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Metabolic impact of extrahepatic PCSK9 modulation

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ABSTRACT

The Proprotein Convertase Subtilisin Kexin type 9 (PCSK9) protease is a 692 amino acid glycoprotein which belongs to the proprotein convertase family. PCSK9 binds several receptors of the LDL family, including VLDLR and LRP1 but also CD36, driving their lysosomal degradation. Since the beginning of the 21st century a growing body of interest raised around the opportunity to pharmacologically inhibit PCSK9, and most recently, monoclonal antibodies have been successfully tested for the treatment of severe/genetic forms of dyslipidemia. Despite the majority of circulating PCSK9 being produced by the liver, other organs come into play contributing to its production, such as the heart, the pancreas, and the brain. Nonetheless, extrahepatic PCSK9 may exert a local/paracrine and/or autocrine metabolic impact in the homeostatic regulation of cholesterol metabolism, suggesting that, opposite to the liver, in other tissue PCSK9 deficiency or inhibition could contribute to the development of specific organ and tissue dysfunctions.

Keywords
PCSK9;
Pancreas;
Heart;
Liver and Brain

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Introduction

The most updated statistics of the American Heart Association (AHA) (1) and European Society of Cardiology (ESC) (2) still account for cardiovascular diseases (CVDs) as the principal cause of death in the United States and around 50% of all the deaths in the European countries in 2019/2020. CVDs arise from a plethora of risk factors, such as smoking, diabetes, and obesity but among them, hypercholesterolemia remains the principal cause of atherosclerotic cardiovascular events (3). Of note, hypercholesterolemia can be genetically determined or a consequence of unhealthy lifestyle and dietary habits. Among the genetic forms, familial hypercholesterolemia (FH), which affects 1:1.000.000 subjects in homozygosis and 1:200/250 in heterozygosis, is the most severe form of hypercholesterolemia associated with a high risk of CVD. FH diagnosis is made by the identification of mutations on *LDLR*, *APOB* or *PCSK9* genes that lead to increased circulating LDL cholesterol (LDL-C) levels from 200 mg/dL to 400 mg/dL. FH subjects present high levels of cholesterol from childhood thus magnifying their CVD risk; therefore, the currently available pharmacological treatments play a crucial role in the long-time reduction of cholesterol levels from a young age to prevent CVD events later in adulthood.

Since the eighties, statins have been considered the gold standard pharmacological treatment for all forms of hypercholesterolemia. Indeed, in large-scale clinical trials, statins, in combination with proper dietary habits and lifestyle corrections, have been shown to reduce cardiovascular (CV) morbidity and promote the regression of atherosclerotic plaque (4). However, for the most aggressive forms of hypercholesterolemia, the development of novel pharmacological treatments in combination with the highest tolerated dose of statins has been crucial as statins are ineffective for some categories of patients.

Among them, the proprotein convertase subtilisin/kexin type 9 (PCSK9) emerged as an interesting potential target, monoclonal antibodies against PCSK9 have rapidly risen in the clinical practice for treating subjects with hypercholesterolemia who do not reach the LDL-C levels recommended by the guidelines (5). PCSK9 became of interest from a cardiovascular point of view for the first time in 2003 (6) when two gain of function (GOF) mutations in the *PCSK9* gene were discovered in French families with autosomal dominant hypercholesterolemia. Later on, in 2005, loss of function (LOF) polymorphisms, responsible for two nonsense mutations (Y142X and C679X), were also identified. From a clinical point of view, PCSK9 LOF is associated with reduced LDL-C and protection from coronary artery disease (7), while PCSK9 GOF leads to the classical familial hypercholesterolemia phenotype (6). These discoveries speeded up the study of PCSK9 inhibition as a potential pharmacological strategy to reduce lipid burden and its possible side effects. Moreover, despite the liver being the most relevant production site of PCSK9, this protein is also produced at considerable levels by the brain, pancreas, heart, kid-

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neys and also immune cells, and all these extrahepatic districts are therefore involved in the cardiovascular system regulation. Hence, a deep understanding of the extrahepatic role of PCSK9 would shed the light on the potential effects of its modulation in other tissue than the liver, unmasking side effects or innovative pharmacological routes (8).

Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9)

PCSK9 biology and regulation

PCSK9 is a serine protease with a molecular weight of 62 kDa. PCSK9 was discovered in 2003 as neural apoptosis-regulated convertase 1 (NARC-1) in the brain. PCSK9 belongs to a group of serine proteases that catalyse the hydrolysis of peptide bonds mediating the activation of target proteins. PCSK9 is initially synthesized as a 75 kDa proPCSK9 in the rough endoplasmic reticulum (RER) and is composed of a signal peptide (aa 1-30), a prodomain (aa 31-152), a catalytic domain (aa 153-404), a hinge region (aa 405-454), and the C-terminal domain (aa 452-692) rich in cysteine and histidine (8). The proPCSK9 is then driven to the smooth endoplasmic reticulum where it undergoes an autocatalytic cleavage that facilitates its folding and secretion. Despite this cut, the signal peptide remains attached with hydrogen bound to the catalytic domain of PCSK9 also favouring the proper protein folding (6). This newly formed interaction protects intracellular PCSK9 from protease degradation and allows its delivery/transport to the Golgi apparatus via coat protein complex (COPII) vesicle pathways. The C-terminal domain is crucial during this phase being the binding site between PCSK9 and COPII vesicles, indeed deletion on this part of the protein is associated with reduced PCSK9 release (9). Intracellularly PCSK9 is mainly inactive and needs to be released in the extracellular space to exert its proper function; however, more recent works have hypothesized an intracellular activity of PCSK9 in the endoplasmic reticulum (ER). PCSK9 starts its maturation in the ER where it is under the control of ER protein Glucose Regulated protein 94 (GRP94) which is actively involved in the regulation of PCSK9 and low-density lipoprotein receptor (LDLR) expression. GRP94 binding to the newly formed PCSK9 in the reticulum can prevent PCSK9-mediated LDLR degradation leading to increased ER stress (10, 11). Indeed, ER-retained PCSK9 bound to GRP94 is not able to interact with the LDLR before its secretion thus protecting the complex from degradation (11) (**Figure 1**). This has also been associated with major modification in circulating LDL cholesterol levels that in mice lacking GRP94 are significantly higher, as are the circulating levels of PCSK9. This observation, therefore, suggests a key intracellular function for PCSK9 before its secretion. Then, PCSK9 release from the cell requires its prior internalization into vesicles, a common mechanism shared with the secretion of LDLR. While PCSK9 is rapidly released in the extracellular matrix, LDLR remains attached to the cellular membrane (8).

Upon secretion, the catalytic domain of PCSK9 binds to the epidermal growth factor A (EGF-A) domain on the receptors belonging to the LDLR family, localized on the plasmatic membrane. Once bound to the EGF-A domain, the PCSK9-LDLR complex is internalized in clathrin-mediated endocytosis (12). The low pH characteristic of the late endosome strengthens the binding between PCSK9 and EGF-A and this strong interaction prevents the dissociation and the recycling of the receptor to the cell membrane. In this way, PCSK9 enhances LDLR lysosomal degradation within the cell cytoplasm preventing receptor recycling on the cell membrane (13). In turn, this leads to a reduced LDLR expression on the plasma membrane of hepatocytes with an increase in the plasmatic levels of LDL-C (14).

PCSK9 can also target other receptors, including other members of the LDLR family such as the very low-density lipoprotein receptor (VLDLR) and ApoER2 (15) but it can also interact with CD36 with a similar mechanism. Indeed, PCSK9 deletion associates with an increased expression of CD36 in the liver of experimental models leading to the accumulation of fatty acids and triglycerides within lipid droplets (16).

Given its role in cholesterol and fatty acid metabolism, PCSK9 expression is finely regulated both at the transcriptional and post-transcriptional levels by different metabolic actors.

Circulating PCSK9 is almost entirely produced and released by the liver and its production goes through several intracellular modulations in addition to the extracellular furin cleavage. Indeed, its expression is regulated at different levels and by several factors, including diurnal rhythm, hormones, diet, exercise, cholesterol levels, and hypocholesterolemic drugs. Among cholesterol-lowering drugs, statins, by inhibiting HMG-CoA reductase and reducing intracellular cholesterol levels, increase PCSK9 gene expression *in vitro* and *in vivo*. Patients treated with atorvastatin showed a 34% increase in PCSK9 plasmatic levels (17). Statins, by inhibiting HMG-CoA reductase and reducing cholesterol levels, promote the activation of the sterol-regulatory element-binding protein-2 (SREBP2) and SREBP-1c that bind the sterol regulatory elements (SRE) in PCSK9 and LDLR gene promoters and increase their expression (**Figure 1**). PCSK9 promoter contains also other sequences including the highly conserved

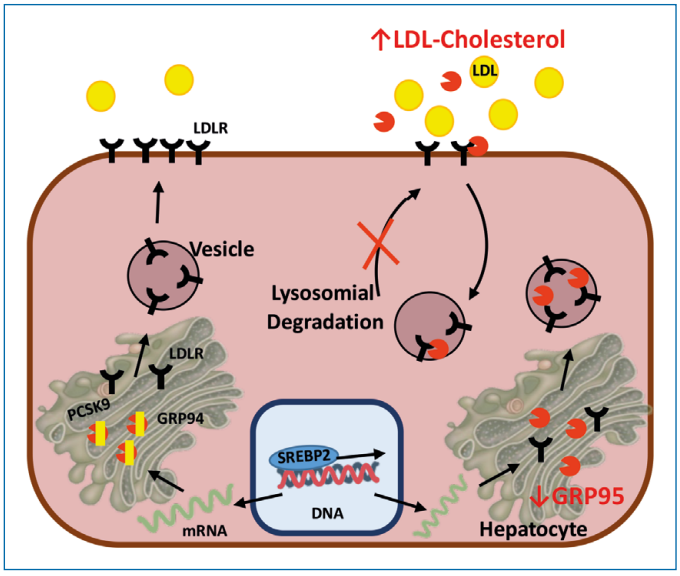

Figure 1 | **Intracellular PCSK9.** The liver is the principal organ involved in PCSK9 production. PCSK9 genetic expression is finely regulated by different transcription factors as SREBP2. PCSK9 is produced as Pre-ProPCSK9 and undergoes different post-transcriptional modification to the mature form. When the mature form reaches the endoplasmic reticulum PCSK9 is packed into vesicle with the LDLR for the release. In the presence of GRP94, PCSK9 is retained into the reticulum leading to an increased recycling of LDLR on the cell membrane. This situation is reverted in the absence of GRP94 and PCSK9 is packed in the same vesicle of the LDLR and can directly mediate vesicle degradation into the lysosome. This is therefore associated with a reduced expression of LDLR on hepatocyte membrane and high cholesterol levels.

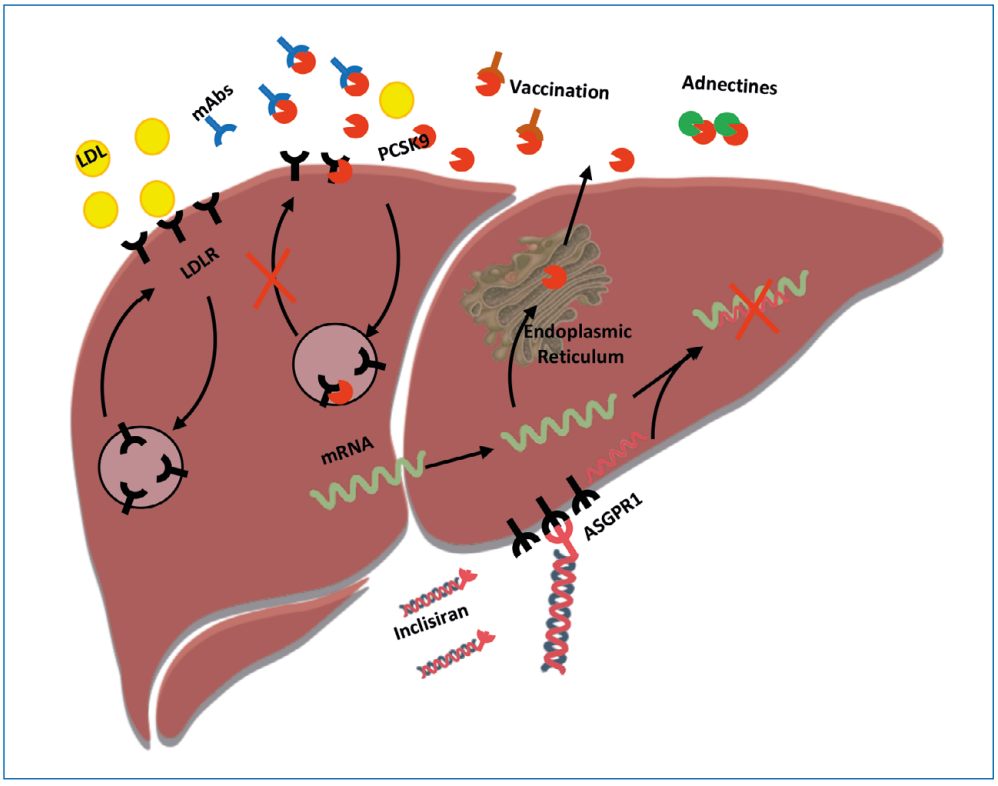

Figure 2 | **Pharmacological approaches for PCSK9 inhibition.** PCSK9 became a pharmacological target in 2015 when monoclonal antibodies have been approved for targeting circulating PCSK9. Circulating PCSK9 can also be targeted by immunization with vaccination that stimulate the production of autoantibodies against PCSK9. Adnectines mimic the 10th domain of fibronectin type III and can bind to circulating PCSK9 avoiding the binding with the LDLR. Other approaches are finalized to inhibit protein transcription of PCSK9 and Inclisiran, a siRNA, modified with three molecules of N-acetylgalactosamine (GalNAc) is selectively driven to the liver by the receptor for asialoglycoproteins (ASGPR).

hepatocyte nuclear factor 1 (HNF-1) and the binding site of transcription factor 1 (SP1) (18). Circulating mature PCSK9 is cleaved by furin producing a truncated protein of about 55 KDa that can still bind the EGF-A domain but is less able to bind the LDLR compared to the full form (19). PCSK9 can also circulate bound to the LDL particles, which contributes to maintaining its uncut form with the highest ability to bind its target (20).

PCSK9 pharmacological treatments

PCSK9 inhibition has been established as the gold treatment for high and very-high CV risk dyslipidemic patients, and many pharmacological approaches are still under development to target different PCSK9 forms.

Monoclonal antibodies (mAbs) targeting PCSK9 (alirocumab and evolocumab) represent the first and most commonly used approach in high-risk patients. Targeting circulating PCSK9 with mAbs was shown to reduce LDL-C, either as monotherapy or in combination with other lipid-lowering drugs, by approximately ~60%. In line with that, cholesterol reduction in subjects already treated with statins showed a reduction of 15% in the incidence of composite cardiovascular death and MI. More recently, a new drug named inclisiran that target PCSK9 hepatic production has been approved for the treatment of high and very high-risk patients. Inclisiran is a small interfering RNA (siRNA), optimized by the conjugation with three molecules of N-acetylgalactosamine (GalNAc) that selectively bind to the receptor for asialoglycoproteins (ASGPR) in the liver (21). Other pharmacological approaches that exploit others pathways include adnectins, synthetic proteins containing the 10th domain of fibronectin type III (22). Adnectins can bind PCSK9 inhibiting the interaction between PCSK9 and the EGF-A domain of LDLR, thus avoiding receptor degradation. Other pharmacological approaches include vaccination with peptide-based immunization that triggers B lymphocytes to produce anti-PCSK9 antibodies, and oral PCSK9 inhibitors (23). The pharmacological targeting of circulating PCSK9 is therefore one of the main therapeutic approaches to strongly reduce cholesterol levels by favouring LDL uptake in the liver and other organs (**Figure 2**). While great efforts have been made to inhibit hepatic PCSK9 production, many studies have been addressed to investigate the possible side effects in extrahepatic tissue metabolism and function.

Extrahepatic effects of PCSK9 inhibition

PCSK9 and the pancreas

Despite the effectiveness in reducing CVD risk, the use of statins has been associated with the development of new-onset diabetes; this has questioned whether other hypocholesterolemic pharmacological strategies, such as PCSK9 inhibitors, would affect pancreatic function and glucose metabolism modulation. This evidence rises the need to test the impact of PCSK9 inhibition on pancreatic function, focusing on endocrine pancreatic β-cells, those involved in insulin production. To deeply investigate the interconnection between PCSK9 inhibition and the risk of developing diabetes, clinical trials with monoclonal antibodies, meta-analysis and mendelian randomization studies have been performed. Despite clinical trials did not show any correlation, meta-analysis and mendelian randomization studies showed a positive correlation between genetic polymorphisms in genes involved in cholesterol metabolism, such as HMG-CoA reductase (confirming the involvement of statins in this pathway), NPC1L1, and PCSK9 with the risk of developing type 2 diabetes (24). For this reason, many studies have focused their attention on the metabolic impact of PCSK9, cholesterol, and lipids selectively in the pancreas. Indeed, lipid metabolism is crucial at the pancreatic level as lipotoxicity has been deeply connected with pancreatic ß-cell dys-

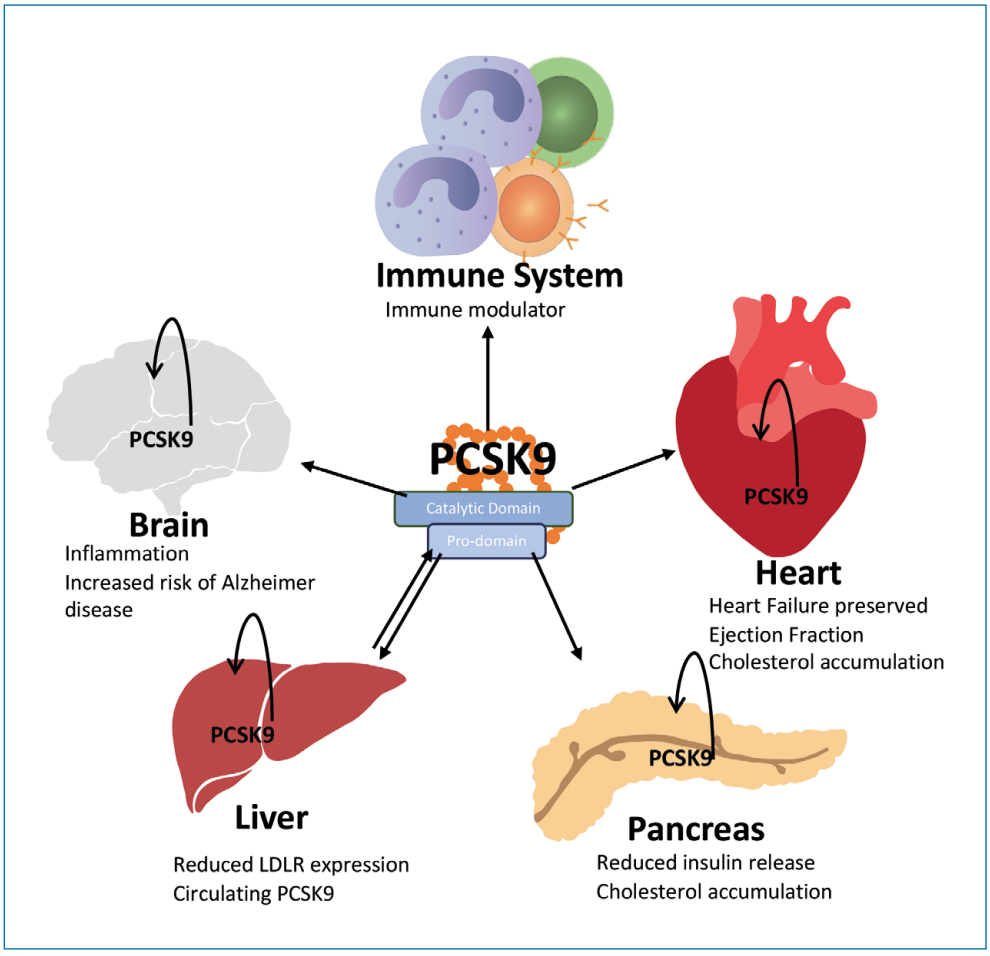

Figure 3 | **Extrahepatic role of PCSK9.** Circulating PCSK9 is mainly produced in the liver where it can modulate LDLR expression. PCSK9 is also produced by many other tissues such as the heart, brain, pancreas and macrophages, where can regulate lipid metabolism and inflammatory response. PCSK9 deficiency could therefore lead to cellular cholesterol accumulation in the pancreas, increasing the risk of diabetes, and in the heart leading to cardio-lypotoxicity and heart failure with preserved ejection fraction. In the brain, PCSK9 deficiency, by affecting apoptotic pathways, plays a role in the modulation of inflammation and the development of Alzheimer disease. PCSK9 has also direct and indirect immunomodulatory functions while PCSK9 inhibition can improve anti-cancer therapy.

function (25). Among lipids, cholesterol accumulation in different cell compartments may affect cellular flexibility leading to the impaired release of insulin granules by β-cells (26). In fact, alterations of the cellular tridimensional structure of β-cells, due to cholesterol accumulation, affect the regulation of membrane calcium channel and the transduction of vesicles containing insulin, with a consequent reduction in insulin release. To note, in transgenic animal models PCSK9 locally produced in the pancreas causes an increased expression of LDLR in pancreatic β-cells leading to cholesterol accumulation and reduced insulin trafficking and release (27). However, other studies have limited the impact of selective β-cell PCSK9 deficiency on glucose intolerance and diabetes development suggesting the possible role of PCSK9 released by other pancreatic islet subpopulations (**Figure 3**).

Cardiac impact of PCSK9 modulation

The heart is the tissue that requires the highest energy to perform continuous contraction. To overcome this need, aerobic metabolism plays a crucial role in the production of adenosine triphosphate (ATP). A healthy heart relies on glycolysis for almost 30% while the remaining part, 70%, is up to fatty acid oxidation (28). Unlike the liver, the heart is less prepared to synthesize fatty acids starting from glucose and amino acids, so the energetic requirement is mainly fed by lipids picked up from the bloodstream. In the heart lipid receptors including VLDLR, LRP1 and LDLR, are the main receptors involved in the uptake of triglyceride (TG)-rich lipoproteins that have been previously metabolized by the lipoprotein lipase (LPL). Scavenger receptors such as CD36 are also involved in the uptake of non-esterified fatty acids, mainly carried by albumin or released by lipoproteins following LPL activity (29, 30). Cardiac lipid metabolism is finely balanced between lipid uptake and mitochondrial oxidation to prevent excess lipid accumulation in the cardiomyocyte. The accumulation of lipids in the heart, and the consequent lipotoxicity, is associated with the development of cardiac dysfunction and heart failure in humans and experimental models. Therefore, these lipotoxic effects contribute to the development of cardiovascular metabolic complications, such as diabetes mellitus and metabolic syndrome. Heart failure (HF) in particular, is a pathological condition in which the heart is no longer able to pump enough blood to support the body's demands (31). More specifically, HF with preserved ejection fraction (HFpEF) is characterized by metabolic changes due to dysfunctional mitochondria that are unable to perform oxidative phosphorylation and generate enough ATP (32). Mitochondrial dysfunction is crucial for different diseases besides HFpEF including atrial fibrillation (33) and diabetic cardiomyopathy (34). In this context, through its ability to modulate LDLR and CD36 expression on the plasma membrane and therefore cellular lipid uptake, PCSK9 plays a key role in the modulation of cardiac function. It has been observed that the lack of PCSK9 in the heart is associated with an increased accumulation of cholesterol in the form of lipid drops and the consequent mitochondrial damage leads to energy depletion in the heart. As a consequence of these alterations, experi-

mental models lacking PCSK9 develop heart failure with preserved ejection fraction (35) (**Figure 3**).

PCSK9 and the central nervous system

PCSK9 was discovered in the brain as neural apoptosis-regulated convertase-1 (NARC-1) as its mRNA expression is mainly localized in the telencephalon neurons and is lower in a steady state but its expression is up-regulated during apoptosis (6). While the PCSK9-LDLR pathway is crucial all over the body, in the brain PCSK9 modulation mainly involves the ApoER2 receptor that promotes neural apoptosis through the increase in caspase activity (36). Indeed, cholesterol metabolism is complex in the brain and while all around the body cholesterol is mainly transported in LDL, in the brain it is transported in apoE-rich particles. PCSK9 has also been shown to promote apoptotic events through the JNK pathway and the activation of both extrinsic and intrinsic apoptotic pathways (37). Despite this activity, no signs of neurological effects have been reported in carriers of PCSK9 polymorphisms, while circulating PCSK9 – as well as LDL particles – cannot pass the intact blood-brain barrier in physiological conditions, limiting the impact of lipid-lowering therapies as well as pharmacological inhibition of PCSK9. PCSK9 in the brain is expressed mainly by proliferative cells in the adult brain and during the neurodevelopment of the telencephalon and cerebellum (38). As main the player in regulating lipid metabolism and apoptotic pathways, the role of PCSK9 has been investigated in different neuropathologies including Alzheimer's disease but the results are unclear (39). Mouse models of the disease show that PCSK9 may regulate Aβ clearance by controlling the expression of LRP1 and CD36. Concordantly, Pcsk9$^{-/-}$ mice show an upregulation of β-secretase-1 (BACE1) production, suggesting an increased production of β-amyloid (40). In line with this observation in the cerebrospinal fluid of human patients with Alzheimer's disease, higher PCSK9 levels have been reported compared to healthy subjects (39) (**Figure 3**).

Role of PCSK9 in inflammation and immunity

The inflammatory nature of atherosclerotic cardiovascular diseases and the recognition that immune responses are influenced by the modulation of systemic and cellular lipid metabolism has brought attention to the contribution of PCSK9 to the immune-inflammatory processes. Whether this effect is mediated directly by PCSK9 or through its modulation of systemic dyslipidemia is still questioned. Evidence coming from subjects affected by immune-inflammatory diseases suggests a link between PCSK9 and inflammation; indeed, patients with systemic lupus erythematosus (SLE) show an increase in circulating levels of PCSK9 that positively correlates with C-reactive protein (CRP) levels – a highly sensitive but unspecific marker of inflammation (41), while subjects infected with HIV present a positive correlation between the increased levels of PCSK9 and markers of monocyte activation (42). In line with this, also in patients with stable coronary artery disease, the elevation in plasmatic PCSK9 levels was positively correlated with those of CRP (43) and associated with disease severity (44). While the impairment of lipid metabolism, reported also under inflammatory conditions, might guide these effects, inhibition of circulating PCSK9 by a monoclonal antibody – despite dramatically reducing systemic cholesterol levels – had limited impact on systemic markers of inflammation (45), in contrast to statins that, in parallel to LDL-C, reduce plasmatic CRP levels (46); this evidence might suggest that different routes of pharmacological targeting of cholesterol metabolism would differently affect immune-inflammatory response.

Despite this clinical evidence, molecular studies have reported a direct pro-inflammatory effect of PCSK9 on cells typically associated with the atherosclerotic process, such as smooth muscle cells and macrophages. Vascular smooth muscle cells express PCSK9 that regulates LDLR expression in macrophages (47), an effect associated *in vitro* with the release of pro-inflammatory cytokines (48). Furthermore, PCSK9 expressed by bone-marrow-derived macrophages has been shown to accentuate vascular inflammation (49) independently on the modulation of cholesterol levels but instead involving the activation of the toll-like receptor 4 (TLR4)/NF-kB signalling pathway (50).

It is well documented that lipids, and in particular cholesterol, shape adaptive immune responses. Indeed, the polarization of CD4+ T-lymphocytes toward an activated phenotype (T-effector memory cells, TEM) is directly correlated to systemic cholesterol levels and the severity of coronary artery diseases in humans and has been confirmed in mouse models of atherosclerosis (51). To note, dyslipidemia triggers T cell proliferation and expansion of less functional immunosuppressive T regulatory cells (52) also by affecting the reactivity of antigen-presenting cells to prime lymphocytes (53-55). In line with this, immune system humanized mice, where hypercholesterolemia has been induced by PCSK9 overexpression in the liver, show a similar pro-inflammatory phenotype of T-cells (56). Altogether this evidence suggests that the increased levels of systemic cholesterol are more likely to mediate the polarization of adaptive immune response in the context of cardiovascular disease.

Different is the role played by cellular lipid metabolism on the activation of cell-mediated immune-inflammatory response, a field of investigation known as immunometabolism (57) that is particularly relevant in the context of cancer immunotherapy. It has been recently shown that PCSK9 inhibition potentiates checkpoint therapy for cancer by blocking the recycling of major histocompatibility protein class I (MHC I) proteins on the tumour cell surface, thus increasing its expression and promoting robust intratumoral infiltration of cytotoxic T-cells (58). Furthermore, PCSK9 inhibition in tumour cells directly enhances the activation of CD8+ T-cells by increasing the expression of LDLR that in this context favours the recycling of the T-cell receptor and its intracellular signalling. These findings broaden the role of PCSK9-mediated cholesterol metabolism in the modulation of T-cell response by increasing the expression of the LDLR; to note, LDLR has been recently involved in the immunometabolic response of CD8+ but not CD4+ T-cells (unpublished data), suggesting that the axis PCSK9 inhibition-LDLR expression could be directly targeted to selective T-cell subsets.

Conclusions

The gradual increase in the use of PCSK9 inhibitors in clinical practice raises interest in the possible side effects of circulating protein inhibition. Recent approaches of PCSK9 gene silencing partially exclude the possibility of side effects due to the hepatic selective targeting through ASGR1-mediated uptake. However, concerns are still made about extrahepatic modulation of lipid metabolism. Indeed, PCSK9 is also expressed in other tissues and the characterization of subjects with loss-of-function mutations of PCSK9 has allowed studying the effects of whole protein deficiency. For example, while the risk of developing type 2 diabetes has been reported in subjects with PCSK9, this has not been confirmed in patients treated with inhibitors of circulating PCSK9. This apparent discrepancy was explained by *in vivo* and *in vitro* studies that addressed the role of locally produced PCSK9 in different tissues including the pancreas, heart, and brain. Being PCSK9 crucial for apoptotic pathways modulation its role has been investigated in the field of immunity and cancer showing the possibility of PCSK9-mediated immune modulation. Even

though many works have been focused on investigating the extrahepatic impact of PCSK9 inhibition information is still incomplete and many questions are left unanswered.

Conflict of interest

All authors have no conflict of interest to disclose

Authors' contributions

All authors have made equal intellectual contributions to the writing of this manuscript. All authors read and approved the final manuscript.

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