PCSK9
A Key Modulator of Cardiovascular Health

Nabil G. Seidah, Zuhier Awan, Michel Chrétien, Majambu Mbikay

Abstract: Since the discovery of proprotein convertase subtilisin kexin 9 (PCSK9) in 2003, this PC has attracted a lot of attention from the scientific community and pharmaceutical companies. Secreted into the plasma by the liver, the proteinase K–like serine protease PCSK9 binds the low-density lipoprotein (LDL) receptor at the surface of hepatocytes, thereby preventing its recycling and enhancing its degradation in endosomes/lysosomes, resulting in reduced LDL-cholesterol clearance. Surprisingly, in a nonenzymatic fashion, PCSK9 enhances the intracellular degradation of all its target proteins. Rare gain-of-function PCSK9 variants lead to higher levels of LDL-cholesterol and increased risk of cardiovascular disease; more common loss-of-function PCSK9 variants are associated with reductions in both LDL-cholesterol and risk of cardiovascular disease. It took 9 years to elaborate powerful new PCSK9-based therapeutic approaches to reduce circulating levels of LDL-cholesterol. Presently, PCSK9 monoclonal antibodies that inhibit its function on the LDL receptor are evaluated in phase III clinical trials. This review will address the biochemical, genetic, and clinical aspects associated with PCSK9’s biology and pathophysiology in cells, rodent and human, with emphasis on the clinical benefits of silencing the expression/activity of PCSK9 as a new modality in the treatment of hypercholesterolemia and associated pathologies. (Circ Res. 2014;114:1022-1036.)

Key Words: clinical trials, phase III as topic ■ hyperlipoproteinemia type II ■ LDL cholesterol

During 1960 to 1970s, it was firmly established that many bioactive secretory proteins, including polypeptide hormones and enzymes, are initially produced as inactive precursors that are transformed into bioactive moieties by limited proteolysis, as illustrated by the processing of β-endorphin3 and proinsulin that are transformed into bioactive moieties by limited proteolysis, resulting in reduced LDL-cholesterol clearance. Surprisingly, in a nonenzymatic fashion, PCSK9 enhances the intracellular degradation of all its target proteins. Rare gain-of-function PCSK9 variants lead to higher levels of LDL-cholesterol and increased risk of cardiovascular disease; more common loss-of-function PCSK9 variants are associated with reductions in both LDL-cholesterol and risk of cardiovascular disease. It took 9 years to elaborate powerful new PCSK9-based therapeutic approaches to reduce circulating levels of LDL-cholesterol. Presently, PCSK9 monoclonal antibodies that inhibit its function on the LDL receptor are evaluated in phase III clinical trials. This review will address the biochemical, genetic, and clinical aspects associated with PCSK9’s biology and pathophysiology in cells, rodent and human, with emphasis on the clinical benefits of silencing the expression/activity of PCSK9 as a new modality in the treatment of hypercholesterolemia and associated pathologies. (Circ Res. 2014;114:1022-1036.)

Key Words: clinical trials, phase III as topic ■ hyperlipoproteinemia type II ■ LDL cholesterol

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### Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ApoER2</td>
<td>apolipoprotein E receptor 2</td>
</tr>
<tr>
<td>CHRD</td>
<td>cys-his-rich domain</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>GOF</td>
<td>gain-of-function</td>
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<tr>
<td>LDL-C</td>
<td>low-density lipoprotein-cholesterol</td>
</tr>
<tr>
<td>LDLR</td>
<td>low-density lipoprotein receptor</td>
</tr>
<tr>
<td>LOF</td>
<td>loss-of-function</td>
</tr>
<tr>
<td>LRP1</td>
<td>lipoprotein receptor–related protein 1</td>
</tr>
<tr>
<td>PCSK9</td>
<td>proprotein convertase subtilisin kexin 9</td>
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<tr>
<td>VLDLR</td>
<td>very LDLR</td>
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CVD risk when compared with noncarriers. Such LDL-C-lowering effects are also found in French Canadian subjects with a dominant negative Q152H mutation, which prevents proPCSK9 autocatalytic cleavage into PCSK9 in the endoplasmic reticulum (ER). In addition, 2 complete LOF mutations causing marked hypocholesterolemia were found to be compatible with life and result in an amazingly low level of circulating LDL-C levels of 0.4 mmol/L. Indeed, mammals can survive and stay healthy without PCSK9, as also confirmed in Pcsk9-knockout mice. However, this does not seem to be the case in some lower vertebrae, where knockdown of PCSK9 mRNA in zebrafish leads to disorganization of the nervous system and lethality.

A third of the adult population in the United States had elevated LDL-C and as a consequence is at risk for CVD. Furthermore, cholesterol-lowering treatment solely based on statins has proven futile in a significant number of patients that are either resistant to statins and do not respond adequately or present serious side effects to these drugs. PCSK9 inhibitors have recently emerged as an alternative new class of cholesterol-lowering drugs. To date, the best studied property of PCSK9 is to bind the hepatocyte-derived LDLR leading to its intracellular degradation. Disrupting this [PCSK9=LDLR] protein–protein interaction prevents LDLR degradation, thereby raising LDL levels, lowers LDL-C, and is thought to protect from the development of atherosclerosis.

### Structural and Cellular Biology of PCSK9

**PCSK9 Ontogeny, Biosynthesis, Structure, and Degradation of the LDLR**

The human 22-kb gene PCSK9 is located on the small arm of chromosome 1p32 and contains 12 exons and 11 introns. The gene encodes a 692–amino acid (aa) proteinase K–like serine protease named PCSK914 (originally called neural apoptosis regulated convertase). During rodent development, PCSK9 was shown to be transiently expressed in brain centers, such as the telencephalon, olfactory bulb, and cerebellum. Recent in situ hybridization showed that PCSK9 mRNA is also abundant in the embryonic umbilical artery wall, including presumptive smooth muscle cells and in embryonic membranes. In the adult, PCSK9 remains highly expressed in liver hepatocytes and less so in the small intestine and kidney.

PCSK9 exhibits an atypical zymogen activation pathway when compared with the 8 other members of PC family and its associated activity and biology made it an outlier to classical PCs. The proteinase K–like PCSK9 shares sequence similarity with many vertebrate species, including chimpanzee, rhesus monkey, mouse, rat, chicken zebrafish, and ≥40 other species. However, although the PCSK9 gene is not found in most invertebrates, it is found in some, such as Branshiostoma floridae, a cephalocordate. Within vertebrates, some species, such as the bovine, do not express the protein. Analysis of the bovine genome revealed the presence of a nonfunctional PCSK9 gene with the absence of 3’ end exons and an early termination at exon 10, suggesting a selection by deletion in some vegetarian species. Practically, this would mean that the fetal bovine serum universally used in cell culture media would not have endogenous PCSK9.

After cleavage of its signal peptide (aa 1–30) in the ER, the zymogen proPCSK9 (aa 31–692) cannot exit this compartment until it intramolecularly cleaves itself at the sequence Val-Asn-His-Ala-Gln152-Ser-Ile-Pro (VFAQ152SIP) to release the mature enzyme (aa 153–692). Interestingly, proPCSK9 has a tendency to oligomerize in the ER in a disulfide-dependent manner. Analysis of the specificity of PCSK9 for autocatalytic cleavage at the P1 Gln152 demonstrated that the only P1 residues that can be recognized by PCSK9 are Glulyn→Asn→Ser→Thr→Asn, revealing an unsuspected cleavage specificity. However, different from the other PCs, mature PCSK9 remains noncovalently bonded to its inhibitory prosegment (aa 32–152) and is secreted as a [prosegment]=PCSK9 complex (Figure 1). The latter is enzymatically inactive because the prosegment occupies the active site cleft of the protease and shields it from interacting with other substrates (Figure 1). Thus, PCSK9 has no other substrate than itself, and its activity is related to its binding to specific target proteins and to escort the resulting complex toward intracellular degradation compartments. The catalytic subunit of PCSK9 (aa 153–421) contains the active sites Asp109, His294, and Ser106 and the oxyanion hole Asn137, which are typical of all subtilisin-like serine proteases. A small 18-aa hinge region (H; aa 422–439) links the catalytic subunit to the C-terminal cys-his–rich domain (CHRD; aa 440–692; Figure 1). The first crystal structure of PCSK9 revealed that the CHRD is composed of 3 modules termed M1 (aa 453–531), M2 (aa 530–605), and M3 (aa 604–692), and most of the His residues within the CHRD (9 of a total of 14 His) are found in the M2 domain lining up a groove-like structure.

The best characterized activity of the [prosegment]=PCSK9 complex is its ability to bind to specific target proteins and to escort them toward intracellular degradation compartments. The first PCSK9 target to be identified is the LDLR at the surface of liver hepatocytes. The catalytic subunit of PCSK9 was shown to bind the epidermal growth factor-A (EGF-A) domain of the LDLR (Figure 2), as well as the similar domain found in other LDLR superfamily members (eg, very LDLR [VLDLR], apolipoprotein E receptor 2 [ApoER2], and lipoprotein receptor–related protein 1 [LRP1]). Normally, the [LDLR]=LDL-C complex enters cells via clathrin heavy chain–coated vesicles, and when internalized, the acidic pH of endosomes causes the allosteric dissociation of the LDLR and its recycling to the cell surface, whereas the LDL-C is directed
to lysosomes for degradation, where cholesterol is recovered and distributed in the cell (Figure 2).\textsuperscript{45} In contrast, the complex [PCSK9\(\equiv\)LDLR], although also entering the cells via clathrin-coated vesicles,\textsuperscript{46,47} does not dissociate at acidic pHs but is rather more tightly associated,\textsuperscript{36} and, through some unknown mechanism it is escorted to lysosomes for degradation by as yet undefined proteases.\textsuperscript{35,46} An added complication is the observation that PCSK9 can enhance the degradation of the LDLR either by a direct intracellular pathway not requiring its secretion\textsuperscript{48} or extracellularly upon binding the LDLR at the cell surface (Figure 2).\textsuperscript{49} Clathrin light chains are not required for clathrin-mediated endocytosis but are critical for

Figure 1. Schematic representation of proprotein convertase subtilisin kexin 9 (PCSK9) zymogen processing and binding to the low-density lipoprotein receptor (LDLR). A, The autocatalytic zymogen processing of proPCSK9 (75 kDa) at Val-Phe-Ala-Gln\textsubscript{152}↓Ser-Ile-Pro (VFAQ\textsubscript{152}↓SIP) into the [prosegment (15 kDa)=PCSK9 (62 kDa)] complex is emphasized, together with the positions of the active site Asp\textsubscript{186}, His\textsubscript{226}, and Ser\textsubscript{386} and the oxyanion hole Asn\textsubscript{317}. The C-terminal hinge domain (H) and cysteine-rich domain (CHRD) are shown. B, Cartoon representation of the cell surface interaction of the catalytic domain of PCSK9 with the epidermal growth factor-A (EGF-A)–like domain of the LDLR, as well as the suspected interaction of the prosegment with the \(\beta\)-barrel domain of the LDLR and the CHRD with a putative membrane-bound protein X. C, Crystal structure of the ectodomain of the LDLR with PCSK9 emphasizing the interaction between them and the 3 subdomains in the CHRD (M1, M2, and M3). The interaction of protein X is presumed to be with one of the latter subdomains, possibly M2.

Figure 2. Schematic representation of the intracellular and extracellular pathways of proprotein convertase subtilisin kexin 9 (PCSK9) induced degradation of the low-density lipoprotein receptor (LDLR). When PCSK9 levels are high or if it has a gain-of-function, it will enhance the degradation of the LDLR using both the intracellular and the extracellular pathways leading to the degradation of the (PCSK9\(\equiv\)LDLR) complex in lysosomes. This results in low levels of the LDLR at the cell surface and increased levels of circulating LDL-C. In absence or under low levels of PCSK9, cell surface LDLR levels are high and the LDLR can be recycled back to the surface after delivery of LDL particles to acidic endosomes. The evidence for the intracellular pathway is based on the knockdown of clathrin light chains in the human hepatocellular carcinoma HepG2 cells.\textsuperscript{48,50} The extracellular pathway–specific treatments include the use of a monoclonal antibody (mAb), an inhibiting adnectin or a small-molecule epidermal growth factor-A (EGF-A)–like inhibitor. TGN indicates Trans Golgi Network.
clathrin-mediated trafficking between the trans Golgi network and the endosomal system.30 Indeed, clathrin light chain siRNAs that block direct intracellular trafficking from the trans Golgi network to lysosomes rapidly increased LDLR levels within the human hepatocellular carcinoma cell line HepG2 cells in a PCSK9-dependent fashion, without affecting the ability of exogenous PCSK9 to enhance LDLR degradation.46 Whether these 2 pathways are operative in all tissues is still not clear. In support of this model, a recent observation revealed that PCSK9 lacking the M2 domain of the CHRD can still degrade the LDLR intracellularly but not when added outside cells, supporting the presence of 2 distinct sorting and regulatory pathways of the [PCSK9=LDLR] complex.31

Interestingly, both the intracellular and the extracellular LDLR degradation activities of PCSK9 require the presence of the CHRD because the [PCSK9-ΔCHRD=LDLR] complex that lacks this domain, although still capable of internalization into endosomes, does not traffic to lysosomes and is likely recycled to the cell surface.53,55 This has led to the search of other proteins that may interact with the CHRD and the LDLR and drive the [PCSK9=LDLR] complex to lysosomes.

Recent reports shed some light on this still obscure mechanism. Two studies suggest that the CHRD binds weakly ≥1 of the 7 Ca2+-coordinating repeats in the N-terminal ligand-binding domain of the LDLR53 and that such binding may be necessary to prevent the dissociation of the [PCSK9=LDLR] complex.54 Furthermore, it was suggested that the binding of the positively charged CHRD to the negatively charged LDLR’s ligand-binding domain is favored at acidic pHs,33,54 where the His residues of the CHRD would be positively charged. These data support the finding that aside from the EGF-A domain, ≥3 of the 7 ligand-binding repeats of the LDLR and the β-propeller domain are necessary for the LDLR to be degraded in the presence of PCSK9.55 However, such a binding is likely to be weak because all reported crystal structures of PCSK9 at either neutral or acidic pH do not reveal any interaction of the CHRD with the N-terminal repeats (aa 25–313) of the LDLR. One exception is the predicted weak hydrophobic interaction of Leu108 of the β-propeller domain of the LDLR with Leu518 of the prosegment of PCSK9,41 which was also deduced from a GOF L108R PCSK9 mutant that possibly strengthens this interaction by favoring the electrostatic binding of Glu505 of the LDLR to the mutant Arg108 of PCSK9.17

Nevertheless, because the cytosolic tail of the LDLR is not necessary for the sorting of the [PCSK9=LDLR] complex to lysosomes,44,55 this suggests that another protein must bind the luminal CHRD domain and that such protein X would also have a transmembrane domain and cytosolic tail linking motor proteins in the cytosol to direct the complex to lysosomes (Figure 1).44 In that context, a recent report proposed that the amyloid precursor–like protein-2 (APLP-2) can bind the CHRD at the surface of cells and in endosomes, and that this [LDLR=PCSK9=APLP-2] tripartite complex is then targeted to lysosomes (Figure 2),46 by a still undefined mechanism.37 Does APLP-2 and other members of the family, such as amyloid precursor protein and APLP-1, represent the missing link(s) to understand the cellular trafficking of the [PCSK9=LDLR] complex to lysosomes? It is still too early to settle this point because in vivo analyses of Aplp2- knockout mice are still lacking, and our preliminary data suggest that knockdown of APLP-2 expression in cells does not affect the ability of PCSK9 to degrade the LDLR or LRP144 both intracellularly and extracellularly (M. Canuel, et al, unpublished data, 2014).

What is the role of the prosegment in the sorting or activity of the [prosegment=PCSK9] complex? First, the human Q152H-dominant negative variant confirmed that the autocatalytic cleavage of proPCSK9 into PCSK9 is an absolute requirement for exit of this protein from the ER72 and its subsequent enhancement of the LDLR degradation.22,34 Second, because the [Δ33,54-prosegment=PCSK9] complex lacking the N-terminal acidic region of the prosegment (aa 31–58) is ≥4- to 7-fold more active in degrading the LDLR,58,59 this suggests that this acidic region, which is likely not stabilized on its own because it is not seen in any crystal structure reported, is a negative regulator of the activity of PCSK9 on the LDLR. Recent evidence suggested that the association of PCSK9 with LDL particles in plasma lowers the ability of PCSK9 to bind to cell surface LDLR, thereby blunting PCSK9-mediated LDLR degradation.40 Because ApoB is the major protein in LDL, this suggests that it is the active component that binds PCSK9 in plasma and blunts its function on the LDLR. Whether such binding implicates the interaction of the acidic aa 31 to 58 at the N terminus of the prosegment of PCSK9 with a positively charged domain in ApoB has yet to be demonstrated. Cocrystallization of PCSK9 with the ApoB-binding domain should shed light on this model.

The secretory pathway of eukaryotic cells packages cargo proteins into Coat Protein-II–coated vesicles for transport from the ER to the Golgi, which are then sorted into other organelles or secreted. What are the other cellular partners of PCSK9 that regulate its exit from the ER and trafficking? It was recently reported that the exit of the complex [prosegment=PCSK9] from the ER requires an interaction with a putative membrane-bound protein that links via its tail the cytosolic protein sec24a that is associated with COP-II vesicles.61 The absence of the latter results in markedly lower levels of secreted PCSK9, leading to higher levels of hepatic LDLR protein levels because of decreased degradation. It would be stimulating to find out if the opposite also exists (ie, that some mutations in PCSK9 or its putative ER partner may enhance PCSK9 secretion from the ER).

In conclusion, the escort and degradation of the [PCSK9=LDLR] complex is regulated by a variety of proteins, including PCSK9 and LDLR themselves, protein X, ApoB, sec24a, and most likely, other undefined and transitory partners that would interact with this complex along the secretory route, even as early as the ER.66,62 Furthermore, the PC Furin cleaves PCSK9 at Arg218 at the surface of hepatocytes and likely results in its in vivo inactivation.63,64 suggesting that some proteases could regulate PCSK9 activity. PCSK9 seems to be unique when compared with other PCs in the sense that it is the only convertase that has only 1 enzymatic substrate, itself. It seems that this relatively more recent and polymorphic convertase was selected for its protein–protein interaction with LDLR-like receptors, rather than as a protease, because the inhibitory prosegment remains tightly bound to the catalytic subunit. Whether factors exist, other than the prosegment, that could also inhibit the enzymatic activity of
PCS\textsubscript{9} is not yet known. The evolutionary conservation of this enzyme suggests that such a regulatory mechanism has been maintained in most vertebrates but not in invertebrates that express other PC-like proteases.

**Other PCS\textsubscript{9} Target Proteins**

Although the LDLR is no doubt the best studied target of PCS\textsubscript{9} and probably relevant physiologically because it controls the levels of circulating LDL-C, PCS\textsubscript{9} was also found to escort other receptor members of the LDLR superfamily toward endosomal/lysosomal degradation. Thus, PCS\textsubscript{9} was first found to enhance the extracellular and intracellular degradation of the closest LDLR family members, namely the VLDLR and ApoER2 (LRP8).

However, although the interaction of the catalytic domain of PCS\textsubscript{9} with the EGF-A-like domains of these receptors was confirmed, the specific aa in each protein implicated in such interactions are not the same as for the LDLR. For example, in contrast to the LDLR, the GOF D374Y PCS\textsubscript{9} does not degrade these other receptors more efficiently than wild-type PCS\textsubscript{9}. The receptors LDLR, VLDLR, and ApoER2 have been confirmed as PCS\textsubscript{9} target proteins in mice and the LDLR in monkeys and human. Recently, we showed that PCS\textsubscript{9} can enhance the degradation of LR1P1 in various cells although proof of this activity in vivo is still lacking. Finally, CD36, a scavenger receptor with multiple ligands and cellular functions, including facilitating cellular uptake of free fatty acids (FFA), was also suspected to be a PCS\textsubscript{9} target in intestinal epithelial cells and adipose tissue. Recently, this PCS\textsubscript{9} activity on CD36 was elegantly proven in cells and adipocytes because the structures of CD36 or CD81 (see below) do not exhibit an EGF-A-like domain, their respective sequences that bind to ESC\textsubscript{9} either directly or indirectly have yet to be defined.

The fact that many PCs can process surface glycoproteins of infectious viruses prompted us to test the effect of the lack of PCS\textsubscript{9} on the titer and infectivity of viruses that infect the liver (richest source of PCS\textsubscript{9}), such as the hepatitis C virus (HCV). The data showed that PCS\textsubscript{9} targets 2 hepatic HCV receptors for degradation, namely the LDLR and the tetraspanin protein CD81. Furthermore, it was recently reported that other viruses bind to cell surface LDLR family members to enter and infect cells, including vesicular stomatitis virus that likely uses the LDLR and LR1P1 as entry receptors. These results suggested that although inhibiting PCS\textsubscript{9} may be beneficial to reduce the levels of circulating LDL-C, it potentially could enhance the infectivity of certain viruses, such as HCV, vesicular stomatitis virus, the common cold rhino virus, and rous sarcoma virus.

It was suggested that PCS\textsubscript{9} could enhance the degradation of certain targets within the ER/ER-Golgi intermediate compartment. Two examples were reported: (1) The Alzheimer disease-associated aspartyl protease $\beta$-secretase $\beta$-amyloid precursor protein cleaving enzyme-1 (BACE1) is transiently acetylated on 7 Lys residues in the lumen of the ER/ER-Golgi intermediate compartment. The acetylated intermediates of the nascent protein are able to reach the Golgi apparatus, whereas the nonacetylated ones are retained and degraded in a post-ER compartment. PCS\textsubscript{9} was reported to contribute to the disposal of nonacetylated BACE1. This interesting observation still requires in vivo validation. (2) In the second example, within the ER/ER-Golgi intermediate compartment of cells, PCS\textsubscript{9} was reported to enhance the degradation of the epithelial Na"a channel (EhNaC) that is critical for Na"a homeostasis and blood pressure control. This observation was intriguing, especially in view of the expression of PCS\textsubscript{9} in the kidney. However, our data revealed that in PCS\textsubscript{9}-knockout mice, the basal and angiotensin-II--induced blood pressure rise is not different from wild-type controls, suggesting that EhNaC levels are not appreciably increased in the absence of PCS\textsubscript{9}, at least in mice (N.G. Seidah and T. Reudelhuber, unpublished data, 2014). These results cast some doubt as to the physiological relevance of the reported cellular role of PCS\textsubscript{9} on epithelial Na"a channel.

**Gene Regulation of PCS\textsubscript{9}**

**Transcriptional Regulation of PCS\textsubscript{9} Expression**

As for any other gene, regulation of PCS\textsubscript{9} gene expression begins at transcription. A stringent scanning of the proximal promoter of the PCS\textsubscript{9} gene (600 bp upstream and first exon) was conducted to search for probable transcriptional regulatory elements using the Nsite online algorithm (http://linux1.softberry.com). The functional characterization of these elements have focused mostly on the human and mouse PCS\textsubscript{9} gene promoter.

**Nuclear-Binding Factors**

The sterol regulatory element (SRE) is the most conserved of these transcriptional motifs, consistent with a modulatory role of this gene in cholesterol metabolism. SRE is the binding site for SRE-binding proteins (SREBPs), the master transcriptional factors in lipid biosynthetic pathways. Shortly after the discovery of PCS\textsubscript{9}, an unbiased analysis of the hepatic transcriptome of mice showed that the level of PCS\textsubscript{9} mRNA was strongly downregulated when mice were fed a cholesterol-rich diet and upregulated in transgenic mice overexpressing nuclear SREBP-1a or SREBP-2. Later, Dubuc et al.

showed that statins, which inhibit 3-hydroxy-3-methyl-glutaryl-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, resulting in a feedback activation of nuclear SREBP-2, increased the level of PCS\textsubscript{9} mRNA in HepG2 human hepatocytes in culture. The statin induction could be abrogated by mevalonate, a post-3-hydroxy-3-methyl-glutaryl-CoA reductase cholesterol precursor, confirming the link of this regulation to cholesterol metabolism. SREBP-1c was also implicated in postprandial insulin upregulation of PCS\textsubscript{9} gene expression in hepatocytes because constitutive expression or inactivation of this transcriptional factor increased or reduced the level PCS\textsubscript{9} mRNA in these cells in culture, respectively. SREBP-1 and SREBP-2 have been shown to bind to the PCS\textsubscript{9} gene promoter SRE in vitro specifically.

SREBP activation of the PCS\textsubscript{9} gene promoter is potentiated by the hepatocyte nuclear factor-1 (HNF1$\alpha$), which binds to an element located 28 nucleotides (nts) upstream of the SRE. This site is conserved between primate and rodent PCS\textsubscript{9} promoters and is absent in the LDLR promoter. Its invalidation by site-directed mutagenesis dramatically reduces PCS\textsubscript{9} gene promoter-driven expression of a reporter gene in transfected HepG2 cells. Upregulation of HNF1$\alpha$ expression...
by statins\(^6\) contributes to sustained PCSK9 production/secre-
tion that attenuates the LDLR-mediated clearance of plasma
LDL-C induced by these drugs. Its expression is downregu-
lated by the phytochemical berberine\(^7\) and by activators of he-
patic mechanistic target of rapamycin complex 1 (mTORC1)
signaling pathway,\(^3\) making such compounds potential en-
hancers of the LDL-C clearance.

Situated between the SRE and HNF1\(\alpha\)-binding motif is a
histone 1 nuclear factor P(H1NFP)–binding motif that has
been shown to critical to the functionality of the SRE in the
PCSK9 promoter, being required for both basal and enhanced
activation of this promoter by SREBP-2. Binding to this motif,
H1NFP cooperates with the cofactors nuclear protein of the
ataxia telangectasia mutated mutant locus and transformation/trans-
activation domain–associated protein to activate the PCSK9
promoter. Transformation/transactivation domain–associated
protein is a cofactor of histone acetyltransferase, which me-
diates histone H4 acetylation on the promoter, facilitating its
activation.\(^2\) Inversely, impaired acetylation or deacetylation
of histone at the PCSK9 promoter can hamper its activation.
Sirtuin 6, an NAD\(^+\)–dependent histone deacetylase is a tran-
scriptional repressor of the PCSK9 because its absence in
mouse liver leads to increased expression of this gene, whereas
its overexpression reduces it.\(^8\) The insulin-responsive ele-
ment–binding factor known as FoxO3 recruits Sirtuin 6 to the
PCSK9 promoter, where it deacetylates histone 3 and causes
local chromatin changes that negate promoter activation.
Interestingly, the recognition element of FoxO3 is embedded
within that of HNF1\(\alpha\). Interactions between the 2 oppositely
acting factors have been demonstrated in coimmunoprecipita-
tion studies.\(^3\) The outcome of this competitive interaction may
depend on the relative amounts of these factors and their re-
quired cofactors.

### Nuclear Receptors

The PCSK9 promoter is also regulated by ligand-activated
nuclear receptors, such as Farnesoid X receptor and peroxi-
some proliferator–activated receptors (PPARs). Farnesoid X
receptor, which binds chenodeoxycholic acid, has been impli-
cated in the fall of PCSK9 mRNA in cultures of hepatocytes
after exposure to this bile acid component. This implication is
probably indirect because siRNA-induced knockdown of the
receptor was ineffective.\(^8\)

PPAR\(\alpha\) agonists, such as fenofibrate, are commonly used to
treat hypercholesterolemia and mainly hypertriglyceridemia.
Their effects on PCSK9 mRNA levels have been explored
with mixed results. They have been shown to repress PCSK9
gene expression in a human hepatocyte cell line and isolated
mouse hepatocytes.\(^8\)\(^,\)\(^9\) but the WY14643 agonist had no ef-
fect on the level of PCSK9 mRNA in isolated hamster hepa-
tocytes.\(^8\) The reasons for this discrepancy are unclear as are
those from clinical studies in fibrate-treated patients, showing
increased plasma PCSK9 levels in most studies\(^8\)\(^,\)\(^9\)\(^,\)\(^10\) and a
decreased levels in another.\(^8\)

PPAR\(\gamma\) ligands, 15d-PGJ1 or pioglitazone, have been
shown to augment the level of PCSK9 mRNA in HepG2 cells.
This induction can also be achieved by preventing phosphory-
lation of the receptor by extracellular-regulated kinases 1 and
2 with such specific inhibitors PD98059 or U0126.\(^9\)

It should be noted that, in this transcriptional regulatory
network, the SREBP-2 and HNF1\(\alpha\) genes themselves are of-
ten downstream targets of other factors. For example, PPAR
(\(\alpha\) or \(\gamma\)) activation in liver, hepatocytes, or enterocytes has
been shown to reduce the level of nuclear SREBP-2 and ul-
timately of cholesterol biosynthesis.\(^9\)\(^,\)\(^10\) mTOR1 also down-
regulates HNF1\(\alpha\), resulting in reduced PCSK9 transcription.\(^8\)
Sirtuin 6 had similar inhibitory effects on SREBP-2 and its
target genes.\(^9\)\(^,\)\(^10\) Thus, SREBP-2 and HNF1\(\alpha\) seem to stand at
a crossroad of transcriptional pathways regulating expression
of genes that could influence intracellular cholesterol homeo-
stakes, including PCSK9 and LDLR genes.

Thus, transcriptional up- or downregulation of PCSK9 is
determined by the relative abundance and activity of a vari-
ety of nuclear factors acting in cooperation or in competition
on cis regulatory elements. The elements described above are
located in the proximal promoter, but the possibility of more
distal ones cannot be discounted as yet. The functionality has
been explored primarily in liver cells. A better understanding
of transcriptional regulation may allow one to anticipate the
direction of PCSK9 expression in these cells under certain
physiological or therapeutic conditions. Furthermore, it may
clarify why some mutations in the cognate gene are associated
with altered expression of the protein, as has been observed
with c.-332C>A variant located near the SRE and associated
with a GOF phenotype in a Spanish population.\(^8\)

### Post-Transcriptional Regulation of PCSK9

**Expression**

Regulation of mRNA stability and translation into protein are
more immediate response mechanisms to physiological de-
mands than transcription. To our knowledge, there has been
no experimental evidence of PCSK9 expression regulation at
these levels. However, the sequences of the 3′ untranslated
region (3′-UTR) of its mRNA exhibit features suggestive of
such a regulation. Structurally unstable mRNAs are typically
characterized by a high AU content in their 3′-UTR.\(^9\)\(^,\)\(^10\) At the
extreme end of 1.3-kb long 3′-UTR of human PCSK9, mRNA
is an island of \(\approx\)120 nts (nts 575–3692; NM_174936.3), which
exhibits 71% AU content, contains 2 AUUUU canonical
AU-rich elements commonly associated with mRNA instability,
and is relatively conserved among primates and rodents.
The relevance of this AU island in mRNA stability remains to
be functionally determined. This is further justified by the fact
that the mRNA of the LDLR, the opposing partner of PCSK9 in
lipid homeostasis, is itself, subject to downregulation through
its 3′-UTR by heterogeneous nuclear ribonucleoprotein D.\(^9\)\(^,\)\(^10\)
In addition, the presence of a potential miRNA-24 recognition
in the 3′-UTR of human PCSK9 mRNA warrants investiga-
tion of its possible regulatory role, considering the growing
implications of miRNAs in cholesterol metabolism.\(^10\)

**PCSK9 Animal Models**

### Hypercholesterolemia and Atherosclerosis

**(Transgenic Models)**

Adenoviral-mediated expression of human PCSK9 in mice
results in an intermediate LDLR-knockout phenotype.\(^8\)
PCSK9 effect is mostly exerted in a paracrine/endocrine
fashion as shown by parabiosis experiments.\(^10\) As expected,
transgenic mice overexpressing PCSK9 under an Apoe promoter \([Tg(Apoe-PCSK9)]\) are viable, fertile, and severely hypercholesterolemic.\(^{26,39}\) Interestingly, although the LDLR is not detected in the liver of these mice, the circulating LDL-C is only increased \(\approx 5\)-fold when compared with \(\approx 15\)-fold in \(Ldlr\)-knockout mice.\(^{23}\) This suggests that the LDLR in tissues other than liver contribute to the clearance of circulating LDL-C, and that the LDLR in these extrahepatic tissues are not regulated by, or accessible to, circulating PCSK9. Possible mechanisms are described in the section below describing the role of PCSK9 in extrahepatic tissues.

On an Apoe-knockout genetic background, \(Tg(Pcsk9)\) mice develop accelerated atherosclerosis with larger plaque size, when fed a regular chow diet, and a protective effect was seen with \(Pcsk9\)-knockout mice on the same background.\(^{31}\) The late complications of atherosclerosis in the form of vascular calcified plaques were also observed in these Apoe-knockout/\(Tg(Pcsk9)\) mice but to a lesser degree and with a longer latency when compared with \(Ldlr\)-knockout mice.\(^{103}\) Thus, these ApoE-deficient \(Tg(Pcsk9)\) mice exhibit an intermediate phenotype to the complete absence of the LDLR and are, therefore, more relevant to the majority of individuals with an attenuated LDLR activity and dyslipidemia. Finally, hypercholesterolemia and atherosclerosis were recapitulated in a pig model of a human PCSK9 GOF D374Y mutant. A liver-specific expression of this mutant in minipigs resulted in markedly reduced levels of hepatic LDLR, impaired LDL-C clearance, severe hypercholesterolemia, and eventually these minipigs developed spontaneous progressive atherosclerotic lesions that could be visualized by noninvasive imaging.\(^{104}\) Two conclusions can be drawn from these observations: first, inhibition of PCSK9 would be a potential therapeutic modality; second, the use of large species models will better advance the translational research aspect of treating atherosclerosis from animals to human.\(^{104}\)

**Hypcholesterolemia and Fat Metabolism (Knockout Models)**

One of the classical experiments aimed at understanding the functions of a given gene is to delete it from the genome and observe the consequences of such lack of expression. Complete \(Pcsk9\)-knockout resulted in viable and fertile mice exhibiting severe hypcholesterolemia,\(^{25,26}\) with an \(\approx 40\%\) and \(\approx 80\%\) drop in total cholesterol and LDL-C, respectively.\(^{18}\) In comparison, liver-specific \(Pcsk9\)-knockout mice exhibit \(\approx 27\%\) less circulating cholesterol, suggesting that liver PCSK9 contributes to \(\approx 70\%\) of the cholesterol phenotype.\(^{18}\) Interestingly, the liver-specific loss of PCSK9 expression\(^{26}\) resulted in its complete absence from circulation, demonstrating that hepatocytes are the primary source of plasma PCSK9.\(^{65}\) Notably, lipidomics analyses of the plasma of \(Pcsk9\)-knockout and transgenic mice revealed that \(Pcsk9\)-knockout mice result in a marked reduction in the plasma levels of sphingomyelin and ceramides, which are known risk factors for coronary artery disease.\(^{105}\) These lipid biomarkers should now also be measured in the plasma of individuals with the R46L LOF mutation\(^{105}\) or those treated with simvastatin.\(^{106}\)

As mentioned above PCSK9 may target other receptors, and VLDLR is upregulated in perigonadal depots of female \(Pcsk9\)-knockout mice. As a consequence, adipocytes became hypertrophic because of higher levels of adipocyte VLDLR and increased uptake of FFA,\(^ {65}\) possibly via an associated increased level of CD36.\(^ {70}\) Conversely, plasma triglycerides were slightly increased in \(Pcsk9\)-knockout males (\(\approx 35\%;\) not significant) and females (\(\approx 46\%;\) \(P=0.03\)). Furthermore, in vivo studies revealed that PCSK9 deficiency was associated with a 2-fold decrease in postprandial triglycerides levels, suggesting improved triglycerides clearance and a role for PCSK9 in triglycerides metabolism,\(^ {107}\) possibly via its ability to enhance the degradation of VLDLR and CD36. PCSK9 is thus essential in fat metabolism because it maintains high circulating LDL-C levels via hepatic LDLR degradation, but at the same time it downregulates triglycerides and FFA entry into visceral adipocytes, possibly via adipose tissue VLDLR\(^ {65}\) and CD36 degradation.\(^ {69}\)

**Role of PCSK9 in Extrahepatic Tissues**

**Adipocytes, Muscles, and Myocytes**

PCSK9 is mainly expressed in adult liver, small intestine, kidneys,\(^ {12,26}\) and pancreas.\(^ {12,108}\) Although not expressed in adipocytes, circulating PCSK9 produced by the liver can regulate the levels of cell surface receptors in this tissue.\(^ {65}\) Because human PCSK9 targets ex vivo human VLDLR\(^ {42}\) and binds in vitro mouse VLDLR,\(^ {65}\) PCSK9 has the capacity to target human VLDLR in vivo. Indeed, visceral fat accumulates in PCSK9-deficient mice because of the high uptake of chylomicrons and VLDL-C in tissues that predominately express the VLDLR (such as adipose tissue followed by heart and muscles). Lack of PCSK9 increases the surface expression of the VLDLR that facilitates triglycerides hydrolysis and FFA uptake in the visceral adipocytes. In theory, muscle and heart tissues would have been affected too, but muscles burn fat continuously, whereas adipose tissues tend to store energy in the form of fat droplets for later usage. Lack of PCSK9 in mice or LOF PCSK9 variants in humans are suggested to affect postprandial lipemia,\(^ {109}\) which further establishes the association between serum PCSK9 and lipid metabolism. At least in \(Pcsk9\)-knockout mice, no upregulation of cytokines, associated with the metabolic syndrome, was observed.\(^ {65}\) Whether an increased visceral fat deposition also occurs in humans lacking functional PCSK9 remains to be elucidated. Perigonadal fat is part of what is called the visceral adipose tissue, which correlates directly with obesity-related metabolic disease and coronary heart disease. However, it was recently reported that in patients with obesity with similar levels of visceral adipose tissue, metabolic complications were more prevalent in those exhibiting higher intrahepatic triglycerides.\(^ {109}\) In a clinical perspective, because \(Pcsk9\)-knockout mice do not develop liver steatosis\(^ {26}\) and are not prone to obesity, administration of a PCSK9 inhibitor for the treatment of hypercholesterolemia is not expected to result in adverse effects.

**Adrenals and Kidneys**

Like liver, adrenals require synthesis and uptake of cholesterol for proper function. It was found that annexin A2 is a natural extrahepatic inhibitor of PCSK9\(^ {110}\) and that in \(Anxa2\)-knockout
mice, plasma PCSK9 doubles and LDLR decreases by \(\approx 50\%\) in some extrahepatic tissues, such as adrenals, small intestine, and colon but not in liver.\(^{111}\) In addition, transgenic mice expressing human PCSK9 mainly in the kidney resulted in LDLR degradation mostly in the liver but not in adrenals.\(^{112,113}\) Thus, it is probable that the high levels of annexin A2 in adrenals are responsible for the refractoriness of the LDLR in this tissue to the action of PCSK9.\(^{111,112}\) Another, not mutually exclusive, possibility is that in refractory tissues, such as the adrenals, the complex [PCSK9\(\approx \)LDLR] enters endosomes but does not traffic to lysosomes and may actually recycle back to the cell surface. Accordingly, a possible explanation for the tissue-specific activity of PCSK9 could be that extrahepatic tissues that do not respond to PCSK9 are unable to sort the [PCSK9\(\approx \)LDLR] complex to lysosomes, likely because of unfavorable cellular response or absence of functional specific protein(s) that control lysosomal targeting of the complex (Figure 2). In support of this notion, it was recently shown that fibroblasts, which are resistant to PCSK9-induced degradation of the LDLR, can bind PCSK9 but that the endocytosed PCSK9 dissociates from the LDLR within early endosomes, and the latter is rapidly recycled back to the cell surface and not sent to lysosomes for degradation.\(^{114}\) Alternatively, some tissues, such as adrenals, are enriched in an endogenous inhibitor of PCSK9 (eg, annexin A2), that prevent its function on the LDLR.\(^{111}\)

The physiological role of PCSK9 in kidney is still not clear, especially because its expression therein shifts from the cortex to the embryonic in the lumen in adult rodents.\(^{12}\) It is also possible that PCSK9 acts locally in some extrahepatic tissues that express this protein (eg, small intestine, pancreas, and kidney), and that its circulating levels would not affect the LDLR in these tissues. The reported possible PCSK9-induced degradation of epithelial Na\(^+\) channel in kidney cells\(^{74}\) needs in vivo confirmation because basal blood pressure and angiotensin-II induced rise in blood pressure in mice are not modified by the lack of expression of PCSK9 (N.G. Seidah and T. Reudelhuber, unpublished data, 2014).

**Small Intestine and Pancreas**

Findings from cellular and tissue expressions from rat, mouse, and human showed that not only the liver and cerebellum are rich in PCSK9 but also the small intestine, especially the ileum.\(^{13}\) PCSK9 regulation of the LDLR protein levels in intestinal cell lines has been reported.\(^{49,115}\) The possible implication of PCSK9 in the transintestinal reverse cholesterol excretion from the small intestine has been recently evoked.\(^{116}\) Although the LDLR is involved in this process, it seems that yet another receptor(s) sensitive to PCSK9 is/are also implicated.\(^{116}\) Could this receptor be one of the known targets of PCSK9, such as VLDLR, apoER2, LRP1, CD36, or yet another receptor to be identified in the future? Finally, it was reported that the N-terminal negatively charged sequence of the prosegment of PCSK9 is critical for the binding of the [prosegment\(\approx \)PCSK9] to LDL-C (likely to a positively charged sequence within ApoB).\(^{49}\) Because liver expresses mostly ApoB-100 and the small intestine ApoB-48, and the latter does not bind the LDLR, it is possible that the difference between liver and small intestine in relation to responsiveness to circulating PCSK9 may be affected by these 2 isoforms of ApoB.

PCSK9 was originally observed to be highly expressed in pancreatic \(\beta\)-cell lines.\(^{12}\) When compared with controls, older male Pcsk9-knockout mice express more LDLR in pancreatic islet cells and are glucose-intolerant.\(^{108}\) It remains to be seen whether circulating PCSK9 affects the LDLR and other receptors at the surface of \(\beta\)-cells, and whether the upregulation of these in the absence of endogenous PCSK9 or from circulation results in lipotoxicity or impairment of \(\beta\)-cell function.

**PCSK9 and Pathological Conditions**

**Liver Regeneration**

Given that PCSK9 is mainly produced by the liver and that the latter is a regenerating organ, it was expected that conditions that perturb liver function may influence PCSK9 levels and that the latter may in turn affect the ability of the liver to recover from certain insults. In vivo studies revealed that after partial hepatectomy, regenerating livers of Pcsk9-knockout mice exhibited the presence necrotic lesions and a significant delay in their regenerating capacity.\(^{26}\) Fortunately, this lesion phenotype and regenerating capacity were reversed when a high-cholesterol diet was supplemented to mice before and after partial hepatectomy.\(^{26}\) These results were the first indication that on hepatic damage, patients lacking PCSK9 could be at risk. However, under normal circumstances, lipid accumulation in hepatocytes of these mice was markedly reduced under both regular and high-cholesterol diets, revealing that PCSK9 deficiency confers resistance to liver steatosis.\(^{26}\) In conclusion, although PCSK9 inhibition may protect against the development of liver steatosis, it may also result in an increased risk of liver damage after a liver insult. It remains to be seen what kind of insults are more deleterious to liver function in the absence of PCSK9 (eg, hepatitis, liver cirrhosis, or hepatocellular carcinoma).

**Insulin Resistance**

Adult PCSK9-deficient male mice exhibit impaired glucose tolerance and may be at risk to develop diabetes mellitus on aging.\(^{108}\) However, the underlying mechanism is largely unknown. In 1 population study, it was shown that subjects with PCSK9 LOF R46L have a significant increment in markers of insulin resistance, namely insulin, homeostasis model assessment of insulin resistance, and the adipokine hormone leptin in individuals carrying 1 copy of apoE2 when compared with those carrying other apoE isoforms.\(^{117}\) This indicates that heterozygote LOF of APOE and PCSK9 may lead to insulin resistance. To date, the only woman analyzed that lacks completely PCSK9 because of compound heterozygous null mutations\(^{118}\) has not been reported to have insulin resistance.

**Viral Infections**

HCV particles interact with a number of putative HCV receptors, including CD81, scavenger receptor class B type I, and Claudin-1.\(^{116}\) Circulating HCV particles are associated with VLDL and LDL of infected patients, suggesting that the LDLR is critical for hepatic viral infection. Recently, it was shown that liver expression of CD81 is markedly increased in Pcsk9-knockout mice and that the HCV receptor CD81 protein is downregulated independently from the LDLR.\(^{71}\) Therefore, it was proposed that the plasma level and activity
of PCSK9 could modulate HCV infectivity in humans. In summary, the LDLR and CD81, 2 HCV entry receptors are dose dependently downregulated by PCSK9, resulting in the reduction of the cellular infectivity of HCV in mice. Although never shown in humans yet, PCSK9 has the potential to protect against HCV. Therefore, caution must be exercised when administering PCSK9 inhibitors to subjects that could be potentially infected with HCV or other viruses that use ≥1 of the LDLR superfamily members as entry receptors.72

**Physiological Modulation of PCSK9 Levels**

**Variation with Age, Sex, and Pregnancy**

A cross-sectional pediatric study of ~1700 subjects revealed that in boys, plasma PCSK9 levels continuously decreased from age 9 to 16 years, correlating with mean total cholesterol levels that continuously decreased with age. In contrast, in girls, PCSK9 levels peaked at the age of 13 years and then decreased to higher levels than boys at 16 years, and total cholesterol levels were higher in 9 and 16 years olds than in 13 years olds.119 It is thought that this regulation follows a growth hormone pattern.119 In the multiethnic Dallas Heart Study of 3138 patients, plasma PCSK9 levels were significantly higher in women (n=1863) than in men (n=1489). This difference persisted after adjusting for most variables.120 In the same study, premenopausal women had considerably higher levels of plasma PCSK9 than postmenopausal women. Estrogen treatment did not significantly affect fasting PCSK9 levels in postmenopausal women. In comparison, there was no difference in plasma PCSK9 levels in men >50 versus those <50 years of age. Similar findings were obtained in older men and postmenopausal women, with 9% to 14% higher PCSK9 levels observed in women. Finally, serum PCSK9 levels were increased in pregnancy at term. However, recent data also showed that the human fetus has ~2-fold lower PCSK9 levels than the mother, which may be necessary to provide LDL-C to the growing fetus.121

**Variation With Diet and Medication**

It was found that a 5-week Mediterranean diet in normal subjects can lower plasma LDL-C and PCSK9 by as much as ~10% and ~15%, respectively.122 Moreover, it was shown that PCSK9 transcription could be suppressed by fasting and induced by insulin, likely by activating liver X receptor and SREBP-1c.77 Like the LDLR, PCSK9 is also upregulated by intracellular sterol depletion and statin treatment. Thus, in hepatic cell lines, statins coordinately upregulated the mRNA expression of LDLR and PCSK9.76 This suggested that statins would have a higher capacity to decrease LDL-C if not for the associated rise in PCSK9 level after statin. Indeed, statin treatment of Pcsk9-knockout mice resulted in a higher reduction in LDL-C than that of wild-type mice, signifying a hypersensitivity state.25 This was confirmed in individuals with FH harboring the common LOF R46L variant who seem to be more responsive to statin.123 Although statins directly increase PCSK9 mRNA expression, PPARα agonists, such as fibrates, indirectly affect PCSK9 expression through modulation of cholesterol levels.88 In a randomized trial, although statins were shown to increase the levels of circulating PCSK9, the cholesterol absorption inhibitor ezetimibe had no effect on PCSK9.124 Finally, in a study in which women underwent in vitro fertilization, high estrogens resulted in a reduction in VLDL, LDL, and PCSK9 levels.125 However, this might have been an effect of the 3-fold increase in growth hormone in response to induction of ovulation. Thus, growth hormone may negatively affect PCSK9 to favor increased uptake of circulating cholesterol by growing cells.

**PCSK9 Genetic Variance**

Family studies of patients with coronary heart disease led to the mapping of PCSK9 gene to FH, with GOF mutations exhibiting high serum levels of LDL-C;14; likewise LOF mutations were later found to lower LDL-C and could protect from coronary heart disease.19 Until now, only 2 women were reported to lack PCSK9 completely and to have low levels of circulating LDL-C: a subject with compound heterozygote LOF mutations22 and 1 homozygote LOF C679X mutation.23 A continuously updated list of all natural mutations of PCSK9 can be found in (http://www.ucl.ac.uk/ldlr/LODVH.1.10/index.php?select_db=PCSK9), and some of them were recently summarized.126

**Natural Mutations Determining PCSK9 Levels**

By screening a hypercholesterolemic cohort of individuals lacking mutations in the LDLR and APOB, FH cases attributed to PCSK9 mutations were estimated at ~2% of patients with FH.14,30 However, because the LDLR is the major route for PCSK9 uptake, its circulating levels are higher in patients with FH with LOF LDLR mutations, which may contribute to the wide spectrum of FH.127 PCSK9 GOF was associated with increased severity of coronary atherosclerosis in patients with polygenic hypercholesterolemia.128 Plasma PCSK9 levels are increased not only in patients with FH but also in patients with Familial Combined Hypercholesterolemia.129 These suggest that rare missense mutations in PCSK9 may worsen the clinical phenotype of patients carrying LDLR mutations. Similarly, the APOE genotype will influence the lipid phenotype of PCSK9 mutations as suggested in 1 study, with special emphasis on the E3/E2 genotype.117

**Variations With Exceptional Mechanisms**

Some mutations allowed a better understanding of the biosynthesis and secretion of PCSK9 biology and are worth highlighting. (1) The French Canadian LOF Q152H mutation prevents the autocatalytic processing of proPCSK9, resulting in a dominant negative form of the protein that in a heterozygote state reduces the circulating levels of PCSK9 and LDL-C by as much as ~80% and ~50%, respectively.22 This mechanism was later confirmed after an exhaustive analysis of all possible Gln₁₁₉ mutations.34 (2) Intriguingly the GOF R218S mutation significantly decreases PCSK9 catabolism, allowing it to circulate longer and negatively affect the LDLR to promote high cholesterol.130 This is likely because of the resistance of this mutant to Furin inactivation.61 (3) Conversely, the LOF A443T9 mutation likely results in a novel PCSK9 O-glycosylation site that favors Furin-induced degradation of the protein.61 (4) The most severe Anglo-Saxon GOF mutation D374Y,30 which despite lower circulating levels, results in a 10- to 25-fold higher binding affinity of PCSK9 to the LDLR.90 (5) A few variations in PCSK9 have also been reported in the hinge region (Figure 2; eg, the LOF R434W mutation resulting in lower secretion levels of PCSK9 and reduced
circulating LDL-C levels, likely because of a negative effect on the folding of the protein in the ER).131 (6) On the opposite side, the LDLR GOF H306Y mutation in the EGF-A domain associated with PCSK9-binding results in an enhanced PCSK9-mediated cellular degradation.132

PCSK9-Based Therapies and Safety Considerations

Companies are racing to develop a drug that mimics the effects of LOF PCSK9 mutations. As previously mentioned, PCSK9 enhances the post-translational degradation of the LDLR, therefore decreasing its capacity to lower LDL-C. This makes PCSK9 a promising therapeutic target6,30 and several pharmaceutical companies in active clinical or preclinical trials are testing various approaches to inhibit PCSK9.15,133,134 The best approach to date is the use of a monoclonal antibody (mAb) to PCSK9 that blocks its binding to the LDLR, via an allosteric mechanism. Antibodies targeted against PCSK9 showed remarkable cholesterol lowering in mice and monkeys, where a single injection results in an amazing reduction in LDL-C levels by ≈80% for more than a week.67

Phase I and II clinical trials in humans have been conducted by many pharmaceutical companies like Sanofi/Regeneron and Amgen; phase I trials confirmed safety and efficacy. In phase II trials, both companies report an LDL-C reduction varying between 60% and 70% on subcutaneous injection of ≈140 to 150 mg of the mAb every 2 weeks, with no significant elevation in liver enzymes.15,113 Interestingly, the combination of 80 mg atorvastatin with a 150 mg PCSK9 mAb had no >7% extra lowering effect on LDL-C achieved by the mAb alone.138 However, we will have to await the results of long-term administration of a mAb with or without a statin before deciding whether a mAb monotherapy is sufficient. As an added benefit, such mAbs also reduced by ≈30% the levels of the highly atherogenic Lp(a), suggesting that PCSK9 also targets the hepatic Lp(a) receptor(s) for degradation. The 2-fold lower reduction of circulating Lp(a) by a PCSK9 mAb when compared with LDL-C suggests that >1 Lp(a) receptor exists,136 one of which may be targeted for degradation by PCSK9. The identification of the putative PCSK9-sensitive receptor(s) and the segment(s) within that interacts with PCSK9 will surely have an important effect on our understanding of the Lp(a) and PCSK9 biology. Furthermore, because reduction of Lp(a) is currently limited to the use an antisense oligonucleotides to ApoB mRNA or apheresis,136 defining the PCSK9-sensitve Lp(a) receptor will surely improve current antitherogenic therapies. The mAb that will move to routine clinical application will depend on long-term safety data, ease of administration, and price.126 Online Table I summarizes some of the results of past and ongoing clinical trials. Although injections are not particularly attractive for lifelong treatment, such an approach would likely be embraced by patients having side effects from current lipid-lowering agents or by high-risk subjects striving to achieve lower LDL-C, as indicated by recent guidelines.137,138 For example, homozygous patients with FH, for whom initial LDL-C levels starts ≈3 to 4x that of the general population, are usually unable to achieve a 50% reduction on available oral agents and thus require LDL-apheresis, a form of dialysis to eliminate the LDL-C from blood.139 Amazingly, administration of a blocking PCSK9 mAb to homozygote patients with FH with some residual LDLR activity resulted in ≈30% reduction in LDL-C,140 giving some new hope for these patients that have to often undergo apheresis.

Another approach for inhibiting the PCSK9-LDLR extracellular interaction is to use inhibitory adnectins that are specific for PCSK9. Indeed, Bristol-Myers Squibb and Adnexus are currently investigating the efficacy of such an approach in phase I clinical studies. The isolated adnectins are similar to mAbs (which are ≈150 kDa), but smaller in size (≈12 kDa), and comprise a scaffold of the tenth extracellular human fibronectin type III domain that exposes a PCSK9-binding loop.

Because PCSK9 enhances the degradation of the LDLR by an intracellular and extracellular pathway (Figure 2),48 and unless the liver mostly uses the extracellular pathway, a therapeutical approach that blocks both processes may be more effective than one inhibiting only the extracellular pathway, as with mAbs. The use of antisense oligonucleotides should abrogate both pathways because it reduces mRNA levels of PCSK9 directly. However, both small 2′-O-methoxyethyl-modified phosphorothioate antisense oligonucleotide141 and locked nucleic acid (LNA) antisense oligonucleotides142,143 against PCSK9 had a rough start. Although promising results were obtained in mice and monkeys, both phase I clinical trials had to be terminated (http://rnaitherapeutics.blogspot.ca/2011/10/santaris-terminates-pcsk9.html); the modified phosphorothioate antisense oligonucleotide trial was terminated for unknown reasons likely because of serious side effects. Thus, although antisense LNA, which targets both human and mouse PCSK9 with similar efficiency in reducing PCSK9,142 kidney toxicity led LNA-targeted PCSK9 treatment to be terminated from further clinical trials.144

A small interfering RNA (siRNA) clinical trial involving siRNA-targeting PCSK9 has been evaluated in a randomized, single-blind, placebo-controlled, phase 1 dose-escalation study in healthy adult volunteers with serum LDL-C of ≥3·mmol/L or higher.145 The data showed that at a dose of 0.4 mg/kg, this relatively safe treatment resulted in a mean 70% reduction in circulating PCSK9 plasma protein and a mean 40% reduction in LDL-C from baseline relative to placebo. Phase II clinical trials are underway. Although mAbs seem to block close to 100% of free circulating PCSK9, the siRNA approach still left ≈30% PCSK9 in circulation, suggesting limited efficacy of the current siRNA method. Although a direct comparison of this approach with the mAb one is yet to be tested in human, the efficacy of the reduction of LDL-C observed with siRNA (40%)145 is still not better than the one achieved with mAbs (50%–70%).15,133 It is thus possible that the intracellular pathway in liver may have a relatively minor contribution to the overall activity of PCSK9 on LDLR.

Other anti-PCSK9 approaches may involve the use of Fc-fusion proteins to either the PCSK9 prosegment,146 or even the R1 domain of annexin A2.111 None of the published therapeutic anti-PCSK9 approaches in humans used a small-molecule inhibitor, possibly because of the relative flatness of the surface of interaction of the [PCSK9=LDLR] complex, and thus the absence of a targetable groove that could accommodate a small molecule inhibitor.38 However, we will have to await the
results of some novel approaches toward the identification and testing of small-molecule inhibitors, including those that block either the PCSK9 interaction with the LDLR using EGF-A mimetics or prevent the autoprocessing of proPCSK9 and the secretion of PCSK9, thus mimicking the mutants lacking either an active site residue, the C672X or the Q152H mutation. Such novel approaches may overcome the obstacles to small-molecule therapy, especially if they will result in an orally active inhibitor of liver PCSK9.

Because PCSK9 seems to target a number of LDLR family members for degradation, some of which act as entry receptors for infectious viruses, it is recommended that those individuals harboring a viral infection be carefully monitored for viral titers or excluded from anti–PCSK9-based therapy. Furthermore, in view of the critical importance of PCSK9 in liver regeneration, patients undergoing liver resection should not take anti-PCSK9 medication. In addition, chronic administration of an anti-PCSK9 therapy in patients must be monitored for the possible occurrence of insulin resistance and glucose intolerance. However, it must be noted that the glucose intolerance was to date observed only in genetic mutants with global LOF, including male Pcsk9-knockout mice and humans carrying the R46L variant. The use of a mAb may not have similar effects in human. To date, with the short-term treatment (from 12 weeks to 1 year) with either a mAb or siRNA, no evidence was reported for the development of type II diabetes mellitus. However, the gold standard inhibition by statins, it has been a long time that a new avenue has come about to reduce substantially cholesterol levels. The realization that a complete LOF PCSK9 mutation and inhibition of plasma PCSK9 result in rock-bottom cholesterol levels and improves other lipid parameters, such as ceramides and Lp(a). Monoclonal antibodies are at present the most advanced PCSK9 inhibitors in terms of pharmacological development and clinical response. Long-term studies will establish whether the beneficial effects of PCSK9 inhibition on LDL-C levels directly translate into safe and effective CVD risk reduction. Despite its apparent safety, there are concerns about the inhibition of PCSK9 because we still know little about its global physiological functions. Nevertheless, since the discovery of the LDLR and its importance in hypercholesterolemia and regulation by statins, it has been a long time that a new avenue comes about to reduce substantially cholesterol levels. The realization that a complete LOF PCSK9 mutation and inhibition of plasma PCSK9 result in rock-bottom cholesterol levels suggests that PCSK9 inhibitors could be the next blockbuster drug to combat hypercholesterolemia, which would be a harbinger of things to come.

Conclusions
With the discovery of PCSK9 in 2003 the lipid field took a sharp turn, with PCSK9 inhibitors becoming an undeniable therapeutic reality (Figure 3). The molecular basis for PCSK9 action supports a model in which PCSK9 is self-cleaved, secreted, and tightly bound to the EGF-A-like domain of the LDLR. This reduces LDLR recycling and downregulates LDLR activity, thereby increasing the levels of LDL-C in the blood. Thus, PCSK9 plays a key role in cholesterol homeostasis. Humans with high levels of PCSK9 or GOF mutations have increased levels of plasma LDL-C and significantly enhanced CVD risk during their lifetime. Humans with low levels of PCSK9 or LOF mutations have reduced levels of plasma LDL-C and significantly lower risk of developing a CVD. In addition, PCSK9 exhibits pleiotropic metabolic effects that need to be further explored. Statin therapy results in an increased plasma PCSK9 levels but lower overall LDL-C levels. This suggests that lowering PCSK9 levels may enhance the efficacy of statins to reduce LDL-C. Animal models have proven invaluable to screen a new drug’s modality because atherosclerosis and vascular calcification are enhanced in transgenic mice overexpressing PCSK9 and reduced in Pcsk9-knockout mice. In humans, loss of 1 copy of PCSK9 prevents 88% of cardiovascular events in the Atherosclerosis Risk in Communities (ARIC) trial on >3000 individuals followed >15 years.

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Table S1: Ongoing therapeutic approaches targeting PCSK9

<table>
<thead>
<tr>
<th>Type of agent</th>
<th>Company</th>
<th>Stage</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb-REGN727 / SAR236553</td>
<td>SA-Regeneron</td>
<td>Ph 3 Odyssey</td>
<td>~22,000 subjects injected (by 2018)</td>
</tr>
<tr>
<td>mAb-REGN727 / SAR236553</td>
<td>SA-Regeneron</td>
<td>Ph 2</td>
<td>40-72% LDL-C↓ vs 17% Atorvastatin alone</td>
</tr>
<tr>
<td>mAb-AMG145</td>
<td>Amgen</td>
<td>Ph 2/3</td>
<td>Subcut. injections/4 wks; 43-55% LDLc↓ at wk 12</td>
</tr>
<tr>
<td>mAb-RN316 (PF-04950615)</td>
<td>Pfizer-Rinat</td>
<td>Ph 2</td>
<td>Completed</td>
</tr>
<tr>
<td>LGT209</td>
<td>Novartis</td>
<td>Ph 2</td>
<td>Completion: 2015</td>
</tr>
<tr>
<td>RG7652</td>
<td>Roche/Genentech</td>
<td>Ph 2</td>
<td>Completion: 2015</td>
</tr>
<tr>
<td>Ad. BMS-962476</td>
<td>BMS-Adnexus</td>
<td>Ph 2</td>
<td>Started: 2013</td>
</tr>
<tr>
<td>siRNA</td>
<td>Alnylam</td>
<td>Ph 1</td>
<td>Cationic lipidoid formula</td>
</tr>
<tr>
<td>Small molecule</td>
<td>Shifa Biomedical Corp</td>
<td>Pre-Clinical</td>
<td>In preparation for Ph 1</td>
</tr>
<tr>
<td></td>
<td>Serometrix</td>
<td></td>
<td>In develop.</td>
</tr>
<tr>
<td></td>
<td>NuChem Therapeutics / AmorChem</td>
<td></td>
<td>In develop.</td>
</tr>
</tbody>
</table>