JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 8, NO. 8, 2023

EDITORIAL COMMENT

Sailing the Uncharted Waters of Familial Hypercholesterolemia LDL Receptor Genetic-Related Variants

Land in Sight?*

Raul D. Santos, MD, PhD, MSc

Heterozygous familial hypercholesterolemia is a common autosomal codominant disorder[1] affecting 1 in 300 individuals characterized by elevation of blood low-density lipoprotein (LDL) cholesterol and early atherosclerotic cardiovascular disease onset. Reduction of LDL clearance by hepatocytes is the main mechanism behind LDL cholesterol blood accumulation in familial hypercholesterolemia (FH). LDL binds the low-density lipoprotein receptor (LDLR) by apolipoprotein B and this complex is internalized into clathrin-coated pits by endocytosis. After that, LDL is released from the receptor for degradation and the latter is recycled to hepatocyte surface.[2] Loss of function variants in *LDLR* are responsible for 60%-80% of proven FH cases, whereas 5%-10% are caused by abnormalities in the apolipoprotein B gene (*APOB*), and <1% by gain of function variants in the *PCSK9*.[2,3] The heterozygous FH phenotype may rarely be caused by variants in the apolipoprotein E gene. When no genetic defects in the canonical genes are encountered, the phenotype may be caused in 20%-40% of cases by polygenic effects (polygenic hypercholesterolemia).

FH is a genetic disease, and an accurate molecular diagnosis has important implications for index cases and their first-degree relatives considering, respectively, the associated higher risk of atherosclerosis in comparison with other forms of hypercholesterolemia and the autosomal dominant transmission pattern of this disease.[4] The onset of next-generation sequencing has facilitated FH molecular diagnosis; however, it brought challenges considering that many variants encountered in the canonical genes were recently described and therefore not proven to cause the phenotype. This is especially true for those occurring in *LDLR*.

Variant validation may be done by cosegregation analyses in possibly affected families, by testing the impact of the defect on protein structure by computerized in silico models, or by in vitro or ex vivo functional testing.[3] Cosegregation analysis is performed by checking concomitantly genetic defects and blood LDL cholesterol concentrations, usually this needs a great number of individuals from the same family, and in many situations is not feasible. Therefore, laboratory techniques are of extreme importance in clinical practice. However, variant validation is a rather complex process because defects may be analyzed from either the gene or the protein side. The *LDLR* variants are classified as nonsense, missense, synonymous, variants in the promoter and splice sequences, frameshift (small deletions or insertions), and large DNA rearrangements.[4] In silico models are usually adequate for nonsense, frameshift, or large DNA rearrangements validation. Missense variants are the most common (40%-50% of the newer described ones) and the hardest to prove their pathogenicity because they may cause a change on a single amino acid in the protein sequence.[3,4] These usually need further testing. On the protein side, variants may cause defects in LDLR recycling on

*Editorials published in *JACC: Basic to Translational Science* reflect the views of the authors and do not necessarily represent the views of *JACC: Basic to Translational Science* or the American College of Cardiology.

From the Lipid Clinic, Heart Institute University of São Paulo Medical School Hospital, São Paulo, Brazil; and the Academic Research Organization, Hospital Israelita Albert Einstein, São Paulo, Brazil.

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ISSN 2452-302X https://doi.org/10.1016/j.jacbts.2023.05.014

hepatocytes by affecting LDLR expression or biosynthesis. Class I defects lead to no protein synthesis and are called null variants; class II lead to complete (IIa) or partial (IIb) retention of the protein at the endoplasmic reticulum; class III affect LDL particle binding; class IV lead to reduction in LDL internalization; class V cause defects in recycling; and class VI cause defects in LDLR transport to basolateral membrane.[1,2]

Functional testing is usually performed by comparing cells containing a suspected variant with normal or wild-type cells to test LDLR activity by flow cytometry with fluorescently labeled LDL.[2] For that, one can use patient cells that express the LDLR (classically fibroblasts and more recently lymphocytes or lymphoblasts) or cells not expressing the LDLR that are transfected with plasmids containing *LDLR* with the suspected variant. As one may suppose these are complex and laborious laboratory experiments and are not widely available. Things may get more complex considering the need for experiment reproducibility and possible impact of epigenetic effects on protein expression.

In this issue of *JACC: Basic to Translational Science*, Graça et al[5] propose an elegant way to advance testing the functionality of *LDLR* variants and help guide navigation on uncharted waters of FH diagnosis. Instead of using the classic flow cytometry studies, they developed a model where high-throughput microscopy was used in association with labeled LDL binding and uptake assays. The *LDLR*-deficient cells were transfected with wild-type and mutant variables. LDLR of transfected cells was overexpressed, then cells were incubated with labeled LDL and LDLR expression, binding, and activity were measured. Initially, the investigators tested normal wild-type cells, and previously proven pathogenic *LDLR* variants of different class defects (I, IIa, III, IV, or V). Normal and abnormal variants were respectively considered if labeled LDL uptakes were, respectively, >90% or <70% of the ones from wild-type cells. After that, 19 uncharacterized *LDLR* variants of Portuguese patients with clinical diagnosis of FH (mean LDL cholesterol 252.2 ± 67.9 mg/dL) were tested. Findings were normalized against those of wild-type control cells. Two sets of assays were performed respectively with 24 and 96 wells. In the control set, all 5 variants codifying null defects as well as 5 of 6 normal variants were identified with both 24- and 96-well assays (with the exception of 1 control variant with the latter). Class III variants that affect LDL binding were correctly identified by both assays; however, the 2 class IV variants were not. Only 2 of 9 variants (22%) with partial expression at cell surface or recycling defects were identified by both assays.

In the test set, 11 variants showed abnormal function, 5 were normal, and 3 were inconclusive in both 24- and 96-well assays. According to the American College of Medical Genetics FH Expert Panel *LDLR* criteria, 11, 7, and 1 had been previously classified as pathogenic/likely pathogenic, variants of uncertain significance, and likely benign, respectively. Four variants previously classified as uncertain were reclassified with the functional testing. According to Graça et al[5] their experiments would have taken one-third and one-sixth of the time needed for the flow-cytometry studies for the same output.

Overall, this is an elegant proof-of-concept study suggesting a promising new methodology to identify *LDLR* variants that may or may not cause the FH phenotype. However, the study is limited by the relatively small number of variants tested and by the fact that 43% of variants of unknown significance were still not reclassified because assays did not unequivocally distinguish class IIB and V or class III and IV variants (variants coding a milder FH phenotype). Graça et al[5] recognized that further testing in bigger samples should be performed and that the experimental protocol should be technically improved. In addition, results should be reproduced by other laboratories.

Graça et al[5] propose a novel method that has potential to help improve validation of FH-related variants, but further studies are still necessary. The Portuguese were bold navigators in the 16th century and discovered the ways to navigate in uncharted waters to distant and rich lands. Hopefully Graça et al[5] are on the same path where FH diagnosis is concerned.

FUNDING SUPPORT AND AUTHOR DISCLOSURES

Dr Santos has received honoraria related to consulting, research, and speaker activities from Amgen, Amryt, Abbott, Biolab, Esperion, Getz Pharma, Kowa, Libbs, PTC Therapeutics, Pfizer, Novartis, Novo-Nordisk, and Sanofi/Regeneron.

ADDRESS FOR CORRESPONDENCE: Dr Raul D. Santos, Unidade Clínica de Lipides InCor HCMFUSP, Av Dr Eneas Carvalho Aguiar 44, CEP 05403-000, São Paulo, São Paulo, Brazil. E-mail: raudlsf@gmail.com.

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 8, NO. 8, 2023
AUGUST 2023:1022–1024

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KEY WORDS ACMG classification, familial hypercholesterolemia, high-throughput characterization, LDL cholesterol, LDL receptor, molecular diagnosis, variants