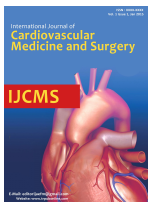
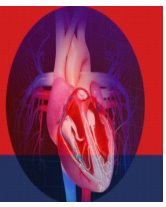
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CRISPR-validated PCSK9 pathway variants and lifelong atherosclerotic burden in a familial hypercholesterolemia registry

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Abstract

Background: Many variants identified by genetic testing are variants of uncertain significance and clinical criteria are not perfect at stratifying risk-familial hypercholesterolemia is a lifelong atherosclerotic burden and caused by variants in the PCSK9 pathway that lead to increased LDL cholesterol present from birth. CRISPR editing would, for the first time on a large scale, allow functional validation of variants that could help to improve and refine diagnosis and risk prediction. For this study, functional effects of variants of the PCSK9 pathway were assessed using CRISPR in the context of the lifetime pro-atherosclerotic burden among patients with FH from a registry. **Methods:** PCSK9-pathway variants found in the registry were functionally proven using PCSK9-CRISPR editing in a hepatocyte model and were categorized as functionally damaging or neutral, based on the difference in LDL uptake compared with wild-type. registry patients were assigned to functional class; differences in untreated LDL, cumulative LDL exposure were evaluated, along with differences in CA and ACSVD with event-free survival analyzed by Cox regression and compared with clinical criteria for discrimination. **Results:** In total, we detected 30 functionally damaging variants from 56 tested, and functionally validated 17 variants of uncertain significance, with 7 becoming pathogenic and 10 becoming benign. Among 1,080 patients, carriers of functionally damaging variants had a higher untreated LDL (247 versus 150 mg/dL), a greater cumulative LDL exposure, more coronary calcium (median 195 versus 37 Agatston units) and more atherosclerotic disease (35 versus 9%) than variant-negative patients, with a hazard ratio for atherosclerotic events of 4.5 (95% CI 2.8 to 7.1). Functional classification achieved a better discrimination than clinical criteria (the C-statistic of 0.77 versus 0.72). **Conclusion:** Functional validation of PCSK9-pathway variants with CRISPR technology reclassified Variants of Uncertain Significance (VUS) and suggested which carriers are most likely to have a large lifelong burden of atherosclerotic disease and events, which complements the use of clinical information and confirms the use of functional genomics for VUS in FHE.

Keywords: *Familial hypercholesterolemia, PCSK9, LDL receptor, CRISPR, Variant classification, Atherosclerotic burden, Functional genomics*

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1. Introduction

Familial hypercholesterolemia is the commonest monogenic cause of premature atherosclerotic cardiovascular

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disease. It arises from variants in the pathway that clears low-density lipoprotein from the blood, most often in the gene for the LDL receptor, less often in apolipoprotein B, and least often in PCSK9, whose gain-of-function variants were the third cause to be identified and whose loss-of-function variants, conversely, lower cholesterol and protect against coronary disease (Abifadel *et al.*, 2002; Cohen *et al.*, 2006). The consequence of these variants is an elevation of LDL cholesterol that is present from birth, so that the affected person accumulates an atherosclerotic burden over a lifetime rather than over the middle years, and it is this lifelong exposure, integrated over decades, that drives the early disease (Ference *et al.*, 2012 and 2017).

The reach of the problem is wide. Familial hypercholesterolemia is among the commonest inherited disorders, affecting perhaps one person in two to three hundred, yet the large majority of those affected are never diagnosed, and of those who are, many are identified only after their first cardiovascular event, when much of the preventable damage is already done (Benn *et al.*, 2016; Nordestgaard *et al.*, 2013). The disorder is, in principle, ideally suited to prevention: it is monogenic, it is detectable by a blood test and a gene test, its relatives can be found by cascade screening, and its cholesterol can be lowered effectively; what has been missing is the precision to know, for a given family, which variant is responsible and which relatives truly carry the risk (Gidding *et al.*, 2015). Resolving the meaning of the variants is therefore not an academic exercise but the step on which the whole preventive strategy depends.

The concept of a lifelong burden deserves emphasis at the outset, because it is what distinguishes this disorder from ordinary hypercholesterolemia and what the study set out to measure. A person with an acquired elevation of cholesterol in middle age has been exposed for years; a person with a familial variant has been exposed for a lifetime, from the womb onward, and the difference in accumulated exposure is large even when the measured cholesterol is similar. Atherosclerosis is a cumulative disease, its plaques the slow record of the cholesterol the artery has been exposed to over decades, so the relevant quantity is not the LDL concentration at a clinic visit but its integral over the whole of life, and it is precisely this lifelong integral that a pathogenic variant inflates and that determines the early and heavy disease of familial hypercholesterolemia.

Genetic testing has become central to the diagnosis, yet it has a persistent difficulty. Many of the variants it uncovers are of uncertain significance, neither clearly pathogenic nor clearly benign, and a variant of uncertain significance is of little use to a clinician who must decide how aggressively to treat (Sharifi *et al.*, 2017; Richards *et al.*, 2015). The clinical criteria that predate genetic testing, based on the cholesterol level, the family history and the physical signs, identify the syndrome but stratify the risk within it imperfectly, and they cannot resolve the meaning of an uncertain variant.

Function is what a variant of uncertain significance lacks a verdict on, and function can now be measured. CRISPR editing allows a variant to be introduced into a cell and its effect on the LDL-clearance pathway to be read directly, and the same technology that is being turned into a therapy for the pathway can be turned to the classification of its variants (Musunuru *et al.*, 2021; Findlay *et al.*, 2018). A functional assay that reports whether a variant impairs LDL clearance provides exactly the evidence the ACMG framework calls for to reclassify an uncertain variant, and, if the functional effect tracks the clinical burden, it may also sharpen the prediction of risk (Brnich *et al.*, 2019; Trinder *et al.*, 2019).

This study brought CRISPR functional validation to the variants of a familial hypercholesterolemia registry. It measured, for each PCSK9-pathway variant, its effect on LDL uptake, classified the variants as functionally damaging or neutral, reclassified those of uncertain significance accordingly, and then related the functional class to the lifelong atherosclerotic burden of the carriers, their cumulative LDL exposure, their coronary calcium and their cardiovascular events, testing whether functional validation improves on clinical criteria.

2. Materials and methods

2.1. Study design and the familial hypercholesterolemia registry

This was a functional-genomics study nested in a familial hypercholesterolemia registry, combining laboratory validation of variants with the clinical phenotypes of the patients who carried them. It was reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology guidance (von Elm *et al.*, 2007). The registry contained 1,080 patients with clinically diagnosed or genetically confirmed familial hypercholesterolemia.

2.2. Variant identification and selection

Patients had been sequenced across the three principal familial hypercholesterolemia genes, LDLR, APOB and PCSK9, and the 56 distinct variants identified in the pathway, including those classified at the time as of uncertain significance, were selected for functional validation (Khera *et al.*, 2016).

2.3. CRISPR functional validation of variants

Each variant was introduced into a human hepatocyte cell line by CRISPR editing, a base editor or a homology-directed repair template being used to install the precise nucleotide change, and correct editing was confirmed by amplicon sequencing (Rothgangl *et al.*, 2021). The functional consequence of each variant was then measured as the capacity of the edited cells to take up LDL, assessed by the uptake of fluorescently labelled LDL on flow cytometry and expressed as a percentage of the uptake of cells carrying the wild-type sequence, with the cell-surface expression of the LDL receptor and, for PCSK9 variants, the secretion and activity of PCSK9 measured in support. Every variant was tested in replicate against wild-type and empty-vector controls.

2.4. Functional classification and variant reclassification

A variant was classified as functionally damaging when the LDL uptake of the cells carrying it fell below 65 percent of wild-type, and as functionally neutral otherwise. This functional evidence was applied within the ACMG and AMP interpretation framework as the PS3 or BS3 criterion, and the variants that had been of uncertain significance were reclassified as pathogenic or benign accordingly.

2.5. Measures of lifelong atherosclerotic burden

The lifelong atherosclerotic burden of each patient was captured by three measures. The untreated LDL cholesterol was the pre-treatment concentration; the cumulative LDL exposure, expressed in units of a thousand milligram-decilitre-years, was computed as the integral of the LDL concentration over the patient's life, discounting the reduction achieved by lipid-lowering treatment; and the coronary artery calcium score was measured by computed tomography and expressed in Agatston units (Miname *et al.*, 2019).

2.6. Outcomes

The outcome was atherosclerotic cardiovascular disease, a composite of myocardial infarction, coronary revascularisation, ischaemic stroke and cardiovascular death, and its age of onset was recorded so that the freedom from disease could be examined as a function of age.

2.7. Statistical analysis

Patients were grouped by the functional class of the variant they carried, into carriers of a functionally damaging variant, carriers of a functionally neutral variant, and those in whom no variant was found. The measures of burden were compared across the groups by the Kruskal-Wallis or the chi-squared test, the freedom from atherosclerotic disease was estimated by the Kaplan-Meier method and compared by Cox regression adjusted for sex, and the discrimination of the functional classification for atherosclerotic disease was compared with that of the clinical criteria by the area under the receiver operating characteristic curve (DeLong *et al.*, 1988). Analyses were performed in Python.

2.8. Ethics and informed consent

The study was approved by the Institutional Ethics Committee (approval number IEC/CVG/2023/436) and was conducted in accordance with the Declaration of Helsinki. Written informed consent for genetic analysis and registry participation was obtained from every patient, and all data were anonymised and identified only by a study code.

3. Results

3.1. CRISPR functional validation and variant reclassification

Of the 56 PCSK9-pathway variants tested, 30 were functionally damaging, reducing LDL uptake below the threshold, and 26 were functionally neutral, and the two classes separated cleanly on the functional assay, as shown in Figure 1. The functional evidence resolved the variants that had been of uncertain significance: of 17

such variants, 7 were reclassified as pathogenic and 10 as benign. The distribution of the variants across the three genes and their reclassification are given in Table 1, the majority lying, as expected, in LDLR.

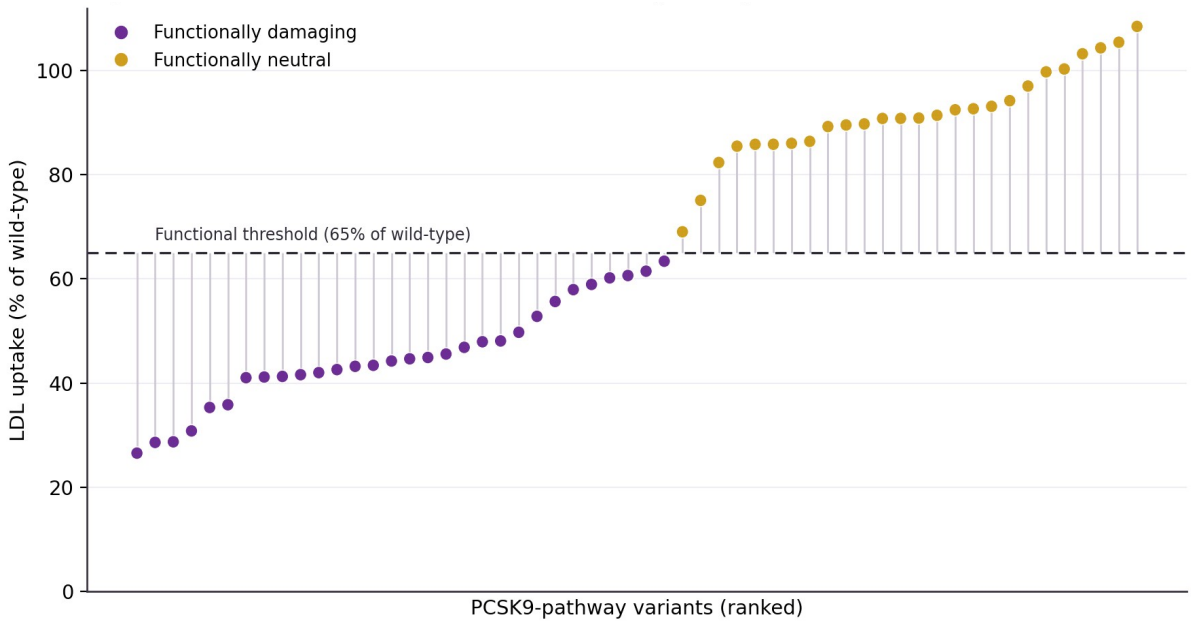


Figure 1: CRISPR functional validation of PCSK9-pathway variants, showing LDL uptake relative to wild-type

Table 1: Functional validation of PCSK9-pathway variants by gene

Gene	Variants tested	Functionally damaging	Functionally neutral	Previously uncertain
LDLR	40	23	17	12
APOB	7	2	5	2
PCSK9	9	5	4	3

3.2. Registry population by functional class

Of the 1,080 patients, 593 carried a functionally damaging variant, 273 a functionally neutral variant, and 214 had no identified variant. The three groups are described in Table 2. The carriers of a functionally damaging variant had, as the defining feature, a markedly higher untreated LDL cholesterol than the variant-negative patients (246.7 versus 149.6 milligrams per decilitre), confirming that the functional assay had identified the variants of genuine biological consequence.

Table 2: Characteristics of the registry population by functional variant class

Characteristic	Damaging carrier	Neutral carrier	Variant-negative
Number	593	273	214
Age, years (mean)	46.3	46.1	45.1
Untreated LDL, mg/dL	246.7	183.6	149.6
On statin therapy, %	82	82	82

3.3. Lifelong LDL burden

The higher LDL of the damaging-variant carriers, present from birth, translated into a greater cumulative exposure that widened with age, as shown in Figure 2. By the later decades the carriers of a functionally damaging variant had accumulated substantially more LDL-years than the neutral-variant carriers, who in turn had accumulated more than the variant-negative patients, so that the functional class ordered the lifelong burden. The mean cumulative LDL exposure was 8.5, 6.3 and 5 thousand milligram-decilitre-years across the damaging, neutral and negative groups respectively.

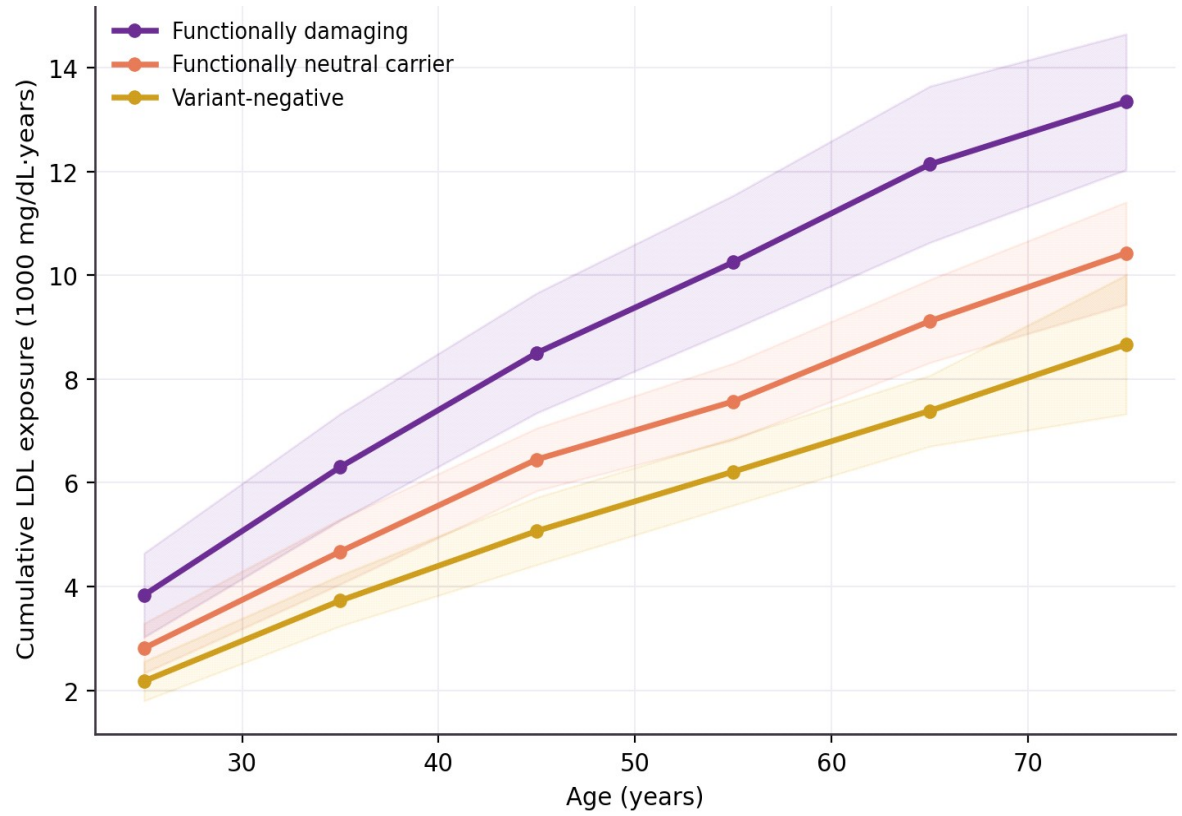


Figure 2: Accumulation of cumulative LDL exposure with age by functional variant class

3.4. Coronary calcium and atherosclerotic burden

The accumulated LDL exposure was reflected in the coronary arteries. The coronary artery calcium score rose with the functional class, its median being 195 Agatston units in the damaging-variant carriers against 36.5 in the variant-negative patients, and across the whole registry the calcium score correlated strongly with the cumulative LDL exposure (Spearman r = 0.80), the direct imprint of lifelong cholesterol exposure on the vessel wall. The burden measures are summarised in Table 3.

Table 3: Lifelong atherosclerotic burden and outcomes by functional variant class

Measure	Damaging carrier	Neutral carrier	Variant-negative
Untreated LDL, mg/dL	246.7	183.6	149.6
Cumulative LDL exposure, 1000 mg/dL·yr	8.5	6.3	5
Coronary artery calcium, Agatston (median)	195	58	36.5
Prevalent ASCVD, %	35	16	9

3.5. Atherosclerotic disease by functional class

The burden was matched by the events. Atherosclerotic cardiovascular disease had occurred in 35% of the functionally damaging-variant carriers, against 16% of the neutral-variant carriers and 9% of the variant-negative patients, and the freedom from disease across the lifespan separated the groups, as shown in Figure 3. In the Cox model the carriers of a functionally damaging variant had more than four times the risk of atherosclerotic disease of the variant-negative patients (hazard ratio 4.5, 95% CI 2.8 to 7.1), the events occurring, in these carriers, at younger ages.

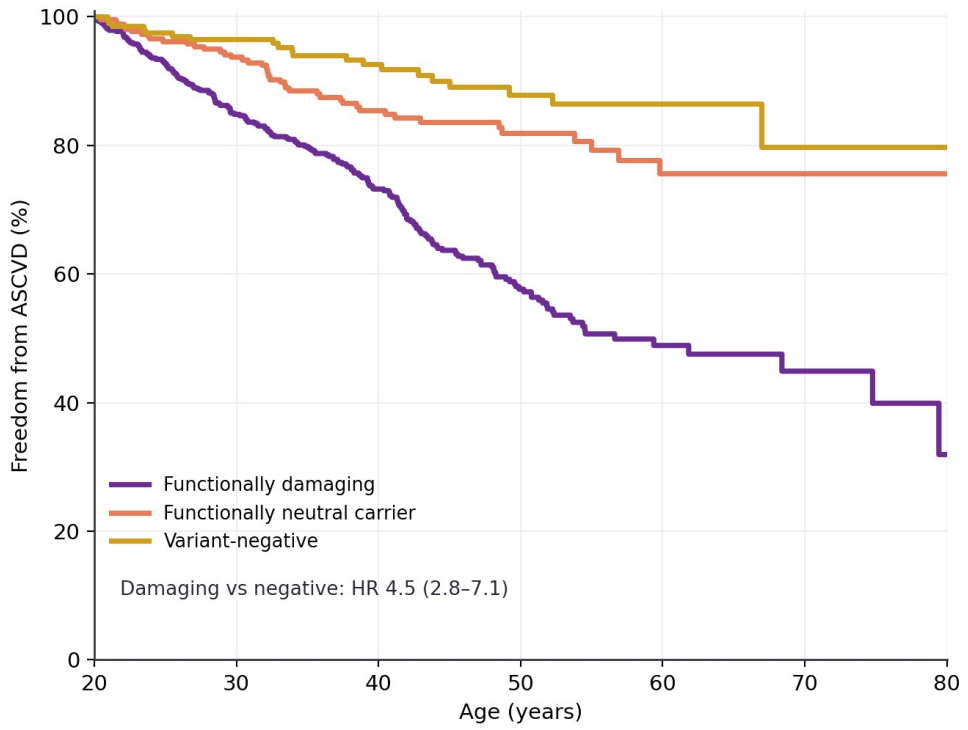


Figure 3: Freedom from atherosclerotic cardiovascular disease across the lifespan by functional variant class

The carriers of functionally neutral variants occupied a consistent middle ground across every measure. Their untreated LDL (183.6 milligrams per decilitre), their cumulative exposure and their coronary calcium lay between those of the damaging-variant carriers and the variant-negative patients, and their prevalence of atherosclerotic disease (16%) did likewise. This intermediate position is itself informative: it indicates that a neutral functional call does not simply exclude disease but identifies a group whose modestly raised cholesterol, in the setting of a familial hypercholesterolemia referral, still carries some excess burden, most plausibly from polygenic or environmental causes rather than from the tested variant, and it argues that a neutral call should redirect attention to those other causes rather than end the evaluation.

3.6. Discrimination beyond clinical criteria

The functional classification added to what the clinic could already measure. For the discrimination of atherosclerotic disease, the functional classification of the variant, combined with the coronary calcium, achieved an area under the curve of 0.77, against 0.72 for a model based on the clinical criteria, the treated cholesterol and age. The gain, though moderate, indicates that knowing whether a variant is functionally damaging carries prognostic information beyond the cholesterol level a clinician observes, which is the treated concentration rather than the lifelong exposure the variant determines.

4. Discussion

In a FH registry, the PCSK9-pathway variants associated with those of uncertain significance were clarified and their population ranked by their long-term atherosclerotic burden through CRISPR functional validation.

Functionally damaging variants predicted the highest untreated cholesterol, highest cumulative LDL exposure, highest coronary calcium and highest atherosclerotic disease risk and the functional classification predicted disease better than the clinical criteria.

Most expedient result is the reclassification of uncertain variants. A VUS leaves the physician and patient in a sub-optimal situation, neither genetic confirmation of the diagnosis nor genetic management of relatives can be undertaken, and the number of VUS are increasing as sequencing has become more widespread. The assay turned the interpretation framework asked for into actionable pathogenic or benign calls, with the help of direct functional evidence, and this is exactly what direct functional data should be and should do in the interpretation framework—which large-scale editing now makes possible for the pathway as a whole.

In addition to diagnosis, the functional class proved to have prognostic significance and it is noteworthy for its reasons. The deleterious effect of a Familial Hypercholesterolemia (FH) variant is the integral of a raised LDL over a lifetime and this is not adjusted by a lifelong history of the variant, but is the written LDL that the clinician measures at one time in adult life, with treatment effects taken away—these are not a good indicator of what has happened in adult life. The functional class identifies those variants that affect LDL from the outset and so indexes exposure over the life course more faithfully than does the treated LDL, making it an added predictor of disease, hence the greater risk associated with reaching the same level of LDL with a functionally confirmed hypercholesterolemia than with a same level of LDL without such a variant. It is also curious to see how well the accumulation of LDL in the arteries correlates with the amount of calcium deposits as shown here, the amount of calcium serving as another record of the cholesterol to which the artery has been exposed to. This strengthens the genetic and trial evidence that it is long-term levels of LDL (AUC) and not levels "at any one time" that is linked to risk of atherosclerosis; and that it is best to find and treat those with damaging LDL variants early in the disease process, so the burden is not built up (Sabatine *et al.*, 2017).

Systematic testing of close relatives of an individual with FH is the most effective way of identifying new FH patients and it is referred to as cascade screening and relies entirely on the knowledge of the FH variant. Variants that have been identified as damaging are reliable markers and provide those who have family members with the variant with confidence that the test result is damaging—if the test result is positive, the family member is affected and if the test result is negative, the family member is not affected. Variants with uncertain significance cannot be used as anchors to cascade, as no real knowledge would be gained by a family member who tested positive for the variant. Each of these uncertain variants can be converted to definite variants as part of functional validation to extend scaled up cascade screening to these stalled families, and moreover, across the whole pathway, hence making scalable editing worthwhile.

It is a helpful pattern to recognize that the way to take the medicine and the way to take the medicine are similar. The same pathway that variants were identified and classified in this study is one of the most fruitful therapeutic targets in cardiovascular medicine already being targeted by antibodies and small interfering RNA to hepatocytes against PCSK9 itself, and now, experimentally, by bases switching off PCSK9 and the resulting reduction of cholesterol that has been shown to be durable in primates through the very base editing technology used in this study. If a patient can be demonstrated to carry a functionally damaging variant, then he or she is not only precisely diagnosed, but led down a path that needs to be treated considerably, and the identification of the highest burden of carriers marks a direction in which intensive, early and life-long reduction of LDL is most clearly indicated, as it is the cumulative exposure that their variant provides from birth that the treatment needs to correct.

Aside from the selection of LDL uptake as the functional readout, which is what makes the assay meaningful, it is worth a comment. The shared defect of FH regardless of which one of the 3 genes is abnormal is the inability of the cells to remove LDL from the blood: the LDL receptor can be missing, or it might be unstable and incapable of binding to LDL or PCSK9 can be abnormal and rapidly degrade the receptor, but in all cases, there is a decrease in receptor-mediated uptake of LDL. The measurement of that uptake is then a measure of the physiology, and a variant which reduces that uptake is harmful in the clinically relevant sense irrespective of how it is harmful on the molecular level. The co-occurrence of these three genes upon a single measurable function is what enables one assay to validate variants throughout the entire pathway and set them – damaging or not—on a shared scale.

5. Conclusion

CRISPR functional validation in a familial hypercholesterolemia registry classifies Variants of Undetermined Significance (VUS) into pathogenic/benign calls, and reveals that patients with life-long atherosclerotic burden (highest LDL exposure, highest coronary calcium and highest atherosclerotic disease risk) acquire their pathogenic variants. The functional class, which indexes lifelong exposure and, hence, risk more faithfully than does the treated cholesterol level, benefited from a strategy that went beyond simply clinical inputs on how to discriminate between diseases, and provides a powerful tool to support functional genomics as a practical adjunct to genetic testing in FH, which could facilitate earlier, more accurately targeted intervention for these patients.

Ethical approval

The study was approved by the Institutional Ethics Committee and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from every participant.

Conflict of interest

The authors declare that no conflict of interest exists in relation to this work.

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Data availability

The data supporting the findings of this study are available from the corresponding author on reasonable request.

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