PCSK9 and atherosclerosis: looking beyond LDL regulation

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ABSTRACT

Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) is involved in cholesterol homeostasis. After binding to the complex low-density lipoprotein (LDL)-LDL receptor, PCSK9 induces its intracellular degradation, thus reducing serum LDL clearance. In addition to the well-known activity on the hepatic LDL receptor-mediated pathway, PCSK9 has been, however, associated with vascular inflammation in atherogenesis. Indeed, PCSK9 is expressed by various cell types that are involved in atherosclerosis (e.g., endothelial cells, smooth muscle cells, and macrophages) and is detected inside human atherosclerotic plaques. We here analyze the biology of PCSK9 and its possible involvement in molecular processes involved in atherosclerosis, beyond the regulation of circulating LDL cholesterol levels.

Key words: Proprotein Convertase Subtilisin/Kexin type 9; atherosclerosis; low-density lipoproteins; inflammation.

Content

Introduction

Atherosclerosis may be defined as an inflammatory pathological process of large arteries leading to coronary artery disease (CAD), ischemic stroke, and other cardiovascular diseases [1-3]. Development and progression of the atherosclerotic plaque involve the interplay between circulating levels of low-density lipoproteins (LDL), rich in cholesterol (LDL-C), and the activation of various cellular processes including, among others, endothelial cell (EC) dysfunction/activation and apoptosis, smooth muscle cell (SMC) proliferation and migration into the intima, T-cell and macrophage activation and foam cell formation (Figure 1) [2;4-6].

The role of Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) in this context has rapidly evolved from its discovery as a genetic determinant of some forms of familial hypercholesterolemia to its use as the target of new lipid lowering drugs now tested or being tested in clinical trials [3;7-8]. PCSK9 is an enzyme able to regulate serum LDL-C levels through the degradation of the LDL receptor (LDLR) [3]. Blocking PCSK9 with specific inhibitors thus causes a significant decrease of LDL-C blood levels both in healthy volunteers and in individuals with hypercholesterolemia, with and without statin cotreatment [8-12].

Although the majority of studies related to PCSK9 have focused on its expression in the liver and its local function on the LDLR, several investigators have shown that PCSK9 is also expressed in other cells and tissues, including all cellular components of the vessel wall [13-16]. In particular, the expression of PCSK9 is present in the two constituent cellular types regulating vascular function, i.e., ECs and SMCs, as well as in inflammatory cells, such as macrophages. This raises the question of whether PCSK9 may have pro-atherogenic effects beyond its well-known activity on the hepatic LDLR. Thus, a deep understanding of mechanisms regulated by PCSK9 in cellular elements different from hepatocytes and potentially directly contributing to atherosclerosis, could be relevant to understand the effects of drugs targeting PCSK9 in slowing down the progression of vascular disease and reducing vascular outcomes.

To preliminary screen the role of PCSK9 in cardiovascular disease, we have here analyzed literature data on this topic enquiring PubMed with the following terms: “cardiovascular disease” AND “atherosclerosis” AND “PCSK9”. The resulted 352 papers were analyzed by the bibliometric mapping tool VOSviewer [17]. Bioinformatic analysis of medical subject headings (MeSH) associated to retrieved papers, returned a total number of 592 MeSH keywords, of which 68 met the threshold levels (minimum number of occurrence of a keyword = 10). In terms of “occurrence”, the MeSH term “PCSK9” resulted as the second cited key word, only preceded by the term “humans”. Next, we built up a term map based on the strength of the co-occurrence links with other keywords. The keywords with greatest total link strength were selected and highlighted as bubbles (Figure 2). As expected, among the retrieved MeSH keywords significantly connected to “PCSK9”, those linked with CAD and lipid metabolism resulted with a strong association. Other keywords linked with CAD beyond LDL-C, such as “inflammation” and “atherosclerotic plaques”, were significantly connected to “PCSK9”, and

precisely these were chosen as the search objects of the present review. Thus, this review outlines the synthesis and biology of PCSK9 and its effects on atherosclerosis, and summarizes the experimental and clinical data supporting a role for PCSK9 in atherosclerosis beyond the regulation of circulating LDL levels. In particular, at the experimental level, we focused on the action of PCSK9 in the different cell types potentially involved in the formation and progression of atherosclerotic plaques. Moreover, al the clinical level, data on PCSK9 circulating levels in subjects at risk of or with atherosclerotic cardiovascular disease (ASCVD) were also reported.

PCSK9: gene expression and protein maturation

PCSK9, also called neural-apoptosis-regulated convertase 1 (NARC-1), belongs to the family of secretory serine proteinases known as proprotein convertases (PCs) [18-19]. Although PCSK9 is principally synthesized and secreted by the liver, its expression is also found in the kidney, lung, intestine, central nervous system, inflammatory and blood vessel cells, such as ECs and SMCs [13-18].

The PCSK9 gene, mapped on the short arm of chromosome 1 (chr1p32.3), has a length of 22 kb, and includes 12 exons and 11 introns [20]. The PCSK9 gene expression is regulated by different transcription factors, among which sterol-response element binding proteins 1 and 2 (SREBP1 and SREBP2) have been well studied [21]. In particular, studies *in vitro* and *in vivo* have described that high levels of sterols increase PCSK9 gene expression in the post-prandial period by SREBP2 [22]. Similarly, the increased levels of circulating insulin after feeding induce PCSK9 mRNA synthesis through SREBP1 activation [23]. However, clinical evidences suggest that the PCSK9 gene expression mediated by insulin is irrelevant in healthy subject [24]. In addition to SREBPs, peroxisome proliferator-activated receptor α and γ (PPARα and PPARγ) transcription factors regulate PCSK9 gene expression. PPARγ increases the gene expression of proprotein convertase in the liver [28]. While PPARα reduces its expression [20]. Finally, also sirtuin 1 and 6 (SIRT1 and SIRT6) can reduce the expression of PCSK9 by silencing its gene [25].

The PCSK9 gene encodes for a 692 amino acid protein with a molecular weight of 72 kDa (pro-PCSK9) [13-14]. Pro-PCSK9 is synthesized in the endoplasmic reticulum (ER), similar to its main target, the LDLR [14]. Pro-PCSK9 includes five segments: a signal peptide (aa 1-30); an N-terminal prodomain (aa 31-152) that is cleaved in the ER; a catalytic domain (aa 153-421) that contains the active sites Asp_{186}, His_{226}, and Ser_{386}; a small region of 18 amino acids that links the catalytic domain to the C-terminal cys-his-rich domain (CHRD) (aa 440-692) [13,19,26]. Post-translational modifications are necessary for pro-PCSK9 maturation. In particular, in the ER the N-terminal prodomain of pro-PCSK9 is autocatalytically cleaved at the Val-Phe-Aln-Gln_{152}Ser-Ile-Pro ($VFAQ_{152}SIP$) site, but remains non-covalently associated with the rest of the mature protein, assisting the folding of PCSK9, and blocking its catalytic domain [21,27-28].

After the synthesis and cleavage, mature PCSK9 is transferred from the ER to the Golgi apparatus through interaction with the transport protein SEC24 homolog A, COPII coat complex component (Sec24A) [14,29]. Similarly, the bond between PCSK9 and sortilin 1 (SORT1) in the *trans*-Golgi is required for the secretion of inactive protease trough the blood [23,30].

PCSK9 and the LDL receptor

Several studies have found that the principal role of mature PCSK9 is to reduce levels of LDLR expressed in the liver or in peripheral tissues, thus indirectly preventing hepatocyte and tissue LDL uptake [31]. PCSK9 contributes to LDLR degradation by two different pathways: an intracellular and an extracellular regulation. Early on, it was suggested that, after protein synthesis, mature PCSK9 mediates the transport of immature LDLR from the ER to Golgi membranes [32]. In the Golgi apparatus, LDLR is glycosylated and converted into the mature form. At this point, the catalytic domain of mature PCSK9 can bind the mature LDLR and induce its degradation by clathrin light chain/lysosome activation [33]. Indeed, the clathrin *light* chain is responsible for the intracellular trafficking from the trans-Golgi network to the lysosomes [34]. The fraction of LDLR that manages to achieve the cell surface is then able to bind LDL present in the blood. At this point, the complex LDL/LDLR is internalized in the cells via clathrin *heavy* chain-coated vesicles. The change in pH within the endosomal particle leads to the dissociation of LDLR from LDL that will be degraded in the lysosome, while the LDLR will be recycles in the cell surface [14].

The second pathway that leads to LDLR degradation depends on circulating PCSK9. The catalytic domain of circulating PCSK9 can bind the epidermal growth factor A (EGF-A) domain expressed in LDLR, inducing its degradation by clathrin *heavy* chain-mediated endocytosis [35]. The acidic pH of vesicles increases the interaction between PCSK9 and LDLR [36]. Therefore, in endosomes/lysosomes, PCSK9 is still bound to its ligand, and LDLR cannot assume the right conformation required to its recycling on the cellular surface, and is thus degraded in the lysosome [37].

In addition to LDLR degradation and the resulting increase in circulating levels of LDL-C, PCSK9 may affect lipid metabolism by interacting with other receptors. Circulating PCSK9 can modify triglyceride metabolism in the heart, skeletal muscle, and adipose tissue by degradation of the very low-density lipoprotein receptor (VLDLR) [38] and the apolipoprotein E receptor 2 (ApoER2 or LRP8) [33]. Instead, in the liver and in the adipose tissue, PCSK9 can interfere with triglyceride metabolism by interacting with scavenger receptors, such as cluster of differentiation 36 (CD36) [39-40].

PCSK9 is also associated with degradation of the lipoprotein receptor related protein 1 (LRP-1). This receptor expresses the same EGF-A domain of LDLR [41-42]. It has been suggested that, in the atherosclerotic plaque, the degradation of LPR1 results in both increased tissue factor expression by ECs [41] and a pro-inflammatory response by macrophages [42].

Alternative roles of PCSK9 in the various cell types involved in atherosclerosis

Several years ago, it was demonstrated that expression levels of PCSK9 were higher in various cellular components of the atherosclerotic plaque (SMCs and monocyte/macrophages). These data first suggested that PCSK9 was involved in the pathophysiology of atherosclerosis also through actions independent from LDLR cleavage [15,43]. The alternative roles of PCSK9 in these cellular elements involved in atherosclerosis are described below.

Endothelial cells

Dysregulation in the normal hemodynamic shear stress, high circulating levels of pro-inflammatory mediator (oxLDL, TNFα, IL-1β), excessive production of reactive oxygen species (ROS) and cell apoptosis may all contribute to tamper the integrity to endothelial monolayer and to promote the sequence of events leading to advanced atherosclerosis. In physiological conditions, the arterial wall is continuously subject to hemodynamic shear stress changes, and its cellular components respond with structural and functional adaptive actions [15]. Generally, a high shear stress is considered atheroprotective because it inhibits the adhesion of inflammatory cells to vessel wall, regulates the production of nitric oxide (NO) and the production or activation of prothrombotic factors [44]. Conversely, the low shear stress typically occurring in areas of arterial bending, on the convex site, and at bifurcations, contributes to an increased expression of transcription factors, such as activator protein-1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which both promote the expression of proteins involved in a pro-oxidant and pro-inflammatory state (Figure 3A) [15,45]. Ding et al. found that ECs and SMCs express high levels of PCSK9 under low shear stress conditions and/or in the presence of inflammatory mediators, such as those involved in the lipopolysaccharide(LPS)/toll like receptor 4 (TLR-4) or oxLDL/ lectin-like oxidized LDLR-1 (LOX-1) pathway activation (Figure 3A) [15, 43, 46-47]. Data suggested a strong relation and a bidirectional positive feedback loop among PCSK9 expression, LOX-1 synthesis, ROS production and a pro-inflammatory state. The relationship between LOX-1 and ROS, in particular, has been greatly debated in the literature. Generally, the oxLDL/LOX-1 pathway induces the activation of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidases, providing an intracellular source of ROS. Increased ROS production induces NF-κB translocation in the nucleus, with activation of LOX-1 and pro-inflammatory cytokines, and the gene transcription of adhesion molecules.

Studies *in vivo* and *in vitro* have shown that, in ECs, the use of NADPH oxidase inhibitors or NF-κB knockout with the use of small interfering RNA (siRNA) decrease the production of ROS and LOX-1, but also reduce expression levels of PCSK9 (Figure 3B) [15;43;46], suggesting the involvement of these pathways in the local synthesis of the enzyme. In the same way, these data support the hypothesis of a possible regulatory mechanism by a positive feedback between PCSK9 and the LOX-1/ROS pathway. Indeed, it was found that the rise in PCSK9 levels in the presence of

low shear stress and/or after inflammatory stimuli, such as LPS/TLR4/myeloid differentiation primary response 88 (MyD88), induce ROS production through NADPH oxidase activation and the consequent expression of LOX-1 mediated by NF-κB [15;43;46-47].

A relationship between PCSK9 and apoptosis in ECs has also been reported. As described in Figure 3B, endothelial monolayers pre-treated with a siRNA against PCSK9 decreased the number of apoptotic cells in response to oxLDL [46]. In particular, the authors observed that mRNA and protein levels of pro-apoptotic molecules [Bcl-2-associated X protein (Bax), caspase 3 and 9] were lower in the PCSK9-siRNA/oxLDL co-transfected cells compared with control [46]. Conversely, levels of the anti-apoptotic element Bcl-2 were found to be increased [46]. In agreement with this study, other investigators found that, in ECs treated with oxLDL, PCSK9 can regulate apoptosis through a modulation of the expression levels of Bcl-2/Bax and caspase-3, in addition to the activation of p38/JNK/MAPK pathway [48].

Smooth muscle cells

The migration of SMCs into the intima together with the deposition of extracellular matrix contributes to the accrual of the fibrous cap, separating the lipid core of the plaque from flowing blood, and plays an important role in atherosclerotic plaque progression. SMCs within the arterial intima feature high levels of proliferation and motility, low contractile function, and a high capacity of synthesis and secretion of extracellular matrix components and cytokines compared to SMCs in the media [49]. Various atherogenic stimuli, including shear stress and inflammatory mediators, contribute to SMC differentiation from a “normal” to a “migrant” phenotype, although the trigger mechanisms for this change are still unknown [15, 49-50].

PCSK9 knockdown (PCSK9 $^{-/-}$) mice are partially protected in the differentiation of SMCs from normal to migrant cells and, consequently, toward the formation of an atherosclerotic fibrous cap [49]. Indeed, SMCs isolated from PCSK9 $^{-/-}$ mice express high levels of contractile proteins, such as alpha-actin and myosin heavy chain II, accompanied by a lower degree of proliferative and migrating capacity compared to SMCs obtained from PCSK9 wild type (PCSK9 $^{+/+}$) mice (Figure 4 A-B) [49]. It has been hypothesized that molecular mechanisms involving PCSK9 in SMC proliferation and migration depend on the regulatory effects of PCSK9 on LDLR, LPR-1, VLDLR, and CD36, as well as on the consequent changes in cholesterol membrane homeostasis and composition [39,49,51]. In particular, in PCSK9 $^{+/+}$ mice the reduced amount of cholesterol in the membrane lipid composition raise the activity of the specific small G proteins Rac-1 and Rho-A. These molecules are involved in the recruitment of integrins and in the creation of focal adhesion and lamellipodia, necessary for cell motility [39;49;51]. Conversely, PCSK9 $^{-/-}$ cells showed impaired migratory capacity both under basal conditions and after platelet-derived growth factor-BB (PDGF-BB) stimulation [49].

The role of PCSK9 in SMC proliferation may also consist in the activation of mammalian target of rapamycin (mTOR), a kinase involved in cell growth, survival and proliferation (Figure 4 A-

B) [50]. Studies *in vitro* have found that treatment of SMCs with siRNAs against PCSK9 leads to a lower activation of mTOR. On the contrary, the administration of recombinant PCSK9 induces the genes expression of mTOR and of a component of the mTOR complex (mTORC1), with the consequent boosting of cell growth [50].

Together with EC apoptosis, SMC apoptosis is a hallmark of atherosclerosis progression, and - in particular - is an essential element for plaque rupture [50]. Studies *in vitro* have shown that treatment of SMCs with LPS increases the expression and secretion of PCSK9 in parallel with increased ROS levels, mitochondrial DNA (mtDNA) damage, and activation of the apoptotic mechanisms (Figure 4 B) [51]. Moreover, data obtained under inflammatory conditions suggest that high levels of mtDNA damage are sufficient to induce the expression of PCSK9. Likewise, treatment of SMCs with recombinant PCSK9 enhances mtDNA damage and apoptosis activation (Figure 4 A) [50]. Studies performed in PCSK9 $^{+/+}$ and PCSK9 $^{-/-}$ animal models have confirmed the results *in vitro*: indeed, PCSK9 $^{+/+}$ mice showed a dramatic release of PCSK9 and mtDNA damage after LPS treatment, at variance from what observed in PCSK9 $^{-/-}$ mice [50].

Finally, although the vast majority of studies suggest that gene expression of PCSK9 is detectable in ECs and SMCs obtained from the arterial wall, it was observed that only SMCs are able to secrete a functionally active PCSK9. Indeed, conditioned media obtained from SMCs significantly decreased LDLR levels on macrophage membranes [16], thus contributing to the processes described here below.

Inflammatory cells

The traditional paradigm for atherogenesis has focused on monocyte (Ly6C^{hi}) infiltration in the intima, differentiation in pro-inflammatory macrophages (M1), increased expression of cytokines such as IL-1β, IL-6, TNFα and proteolytic enzymes, macrophage uptake of LDL and modified LDL (oxLDL), with the consequent foam cell formation in arterial walls and progression of disease [52]. As described above, SMCs synthetize and release in the atheroma a functionally active PCSK9, which exerts a paracrine function on M1 present in the intima [16]. Studies *in vitro* have found that incubation of macrophages for 24 h with PSCK9 significantly increases expression levels of pro-inflammatory cytokines compared to control (Figure 5 A) [52]. Moreover, bone marrow macrophages isolated from LDLR $^{-/-}$ mice and treated with PCSK9 (2.5 μg/mL) expressed moderate levels of TNFα mRNA compared to control mice, suggesting that PCSK9 promotes a pro-inflammatory response in macrophages mainly – but not exclusively – dependent on LDLR (Figure 5 B) [52]. In addition, the results of the effect of PCSK9 on ApoER2 are particularly interesting [53]. Within the plaque, the interaction between ApoE and ApoER2 tends to quench atherosclerotic processes by inducing macrophage differentiation from M1 to M2 (anti-inflammatory macrophages), decreasing the production of IL-1β, IL-6 and TNFα, lipid accumulation and apoptosis [53-54]. By interacting with the EGF-A domain of ApoER2, PCSK9 prevents the binding with ApoE and thus its

overall anti-inflammatory effects [56]. Moreover, PCSK9 can drive inflammation responses in macrophages through the TLR4/NF-κB signaling pathway. The overexpression of PCSK9 in TNFα-treated macrophages stimulates the expression of TLR4, a transmembrane receptor that promotes NF-κB nuclear translocation and, by doing so, the expression and release of pro-inflammatory cytokines in the atherosclerotic lesion (Figure 5 A) [55].

In addition to cytokine synthesis, endogenous and exogenous PCSK9 in macrophages can modify the uptake of oxLDL and increase the number of foam cells by regulating scavenger receptors LOX-1, CD36 and SRA [58]. In PCSK9 transgenic macrophages overexpressing PCSK9, the uptake of oxLDL was increased compared to control. In the same cells, the use of siRNA against LOX-1 dramatically decreases oxLDL uptake compared to macrophages treated with siRNAs against CD36 or SRA, suggesting that the endogenous PCSK9 can differentially regulate the activity of the various scavenger receptors [56]. Consistently, in macrophages pretreated with TNFα and then incubated with recombinant PCSK9, the uptake of oxLDL was higher, due to the expression of scavenger receptors in a concentration-depended manner (Figure 5 A) [56].

PCSK9 can modify lipid homeostasis in macrophages also through regulation of the ATP binding cassette transporter A1 (ABCA1) and G1 (ABCG1) [57-58]. The ABC transporters are involved in cholesterol efflux from the cells into the extracellular milieu. Thus, inhibition of this process may favor foam cell formation in the atherosclerotic plaque. Treatment of macrophages with exogenous PCSK9 reduces the efflux of cholesterol by ABCA1 [58]. In particular, data suggest that PCSK9 inhibits ABCA1-dependent cholesterol efflux by affecting its protein expression in an LDLR-dependent fashion (Figure 5 A) [58]. However, in LDLR $^{-/-}$ macrophages PCSK9 is still able to regulate cholesterol efflux preventing the ABCA1 gene expression [58]. This condition could be related to the binding of PCSK9 with other receptors, such as VLDR, CD36 or ApoER2 (Figure 5 B) [58]. However, other studies need to be performed to clarify this regulatory effect of PCSK9.

Circulating PCSK9 and atherosclerotic risk

Besides experimental studies, over the last years, several lines of clinical research have investigated the relationship between circulating levels of PCSK9 and the presence and severity of ASCVD. Overall, these studies did not show clear or concordant results, possibly depending on multiple reasons. Circulating PCSK9 levels not necessarily reflect the complex regulatory mechanisms involving PCSK9 at hepatic and other cellular levels involving or not LDL-R. Moreover, the complexity and multiplicity of factors contributing to atherosclerotic disease severity and progression, the different methods to assess atherosclerosis and vascular districts evaluated, and the variability in clinical study cohorts could influence the relationships between circulating PCSK9 and ASCVD [59-60]. In asymptomatic subjects an association between circulating PCSK9 levels and carotid intima-media wall thickness (CIMT) or coronary artery calcium (CAC) score was found, and confirmed in familial hypercholesterolemia (FH) patients [61-62]. In patients with known stable CAD,

an association between blood PCSK9 concentration and the extent of the necrotic core fraction of atherosclerotic plaques (an index of plaque vulnerability) was documented by intravascular ultrasound (IVUS) virtual histology [63]. In patients with acute coronary syndromes, circulating levels of PCSK9 were associated both with coronary plaque inflammation [56,64] and serum levels of high-sensitivity C-reactive protein (h-CRP) [65].

All these findings are consistent with the concept that PCSK9 is associated with presence and severity of ASCVD but do not clarify to which extent these association is exclusively related to the effects on LDLR and circulating LDL-C levels or also to other mechanisms. Moreover, the clinical phenotypes associated with ASCVD are disparate and include conditions of cardio-metabolic risk that are not directly related with LDL-C levels and possible PCSK9 effects [66]. As a matter of fact, in a large European population of patients with stable angina and intermediate-low risk of CAD, we observed the expected correlation of circulating PCSK9 with LDL-C (60,67) but we could not demonstrate a relationship with the presence and extent of obstructive CAD. Conversely, patients with lower values of PSCK9 demonstrated the clinical features of the metabolic syndrome and a higher coronary atherosclerotic burden as assessed by CTA [60].

PCSK9 and pharmacological approaches

The established evidence that PCSK9 degrades LDLR in the liver resulting in increased levels of LDL-C [3] led to the rapid development of effective anti-PCSK9 treatments (anti-PCSK9 monoclonal antibody). Currently, two human monoclonal antibodies, evolocumab and alirocumab, are approved for use in patients at high-risk of coronary artery disease [8,11-12]. The data collected from FOURIER and ODYSSEY-Outcomes studies have demonstrated that the two anti-PCSK9 drugs reduce major adverse cardiovascular events in patients at high risk [68]. In addition to monoclonal antibodies, specific siRNAs have been proposed as new-generation drugs targeted at this enzyme. The ORION program is currently underway to verify the efficacy of the synthetic siRNA inclisiran. It has been found that inclisiran effectively prevents the translation of mRNA for PCSK9, with consequently decreased concentrations of the protein in the liver and profoundly lower concentrations of LDL-C in the blood [69]. Other promising innovative strategies are under consideration, including antibodies targeting the C-terminal domain of PCSK9, thereby inhibiting the trafficking of PCSK9-LDLR to lysosomes; small molecules that either prevent PCSK9 binding to the LDLR, its trafficking to lysosomes or its secretion from cells; and complete silencing of PCSK9 by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 strategies; and PCSK9 vaccines that inhibit the activity of circulating PCSK9 [70].

At present, PCSK9 inhibitors are included in the guidelines for the management of CCS and dyslipidemias besides the established lipid lowering treatments with the goal of achieving progressively lower levels of LDL-C in patients with higher CV risk [71-72]. Nevertheless, treatment of patients with atherosclerosis is complex due to the multifactorial nature of the disease and this

may explain why adverse events in clinical ASCVD have been reduced by current treatments but not abolished.[2]. Considering in particular the role of inflammation in atherosclerosis, new anti-inflammatory drugs have been developed in an attempt to reduce the residual cardiovascular risk related to disease progression and adverse events (stroke, myocardial infarction and cardiovascular death) associated with the inflammatory process and independent on LDL-C levels [2]. In the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) trial, it was shown that canakinumab, a human monoclonal antibody against IL-1β, reduced cardiovascular events by its effects on circulating levels of inflammatory mediators, independent of LDL-C [73]. According to these results, it has been suggested that the simultaneous use of lipid lowering and anti-inflammatory drugs could provide a favorable synergistic strategy for the management of atherosclerotic patients [69]. In this context, drugs capable of regulating both cholesterol homeostasis and the inflammatory response in atherosclerotic patients could be particularly helpful. Based on the pleiotropic effects demonstrated for PCSK9 on molecular pathways involved in the vascular inflammatory response of atherogenesis [74]. PCSK9 inhibitors may here play a pivotal role (7). PCSK9 inhibitors have the potential to be integrated in a therapeutic approach addressing the “residual risk” of cardiovascular events in ASCV patients despite current lipid lowering therapy [8,11-12,68]. Other cardiometabolic risk conditions, not directly linked to LDL-C levels, are known to contribute to the pathophysiology and the clinical manifestations of ASCVD. Whether these conditions, such as the insuline resistance state, characteristic of the metabolic syndrome, and the so called “diabetic dyslipidemia” [75] may also contribute to the effects of PCSK9 inhibitors on ASCVD outcomes is to be established.

Conclusions

Studies on the role played by PCSK9 in the different cell types involved in the initiation and progression of atherosclerotic plaque, namely ECs, SMCs and macrophages, have shown that the action of PCSK9 on atherosclerosis goes beyond the degradation of the LDLR receptor, and occurs by interfering with other molecular mechanisms ranging from EC function and apoptosis to SMC migration and the activation of inflammatory pathways. Moreover, clinical studied in patients with ASCVD confirmed that the relationships among circulating PCSK9 levels, coronary disease and risk is complex and might be partially independent of LDL cholesterol. Therefore, therapies based on PCSK9 inhibition might lead to a variety of additional favorable effects beyond lowering LDL cholesterol levels. These are not yet fully investigated.

Thus, on the basis of both experimental and clinical evidence, further research is needed to unfold PCSK9 actions in atherosclerosis by unveiling its underlined molecular mechanisms. This may allow the development and use of highly effective drugs targeting this molecule. The challenge here ahead is to prove their efficacy in several contexts even once sufficient LDL reduction has been achieved.

REFERENCES

1. Ross R. Atherosclerosis-an inflammatory disease. N Engl J Med. 1999 Jan 14;340(2):115-26.
2. Libby P, Buring JE, Badimon L, Hansson GK, Deanfield J, Bittencourt MS, Tokgözoğlu L, Lewis EF. Atherosclerosis. Nat Rev Dis Primers. 2019 Aug 16;5(1):56.
3. Yurtseven E, Ural D, Baysal K, Tokgözoğlu L. An Update on the Role of PCSK9 in Atherosclerosis. J Atheroscler Thromb. 2020 Jul 23.
4. Gu HF, Tang CK, Yang YZ. Psychological stress, immune response, and atherosclerosis. Atherosclerosis. 2012 Jul;223(1):69-77.
5. Stary H. Natural history and histological classification of atherosclerotic lesions: An update. Arterioscler Thromb Vasc Biol 2000;20:1177–8.
6. Momtazi-Borojeni AA, Sabouri-Rad S, Gotto AM, Pirro M, Banach M, Awan Z, Barreto GE, Sahebkar A. PCSK9 and inflammation: a review of experimental and clinical evidence. Eur Heart J Cardiovasc Pharmacother. 2019 Oct 1;5(4):237-245.
7. Abifadel M, Varret M, Rabès JP, Allard D, Ouguerram K, Devillers M, Cruaud C, Benjannet S, Wickham L, Erlich D, Derré A, Villéger L, Farnier M, Beucler I, Bruckert E, Chambaz J, Chanu B, Lecerf JM, Luc G, Moulin P, Weissenbach J, Prat A, Krempf M, Junien C, Seidah NG, Boileau C. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet. 2003 Jun;34(2):154-6.
8. Landmesser U, Chapman MJ, Farnier M, Gencer B, Gielen S, Hovingh GK, Lüscher TF, Sinning D, Tokgözoglu L, Wiklund O, Zamorano JL, Pinto FJ, Catapano AL; European Society of Cardiology (ESC); European Atherosclerosis Society (EAS). European Society of Cardiology/European Atherosclerosis Society Task Force consensus statement on proprotein convertase subtilisin/kexin type 9 inhibitors: practical guidance for use in patients at very high cardiovascular risk. Eur Heart J. 2017 Aug 1;38(29):2245-2255.
9. Reyes-Soffer G, Pavlyha M, Ngai C, Thomas T, Holleran S, Ramakrishnan R, Karmally W, Nandakumar R, Fontanez N, Obunike J, Marcovina SM, Lichtenstein AH, Matthan NR, Matta J, Maroccia M, Becue F, Poitiers F, Swanson B, Cowan L, Sasiela WJ, Surks HK, Ginsberg HN. Effects of PCSK9 Inhibition With Alirocumab on Lipoprotein Metabolism in Healthy Humans. .Circulation. 2017 Jan 24;135(4):352-362.
10. Sabatine MS, Giugliano RP, Keech AC, Honarpour N, Wiviott SD, Murphy SA, Kuder JF, Wang H, Liu T, Wasserman SM, Sever PS, Pedersen TR; FOURIER Steering Committee and Investigators. Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease. .N Engl J Med. 2017 May 4;376(18):1713-1722.
11. Sever P, Gouni-Berthold I, Keech A, Giugliano R, Pedersen TR, Im K, Wang H, Knusel B, Sabatine MS, O'Donoghue ML. LDL-cholesterol lowering with evolocumab, and outcomes

according to age and sex in patients in the FOURIER Trial. Eur J Prev Cardiol. 2020 Feb 4:2047487320902750.

12. Diaz R, Li QH, Bhatt DL, et al. Intensity of statin treatment after acute coronary syndrome, residual risk, and its modification by alirocumab: insights from the ODYSSEY OUTCOMES trial [published online ahead of print, 2020 Jul 27]. Eur J Prev Cardiol. 2020;2047487320941987.
13. Seidah NG. The proprotein convertases, 20 years later. Methods Mol Biol. 2011;768:23-57
14. Schulz R, Schlüter KD, Laufs U.Molecular and cellular function of the proprotein convertase subtilisin/kexin type 9 (PCSK9). Basic Res Cardiol. 2015 Mar;110(2):4.
15. Ding Z, Liu S, Wang X, Deng X, Fan Y, Sun C, Wang Y, Mehta JL. Hemodynamic shear stress via ROS modulates PCSK9 expression in human vascular endothelial and smooth muscle cells and along the mouse aorta. Antioxid Redox Signal. 2015 Mar 20;22(9):760-71.
16. Ferri N, Tibolla G, Pirillo A, Cipollone F, Mezzetti A, Pacia S, Corsini A, Catapano AL. Proprotein convertase subtilisin kexin type 9 (PCSK9) secreted by cultured smooth muscle cells reduces macrophages LDLR levels. Atherosclerosis. 2012 Feb;220(2):381-6.
17. van Eck NJ, Waltman L. Software survey: VOSviewer, a computer program for bibliometric mapping. Scientometrics. 2010;84(2):523-538.
18. Norata GD, Tibolla G, Catapano AL. Targeting PCSK9 for hypercholesterolemia. Annu Rev Pharmacol Toxicol. 2014;54:273-93.
19. Benjannet S, Rhainds D, Essalmani R, Mayne J, Wickham L, Jin W, Asselin MC, Hamelin J, Varret M, Allard D, Trillard M, Abifadel M, Tebon A, Attie AD, Rader DJ, Boileau C, Brissette L, Chrétien M, Prat A, Seidah NG. NARC-1/PCSK9 and its natural mutants: zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol. J Biol Chem. 2004 Nov 19;279(47):48865-75.
20. Seidah NG, Prat A. The proprotein convertases are potential targets in the treatment of dyslipidemia.J Mol Med (Berl). 2007; 85:685–696.
21. Seidah NG, Awan Z, Chrétien M, Mbikay M. PCSK9: a key modulator of cardiovascular health. Circ Res. 2014 Mar 14;114(6):1022-36.
22. Wu M, Dong B, Cao A, Li H, Liu J. Delineation of molecular pathways that regulate hepatic PCSK9 and LDL receptor expression during fasting in normolipidemic hamsters. Atherosclerosis. 2012 Oct;224(2):401-10.
23. Costet P, Cariou B, Lambert G, Lalanne F, Lardeux B, Jarnoux AL, Grefhorst A, Staels B, Krempf M. Hepatic PCSK9 expression is regulated by nutritional status via insulin and sterol regulatory element-binding protein 1c. J Biol Chem. 2006 Mar 10;281(10):6211-8.
24. Brouwers MC, van Greevenbroek MM, Troutt JS, Bonner Freeman A, Lu A, Schaper NC, Konrad RJ, Stehouwer CD. Plasma proprotein convertase subtilisin kexin type 9 is a heritable trait of familial combined hyperlipidaemia. Clin Sci (Lond). 2011 Nov;121(9):397-403.

25. Duan Y, Chen Y, Hu W, Li X, Yang X, Zhou X, Yin Z, Kong D, Yao Z, Hajjar DP, Liu L, Liu Q, Han J. Peroxisome Proliferator-activated receptor γ activation by ligands and dephosphorylation induces proprotein convertase subtilisin kexin type 9 and low density lipoprotein receptor expression. J Biol Chem. 2012 Jul 6;287(28):23667-77.
26. Tao R, Xiong X, DePinho RA, Deng CX, Dong XC. FoxO3 transcription factor and Sirt6 deacetylase regulate low density lipoprotein (LDL)-cholesterol homeostasis via control of the proprotein convertase subtilisin/kexin type 9 (Pcsk9) gene expression. J Biol Chem. 2013 Oct 11;288(41):29252-9.
27. Denis M, Marcinkiewicz J, Zaid A, Gauthier D, Poirier S, Lazure C, Seidah NG, Prat A. Gene inactivation of proprotein convertase subtilisin/kexin type 9 reduces atherosclerosis in mice. Circulation. 2012 Feb 21;125(7):894-901.
28. Benjannet S, Hamelin J, Chrétien M, Seidah NG. Loss- and gain-of-function PCSK9 variants: cleavage specificity, dominant negative effects, and low density lipoprotein receptor (LDLR) degradation. J Biol Chem. 2012 Sep 28;287(40):33745-55.
29. Chen XW, Wang H, Bajaj K, Zhang P, Meng ZX, Ma D, Bai Y, Liu HH, Adams E, Baines A, Yu G, Sartor MA, Zhang B, Yi Z, Lin J, Young SG, Schekman R, Ginsburg D. SEC24A deficiency lowers plasma cholesterol through reduced PCSK9 secretion. Elife. 2013 Apr 9;2:e00444.
30. Gustafsen C, Kjolby M, Nyegaard M, Mattheisen M, Lundhede J, Buttenschøn H, Mors O, Bentzon JF, Madsen P, Nykjaer A, Glerup S. The hypercholesterolemia-risk gene SORT1 facilitates PCSK9 secretion. Cell Metab. 2014 Feb 4;19(2):310-8.
31. Liu X, Suo R, Chan CZY, Liu T, Tse G, Li G. The immune functions of PCSK9: Local and systemic perspectives. J Cell Physiol. 2019 Nov;234(11):19180-19188.
32. Strøm TB, Tveten K, Leren TP. PCSK9 acts as a chaperone for the LDL receptor in the endoplasmic reticulum. Biochem J. 2014 Jan 1;457(1):99-105.
33. Poirier S, Mayer G, Poupon V, McPherson PS, Desjardins R, Ly K, Asselin MC, Day R, Duclos FJ, Witmer M, Parker R, Prat A, Seidah NG. Dissection of the endogenous cellular pathways of PCSK9-induced low density lipoprotein receptor degradation: evidence for an intracellular route. J Biol Chem. 2009 Oct 16;284(42):28856-64.
34. Poupon V, Girard M, Legendre-Guillemin V, Thomas S, Bourbonniere L, Philie J, Bright NA, McPherson PS. Clathrin light chains function in mannose phosphate receptor trafficking via regulation of actin assembly. Proc Natl Acad Sci U S A. 2008 Jan 8;105(1):168-73.
35. Park SW, Moon YA, Horton JD. Post-transcriptional regulation of low density lipoprotein receptor protein by proprotein convertase subtilisin/kexin type 9a in mouse liver. J Biol Chem. 2004 Nov 26;279(48):50630-8.
36. Du F, Hui Y, Zhang M, Linton MF, Fazio S, Fan D. Novel domain interaction regulates secretion of proprotein convertase subtilisin/kexin type 9 (PCSK9) protein. J Biol Chem. 2011 Dec 16;286(50):43054-61.

37. Leren TP. Mutations in the PCSK9 gene in Norwegian subjects with autosomal dominant hypercholesterolemia. Clin Genet. 2004 May;65(5):419-22.
38. Poirier S, Mayer G, Benjannet S, Bergeron E, Marcinkiewicz J, Nassoury N, Mayer H, Nimpf J, Prat A, Seidah NG.The proprotein convertase PCSK9 induces the degradation of low density lipoprotein receptor (LDLR) and its closest family members VLDLR and ApoER2. J Biol Chem. 2008 Jan 25;283(4):2363-72.
39. Canuel M, Sun X, Asselin MC, Paramithiotis E, Prat A, Seidah NG. Proprotein convertase subtilisin/kexin type 9 (PCSK9) can mediate degradation of the low density lipoprotein receptor-related protein 1 (LRP-1). PLoS One. 2013 May 13;8(5):e64145.
40. Levy E, Ben Djoudi Ouadda A, Spahis S, Sane AT, Garofalo C, Grenier É, Emonnot L, Yara S, Couture P, Beaulieu JF, Ménard D, Seidah NG, Elchebly M. PCSK9 plays a significant role in cholesterol homeostasis and lipid transport in intestinal epithelial cells. Atherosclerosis. 2013 Apr;227(2):297-306.
41. Wang M, Li YF, Guo YG, Chen MM, Jiang ZL, Song JY. Positive correlation between plasma PCSK9 and tissue factors levels in patients with angiographically diagnosed coronary artery disease and diabetes mellitus. J Geriatr Cardiol. 2016 May;13(4):312-5.
42. Overton CD, Yancey PG, Major AS, Linton MF, Fazio S. Deletion of macrophage LDL receptor-related protein increases atherogenesis in the mouse. Circ Res. 2007 Mar 16;100(5):670-7.
43. Ding Z, Liu S, Wang X, Deng X, Fan Y, Shahanawaz J, Shmookler Reis RJ, Varughese KI, Sawamura T, Mehta JL. Cross-talk between LOX-1 and PCSK9 in vascular tissues. Cardiovasc Res. 2015 Sep 1;107(4):556-67.
44. Berk BC, Min W, Yan C, Surapisitchat J, Liu Y, Hoefen R. Atheroprotective Mechanisms Activated by Fluid Shear Stress in Endothelial Cells. Drug News Perspect. 2002 Apr;15(3):133-139.
45. Mohan S, Koyoma K, Thangasamy A, Nakano H, Glickman RD, Mohan N. Low shear stress preferentially enhances IKK activity through selective sources of ROS for persistent activation of NF-kappaB in endothelial cells. Am J Physiol Cell Physiol. 2007 Jan;292(1):C362-71.
46. Wu CY, Tang ZH, Jiang L, Li XF, Jiang ZS, Liu LS. PCSK9 siRNA inhibits HUVEC apoptosis induced by ox-LDL via Bcl/Bax-caspase9-caspase3 pathway. Mol Cell Biochem. 2012 Jan;359(1-2):347-58.
47. Liu S, Deng X, Zhang P, Wang X, Fan Y, Zhou S, Mu S, Mehta JL, Ding Z. Blood flow patterns regulate PCSK9 secretion via MyD88 mediated proinflammatory cytokines. Cardiovasc Res. 2019 Oct 8. pii: cvz262.
48. Li J, Liang X, Wang Y, Xu Z, Li G. Investigation of highly expressed PCSK9 in atherosclerotic plaques and ox-LDL-induced endothelial cell apoptosis. Mol Med Rep. 2017 Aug;16(2):1817-1825.

49. Ferri N, Marchianò S, Tibolla G, Baetta R, Dhyani A, Ruscica M, Uboldi P, Catapano AL, Corsini A. PCSK9 knock-out mice are protected from neointimal formation in response to perivascular carotid collar placement. Atherosclerosis. 2016 Oct;253:214-224.
50. Ding Z, Liu S, Wang X, Mathur P, Dai Y, Theus S, Deng X, Fan Y, Mehta JL. Cross-Talk Between PCSK9 and Damaged mtDNA in Vascular Smooth Muscle Cells: Role in Apoptosis. Antioxid Redox Signal. 2016 Dec 20;25(18):997-1008.
51. Navarro-Lérida I, Sánchez-Perales S, Calvo M, Rentero C, Zheng Y, Enrich C, Del Pozo MA. A palmitoylation switch mechanism regulates Rac1 function and membrane organization. EMBO J. 2012 Feb 1;31(3):534-51
52. Ricci C, Ruscica M, Camera M, Rossetti L, Macchi C, Colciago A, Zanotti I, Lupo MG, Adorni MP, Cicero AFG, Fogacci F, Corsini A, Ferri N. PCSK9 induces a pro-inflammatory response in macrophages. Sci Rep. 2018 Feb 2;8(1):2267.
53. Waltmann MD, Basford JE, Konaniah ES, Weintraub NL, Hui DY. Apolipoprotein E receptor-2 deficiency enhances macrophage susceptibility to lipid accumulation and cell death to augment atherosclerotic plaque progression and necrosis. Biochim Biophys Acta. 2014 Sep;1842(9):1395-405
54. Shan L, Pang L, Zhang R, Murgolo NJ, Lan H, Hedrick JA. PCSK9 binds to multiple receptors and can be functionally inhibited by an EGF-A peptide. Biochem Biophys Res Commun. 2008 Oct 10;375(1):69-73.
55. Tang ZH, Peng J, Ren Z, Yang J, Li TT, Li TH, Wang Z, Wei DH, Liu LS, Zheng XL, Jiang ZS. New role of PCSK9 in atherosclerotic inflammation promotion involving the TLR4/NF-κB pathway. Atherosclerosis. 2017 Jul;262:113-122.
56. Ding Z, Liu S, Wang X, Theus S, Deng X, Fan Y, Zhou S, Mehta JL. PCSK9 regulates expression of scavenger receptors and ox-LDL uptake in macrophages. Cardiovasc Res. 2018 Jul 1;114(8):1145-1153.
57. Adorni MP, Zimetti F, Billheimer JT, Wang N, Rader DJ, Phillips MC, Rothblat GH. The roles of different pathways in the release of cholesterol from macrophages. J Lipid Res. 2007 Nov;48(11):2453-62. Epub 2007 Aug 29.
58. Adorni MP, Cipollari E, Favari E, Zanotti I, Zimetti F, Corsini A, Ricci C, Bernini F, Ferri N. Inhibitory effect of PCSK9 on Abca1 protein expression and cholesterol efflux in macrophages. Atherosclerosis. 2017 Jan;256:1-6.
59. Shapiro MD, Tavori H, Fazio S. PCSK9: from basic science discoveries to clinical trials. Circ Res. 2018;122(10):1420–38.
60. Caselli C, Del Turco S, Ragusa R, Lorenzoni V, De Graaf M, Basta G, Scholte A, De Caterina R, Neglia D. Association of PCSK9 plasma levels with metabolic patterns and coronary atherosclerosis in patients with stable angina. Cardiovasc Diabetol. 2019 Oct 31;18(1):144.

61. Chan DC, Pang J, McQuillan BM, Hung J, Beilby JP, Barrett PH, Watts GF. Plasma Proprotein Convertase Subtilisin Kexin Type 9 as a Predictor of Carotid Atherosclerosis in Asymptomatic Adults. Heart Lung Circ. 2016; 25:520–5.
62. Alonso R, Mata P, Muniz O, Fuentes-Jimenez F, Diaz JL, Zambon D, Tomas M, Martin C, Moyon T, Croyal M, Thedrez A, Lambert G. PCSK9 and lipoprotein (a) levels are two predictors of coronary artery calcification in asymptomatic patients with familial hypercholesterolemia. Atherosclerosis. 2016; 254:249–253.
63. Shapiro. Cheng JM, Oemrawsingh RM, Garcia-Garcia HM, Boersma E, van Geuns RJ, Serruys PW, Kardys I, Akkerhuis KM. PCSK9 in relation to coronary plaque inflammation: Results of the ATHEROREMO-IVUS study. Atherosclerosis. 2016; 248:117–22.
64. Cheng JM, Oemrawsingh RM, Garcia-Garcia HM, Boersma E, van Geuns RJ, Serruys PW, Kardys I, Akkerhuis KM. PCSK9 in relation to coronary plaque inflammation: Results of the ATHEROREMO-IVUS study. Atherosclerosis. 2016 May;248:117-22.
65. Gencer B, Montecucco F, Nanchen D, Carbone F, Klingenberg R, Vuilleumier N, Aghlmandi S, Heg D, Räber L, Auer R, Jüni P, Windecker S, Lüscher TF, Matter CM, Rodondi N, Mach F. Prognostic value of PCSK9 levels in patients with acute coronary syndromes. Eur Heart J. 2016 Feb 7;37(6):546-53.
66. Ferri N, Ruscica M. Proprotein convertase subtilisin/kexin type 9 (PCSK9) and metabolic syndrome: insights on insulin resistance, inflammation, and atherogenic dyslipidemia. Endocrine, 2016;54:588–601.
67. Zhu YM, Anderson TJ, Sikdar K, Fung M, McQueen MJ, Lonn EM, Verma S. Association of proprotein convertase subtilisin/kexin type 9 (PCSK9) with cardiovascular risk in primary prevention. Arterioscler Thromb Vasc Biol. 2015;35(10):2254–9.
68. Furtado RHM, Giugliano RP.What Lessons Have We Learned and What Remains to be Clarified for PCSK9 Inhibitors? A Review of FOURIER and ODYSSEY Outcomes Trials. Cardiol Ther. 2020 Feb 5.
69. German CA, Shapiro MD. Small Interfering RNA Therapeutic Inclisiran: A New Approach to Targeting PCSK9.BioDrugs. 2020 Feb;34(1):1-9. doi: 10.1007/s40259-019-00399-6.
70. Seidah NG, Prat A, Pirillo A, Catapano AL, Norata GD. Novel strategies to target proprotein convertase subtilisin kexin 9: beyond monoclonal antibodies. Cardiovasc Res. 2019 Mar 1;115(3):510-518. doi: 10.1093/cvr/cvz003.
71. Knuuti J, Wijns W, Saraste A, et al. 2019 ESC Guidelines for the diagnosis and management of chronic coronary syndromes. Eur Heart J. 2020;41(3):407-477.
72. François Mach, Colin Baigent, Alberico L Catapano, et al. 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk. Eur Heart J 2020 Volume; 41(1):111–188.

73. Dhorepatil A, Ball S, Ghosh RK, Kondapaneni M, Lavie CJ. Canakinumab: Promises and Future in Cardiometabolic Diseases and Malignancy. Am J Med. 2019 Mar;132(3):312-324.
74. Angelos D Karagiannis, Martin Liu, Peter P Toth, Shijia Zhao, Devendra K Agrawal, Peter Libby, Yiannis S Chatzizisis. Pleiotropic Anti-atherosclerotic Effects of PCSK9 Inhibitors. Curr Atheroscler Rep. 2018 Mar 10;20(4):20
75. Ishwarlal Jialal, Gurdeep Singh. Management of diabetic dyslipidemia: An update. World J Diabetes. 2019 May 15; 10(5): 280–290.

FIGURE LEGEND

Figure 1. The various cell types involved in atherosclerosis. In vessels with damaged/dysfunctional ECs, high levels of LDL-C (yellow and black circle) can be transported and accumulated in the intima by trans-cytosis or open endothelial junction. After translocation in sub-endothelial space, LDL-C is trapped by proteoglycans and oxidized in oxLDL (yellow and red circle). oxLDL is an immunogenic factor able of inducing oxidative stress on ECs with consequence expression of cell surface adhesion molecules; promoting migration and proliferation of monocytes/macrophages and SMCs within the intima. oxLDL accumulate in the intima are rapidly recognized by scavenger receptors and phagocytized by macrophages and SMCs leading to formation of lipid-laden "foam cells". Once created, the foam cells support the progression of atherosclerotic lesion by the expression and release of pro-inflammatory cytokines (green, red and black stars). These molecules facilities the migration and proliferation of other monocytes in the intima, and promote the production of fibrous cap through the synthesis of extracellular matrix molecules, as collagen and proteoglycans by SMCs. Meantime, apoptosis and necrosis of foam cells and SMCs contribute to necrotic core development and atherosclerosis progression.

Figure 2. MeSH Network. The bubble map visualizes the medical subject headings (MeSH) keywords selected from papers published and retrieved in PubMed under the search terms term "cardiovascular disease" "atherosclerosis" and "PCSK9". The bubble size indicates the frequency of occurrence of the words, while the bubble color represents the cluster of belonging.

Figure 3. Role of PCSK9 in Endothelial cells. Among the pathophysiological mechanisms that contribute to plaque creation and progression during atherosclerosis, the dysfunction of endothelial cells (ECs), carried out by high levels of oxLDL, low shear stress, and presences of inflammatory stimuli, is depicted. **A**. In ECs, after the interaction among oxLDL and LOX-1 the levels of ROS increased by NADPH oxidase effect, resulting in rise of mitochondrial DNA damage (pro-apoptotic stimulus) and NF-κB activation. In addition to oxLDL, also low shear stress and inflammatory stimuli (such as LPS) contributed to NF-κB activation and translocation into the nucleus. The LPS/TLR4 pathway also contributes to the activation of the AP-1 transcription factor. In the nucleus, NF-κB and AP-1 activate the expression of pro-apoptotic and pro-inflammatory mediators, and also the synthesis of mRNA-PCSK9. PCSK9, in turn, promotes NF-κB and AP-1 activation by a positive feedback mechanism. **B**. The use of NADPH oxidase inhibitors or siRNA for NF-κB decrease the expression of PCSK9, pro- apoptotic and inflammatory factors.

Figure 4. **Role of PCSK9 in Smooth muscle cells. A.** Smooth muscle cells (SMCs) were collected from the arterial vessel of PCSK9 knockdown mice ($PCSK9^{-/-}$). In the presence of vascular injury (in the blue box), $PCSK9^{-/-}$ mice resulted partially protected by differentiation of SMCs from a normal to

a migrant phenotype and showed high contractile capacity. Treatment of SMCs from PCSK9$^{-/-}$ mice with recombinant PCSK9 (hPCSK9) red box) increases the activation of mTOR and the proliferating capacity of cells. Moreover, treatment of SMCs from PCSK9$^{-/-}$ mice with hPCSK9 (green box) increases the expression of pro-apoptotic factors and supports the mithocondrial DNA (mtDNA) damage. **B**. SMCs collected from the arterial vessel of PCSK9 wild-type mice (PCSK9 +/+). In the presence of vascular injury (in the blue box), SMCs showed high motility, high proliferating capacity and low degree of differentiation. Treatment of control SMCs (PCSK9$^{+/+}$) with siRNA against PCSK9 (red box) reduces the activation of mTOR and the proliferating capacity of the cells. Treatment of control SMCs (PCSK9$^{+/+}$) with LPS (green box) increases the production of ROS, mtDNA damage, pro-apoptotic factor synthesis, as well as the expression and secretion of PCSK9. In addition to LPS, increased levels of ROS and mtDNA obtained from different stress conditions were able to activate the synthesis of PCSK9.

Figure 5. **Role of PCSK9 in macrophages. A.** Treatment of control mouse peritoneal macrophages (LDLR$^{+/+}$) with TNFα (red box) increases the expression of pro-inflammatory mediators and PCSK9 by NF-κB activation. PCSK9, in turn, promotes NF-κB activation by a positive feedback mechanism; co-treatment with TNFα and PCSK9 (red and yellow boxes) increases the expression of LOX-1 and consequently the uptake of oxLDL and the expression and release of cytokines; cell treatment with PCSK9 (yellow box) increases the production of pro-inflammatory cytokines and reduce cholesterol efflux by ABCA1 from cells. These effects are mainly mediated by the LDLR. **B.** In macrophages collected from LDLR knockdown (LDLR$^{-/-}$) mice, treatment with PCSK9 still decreases ABCA1-mediated cholesterol efflux. This effect is mediated by the VLDLR, but also by SRA and CD36. In LDLR$^{-/-}$ macrophages treated with PCSK9 the synthesis of pro-inflammatory cytokines is only moderately increased compared to control.

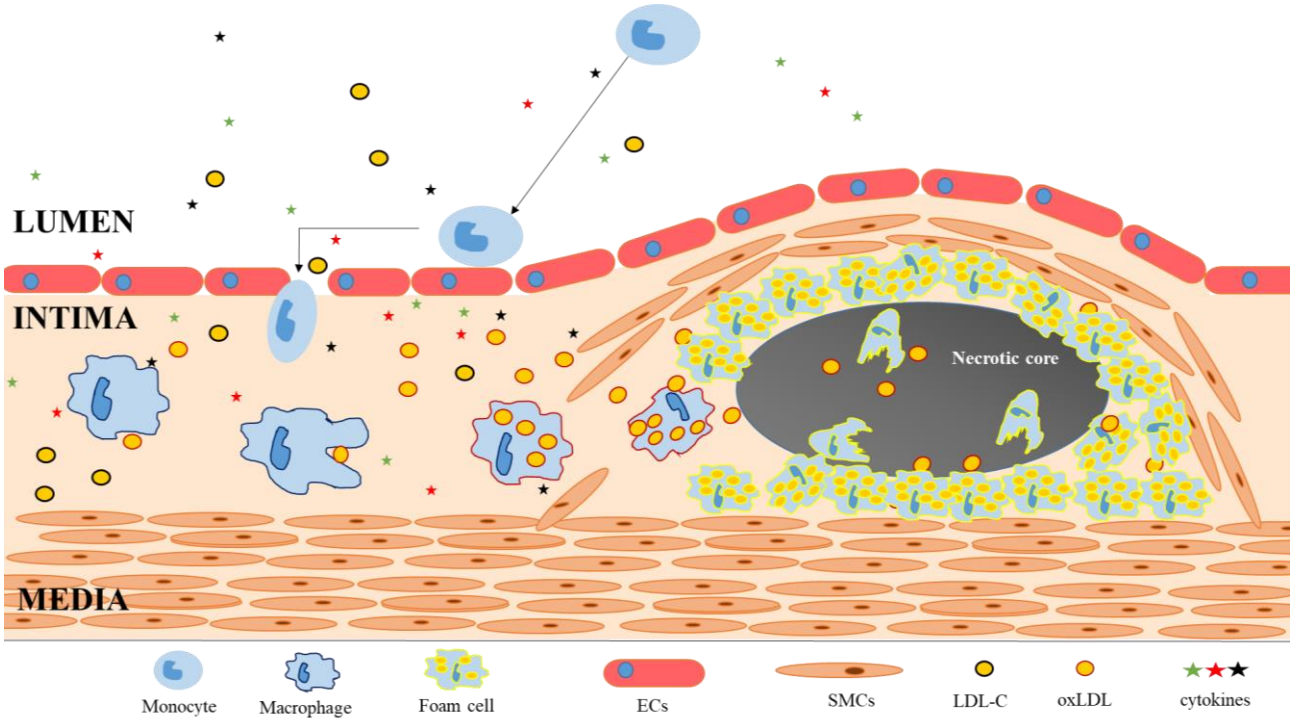


Figure 3A-B

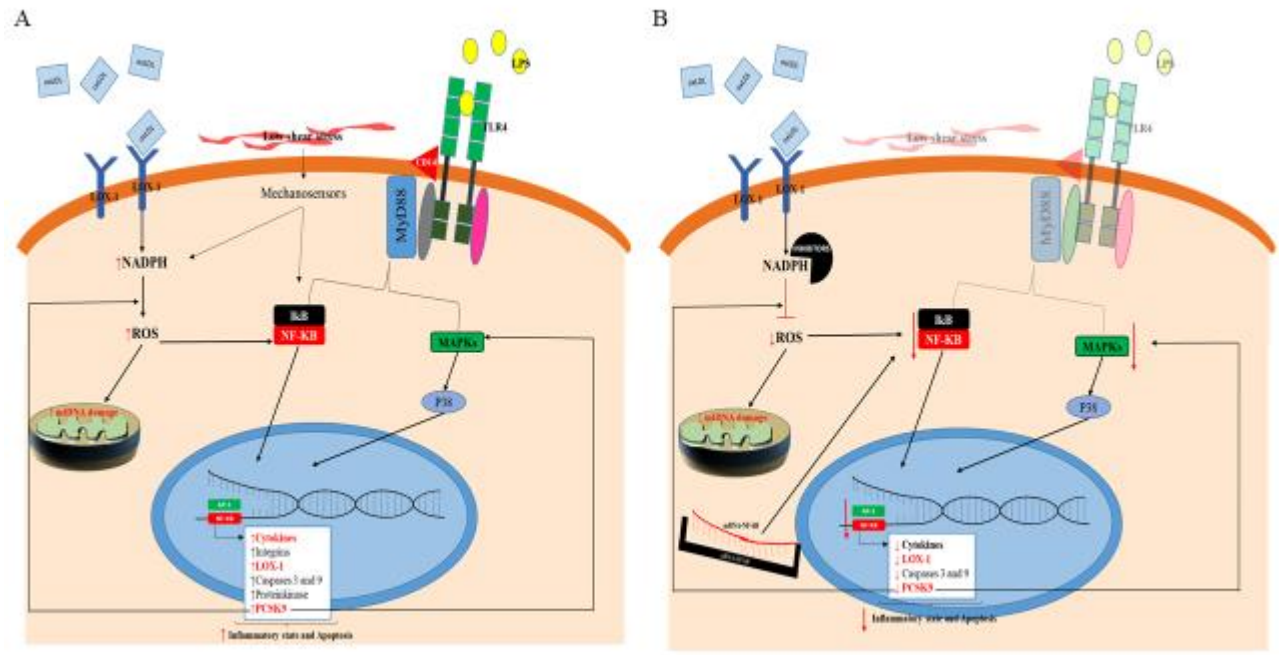
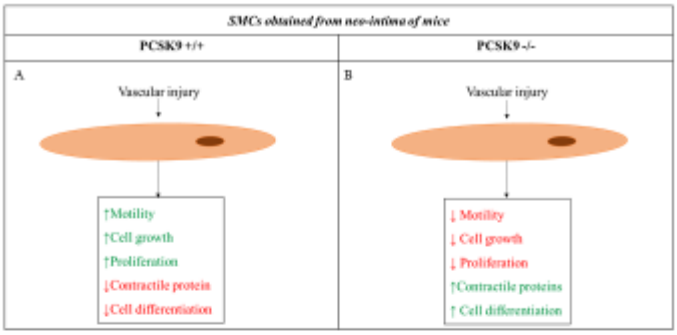
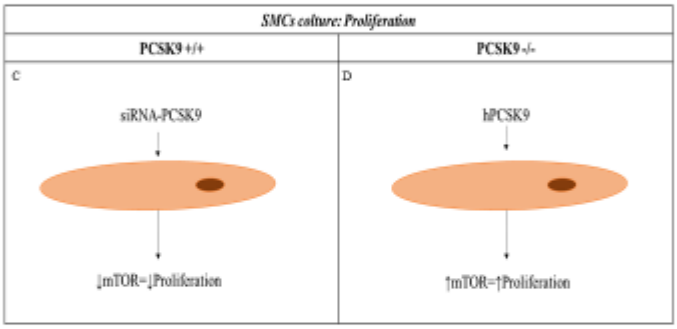


Figure 4A-B


SMCs obtained from neo-intima of mice
PCSK9 +/+
PCSK9 -/-
A
Vascular injury
↑Motility
↑Cell growth
↑Proliferation
↓Contractile protein
↓Cell differentiation
B
Vascular injury
↓ Motility
↓ Cell growth
↓ Proliferation
↑Contractile proteins
↑ Cell differentiation


SMCs culture: Proliferation
PCSK9 +/+
PCSK9 -/-
C
siRNA-PCSK9
↓mTOR=↓Proliferation
D
hPCSK9
↑mTOR=↑Proliferation

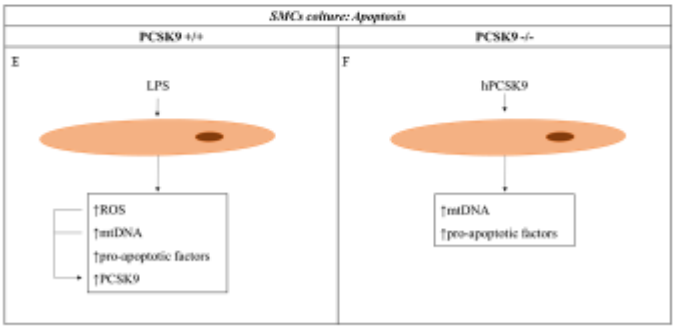
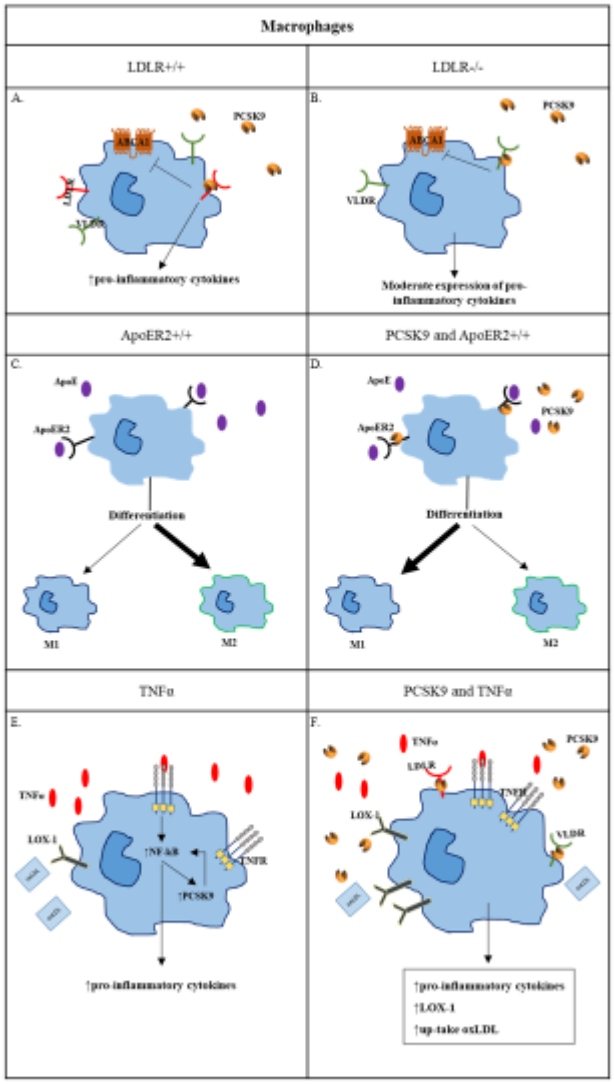

SMCs culture: Apoptosis
PCSK9 +/+
PCSK9 -/-
E
LPS
↑ROS
↑mtDNA
↑pro-apoptotic factors
↑PCSK9
F
hPCSK9
↑mtDNA
↑pro-apoptotic factors

Figure 5A-B


Macrophages
LDLR+/+
LDLR-/-
A.
PCSK9
ABCA1
LDLR
VLDR
↑pro-inflammatory cytokines
B.
PCSK9
ABCA1
VLDR
Moderate expression of pro-inflammatory cytokines
ApoER2+/+
PCSK9 and ApoER2+/+
C.
ApoE
ApoER2
Differentiation
M1
M2
D.
ApoE
ApoER2
PCSK9
Differentiation
M1
M2
TNFα
PCSK9 and TNFα
E.
TNFα
LOX-1
↑NF-kB
TNFR
↑PCSK9
↑pro-inflammatory cytokines
F.
TNFα
PCSK9
LDLR
TNFR
LOX-1
VLDR
↑pro-inflammatory cytokines
↑LOX-1
↑up-take oxLDL