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REVIEW ARTICLE

PCSK9 INHIBITORS AS LDL CHOLESTEROL-LOWERING AGENTS: RATIONALE, CONCERNS AND PRELIMINARY OUTCOMES

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CONTENTS

SUMMARY

Lowering cholesterol associated with low-density lipoprotein (LDL) is an efficient strategy to prevent cardiovascular diseases or attenuate their progression and damage. Statins have been successfully used for this purpose, but many patients still need alternative or additional, more aggressive therapy. Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibition may be the solution. The hypercholesterolemic effect of PCSK9 is due to its interaction with the LDL receptor (LDLR). Its absence leads to lower LDL cholesterol and reduced cardiovascular risk. However, the interaction of PCSK9 with other lipoprotein receptors and its presence in organs such as the brain raise concerns about its inhibitory effects. Several strategies have been developed to inhibit PCSK9 synthesis or its binding to the LDLR, and it is now being evaluated in clinical trials. Preliminary results are promising.

Key words: LDL cholesterol – PCSK9 inhibitors – Cardiovascular disease – Hypercholesterolemia

CURRENT CONTEXT OF LDLC MANAGEMENT

Cardiovascular disease is a leading cause of death in the world (1). There is compelling evidence from population-based data and clinical trials that reducing cholesterol associated with low-density lipoproteins (LDLs) prevents coronary heart disease (CHD), slows its progression or attenuates the damage (2). LDLs are cleared by the liver mainly via the LDL receptor (LDLR). Cholesterol is then secreted into the bile and excreted into feces or reabsorbed by the intestine. In plasma, cholesterol from modified LDL, such as oxidized LDL, is taken in by cells of the arteries via specific receptors. These early events initiate and maintain the atherogenic process, an inflammatory disease present in adolescents and that develops throughout life (3).

Statins are powerful LDL cholesterol (LDLC)-lowering agents that represent the therapy of choice and continue to give excellent results by inhibiting the first and rate-limiting enzymatic reaction of endogenous cholesterol synthesis. New trials led experts to lower LDLC goal therapies to < 70 mg/dL in the U.S. and Europe in patients with existing CHD and additional risk factors (4-6). Unfortunately, recent surveys have shown that patients who are at highest cardiovascular risk are also those that fail more often to achieve their therapeutic goal, in particular diabetics (7). Some patients also require larger reductions of LDLC due to high baseline levels, such as those with familial hypercholesterolemia. Others present adverse events and stop or discontinue the therapy (8, 9). This illustrates the need for additive or replacement therapy to statins (as reviewed in 10).

RATIONALE BEHIND PCSK9 INHIBITION

PCSK9 and gain-of-function mutations increase LDLC

Proprotein convertase subtilisin/kexin type 9 (PCSK9; *PCSK9*) was identified in 2003 as the third gene associated with autosomal-dominant hypercholesterolemia (11). This disease (OMIM #144010) is characterized by high concentrations of LDLC and premature atherosclerosis. It was rapidly shown in mice that wild-type *Pcsk9* per se is hypercholesterolemic (12-15). Conversely, mice deficient in *Pcsk9* present with approximately 50% lower cholesterolemia (16, 17). PCSK9 opposes hepatic LDL endocytosis by the LDLR, and therefore cholesterol excretion. LDL particles bind to the LDLR via its ligand apolipoprotein B-100 (Apo B-100) and are directed toward lysosomal degradation, before the LDLR is recycled to the cell surface. Plasma PCSK9 binds to the LDLR and prevents this recycling (18-20). Both proteins are endocytosed and degraded into the lysosomal compartment, a process that probably involves LDLR ubiqui-

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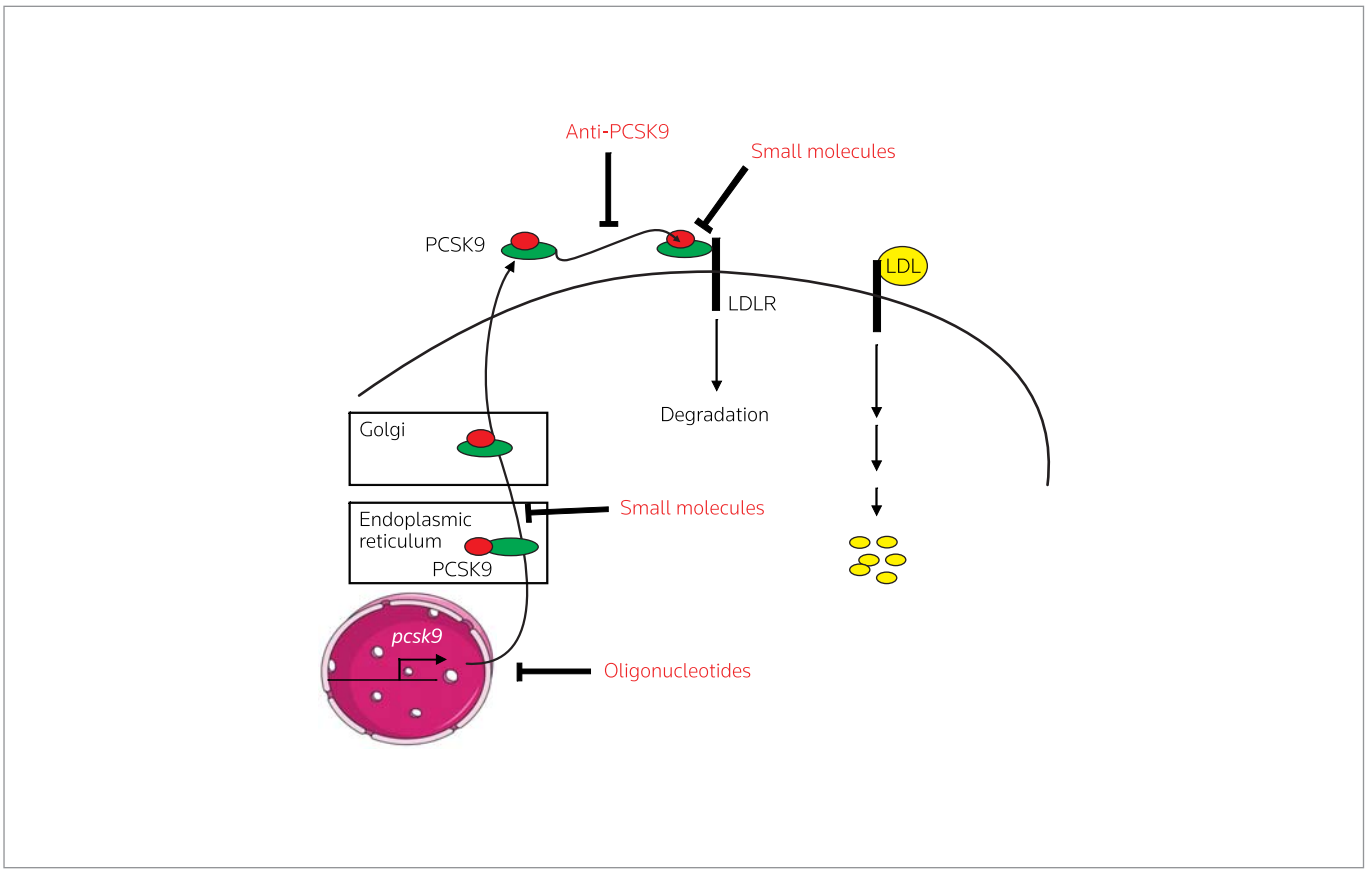

Figure 1. Proprotein convertase subtilisin/kexin type 9 (PCSK9) induces degradation of LDL receptor (LDLR) and prevents low-density lipoprotein (LDL) clearance. PCSK9 is cleaved in the endoplasmic reticulum and secreted together with its prodomain (red), which blocks its catalytic site. This cleavage is necessary for PCSK9 to reach the LDLR. PCSK9 binds to the LDLR at the cell surface of hepatocytes and impairs its recycling. As a consequence, LDL clearance is reduced. There are two strategies currently evaluated in clinical trials: oligonucleotides that reduce PCSK9 synthesis or anti-PCSK9 antibodies preventing its interaction with the LDLR. In theory, small molecules that would inhibit PCSK9 autocatalytic activity in the endoplasmic reticulum or prevent its interaction with the LDLR will also protect the receptor from PCSK9.

tinylation (21). In patients, gain of function mutations increase PCSK9 affinity for the LDLR (18, 22-24), prevent PCSK9 degradation by furin (25, 26), or modify the pool of PCSK9 multimers, thereby modifying its binding to the LDLR (27). The most severe form of hypercholesterolemia is due to variant D374Y, which increases the affinity of PCSK9 toward the LDLR. These patients have concentrations of LDLC comparable or superior to those seen in heterozygous familial hypercholesterolemia (FH) patients (mutations in *LDLR*), resistance to statins and a very early onset of CHD (28).

One of the obvious populations that could benefit from PCSK9 inhibitors are heterozygous FH patients, assuming that neutralizing PCSK9 would increase the activity of intact or mutated enzyme. Such a strategy might actually pay off in homozygous FH whose LDLR is not totally inactivated. Three Japanese subjects with double heterozygotes for the *PCSK9* gain of function mutation E32K and *LDLR* mutations (C183S, C292X and K790X) presented with high LDLC and severe cutaneous xanthomatosis (29). A 1-year-old subject presented an LDLC of 581 mg/dL. In a recent study in FH patients not yet treated with hypocholesterolemic drugs, the mean plasma PCSK9 concentration was higher in FH patients with LDLC above the 90th percentile than in patients with LDLC below the 75th percentile. Thus, PCSK9 probably worsens the phenotype of FH patients (30).

In the current context of *PCSK9* inhibitor trials, this review will focus on *PCSK9* loss. More information on *PCSK9* gain of function mutations and to what extent they affect cholesterolemia and atherosclerosis has been described elsewhere (31).

PCSK9 life-long reduction in humans

PCSK9 loss-of-function mutations are associated with lower LDLC and a reduced risk of coronary artery disease (CAD). Genotyping Afro-Americans from the Atherosclerosis Risk In Communities studies revealed that 2.6% had nonsense mutations in *PCSK9*, associated with a decrease of 29% in LDLC and a reduction in the risk of CHD (myocardial infarction, fatal CHD or coronary revascularization) by 88% over a period of 15 years (32). In the same study, 3.2% of

Caucasians had a mutation in *PCSK9* associated with a reduction of 15% in LDLC and reduction in the risk of CHD of 47%. This R46L variation results in less circulating PCSK9 (33, 34). The same variation was later associated with a reduced risk of early-onset myocardial infarction in a cohort of European ancestry (35) and in Italians (36). The variant was also significantly associated with a 46% decrease in ischemic heart disease in a very large study of the Danish general population (N = 26,013), but not in two smaller studies (N = 1,032 and N = 9,654) (37). The variant was not predictive of mortality. The decrease in LDLC was observed in patients aged 20 to 80+ years. Interestingly, in a meta-analysis, the 28% reduction in the risk of ischemic heart disease was superior to the 5% reduction predicted by the 12% reduction of LDLC associated with R46L. As discussed by the authors of the study, this suggests that the genotype is more predictive of the risk than the value of LDLC measured in adults.

PCSK9 deficiency and atherosclerosis in mice

As described above, life-long reduction of PCSK9 and LDLC decreases cardiovascular risk dramatically (32). However, there have been conflicting results on the level of expression of *PCSK9* in atherosclerotic plaque (38, 39). One recent study showed that it is secreted by smooth muscle cells and degrades the LDLR in macrophages in vitro. The authors suggested that PCSK9 might have a protective role against cholesterol accumulation in these cells (38). Mice deficient in Pcsk9 have roughly 50% less cholesterolemia (16, 17). Pcsk9 deficiency in mice does not lead to an obvious decrease in plaque size or intima–media thickness under a 12-month-long high-fat diet, nor in the absence of apolipoprotein E (Apo-E), a background favorable to atherosclerosis (40). Nevertheless, when fed a high-fat diet, *Pcsk9*$^{-/-}$ mice accumulated 4-fold less cholesteryl esters in their aorta than wild-type mice on a 12-month Western diet. Mice with double knockouts for *Pcsk9* and *Apoe* exhibited less aortic cholesteryl esters compared to *Apoe*$^{-/-}$ mice. Interestingly, the absence or overexpression of *Pcsk9* does not affect cholesterolemia nor the extent of atherogenesis in *Ldlr*$^{-/-}$ mice, suggesting that the contribution of PCSK9 to the disease is entirely based on its interaction with this receptor.

There have been numerous publications showing that inhibiting PCSK9 synthesis using oligonucleotides (41-45) or preventing its interaction with the LDLR with injectable antibodies (45-48) reduces cholesterolemia to a great extent. Unfortunately, to our knowledge, there has been no report on an antiatherogenic effect for PCSK9 inhibitors in preclinical models.

PCSK9 interactions with hypolipidemic drugs

By inducing LDLR degradation, PCSK9 impairs the hypocholesterolemic effect of several drugs. This makes it an attractive target to enhance those drugs.

Statins

Statins increase LDL clearance by inhibiting endogenous cholesterol synthesis, which in turn also activates the transcription of the LDLR via sterol regulatory element-binding protein 2 (SREBP-2). PCSK9 is also regulated by intracellular cholesterol concentrations and statins (49), because it is also a target for SREBP-2 (50, 51). PCSK9 transcriptional activation by statins is superior to that of the LDLR because the PCSK9 promoter contains an additional response element for hepatocyte nuclear factor 1-alpha (HNF-1-alpha), which accumulates in response to statins (52, 53). Of note, the immunosuppressive agent rapamycin induces PCSK9 by decreasing HNF-1-alpha cell content via suppression of mTORC1 activity (54). This mechanism could explain the hypercholesterolemic effect of rapamycin in transplanted patients. Plasma PCSK9 is also increased by statins (49, 55-58), with the exception of simvastatin at a low dose (10 mg) in one study (59). We showed that standard doses of atorvastatin (10 mg) increase plasma PCSK9 within a day in diabetics and that this effect is sustainable (+14% at 6 weeks of treatment). A recent analysis of PCSK9 and LDLC variations in the Justification for Use of Statins in Prevention: an intervention Trial Evaluating Rosuvastatin (JUPITER) trial showed that induction by rosuvastatin is sustained after a year of treatment (60). It appears that PCSK9 induction by statins is dose-dependent. A high dose of atorvastatin (80 mg) increased plasma PCSK9 by 47% within 4 weeks of treatment and the the effect was sustained for 16 weeks (58).

The immediate consequence of PCSK9 induction by statins is that the association between PCSK9 and LDLC is abolished in patients treated with statins (58). Another consequence is that PCSK9 is a brake on the effect of statins. Thus, PCSK9 inhibitors should amplify the effect of statins. This concept was validated in *Pcsk9* knockout mice that are more responsive to lovastatin (16) and in recent clinical trials with the PCSK9 antibody REG-727 (61, 62). However, in the JUPITER trial, there was a positive relationship between the increase of PCSK9 and the decrease in LDLC in response to rosuvastatin (60). Similar findings were observed in hypercholesterolemic patients treated with statins and ezetimibe (63). These observations remain unexplained. It is unclear whether the baseline PCSK9 level is predictive of the response to statins (58, 64), or not (59). Given the interindividual variations of PCSK9 and of LDLC in response to statins, it is still difficult to imagine that measuring total plasma PCSK9 as we currently do could be a very helpful tool for personalized medicine.

Fibrates

We initially reported that fibrates, hypotriglyceridemic agents, repress PCSK9 in mice and human hepatocytes (51). It was then reported that fenofibrate decreases PCSK9 concentrations in diabetics (65, 66) and in diabetics treated with statins (65). However, we observed an important elevation in plasma PCSK9 (+26% at week 6 of treatment) in diabetic patients with fenofibrate (160 mg) and no additive effect with atorvastatin (56). Several other teams had also observed an increase in plasma PCSK9 in response to fenofibrate or bezafibrate (57, 67, 68). There was no obvious difference in the populations tested, the dose used or the duration of the treatments that could explain these discrepancies between studies. One possibility would be that fibrates specifically induce various forms of PCSK9 in mice and humans and/or that the ELISA assays do not detect the same form of PCSK9. However, there is no debate concerning the existence of PCSK9 induction by statins using various assays.

Ezetimibe

Ezetimibe inhibits cholesterol absorption (10). By decreasing the flux of cholesterol to the liver, it secondarily activates the LDLR pathway

via the SREBP-2 transcription factor, similar to the action of statins. Thus, ezetimibe should increase PCSK9 expression. One study showed that hypercholesterolemic patients with ezetimibe on top of statins had higher PCSK9 than patients on statin therapy alone (63). Another study found no change in plasma PCSK9 in response to low-dose (10 mg) simvastatin or ezetimibe (10 mg), or both in combination (59). More studies are needed to clarify these aspects.

QUESTIONS ABOUT THE SAFETY OF PCSK9 INHIBITION

Data from subjects deficient in PCSK9 are reassuring concerning the safety of its inhibition, but remain scarce. Most concerns come from a simple question that remains unanswered: is PCSK9 good for anything? We will review the various pieces of the puzzle gathered by a relatively limited number of teams.

Data in humans

There is currently no obvious danger concerning PCSK9 inhibition based on clinical observations in patients with *PCSK9* loss of function mutations, but the amount of information available in patients with no detectable PCSK9 is scarce and does not inform us about how PCSK9 deficiency affects subjects over the age of 50. The decrease in cardiovascular risk observed in patients heterozygous for *PCSK9* loss of function mutations (32) and the absence of obvious pathology in a 32-year-old female heterozygote with no circulating PCSK9 (69) are reassuring. We identified a 49-year-old man with 16 mg/dL of LDLC who had no detectable circulating PCSK9 due to a dominant-negative effect of the variant (70). This subject was also diabetic, but was attending the endocrinology clinic for this reason and therefore no conclusions can be drawn concerning a possible causal link between the absence of PCSK9 and the presence of diabetes. Despite low LDLC (7 mg/mL at the first visit, before his diabetes was controlled), he presented with normal liver function but moderate steatosis, probably irrelevant to the extreme forms observed in abetalipoproteinemia due to a defect in VLDL secretion.

PCSK9 affects other lipoprotein receptors

At first, It was shown that PCSK9 induces degradation of LDLR exclusively, and not of any other members of this receptor family (20). However, data obtained from cell-free-based assays (71, 72), as well as in vitro (73), were later published showing that PCSK9 can degrade very-low-density lipoprotein receptor (VLDLR), as well as apolipoprotein E receptor 2 (APOER2; *APOER2*). Further studies in mice showed that Pcsk9 deficiency results in increased VLDLR in the adipose tissue, in particular, in females, larger visceral fat pads (+70% to +90%) and hypertrophic adipocytes. This was accompanied by increased uptake of orally delivered fatty acids and increased triglyceride synthesis, but a limited effect on body weight (74). Thus, it could be speculated that PCSK9 removal in patients would increase adipocyte storage. This does not fit with the observation that PCSK9 is positively associated with body mass index (BMI) in humans, but it would be interesting to test the association between PCSK9 and waist circumference in both sexes.

Another legitimate question is relative to the recent observation that myocardial VLDLR is induced by hypoxia during myocardial infarction and worsens its outcome by promoting lipotoxicity (75). The study was conducted in mice, but some findings in humans corroborate the results. A higher expression of VLDLR in ischemic compared with non-ischemic ventricles was observed in humans and VLDLR mRNA levels correlated with the extent of lipid storage in biopsies of ischemic hearts. In this context, can we imagine that PCSK9 actually protects the heart from excessive lipid intake during or after myocardial infarction? Since these patients are obvious candidates for PCSK9 inhibitors, this point needs clarification.

PCSK9 role in the brain

PCSK9 was first cloned in neurons and named neural apoptosis-regulated convertase 1 (NARC-1). PCSK9 favors neuronal differentiation in primary cultures of embryonic telencephalon neurons (76). PCSK9 is transiently expressed in the embryonic telencephalon in the frontal cortex and is found in adults in the cerebellum and the rostral extension of the olfactory peduncle (76, 77). The LDLR co-localizes with PCSK9 in these tissues and its expression, together with Apo-E protein levels, was increased in *Pcsk9*$^{-/-}$ embryos. There were no gross abnormalities of the telencephalon at E12.5 or of the cerebellum at P7, and no change in the markers of cell proliferation, cell differentiation and synapse (77). In the adult mouse, Pcsk9 deficiency did not impact LDLR expression in the rostral extension of the olfactory peduncle or the olfactory bulb, nor the organization of these regions. Following an ischemic stroke, PCSK9 (mRNA) was expressed in the dentate gyrus and the LDLR and Apo-E levels were decreased in the hippocampus. However, behavioral assessments and lesion volumes were identical in *Pcsk9*$^{-/-}$ and wild-type mice and there was no indication of a defect in neurogenesis.

There were some legitimate concerns about the role of PCSK9 in neurodegenerative diseases because LDLR, VLDLR and APOER2 are expressed in the central nervous system and have been implicated in pathologies such as Alzheimer's disease, mainly because they regulate the level of Apo-E (78). One team also showed that PCSK9 is involved in the disposal of non-acetylated forms of beta-secretase 1 (BACE1; *BACE1*) (79). BACE1 is the rate-limiting enzyme in the generation of Alzheimer's disease β-amyloid peptide (Aβ). They showed that 2.5-month-old *Pcsk9*$^{-/-}$ mice have increased levels of BACE1, of direct APP cleavage product C99 and Aβ in their neocortex. However, these results were not confirmed by another team in a different strain of *Pcsk9*$^{-/-}$ mice. Compared with wild-type mice, they found no difference in 6-month-old *Pcsk9*$^{-/-}$ mice in LDLR, VLDLR, APOER2 or BACE1 protein levels in brain homogenates and in $A\beta_{40}$ in homogenates of both cortex and hippocampus (72). The lack of increase in LDLR in the absence of Pcsk9 fits with the results of immunohistochemistry in the adult mice by Rousselet et al. (77). They also found no change in mice overexpressing *Pcsk9* in the brain, but we should take into account that the level of overexpression seemed rather low, possibly because the transgene was under the control of an Apo-E liver-specific enhancer (80). Although these mice were previously shown to have very high concentrations of plasma Pcsk9 because of major overexpression in the liver (80), it was recently shown that circulating PCSK9 probably does not cross the blood–brain barrier (77) and should not affect LDLR in the brain.

There is a clear lack of information concerning the evaluation of the association of PCSK9 with neurodegenerative diseases in patients. A recent analysis of 25 genes involved in lipid metabolism by dense

linkage disequilibrium mapping of 1,554 dementia cases, including 1,270 with Alzheimer's disease, identified an association with Apo-E, but not with PCSK9 (81).

PCSK9 and hepatic function

PCSK9 contributes to proper liver regeneration (17, 76). Following partial hepatectomy, mortality in *Pcsk9*$^{-/-}$ mice was higher than in controls, necrotic lesions were more abundant and mice presented with delayed liver regeneration (17). In diabetic patients, we determined that PCSK9 is also independently associated with gamma-glutamyl transferase (82), an indicator of hepatic dysfunction in insulin-resistant and non-alcoholic fatty liver disease (83). Thus, it is possible that PCSK9 removal would be detrimental in patients with liver dysfunction.

PCSK9 and hepatitis C virus

Plasma PCSK9 may be protective against hepatitis C virus (HCV) infection. HCV is transported on triglyceride-rich particles containing Apo-E and Apo B-100 lipoproteins. There are several receptors and co-receptors that might mediate HCV entry into cells, including CD81 and LDLR (84). In cell cultures, expression of PCSK9 or addition of purified PCSK9 at high concentrations to cell culture media impaired HCV infection and removed HCV receptor CD81 from the cell surface, independently of the LDLR (85). CD81 expression was also higher in *Pcsk9*$^{-/-}$ mice. In another study, gain of function variant D374Y did not affect CD81 expression, but the concomitant LDLR degradation did prevent HCV infection (86). Studies with other variants would help identify the residues specifically affecting CD81 or LDLR.

PCSK9 and its association with metabolic factors

Plasma PCSK9 is associated with many metabolic factors without any definite understanding about a possible causal link. In particular, PCSK9 has been found to be associated with BMI, plasma triglycerides (TGs), glycemia and insulin resistance, as well as blood pressure (34, 64, 87, 88). Afro-Americans in the ARIC study with loss of function mutations had lower TG and fewer of them presented with hypertension compared to non-carriers (32). To our knowledge, there has been no further information about a potential role of PCSK9 in blood pressure (its role in the kidney remains unknown). We will focus here on triglyceridemia and insulin resistance.

Triglyceridemia

There have been several findings in mice that indicate a causal link between PCSK9 and TG. We showed very early that increasing hepatic *Pcsk9* expression in mice results in hypertriglyceridemia upon fasting, when the Pcsk9 level is normally low, and that the liver is flowed with free fatty acid (89), but this was not observed in randomly fed mice (13, 89). It is unclear whether this is due to a lack of reuptake of nascent VLDL by the LDLR, which was virtually absent following *Pcsk9* overexpression during fasting, or to additional mechanisms. Non-fasted TGs are not changed in *Pcsk9*$^{-/-}$ mice (16). However, we measured dramatically decreased postprandial lipemia after a lipid oral load in *Pcsk9*$^{-/-}$ mice due to a change in chylomicron production, size and catabolism (90). Using the same approach in mice with a different genetic background, Roubstova et al. showed that dietary fatty acid uptake by adipose tissue is increased in *Pcsk9*$^{-/-}$ mice (74).

In humans, circulating PCSK9 is associated with fasted TGs (34, 64, 87, 88). The association of PCSK9 with TGs observed in various cohorts might result from a defect in TG-rich lipoprotein clearance due to the LDLR or the VLDLR. Using lipoprotein kinetics, we published that variant S127R is associated with an increased VLDL production rate, but this is probably a specific trait of this variant (91). Since then, it has been showed that wild-type *PCSK9* concentrations are not associated with hepatic VLDL and LDL–Apo B-100 production rates (92, 93). PCSK9 is a heritable trait of familial combined hyperlipidemia, characterized by high Apo B-100 and TG levels. However, PCSK9 is associated with LDL–Apo B-100 and not VLDL–Apo B-100 in these patients (94). To our knowledge, there has been no report of a robust change in TGs in mice, hamsters, monkeys or humans treated with PCSK9 inhibitors, causing doubt about a major role for PCSK9 in TG metabolism.

PCSK9 and insulin resistance, diabetes

Circulating PCSK9 was found to be positively correlated with fasting plasma glucose and homeostatic model assessment-insulin resistance (HOMA-IR), an index of insulin sensitivity, in several cohorts of nondiabetic subjects (34, 64, 87, 88). There is some evidence of a link between statin treatment and diabetes, but no obvious mechanistic explanation (95, 96). Since PCSK9 inhibitors should amplify the effect of statins, it is important to verify whether PCSK9 modulates glucose metabolism or insulin signaling. Highly contradictory results were reported in mice. We studied PCSK9 in human and mouse pancreas and found that it is not expressed in beta cells but in delta cells. While recombinant PCSK9 is able to reduce LDLR in pancreatic islets, PCSK9 deficiency does not alter the function of the islets, with no particular phenotype regarding glucose homeostasis (97). Conversely, Mbikay et al. found that *Pcsk9*$^{-/-}$ mice on a different genetic background were glucose intolerant, with an increased rate of beta cell apoptosis (98). Thus, more work is needed to clarify these aspects. It would be interesting to verify how VLDLR is regulated by PCSK9 in the pancreas. As described above, more studies are also needed to verify that PCSK9 removal will impact the LDLR pathway to the same extent in a context of insulin resistance.

PCSK9: A NONCLASSICAL TARGET

Biological facts that motivated current strategies of inhibition

Clinical trials are currently evaluating nonclassical agents to inhibit PCSK9. This choice was driven by several biological facts that we will review here.

PCSK9 is abundant in the liver, intestine and kidney (17). Seventy percent of circulating PCSK9 originates from the liver, which makes this organ the main target of synthesis inhibitors. PCSK9 is autocleaved in the endoplasmic reticulum (ER) and secreted together with its prodomain. If not cleaved, PCSK9 cannot reach the LDLR (99). The idea of inhibiting PCSK9 within the ER has been rapidly dismissed by many companies, because of the difficulty of finding an inhibitor that would compete with the kinetics of self-cleavage. It was also quickly established that inhibiting PCSK9 enzymatic activ-

ity in the plasma would not impair its interaction with the LDLR. Indeed, the plasma PCSK9 catalytic site is occupied by the prodomain (18, 24), and it binds like a chaperone to the extracellular epidermal growth factor homology domain repeat A (EGF-A) of the LDLR, preventing its recycling (18-20). At neutral plasma pH, LDL binds to LDLR in an open and extended position. Within the endosomes, at an acidic pH, the LDLR adopts a closed conformation where the beta propeller interacts with the ligand binding domain and LDL is released. Lo Surdo et al. proposed a new model where the PCSK9 catalytic site binds to the LDLR and the prodomain binds to the beta propeller (100). This maintains the LDLR in an extended position, preventing a closed conformation and recycling. The *C*-terminal domain is solvent-exposed and might interact with cofactors or a coreceptor such as annexin A2 (101). PCSK9 interaction with the LDLR and the degradation of the receptor were experimentally prevented in cell cultures with peptides that mimic the EGF-A domain (102) or portions of the prodomain (103). Small molecules disrupting the contact between the EGF domain and the catalytic site or between the prodomain and the beta propeller should also be effective (100, 103). Anti-PCSK9 antibodies that would specifically bind to these residues should also prevent the interactions between the two proteins. There have been several publications with monoclonal antibodies that successfully bound PCSK9 in mice or monkeys and lowered cholesterolemia (45-48). These antibodies are cleared by PCSK9 and therefore have a relatively short half-life. By modifying residues of the cross-reacting determinant, Chaparro-Riggers et al. were able to design a pH-sensitive antibody with high affinity for PCSK9 at neutral pH and low affinity at acidic pH (48). This modified antibody binds to the endosomal neonatal Fc receptor and is released into the circulation. Plasma PCSK9 has little effect on its half-life. This antibody presented an extended half-life in vivo. In monkeys, the antibody induced a similar magnitude of LDLC lowering but the duration of the effect was more than doubled.

Circulating PCSK9 remains poorly characterized

The range of concentrations of PCSK9 is wide open in the general population, with up to a 100-fold difference between individuals (34). Several studies have reported higher concentrations in women than in men and an increase with age (34, 64, 88). PCSK9 follows a diurnal rhythm synchronous with cholesterol synthesis, with changes of roughly +15% and –15% from the mean (104). PCSK9 decreases during fasting (104-106).

There are some indications that PCSK9 does not constitute a homogeneous pool of circulating proteins. One study reported that PCSK9 circulates as mono- and multimers that have different affinity for the LDLR (27). PCSK9 is also subject to post-translational modifications (107, 108). A recent patent by Bristol-Myers Squibb describes PCSK9 polypeptides corresponding to alternative splicings of PCSK9, secreted by cells in vitro and active towards the LDLR (US 8105804). How these isoforms are detected by various ELISA assays and how their synthesis, secretion and clearance are regulated are important questions that must be answered if we want to correctly interpret the measures of circulating PCSK9. When injected into mice, the half-life of recombinant purified Pcsk9 is very short (in the range of minutes), as it is rapidly cleared by the liver (109), but this might represent only a portion of plasma Pcsk9. Whether the composition of plasma PCSK9 pool would significantly affect the effect of antibodies from one patient to another remains to be determined.

Plasma PCSK9 is associated with LDLC in adults (34, 64, 88), children and adolescents (87, 110). However, the association is modest and the PCSK9 variation explained only 7% of the LDLC variation in the largest cohort (34). PCSK9 is inversely associated with LDL catabolism (92, 93). We showed that plasma PCSK9 explained 37% of Apo B-100 fractional catabolic rate variance in healthy individuals (93). The association with LDLC disappears when patients are treated with statins because the drug increases PCSK9 and lowers LDLC. The association also seems to be less reproducible in diabetic patients (93, 111), and it will be interesting to verify whether PCSK9 removal will affect LDLC to the same extent in the context of insulin resistance. Plasma PCSK9 was recently associated with increased intima–media thickness in non-treated individuals, even after adjustment for the lipid profile and other cardiovascular risk factors (30).

Strategies of inhibition and current clinical trials

The first approach to lower PCSK9 (and LDLC) is an appropriate diet. The Mediterranean diet induced an 11% decrease in PCSK9 and a 9.9% decrease in LDLC in men with metabolic syndrome, without weight loss (112). Of course, such a level of inhibition is not sufficient for many patients. Drugs that inhibit PCSK9 synthesis or neutralize the plasmatic protein are now under clinical evaluation.

Oligonucleotides

Strategies aimed at silencing *PCSK9* in preclinical models were mainly based on the two classes of oligonucleotide-based approaches of gene silencing: single-stranded (antisense) (42-44) and double-stranded (small interfering RNA [siRNA]) (113). Both strategies induced degradation of targeted mRNA. These technologies present with advantages and issues, but new developments have made them serious candidates for therapy. There has been a constant evolution in the design of the oligonucleotides and delivery systems. For example, modification of the antisense composition by replacing one of the non-bridging oxygens by a sulfur oxygen (phosphorothioates) was shown to prevent excessive degradation. Locked nucleic acid (LNA) technology has increased the affinity of antisense compounds for their target and reduced the need for complex vehicles. For large molecules, such as siRNAs, a new generation of liposomal vehicles has improved the major issue of intracellular delivery and toxicity (113, 114).

Santaris and Bristol-Myers Squibb/ISIS Pharmaceutical have terminated their clinical trials on antisense oligonucleotides targeting PCSK9 (SPC-5001 and BMS-844421, respectively). They used the technology of phosphorothioate LNA RNAse H antisense. To our knowledge, the exact causes of this termination were not communicated.

In September 2011, Alnylam commenced a phase I evaluation of its compound ALN-PSC in the U.K. ALN-SPC is based on a second-generation stable nucleic acid–lipid particle (SNAPL) from Tekmira, using the MC3 lipid and aimed at systematically delivering siRNA targeting PCSK9. Safety and tolerability were evaluated during a single-escalating-dose study (0.015-0.250 mg/kg) in a total of 32 healthy volunteers with elevated baseline LDLC (> 116 mg/dL) (NCT01437059). The company reported results from the initial 20 subjects (115). There were no serious adverse events at the time. At

the maximal dose, a statistically significant mean reduction of plasma PCSK9 of 60% was obtained. There was a dose-dependent decrease in LDLC of up to 50% relative to baseline (statistically significant mean reduction of 39% at day 4 at the highest dose).

Monoclonal antibodies

Pfizer has moved its humanized monoclonal antibody (MAb) into phase II (NCT01350141). The trial will test the efficacy of PF-04950615 at reducing LDLC on top of a maximal dose of statins (atorvastatin 80 mg or rosuvastatin 45 mg) in hypercholesterolemic patients (fasting LDLC ≥ 80 mg/dL and fasting TG < 400 mg/dL). Of note, the duration of infusion is 60 minutes.

Amgen's fully human MAb AMG-145 is being evaluated in phase II trials. A phase I study in healthy subjects with LDLC > 100 mg/dL administered single i.v. doses of AMG-145 has shown a reduction of 64% in LDLC. No serious adverse events were reported.

Sanofi and Regeneron published the clinical evaluation of a fully human anti-PCSK9 MAb, REG-727/SAR-236553. In two randomized, single-ascending-dose studies, the antibody or placebo was injected in healthy adults with LDLC > 100 mg/dL s.c. (32 subjects; doses: 50, 100, 150 or 200 mg/kg) or i.v. (40 subjects; doses: 0.3, 1.0, 3.0, 6.0 or 12.0 mg/kg). A multiple-dose study was performed in adults with FH who were receiving atorvastatin (21 subjects) and in adults with non-FH who were receiving atorvastatin (30 subjects) or a modified diet alone (10 subjects). The antibody was injected s.c. at doses of 50, 100 or 150 mg/kg on days 1, 29 and 43. Results from these three small phase I studies indicated no discontinuation due to adverse events (62). In the single-dose studies, the maximum LDLC-lowering effect was observed at the maximal i.v. dose of 12.0 mg/kg. There was a −65% change from baseline versus placebo in measured LDLC. A maximum percent change of 45% from baseline versus placebo was observed with the maximum dose (250 mg) injected s.c. The extent and duration of LDLC lowering were dose-dependent, lasting up to 64 days for the i.v. injection. In the multiple-dose study, REG-727/SAR-236553 decreased LDLC up to −61% in the combined atorvastatin patients at the dose of 150 mg. The effect was dose-dependent. Interestingly, the extent and duration of LDLC paralleled that of free PCSK9. Similar results were obtained in FH and non-FH patients, as well as in non-FH patients not taking atorvastatin.

A phase II study addressed the safety and efficacy of s.c. injections of REG-727/SAR-236553 in patients with primary hypercholesterolemia receiving atorvastatin over a period of 12 weeks (61). One patient had a serious event of leukocytoclastic vasculitis. PCSK9 neutralization resulted in a further reduction of LDLC by 40-72%, in a dose- and dose frequency-dependent fashion. Injections of 100 and 150 mg REG-727/SAR-236553 every 2 weeks were more effective than 200 and 300 mg every 4 weeks. All patients receiving 150 mg REG-727/SAR-236553 every 2 weeks reached LDLC < 70 mg/dL.

These studies suggest that PCSK9 neutralization might provide a cardiovascular risk reduction by other means than just LDLC lowering. As discussed by the authors, REG-727/SAR-236553 injected every 2 weeks at 100 and 150 mg significantly decreased non-HDLC and Apo B-100 levels, two parameters that might constitute better markers of outcome than LDLC in secondary prevention and high-risk patients (61, 116). The decrease in Apo B-100 on top of statins (−48% and −56%, respectively) enabled patients to meet both their LDLC and Apo B-100 therapeutic goals. Unexpectedly, REG-727/SAR-236553 also decreased apolipoprotein(a) (Lp[a]) in both phase I and II studies. This remains unexplained. This is an interesting observation, since Lp(a) is associated with cardiovascular risk and because other LDLC-lowering agents do not decrease its plasma concentrations, except for niacin (117). There was also a trend toward an increase in HDLC and Apo-AI that might be explained by a lack of transfer of cholesterol from HDL towards LDL by cholesterol ester transfer protein (CETP). Of note, TG reductions were modest, but the study did not enroll patients with high baseline TG levels.

CONCLUSION

Within less than 10 years, our knowledge about PCSK9 has tremendously increased. Its role as a major regulator of LDLC is not contested. However, entire aspects of this protein remain in the dark, such as its role in the kidney, the intestine or the brain, the exact nature of the circulating forms or the physiological relevance of its interaction with lipoprotein receptors other than the LDLR. The current clinical strategies are relying on siRNA to inhibit PCSK9 synthesis or MAbs to neutralize it in the plasma. Preliminary results are encouraging. These crucial steps will also provide us with an opportunity to compare both approaches.

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