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PCSK9 in extrahepatic tissues: What can we expect from its inhibition?

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ABSTRACT

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is an enzyme that belongs to the serine protease family and plays a key role in regulating low-density lipoprotein cholesterol (LDL-C) levels in the blood. PCSK9 binds to the LDL receptor (LDLR), targeting it for degradation, resulting in an increase in circulating LDL-C levels. Loss-of-function mutations in the PCSK9 gene are associated with lower LDL-C levels and lower cardiovascular risk; in contrast, gain-of-function mutations are a cause of familial hypercholesterolaemia. The identification of PCSK9 as a pharmacological target led to the development of inhibitors for the treatment of hypercholesterolaemia. To date, the monoclonal antibodies evolocumab and alirocumab (which target plasma PCSK9) and the small-interfering RNA inclisiran (which targets hepatic PCSK9 mRNA) have been approved for the treatment of hypercholesterolaemia. Although hepatic PCSK9 plays a central role in regulating plasma LDL-C levels, this protein is also expressed in other tissues, including the brain, pancreas, heart, kidney, intestine and adipose tissue. In extrahepatic tissues, the functions of PCSK9 are both dependent and independent of LDLR and not necessarily harmful. For this reason, it is essential to uncover any potentially harmful effects of therapies that inhibit PCSK9, beyond their known LDL-C-lowering and CV risk-reducing effects.

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Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is an enzyme that belongs to the serine protease family and is an important regulator of low-density lipoprotein cholesterol (LDL-C) levels (1). PCSK9 binds to the LDL receptor (LDLR) and initiates endocytosis and subsequent lysosomal degradation of the LDLR, preventing the receptor from returning to the cell surface (1). This leads to an increase in circulating LDL-C levels. The *PCSK9* gene contains several polymorphisms, including gain-of-function and loss-of-function mutations, which significantly affect normal PCSK9 signalling and cholesterol metabolism (2). PCSK9 is predominantly expressed in the liver, which produces the bulk of circulating PCSK9, which in turn regulates plasma LDL-C levels.

The role of PCSK9 in determining plasma LDL-C levels and its association with cardiovascular disease has been suggested by the observation that loss-of-function mutations in the PCSK9 gene are associated with lower LDL-C levels and lower cardiovascular risk (3-6); in contrast, gain-of-function mutations have been identified as a cause of familial hypercholesterolaemia, with elevated LDL-C levels from birth and high cardiovascular risk (2, 7-11). Once PCSK9 was identified as a pharmacological target, research focused on the development of inhibitors to control hypercholesterolaemia. To date, two monoclonal antibodies (mAbs, evolocumab and alirocumab) targeting circulating PCSK9 and a small interfering ribonucleic acid (siRNA, inclisiran) targeting hepatic PCSK9 mRNA have been approved for the treatment of hypercholesterolaemia. Both approaches have

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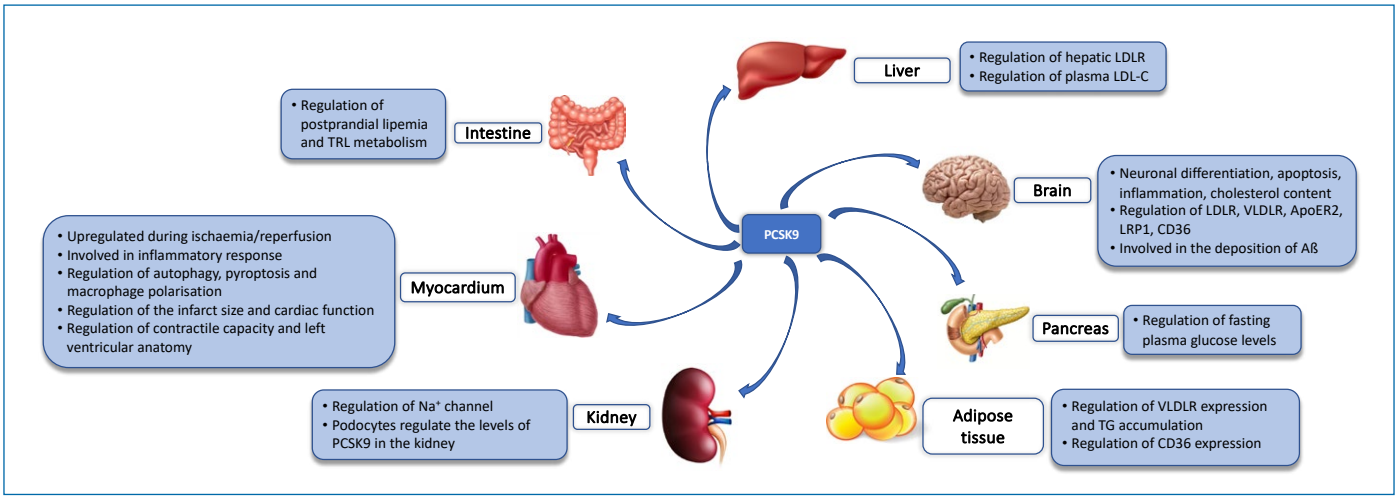

Figure 1 | Effects of PCSK9 in the liver and extra-hepatic tissues.

shown great efficacy in lowering LDL-C levels (~50-60% for mAbs and ~50% for siRNA); outcome trials have reported clinical benefit for both mAbs (12, 13); the ongoing ORION-4 is investigating the effect of inclisiran on clinical outcomes in patients with cardiovascular disease (NCT03705234).

Although PCSK9 has been studied primarily in the liver due to its important role in regulating plasma LDL-C levels, it is also expressed in other tissues, albeit to a lesser extent, such as the brain, pancreas, heart, kidneys, intestine, and adipose tissue. In these tissues, most of the effects exerted by PCSK9 are associated with metabolic pathways involving LDL-C, but relevant effects independent of LDLR metabolism have also been described (**Figure 1**). Based on this observation, questions have been raised about the possible effects of PCSK9 inhibitors in extrahepatic tissues. In this review, we aim to discuss the evidence available to date on this topic.

PCSK9 and the brain

PCSK9 was first discovered in neuronal cells undergoing apoptosis, and it appears to play a role in neuronal differentiation, cholesterol regulation, apoptosis, and inflammation in the brain (14). The brain is the most cholesterol-rich organ, but its cholesterol metabolism is uncoupled from peripheral tissues, as neither cholesterol nor PCSK9 can cross the blood-brain barrier under physiological conditions (15). In addition to the LDLR, PCSK9 regulates the levels of other receptors involved in the transport of cholesterol into neurons, including very low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2), as well as LDL receptor-related protein-1 (LRP1) and the scavenger receptor CD36 (16), which are highly expressed in the central nervous system (CNS). PCSK9 is present in cerebrospinal fluid at a low but constant level, in contrast to serum PCSK9 levels, which show large diurnal fluctuations (17), suggesting that the regulation of PCSK9 in cerebrospinal fluid may be different from that of PCSK9 in the bloodstream.

Although lowering LDL-C levels to very low levels is associated with clinical cardiovascular benefits, concerns have been raised about possible negative effects on cognitive function, as cholesterol is an essential component of myelin. This interaction between cholesterol homeostasis and cognition appears to be of particular importance in dementia.

Alzheimer's disease (AD), the most common cause of dementia, is a neurodegenerative disease characterised by continuous cognitive decline leading to poor quality of life (18). A body of evidence suggests that PCSK9 plays a role in AD, although our understanding of it is still incomplete. The accumulation of ß-amyloid (Aß) is a hallmark of AD; it arises from the amyloid precursor protein through the action of ß-site amyloid precursor protein-cleaving enzyme 1 (BACE1). Overexpression or inhibition of PCSK9 leads to decreased or increased expression of BACE1, respectively, and thus to higher or lower deposition of Aß (19). An indirect effect of PCSK9 has also been suggested, through the increase in systemic LDL-C levels, which may increase Aß deposition either by disturbing the balance of oxysterols (which can efficiently cross the blood-brain barrier) or by impairing Aß transport and degradation or by damaging the blood-brain barrier through an inflammatory process (20). Impaired Aß clearance in the brain promotes the onset and progression of AD. LRP1 and CD36 are two lipoprotein receptors that play a crucial role in Aß clearance by transporting Aß from the brain into the blood (21, 22). PCSK9 could therefore interfere with this clearance process by negatively regulating these receptors. In mice, mAbs against PCSK9 were able to reduce cerebral Aß burden, an effect that did not occur in mice lacking LRP1 (23). Another mechanism likely linking PCSK9 and AD is the induction of a pro-apoptotic effect in neuronal cells through the degradation of ApoER2 (24).

It is noteworthy that monoclonal antibodies, due to their size, cannot cross the intact blood-brain barrier under physiological conditions. However, under certain pathological conditions (such as ischaemic stroke) the permeability of the blood-brain barrier may be impaired. Short-term clinical trials with mAbs targeting PCSK9 found no association between very low LDL-C levels and cognitive impairment (13, 25-29). The EBBINGHAUS trial was specifically designed to investigate cognitive function in patients enrolled in FOURIER who received either evolocumab or placebo in addition to statins (25). No significant differences in cognitive function were observed between the two groups over a median of 19 months (25). In the open-label extension study (FOURIER-OLE), patients were followed up for a median of 5 years: there was no clear monotonic trend between a lower achieved LDL-C level and the risk of neurocognitive events (30). Genetic studies support the lack of association between PCSK9 inhibition and impaired cognitive function. PCSK9 LOF variants, which determine lifelong exposure to low LDL-C levels, were not found to be associated with neurocognitive abnormalities in blacks

participating in the REGARDS study (31). Mendelian randomisation analyses using data from a combined sample of ~740,000 participants showed no significant effects on cognition associated with genetic inhibition of PCSK9 (32), which was confirmed by another Mendelian randomisation analysis (33). In contrast, genetic HMGCR inhibition was associated with reduced cortical surface area, worsened reaction time, and impaired cognitive performance (32), which is consistent with the results of some studies on statins (34, 35). However, some studies have suggested a possible association between PCSK9 inhibition and cognitive impairment. The results of a Mendelian randomisation study have raised the possibility that exposure to PCSK9 inhibitors may predispose individuals to AD (36). A recent analysis of a large pharmacovigilance database found a disproportionality signal related to PCSK9 inhibitors (either as a class or as a single drug) and mental impairment (including memory impairment and amnesia) (37). However, it must be emphasised that this study was conducted with a database of spontaneous reports. Adequate long-term clinical trials could definitely shed more light on this topic.

PCSK9 and the pancreas

Cholesterol homeostasis appears to be essential for pancreatic ß-cell function (38). These cells express both LDLR and PCSK9, which may thus modulate LDLR expression and influence cell function. It is noteworthy that patients with familial hypercholesterolaemia who carry genetic defects leading to reduced LDLR expression/function are less likely to develop diabetes. On the other hand, statins have been shown to increase the risk of new-onset diabetes, especially in pre-diabetics or patients with established risk factors for diabetes (39), although the clinical benefit outweighs the risk. Based on its mechanism of action, PCSK9 inhibition has therefore been suspected of promoting the onset of diabetes, like statins.

PCSK9 loss-of-function variants have shown differential effects on glucose homeostasis, likely related to the genetic background of individuals and the type of the effect on PCSK9 (40). A Mendelian randomisation study showed that PCSK9 variants associated with lower LDL-C levels were also associated with higher fasting plasma glucose levels and increased risk of new-onset type 2 diabetes (41). This confirms a previous observation suggesting that exposure to LDL-C-lowering genetic variants is associated with a higher risk of type 2 diabetes (42). Variants in the *PCSK9* gene and variants in the *HMGCR* gene had approximately the same effect on diabetes risk per unit lower LDL-C level (43). However, pharmacological inhibition of PCSK9 does not appear to increase the risk of new-onset diabetes. A prespecified analysis of the FOURIER trial showed that evolocumab was effective in diabetic and non-diabetic patients and did not increase the risk of new-onset diabetes or worsen blood glucose levels during a median follow-up of 2.2 years (44). The FOURIER-OLE study showed that long-term LDL-C lowering with evolocumab was safe and well tolerated, and resulted in a further reduction in cardiovascular events compared with delayed treatment initiation (45). Interestingly, the rate of new-onset diabetes was not higher in patients who achieved very low LDL-C levels (<20 mg/dL) (30). A meta-analysis of 39 randomised clinical trials involving 66,748 patients treated with alirocumab or evolocumab showed that the use of these PCSK9 inhibitors was not associated with an increased risk of new-onset diabetes (27).

To better explore the potential impact of inhibiting PCSK9 on the development of diabetes, some studies have investigated the role of circulating versus locally produced PCSK9 in animal models. PCSK9 deficiency in mice has been shown to be associated with impaired glucose tolerance due to abnormalities in pancreatic islets, likely due to cholesterol overload of ß-cells and decreased pancreatic insulin secretion (46). However, this effect seems to be independent of circulating (liver-derived) PCSK9 but rather related to locally produced PCSK9 (46): liver-selective PCSK9 knockout mice, mimicking the condition of patients treated with a PCSK9 inhibitor, retain extrahepatic production of PCSK9 (in contrast to the condition of a loss-of-function mutation in the *PCSK9* gene, which affects all sites of production), with normal insulin production, LDLR expression and cholesterol levels in pancreatic islets (46). The ß-cell-specific knockout of PCSK9 resulted in unchanged circulating LDL-C levels with concomitant down-regulation of cholesterogenic genes, which should prevent cholesterol load and toxicity in ß-cells as well as alteration of glucose homeostasis (47). Of note, in this study PCSK9 was selectively inactivated only in mature ß-cells, resulting in residual PCSK9 expression in pancreatic islets (~30%) (47). Silencing PCSK9 expression in endocrine pancreas precursors and mature ß-cells and δ-cells resulted in a 90% reduction in PCSK9 expression in the pancreas (48). Circulating PCSK9 levels remained unchanged, but glucose intolerance was observed in mice due to defective insulin secretion (48). Increased LDLR expression and the resulting cholesterol accumulation were identified as the cause of the observed effect (48). Based on these observations, therapeutic inhibition of PCSK9 should not impair ß-cell function. Indeed, mAbs targeting PCSK9 act against circulating protein derived from the liver. Inclisiran, the siRNA approach that specifically targets PCSK9 mRNA in the liver (thanks to its structure that ensures specific recognition at the hepatic level), should not have diabetogenic properties.

PCSK9 and the heart

Adult differentiated cardiomyocytes constitutively express and release PCSK9 (49). PCSK9 expression is upregulated by inflammation and hypoxia (characteristic of an ischaemic heart) in cardiomyocytes (50, 51). Oxidised LDL, a marker of oxidative stress associated with reduced cardiac function, also increase the expression and release of PCSK9 (49), which appears to affect cardiomyocyte function in an autocrine manner, leading to reduced contraction and relaxation velocity, with the LOX-1 receptor being the most likely candidate to trigger the action of OxLDL (52). During ischaemia/reperfusion (such as acute myocardial infarction, MI), PCSK9 is also upregulated in immune cells, such as neutrophils, monocytes, and macrophages, which are immediately recruited to the ischaemic tissue. PCSK9 promotes the release of pro-inflammatory cytokines from macrophages, leading to a further reduction in cardiomyocyte viability and induction of cardiac cell apoptosis (51).

PCSK9 has been shown to be upregulated in the ischaemic heart of mice, with the zone adjacent to the infarcted areas showing the highest PCSK9 expression and intense autophagy activity, a self-degradation process activated during stress to promote cell survival and cardiac homeostasis (50). PCSK9 released by these cells contributes in determining the infarct size and cardiac function (50): mice lacking the PCSK9 gene or treated with a PCSK9 inhibitor showed better heart function and smaller infarct size (50). PCSK9 expression and release, as well as autophagy, were highest one week after the ischaemic event and then declined (50, 53). Analysis of heart sections from patients who had died of acute myocardial infarction showed PCSK9 and markers of autophagy being strongly expressed in the border zone between ischaemic and normal areas (50). It is likely that the release of PCSK9 early after the onset of myocardial ischaemia may be considered a protective response by stimulating autophagy, at least in the short term. It remains to be elucidated whether sustained PCSK9 release and autophagic response during hypoxia may have deleteri-

ous effects by inducing cell self-digestion and cell death. It is noteworthy that high expression of PCSK9 after myocardial infarction promotes pro-inflammatory M1 macrophage polarisation associated with poor myocardial repair, whereas PCSK9 deficiency promotes anti-inflammatory M2 macrophage polarisation and better protection against myocardial injury (54). Inhibition of PCSK9 has been shown to ameliorate myocardial injury after ischaemia/reperfusion by inhibiting autophagy and inflammation (55).

During acute cardiac ischaemia/reperfusion, high levels of circulating PCSK9 may trigger inflammatory and oxidative processes in ventricular cardiomyocytes, leading to cardiac dysfunction. Therefore, inhibition of PCSK9 could have a cardioprotective effect against ischaemia/reperfusion injury. Indeed, administration of a PCSK9 inhibitor prior to ischaemia resulted in a cardioprotective effect by inhibiting apoptosis, improving cardiac mitochondrial function, reducing infarct size and improving left ventricular function in rats (53, 56). When the PCSK9 inhibitor was administered during ischaemia or reperfusion, no benefits were observed (56). These results are not related to the lipid-lowering effect of the PCSK9 inhibitor, but rather to attenuated cardiac mitochondrial dysfunction and mitochondrial fission, and reduced apoptosis in the ischaemic myocardium (56). Another relevant observation is that PCSK9 regulates pyroptosis in cardiomyocytes during chronic myocardial ischaemia. Pyroptosis is a form of inflammatory programmed cell death that is closely associated with activation of the NLRP3 inflammasome (57). Both PCSK9 and pyroptosis-related proteins have been found to be highly expressed in the zone adjacent to the infarct (58). In *vitro*, PCSK9 activated the NLRP3 inflammasome and further enhanced pyroptosis in cardiomyocytes (58). Consistent with the results in mice, serum levels of PCSK9 and proteins related to pyroptosis were higher in patients with chronic myocardial ischaemia than in healthy subjects (58). Analysis of heart sections from patients who had died of acute MI showed that all these proteins were present in high concentrations in the zone adjacent to the infarcted area (58).

PCSK9 deficiency is associated with increased LDLR and CD36 expression in the heart, leading to lipid accumulation, and altered mitochondrial metabolism (59). These effects manifest as impaired cardiac function and heart failure with preserved ejection fraction (59). However, circulating PCSK9 does not affect cardiac metabolism: mice selectively lacking PCSK9 in the liver exhibit normal cardiac function (59). On the other hand, cardiomyocyte-specific deficiency of PCSK9 resulted in reduced contractile capacity, impaired cardiac function and left ventricular dilatation (60). Interestingly, individuals carrying a loss-of-function mutation in the *PCSK9* gene have increased epicardial fat accumulation and increased left ventricular mass index without alterations in the ejection fraction (59, 61). However, this finding could not be replicated in another study (62). Overall, these observations suggest that therapies targeting PCSK9 should not have negative effects on cardiac metabolism.

Accordingly, clinical trials have shown that therapy with the PCSK9 inhibitors evolocumab and alirocumab can significantly reduce the risk of myocardial infarction. The ODYSSEY OUTCOMES trial showed that alirocumab added to statin therapy reduced the overall incidence of MI, particularly the risk of type 1 (atherothrombotic, the most common form) and type 2 (myocardial oxygen supply-demand mismatch) MI (by 13% and 23%, respectively), but not type 4 (associated with percutaneous coronary intervention) MI (63). The benefit of alirocumab in reducing these types of MI was more pronounced when the increase in biomarkers (as a measure of infarct size), exceeded three times the upper normal limit (63). As an explanation for the effect on type 2 MI, the authors suggested that alirocumab may have improved myocardial oxygen supply (63). On the other hand, a prespecified analysis of the FOURIER trial showed that evolocumab significantly reduced the risk of the first MI by 27%; more specifically, type 1 MI was reduced by 32% and type 4 MI by 35%, while no effect was observed for type 2 MI (64). Evolocumab significantly reduced the risk of non-STEMI and STEMI (by 23% and 36%, respectively (64). A meta-analysis of data from 3 clinical trials of inclisiran failed to demonstrate difference in the risk of MI between patients randomised to inclisiran or placebo (65); the ongoing outcomes trial ORION-4 will shed light on this point.

A post-hoc analysis of the ODYSSEY OUTCOMES trial showed that, among post-ACS patients, alirocumab reduced the risk of MACE in patients without a history of heart failure, but not in patients with a history of heart failure, despite comparable reductions in LDL-C levels (66). In addition, there was a significant increase in non-fatal MI (66), and no effect of alirocumab on hospitalisations for HF, either overall or in the two subgroups (66). This finding is consistent with previous observations that statins do not reduce cardiovascular events in HF patients (67). This suggests the hypothesis that the clinical course of advanced heart failure does not appear to be influenced by anti-atherosclerotic therapies, as deterioration of myocardial function drives disease progression rather than atherosclerotic cardiovascular events (67). The limitations of this subgroup analysis do not allow any conclusions to be drawn, only the need to investigate the clinical efficacy of PCSK9 inhibitors in specific trials. The ongoing EVO-HF pilot trial is investigating whether evolocumab is effective in stable HF patients with reduced ejection fraction of ischaemic origin (NCT03791593).

PCSK9 and the kidney

Lipid and lipoprotein abnormalities are common features in patients with chronic kidney disease (CKD), in whom ASCVD is an important cause of mortality and morbidity. Each type of CKD has a typical phenotype, but overall, at least in the early stages, there are increased triglycerides, decreased high-density lipoproteins, and an excess of small, dense low-density lipoprotein particles. In the kidney, PCSK9 is involved in the regulation of the epithelial Na^+ channel (ENaC) by reducing its expression on the cell surface; this regulation appears to be independent of PCSK9 protease activity (68).

Podocytes (also known as visceral epithelial cells) are highly specialised cells lining the outer surface of the glomerular capillary. Dysfunction of these cells, as seen in patients with nephrotic syndrome, is associated with hypercholesterolaemia, mainly due to increased production and decreased clearance of apoB-containing lipoproteins. Studies in patients with kidney disease have shown that circulating PCSK9 levels are significantly increased compared to healthy subjects, but decrease during remission of the disease (69-72). This finding has also been confirmed in animal models of nephrotic syndrome (71). Injection of a nephrotoxic serum into C57BL/6J mice resulted in an increase in plasma PCSK9 and hypercholesterolaemia associated with a decrease in LDLR (71); this increase in PCSK9 is due to increased expression, increased secretion and decreased clearance (71). Interestingly, PCSK9 clearance was only reduced twofold after podocyte injury, and PCSK9 mRNA was generally not increased, suggesting a post-transcriptional mechanism by which damaged podocytes trigger a signal to the liver that leads to increased PCSK9 secretion (71). On the other hand, mice lacking PCSK9 show a reduced response to the treatment with nephrotoxic serum, with hypercholesterolaemia induced to a lesser extent, suggesting that multiple mechanisms are likely involved in the dyslipidaemia associated with nephrotic syndrome (71).

It is noteworthy that CKD patients receiving haemodialysis have

lower blood LDL-C and PCSK9 levels than healthy people, but those receiving statin therapy have comparable PCSK9 levels to healthy people (73).

Statins are widely prescribed to treat hypercholesterolaemia in patients with CKD. However, it must be emphasised that the clearance of most statins is affected by renal function, leading to excess of drug-drug interactions. In addition, statin therapy is effective in patients with mild-to-moderate CKD, while patients with advanced CKD benefit less (74-76). Evolocumab and alirocumab have been tested for efficacy in CKD patients and provided consistent results in both patients with preserved and impaired renal function (77). An analysis of the efficacy of evolocumab according to the renal function in the FOURIER trial showed that the reduction in LDL-C levels and relative risk reduction were similar for both primary and secondary endpoints in all stages of CKD (including patients with preserved function, stage 2 CKD and ≥stage 3 CKD) (78). The absolute risk reduction for the composite of cardiovascular death, MI, or stroke with evolocumab was numerically greater in patients with more advanced CKD (78). In an analysis of data from ODYSSEY phase 3 trials, alirocumab was shown to lower LDL-C levels independent of the presence or absence of impaired renal function (79). A pre-specified analysis of the ODYSSEY OUTCOMES trial of alirocumab found that alirocumab was effective in reducing LDL-C levels, major cardiovascular events and death across the range of renal function evaluated in patients with recent ACS and dyslipidaemia despite intensive statin therapy (80). However, it should be emphasised that eGFR <30 mL/min/1.73 m^2 was an exclusion criterion in this trial, and eGFR <20 mL/min/1.73 m^2 was an exclusion criterion in FOURIER. Based on this last observation, it is clear that further specifically designed trials are needed to assess whether therapy with mAbs against PCSK9 can have a negative impact on kidney disease and whether it is as effective in patients with advanced kidney disease.

PCSK9 and the intestine

The gut is involved in maintaining cholesterol homeostasis in the body through balanced metabolic cross-talk with the liver (81). PCSK9 is expressed in the small intestine of mice and humans (82). In mice, PCSK9 is expressed throughout the digestive tract and in the colon at levels similar to the liver. In the human intestine, PCSK9 is localised in the cytoplasm and accumulates in the subapical and basolateral compartments of the enterocytes. The cellular distribution of PCSK9 appears to be heterogeneous depending on the intestinal tract: in the duodenum, PCSK9 is expressed at both the apical and basolateral poles, whereas in the ileum it is mainly expressed at the apical pole. This heterogeneity is probably related to the function of PCSK9: in the upper part of the small intestine, PCSK9 is expressed at both poles of the enterocyte according to the absorption process and lipoprotein secretion, whereas in the ileum, which secretes less lipoproteins, PCSK9 is expressed only at the apical side. Furthermore, PCSK9 is not secreted from mature enterocytes in vitro and does not contribute to circulating PCSK9 levels (83), although this remains to be confirmed in vivo. PCSK9 appears to play a critical role in postprandial lipaemia. Indeed, PCSK9-deficient mice are protected from postprandial lipaemia (82). However, in mice, circulating PCSK9 rather than intestinal PCSK9 regulates postprandial lipaemia: both PCSK9-deficient and wild-type mice treated with alirocumab showed reduced postprandial lipaemia (via an LDLR-dependent pathway), an effect not observed in mice specifically lacking intestinal PCSK9 (84). Accordingly, subjects carrying PCSK9 loss-of-function variants had a more favourable lipid profile on fasting and attenuated levels of postprandial TG, apoB48, and total apoB (85), suggesting a role for PCSK9 in regulating TG-rich lipoproteins (TRLs) metabolism. In addition, treatment with evolocumab significantly reduced the postprandial lipaemic response to a mixed high-fat meal in type 2 diabetics, although the production and release of chylomicrons from intestine were not affected (86). Similarly, alirocumab reduced fasting plasma levels of TG and apoB48 and postprandial plasma response of TG and apoB48 in patients with type 2 diabetes on intensive insulin treatment (87). Of note, two studies found no effect of PCSK9 inhibition on the postprandial response in healthy normolipidaemic subjects (88, 89), suggesting that inhibition of PCSK9 may be of particular importance in diabetics who have increased production and impaired clearance of TRLs.

Treatment of human enterocytes with recombinant human PCSK9 markedly increased intestinal production and secretion of apoB and apoB48 by 50%; this effect was due to both an increase in apoB mRNA and an enhanced post-transcriptional apoB protein stability (90). Considering that there is one apoB molecule per TRL particle, this increase in TRL-apoB by PCSK9 suggests a potential doubling of the amount of pro-atherogenic TRL intestinal remnant particles in the circulation after a meal. LDLR expression in enterocytes was reduced by 50%, with concomitant increases in NPC1L1 protein levels, MTP protein levels and lipid transfer activity (90). Of note, treatment of enterocytes with PCSK9 siRNA reversed all observed effects (90). Accordingly, delivery of wild-type PCSK9 or a gain-of-function mutant to epithelial cells in the basolateral medium reduced LDLR at the basolateral membrane and caused marked perturbations in cholesterol homeostasis, including increased cholesterol uptake from the apical membrane via upregulation of NPC1L1, CD36 and ACAT2 and downregulation of HMG-CoAR activity (91).

In vivo, PCSK9 expression significantly reduced LDLR levels in the small intestine, but not in the large intestine in transgenic mice expressing human PCSK9 in multiple tissues (90); MTP expression and activity were increased in the small intestine (where chylomicrons are assembled), but were not detected in the large intestine (90). Similarly, plasma PCSK9 levels correlated positively with the pool size and production rate of intestinal TG-rich lipoproteins (containing apoB48), but not the fractional catabolic rate in men with varying degrees of insulin resistance (92). In addition, intestinal expression of the *PCSK9* gene was positively associated with genes involved in de novo cholesterol synthesis (*HMGCR* and *ACAT2*) and lipoprotein uptake (*LDLR*) (92).

Altogether, these observations suggest that pharmacological inhibition of PCSK9 may have a beneficial effect on postprandial lipaemia, an effect that may be particularly relevant in diabetic patients who have excessive postprandial lipaemia.

PCSK9 and the adipose tissue

Adipose tissue plays a central role in energy balance and storage, but is also involved in the metabolism of TG-rich lipoproteins (93). Adipose tissue also contains a very large pool of free cholesterol and promotes the transfer of cholesterol to HDL (94). Previous studies have shown that circulating PCSK9 regulates VLDLR expression and TG accumulation in visceral adipose tissue: $PCSK9^{-/-}$ mice exhibited significant visceral adipose tissue accumulation compared to wild-type mice, which was associated with adipocyte hypertrophy and increased fatty acid uptake as well as greater cell surface expression of VLDLR, with a mechanism being independent of LDLR (61, 95). Similar effects were observed following specific inactivation of hepatic PCSK9 in wild-type animals (95). In addition, carriers of the PCSK9 R46L loss-of-function variant had higher body mass index and increased percentage of total and android fat mass compared to

non-carriers (61). Circulating PCSK9 is also involved in the degradation of CD36, an important receptor participating in the metabolism of fatty acids and triglycerides in the liver and visceral adipose tissue: PCSK9$^{-/-}$ mice showed high expression of CD36 in adipose tissue, whereas adipocytes treated with PCSK9 showed a strong reduction in cell surface expression of CD36 (96).

PCSK9 is also abundantly expressed in the visceral adipose tissue (97). Of note, statins upregulate PCSK9 expression (98), an effect that has also been demonstrated in adipose tissue (99). PCSK9 expression in adipose tissue is positively correlated with body mass index in humans, suggesting that obesity and adiposity promote PCSK9 expression (97). Insulin and LDL upregulated the expression of PCSK9, LDLR, SREBP-1c and SREBP2 in human adipocytes, and atrial natriuretic peptide partially reversed these effects; this latter observation should be of interest in patients with obesity and hypertension (97). An analysis in overweight/obese individuals with normal LDL-C levels showed that individuals with lower than median plasma levels of PCSK9 had higher expression of LDLR in their white adipose tissue, accompanied by increased expression of CD36, IL-1ß secretion, postprandial hypertriglyceridaemia, lower white adipose tissue function and a lower disposition index, indicating a predisposition to type 2 diabetes (100). Increased LDL uptake is thought to impair adipocyte differentiation and consequently lead to white adipose tissue dysfunction (101), with a concomitant upregulation of MCP-1 expression that promotes the cross-talk between adipocytes and macrophages (100).

Among the various body fat depots, visceral epicardial adipose tissue (EAT) is a proxy of total visceral adiposity and a reliable marker of cardiovascular risk (102, 103). Under physiological conditions, it provides mechanical protection and also functions as an energy supplier, thermoregulator, and endocrine organ. However, under pathological conditions, EAT dysfunction can be detrimental and promote CVD progression (103). EAT is a source of PCSK9; local PCSK9 levels (but not plasma PCSK9 levels) correlated with EAT thickness and local inflammation (104); conversely, PCSK9 R46L carriers have higher EAT thickness compared to non-carriers (61). Further studies are needed to better define the role of PCSK9 in this specific tissue and the potential consequences of PCSK9 inhibition.

Conclusion

Since its discovery, PCSK9 has been shown to be a major determinant of circulating LDL-C levels through its main function in the liver, but also plays a key role in other tissues and organs. The rapid development and approval of PCSK9 inhibitors for the treatment of hypercholesterolaemia has paved the way for many unanswered questions related to the potential adverse effects of inhibiting PCSK9 in tissues and organs other than the liver. Many studies have attempted to answer these questions and provide evidence that inhibition of PCSK9 has not adverse effects, but many questions remain unresolved. Although PCSK9 inhibitors have been shown to be beneficial (the results for inclisiran are awaited), particularly in the context of cardiovascular health and metabolic disorders, the use of PCSK9 inhibitors could have effects on several other organs and tissues, particularly in the context of neurocognitive disorders, ß-cell function and diabetes, cardiac metabolism, heart failure and chronic kidney disease. To date, there is no evidence that PCSK9 inhibitors have negative effects on neurocognitive disorders or ß-cell function. In addition to the proven benefit for ASCVD, PCSK9 inhibition does not appear to negatively affect cardiac metabolism or have a negative impact on the clinical course of advanced heart failure. Monoclonal antibodies targeting circulating PCSK9 have been shown to be effective in reducing the risk of ASCVD in both patients with preserved and impaired renal function, although there are no specifically designed trials to assess their efficacy in patients with advanced kidney disease.

Results from experimental models are critical to understanding the underlying molecular mechanisms, but have limited translatability to humans. Similarly, observations in individuals carrying genetic variants of PCSK9 do not always translate to clinical trials of pharmacological inhibition of PCSK9, although they are critical in defining the precise role of this protein. Notwithstanding the role that PCSK9 inhibitors play in controlling hypercholesterolaemia, specific studies are needed to understand the long-term effects of PCSK9 inhibition.

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Competing interests

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