REVIEW**Proprotein Convertase Subtilisin/Kexin Type 9** ***A View beyond the Canonical Cholesterol-Lowering Impact***Chiara Macchi,^{*} Nicola Ferri,[†] Cesare R. Sirtori,^{*} Alberto Corsini,^{*‡} Maciej Banach,^{§¶||} and Massimiliano Ruscica^{*}

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Proprotein convertase subtilisin/kexin type 9 (PCSK9), mainly synthesized and released by the liver, represents one of the key regulators of low-density lipoprotein cholesterol. Although genetic and interventional studies have demonstrated that lowering PCSK9 levels corresponds to a cardiovascular benefit, identification of non-cholesterol-related processes has emerged since its discovery. Besides liver, PCSK9 is also expressed in many tissues (eg, intestine, endocrine pancreas, and brain). The aim of the present review is to describe and discuss PCSK9 pathophysiology and possible non-lipid-lowering effects whether already extensively characterized (eg, inflammatory burden of atherosclerosis, triglyceride-rich lipoprotein metabolism, and platelet activation), or to be unraveled (eg, in adipose tissue). The identification of novel transcriptional factors in the promoter region of human PCSK9 (eg, *ChREBP*) characterizes new mechanisms explaining how controlling intrahepatic glucose may be a therapeutic strategy to reduce cardiovascular risk in type 2 diabetes. Finally, the evidence describing PCSK9 as involved in cell proliferation and apoptosis raises the possibility of this protein being involved in cancer risk. (*Am J Pathol* 2021, 191: 1385–1397; <https://doi.org/10.1016/j.ajpath.2021.04.016>)

The story of proprotein convertase subtilisin/kexin type 9 (PCSK9) started in 2003 when Abifadel et al¹ described two gain-of-function (GOF) mutations in the *PCSK9* gene (*S127R* and *F216L*), both associated with autosomal dominant hypercholesterolemia. Since then, >220 PCSK9 variants have been reported. *PCSK9* has been classified as the third gene associated with familial autosomal dominant hypercholesterolemia, with an estimated incidence around 2.3%; and for variants for low-density lipoprotein receptor (*LDLR*) and apolipoprotein B (*apoB*) an incidence of approximately 67% and 14%, respectively. Three loss-of-function (LOF) nonsense mutations (*Y142X*, *C679X*, and *R46L*) have been identified.² On the basis of genetic studies reporting that carriers of LOF variants for *PCSK9* exhibit low levels of LDL with a reduction in the risk of myocardial infarction, fatal coronary heart diseases, or coronary revascularizations,² the Food and Drug Administration approved two monoclonal antibodies (evolocumab and alirocumab) for the treatment of hypercholesterolemias,

<12 years after the gene discovery. In 2020, the European Medical Agency approved the first silencing RNA therapy against PCSK9 (inclisiran).

Besides regulating LDL cholesterol homeostasis, a role of PCSK9 in non-cholesterol-related processes has emerged. Although mainly derived from the liver,³ PCSK9 is expressed in other tissues (eg, intestine, pancreas, and central nervous system) and cell types (eg, macrophages). The use of transgenic animal models has provided evidence that, in addition to *LDLR*, PCSK9 seems to degrade other receptors (eg, *CD36*), thus implying a direct involvement in endothelial function, inflammation, and hemostasis. In spite of safety data from clinical trials, mendelian randomization analyses have highlighted a higher risk of developing new-onset diabetes in carriers of *PCSK9* LOF variants.⁴ Finally, considering that

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PCSK9 was initially identified as an uncharacterized brain subtilase called neural apoptosis-regulated convertase-1 (NARC1),⁵ transiently expressed in the growing telencephalon and cerebellum, its potential effects on the central nervous system are as yet not clear.

Thus, the aim of the present review is to describe the molecular mechanisms beyond cholesterol lowering and to discuss the clinical implications of expected and unexpected effects of a therapeutic approach leading to undetectable circulating PCSK9 levels. Research strategies comprised the use of pubmed.gov and selection of articles in English. The following search parameters were used: *PCSK9* or *NARC1* AND *adipose tissue* AND *atherosclerosis* AND *brain* AND *coagulation* AND *inflammation-related atherosclerosis* AND *intestine* AND *triglyceride-rich lipoproteins* AND *major adverse cardiovascular events* AND *pancreas* AND *platelet* AND *tumors*. C.M. and M.R. screened titles and full text of articles identified in this search.

The Biology

Human *PCSK9* encodes a 692–amino acid glycoprotein with an overall domain structure similar to other proprotein convertase family members. PCSK9 includes a signal peptide, a prodomain, a subtilisin-like catalytic domain, and a variable C-terminal domain with a fold that has never been observed in subtilisin-like serine proteases.⁶ As depicted in

Figure 1, PCSK9 contains a catalytic triad (Asp¹⁸⁶, His²²⁶, and Ser³⁸⁶) superimposable on the catalytic triads of other subtilisins.^{5,7} PCSK9 processing occurs in the secretory pathway, and the autocleavage generates a stable PCSK9 heterodimer composed of a prodomain fragment (amino acids 32 to 152; 13 kDa) and a mature 62-kDa fragment containing the catalytic and C-terminal domains.⁸ The prodomain serves a dual role: as a chaperone for folding and as an inhibitor of catalytic activity. The cysteine-histidine rich domain of the C-terminal domain is a critical determinant for cellular degradation of LDLR⁹ and PCSK9 secretion.¹⁰ Once secreted at the cell surface, PCSK9 interacts directly with the epidermal growth factor repeat A domain of the LDLR,¹¹ thus favoring its degradation through an extracellular route implicating clathrin heavy chain–mediated endocytosis. An intracellular route has also been identified: PCSK9 fosters LDLR degradation by binding and shuttling LDLR from the trans-Golgi network directly to lysosomes involving clathrin light chains.¹² PCSK9-induced endocytosis and lysosomal degradation of LDLR are also mediated by caveolin but not by clathrin, in a cyclase-associated protein 1–dependent manner¹³ (**Figure 2**). PCSK9 can also play a role in LDLR maturation.¹⁴ In more extreme experimental conditions (ie, in the case of PCSK9 adenoviral infections), a second intracellular pathway associated with LDLR degradation has been observed within the post–endoplasmic reticulum (ER) compartments

Figure 1 The mature form of proprotein convertase subtilisin kexin type 9 (PCSK9) consists of a signal peptide [amino acids (aa) 1 to 30], a prodomain (aa 31 to 152), a catalytic domain (aa 153 to 452), and a C-terminal domain (aa 453 to 692). Once in the endoplasmic reticulum, the signal peptide is cleaved and autocatalysis of the PCSK9 zymogen occurs between Gln152 and Ser153 [valine-phenylalanine-alanine-glutamine (VFAQ)₁₅₂/serine-isoleucine-proline (SIP)]. Mature PCSK9 (aa 153 to 692; roughly 60 kDa) undergoes a furin enzymatic inactivation, leading to a 55-kDa–truncated form. The **orange boxed area** represents the signal peptide (SP); the **yellow boxed area** represents the prodomain; the **green boxed area** represents the catalytic domain; and the **blue boxed area** represents the C-terminal domain.

Figure 2 Cyclase-associated protein 1 (CAP1) has been described as a new binding partner of proprotein convertase subtilisin kexin type 9 (PCSK9). CAP1 contributes to mediate PCSK9-driven endocytosis and lysosomal degradation of low-density lipoprotein receptor (LDLR) through a process mediated by caveolae and not by clathrin.¹³ On the cell surface at neutral pH, the LDLR adopts an open extended conformation and binds to low-density lipoprotein (LDL) predominantly through the L4 and L5 domains of the LDL repeats. CTD, C-terminal domain; L, LDL repeats; SH3 BD, Src homology 3 binding domain.

without any involvement of proteasome or lysosomal cysteine proteases.¹⁵

Besides LDLR, PCSK9 promotes the degradation of other LDLR family members (eg, the very-low-density lipoprotein receptor (VLDLR)), although with a much lower efficiency.¹⁶ Kilodaltons (K_d) for VLDLR and apolipoprotein E (apoE) receptor 2 are 379 and 516 nmol/L, respectively. PCSK9 directly interacts with CD36, with a K_d of 1.2 μ mol/L, and elicits its degradation through a lysosomal and proteasomal pathway.¹⁷ A proportion of circulating PCSK9 is bound to lipoproteins, LDL, high-density lipoprotein, or lipoprotein(a), although binding to lipoprotein(a) is not supported by *in vitro* data.¹⁸

Pulse-chase experiments show that PCSK9 is processed from the precursor form of 74 kDa to the mature catalytic fragment of 62 kDa in under 2 hours, and is ultimately secreted from the cells.¹⁹ This fragment can also undergo a second proteolytic cleavage mediated by the protease furin, which cleaves PCSK9 at the N-terminal region, after residue 218, releasing an approximately 7-kDa peptide and generating a PCSK9 residue of 55 kDa. The mature (62-kDa) and furin-cleaved forms are both present in circulation. Overall, furin-cleaved PCSK9 is formed extracellularly, has a plasma clearance faster than the mature form, and once internalized, cannot get secreted back because of the lack of the prodomain, but retains a reduced efficiency to cause LDLR degradation.²⁰

A precise mechanism for secretion is still lacking. The coat protein II adaptor protein SEC24A (part of an unknown sorting receptor complex) facilitates the ER-to-Golgi transport of PCSK9. Experiments in mice show that deficiency of SEC24A reduces the secretion of PCSK9, leading to a hypocholesterolemic phenotype.²¹ In this context, the C-terminal domain of PCSK9 might interact with a potential cargo receptor to mediate PCSK9 secretion, with SEC24A, SEC24B, and SEC24C, but not SEC24D, involved in endogenous secretion.²² This suggests a receptor-mediated mechanism for the recruitment of PCSK9 into coat protein II vesicles. The cargo receptor sorts soluble proteins into the coat protein II complex. *In vitro* studies in 293T cells show that a cargo receptor, surfeit locus protein 4, facilitates secretion of PCSK9,²³ an unconfirmed finding in cultured human hepatocytes (HEK293, Huh7, and HepG2 cell lines).²⁴ Another hypothesis relies on the role of cytosolic dynamin-related protein 1. Primarily localized in mitochondria, dynamin-related protein 1 is associated with ER tubules in the liver and its inhibition promotes hepatic proteasomal degradation of PCSK9 in the early secretory pathway. Dynamin-related protein 1 inhibition raises the cytosolic localization of glucose-regulated protein 94, an ER chaperone that binds PCSK9 and prevents its interaction with LDLR in the ER.²⁵ Finally, sortilin facilitates the transport of PCSK9 from the *trans*-Golgi network to plasma membranes.²⁶

Transcriptional Regulation of PCSK9

PCSK9 was identified as one of the 33 genes regulated by the sterol regulatory element binding protein (SREBP) family, as shown by a combined analysis of oligonucleotide microarray data from SREBP1a and SREBP2 transgenic mice and SREBP cleavage-activating protein knockout mice.²⁷ Thus, identification of a sterol-regulatory element in the promoter region of *PCSK9* explains its SREBP dependency and the reason why *PCSK9* promoter activity is induced in response to cellular cholesterol depletion.²⁸ The expression of *PCSK9* is strongly dependent on a second transcription factor, the hepatocyte nuclear factor 1 α , whose binding site is in proximity (28 bp upstream) to that of the sterol-regulatory element in the promoter region of *PCSK9*.²⁹ It is worth mentioning that mammalian target of rapamycin complex 1 reduces the expression of *PCSK9* by activating protein kinase C δ and reducing hepatocyte nuclear factor 4 α and hepatocyte nuclear factor 1.³⁰ Finally, the carbohydrate-responsive element-binding protein, a critical glucose-sensing factor that regulates gene transcription in glycolysis and *de novo* lipogenesis, has been identified as a novel transcriptional factor of human *PCSK9*.³¹ This puts forth new mechanisms explaining how controlling intrahepatic glucose could be a therapeutic strategy to reduce cardiovascular (CV) risk in type 2 diabetes.

PCSK9 and Brain

Even though the central nervous system accounts for 2.1% of body weight, it contains 23% of the sterol present in the whole-body pool. Considering that there is no detectable uptake of cholesterol carried by lipoproteins across the blood-brain barrier, high rates of *de novo* cholesterol synthesis in the glia and neurons provide the necessary sterols for early brain development.³² Although *PCSK9* was first identified in the brain, its exact role in the central nervous system still needs to be clarified. *In vitro* and *in vivo* studies suggest that *PCSK9* (NARC-1) is involved in the following: i) differentiation of neural progenitor cells to neurons, ii) regulation of neuronal apoptosis, and iii) activation of astrocytes and microglia, all facilitating *PCSK9* expression in the cerebellum of adult humans.³³

In zebrafish, *PCSK9* is expressed in areas of continued neurogenesis, such as cortical, intracranial, and cerebellar granule neurons.³⁴ In the mouse brain, *PCSK9* is expressed in the telencephalon at the embryonic stage 12.5, in the cerebellum from embryonic stage 17.7 to postnatal day 7, and in adults, in the rostral extension of the olfactory peduncle.⁵ *PCSK9* regulates expression of the LDLR in the same brain areas during development and not in adulthood without affecting brain morphology, cell differentiation, or synapses.³⁵ It is worth mentioning that *Ldlr*^{-/-} mice exhibit impaired learning and memory,³⁶ whereas overexpression of

the LDLR in brain markedly inhibits amyloid deposition and increases extracellular $\alpha\beta$ clearance.³⁷

Alterations of cholesterol homeostasis associate with various neurodegenerative disorders, including Alzheimer disease. Specifically, cholesterol produced by astrocytes undergoes efflux to apoE-containing particles to be delivered to neurons by binding to specific receptors such as the LDLR, LDL receptor-related protein 1 (LRP1), VLDLR, and apoE receptor 2. These receptors, implicated in Alzheimer disease pathology, are degraded by *PCSK9*. apoE, mainly produced in and secreted from astrocytes in the brain, is lipidated by the ATP-binding cassette transporter A1 to supply cholesterol/lipids to neurons and other cells through LRP1 and CD36. *PCSK9* down-regulates the protein expression of ATP-binding cassette transporter A1, thus leading to a possible modulation of the release of apoE-containing lipoproteins. apoE isoforms may affect LRP1-mediated A β metabolism by directly interacting with A β or competing with A β for receptor binding³⁸ (Figure 3).

When overexpressed in CHO and human neuroglioma (H4) cells, *PCSK9* reduces the expression of membrane protein β -site amyloid precursor protein–cleaving enzyme 1 (BACE1),³⁹ responsible for the cleavage of amyloid precursor protein into the toxic amyloid β . This was confirmed in *Pcsk9*^{-/-} mice expressing low levels of BACE1 in the neocortex.³⁹ However, there is no confirmation of the involvement of *PCSK9* in the degradation of LDLR and BACE1 in the adult mouse brain.⁴⁰ *PCSK9* does not regulate either BACE1 levels or the predominant amyloidogenic fragment A β ₄₂, released after BACE1 and γ -secretase activity.

Although a mendelian randomization analysis reported that the inhibition of *PCSK9* might predispose individuals to Alzheimer disease, a clear mechanism and the consequent causality have not been confirmed.⁴¹ Conversely, negative conclusions were reported in the Reasons for Geographic and Racial Differences in Stroke (REGARDS) study, in which carriers of *PCSK9* LOF variant C697X or Y142X did not show any change in neurocognitive performance, unlike in the case of carriers of the *PCSK9* R46L variant.³⁸ Interestingly, considering that *APOE4* is the major genetic risk factor for late-onset Alzheimer disease,⁴² increased levels of *PCSK9* in APOE ϵ 4 carriers have been found.⁴³

Because drug therapies targeting *PCSK9* (both monoclonal antibodies and silencing RNA) reduce LDL cholesterol by >50%, understanding its role in cognitive impairment is important. Although genetic studies⁴⁴ and major clinical trials did not find any impact on cognitive performance,⁴⁵ data from the EudraVigilance database reported that administration of *PCSK9* monoclonal antibodies is associated with a higher probability of nervous and psychiatric adverse drug reactions.⁴⁶ Another important aspect is the link between *PCSK9* and depressive mood. As reported in a retrospective study, depression increases *PCSK9* levels,⁴⁷ although a mutual effect cannot be excluded. Among 479,522 individuals of the UK Biobank, the ones

Figure 3 Role of proprotein convertase subtilisin kexin type 9 (PCSK9) on A β amyloid deposition in central nervous system (CNS) and on platelet activation. Once released from the liver into the bloodstream, PCSK9 contributes to platelet activation by interacting with the CD36 receptor. PCSK9 is also present in the cerebrospinal fluid, and emerging evidence suggests its role in A β deposition, potentially through modulation of low-density lipoprotein receptor-related protein 1 (LRP1) receptor. Considering that under physiological conditions, plasma PCSK9 does not cross the blood-brain barrier (BBB), PCSK9 in the central nervous system should be synthesized locally by neurons. APP, amyloid precursor protein.

carrying the LDL-lowering T allele *rs1159147* variant had a concomitant higher risk of depression.⁴⁸ Although genetic analyses found a positive association between increased risk of depressive mood and circulating levels of PCSK9, the notion that lipid-lowering drugs might increase the risk of depression has not been confirmed in trials with PCSK9 inhibitors.³³

PCSK9 and the Inflammatory Burden of Atherosclerosis

Atherosclerosis is characterized by the retention of modified lipoproteins in the arterial wall, a burden initiated by the intramural retention of atherogenic lipoproteins, activating resident macrophages and recruitment of monocyte-derived cells. However, native LDL can be taken up by macrophages by macropinocytosis, especially when LDL is elevated.⁴⁹

Compelling evidence implicates PCSK9 in plaque development. It is expressed in endothelial cells, vascular smooth muscle cells, and macrophages. In macrophages, there is a stepwise increase in PCSK9 gene expression while transitioning from monocytes (PCSK9 being almost undetectable), to differentiating monocytes (significantly up-regulated), to fully differentiated macrophages.⁵⁰ In vascular smooth muscle cells, PCSK9 expression is instead regulated in response to reactive oxygen species⁵¹ and shear stress.⁵² Experiments aimed at investigating the impact of different blood flow patterns on *PCSK9* expression showed that low-flow state at arterial branch points

increased PCSK9 (both gene and protein) compared with normal flow, whereas laminar flow (preventing the accumulation of atherogenic lipids) reduced *PCSK9*.⁵³ Interestingly, PCSK9 inhibition by evolocumab (a monoclonal antibody) rapidly reversed coronary artery endothelial dysfunction in HIV⁺ individuals and people with dyslipidemia.⁵⁴

The hypothesis that PCSK9 might mediate inflammation in atherogenesis comes from the seminal article by Giunzioni et al,⁵⁵ demonstrating that human PCSK9 alters plaque morphology, increasing infiltration of inflammatory bone marrow-derived Ly6C^{high} monocytes into the lesion, leading to their differentiation into macrophages, a process entirely dependent on the LDLR. In line with these findings, expression of human PCSK9 in lipopolysaccharide-stimulated murine peritoneal macrophages increases mRNA expression of tumor necrosis factor (TNF)- α and IL-1 β , as later confirmed in human primary macrophages.⁵⁶ Exposure to human recombinant PCSK9 up-regulates the mRNA expression of proinflammatory cytokines and chemokines (eg, IL-1 β , IL-6, TNF- α , CXCL2, and monocyte chemoattractant protein-1). Accordingly, atherosclerotic lesions of mice injected with short hairpin RNA (shRNA) for PCSK9 have fewer macrophages, and a reduced mRNA expression of vascular inflammatory regulators such as TNF- α , IL-1 β , monocyte chemoattractant protein-1, toll-like receptor 4, and NF- κ B.⁵⁷ NF- κ B regulates PCSK9 aortic expression through the toll-like receptor 4–MyD88 pathway but not toll-like receptor 4–TRIF.⁵³

Interestingly, in an atherosclerosis-prone mouse model, *Pcsk9* deletion led to a phenotype with fewer atherogenic lipoproteins, via mechanisms independent of the LDLR. Compared with mice lacking *Ldlr* and *Apobec1*, endothelial cells exposed to lipoproteins from animals simultaneously lacking *Ldlr*, *Apobec1*, and *Pcsk9* expressed fewer adhesion molecules (*Icam-1*), inflammatory cytokines (*IL-1 β*), and chemotactic factors [eg, *Ccl2* (*Mcp-1*) and *Ccl-7* (*Mcp-3*)], all promoting monocyte adhesion and infiltration into the vessel wall.⁵⁸

Among the plethora of inflammatory mediators and scavenger receptors implicated in the inflammatory burden of atherosclerosis, lectin-like oxidized lipoprotein-1 (LOX-1) plays a prominent role. In arterial tissues, cultured endothelial cells, and vascular smooth muscle cells, mitochondrial reactive oxygen species generation exacerbates a positive cross talk between PCSK9 and LOX-1, in which LOX-1 increases PCSK9 and PCSK9 promotes LOX-1 and oxidized LDL uptake.⁵¹ Moreover, in TNF- α -primed macrophages, treatment with exogenous murine PCSK9 up-regulates expression of scavenger receptors (the class A macrophage scavenger receptor, CD36, and LOX-1) and enhances oxidized LDL uptake.⁵⁹

Internalization of lipids into macrophages is also facilitated by the LDLR-related protein family of receptors (eg, LRP5).⁵⁰ Although PCSK9 and LRP5 do not affect each other's expression, LRP5 and PCSK9 interact directly,

being colocalized at the perinuclear area. Immunoprecipitation experiments have shown that exposure to aggregated LDL favors the formation of a complex in the cytoplasm of macrophages, which is stronger in lipid-loaded macrophages. Silencing of LRP5 results in a reduction in PCSK9 release.⁵⁰

As previously reviewed, accumulation of lipoprotein-derived lipids in macrophages or dendritic cells induces activation of a multiprotein complex termed inflammasome.⁶⁰ Human atherosclerotic lesions show increased expression of the major components of the NOD-, LRR- and pyrin domain-containing protein (NLRP3) inflammasome, characterized by three domains: a C-terminal lysine-rich domain, a central nucleotide domain (the NACHT domain), and a terminal pyrin domain. Formation of the NLRP3 complex induces autocleavage and activation of caspase 1.⁶¹ In the intertwined relationship between PCSK9 and inflammation,⁶² interferon- γ increases mRNA expression of PCSK9⁶³; NLRP3 and its downstream signals caspase-1, IL-18, and IL-1 β all participate in PCSK9 secretion.⁶⁴

Vascular inflammation, T-cell accumulation in aortic plaques, and necrotic core formation are attenuated by treatment with the PCSK9 monoclonal antibody alirocumab⁶⁵ or an anti-PCSK9 vaccine.^{66,67} Immunization with the AT04A vaccine, recognizing the PCSK9 epitope (amino acids 153 to 692), reduces the number of monocytes adhering to the activated endothelium, as well as the expression of intercellular adhesion molecule 1 and NLRP3 in macrophages.⁶⁶ A similar pattern was found when the PCSK9Q β -003 vaccine was tested in *apoE*^{-/-} mice.⁶⁷

PCSK9 Regulates TG-Rich Lipoproteins

Relative to the so-called CV residual risk, epidemiologic and genetic studies have reported a causal role for triglyceride (TG)-rich lipoproteins. Carriers of the GOF S127R variant show a lipid profile characterized by a dramatic increase in the production rate of apoB100 (threefold), VLDL (threefold), and intermediate-density lipoprotein (threefold) compared with matched control subjects or carriers of LDLR mutations.⁶⁸ One of the leading hypotheses relies on the ability of PCSK9 to promote apoB (and VLDL) secretion by protecting apoB from LDLR-induced intracellular catabolism.⁶⁹ A direct involvement of the LDLR in the PCSK9-driven apoB secretion is supported by experiments in a mouse model overexpressing PCSK9.⁷⁰ A 24-hour fasting leads to a 2.5-fold increment in plasma TG levels, with a sharp increase in the apoB100 and apoB48 content of VLDL, intermediate-density lipoprotein, and LDL. These increases do not involve changes in lipase activity, but rather correspond to a faster catabolism: the fractional catabolic rate of VLDL/intermediate-density lipoprotein apoB is maximal in fasted mice overexpressing PCSK9. To disentangle the role of LDLR in this process, PCSK9 was

overexpressed in a background of *Ldlr*^{-/-} mouse model. On fasting, both genotypes showed hypertriglyceridemia, highlighting that the modulation of LDLR is required for the VLDL output.⁷⁰

Concerning the LDLR-independent pathway, Sun et al⁷¹ found that overexpression of PCSK9 in livers of C57BL/6, *Ldlr*^{-/-}, or *Ldlr*^{-/-}*ApoBec1*^{-/-} mice (models for human hyperlipidemia), led to an increase in plasma VLDL, LDL apoB100, and apoB48.⁷¹ PCSK9 interacts with apoB to prevent/inhibit/decrease the mobilization of apoB toward autophagosomes for degradation via the autophagosome/lysosome pathway. Finally, a mechanism of action outside of the lack of LDLR activity⁷² involves overexpression of PCSK9 GOF D374Y in mice leading to a phenotype with a large increase in TG-rich lipoproteins compared with wild-type or *Ldlr*^{-/-} mice.

Role of PCSK9 in Platelet Activation

Platelet aggregation and activation of the coagulation cascade are common etiologies of arterial and venous thrombotic events. Thrombosis in the arterial circulation is the main cause of acute coronary syndromes and ischemic stroke.⁷³ PCSK9 levels increase after acute coronary syndrome⁷⁴ and are positively associated with severity of coronary artery lesions.⁷⁵ The results of the Association of PCSK9 Serum levels and Platelet Reactivity in Patients with Acute Coronary Syndrome Treated with Prasugrel or Ticagrelor (PCSK9-REACT) study are in line with this evidence and show a positive association between circulating PCSK9 and platelet reactivity in acute coronary syndrome patients undergoing percutaneous coronary intervention.⁷⁶

The possible link between PCSK9 and platelet activation was first explored in *Pcsk9*^{-/-} mice, showing a reduced formation and stability of arterial thrombus.⁷⁷ These findings indicate that the *Pcsk9*^{-/-} mouse model develops smaller venous thrombi after ligation of the inferior vena cava with reduced P-selectin levels.⁷⁸ An improved understanding of the mechanisms explaining the impact of PCSK9 on platelet activation and thrombosis came from Qi et al⁷⁹ (Figure 3). They used pharmacologic, immunologic, and genetic approaches to show that enhanced platelet responsiveness, arterial thrombosis, and myocardial infarction expansion after myocardial infarction driven by PCSK9 are dependent on platelet CD36 expression and signaling.⁷⁹ Platelet activation and *in vivo* thrombosis were antagonized by both evolocumab and aspirin. Furthermore, this study was the first one supporting PCSK9 as a CD36 ligand, similar to oxidized LDL, advanced glycosylated proteins, and cell-derived microvesicles.⁷⁹

PCSK9 and Intestine

Early studies identified *Pcsk9* mRNA expression substantially in the small intestine in mice and in the human

colon carcinoma cell line LoVo-C5. Subsequently, *Pcsk9* was shown to express homogeneously along the intestinal cephalon-caudal axis in the mouse, whereas immunohistochemical analysis of jejunal and ileal biopsies showed that PCSK9 was present in enterocytes and goblet cells.⁸⁰

After intracellular re-esterification, long-chain fatty acids and cholesterol esters are associated with phospholipids and apolipoproteins (eg, apoB48) to produce TG-rich lipoproteins. The lack of PCSK9 leads to a reduction in postprandial lipemia, caused partially by an increased hepatic clearance of chylomicrons and their remnants without changes in gastric emptying and intestinal transit.⁸¹ Studies using the first intestine-specific PCSK9-deficient (*i-Pcsk9*^{-/-}) mouse model⁸² show that liver-derived PCSK9, and not the intracellular intestinal form, through its canonical regulation on LDLR expression, is the key regulator of postprandial lipemia. Indeed, administration of a specific monoclonal antibody (alirocumab) did not reduce postprandial lipemia in *Ldlr*^{-/-} mice.⁸² These findings are in line with the impact of evolocumab on postprandial lipemia [namely, TG (-21%), apoB48 (-17%), and VLDL₁ (-15%) in patients with type 2 diabetes mellitus or carriers of *PCSK9* LOF mutations].⁸³

Studies in CaCo2 cells show that recombinant PCSK9 directly stimulates, by roughly 50%, the intestinal production of TG-rich lipoproteins, apoB48, and apoB100, as well as that of fatty acid synthase (*FAS*), stearoyl-CoA desaturase (*SCD*), and diacylglycerol O-acyltransferase (*DGAT*) 2.⁸⁴ In human PCSK9 (hPCSK9) transgenic mice overexpressing human PCSK9 at the physiological levels, LDLR levels in the small intestine were reduced by 54% compared with *Pcsk9*^{-/-} or wild-type models, whereas the absence of mouse PCSK9 (mPCSK9) increased LDLR levels by nearly 70%. Moreover, hPCSK9 transgenic mice has increased intestinal microsomal triglyceride transfer protein levels and activity regardless of LDLR expression, whereas the absence of mPCSK9 does not alter microsomal triglyceride transfer protein at any level, mRNA, protein, or activity.⁸⁴ However, in *i-Pcsk9*^{-/-} mice, no significant changes were found in the mRNA expression of *Mtp* and other lipogenic genes in the small intestine compared with control mice.⁸²

Experiments in Caco-2/15 cells show that PCSK9 increases the expression of Niemann-Pick C1 like 1, a key protein in cholesterol absorption. However, this effect is consequent only to a reduced LDL uptake and a decrement in cholesterol pool inside the cell, as a result of the PCSK9-driven degradation of LDLR at the basolateral membrane of the enterocytes.⁸⁵

In addition to its effect on postprandial lipemia, PCSK9 acts as a modulator of transintestinal cholesterol excretion, an alternative route for plasma cholesterol excretion. Transintestinal cholesterol excretion is increased in *Pcsk9*^{-/-} mice and rapidly reduced when PCSK9 is replaced by i.v. injection. The effect on transintestinal cholesterol excretion

seems fully dependent on LDLR function because it is lost in *Ldlr*^{-/-} mice.⁸⁶

PCSK9 and Pancreas

In pancreatic β cells, the accumulation of cholesterol occurs mainly via LDLR. Any genetic or pharmacologic intervention that increases LDLR expression is virtually associated with cholesterol overload in β cells, a process dampening glucose-stimulated insulin secretion.

The first evidence of the expression of PCSK9 in the pancreas came from *in vitro* experiments on RIN-m5F and bTC-3 β cells and MIN6 pancreatic islet cells.^{5,87} Later studies in *in vivo* murine models show that PCSK9 mRNA is expressed in isolated pancreatic islets and colocalizes with somatostatin in human pancreatic δ cells. While PCSK9 is detectable in α cells, β cells, or in the exocrine tissue, LDLR expression is significantly increased in the β cells of the *Pcsk9*^{-/-} mice.⁸⁸ *Ex vivo* experiments demonstrate that recombinant PCSK9 reduces the expression of LDLR in the pancreatic islets, whereas those isolated from *Pcsk9*^{-/-} mice show a significant increase in the LDLR content (200%). Similarly, Mbikay et al⁸⁷ found a higher content of LDLR in the whole pancreas of *Pcsk9*^{-/-} mice compared with wild type. These effects were mostly mediated in an endocrine rather than in an autocrine manner.⁸⁸

Relative to glucose homeostasis, 4-month-old male *Pcsk9*^{-/-} mice exhibit impaired glucose tolerance with an altered glucose-stimulated insulin secretion.⁸⁷ The lack of PCSK9 leads to significantly higher fasting glucose, with reduced pancreatic and fasting plasma insulin. Down-regulation of levels of PC1/3 proteins involved in insulin biosynthesis is observed in *Pcsk9*^{-/-} mice fed a Western diet.⁸⁷ In line with these findings, the absence of PCSK9 in mice fed a standard or a high-fat diet is associated with an impaired glucose tolerance. Despite a normal insulin sensitivity, plasma insulin and C-peptide levels are significantly reduced, and the pancreatic insulin content is increased. These data suggest a defective insulin secretion, a feature confirmed in *PCSK9* R46L carriers, who present a lower homeostasis model assessment of β -cell function, indicating an impaired β -cell functionality.⁸⁹ In pancreatic islets, the increased expression of LDLR is associated with PCSK9 deficiency and paralleled by an accumulation of cholesterol esters with a down-regulation of genes involved in cholesterol biosynthesis and uptake. Herein, the role of LDLR, the main target of PCSK9, is crucial. It is responsible for the phenotype, because the knockdown of both LDLR and PCSK9 completely revert the metabolic and pancreatic derangements seen in the *Pcsk9*^{-/-} mouse model. Further evidence comes from the analysis of liver from the *Pcsk9*^{-/-} mouse model that unveils the marginal role of circulating PCSK9 on glucose metabolism and β -cell function.⁸⁹ Pancreatic islet morphology indicates PCSK9 deficiency leading to alterations associated with signs of

inflammation and early apoptosis.^{87,89} In particular, the Western diet significantly increases both islet mass and numbers in *Pcsk9*^{-/-} mice, also showing larger and possibly immature secretory granules, suggesting an abnormal biogenesis of insulin secretory granules.⁹⁰ Finally, different conclusions have been reached by using human pancreatic sections and EndoC-βH1 cells or β-cell-specific *Pcsk9*^{-/-} KO mice. Inhibition of exogenous or endogenous PCSK9 did not affect insulin secretion and specific PCSK9 deletion in β-cells did not lead to any toxic effect.^{91,92}

PCSK9 and Adipose Tissue

Human adipose tissue is among the numerous tissues that express PCSK9.⁹³ A significant accumulation of visceral adipose tissue is found in *Pcsk9*^{-/-} mice⁹⁰ fed a Western diet. In female mice, this diet leads to an increase in perigonadal white adipose tissue.⁹⁰ Similarly, carriers of the *R46L* LOF variant show an increased ectopic fat accumulation.⁹⁴

The deletion of PCSK9 leads to adipocyte hypertrophy, independent of the presence of LDLR, with a higher fatty acid uptake and an increase in TG synthesis in perigonadal and inguinal pads. Furthermore, circulating PCSK9 down-regulates the expression of the surface VLDLR, especially in females, as confirmed by higher protein levels of this receptor in perigonadal depots of *Pcsk9*^{-/-} mice. Interestingly, this effect does not implicate LDLR involvement.⁹⁵ Further analysis shows interaction and colocalization of PCSK9 with the endogenous CD36 at the adipocyte cell surface as well as in lysosomes. Indeed, PCSK9 directly interacts with CD36, inducing degradation through a mechanism involving both lysosomes and proteasome, with a consequent reduced uptake of CD36 ligands.¹⁷ CD36 protein levels are up-regulated in the visceral adipose tissue of *Pcsk9*^{-/-} mice.⁹⁵

Clinical studies also corroborate these findings by the characterization of subjects with low circulating PCSK9 levels. Their white adipose tissue depots present a reduced function, as assessed by the lower lipoprotein lipase activity and high surface expression of LDLR and CD36, with increased NLRP3 inflammasome activation.⁹⁶ As to possible mechanisms regulating PCSK9 expression, insulin treatment increases PCSK9 expression in human adipocytes, an effect partially counteracted by the atrial natriuretic peptide. Interestingly, incubation of human adipocytes with LDL isolated from human plasma lead to an initial increase of intracellular PCSK9 levels, together with an increment of *LDLR*, *SREBP-1c*, and *SREBP-2* expression. In the following 18 hours, increase in the secreted mature form of PCSK9 is associated with a reduced LDLR expression, suggesting that PCSK9 in adipocytes are fully functional. In support of this hypothesis, the decrement in PCSK9 expression on atrial natriuretic peptide treatment is paralleled by reduced LDLR degradation.⁹³

Among the different white adipose tissue loci, the visceral epicardial adipose tissue may play an important role in the pathogenesis of CV disease. In patients with coronary artery or valvular diseases, PCSK9 was positively correlated with epicardial adipose tissue thickness and inflammation.⁹⁷ A positive association was also found between PCSK9 and several local proinflammatory mediators such as chemokines, chemokine receptors, ILs, and cytokines.⁹⁷ In line with this finding, PCSK9 GOF Ossabaw mini-pigs showed an up-regulated pericardial fat mRNA expression of TNF-α.⁹⁸ However, the PCSK9 content in epicardial adipose tissue was not associated with plasma PCSK9 concentrations, suggesting that epicardial adipose tissue inflammation could be associated with the local PCSK9 levels, rather than with the circulating ones.⁹⁷

Finally, considering the link between adipose tissue and CV health,⁹⁹ it is worth mentioning that adiponectin, leptin, and resistin regulate PCSK9 expression. In wild-type and *apoE*^{-/-} mice, adiponectin have an opposite effect by increasing and reducing *PCSK9* expression, respectively, through the involvement of peroxisome proliferator-activated receptor γ and AMP-activated protein kinase α. This opposing behavior may be explained by a specific interaction between PCSK9 and apoE.¹⁰⁰ Relative to leptin, in HepG2 cells, this adipokine increases PCSK9, an effect that depends on the STAT3 pathway,¹⁰¹ whereas in leptin-deficient ob/ob mice, the effects of leptin on PCSK9 are strongly modified by sexual hormones.¹⁰² Finally, resistin increases PCSK9 mRNA and protein expressions, possibly through the up-regulation of *SREBP-2* or a post-transcriptional stabilization process.¹⁰³

PCSK9 and Tumors

Preclinical and clinical studies have shown that manipulating cholesterol metabolism inhibits tumor growth, reshapes the immunologic landscape, and reinvigorates anti-tumor immunity.¹⁰⁴ The evidence describing the involvement of PCSK9 in cell proliferation and apoptosis raises the question of whether this protein is involved in cancer risk. Although a partial hepatectomy increases the expression of PCSK9 in mice, the same procedure leads to an impaired hepatocyte proliferation and enhanced apoptosis in *Pcsk9*^{-/-} mice.³ Carriers of LOF variants show a reduced incidence of breast cancer, whereas those carrying GOF variants have an increased risk.¹⁰⁵ Inoculation of nanoliposomal anti-PCSK9 vaccine in mice bearing breast or colorectal cancers results in a moderate reduction in tumor growth and an increase in lifespan.^{106,107} In an *in vitro* model of human lung adenocarcinoma A549 cells, PCSK9 silencing exhibits anti-tumor activity by inducing apoptosis.¹⁰⁸ Finally, in the context of immune therapy and cancer, many efforts are underway to improve the effectiveness of immune checkpoint therapy. Inhibition of PCSK9 can boost the response of tumors to immune checkpoint therapy in a cholesterol-independent

manner. The reduction of PCSK9 by genetic or pharmacologic approaches has the ability to increase the expression of major histocompatibility protein class I presentation on tumor cell surfaces, thus favoring entry of CD8⁺ T lymphocytes and reducing tumor growth.¹⁰⁹

Future Perspectives and Conclusions

Although there is a huge escalation of experimental and clinical data describing PCSK9 pathophysiology and possible effects besides lipid lowering, other effects such as the case of calcific aortic valve stenosis and the regulation of the hypothalamic-pituitary-gonadal axis need to be explored further. Although calcific aortic valve stenosis is a condition conferring an increased risk of major cardiac events, far less is known about PCSK9 and calcific aortic valve stenosis. Carriers of *PCSK9* R46L LOF variant compared with noncarriers have a reduced prevalence of this disease (odds ratio, 0.80; 95% CI, 0.70-0.91).¹¹⁰ Relative to sex hormones, administration of estradiol in ovariectomized *apoE*^{-/-} mice (a model developing aortic atherosclerosis) increases plasma PCSK9 and hepatic mRNA expression versus specific controls.¹¹¹ In HepG2 cells, estradiol, by binding to a specific estrogen-responsive element, increases PCSK9 activity in a dose-dependent manner.¹¹¹

Overall, unveiling noncholesterol pathways related to PCSK9 is the key to understanding long-term PCSK9 pharmacologic inhibition. PCSK9 inhibition may increase the risk of new-onset diabetes but can also provide a large CV benefit driven by LDL lowering. The possibility of categorizing PCSK9 among danger-associated molecular patterns needs to be explored further. This broad family of modified endogenous structures, generated during inflammation, tissue injury, and oxidant stress, interacts with specific scavenger receptors and toll-like receptors to trigger proinflammatory and prothrombotic responses by the innate immune system.¹¹² These findings suggest PCSK9 inhibition as a potentially safe therapeutic target to prevent arterial thrombosis in individuals with elevated PCSK9 levels. Identification of new PCSK9 signaling pathways may constitute a significant challenge for molecular biology, specially considering that PCSK9 is a mediator of many metabolic processes. Finally, involvement in immune checkpoint therapy for cancer provides a compelling rationale to conduct future clinical trials in patients with cancer by combining checkpoint inhibitor anticancer drugs with inhibitors of PCSK9.

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