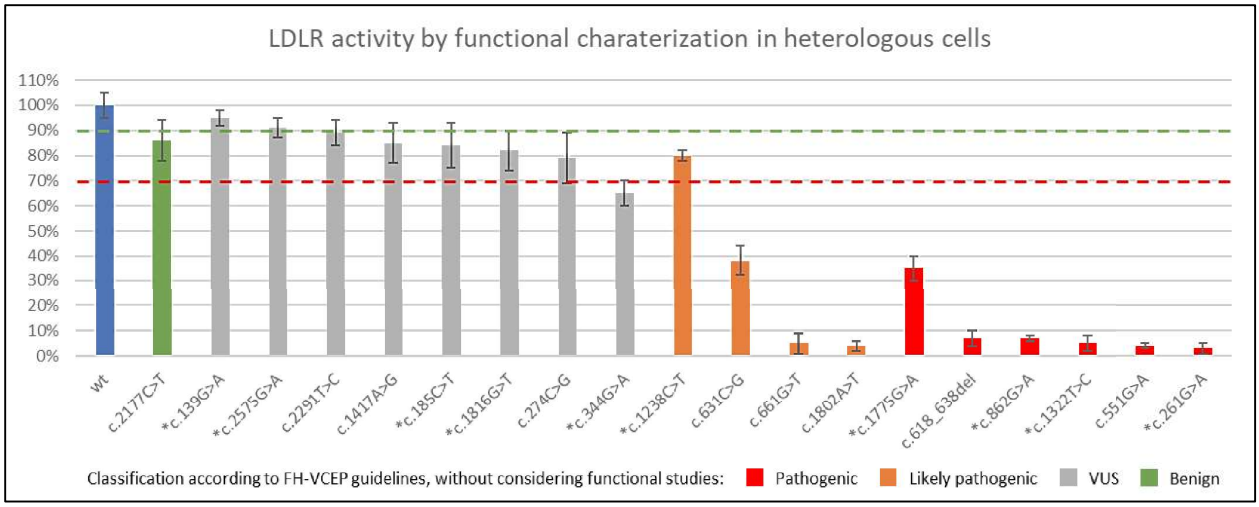


Figure S1. Functional characterization of LDLR activity in heterologous cells transfected with mutant plasmids. LDLR activity levels adapted from references[2–5]. The lowest activity value among LDLR expression, LDL binding and LDL internalization is displayed. The green dotted line represents the recommended LDLR activity threshold (>90% compared to wild-type) for application of Level 1 BS3 functional study evidence, and the red dotted line represents the recommended LDLR activity threshold (<70% compared to wild-type) for application of Level 1 PS3 functional study evidence. Note: the star (*) represents variants included our pilot study, where the associated classification does not require the application of PS3/BS3 criteria. For the remaining variants, classifications were performed by only one curator and using internal case-level data, so final FH VCEP classifications which appear in ClinVar may be different.

Table S4. *LDLR* cysteine residues involved in disulfide bond formation (residues eligible for PM1 when mutated).

Residue	Domain	Structure analysis	Predicted impact on LDLR structure and/or function
p.Cys27	LDL-receptor class A 1	disulfide bond	folding defect
p.Cys34	LDL-receptor class A 1	disulfide bond	folding defect
p.Cys39	LDL-receptor class A 1	disulfide bond	folding defect
p.Cys46	LDL-receptor class A 1	disulfide bond	folding defect
p.Cys52	LDL-receptor class A 1	disulfide bond	folding defect
p.Cys63	LDL-receptor class A 1	disulfide bond	folding defect
p.Cys68	LDL-receptor class A 2	disulfide bond	folding defect
p.Cys75	LDL-receptor class A 2	disulfide bond	folding defect
p.Cys82	LDL-receptor class A 2	disulfide bond	folding defect
p.Cys89	LDL-receptor class A 2	disulfide bond	folding defect
p.Cys95	LDL-receptor class A 2	disulfide bond	folding defect
p.Cys104	LDL-receptor class A 2	disulfide bond	folding defect
p.Cys109	LDL-receptor class A 3	disulfide bond	folding defect; LDL binding defect
p.Cys116	LDL-receptor class A 3	disulfide bond	folding defect; LDL binding defect
p.Cys121	LDL-receptor class A 3	disulfide bond	folding defect; LDL binding defect
p.Cys128	LDL-receptor class A 3	disulfide bond	folding defect; LDL binding defect
p.Cys134	LDL-receptor class A 3	disulfide bond	folding defect; LDL binding defect
p.Cys143	LDL-receptor class A 3	disulfide bond	folding defect; LDL binding defect
p.Cys148	LDL-receptor class A 4	disulfide bond; acidic pH intramolecular binding interface	folding defect; receptor-recycling defect; LDL binding defect
p.Cys155	LDL-receptor class A 4	disulfide bond	folding defect; LDL binding defect
p.Cys160	LDL-receptor class A 4	disulfide bond; acidic pH intramolecular binding interface	folding defect; receptor-recycling defect; LDL binding defect
p.Cys167	LDL-receptor class A 4	disulfide bond	folding defect; LDL binding defect
p.Cys173	LDL-receptor class A 4	disulfide bond; acidic pH intramolecular binding interface	folding defect; receptor-recycling defect; LDL binding defect
p.Cys184	LDL-receptor class A 4	disulfide bond	folding defect; LDL binding defect
p.Cys197	LDL-receptor class A 5	disulfide bond	folding defect; LDL binding defect
p.Cys204	LDL-receptor class A 5	disulfide bond	folding defect; LDL binding defect
p.Cys209	LDL-receptor class A 5	disulfide bond	folding defect; LDL binding defect
p.Cys216	LDL-receptor class A 5	disulfide bond	folding defect; LDL binding defect
p.Cys222	LDL-receptor class A 5	disulfide bond; acidic pH intramolecular binding interface	folding defect; receptor-recycling defect; LDL binding defect
p.Cys231	LDL-receptor class A 5	disulfide bond	folding defect; LDL binding defect
p.Cys236	LDL-receptor class A 6	disulfide bond	folding defect; LDL binding defect
p.Cys243	LDL-receptor class A 6	disulfide bond	folding defect; LDL binding defect
p.Cys248	LDL-receptor class A 6	disulfide bond	folding defect; LDL binding defect

p.Cys255	LDL-receptor class A 6	disulfide bond	folding defect; LDL binding defect
p.Cys261	LDL-receptor class A 6	disulfide bond	folding defect; LDL binding defect
p.Cys270	LDL-receptor class A 6	disulfide bond	folding defect; LDL binding defect
p.Cys276	LDL-receptor class A 7	disulfide bond	folding defect; LDL binding defect
p.Cys284	LDL-receptor class A 7	disulfide bond	folding defect; LDL binding defect
p.Cys289	LDL-receptor class A 7	disulfide bond	folding defect; LDL binding defect
p.Cys296	LDL-receptor class A 7	disulfide bond	folding defect; LDL binding defect
p.Cys302	LDL-receptor class A 7	disulfide bond	folding defect; LDL binding defect
p.Cys313	LDL-receptor class A 7	disulfide bond	folding defect; LDL binding defect
p.Cys318	EGF-like 1	disulfide bond	folding defect; LDL binding defect
p.Cys325	EGF-like 1	disulfide bond	folding defect; LDL binding defect
p.Cys329	EGF-like 1	disulfide bond	folding defect; LDL binding defect
p.Cys338	EGF-like 1	disulfide bond	folding defect; LDL binding defect
p.Cys340	EGF-like 1	disulfide bond	folding defect; LDL binding defect
p.Cys352	EGF-like 1	disulfide bond	folding defect; LDL binding defect
p.Cys358	EGF-like 2; calcium-binding	disulfide bond	folding defect; receptor-recycling defect
p.Cys364	EGF-like 2; calcium-binding	disulfide bond	folding defect; receptor-recycling defect
p.Cys368	EGF-like 2; calcium-binding	disulfide bond	folding defect; receptor-recycling defect
p.Cys377	EGF-like 2; calcium-binding	disulfide bond	folding defect; receptor-recycling defect
p.Cys379	EGF-like 2; calcium-binding	disulfide bond	folding defect; receptor-recycling defect
p.Cys392	EGF-like 2; calcium-binding	disulfide bond	folding defect; receptor-recycling defect
p.Cys667	EGF-like 3	disulfide bond	folding defect; receptor-recycling defect
p.Cys677	EGF-like 3	disulfide bond	folding defect; receptor-recycling defect
p.Cys681	EGF-like 3	disulfide bond	folding defect; receptor-recycling defect
p.Cys696	EGF-like 3	disulfide bond	folding defect; receptor-recycling defect
p.Cys698	EGF-like 3	disulfide bond	folding defect; receptor-recycling defect
p.Cys711	EGF-like 3	disulfide bond	folding defect; receptor-recycling defect

Adapted from Guo et al., 2019.[6] Residues correspond to *LDLR* transcript NM_000527.5. Abbreviations: Cys, cysteine; EGF, epidermal growth factor

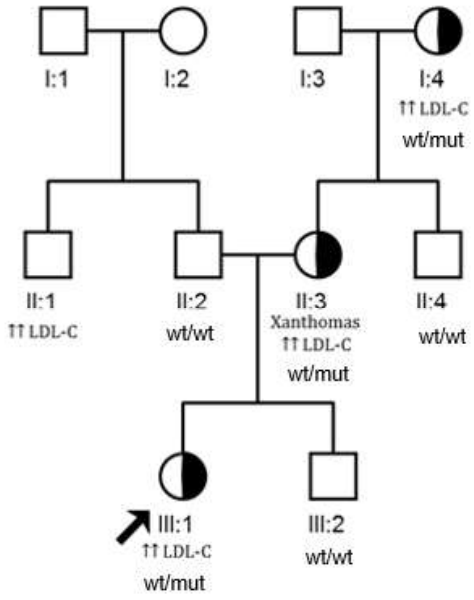


Figure S2. Pedigree of a FH family. Index case is identified with an arrow. Half-filled symbols represent heterozygous individuals. Index case III:1 inherited her *LDLR* variant from the maternal (II:3) side of the family. Her father (II:2) has normal cholesterol, no cardiovascular disease history, and is negative for the *LDLR* variant; therefore, her father (II:2) and paternal uncle (II:1) should not be considered in the co-segregation study. Similarly, the maternal grandfather (I:3) should not be considered. In this family the individuals that can be considered informative meioses are the index case's brother (III:2), mother (II:3), maternal uncle (II:4) and maternal grandmother (I:4). Index cases should not be counted as positive cases for co-segregation results.

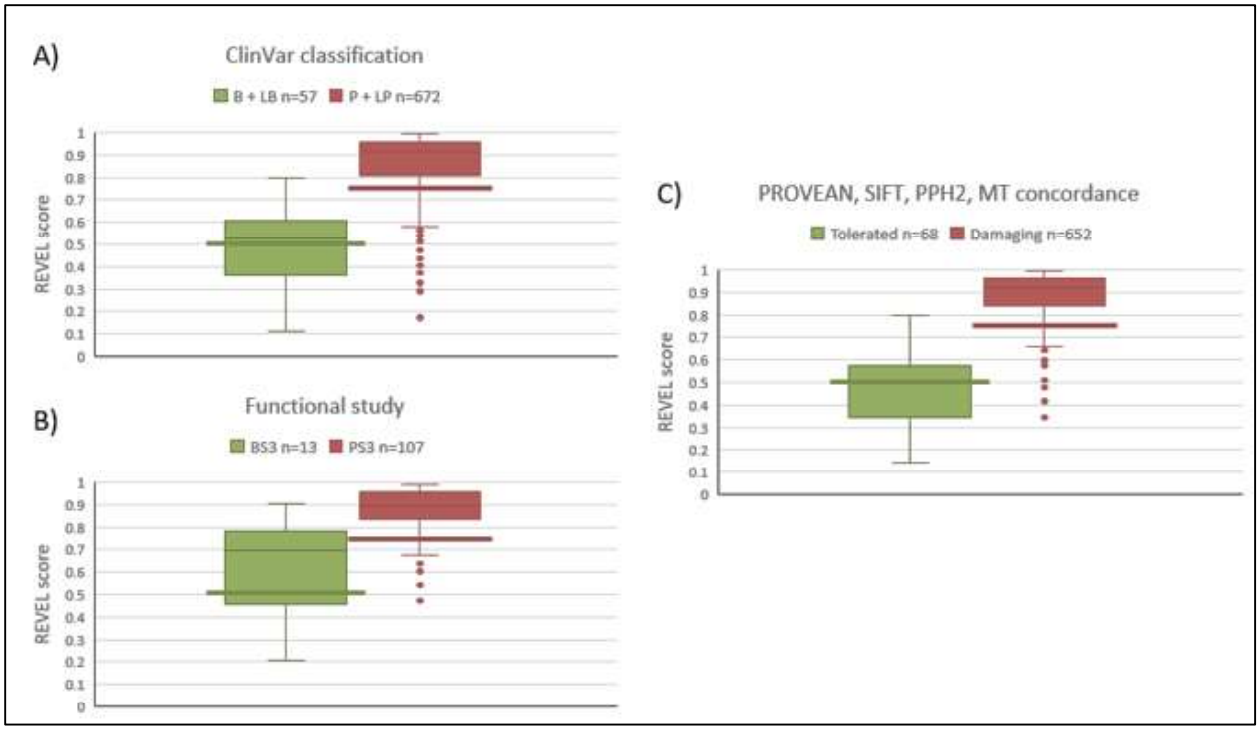


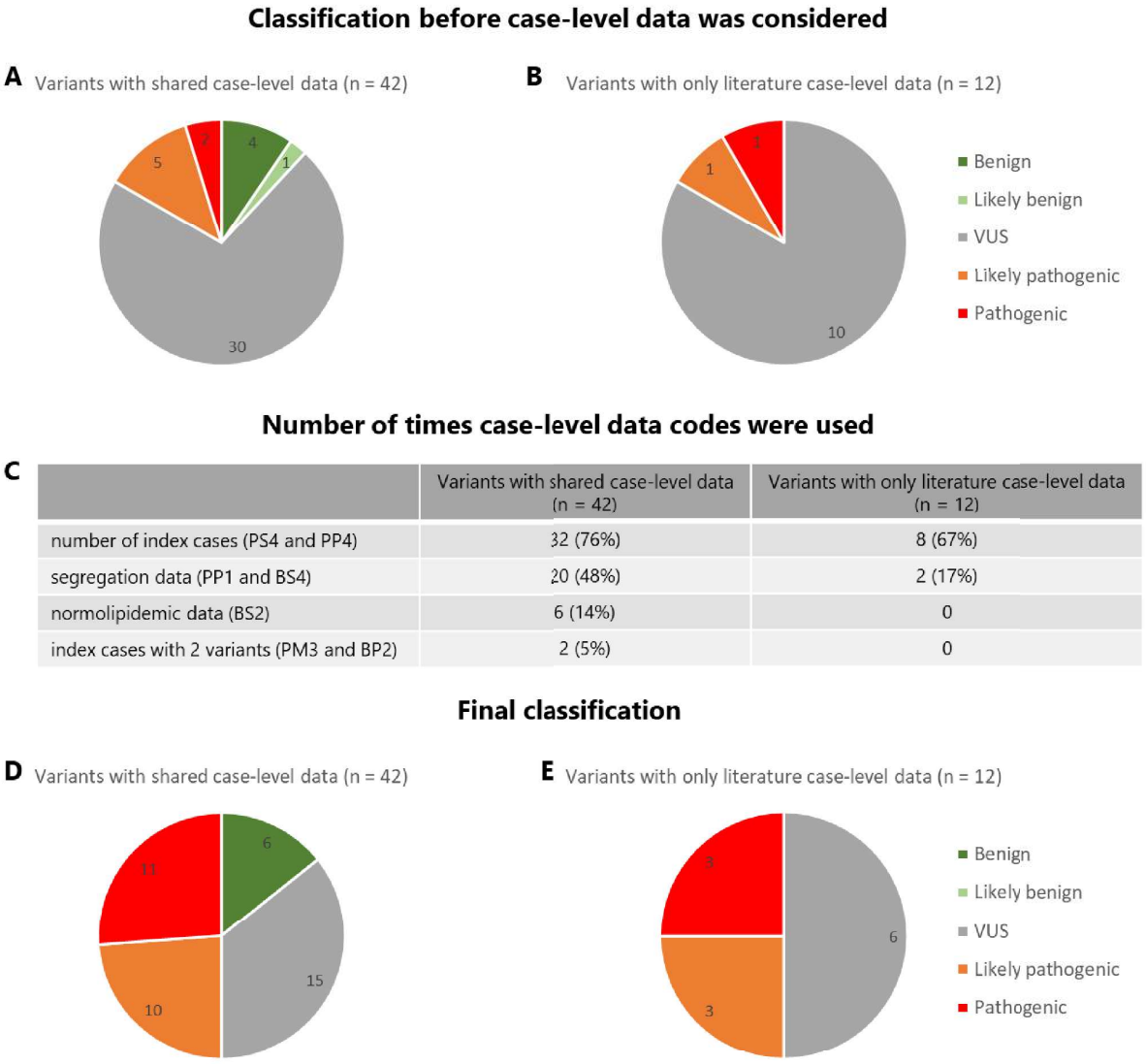
Figure S3. REVEL score distributions for determining missense PP3 and BP4 thresholds in *LDLR*. **A)** REVEL scores for *LDLR* missense variants classified as Benign/Likely benign (B+LB) or Pathogenic/Likely pathogenic (P+LP) in ClinVar. **B)** REVEL scores for *LDLR* missense variants with neutral or damaging BS3/PS3 evidence from functional studies. **C)** REVEL scores for *LDLR* missense variants with concordant *in silico* results for Poly-Phen-2 (PPH2), SIFT, PROVEAN and MutationTaster (MT). The green line represents the suggested threshold score of <0.50 used in the applicability of ACMG/AMP criterion BP4, and the red line represents the suggested threshold score of >0.75 used in the applicability of ACMG/AMP criterion PP3.

Table S5. *LDLR* pilot study variants.

***LDLR* Variant (NM_000527.5)**	**ClinVar ID**	**Previous ClinVar Status***	**ClinVar Classification**	**Pilot Study Classification**
c.1061-?_1845+?del	265901	1-star	P	Pathogenic
c.1A>T (p.Met1Leu)	250968	1-star	LP	VUS
c.58G>A (p.Gly20Arg)	161272	1-star	Conflicting: B (1); LB (5); VUS (5)	Benign
c.139G>A (p.Asp47Asn)	251034	1-star	Conflicting: LP (1); VUS (2)	VUS
c.185C>G (p.Thr62Arg)	375775	1-star	LP	VUS
c.185C>T (p.Thr62Met)	161273	1-star	Conflicting: LB (1); VUS (7)	VUS
c.232C>T (p.Arg78Cys)	161289	1-star	Conflicting: LP (1); VUS (1)	VUS
c.259T>G (p.Trp87Gly)	3685	2-star	P/LP	Pathogenic
c.261G>A (p.Trp87Ter)	251100	2-star	P	Pathogenic
c.268G>T (p.Asp90Tyr)	251106	2-star	LP	Likely pathogenic
c.296C>G (p.Ser99Ter)	161269	2-star	P	Pathogenic
c.313+1G>A	3736	1-star	Conflicting: LB (1); LP (2); P (12)	Pathogenic
c.343C>T (p.Arg115Cys)	251162	1-star	Conflicting: LP (1); P (1); VUS (1)	Likely pathogenic
c.344G>A (p.Arg115His)	225402	1-star	Conflicting: LB (1); LP (2); P (1); VUS (3)	VUS
c.693C>G (p.Cys231Trp)	251400	2-star	LP	Likely pathogenic
c.718G>T (p.Glu240Ter)	251422	2-star	P	Pathogenic
c.757C>T (p.Arg253Trp)	161261	1-star	Conflicting: LB (2); LP (1); P (1); VUS (4)	VUS
c.798T>A (p.Asp266Glu)	161287	1-star	Conflicting: LB (1); LP (9); P (6); VUS (2)	Pathogenic
c.806G>A (p.Gly269Asp)	161279	1-star	Conflicting: B (1); LB (8); LP (1); VUS (1)	Likely benign
c.862G>A (p.Glu288Lys)	161268	1-star	Conflicting: LP (9); P (1); VUS (1)	Pathogenic
c.907C>T (p.Arg303Trp)	161281	1-star	Conflicting: LP (1); VUS (4)	VUS
c.910G>T (p.Asp304Tyr)	251517	2-star	P/LP	Likely pathogenic
c.967G>A (p.Gly323Ser)	161282	1-star	Conflicting: LP (2); VUS (1)	VUS
c.970G>A (p.Gly324Ser)	161263	1-star	Conflicting: B (4); LB (3); LP (1); P (1); VUS (2)	Benign
c.1024G>T (p.Asp342Tyr)	251603	1-star	Conflicting: LP (1); VUS (3)	VUS
c.1055G>A (p.Cys352Tyr)	36450	2-star	P/LP	Likely pathogenic
c.1060+10G>C	226709	2-star	B/LB	Benign
c.1171G>A (p.Ala391Thr)	183138	2-star	B/LB	Benign

c.1186+5G>A	251706	1-star	Conflicting: LP (2); P (1); VUS (2)	Likely pathogenic
c.1216C>A (p.Arg406=)	3746	2-star	P	Likely pathogenic
c.1217G>A (p.Arg406Gln)	228798	1-star	Conflicting: LP (1); P (1); VUS (3)	Likely pathogenic
c.1222G>A (p.Glu408Lys)	36453	2-star	P/LP	Likely pathogenic
c.1238C>T (p.Thr413Met)	161276	1-star	Conflicting: LP (6); VUS (2)	Likely pathogenic
c.1322T>C (p.Ile441Thr)	251783	2-star	P/LP	Pathogenic
c.1323C>T (p.Ile441=)	456650	2-star	B/LB	Benign
c.1576C>T (p.Pro526Ser)	183120	1-star	Conflicting: LP (4); P (2); VUS (1)	VUS
c.1775G>A (p.Gly592Glu)	161271	2-star	P/LP	Pathogenic
c.1783C>T (p.Arg595Trp)	161290	1-star	Conflicting: LP (5); P (3); VUS (3)	Pathogenic
c.1816G>T (p.Ala606Ser)	161264	2-star	VUS	VUS
c.1855T>C (p.Phe619Leu)	252083	1-star	Conflicting: LP (1); VUS (3)	Likely pathogenic
c.1965C>G (p.Phe655Leu)	252135	1-star	Conflicting: LB (1); LP (1); P (1)	Likely pathogenic
c.1966C>A (p.His656Asn)	252136	1-star	Conflicting: B (2); LP (2)	VUS
c.2000G>A (p.Cys667Tyr)	3689	2-star	P/LP	Likely pathogenic
c.2043C>G (p.Cys681Trp)	252188	1-star	LP	Pathogenic
c.2096C>T (p.Pro699Leu)	252219	1-star	Conflicting: LP (7); P (2); VUS (2)	VUS
c.2101G>A (p.Gly701Ser)	183130	1-star	Conflicting: B (1); LB (2); LP (1); VUS (3)	VUS
c.2140+1G>A	3744	2-star	P/LP	Pathogenic
c.2140+5G>A	36460	1-star	Conflicting: B (7); LB (6); VUS (1)	Benign
c.2389+4A>G	252304	1-star	Conflicting: LB (1); VUS (3)	VUS
c.2389+8C>T	413774	2-star	B/LB	VUS
c.2479G>A (p.Val827Ile)	36462	1-star	Conflicting: B (3); LB (3); LP (1); VUS (7)	VUS
c.2531G>A (p.Gly844Asp)	3734	1-star	LP	Likely pathogenic
c.2546C>A (p.Ser849Ter)	252350	2-star	P/LP	Likely pathogenic
c.2575G>A (p.Val859Met)	252360	1-star	Conflicting: LB (2); VUS (2)	Likely benign

Abbreviations: B, benign; LB, likely benign; VUS, variant of uncertain significance; LP, likely pathogenic; P. pathogenic. *ClinVar status prior submitting FH VCEP classifications using the approved *LDLR*-specific ACMG/AMP guidelines; these variants are now at 3-star status.



	Variants with shared case-level data (n = 42)	Variants with only literature case-level data (n = 12)
number of index cases (PS4 and PP4)	32 (76%)	8 (67%)
segregation data (PP1 and BS4)	20 (48%)	2 (17%)
normolipidemic data (BS2)	6 (14%)	0
index cases with 2 variants (PM3 and BP2)	2 (5%)	0

Figure S4. Impact of case-level data in *LDLR* variant classification. Variant classifications prior to considering **A)** shared laboratory (i.e., internal) case-level data and **B)** case-level data available only in the published literature, in a pilot study of 54 *LDLR* variants. **C)** Number of times each *LDLR*-specific ACMG/AMP criteria code involving case-level data was used in variant classification. Final classification of 54 pilot study variants after application of **D)** shared laboratory (i.e., internal) case-level data and **E)** case-level data available only in the published literature.

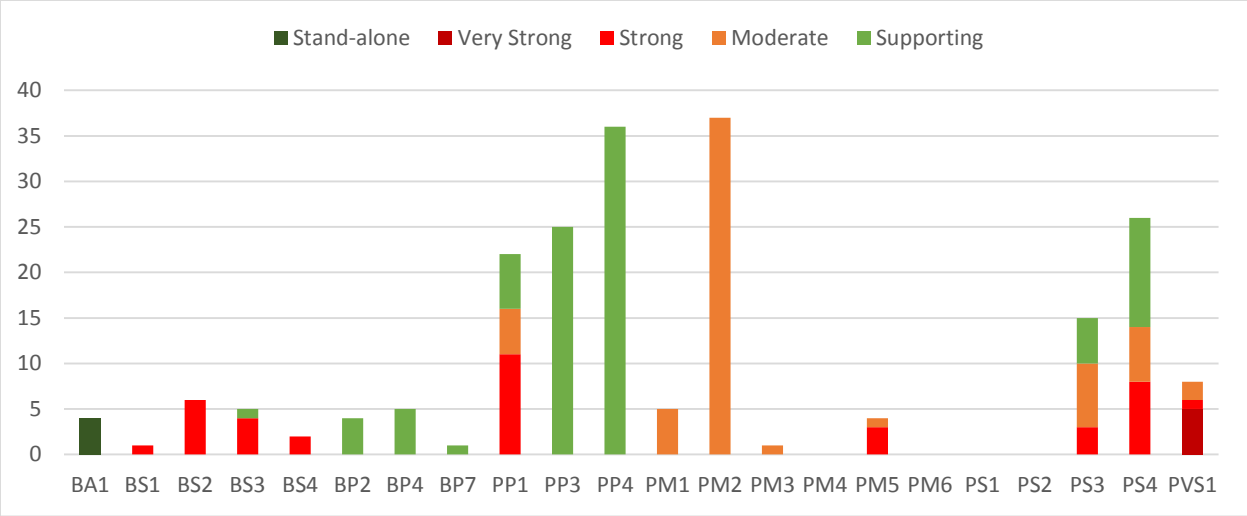


Figure S5. Number of times each ACMG/AMP criteria code was applied in a pilot study of 54 *LDLR* variants.

Supplementary File References

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