

# An Update on the Role of PCSK9 in Atherosclerosis

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Atherosclerosis is initiated by functional changes in the endothelium accompanied by accumulation, oxidation, and glycation of LDL-cholesterol in the inner layer of the arterial wall and continues with the expression of adhesion molecules and release of chemoattractants. PCSK9 is a proprotein convertase that increases circulating LDL levels by directing hepatic LDL receptors into lysosomes for degradation. The effects of PCSK9 on hepatic LDL receptors and contribution to atherosclerosis via the induction of hyperlipidemia are well defined. Monoclonal PCSK9 antibodies that block the effects of PCSK9 on LDL receptors demonstrated beneficial results in cardiovascular outcome trials. In recent years, extrahepatic functions of PCSK9, particularly its direct effects on atherosclerotic plaques have received increasing attention. Experimental trials have revealed that PCSK9 plays a significant role in every step of atherosclerotic plaque formation. It contributes to foam cell formation by increasing the uptake of LDL by macrophages via scavenger receptors and inhibiting cholesterol efflux from macrophages. It induces the expression of inflammatory cytokines, adhesion molecules, and chemoattractants, thereby increasing monocyte recruitment, inflammatory cell adhesion, and inflammation at the atherosclerotic vascular wall. Moreover, low shear stress is associated with increased PCSK9 expression. PCSK9 may induce endothelial cell apoptosis and autophagy and stimulate the differentiation of smooth muscle cells from the contractile phenotype to synthetic phenotype. Increasing evidence indicates that PCSK9 is a molecular target in the development of novel approaches toward the prevention and treatment of atherosclerosis. This review focuses on the molecular roles of PCSK9 in atherosclerotic plaque formation.

**Key words:** PCSK9, Atherosclerosis, Foam cell, Inflammation, Vascular wall

Atherosclerosis is a chronic condition that starts early in life and then continues lifelong. Atherosclerotic process begins with functional changes in the endothelium accompanied by accumulation, oxidation, and glycation of low-density lipoprotein (LDL) cholesterol in the inner layer of the arterial wall and continues with the expression of adhesion molecules and the release of chemoattractants<sup>1</sup>. Monocytes and T cells are recruited in the intimal space, where monocytes engulf the oxidized LDL and become foam cells<sup>2</sup>. Fatty streaks, the earliest lesions of atherosclerosis, can be visualized in the aorta from the first decade of life. They appear in the coronary arteries in the second decade and in the cerebral arteries from the third or fourth decades<sup>3</sup>. Fatty streaks develop into atherosclerotic plaques, which can lead to luminal nar-

rowing of the arteries causing ischemic complaints or plaque may become unstable, leading to acute atherothrombotic events.

Among the various risk factors of atherosclerosis, dyslipidemia—particularly LDL cholesterol—plays the main role in the initiation and progression of the atherosclerotic process<sup>4</sup>. As confirmed by several experimental and clinical studies, there is a clear, log-linear relationship between the LDL cholesterol level and atherosclerosis<sup>5, 6</sup>. Circulating LDL cholesterol level is mainly determined by the number of hepatic LDL cholesterol receptors (LDLR) and the expression or the activity of proprotein convertase subtilisin kexin type 9 (PCSK9) enzyme<sup>7</sup>.

Proprotein convertases are a family of proteins that are responsible for post-translational modifica-

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tions of many functional proteins, including proteolytic cleavage, and activation of most polypeptide hormones such as proinsulin<sup>8</sup>). Hepatic PCSK9 is the circulating protein that regulates the half-life of LDLR in the liver. In the absence of PCSK9, LDLR binds to LDL on the hepatocyte surface, transfers it into endosomes inside the hepatocyte, and recycles back to the cell surface to bind and internalize new LDL particles. Binding with PCSK9 routes the LDLR to lysosomal degradation, and the recycling of the LDLR is blocked<sup>9</sup>). Endocytosis of PCSK9-mediated LDLRs occurs both via clathrin- and caveolae-dependent pathways, whereas recycling of LDLR and PCSK9 complex occurs in clathrin-dependent pathway and degradation occurs in caveolae-dependent pathway. LDLR and PCSK9 complex interaction with adenyl cyclase associated protein-1 (CAP1) leads to caveolae-dependent endocytosis<sup>10</sup>).

PCSK9, synthesized as a 74 kDa zymogen with 692 amino acids, comprises four domains, namely, the N-terminal pro-domain, signal peptide, the catalytic domain, and a C-terminal domain. Autocatalysis occurs between its pro-domain and catalytic domain in endoplasmic reticulum<sup>11</sup>). After autocatalysis, the pro-domain remains non-covalently attached to its catalytic pocket, which is unique to this enzyme<sup>12</sup>). N-terminal of pro-domain inhibits convertase activity of PCSK9, preventing it from interacting with surrounding substrates. Therefore, the pro-domain acts to modulate the effect of PCSK9 by releasing the catalytic domain. A short segment of amino acids in the catalytic domain of PCSK9 (amino acids 367 to 380) interacts with the epidermal growth factor-like A domain of LDLR to divert it to endosomes and lysosomes for degradation<sup>13, 14</sup>). The catalytic domain activity of PCSK9 is not required for its effect on LDLR. PCSK9 also escorts very low-density lipoprotein receptor (VLDLR), apolipoprotein E receptor 2 (ApoER2), and LDLR-related protein-1 (LRP1) to lysosomal degradation<sup>15, 16</sup>).

### PCSK9's Role in Atherosclerotic Process

The atherogenic effects of PCSK9 were initially explained through its role in lipid metabolism; however, plasma PCSK9 level is only modestly correlated with the plasma lipid levels<sup>17</sup>). In recent years, it has been recognized that beyond its effects on lipid metabolism, PCSK9 also has direct atherosclerotic effects on the vascular wall. Several preclinical and clinical studies showed that the relation of PCSK9 with atherosclerosis can be partly independent of its hyperlipidemic effects. In Apo E knock-out mice, PCSK9 accelerated atherosclerosis without affecting plasma lipid

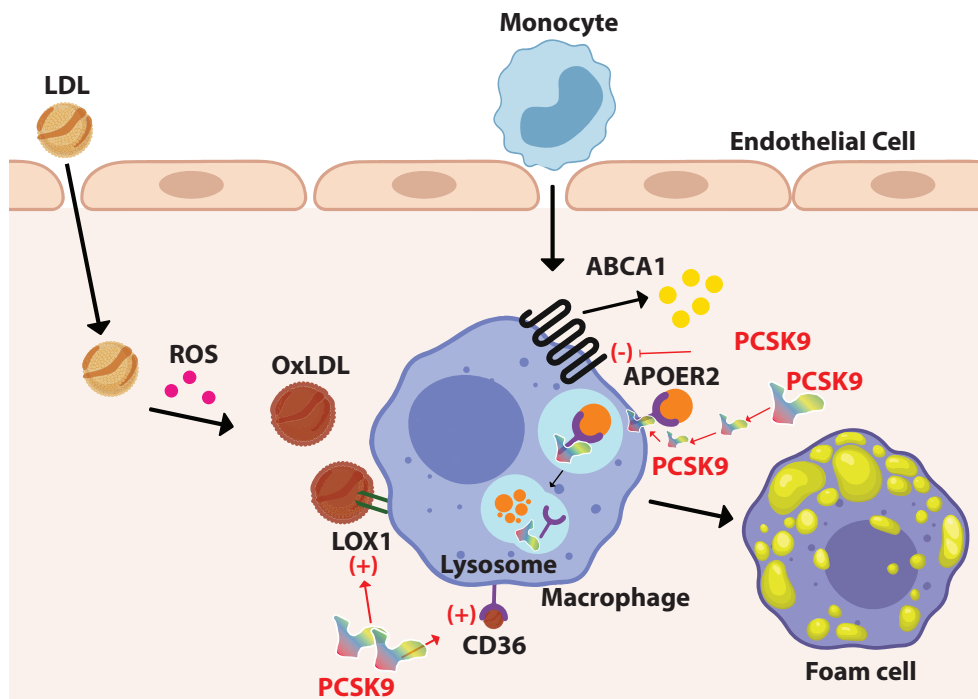
levels, whereas PCSK9 overexpression did not cause an increase in plasma lipids but in atherosclerotic lesion size<sup>18, 19</sup>). The Atheroma IVUS study revealed that the necrotic core volume of the atherosclerotic plaque increases proportionately to serum PCSK9 level independent of LDL cholesterol<sup>20</sup>). A 10 year follow-up study showed that the carotid plaque area and the formation of new carotid plaques are related to LDL levels and also independent to increased PCSK9 levels<sup>17</sup>). Moreover, STAINLAS study cohort revealed that increased PCSK9 level is found to be related to carotid plaque formation<sup>21</sup>). These trials, showing the relation of PCSK9 with atherosclerosis, have drawn attention to direct effects of PCSK9 on atherosclerotic plaques.

Vascular smooth muscle cells (SMCs) are the main PCSK9 secreting cells in the vascular wall. Despite conflicting reports, many studies have shown that endothelial cells also express PCSK9, albeit less than SMC<sup>22, 23</sup>). Macrophages and human atherosclerotic plaques are other sources of PCSK9 expression. The expression of PCSK9 in various components of the arterial wall contributes to its direct effects in the initiation and progression of the atherosclerotic process. This review summarizes current data on the role of PCSK9 in atherosclerotic plaque formation.

### Effects of PCSK9 in Oxidized LDL Uptake and Cholesterol Efflux

Atherosclerosis begins with the formation of foam cells, which are macrophages that engulf oxidized LDL (ox-LDL) (**Fig. 1**). Ox-LDL enters into the monocytes/macrophages and also SMCs and fibroblasts by scavenger receptors (SRs), including class A SR, class B SR type I, CD36, macroscialin (CD68), and lectin-like ox-LDL (LOX-1)<sup>24, 25</sup>). LOX-1 is the major receptor in the engulfment of ox-LDL by macrophages and also increases the production of cell surface adhesion molecules and is involved in the process of oxidative stress<sup>26-28</sup>). PCSK9 induces expression of all SRs, but mostly, LOX-1 on monocytes and SMCs<sup>24</sup>). In inflammatory milieu, PCSK9 and LOX1 induce each other, leading to an increased ox-LDL uptake<sup>29</sup>).

Excess intracellular free cholesterol is toxic for macrophages from which they protect themselves by cholesterol efflux to extracellular acceptors<sup>30</sup>). Inhibiting cholesterol efflux in macrophages causes an increase in foam cell formation. ATP Binding Cassette A1 (ABCA1), ATP Binding Cassette G1 (ABCG1), and Scavenger Receptor Class B Type I (SR-BI) are membrane transporters that provide most cholesterol efflux in macrophages. PCSK9 inhibits ABCA-1-de-



**Fig. 1.** Foam cell formation. LDL enters into the intimal layer of the artery and oxidized by reactive oxygen species. Macrophages engulf oxidized LDL by scavenger receptors such as CD36 and LOX-1

ABCA1 receptor maintains cholesterol efflux from macrophage. PCSK9 contributes to the formation of foam cells by inducing scavenger receptors, inhibiting the ABCA1 receptor, and directing ApoE2 to the lysosomes for degradation.

pendent cholesterol efflux by downregulating the ABCA-1 gene and ABCA-1 protein expression; however, it does not inhibit ABCG1 expression and only slightly inhibits SIR-BI expression<sup>31</sup>.

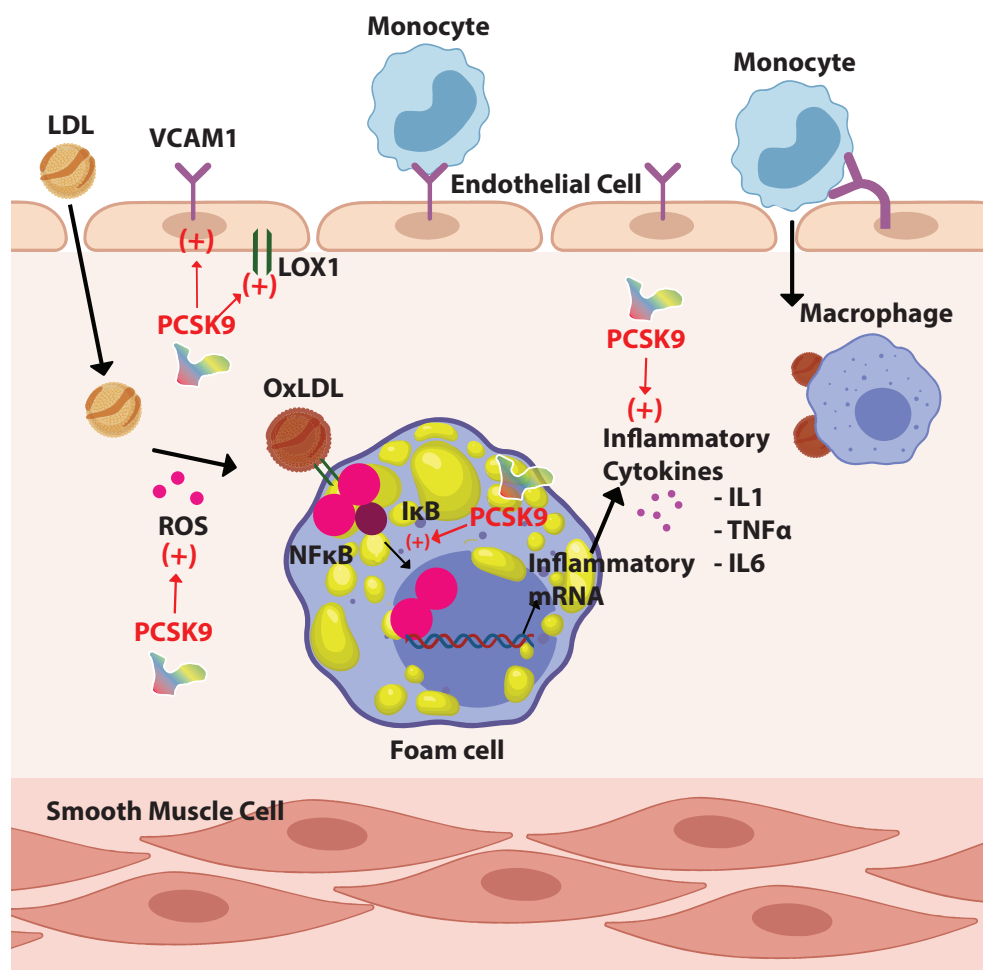
Apolipoprotein (Apo E) is a lipid transport protein and major ligand for LDLR<sup>32</sup>. Parenchymal cells, differentiated macrophages, astrocytic glial cells, and SMCs synthesize and secrete Apo E, which take a role in the transport of cholesterol and other lipids between peripheral tissue and liver, mediating the clearance of chylomicrons and LDL in the liver. Apo E can reduce lipid accumulation in macrophages and inhibit foam cell formation and switching of macrophages from proinflammatory M1 phenotype to anti-inflammatory M2 phenotype<sup>33</sup>. Effects of Apo E are exerted via apoE-receptor-2 (apoER2), which is expressed in platelets, endothelial cells, monocytes, and macrophages<sup>33, 34</sup>. The binding of apoE to apoER2 has protective effect from atherosclerosis by suppressing the inflammatory function of macrophages, reducing lipid accumulation and cell death<sup>35</sup>. ApoER2 is a member of the LDLR family, whose structure is similar to that of LDLR with 46% identity<sup>36</sup>. PCSK9 decreases apoER2 levels with a similar

mechanism used for LDLR, namely without using its catalytic activity, by its coexpression or cell surface internalization.

Thereby, PCSK9 inhibits the atheroprotective effects of apoER2 resulting in increased inflammation and foam cell formation<sup>16</sup>.

### PCSK9 in Vascular Inflammation

PCSK9 induces inflammation in atherosclerosis independently from its hyperlipidemic effect<sup>37</sup>. Disturbed ox-LDL uptake/cholesterol efflux balance and accumulation of ox-LDL stimulates the overlying endothelial cells to produce proinflammatory molecule (Fig. 2)<sup>1, 38</sup>. In addition to ox-LDL accumulation, PCSK9 can directly induce the expression of inflammatory cytokines. Ly6C(hi) monocytes, also called “inflammatory monocytes”, are associated with acute inflammation. They accumulate in inflammatory sites, promoting monocytois and their local recruitment. In the early atherosclerotic plaques, Ly6C(hi) monocytes infiltrate atherosclerotic lesions and produce activated macrophages, responsible for the secretion of proinflammatory cytokines<sup>39</sup>. In Apo



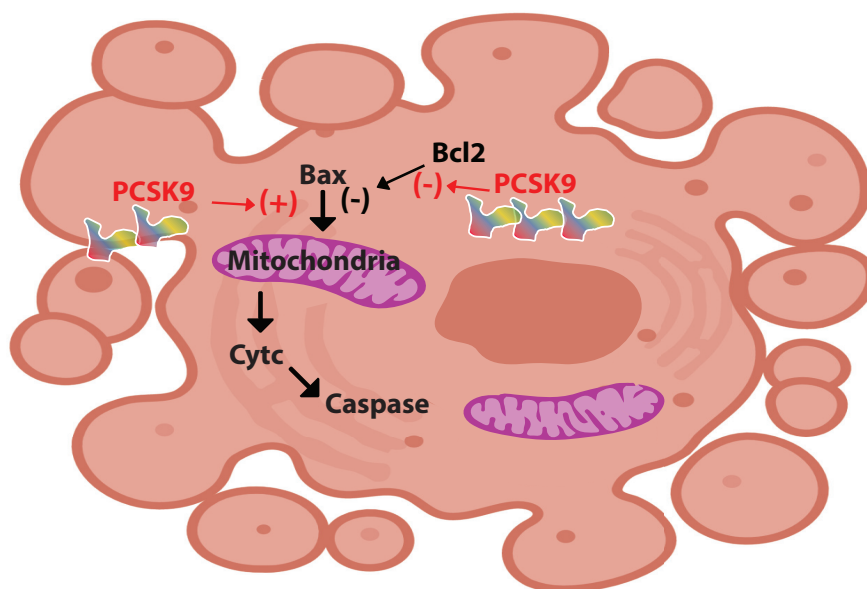
**Fig. 2.** Inflammation

PCSK9 induces the expression of endothelial adhesion molecules. NF-κB nuclear translocation is induced by PCSK9, leading to an increase in mRNA levels and secretion of inflammatory cytokines.

E knock-out mice (Apo E KO), PCSK9 increases Ly6C(hi) monocytes in spleen and enhance their recruitment in arterial wall<sup>40</sup>. It increases macrophage response to lipopolysaccharide (LPS) leading to excessive secretion of inflammatory cytokines, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and interleukin-6 (IL-6) and decreases anti-inflammatory cytokines, including interleukin-10 (IL10) and arginase<sup>41, 42</sup>. Silencing PCSK9 decreases the expression of TNF- $\alpha$ , IL-1b, and monocyte chemoattractant protein 1 (MCP1) in the atherosclerotic aortas of Apo E KO mice<sup>43</sup>. MCP-1 and chemokine (C-X-C motif) ligand 2 (CXCL2) are chemokines related to monocyte recruitment. PCSK9 can directly induce expression of these molecules in macrophages<sup>42</sup>. These proinflammatory effects of PCSK9 are independent of cholesterol but not observed in LDLR -/- mice, suggesting that PCSK9 inflamma-

tory effects on macrophages are mostly via LDLRs<sup>40, 42</sup>.

The nuclear factor kappa B (NF-κB) controls the transcription of many genes that have roles in inflammation and atherosclerosis, including cytokines, chemokines, adhesion molecules, acute phase proteins, regulators of apoptosis, and cell proliferation<sup>44</sup>. NF-κB, mitogen-activated protein kinase, and damaged mitochondrial DNA have significant roles in PCSK9-mediated ox-LDL uptake<sup>29</sup>. NF-κB nuclear translocation is induced by PCSK9 overexpression in macrophages, increasing the mRNA levels of proinflammatory cytokines and toll-like receptor 4 (TLR4) expression. Inversely, NF-κB inhibition decreases LPS, ox-LDL, and TNF- $\alpha$  induced PCSK9 expression<sup>43</sup>. Therefore, it is worth noting that NF-κB has an important signaling role in inflammatory stimulus mediated by PCSK9 expression and PCSK9 accelerates atherosclerotic plaque inflammation through the



**Fig. 3.** Apoptosis

PCSK9 induces apoptosis of HUVECs by inducing Bax and inhibiting Bcl2.

activation of TLR4/NF- $\kappa$ B pathway<sup>29, 43</sup>.

The vascular cell adhesion molecule 1 (VCAM-1) is a protein whose expression increases in inflammatory conditions, mediates the adhesion of lymphocytes, monocytes, eosinophils, and basophils to the vascular wall. PCSK9 induces VCAM-1 expression in vascular SMCs, and PCSK9 inhibition in cultured vascular SMCs significantly decreases VCAM-1 expression<sup>29</sup>.

Dendritic cells that play a role in endothelial inflammation stimulate T cell proliferation and differentiation of naive CD4<sup>+</sup>T cells into Th1 and Th17 in the existence of ox-LDL. Ox-LDL induces the PCSK9 expression in dendritic cells and increases secretion of TNF- $\alpha$ , IL-1, IL-6, transforming growth factor beta and IL-10 from dendritic cells<sup>45</sup>. Differentiation and proliferation of T cells are reduced when PCSK9 is inhibited in dendritic cells<sup>45</sup>.

### PCSK9-Induced Apoptosis

Endothelial cell apoptosis promotes endothelial dysfunction, which is a foundation in the atherosclerosis development. Intrinsic pathway of apoptosis is regulated by B cell lymphoma-2 (Bcl-2) family proteins. Bax and Bcl2 proteins are members of this family with opposite roles: Bax induces apoptosis, whereas Bcl2 inhibits apoptosis. Ox-LDL induces apoptosis of human umbilical vein endothelial cells (HUVECs) and upregulates the expression of PCSK9 and LOX-1

concentration-dependently (Fig.3)<sup>46</sup>. In ox-LDL treated HUVECs, expressions of proapoptotic protein Bax and the activity of caspase9 and caspase3 increase, whereas anti-apoptotic protein Bcl-2 expression decreases<sup>46</sup>. The silencing of PCSK9, with its siRNA, reverses the apoptotic effects of ox-LDL and inhibits apoptosis in endothelial cells<sup>46</sup>. Therefore, it is suggested that PCSK9 increases apoptosis of HUVECs by Bcl/Bax- caspase9-caspase3 pathway<sup>41, 46</sup>. PCSK9 inhibition also decreases the phosphorylation of c-Jun N-terminal kinase and p38, which play an important role in cell apoptosis that is induced by oxidative damage, IL1, TNF- $\alpha$ , and G protein-coupled receptors<sup>41</sup>.

Autophagy is a protective cellular process of self-digestion of the misfolded proteins and dysfunctional organelles. It aims to promote cell survival under unfavorable conditions such as inflammation and oxidative stress. In the early stages of atherosclerosis, autophagy is protective against macrophage engulfment and local inflammation. However, in late stages, it leads to macrophage death, accelerated inflammation, and thinning of fibrous cap by collagen degradation and can cause acute coronary events<sup>47</sup>. The mammalian target of rapamycin (mTOR) is an inhibitor of autophagy, whereas mTOR inhibitor rapamycin is the most commonly used inducer of autophagy, providing a possible anti-atherosclerotic effect<sup>48</sup>. PCSK9 upregulation induces mTOR expression in vascular SMC cultures dose-dependently, whereas inhibition of PCSK9 with its siRNA promotes

autophagy and cellular vitality via decreasing mTOR phosphorylation<sup>49</sup>). Moreover, mTOR pathway is a regulator of LDLR pathway leading to upregulated LDLR expression and inhibited PCSK9 transcription when activated by various signals such as insulin and to decreased LDLR expression and increased PCSK9 expression when inhibited with rapamycin<sup>49</sup>).

### Reactive Oxygen Species-Induced PCSK9 Expression

Reactive oxygen species (ROS) are oxygen-containing reactive molecules and can be produced in oxidation reactions in mitochondria and other cellular locations. In inflammatory states, such as atherosclerosis, increase in mitochondrial ROS production provokes endothelial dysfunction, induces the infiltration and activation of inflammatory cells, and increases apoptosis of endothelial and vascular SMCs. Elevated ROS production increases PCSK9 levels<sup>23</sup>). Mitochondrial ROS leads to PCSK9 and LOX1 increase; the interrelationship of PCSK9 with ROS is strongly supported by the observation that shows a decrease in mtROS in response to LOX-1 or PCSK9 knock-down<sup>29</sup>). Although ROS inducers pyocyanin and superoxide inducer antimycin-A increase PCSK9 expression, ROS inhibitors such as diphenylene iodonium and apocynin decrease PCSK9 expression in macrophages<sup>24</sup>). Increased H<sub>2</sub>O<sub>2</sub> production and Nox2 activation are seen in the platelets that are incubated with PCSK9<sup>50</sup>).

### Shear Stress-Induced PCSK9 Expression

Vascular tone and diameter are under the control of vascular SMCs through the mechanism of contraction<sup>51</sup>). Vascular SMCs are highly specialized cells of which principal functions are contraction and regulation of blood vessel tone, blood pressure, and blood flow. Local susceptibility to atherosclerosis is regulated by blood flow patterns. Endothelial shear stress is one of the mechanical factors that initiate atherosclerotic plaques. It is the tangential stress derived from the friction of the flowing blood on the endothelial surface of the arterial wall. Low shear stress, which typically occurs in regions of branching or bifurcation regions of the arteries, results in high EC turnover, increased accumulation of LDL, increased oxidative stress, increased DNA synthesis, and higher expression of proinflammatory adhesion molecules; therefore, low shear stress is related to atherosclerosis<sup>52</sup>). It has been shown that low shear stress (3–6 dyne/cm<sup>2</sup>) increases PCSK9 expression mostly in the SMCs, and lipid-induced increase in PCSK9 expression is higher

at low shear stress<sup>23</sup>).

### PCSK9's Effects on Vascular SMCs

Vascular SMCs within adult blood vessels exhibit a low rate of proliferation and low synthetic activity and express contractile proteins. SMCs in the media express contraction proteins such as alpha-smooth muscle actin, myosin heavy chain II, and calponin, whereas intimal SMCs synthesize extracellular matrix, proteases, and cytokines and have higher migratory and proliferative capacity<sup>53, 54</sup>). Contractile SMCs can differentiate to a synthetic phenotype with atherogenic stimuli such as shear stress, cytokines, and ROS<sup>55</sup>). Synthetic SMCs proliferate and migrate more rapidly, synthesize more collagen, increase lipid synthesis, and express higher levels of SRs; therefore, differentiation to synthetic SMC contributes to the formation of foam cells<sup>56</sup>). PCSK9, which can be induced by resistin, insulin, and hemodynamic shear stress in SMCs, induces SMC differentiation to synthetic phenotype and increases migration and proliferation of synthetic type SMCs<sup>23, 57, 58</sup>).

PCSK9 deficiency protects arteries partially from neointimal formation<sup>59</sup>). There is an increased number of the synthetic phenotype of SMCs and increased PCSK9 expression in acute aortic dissection samples<sup>60</sup>). In aortic dissection with medial aortic calcification (MAC), PCSK9 expression is higher than in aortic dissection without MAC<sup>60</sup>). Thus, it can be suggested that PCSK9 plays a role in aortic dissection by contributing to the loss of structural integrity of aorta<sup>60</sup>).

### Micro-RNAs Targeting PCSK9 Expression

Micro-RNAs (miRNAs) are short non-coding RNAs (18–22 nucleotides) that function in RNA silencing and controlling post-transcriptional regulation of gene expression<sup>61</sup>). They initiate the degradation or inhibit the translation of their target mRNA by binding to the recognition element on the 3'-UTR<sup>62</sup>). By affecting gene expression, they participate in the regulation of gene expression. Being specific for a given cell and tissue, they influence the phenotype of the cell in which they are expressed. They can be found free in the circulation, bound to proteins or high density lipoprotein (HDL), and in exosomes. By entering body fluids such as blood or urine, they relay signals from the producer cell to distant targets. Their expression has been correlated with various diseases, including coronary artery disease, and they have been reported to be involved in all phases of atherosclerosis, particularly immune cell recruitment and cytokine

production<sup>63, 64</sup>).

Many miRNAs may affect PCSK9 expression<sup>65</sup>. MiR-17-5p, a potential biomarker for the diagnosis of coronary atherosclerosis, inhibits VLDLR expression in vascular SMCs via direct binding to its 3'-UTR<sup>66</sup>. Inhibition of miR-17-5p induces upregulation of VLDLR and downregulation of PCSK9 in atherosclerotic apoE -/- mice<sup>66</sup>. miR-191, miR-222, and miR-224 can bind the 3' end of the PCSK9 mRNA and thus regulate PCSK9 expression<sup>67</sup>. When cells are transfected with vectors overexpressing miR-191, miR-222, and miR-224, significant downregulation of PCSK9 is observed<sup>67</sup>.

Hypomethylation and elevated expression of miR-191 promote epithelial to mesenchymal transition in hepatocellular carcinoma<sup>68</sup>. EGR1 (early growth response protein 1) is a zinc finger transcription factor that has a binding site on the PCSK9 promoter. miR-191 targets EGR1 and can regulate PCSK9 expression in different physiological states<sup>69</sup>.

In a study by Vickers *et al.*, miR-222 level in circulating HDL was found to be 8.2-fold higher in familial hypercholesterolemia patients compared with HDL from normal subjects<sup>64</sup>. miR-222 regulates insulin sensitivity, and miR-222 levels are higher in plasma of obese human patients<sup>70</sup>. It is an important regulator in lipid metabolic pathways and a negative regulator of adipocyte differentiation<sup>71</sup>. It has been reported that miR-222 plays important roles in many physiological and pathological processes in the cardiovascular system; it also plays a role in atherosclerosis and plaque formation<sup>72</sup>. Moreover, miR-222 has been reported to decrease the PCSK9 expression<sup>67</sup>.

miR-27a induces an increase in PCSK9 and consequently decreases LDLR levels by 40% via enhancing LDLR degradation<sup>73</sup>. Silencing PCSK9 repressed miR-27a and, to a lesser extent, let-7c<sup>44</sup>.

Further studies are required to delineate the role of miRNAs in PCSK9 regulation.

## Conclusion

After its discovery in 2003, knowledge on PCSK9 has gone from bench-to bedside in less than 9 years, and PCSK9 monoclonal antibodies entered in dyslipidemia guidelines since 2016. Although the role of PCSK9 in LDL metabolism is well defined, recent studies have revealed that it has various effects on many metabolic pathways, including inflammation, sepsis, sodium metabolism, glucose metabolism, and atherosclerosis<sup>74-76</sup>. Signals of these metabolic pathways also affect PCSK9's level and function<sup>77</sup>.

It is now clear that PCSK9 contributes to every step of the molecular pathway of atherosclerosis.

Despite the rapidly accumulating data on its atherogenic effects, much is still unknown, including its signaling pathways and receptors in atherosclerosis, its acting mechanisms on vascular cells in atherosclerosis, its role in vascular diseases such as aneurysms, its interaction and signal transduction through receptors other than LDLR, the extent of miRNA regulation of PCSK9, and its relevance. Furthermore, it should not be overlooked that, although PCSK9 is a member of the proprotein convertase family, its substrate for convertase activity and why it exists are still unknown. To clarify its role in atherosclerosis and its reason for existence, further research is required.

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## Conflicts of Interest

None.

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