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VASCULAR BIOLOGY (J. HAMILTON, SECTION EDITOR)

Pleiotropic Anti-atherosclerotic Effects of PCSK9 Inhibitors *From Molecular Biology to Clinical Translation*

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Abstract

Purpose of Review Clinical trials with PCSK9 inhibitors have shown a robust decrease in plasma LDL levels and a significant reduction in the incidence of cardiovascular atherosclerotic events. However, the role of PCSK9 in atherosclerosis is not well investigated and it remains unclear whether PCSK9 inhibition has direct, LDL-independent, anti-atherosclerotic effects. This review outlines the molecular pathways and targets of PCSK9 in atherosclerosis and summarizes the experimental and clinical data supporting the anti-atherosclerotic (pleiotropic) actions of PCSK9 inhibitors.

Recent Findings PCSK9 is expressed by various cell types that are involved in atherosclerosis (e.g., endothelial cell, smooth muscle cell, and macrophage) and is detected inside human atherosclerotic plaque. Preclinical studies have shown that inhibition of PCSK9 can attenuate atherogenesis and plaque inflammation.

Summary Besides increasing plasma LDL, PCSK9 appears to promote the initiation and progression of atherosclerosis. Inhibition of PCSK9 may confer atheroprotection that extends beyond its lipid-lowering effects.

Keywords Proprotein convertase subtilisin/kexin 9 · Atherosclerosis · PCSK9 inhibitors · Pleiotropic effects · Anti-atherosclerotic effects · Evolocumab · Alirocumab · Inclisiran

Introduction

Proprotein convertase subtilisin/kexin 9 (PCSK9) is a protein that was discovered in 2003 [1–3]. Before its discovery, there were only two known genes (i.e., *LDL-R* and *ApoB*) [4] linked with familial hypercholesterolemia in humans. A gain-of-function mutation in PCSK9 gene was also found to cause familial hypercholesterolemia [5]. The canonical mode of action of PCSK9 protein involves chaperoning the receptor for low density lipoprotein (LDL) to the intracellular degradative organelles, speeding its breakdown [6]. The consequent reduction in surface LDL receptors impedes LDL clearance, yielding an increase in plasma LDL concentration. At present, many drug candidates that inhibit the PCSK9 pathway have entered preclinical or early phase clinical trials, and two of those drugs (i.e., evolocumab and alirocumab) have already received FDA approval [7, 8]. Preclinical studies showed that PCSK9 exerts pleiotropic effects beyond plasma LDL regulation and could be a key molecule in the pathophysiology of atherosclerosis [9]. The inhibition of PCSK9 attenuates the progression of atherosclerosis and reduces the risk for acute cardiovascular events [10, 11••]. However, the underlying molecular mechanisms through which PCSK9 inhibition might confer atheroprotection beyond LDL lowering remain uncertain. Recognizing the PCSK9 molecular targets other than LDL receptor and their implication on the cellular function

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and phenotype could point the way toward selective PCSK9 inhibition, and more targeted anti-atherosclerotic efficacy.

This review aims to provide an overview of the possible pleiotropic effects of PCSK9 inhibitors in atherosclerosis beyond their lipid-lowering action. We summarize the molecular pathways and targets of PCSK9 in atherosclerosis and discuss the experimental and clinical data supporting the anti-atherosclerotic (pleiotropic) actions of PCSK9 inhibitors. Even though PCSK9 inhibition appears to interfere with systems other than the heart and vasculature (i.e., liver, brain, kidney, intestine, pancreas) [12], those effects exceed the scope of this review.

PCSK9: From Gene Expression to Molecular Targets

Transcription Several transcription factors regulate PCSK9 gene expression including sterol regulatory element binding proteins-1 and -2 (SREBP-1 and SREBP-2) and hepatocyte nuclear factor 1 (HNF1) [3, 13, 14]. Various inflammatory stimuli also augment PCSK9 expression [e.g., lipopolysaccharide (LPS) or oxidized LDL] [15–18] through activation of NF-κB and possible inhibition of peroxisome proliferator activated receptor-α (PPAR-α) [19••, 20].

Translation Following transcription, the PCSK9 mRNA is translated to PSCK9 protein in the endoplasmic reticulum [6]. After leaving the endoplasmic reticulum, PCSK9 protein follows three possible pathways (Fig. 1): (i) Transportation to endosomes/autophagosomes/lysosomes exerting its intracellular actions [21, 22] (ii) Release into the extracellular space exerting its autocrine and paracrine actions [23••] and (iii) Secretion (predominantly from hepatocytes) into the circulation exerting its systemic actions [24].

Post-translational Regulation PCSK9 undergoes post-translational regulation through several mechanisms: (i) In the endoplasmic reticulum, PCSK9 binds with the endoplasmic reticulum-resident chaperone protein, GRP94, limiting its binding with LDL-R intracellularly [25], (ii) In the Golgi apparatus, PCSK9 is truncated and inactivated by furin [26], (iii) On the cellular surface, Annexin A2 binds with PCSK9 and inhibits the interaction of PCSK9 with LDL-R extracellularly [27, 28], and iv) In systemic circulation, > 40% of plasma PCSK9 is bound to apolipoprotein B100 on LDL particles, which interferes with its interaction with the cell surface LDL-R [29].

Molecular Targets of PCSK9 in Atherosclerosis The molecular and cellular targets of PCSK9 are summarized in Table 1. PCSK9 exerts all its actions through enzymatic auto-activation and subsequent tight binding with its molecular targets.

PCSK9 Inhibitors

PCSK9 inhibition can occur at several sites across PCSK9 pathway. The PCSK9 inhibitors and their mechanism of action are summarized in Table 2.

Monoclonal Antibodies Monoclonal antibodies against PCSK9 have been the most well studied and clinically tested PCSK9 inhibitors. It is the only class of PCSK9 inhibitors that gained approval by the FDA and European Medicines Agency [7, 8, 48, 49]. Monoclonal antibodies bind PCSK9 in the extracellular milieu and inhibit its interaction with extracellular molecular targets. Evolocumab and alirocumab - both fully human monoclonal antibodies against PCSK9 - have been studied in several phase 1, 2 and 3 clinical trials, which demonstrated a potent LDL lowering effect without showing safety concerns [50–53]. These two drugs have received FDA approval: (i) As an adjunct to diet and maximally tolerated statin therapy in adult patients with heterozygous familial hypercholesterolemia, and (ii) In high risk patients with clinical atherosclerotic cardiovascular disease who cannot reach the LDL target with statins or are intolerant to statins [7, 8]. In addition, evolocumab is FDA approved for homozygous familial hypercholesterolemia on top of statins [7], and as monotherapy for risk reduction of myocardial infarction, stroke, and coronary revascularization in adults with established cardiovascular disease [54]. Bococizumab is a humanized (not fully human) monoclonal antibody that was tested in phase 3 trials and was withdrawn from further development in November 2016 due to development of antibodies against the drug [37]. LY3015014 and RG7652 are additional anti-PCSK9 monoclonal antibodies currently under investigation in early clinical studies [38, 39, 55].

Small Interfering RNAs (siRNAs) Inclisiran is an siRNA engineered to selectively target hepatocytes where it promotes cleavage of intracellular PCSK9 mRNA [56]. In phase 1 and 2 clinical trials, inclisiran lowered LDL in a dose-dependent and durable fashion [40, 57].

Vaccines To date, two different PCSK9 vaccines have been developed that elicit the production of auto-antibodies against PCSK9 [42, 58, 59]. Both vaccines have demonstrated significant hypolipidemic efficacy, as well as systemic and vascular anti-inflammatory actions in animal studies [41, 42, 58]. Vaccine-mediated inhibition of PCSK9 and its long-term effects warrant further investigation.

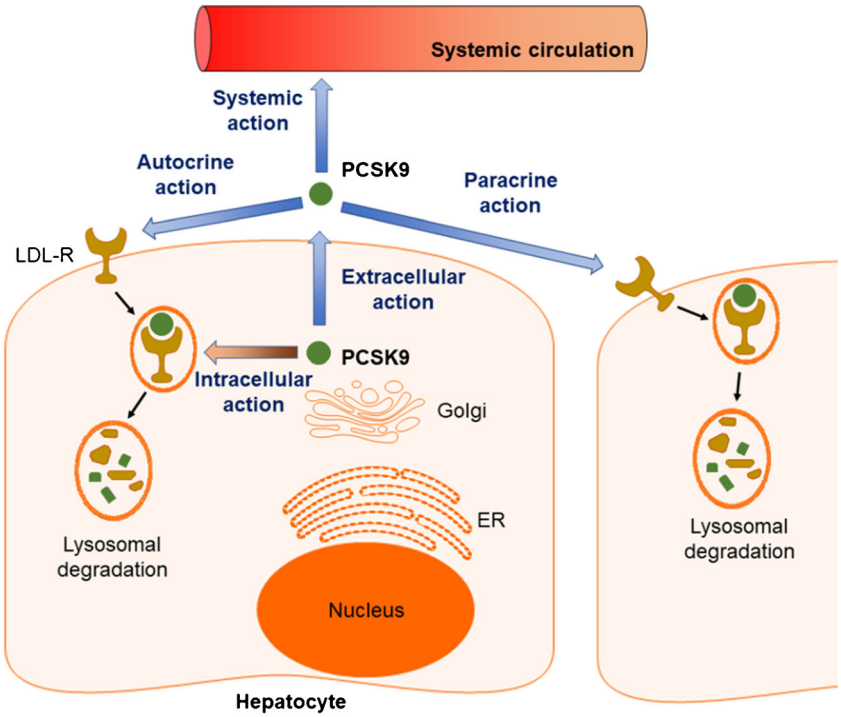

Fig. 1 Intracellular and extracellular pathways of action of PCSK9

Monobodies Adnectin BMS-962476, is a small protein that binds and suppresses free PCSK9. The drug has undergone testing in non-human primates, as well as in a phase 1 clinical trial and showed adequate efficacy in reducing LDL [43].

Preclinical PCSK9 Inhibitors Small, EGF-A mimetic, peptides that competitively block free PCSK9 have been created. In vitro studies showed that those peptides can preserve the levels of surface LDL-R and enhance LDL uptake by hepatocytes [44, 60, 61]. Annexin A2 is a peptide/natural endogenous regulator of PCSK9 that can lower PCSK9 in cell cultures [27, 28, 45]. Furthermore, small molecules that can potentially be given orally have been studied in small animals and decreased PCSK9 levels, LDL, and plaque size [46, 62].

Table 1 Direct molecular targets of PCSK9 relevant to atherosclerosis

Molecular target	Cellular target	Effect of pcsk9 on molecular target levels
LDLR [21]	Hepatocyte	Decreased effect [30]
	Macrophage	Decreased effect [23••]
VLDLR [30]	Adipocyte	Decreased effect [24]
	Smooth muscle cell	Unknown effect
	Endothelial cell	Unknown effect
	Macrophage	Unknown effect
LRP-1 [31]	Hepatocyte	Decreased effect [31]
	Smooth muscle cell	Unknown effect
	Endothelial cell	Unknown effect
	Macrophage	Decreased effect [32]
CD36 [33]	Macrophage	Unknown effect
	Hepatocyte	Decreased effect [33]
	Intestinal cell	Unknown effect
ApoB100 [22]	Hepatocyte	Increased effect [22]
ApoB48 [34]	Intestinal cell	Unknown effect

LDLR, low-density lipoprotein receptor; VLDLR, very low-density lipoprotein receptor; LRP-1, low-density lipoprotein receptor-related protein 1

Table 2 PCSK9 inhibitors

Drug category	Mechanism of action	Pharmacological characteristics	Administration route	Clinical phase	FDA approval status
Monoclonal Antibody	Neutralizes extracellular PCSK9				
Evolocumab [35]		Fully human mAb, efficient and safe profile	SC Q2W or Q4W	Phase 4 (postmarketing surveillance)	Yes [7]
Alirocumab [36]		Fully human mAb, efficient and safe profile	SC Q2W	Phase 4 (postmarketing surveillance)	Yes [8]
Bococizumab [37]		Humanized mAb	SC Q2W	Phase 3 (terminated)	No
LY3015014 [38]		Prolonged duration of action, humanized mAb	SC Q4W or Q8W	Phase 2 (completed)	No
RG7652 [39]		Fully human mAb, prolonged duration of action	SC Q4W or Q8W	Phase 2 (completed)	No
siRNA	Degrades intracellular PCSK9 mRNA				
Inclisiran [40]		Effects last 6 months, targets hepatocytes	SC, Q3M or Q6M	Phase 2 (completed)	No
Vaccine	Elicits production of autoantibodies against PCSK9				
AT04A & AT06A [41]		Long-term effects, cost effective, possible irreversibility?	SC	Phase 1 (completed)	No
Virus-like-particles [42]		Long-term effects, cost effective, possible irreversibility?	SC	Preclinical (macaques)	No
Monobody	Neutralizes extracellular PCSK9				
Adnectin BMS-962476 [43]		100 h half-life	IV or SC	Phase 1 (completed)	No
Small Peptide					
H306Y subfragment [44]	Mutated EGF-A domain—Competitive inhibitor	N/A	N/A	Preclinical (cell cultures)	No
Annexin A2 [28, 45]	Inhibits translation, inhibits PCSK9-LDLR binding	Endogenous molecule	IV	Preclinical (mice)	No
Small Molecule					
K-312 [46]	CETP inhibitor, downregulates PCSK9 transcription	Oral administration, increases HDL	PO	Phase 1 (completed)	No
R-IMPP [47]	Inhibits translation of PCSK9 mRNA	N/A	N/A	Preclinical (cell cultures)	No

SC, subcutaneously; *IV*, intravenously; *Qn^{o} W*, once every n^{o} week; *Qn^{o} M*, once every n^{o} month; *mAb*, monoclonal antibody; *CETP*, cholesterylester transfer protein; *PO*, per os; *N/A*, not available

Table 3 Clinical trials of PCSK9 inhibitors in cardiovascular diseases

Clinical trial	Objectives	Clinical phase	Number of participants	Completion date	Outcome
Evolocumab					
FOURIER [11••]	Effect on cardiovascular morbidity and mortality in patients with vascular disease	III	27,564	Completed	Reduced incidence of MI, coronary revascularization and ischemic stroke
GLAGOV [10]	Effect on coronary atherosclerotic plaque	III	900	Completed	Diminished PAV and normalized TAV, induced plaque regression
ANITSCHKOW [63]	Effect on arterial wall inflammation in patients with high concentrations of Lp(a)	III	120	Apr 2018	
Study of reference [64]	Effect on the intensity of inflammation in the vascular wall	IV	10	Oct 2020	
Study of reference [65]	Effect on platelet reactivity in patients after percutaneous coronary intervention	I	150	Jan 2019	
Alirocumab					
ODYSSEY Outcomes [66]	Effect on cardiovascular outcomes after an ACS	III	18,600	Jan 2018	
ODYSSEY J-IVUS [67]	Effect on coronary artery plaque volume in patients hospitalized for ACS	IV	200	Sep 2018	
Study of reference [68]	Effect on carotid atherosclerotic plaque	IV	30	Aug 2018	
Study of reference [69]	Effect on plaque regression in peripheral arterial disease	IV	54	Jul 2020	
PACMAN-AMI [70]	Effect on PAV reduction in coronary arteries of patients with acute myocardial infarction	III	220	Sep 2019	
Bococizumab					
SPIRE-1 and SPIRE-2 [37]	Effect on reduction of major cardiovascular events in high risk subjects	III	28,000	Terminated	Reduced incidence of cardiovascular events in high risk patients

PAV, percent atheroma volume; *TAV*, total atheroma volume; *ACS*, acute coronary syndrome

PCSK9 Inhibitors and Cardiovascular Outcomes

The effect of PCSK9 inhibition in atherosclerosis and cardiovascular outcomes using monoclonal antibodies (i.e., evolocumab, alirocumab and bococizumab) has been studied in large multicenter randomized clinical trials and is summarized in Table 3. The completed GLAGOV and FOURIER trials showed that evolocumab, on top of statins, in patients with established cardiovascular disease, promotes atherosclerotic plaque regression in coronary arteries [10] and reduces significantly the incidence of myocardial infarction, coronary revascularization and ischemic stroke by 27, 22, and 25%, respectively [11••]. Similar large trials testing the impact of alirocumab on cardiovascular outcomes and atherosclerotic plaque regression (i.e., ODDYSSEY Outcomes, ODYSSEY J-IVUS, PACMAN-AMI) are ongoing [66, 67, 70]. Bococizumab also showed favorable effects on cardiovascular outcomes in the SPIRE-1 and SPIRE-2 trials; however, these studies were terminated prematurely due to the development of anti-bococizumab antibodies in some study participants [37]. Meta-analyses of clinical trials having LDL reduction as primary end-point, showed a mortality benefit with evolocumab and alirocumab [71, 72]. The trials included in the meta-analyses did not have cardiovascular mortality as a primary outcome.

Anti-atherosclerotic Effects of PCSK9 Inhibition Beyond LDL Lowering

The FOURIER trial suggested that the outcome benefit with PCSK9 monoclonal antibodies in patients with established cardiovascular disease related directly to the LDL lowering [11••] as the outcome benefit resembled that achieved by similar lowering of LDL with statins [73]. Based on clinical investigations, loss-of-function mutations of PCSK9, resulting in very low plasma activity of PCSK9, conferred significant reduction in plasma LDL and adverse cardiovascular events [74–77]. This reduction of cardiovascular risk appeared to exceed the anticipated benefit from the LDL reduction and one could hypothesize a possible anti-atherosclerotic (pleiotropic) benefit of PCSK9 inhibition beyond LDL lowering [78]. In fact, in the FOURIER study patients with very low LDL (~20 mg/dl) still

had an event rate of ~7%, suggesting the multifactorial nature of atherosclerosis beyond LDL [11••]. However, the increased cardiovascular risk benefit achieved with PCSK9 loss-of-function mutations could also result from the cumulative beneficial effect of the lifelong LDL reduction, independent of anti-atherosclerotic pleiotropic effects [79].

The strong LDL-dependent effect of PCSK9 inhibition doubtless dominates in improving cardiovascular outcomes. However, concurrent LDL independent pathways of PCSK9 inhibition might also contribute, possibly through direct anti-inflammatory and plaque-stabilizing effects. Preliminary observations from in vitro and animal studies and limited human studies showed the presence of PCSK9 in atherosclerotic plaques [23••], and its production and secretion by various structural and immune cells that participate in the atherosclerotic process [i.e. endothelial cells (ECs) [19••], vascular smooth muscle cells (VSMCs) [23••] and macrophages [18]]. Accordingly, preliminary in vitro and animal studies showed that PCSK9 inhibition interferes with multiple processes in atherosclerosis. The ANITSCHKOW trial, a phase 3 trial investigating the effects of evolocumab on arterial wall inflammation by FDG-PET/CT imaging [63], as well as other similar trials [68, 69], might shed light into the notion of pleiotropic effects of PCSK9 inhibitors.

Furthermore, the observation that PCSK9 monoclonal antibodies lack overt LDL-independent effects on outcomes does not necessarily translate to the whole class of PCSK9 inhibitors, given that the monoclonal antibodies interfere with the plasma PCSK9 and not with intracellular PCSK9. PCSK9 inhibition might affect vascular cell targets other than the LDL receptor (Fig. 2).

Molecular Pathways of Anti-atherosclerotic Pleiotropic Effects of PCSK9 Inhibition

The molecular effects of PCSK9 inhibition in atherosclerosis are summarized in Table 4 and Fig. 3.

PCSK9 Inhibition, Endothelial Shear Stress (ESS) and Vascular Cells Pro-Inflammatory Response In vitro studies showed that low ESS-a major pro-atherosclerotic factor [94]-promotes the expression of PCSK9 by ECs and VSMCs [19••]. In line with those in vitro studies, investigations in mouse atherosclerotic aortas and human atherosclerotic plaques demonstrated that the expression of PCSK9 co-localizes with low ESS [19••, 23••]. Sterol regulatory element binding protein-1 transcription factor, augmented by low ESS can boost PCSK9 expression [95] and thus might link ESS to PCSK9, but this mechanism remains unproven.

Furthermore, a bidirectional positive feedback prevails between PCSK9 and major pro-inflammatory modulators [i.e. NF-κB, reactive oxygen species, lectin-type oxidized LDL receptor-1 (LOX-1)] [16••, 19••, 80], which also respond to low ESS [94]. Transfection of cultured ECs and VSMCs with anti-PCSK9 siRNA suppressed significantly the expression of NF-κB and LOX-1 [16••, 19••], whereas PCSK9 knockout mice treated with LPS – a potent pro-inflammatory mediator - had significantly reduced expression of LOX-1, vascular cell adhesion molecule-1 (VCAM-1) and interleukin 1 (IL-1) in vascular tissues compared to those in wild type mice [16••]. Taken together, these data associate low ESS, pro-inflammatory modulators, PCSK9 and pro-inflammatory EC and VSMC activation, suggesting a role of PCSK9 inhibition in blocking this link (Fig. 4) [16••, 19••, 80].

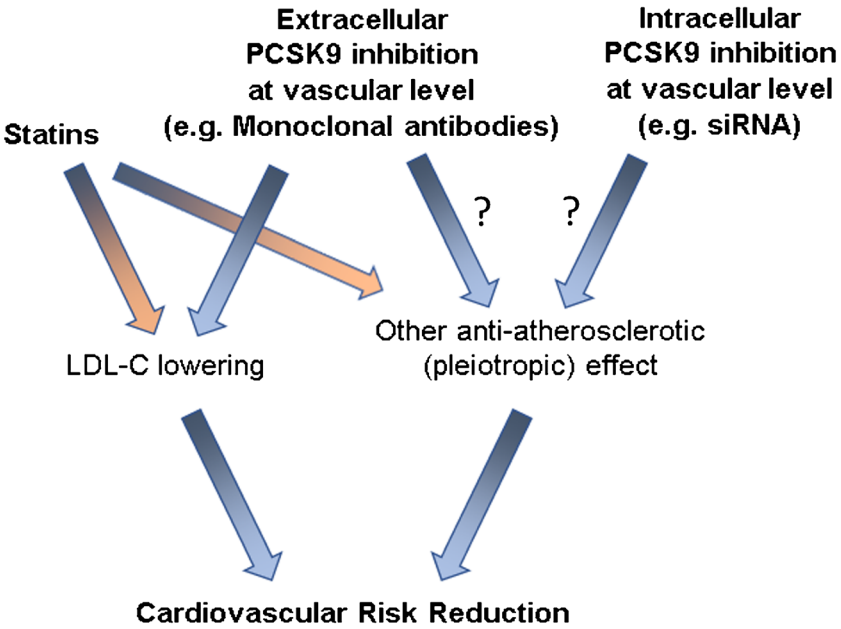

Fig. 2 Conceptual LDL-dependent and LDL-independent implication of PCSK9 inhibitors in cardiovascular risk reduction

Table 4 Pleiotropic effects of PCSK9 inhibition in atherosclerosis

Vascular effect	Molecular effect	Possible target receptor
PCSK9 inhibition and vascular cells pro-inflammatory response		
Reduces expression of pro-inflammatory molecules	Reduces NF-κB, LOX-1 [16••, 19••]	TLR4/NF-κB [80]
Reduces expression of membrane adhesive molecules	Reduces VCAM-1 [16••] and ICAM-1 [81]	NF-κB axis
PCSK9 inhibition and oxidative stress		
Reduces oxidative stress	Reduces NADPH and ROS [19••]	NF-κB axis
PCSK9 inhibition and early plaque inflammation		
Reduces macrophages accumulation	Reduces VCAM-1 [16••], ICAM-1 [41, 81] and MCP-1 [80], TNF-α, IL-1, IL-6 [18, 80]	NF-κB axis
Increases cholesterol efflux in macrophages	Increases ABCA1 cholesterol efflux protein [82•]	NF-κB axis, TLR4/NF-κB [80], LDLR [83]
PCSK9 inhibition and VSMCs		
Promotes contractile phenotype of VSMC	Increases alpha-smooth muscle actin and myosin heavy chain II, Reduces collagen I and caldesmon [84•]	LRP-1 [85], VLDLR [86]
Reduces VSMC proliferation	Unknown	LRP-1 [85], VLDLR [86]
Reduces VSMC migration	Activates lamellipodia and Rac 1 [84•]	LRP-1 [85], VLDLR [86]
PCSK9 inhibition and advanced plaque inflammation		
Reduces apoptosis in endothelial cells [87], VSMCs [88•] and macrophages [89]	Increases Bcl-2 [87, 88•]	Bax - Bcl-2 axis
Promotes autophagy	Reduces mTOR [88•]	mTOR axis [90]
Promotes efferocytosis	Unknown	LRP-1 [91]
PCSK9 inhibition and necrotic core		
Decreases necrotic core size	Decreases the necrotic content of a plaque [81]	
PCSK9 inhibition and thrombosis		
Reduces platelet reactivity	Reduces platelet reactivity in patients with hypercholesterolemia [92]	Reduction in circulating LDL [93]

LOX-1, lectin-type oxidized LDL receptor 1; *TLR4*, toll-like receptor 4; *VCAM-1*, vascular cell adhesion molecule 1; *ICAM-1*, intercellular adhesion molecule 1; *ROS*, reactive oxygen species; *MCP-1*, monocyte chemoattractant protein 1; *ABCA1*, ATP-binding cassette transporter 1; *LRP-1*, low-density lipoprotein receptor-related protein 1; *VSMC*, vascular smooth muscle cell; *mTOR*, mechanistic target of rapamycin

PCSK9 Inhibition and Oxidative Stress The oxidation of LDL in the subendothelial space by locally produced reactive oxygen species may contribute to atherogenesis [96]. Transfection of EC and VSMC cultures with PCSK9 siRNA significantly decreased the production of reactive oxygen species by 30% and 50%, respectively [19••]. Furthermore, in in vivo experimental studies, PCSK9-deficient mice expressed significantly less NADPH oxidase—a major enzyme that generates reactive oxygen species—and subsequently reduced production of reactive oxygen species in aortas compared to wild type mice, suggesting a direct anti-oxidative effect of PCSK9 inhibition [19••].

PCSK9 Inhibition and Early Plaque Inflammation *(i) PCSK9 Inhibition and Plaque Macrophages and T Cells:* PCSK9 promotes monocyte recruitment into the plaque by augmenting the expression of VCAM-1 and monocyte chemoattractant protein-1 (MCP-1) in vitro and in mice [16••, 80]. In addition, mice transplanted with human PCSK9-positive macrophages showed a significant increase in plaque macrophage accumulation compared to controls. Of note, this pro-inflammatory effect of human PCSK9 producing macrophages did not depend on serum LDL levels [32, 97]. PCSK9 inhibition in cultured VSMCs and in mice significantly decreased the expression of VCAM-1 [16••] and intracellular adhesion molecule-1 (ICAM-1) [41, 81]. Likewise, silencing tissue PCSK9 expression with lentivirus-PCSK9 shRNA in mice reduced significantly the expression of MCP-1 and subsequently blunted the accumulation of macrophages in aortic plaques in an LDL independent manner [80]. Similarly to macrophages, aortic plaque T cell accumulation dropped significantly in mice treated with alirocumab [81].

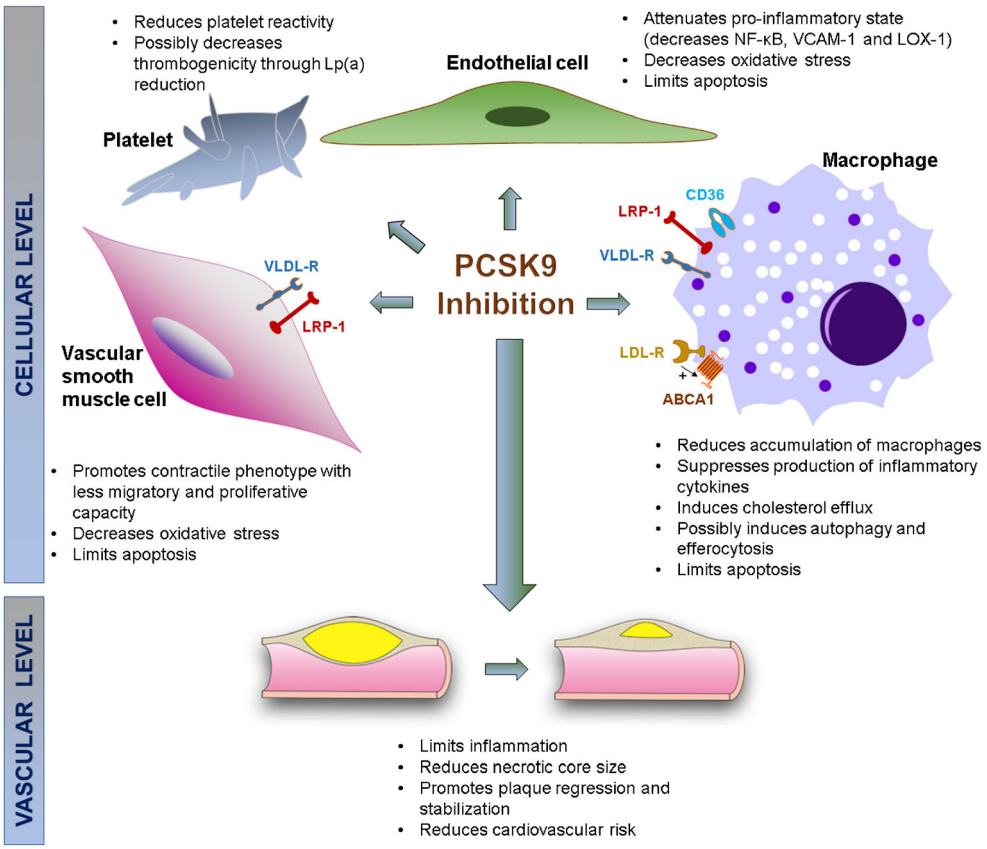

Fig. 3 Cellular and vascular pleiotropic effects of PCSK9 inhibition

(ii) PCSK9 Inhibition and Cytokine Production by Macrophages: Internalized oxidized LDL stimulates the production of PCSK9 and cytokines (e.g., TNF-α, IL-1, IL-6) by macrophages [18] that further aggravate inflammation. PCSK9 inhibition in vitro suppresses the production of inflammatory cytokines (e.g., TNF-α, IL-1, IL-6) in macrophages through downregulation of NF-κB [18, 80]. Furthermore, silencing of PCSK9 expression in vivo in ApoE knockout atherosclerotic mice attenuated significantly the expression of pro-inflammatory molecules (i.e., TNF-α, IL-1, TLR4 and NF-κB) in aortic lesions [80]. More importantly, inhibition of PCSK9 expression in those mice did not interfere with plasma lipid levels, suggesting that the beneficial anti-inflammatory effect of PCSK9 inhibition unlikely depended on plasma LDL [80].

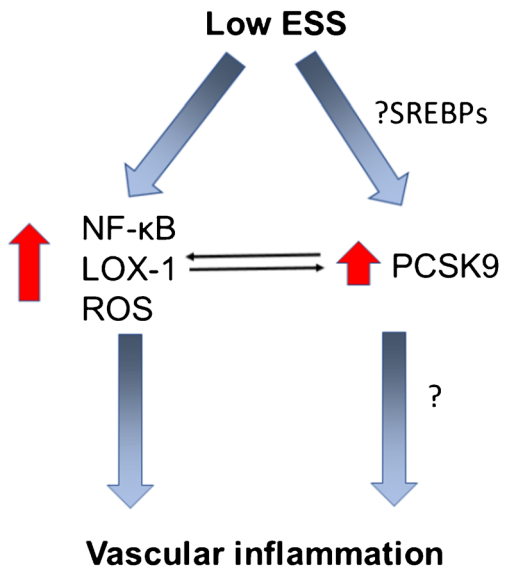

Fig. 4 Conceptual interplay among ESS, PCSK9 and pro-inflammatory vascular cell activation

(iii) PCSK9 Inhibition and Accumulation of Oxidized LDL in Macrophages: LDL accumulation in macrophages is determined by the net balance between LDL influx mediated by CD36, LOX-1 and other scavenger receptors [98] and LDL efflux modulated by several membrane proteins, including the ATP-binding membrane cassette transport proteins A1 and G1 [99]. The role of PCSK9 in this LDL equilibrium in macrophages is not well understood. As PCSK9 leads to degradation of CD36, PCSK9 might prevent LDL loading into macrophages [33, 100]. However, some studies also showed that PCSK9 limits ATP-binding membrane cassette transport protein A1 expression through LDL-R depletion and consequently impairs LDL efflux in macrophages [82•]. Conversely, PCSK9 inhibition appears to stimulate LDL-R and subsequently to increase ATP-binding membrane cassette transport protein A1 in macrophages, thereby promoting cholesterol efflux [82•]. Whether the PCSK9 inhibition-mediated

reduction in LOX-1 attenuates LDL influx in macrophages requires future investigation.

PCSK9 Inhibition and VSMC De-Differentiation, Migration, and Proliferation Following EC activation, local production of platelet-derived growth factor B and matrix metalloproteinases may promote VSMC phenotypic modulation, migration, and proliferation [101]. PCSK9 appears to participate in these pathologic processes [84•]. VSMCs isolated from aortas of PCSK9 knockout mice demonstrated increased contractile VSMC markers, reduced cytoskeletal remodeling and migratory activity, as well as slower proliferation compared to VSMCs from $PCSK9^{+/+}$ mice [84•]. The addition of PCSK9 to cultured $PCSK9^{-/-}$ VSMCs restored migration and proliferation [84•]. In vivo investigations of perivascular carotid collar placement showed that $PCSK9^{-/-}$ mice developed significantly less neointimal thickening than $PCSK9^{+/+}$ mice, consistent with an anti-proliferative effect of PCSK9 inhibition, which could also reduce in-stent restenosis if translatable to humans [84•]. Some of these effects of PCSK9 inhibitors might result from increases in the low-density lipoprotein receptor-related protein 1 (LRP-1) and the very-low-density-lipoprotein receptor (VLDLR) in VSMCs [84•, 85].

PCSK9 Inhibition and Advanced Plaque Inflammation *(i) PCSK9 Inhibition and Apoptosis:* PCSK9 may promote apoptosis of all major cell types involved in atherosclerosis, whereas PCSK9 inhibition exerts the opposite effect [87–89, 102]. Human umbilical vein ECs incubated with oxidized LDL demonstrated attenuated apoptosis after treatment with PCSK9 siRNA [87, 102]. In the same fashion, PCSK9 inhibition of cultured VSMCs decreased mitochondrial DNA damage and increased Bcl-2, thereby reducing VSMC apoptosis [88•]. Also, PCSK9 siRNA attenuated macrophage apoptosis induced by oxidized LDL [89]. Further to in vitro investigations, in vivo experiments in PCKS9 knockout mice showed significantly diminished expression of EC and VSMC apoptotic markers in aortic tissue [88•].

(ii) PCSK9 Inhibition and Autophagy: Autophagy may contribute to atherosclerosis resolution, by clearing dysfunctional or senile cellular components intracellularly thus preventing the expansion of inflammation [103]. Data on the role of PCSK9 inhibitors in autophagy in atherosclerotic plaque are limited. Addition of PCSK9 siRNA to VSMCs cultures seemed to promote autophagy and cellular vitality via the mTOR pathway [88•]. These aspects warrant further research.

(iii) PCSK9 Inhibition and Macrophage Efferocytosis: Efferocytosis is a process of inflammation resolution through which phagocytes remove necrotic cells [104]. Since macrophage LRP-1 deficiency impairs macrophage efferocytosis [91] and PCSK9 inhibition increases membrane LRP-1 in macrophages [32], PCSK9 inhibition might sustain macrophage efferocytosis.

PCSK9 Inhibition and Necrotic Core: Mice treated with alirocumab demonstrated fewer macrophages and a significantly smaller necrotic core size than controls, alterations that might "stabilize" plaque [81]. Of note, these effects appeared to depend on LDL lowering.

PCSK9 Inhibition and Thrombosis In a recent study, high plasma PCSK9 associates with increased platelet reactivity and increased recurrence of acute coronary events at one-year of follow-up [105]. PCSK9 inhibition with monoclonal antibodies in patients with hypercholesterolemia significantly reduced platelet reactivity [92]. Furthermore, in human trials, PCSK9 inhibitors are associated with a robust decrease of lipoprotein a, a highly pro-atherosclerotic and pro-thrombogenic particle [106, 107]. Whether PCSK9 inhibition could reduce the thrombotic events through reduction of platelet reactivity and lipoprotein a warrants further investigation.

Conclusions

The multifaceted protein PCSK9 affects many crucial biological processes, including lipid metabolism. The advent of PCSK9 inhibitors, which have demonstrated clinical efficacy, provide an important new tool in our fight against atherosclerotic diseases, as well as a victory of translational science. The FOURIER study affirmed the notion that PCSK9 inhibition could provide protection against atherosclerotic cardiovascular diseases beyond existing conventional therapy.

Undoubtedly, plasma LDL drives atherogenesis. Consequently, the LDL reduction induced by PCSK9 inhibition likely comprises the main underlying mechanism of the observed atheroprotection. Yet, the mechanism(s) by which PCSK9 inhibitors benefit cardiovascular outcomes remains incompletely unraveled. Other effects in addition to decreased plasma LDL, might also be involved. Potential pleiotropic effects of PCSK9 inhibition merit consideration in this regard. The actions of pharmacologic agents beyond the original supposed specific target often emerge with time of study. Recent evidence links PCSK9 to inflammation, and shows that PCSK9 inhibition affects several processes implicated in plaque formation and complication. Nevertheless, the molecular pathways through which inflammatory stimuli induce PCSK9 expression and PCSK9 promotes atherosclerosis remain incompletely elucidated. Mounting evidence indicates that PCSK9 inhibition blunts the pro-inflammatory activation of EC and VSMC, attenuates plaque oxidative stress and inflammation, decreases VSMC modulation, migration and proliferation, reduces apoptosis of EC, VSMC and macrophage, and might limit thrombosis, ultimately reducing the propensity of a plaque to produce a clinical event.

As we further unfold PCSK9's actions and we develop highly effective and safe-profile drugs that target this

molecule, PCSK9 inhibitors might prove effective in other contexts (e.g., peripheral [108] and carotid artery disease, acute coronary syndromes, stent restenosis, and cardiomyopathies [109]). Consideration of the full palette of the consequences of PCSK9 inhibition could guide this quest and amplify our understanding of the underlying mechanisms of potential benefits.

Compliance with Ethical Standards

Conflict of Interest Angelos D. Karagiannis, Martin Liu, Shijia Zhao, Devendra K. Agrawal, and Yiannis S. Chatzizisis declare no conflicts of interest. Peter P. Toth declares personal fees from Regeneron, personal fees from Amgen, and personal fees from Sanofi, outside the submitted work. Peter Libby declares unpaid consultation to Amgen, Esperion Therapeutics, and Sanofi-Regeneron, outside the submitted work.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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