

Familial Hypercholesterolemia: Why every endocrinologist should know about PCSK9

BY PHILIP W. CONNELLY, PHD

Familial hypercholesterolemia is an established cause of cardiovascular disease. This issue of *Endocrinology Rounds* presents the background for the discovery of the defects in cellular cholesterol uptake that were first described in this disorder, summarizes the latest recommendation for identifying familial hypercholesterolemia, and discusses the insights from the most recent discovery of the role for PCSK9 in cholesterol metabolism.

The discovery of the LDL receptor

For a student working in the area of lipoprotein research, the period from 1974 to 1984 was an exciting time. Familial syndromes causing lipoprotein abnormalities had been clinically described and new tools were being applied to solve the riddles of their molecular and genetic cause. From a cardiovascular perspective, the most important lipoprotein disorder was the syndrome of familial hypercholesterolemia (FH).¹ With the new tool of cell culture, it was discovered that skin fibroblasts from patients with the severest forms of FH were defective in regulating the rate-limiting enzyme of cholesterol synthesis, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase. This defect is expressed when exogenous cholesterol is delivered as low-density lipoprotein (LDL), but not when cholesterol is delivered dissolved in ethanol. This important distinction led to a focus on the uptake of LDL-cholesterol (LDL-C) as the source of the defect in the fibroblasts. These discoveries by Michael Brown and Joseph Goldstein² pushed the entire field forward at a breakneck pace, especially after Wolfgang Schneider and David Russell, via the University of British Columbia, joined them in Dallas. In 1982, using a biochemical approach, Schneider purified (from bovine adrenals) the molecule responsible for the high-affinity uptake of LDLs by skin fibroblasts, a cell receptor called, appropriately, the LDL receptor.³ Others, including Robert Mahley's group at the Gladstone Foundation in San Francisco, recognized that this receptor also had a high affinity for lipoproteins containing apolipoprotein (apo) E and designated the receptor, the apoB, E receptor.⁴ Subsequently, Russell made fundamental contributions to the studies of cloning and mutagenesis in the LDL receptor.⁵

Remarkably, the discovery of the LDL receptor led to more fundamental discoveries about a process called receptor-mediated endocytosis. Now there was an itinerary for the LDL receptor; it involves cycling many times in a vesicular process from the exterior of the cell membrane, delivering LDL for degradation and utilization by the cell. Although this process was discovered by the study of skin fibroblasts, the cycling of LDL is fundamental and universal for cell biology and is essential in organs as diverse as the liver and the adrenal glands.

Brown and Goldstein received a Gairdner Foundation International Award in 1981 and the Nobel Prize in Physiology or Medicine in 1985,⁶ due to their discoveries revealing the distinct parts of the LDL receptor and mapping the essential functional domains. The focus of their research then shifted to the heart of the matter; ie, how does a cell "sense" the balance of cholesterol and the need for uptake or synthesis of this central molecule in cellular metabolism? The result was the discovery of a remarkable



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regulatory system that involved tethering the cholesterol-sensing proteins, ie, sterol regulatory-element binding proteins (SREBPs) 1a, 1c, and 2, in the cellular structure called the endoplasmic reticulum (ER).^{7,8} Both SREBP1 and SREBP2 are deoxyribonucleic acid (DNA)-binding proteins that are released from the ER by a 2-step process requiring proteases. The processing of SREBP2 from membrane bound to a soluble form is increased when cholesterol is low; SREBP2 is also indicated as the primary protein regulating the expression of the LDL receptor and cholesterol biosynthesis.⁷

Familial hypercholesterolemia

Classic FH presents with an isolated elevation of plasma LDL-C and skin findings on physical examination, such as xanthomas and thickening of the Achilles tendon. The homozygous form is rare and unmistakable, exhibiting extremes of LDL-C that can reach 20 mmol/L. The heterozygous form is subtle, giving a wide range of presentation; as a result, objective criteria require the comparison with a reference population that accounts for the effects of age and sex. Subjects with heterozygous FH will consistently have LDL-C >95th percentile; however, only 4% of subjects in the general population with LDL-C >95th percentile will have FH.⁹ The Make Early Diagnosis to Prevent Early Death¹⁰ project proposed a set of criteria, based on patients with genetically proven FH, which were narrower for identifying an index case and then slightly relaxed for identification of family members with the disorder. Recommendations for the detection of FH have recently been updated (Table 1).¹¹

Initially, the genetic studies of the LDL receptor anticipated that a few mutations would explain the majority of affected subjects and, therefore, a genetic test would be a convenient diagnostic approach. The gene for the LDL receptor (*LDLR*) is located on chromosome 19q. Although FH in some populations, such as French Canadians, revealed a small number of founders that carried the genetic defect and it could be ascribed to a small number of inactivating mutations, this is not true for the general population. A database of genetic mutations in the *LDLR* that have been described in patients with FH has now reached over 1000 independent mutations.¹⁰ To be sure, not all of these have been verified at the functional level; however, it is clear that no simple genetic test is of practical value for identifying FH.

Early genetic studies of FH were able to use the information for the chromosomal location of the gene to identify families where the phenotype did not segregate with chromosome 19q. Such genetic studies identified the cause of hypercholesterolemia as a mutation in apoB100, the ligand for the LDLR. Remarkably for such a large protein (>4500 amino acids), familial defective

Table 1: NICE recommendations/definitions for identifying familial hypercholesterolemia¹¹

<p>Definite familial hypercholesterolemia:</p> <ul style="list-style-type: none"> • TC >6.7 mmol/L or • LDL-C >4.0 mmol/L in a child aged <16 years or • TC >7.5 mmol/L or LDL-C >4.9 mmol/L in an adult (levels either pretreatment or highest on treatment) <p>and at least one of the following:</p> <ul style="list-style-type: none"> • Tendon xanthomas in patient or in first-degree relative (parent, sibling or child) or in second-degree relative (grandparent, uncle or aunt) • DNA-based evidence of an LDL-receptor mutation, familial defective apo B-100 or a PCSK9 mutation. <p>Possible familial hypercholesterolemia:</p> <ul style="list-style-type: none"> • TC >6.7 mmol/L or • LDL-C >4.0 mmol/L in a child aged <16 years or • TC >7.5 mmol/L or LDL-C >4.9 mmol/L in an adult (levels either pretreatment or highest on treatment) <p>and at least one of the following:</p> <ul style="list-style-type: none"> • Family history of myocardial infarction: <50 years of age in second-degree relative or <60 years of age in first-degree relative • Family history of raised TC: >7.5 mmol/L in adult first-degree or second-degree relative or >6.7 mmol/L in child or sibling aged <16 years.
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NICE = National Institute for Health and Clinical Excellence; TC = total cholesterol; LDL-C = low-density lipoprotein cholesterol; DNA = deoxyribonucleic acid; apo = apolipoprotein. Reproduced from Minhas R et al. *Heart*. 2009;95(7): 584-587, with permission from BMJ Publishing Group Ltd. Copyright © 2009.

apoB is primarily due to a mutation in amino acid residue 3500, either arginine3500glutamine (in European populations)¹³ or arginine3500tryptophan (in Chinese populations).¹⁴ The Danish population has a frequency of 1/1000 for arginine3500glutamine, but hypercholesterolemia is variable.¹⁵ As a result, while this mutation can be as detrimental as *LDLR* mutations, generally, it has a lower penetrance and, therefore, has a smaller than expected impact at the population level.

The two genetic loci, *LDLR* and *APOB*, accounted for the majority of FH, yet, a small residual number of cases remained unsolved. Typically these cases were not amenable to investigation due to the need to have an informative pedigree – ie, one with a large enough number of affected and unaffected subjects – where known genetic loci could be excluded. During this time, a recessive form of hypercholesterolemia was identified; this recessive form was caused by mutations in an “adaptor” protein required for the vesicular itinerary of the LDLR.¹⁵ Interestingly, this adaptor protein (known as autosomal-recessive hypercholesterolemia [ARH] or LDLR adaptor protein 1 [LDLRAP1]) is not expressed in skin fibroblasts.¹⁶ The first studies of the familial syndrome used transformed lymphocytes to identify this new component of the LDLR pathway,¹⁷ and there was no indication that a major causal factor for FH remained to be described.

PCSK9: from unknown to star performer

This view completely changed with a genetic study and description of an autosomal-dominant FH, which identified a mutation in a serine protease gene, proprotein convertase subtilisin/kexin type 9 (PCSK9).¹⁸ At the time of the initial discovery in 2003, so little was understood about the function of this protease that it was not clear whether the mutation was a *gain* or *loss of function* mutation. However, Nabil Seidah and Michel Chretien,¹⁹ 2 Canadians among the world experts in the field of the proprotein convertases, provided pivotal information about the function of PCSK9. In the same year, Horton, Brown, and Goldstein used mouse transgenic and knockout models to manipulate the expression of SREBP2. In an early application of microarray analysis of hepatic gene expression, they combined the results from 3 experimental states with an elegant use of logic to identify 33 genes as the primary targets for SREBP2.²⁰ One of these targets was PCSK9, and this established PCSK9 as a gene that was turned on in concert with the LDLR and HMG-CoA reductase. From that moment, demonstrating the strength and value of a knowledge base derived from discoveries in basic science, the knowledge of the function of PCSK9 truly leapt forward.

Establishing PCSK9 function

The following reviews some of the most recent findings that illuminated the understanding of the function of PCSK9.

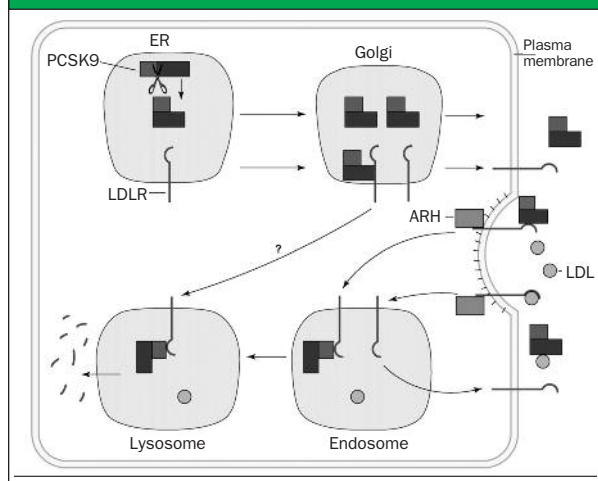
Selective tissue expression

PCSK9 was not identified in detailed studies of skin fibroblasts because it is not expressed in skin fibroblasts; in fact, its expression appears to be highest in liver, kidney, intestine, and neurons.¹⁹

PCSK9 suppresses functional expression of the LDLR

Insights into how PCSK9 raises LDL-C came from the study by Maxwell and Breslow,²¹ where they used an adenoviral expression system to overexpress PCSK9 in mice. This expression suppressed hepatic LDLR protein and raised lipoprotein cholesterol. Lagace et al.²² addressed the question of whether PCSK9 could act from outside hepatocytes with a classic parabiosis experiment, connecting the circulation of a mouse overexpressing PCSK9 with that of a PCSK9 knockout mouse. The liver of the knockout mouse responded to circulating PCSK9 by suppressing the function of the LDLR. The current understanding concerning the functions of PCSK9 is shown in Figure 1 and described in detail below.

Figure 1. PCSK9 undergoes autocatalytic processing within the endoplasmic reticulum (ER)²³



Both PCSK9 and the LDL receptor (LDLR) transit from the ER to the Golgi and then to the plasma membrane. It is uncertain whether PCSK9 and the LDLR interact at this stage. PCSK9 is secreted and binds to the LDLR, possibly competing with circulating LDL. PCSK9/LDLR and LDL/LDLR undergo internalization to the endosome. LDL dissociates from the LDLR, allowing the LDLR to return to the cell membrane. PCSK9/LDLR complex remains and is delivered, along with LDL, to the lysosome.

PCSK9 = proprotein convertase subtilisin/kexin type 9;

ARH = autosomal-recessive hypercholesterolemia adapter protein.

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PCSK9 is a proproteinase that is autocatalytic

The proprotein form of PCSK9 is autocatalytic and is thought to be active within the ER. The peptide released by proteolytic cleavage is bound by the remaining PCSK9, giving an inactive enzyme/peptide complex with a high affinity for binding to the LDLR. It is unclear whether any of the binding occurs within the cell before secretion of PCSK9; however, the secreted form binds to the epidermal growth factor (EGF)-like domain of the LDLR and apparently prevents LDL from binding to the receptor. Once PCSK9/peptide is bound to the LDLR, the receptor is internalized; whereas LDL itself dissociates from the receptor at the stage of the acidic endosomal vesicle, PCSK9 remains tightly bound to the LDLR and the complex is delivered to the lysosome. Thus, PCSK9 disrupts the itinerary of the LDLR and, in a post-translational mechanism, results in reduced expression of the LDLR.

The original experiment of nature – mutation in PCSK9 – is invaluable in understanding an important functional domain of PCSK9. The mutations that cause increasing LDL-C in a dominant pattern are gain of function mutations, because the mutant PCSK9 is present in a form that binds with high affinity to the LDLR and suppresses its return.

How important is PCSK9 if it is a rare cause of FH? One of the interesting facts about LDLR defects is that the “good” allele in a heterozygote is not naturally over-expressed and, therefore, does not compensate for the

“bad” allele. Conversely, 2 “good” copies of the *LDLR* have a maximal effect that is determined by other factors. To date, no “super” expressors of the *LDLR* have been described, hence, the natural benefit of overexpression is unknown.

Nature presents both gain and loss of function mutations in *PCSK9*.^{24,25} The loss of function truncation-mutations were discovered in black subjects. The frequencies of the *PCSK9*^{42X} and the *PCSK9*^{679X} alleles among black participants in the Atherosclerosis Research in Communities (ARIC) study²³ were 0.8% and 1.8%, respectively; another prevalent polymorphism, *PCSK9*^{46L}, was found in 3.2% of white subjects. These mutations presented an opportunity to answer 2 questions:

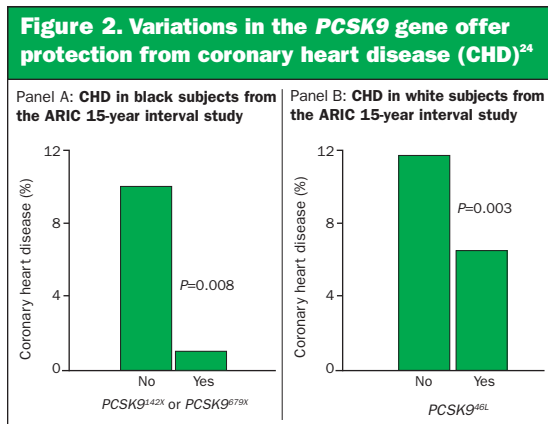
- Would a loss of function in *PCSK9* affect LDL-C?
- Would this affect the incidence of myocardial infarction?

The observational study of naturally occurring mutations has been termed “Mendelian randomization.” The answer to both of these questions is “yes”; loss of function, either heterozygous or homozygous, results in the lowering of LDL-C. The rare homozygote or compound heterozygote carriers²⁵ present with LDL-C of ~0.4 mmol/L; furthermore, the incidence of heart disease is lower in heterozygous carriers of loss of function mutations (Figure 2). Remarkably, the incidence appears to be lower than would have been predicted on the basis of measured LDL-C.

Cholesterol – years

“Cholesterol-years” are better associated with calcific atherosclerosis²⁶ and carotid stenosis²⁷ than are measurements of cholesterol or risk factors at a single time point. Data from FH subjects with varying severity in elevation of LDL-C were consistent with the concept that the cumulative exposure to LDL-C is the contributing factor for cardiovascular disease. From observational data in heterozygotes for FH, it is clear that Mendelian randomization is only represented before age 20.^{28,29} Thus, genetic factors that raise or lower LDL-C early in life and consistently over time should proportionately affect the incidence of cardiovascular disease. The *PCSK9* experiments of nature provide support for this concept.

Contrary to expectation, genetic and *in vitro* functional heterogeneity among the *LDLR* mutations is not a predominant factor in determining the level of LDL-C or coronary heart disease (CHD) in adults with heterozygous FH.²⁹ Koeijvoets et al³⁰ state that “additional factors are of greater relevance towards the burden of FH than hetero-

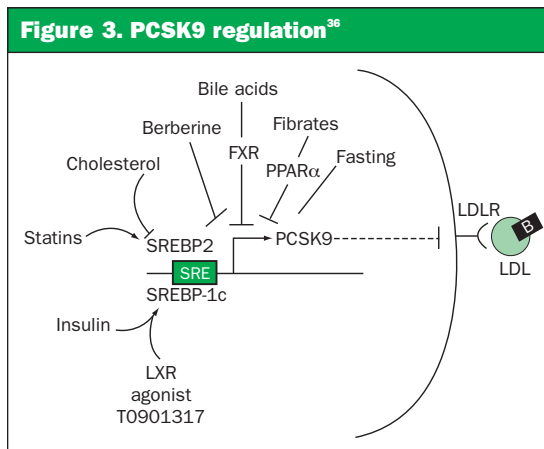


In **black** subjects, plasma levels of LDL-C were lower in those with a mutation in *PCSK9* (81% below 50th percentile) and those with the mutation had a significantly lower percentage of CHD. In **white** subjects, a different mutation also shifted LDL-C to lower levels, but the magnitude was less than with the alleles in the black subjects. Nevertheless, there was a significant decrease in CHD for subjects with the mutant *PCSK9*. ARIC = Atherosclerosis Research in Communities. Reproduced from Cohen JC et al. *N Engl J Med*. 2006;354(12):1264-1272. Copyright © 2006, Massachusetts Medical Society. All rights reserved.

geneity at the LDL-receptor locus.” Thus, among subjects with *LDLR* mutations, the heterogeneity in presentation, response to therapy, and disease outcome are primarily due to other genetic and nongenetic environmental factors. Abifadel et al,³¹ studying FH in Lebanon, provide evidence that one of these genes is *PCSK9*.

Treatment guidelines for heterozygous FH

Ironically, the development of cholesterol-lowering therapeutics was in large measure initially driven by the need for treatment in patients with heterozygous FH,³² yet there is no randomized trial evidence to guide the specifics for treatment in this group. An analysis of registry data for 3382 subjects with heterozygous FH (46 580 person-years) in the United Kingdom²⁹ found a 37% reduction in CHD mortality when comparing events before and after January 1, 1992. This date was chosen to approximate before and after the general use of statins to treat hypercholesterolemia. The National Institute for Healthcare and Clinical Excellence (NICE)^{11,28} recommended that heterozygous FH subjects ages 20-60 be treated with a high-intensity intervention (simvastatin 80 mg, atorvastatin 80 mg or rosuvastatin 40 mg). The interpretation of evidence for efficacy of treatment of subjects >60 years of age is confounded because the significant mortality before age 60 means that healthy survivors are overrepresented in the ≥60 age group. This group would include subjects with fewer risk factors, but would also include subjects with factors that can actively antagonize the detrimental effects of high LDL-C.



PCSK9 expression is downregulated by cholesterol and upregulated by statins via SREBP-2. The PCSK9 SRE was isolated at -336 bp from the transcription start site. Insulin, or the LXR agonist T0901317, activates PCSK9 via SREBP-1c-SRE binding. PCSK9 is transcriptionally repressed by fasting, PPAR α agonists fibrates, bile acids and the FXR agonist GW4064, and the natural compound berberine. *In vitro*, these compounds prevent PCSK9 upregulation by statins and potentiate the positive effect of statins on the LDLR (black) and on LDL (light green) cellular uptake. FXR = farnesoid X receptor; PPAR α = peroxisome proliferator-activated receptor α ; SREBP = sterol regulatory-element binding protein; LXR = liver X receptor.

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The pediatric population presents a special challenge for identification and initiation of treatment. Currently, the most effective means for identification is cascade screening, whereby family members of known index cases are investigated for the FH phenotype.¹¹ Recent consensus statements have been published about choice of therapy and timing of interventions.^{33,34}

Pharmacological regulation of PCSK9

PCSK9 is immediately recognizable as a target for the development of new therapies to lower cholesterol.³⁵ The nature of its regulation presents challenges and opportunities, such as:

- The challenge that any treatment reducing cholesterol and increasing the action of SREBP2 will increase the expression of PCSK9 and antagonize the benefit seen by inducing the expression of LDLR. Consequently, PCSK9 induction may limit the benefit of a statin and may in part explain the plateau response to increasing doses of statin.
- A second challenge is that the machinery contributing to PCSK9 processing and binding to the LDLR may be difficult to selectively target. PCSK9 is a member of a family of related proteases, and as such, is more difficult to target with a small molecule therapeutic agent than HMG-CoA reductase.

- As well, regulating the expression of PCSK9 may not be exclusively through cholesterol-responsive SREBP2; in fact, PCSK9 is the target of multiple regulatory molecules, including peroxisome proliferator activated receptor- α , farnesoid X receptor, insulin, and liver X receptor (Figure 3).^{36,37} In addition, the expression of PCSK9 has been shown to be amenable to new therapeutics, for example, the small interfering ribonucleic acids (siRNAs) that target messenger RNA.³⁸ Given that a half-normal amount of PCSK9 is compatible with health, one can be optimistic that long-term partial suppression of PCSK9 would have a favourable risk/benefit ratio.

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9 – 12 September 2009

8th Joint Meeting of the Lawson Wilkins Pediatric Endocrine Society/European Society for Pediatric Endocrinology

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45th Annual Meeting of the European Association for the Study of Diabetes

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Contact: Website: <http://www.easd.org>

3 – 4 October 2009

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