REVIEW

PCSK9 in Metabolism and Diseases

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Running Title:

Therapeutic Targeting of PCSK9

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Abstract

PCSK9 is a serine protease that regulates plasma levels of low-density lipoprotein (LDL) and cholesterol by mediating the endolysosomal degradation of LDL receptor (LDLR) in the liver. When PCSK9 functions unchecked, it leads to increased degradation of LDLR, resulting in elevated circulatory levels of LDL and cholesterol. This dysregulation contributes to lipid and cholesterol metabolism abnormalities, foam cell formation, and the development of various diseases, including cardiovascular disease (CVD), viral infections, cancer, and sepsis. Emerging clinical and experimental evidence highlights an imperative role for PCSK9 in metabolic anomalies such as hypercholesterolemia and hyperlipidemia, as well as inflammation, and disturbances in mitochondrial homeostasis. Moreover, metabolic hormones – including insulin, glucagon, adipokines, natriuretic peptides, and sex steroids - regulate the expression and circulatory levels of PCSK9, thus influencing cardiovascular and metabolic functions. In this comprehensive review, we aim to elucidate the regulatory role of PCSK9 in lipid and cholesterol metabolism, pathophysiology of diseases such as CVD, infections, cancer, and sepsis, as well as its pharmaceutical and non-pharmaceutical targeting for therapeutic management of these conditions.

Keywords: PCSK9, Lipid and Cholesterol Metabolism, Metabolic Hormones, Inflammation, Cardiovascular Disease (CVD), Atherosclerosis, Clinical Trials.

Significance Statement:

PCSK9 is a promising target for reducing excessive serum LDL levels. This review offers comprehensive guidance on pharmacological strategies aimed at specifically targeting PCSK9 to

lower circulatory LDL, thereby alleviating hypercholesterolemia, atherosclerosis, and related diseases.

Abbreviations:

ABCA1, ATP binding cassette subfamily A member 1; ACE2, angiotensin converting enzyme 2; ADAM10, ADAM metallopeptidase domain 10; ADAM17, ADAM metallopeptidase domain 17; AMPK, AMP-activated protein kinase; AnxA2, annexin A2; APLP2, amyloid precursor-like protein 2; ApoE, apolipoprotein E; ApoER2, apolipoprotein E receptor 2; ARH, autosomal recessive hypercholesterolemia; ASGPR, asialoglycoprotein receptor; ATM, ATM serine/threonine kinase; cAMP, cyclic adenosine monophosphate; Cas9, CRISPR associated protein; CD36, CD36 molecule; CD40L, CD40 ligand; CETP, cholesteryl ester transfer protein; ChREBP, carbohydrate response element binding protein; CRISPR, clustered regularly interspaced short palindromic repeats; E2F1, E2F transcription factor 1; EGF-A, epidermal growth factor; EPAC2, exchange protein directly activated by cAMP 2; FAM20C, FAM20C golgi associated secretory pathway kinase; FOXO3, forkhead box O3; GCGR, glucagon receptor; GPER, G protein-coupled estrogen receptor; GRP94, glucose-regulated protein 94; HINFP, histone H4 transcription factor; HMGCR, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; HNF1α, hepatocyte nuclear factor-1 alpha; hs-CRP, high-sensitivity C-reactive protein; HSP25, heat shock protein 25; HSP27, heat shock protein 27; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon gamma; IFPT, immunogenic fused PCSK9-tetanus; IgG, immunoglobulin G; IgG1, immunoglobulin G1; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-10, interleukin 10; IL-17, interleukin 17; INSR, insulin receptor; IRF7, interferon regulatory factor 7; KLH, keyhole limpet hemocyanin; LKB1, liver kinase B1; LNA, locked nucleic acid; LRP5, LDL receptor related

protein 5; LRPAP1, LDL receptor related protein associated protein 1; MAPK14, mitogen-activated protein kinase 14; MCP-1, monocyte chemoattractant protein-1; MHC, major histocompatibility complex; MHC-I, major histocompatibility complex class I; NF-κB, nuclear factor kappa B; NLRP3, nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3; Nox2, NADPH oxidase 2; NPAT, nuclear protein, coactivator of histone transcription; *PDCD1*/PD-1, programmed cell death 1; PKA, protein kinase A; RAP1A, member of RAS oncogene family; ROS, reactive oxygen species; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; S1P, sphingosine-1-phosphate; S100A10, S100 calcium-binding protein A10; SIRT1, sirtuin1; SIRT6, sirtuin 6; SREBF1, sterol regulatory element binding transcription factor 1; SREBF2, sterol regulatory element binding transcription factor 2; SREBP1, sterol regulatory element-binding protein 1; SREBP2, sterol regulatory element-binding protein 2; STAT3, signal transducer and activator of transcription 3; STING, stimulator of interferon genes; TCR, T-cell receptor; TLR3, Toll-like receptor 3; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor alpha; TRRAP, transformation/transcription domain associated protein; VEGF, vascular endothelial growth factors; VLDL, very-low-density lipoprotein.

Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a positive regulator of circulatory low-density lipoprotein (LDL) and cholesterol, first identified nearly two decades ago [1-3]. Although PCSK9 is produced by multiple organs including brain, kidney, and small intestine, the liver serves as the primary site in human and other primates [4]. Similar to chaperones, PCSK9 binds to the LDL receptor (LDLR) on hepatocytes, facilitating its endocytic internalization and endolysosomal degradation. This process ultimately depletes LDLRs from the hepatocyte cell membrane [5]. As a result, hepatocytes fail to uptake circulatory LDL, leading to its accumulation in the blood, causing hypercholesterolemia associated with foam cell formation and development of atherosclerosis [6]. Indeed, PCSK9 is associated with both atherogenesis and cancer progression [7]. Although this will be discussed in greater detail in the sections below, it is important to note that elevated plasma levels of PCSK9 correlate with increased cholesterol levels, thereby heightening the risk of plaque formation and atherosclerosis [7]. Conversely, PCSK9 can bind to surface antigens on cancer cells, facilitating their lysosomal degradation. This process reduces their visibility to immune cells, contributing to tumor progression [7]. A growing body of clinical and experimental evidence has confirmed a link between hepatic production of PCSK9 and pathophysiology of CVD [8, 9]. Conversely, certain therapeutics can lower hypercholesterolemia and CVD risk by targeting PCSK9. In this in-depth review, we will delineate the biology and regulation of PCSK9, impact of metabolic hormones and transcription factors, as well as its regulatory role in lipid and cholesterol metabolism. Pro-inflammatory circuitries involving PCSK9 in the development of atherosclerosis, as well as clinical implication of PCSK9 in various subsets of diseases, in particularly, CVD will be thoroughly discussed. Finally, we will lay out therapeutic avenues for clinical management of metabolic diseases through regulation and targeting of PCSK9.

PCSK9: biology and function

PCSK9 is a circulatory enzyme predominantly produced by the liver and positively regulates circulatory levels of LDL through degradation of LDL receptor (LDLR), which serves as a LDL sink [10-13]. Therefore, serum concentrations of PCSK9 closely correlate with circulatory levels of LDL and cholesterol [14]. PCSK9 is synthesized in the endoplasmic reticulum (ER) as a 692 amino-acid preproprotein comprising a C-terminal Cys/His-rich domain (amino acids 453-692), a hinge region (amino acids 405-452), a catalytic domain (amino acids 153-404), a pro-domain (amino acids 31-152), and an N-terminal signal peptide (amino acids 1-30) (**Fig 1, 2**) [11, 15]. Autocatalytic cleavage of PCSK9 in the ER splits the propeptide (up to residue 152) and the mature Asn-glycosylated PCSK9 protease [11, 16]. Hence, autocatalytic activity is a pre-requisite for PCSK9 release from the ER. Subsequently, the mature PCSK9 protein and propeptide bind and form an inactive heterodimer transiting through the Golgi apparatus prior to its secretion into the extracellular matrix (ECM) [17]. Finally, the heterodimer binds to LDLR thus eliciting its endocytosis via clathrin-coated vesicles and ultimate degradation in endolysosomes (**Fig 2**) [18, 19]. On mechanistic ground, protease domain of PCSK9 binds to EGF-Adomain of LDLR under acidic milieu in endolysosomes, causing proteolysis of PCSK9-LDLR complex [15, 20]. Upon PCSK9 secretion into the ECM, PCSK9 N-terminal prodomain containing acidic amino acids tightly binds to basic amino acids of catalytic M1-subdomain, rendering autoinhibition in protease activity of PCSK9 [21, 22]. In extrahepatic tissues, annexin A2 inhibits protease activity of PCSK9 by binding to C-terminal M1/M3 subdomains [23, 24]. Hence, PCSK9 primarily acts as a protease during its autocatalytic processing in the ER, and secreted mature PCSK9 is attached with the inhibitory prodomain [11, 25]. PCSK9 prodomain also facilitates the binding of PCSK9 to LDL

cholesterol (LDLc) in the plasma [26-28]. Additionally, the M2 subdomain is critical for directing the PCSK9-LDLR complex toward lysosomal degradation, likely through extracellular interaction between the M1/M3 subdomains and the acidic prosegment of PCSK9 with CAP1 protein [29, 30]. Furthermore, APLP2 protein binds to the C-terminal domain of PCSK9, promoting either its solo endocytosis or that of the PCSK9-LDLR complex for subsequent lysosomal degradation [31]. Of note, there is a some uncertainty regarding the roles of sortilin and APLP2 in regulating PCSK9 [32]. Intriguingly, non-enzymatic formation of the PCSK9-LDLR complex can occur either at the cell surface or within the Golgi apparatus [33] (**Fig 2**). Within cells, PCSK9 can interact with LDLR in the trans-Golgi site before directing it for lysosomal degradation [33, 34]. Alternatively, circulatory PCSK9 reaches the liver, where it binds to the EGF-A domain of LDLR on the cell surface. In the liver, this extracellular binding mode is quantitatively more prominent than the intracellular PCSK9-LDLR interaction. The cell surface biding mode requires ARH adaptor protein that binds to cytoplasmic LDLR motif thus pulling it into clathrin-coated vesicles [33, 35] (**Fig 2**). Hence, *ARH* deficiency prevents LDLR degradation by blocking internalization of PCSK9-LDLR complex [36]. However, the intracellular binding modality does not require ARH for LDLR degradation [37]. Other than LDLR, PCSK9 can bind to and promote degradation of very-low-density lipoprotein receptor (VLDLR), ApoER2, and CD36 [38, 39]. However, a higher concentration of PCSK9 is required to interact with these receptors [40]. VLDLR, which contains an EGF-A domain similar to LDLR, is predominantly expressed in the heart and adipocytes [38]. Intracellular PCSK9 inhibits visceral adipogenesis by regulating VLDLR levels [41]. LRP1, which uptakes apolipoprotein B (ApoB) remnants similar to LDLR [42], has also been shown to be degraded by PCSK9 in cell culture models [42]. ApoB100 serves as the main protein constituent of LDL, possessing positive charges that facilitate LDL-LDLR binding and subsequent

endocytosis [43]. Thus, PCSK9-mediated degradation of LRP1 leads to increased levels of circulatory LDL and a heightened risk of hypercholesterolemia [44].

PCSK9 regulation: role of transcription factors

SREBP2/*SREBF2* is a transcription factor that mediates transcriptional regulation of a subset of cholesterol metabolism genes such as *LDLR* and *PCSK9* [45]. SREBP2 is the primary downstream transactivator of *PCSK9* gene incorporating a sterol-regulatory element (SRE) in its promoter [34, 46]. HNF1α is a liver-sessile transcription factor that binds to the HNF1 site of *PCSK9* promoter in proximity of the SRE, causing its upregulation [47-49] (**Fig 2**). HINFP transcription factor also binds to *PCSK9* promotor between HNF1 and SRE sites to transactivate *PCSK9* [50]. On mechanistic ground, a combination of HINFP and NPAT (a cofactor of HINFP) and TRRAP (a cofactor of histone acetyltransferases) upregulates *PCSK9* expression by mediating acetylation of histone H4 in the promoter [50]. Conversely, histone deacetylase SIRT6 negatively regulates *PCSK9* expression through deacetylation of histone H3 in *PCSK9* promoter. To this end, liver-specific ablation of *SIRT6* upregulates *PCSK9* expression [51]. In addition, SIRT6 and FOXO3 suppress liver expression of *SREBF2*, thereby downregulating *PCSK9* expression [52]. Importantly, fasting promotes LDLc clearance from the plasma through activation of SIRT6 and FOXO3 and downregulation of *PCSK9* expression in the liver [51] (**Fig 2**).

PCSK9 regulation: role of metabolic hormones and diets

Insulin and glucagon

Insulin resistance and type-2 diabetes mellitus contribute significantly to the pathophysiology of CVD [53]. In mice, high carbohydrate intake upregulates *PCSK9* expression, while fasting for 24 hours drastically downregulates both *PCSK9* gene and protein expression [54]. In mouse livers, insulin upregulates *PCSK9* expression through the activation of SREBP1/*SREBF1* transcription factor, independent of glucose metabolism. These findings suggest that a carbohydrate-rich diet and hyperinsulinemia regulate *PCSK9* expression. Thus, it is reasonable to speculate that appropriate fasting or addressing hyperinsulinemia might help restrain *PCSK9* expression, thereby reducing serum LDLc level and prevent CVD. Consistently, insulin upregulates *PCSK9* and LDLR degradation through upregulation of SREBP1, while *INSR* (encoding insulin receptor) deficiency induces PCSK9 downregulation in rat hepatocytes [55]. In mouse β–cells, *PCSK9* deficiency does not influence insulin secretion or islet content of cholesterol, indicating that PCSK9 inhibition may not exacerbate diabetes [56-58] Moreover, the absence of hepatocyte-produced PCSK9 – targeted by anti-PCSK9 monoclonal antibodies - does not impair insulin secretion or pancreatic β-cell function, suggesting no indirect pro-diabetic side effects [59]. However, mendelian randomization studies indicate that *PCSK9* loss-of-function (LOF) variants can reduce serum LDLc while increasing fasting glucose levels and the risk of type 2 diabetes [58]. Therefore, clinical trials involving PCSK9 inhibition should address potential safety concerns related to diabetes risk. In mice, pancreas-deficiency of *PCSK9* induces defects in insulin secretion and modulates cholesterol content and LDLR expression in pancreatic β-cells both *in vitro* and *in vivo* [60]. Furthermore, *PCSK9* knockdown affects the secretome, proteome, and transcriptome, enhancing insulin secretion in human pancreatic β-cell line EndoC-βH1 [61]. Interestingly, neither genetic ablation nor alirocumab-mediated inhibition of PCSK9 notably

affects insulin secretion from EndoC-βH1 cells, countering concerns about diabetes risk following PCSK9 inhibition [62]. These controversies highlight the need for further research and exploration.

Glucagon is another pancreatic hormone that plays a crucial role in glucose and cholesterol metabolism [63]. Emerging evidence suggests that activation of hepatic GCGR signaling regulates PCSK9 levels and cholesterol homeostasis [64]. In mice, genetic ablation of hepatic *GCGR* upregulates PCSK9, resulting in reduced LDLR and elevated circulating LDLc. Furthermore, glucagon regulates PCSK9 homeostasis and degradation via downstream EPAC2 and RAP1 proteins [64]. Collectively, these data denote that glucagon promotes PCSK9 degradation and shortens its half-life through activation of GCGR-EPAC2-RAP1 signaling. In this sense, GCGR activation might be envisaged for PCSK9 downregulation and reduction in circulatory LDLc [64].

Estrogen

Estrogen likely accounts for the "female advantage" in cardiovascular health by preventing atherosclerosis and CVD through suppression of inflammatory responses [65]. It is hypothesized that estrogen levels may correlate with PCSK9 and plasma levels of LDLc. To examine this hypothesis, fasting blood samples were collected from 189 healthy male and 206 healthy female participants to assess circulatory levels of 17β-estradiol/estradiol (E2) and PCSK9. Results indicated that PCSK9 levels in postmenopausal women are higher than those of their male counterparts and premenopausal females, leading to elevated levels of LDLc and CVD prevalence. Conversely, PCSK9 levels are reduced and inversely correlated with estrogen levels in premenopausal women [66], suggesting that estrogen levels negatively regulates PCSK9. However, a study involving a large diverse population found that PCSK9 levels are remarkably

higher in postmenopausal than premenopausal women, seemingly independent of estrogen [67]. Furthermore, in L02 cells, the red wine polyphenol resveratrol blocks *SREBF1* expression through activation of ERα (estrogen receptor alpha), thereby suppressing *PCSK9* expression [68]. This indicates that estrogen may modulate *PCSK9* expression through ERα signaling ~~[68]~~. Given that ERα-*SREBF1* axis plays a regulatory role in *PCSK9* expression, activating ERα signaling could be a new therapeutic paradigm for inhibition of *PCSK9* expression. Testosterone does not modulate circulatory levels of PCSK9 in men, while E2 levels are inversely correlated with PCSK9 levels in women [69]. E2 is believed to trigger receptor-mediated clearance of PCSK9 rather than direct modulation of *PCSK9* expression [69]. In HepG2 cells, E2 prevents LDLR degradation by PCSK9 through activation of GPER, which is closely linked to estrogen-receptor signaling [70]. Therefore, pharmaceutical inhibition or genetic ablation of GPER and estrogen-receptor signaling might prevent LDLR degradation and lower circulatory LDLc. However, a controversial study enrolling 727 healthy elderly women has refuted a link between estrogen and elevated PCSK9 levels in the elderly [71]. Based on this finding, physiological levels of E2 do not modulate *PCSK9* expression in cultured human hepatocytes [71]. On the other hand, endogenous estrogen induces hepatic maintenance of LDLRs through post-transcriptional modifications, thereby reducing circulatory levels of PCSK9 and LDLc [72]. Collectively, these observations indicate that estrogen tends to avert LDLR degradation via hepatic suppression of *PCSK9* expression. In response to E2, female ovariectomized *PCSK9* knockout (KO) mice maintain cholesterol synthesis but release excess LDLR into plasma likely owing to enhanced activity of ADAM17/ADAM10 proteins [73]. Similarly, in female mice under PCSK9 monoclonal antibody (mAb) treatment, excess LDLR appears in plasma [73]. These findings denote that *PCSK9* deficiency promotes LDLR levels in

female and male mice in a different manner, thus explaining the comparatively low efficiency of PCSK9-mAb in females [74].

Adipokines: leptin and resistin

Leptin is an adipokine primarily produced by adipocytes. In the context of adipose tissue damage, the release and production of leptin are altered, contributing to CVD [75]. To uncover the impact of leptin on hepatic expression of PCSK9 and LDLR, HepG2 cells are treated with human recombinant leptin. This treatment results in the upregulation of *PCSK9* and *HNF1α* expression and the downregulation of *LDLR* through activation of MAPK14 [76]. These observations suggest that leptin contributes to dyslipidemia and elevated circulating LDLc via activation of the MAPK14-HNF1α-PCSK9 signaling. Hence, increased levels of adipokines in obesity may imperil cardiovascular health. Therefore, inhibition of MAPK14 signaling through pharmaceutical/genetic measures might reduce *PCSK9* expression. Thus far, numerous efforts have been allocated for the development of MAPK14 inhibitors [77]. In this sense, a pyrazole derivative compound 1 has a promising potential for inhibition of MAPK14 [78]. However, compound 1 is less appreciated due to its unfavorable cell membrane permeability [78]. Hence, pharmacodynamic and pharmacokinetic optimizations may yield more desirable analogues [78]. Compound 5 is another inhibitor of MAPK14 belonging to imidazole family with optimized aqueous solubility [79]. Furthermore, bisamide derivatives such as compound 9 inhibits MAPK14 by competing with ATP and preventing its binding to MAPK14 [80]. Despite challenges for clinical application of these compounds, pharmaceutical targeting of MAPK14 for suppression of *PCSK9* expression seems to be a promising strategy, pending on advances in drug development and application in clinical settings. In HepG2 cells, both leptin and resistin upregulate *PCSK9* expression and protein through

phosphorylation/activation of STAT3 [81]. Hence, targeting STAT3 signaling could be a valuable strategy for the regulation of *PCSK9* expression in the liver. Besides, balanced levels of adipokines seem to be cardioprotective due to regulation of PCSK9 levels. Other than these, metreleptin therapy (a recombinant leptin analog) ameliorates metabolic alterations in patients with lipodystrophy syndromes [72]. Another piece of evidence suggests that metreleptin therapy reduces serum LDLc by reducing PCSK9 levels in female lipodystrophy patients [82]. In a cohort of monogenic lipodystrophies patients, one-year metreleptin therapy has been shown to plummet circulatory levels of PCSK9 and ApoB-containing lipoproteins (e.g., LDL) [83].

Atrial natriuretic peptide

The heart, brain, and other organs release natriuretic peptides to activate homeostatic regulation during disturbances in cardiovascular homeostasis [84]. Human adipocytes participate in lipoprotein metabolism, yet it is unclear how PCSK9 and LDLR are regulated in these cells [85]. Visceral adipose tissue is associated with *PCSK9* expression, and increased body mass index (BMI) has been shown to promote *PCSK9* upregulation in this tissue [85]. In cultured model of human visceral adipocytes, insulin upregulates *SREBF1*, *SREBF2*, *LDLR*, and *PCSK9*, while atrial natriuretic peptide (ANP) downregulates *PCSK9* and prevents LDLR degradation [85]. Hence, human adipocytes express *PCSK9*, which is further upregulated by insulin. However, natriuretic peptides, such as ANP, inhibit *PCSK9* expression, contributing to LDLc reduction and overall cardiovascular health.

PCSK9 in cholesterol metabolism: therapeutic opportunities for cardiovascular health

Timely clearance of plasma LDL through hepatic LDLRs is vital for cardiovascular health (**Fig 3**). Multiple pieces of evidence suggest a regulatory role of PCSK9 in cholesterol metabolism

via ApoB [86]. As a positively charged component of LDL, ApoB100 promotes LDL binding to LDLR, hence facilitating LDL endocytosis [43]. In the ECM, ApoB100 binds to negatively charged proteoglycans and capture LDL from circulation. However, LDL retention in the ECM induces its oxidation and internalization into macrophages through scavenger receptors, ultimately, resulting in foam cell formation [87]. Of note, transgene-enforced overexpression of *PCSK9* increases plasma levels of ApoB100, triacylglycerol, cholesterol, and LDL regardless of *LDLR* levels [88]. From a mechanistic viewpoint, the intracellular interplay between PCSK9 and ApoB100 averts autophagosomal and lysosomal degradation of ApoB100, hence promoting its secretion, which increases plasma levels of LDL [88]. These findings suggest the promises of *PCSK9* inhibition as a new therapeutic paradigm in hypercholesterolemia, a key independent risk factor for CVD. ABCA1 participates in the formation of pre-β-HDL (high-density lipoprotein) by transferring cholesterol to ApoA-I (apolipoprotein A-I) [89]. PCSK9 contributes to hypercholesterolemia and foam cell formation through modulation of ABCA1 [90]. In mouse peritoneal macrophages, PCSK9 inhibits ABCA1-mediated cholesterol transfer through downregulation of *ABCA1* expression and protein, thereby promoting atherogenesis by preventing HDL formation (antiatherogenic cholesterol) [91]. These findings demonstrate a cardinal role of PCSK9-ABCA1 pathway in progression of hypercholesterolemia and deviation of cholesterol metabolism. Interestingly, caffeine is linked to hypercholesterolemia by promoting histone acetylation and upregulation of cholesterol-synthesis genes mainly through modulation of cAMP-PKA pathway and *SIRT1* expression [92]. In addition, caffeine reduces plasma PCSK9 and upregulates *LDLR* expression [93]. In a cohort study of healthy participants, caffeine analogs with improved potency have been developed to decrease PCSK9 levels [93]. From a mechanistic viewpoint, caffeine increases the ER content of Ca^{2+} which deactivates SREBP2, leading to

downregulation of *PCSK9* [93]. These observations imply that the ER Ca^{2+} level regulates cholesterol metabolism and modulates SREBP2-PCSK9-LDLR axis, thereby minimizing CVD risk. Besides, the ER-residing chaperone GRP94 binds to C-terminal domain of PCSK9 to prevent LDLR degradation in the ER[94]. In mice, *GRP94* ablation elicits PCSK9-mediated LDLR degradation, thereby enhancing plasma levels of LDLc [94]. Similar to caffeine, metformin administration remarkably lowers serum levels of PCSK9 and cholesterol via downregulation of *PCSK9*, resulting in upregulation of hepatic LDLR in mice and human hepatocytes *in vitro* [95]. Indeed, metformin suppresses the expression, activation, and nuclear translocation of ChREBP transcription factor that positively regulates *PCSK9* expression (**Fig 2**). As a result, *PCSK9* mRNA declines and increases LDLR production and secretion thus reducing circulatory LDLc [95]. These findings suggest that ChREBP may merit therapeutic targeting for the prevention of CVD. Nonetheless, hypocholesterolemic response of metformin is far from being validated [96]. E2F1 is yet another transcription factor regulating cholesterol homeostasis and metabolism. In mice, liver ablation of *E2F1* promotes LDL uptake through downregulation and upregulation of *PCSK9* and *LDLR*, respectively (**Fig 2**). Mechanistically, E2F1 transactivates *PCSK9* by binding to its promoter [97]. Moreover, *E2F1* deletion leads to liver fibrosis by inducing aberrant accumulation of hepatic cholesterol [97]. These results unveil a novel regulatory role of E2F1-PCSK9 axis in cholesterol metabolism and cardiovascular health.

PCSK9 in atherosclerosis: Role of inflammation

High levels of circulatory cholesterol and chronic inflammation promote the development of atherosclerosis as the leading cause of CVD [98]. Far beyond LDLR degradation, PCSK9 potentiates atherosclerosis through mechanisms involving proinflammatory responses and

associated regulation [99]. In mice, immunohistochemical analysis has detected PCSK9 proteins in atherosclerotic lesions, driving vascular inflammation and disease progression [99]. Macrophage overexpression of *PCSK9* induces IκBα phosphorylation and degradation, NF-κB activation, and upregulation of proinflammatory factors such as *TLR4* [99]. Conversely, *PCSK9* deletion reduces macrophage numbers and downregulates pro-inflammatory factors including NF-κB, IL-1β, MCP-1, TNF-α, and TLR4 [99]. These results indicate that *PCSK9* ablation or NF-κB inhibition in atherosclerotic macrophages may prevent pro-inflammatory circuitry [98]. In macrophages, immunoprecipitation analysis has shown that PCSK9 forms a complex with LRP5, a member of LDLR family, thereby increasing lipid uptake and foam cell formation [100]. Further analyses and exploration are needed to reveal underpinning mechanisms of lipid uptake driven by PCSK9-LRP5 complex. In a clinical study involving 581 patients with stable angina or acute coronary syndrome, serum samples are collected prior to angiography to monitor PCSK9 levels. The authors have noted a linear correlation between serum levels of PCSK9 and necrotic core, independent of LDLc and irrespective of prior use of statins [101]. Hence, in the absence of altered lipid metabolism, a possible correlation among PCSK9, vascular inflammation, and coronary plaque progression can be envisaged. The current literature suggests that PCSK9 is upregulated under proinflammatory conditions, thus creating a vicious cycle due to a plausible role of PCSK9 in inflammatory responses. Lipopolysaccharide (LPS) activates the innate immune system by binding to TLR4, leading to secretion of proinflammatory cytokines [102]. Parenteral administration of LPS markedly upregulates hepatic expression of *PCSK9*. Similarly, turpentine- and zymosan-stimulated inflammation induce hepatic expression of *PCSK9* [103]. These observations denote that inflammation can amplify *PCSK9* expression, resulting in LDLR degradation and increased levels of LDLc as a causative factor for CVD [103]. Therefore,

neutralization of vascular inflammation via pharmaceutical means such as anti-inflammatory agents may suppress hepatic expression of *PCSK9* (**Fig. 4**).

PCSK9 in cardiovascular disease: animal models and clinical relevance

Coronary heart disease and hypertension

PCSK9 and leptin regulate circulatory levels of lipids thus playing a key role in the pathogenesis of coronary heart disease (CHD), also known as disease (CAD) [76, 104]. Until recently, both PCSK9 and leptin have been shown to modulate vascular and coronary artery inflammation independently [105-107]. A clinical study involving 27 CHD patients and 27 healthy volunteers has revealed a remarkable positive association between circulatory lipid and leptin levels in CHD [108]. Also, levels of PCSK9, leptin, and inflammatory factors including ICAM-1 and E-selectin were remarkably elevated in CHD patients compared to controls. Furthermore, a positive association was observed between levels of proinflammatory cytokines (e.g., IL-17, IFN-γ, E-selectin) and PCSK9 in CHD patients [108]. These data indicate that PCSK9 and leptin participate in regulation of not only lipid metabolism but also inflammation and immune responses in CHD.

In an independent cross-sectional study enrolling 251 patients with stable CAD in the absence of lipid-lowering medications, plasma levels of PCSK9 was correlated with white blood cell counts [109]. To explore if circulatory levels of PCSK9 could be proposed as a potential biomarker, 219 CAD patients were enrolled in a clinical study [110]. Findings of this study suggest that a higher circulatory PCSK9 level is positively correlated with increased levels of inflammatory markers such as hs-CRP and fibrinogen, as well as LDLc and total cholesterol [110]. Given the

small size of study and the lack of large-scale data, the correlation between circulatory PCSK9 and inflammatory factors is rather hypothetical, hence necessitating further explorations. In a cross-sectional study involving 330 patients with stable CAD, a positive correlation between PCSK9 plasma levels and platelet count was observed [111]. To assess the value of plasma PCSK9 in predicting the severity of coronary syndrome, a cohort of 121 patients underwent coronary angiography. The results showed that odds ratio of coronary lesions was remarkably greater in patients with the highest PCSK9 levels [112]. Also, serum PCSK9 levels were positively correlated with the number of involved coronary arteries [112].

Meanwhile, result from a study involving 40 patients with idiopathic pulmonary arterial hypertension (IPAH) and 20 healthy controls has revealed a considerably exaggerated circulatory levels of MCP-1, IL-1β, IL-6, TNF-α, and PCSK9 in IPAH patients [113]. Moreover, PCSK9 levels are positively correlated with right ventricular (LV) diameter, pulmonary vasculature resistance, and arterial systolic/diastolic pressure, as well as MCP-1, IL-1β, IL-6, and TNF-α levels [113]. Likewise, these data are retrieved from a small-sized observational study. Thus, the link between circulatory levels of PCSK9 and these proinflammatory factors would require further large-scale validation. Hence, executing double-blind RCT (randomized controlled trials) using a large population of patients to corroborate these findings seems essential. Collectively, these findings suggest a pathological role of PCSK9 in CVD. However, at a fundamental molecular level, they do not adequately elucidate the correlation and pathological role of PCSK9 in CVD. Below, we will carefully examine recent findings regarding the implication of PCSK9 in various CVD diseases and models.

Myocardial ischemia and myocardial infarction

Accumulative number of evidence suggest that PCSK9 inhibition confers cardioprotection against myocardial ischemia/reperfusion (I/R) injury [114-116] by alleviating arrhythmias and infarct size, and improving left ventricular (LV) function through enhanced mitochondrial function, apoptosis inhibition, and connexin 43 phosphorylation [117]. In rats, pre-ischemic administration of PCSK9 inhibitor shrinked infarct size and curbed mitochondrial ROS generation, while ischemic/reperfusion administration of the drug failed to confer protection [117]. This study suggests the prophylactic but not curative benefits of PCSK9 inhibition in myocardial I/R injury, albeit clinical translation of these findings has been notoriously challenging [118].

Nonetheless, PCSK9 inhibition appears to be a promising therapy in patients with myocardial infarction (MI) [119-122]. Several pieces of evidence suggest that *PCSK9* knockout induces defects in liver regeneration following murine hepatectomy [123]. This notion further casts doubts on therapeutic approach involving inhibition/ablation of *PCSK9* in MI [124]. However, in mice, monitoring early response for four weeks after MI reveals that *PCSK9* deficiency does not significantly alter cardiac output, ejection fraction, stroke volume, and diastolic/systolic LV volume [124]. Moreover, *PCSK9* deficiency favors cardiac repair by increasing LV wall thickness and decreasing fibrosis in the remote zones of infarcted areas [124]. Collectively, these data suggest that *PCSK9* deficiency or inhibition does not influence cardiac repair capacity, indicating the efficacy and safety of PCSK9 inhibition in MI. In a mouse model of ischemic heart disease developed by the occlusion of left coronary artery, *PCSK9* expression, autophagy, and cardiac contractile dysfunction were accentuated in infarcted myocardium [125]. In a murine model of MI, pretreatment with PCSK9 inhibitors (e.g., EGF-A and Pep2-8) or genetic ablation of *PCSK9* minimized the infarct size, inhibited autophagy, and refined cardiac function [125]. Besides,

hypoxia exposure promoted *PCSK9* upregulation and constitutive induction of autophagy in cardiomyocytes through the activation of ROS-ATM-LKB1-AMPK signaling [125].

In a 1-year follow-up of 281 patients with acute MI, plasma PCSK9 levels were measured to explore an evident gender bias in adverse cardiac events [126]. Female patients with acute MI and ST-elevation MI (STEMI) exhibited higher levels of PCSK9 and increased mortality compared to their male counterparts [126]. In a pilot study, post reperfusion levels of PCSK9 were measured in 40 first-time STEMI patients with LV ejection fraction (LVEF)<50% and under primary coronary intervention [127]. Through covariance analysis the correlation between PCSK9 levels and LVEF (measured by cardiac magnetic resonance in the first week and then the 6th month of STEMI) was evaluated and the results indicated a significant inverse-correlation between PCSK9 levels and LVEF (P-value = 0.028) [127]. Hence, other than measuring LDLc, increased levels of PCSK9 could be a useful biomarker for evaluation of MI patients.

In a sharp contrast, a case-control study involving 1488 MI patients with an average follow-up of 11.3 years revealed that circulatory PCSK9 might not be a plausible biomarker for the MI risk [128]. A large cohort study of hospitalized patients with acute MI reported that PCSK9 levels do not reflect the risk or severity of CVD [129]. Consistently, a study involving 1646 acute MI patients indicated that female gender, premature CHD, high hs-CRP, and triglycerides (TG) are linked with elevated levels of PCSK9 [130]. Nonetheless, plasma levels of PCSK9 do not predict CVD events and recurrence within 1 year [130]. The physiological range of PCSK9 ranges between 170 to 220 ng/ml in human plasma. Meanwhile, any level ≥ 250 ng/ml is projected to be above-normal or supraphysiological level [131]. Taken together, these findings indicate that excessive or supraphysiological levels of plasma PCSK9 are associated with pathogenesis of MI and myocardial I/R injury. Hence, PCSK9 inhibition could serve as a potential therapeutic option

to prevent MI or alleviate its symptoms. However, clinical trials are currently under way or should be executed before establishing PCSK9 targeting as a standard therapy. These concepts are discussed in detail in the therapeutic sections of this review. In compliance, patients with established atherosclerotic CVD under evolocumab (anti-PCSK9 mAb) treatment for 8 years showed 15-20% decrease in stroke, MI, cardiovascular death, coronary revascularization, unstable angina, and hospitalization [132, 133]. **Table 1** summarizes available key large-scale double-blind RCT and their outcomes.

Atherosclerosis

Exaggerated expression of *PCSK9* is linked to the development of atherosclerosis, while its downregulation can alleviate atherosclerosis and the risk of severe CVD (**Fig 4**) [134-139]. In addition, PCSK9 participates in inflammation and atherogenesis [140-142]. In a clinical study enrolling 184 patients (free of statins for 3 months) with atherosclerotic plaques, fasting blood samples were obtained to assess PCSK9 and CD40L levels. As a result, PCSK9 levels were found to be higher in patients with acute myocardial ischemia or unstable angina patients than in non-CHD patients. Moreover, PCSK9 levels were correlated with CD40L levels depending on CHD severity in acute MI, unstable, and stable angina patients [140]. Collectively, this study suggests that PCSK9 contributes to atherosclerosis and CHD progression by inducing inflammation in plaque sites, independent of serum lipid levels.

In an independent study, carotid ultrasound examination of 643 participants without baseline carotid plaques or lipid-lowering drug usage showed a tight correlation between PCSK9 levels and new plaque formation after adjusting other risk factors and LDLc levels [143]. Also, a

linear correlation was identified between plasma PCSK9 levels and plaque area after adjusting for LDLc. Finally, this study revealed that plasma PCSK9 was linked with the progression of atherosclerosis over a 10-year follow-up [143]. Besides, this study implicates the clinical value of PCSK9 level in predicting atherosclerotic progression beyond its established role in LDL and cholesterol metabolism. Accordingly, PCSK9 levels were correlated with subclinical signs of atherosclerosis (including carotid atherosclerotic plaques, carotid artery diameter, and echolucency of carotid plaque) in 3703 European participants asymptomatic for CVD [144]. Nonetheless, following adjustments of drug use, latitude, sex, and age, these correlations were not proven to be statistically significant, shedding doubts on the utility of PCSK9 for monitoring subclinical CVD [144].

Recent evidence has noted a modulatory role for gut microorganisms in the functional link between PCSK9 and atherosclerosis. The human gut hosts a diverse complex of microorganisms, commonly being referred to as “gut microbiota”, exerting homeostatic or pathological effects on the cardiovascular system [145]. In particular, dysbiosis denotes conditions of an altered composition of the microbiota [146]. In gut dysbiosis, intestinal epithelial cells may overproduce PCSK9 and hence favor atherosclerotic CVD through disruption of regulatory system such as LDLR expression and ApoB cholesterol biosynthesis [147]. Therefore, avoiding gut dysbiosis by dietary interventions such as consumption of fermented foods and plant fibers [148] may adjust PCSK9 levels in favor of cardiovascular health. Collectively, all these data indicate a pathological role for PCSK9 in atherosclerosis and its associated complications, necessitating the need for further studies in this area, as well as large-scale clinical studies to establish the therapeutic value of targeting PCSK9 in atherosclerosis. In this review, we have briefly summarized the ongoing trials).

551

552 ***Atrial fibrillation***

553 Evidence suggests that PCSK9 levels are closely linked with arrhythmia [149, 150]. In 907
554 atrial fibrillation (AF) patients under vitamin K antagonists therapy, cardiovascular events,
555 nonfatal/fatal MI, cardiovascular death, and ischemic stroke (IS) were evaluated [151]. As a result,
556 patients with cardiovascular events exhibited a greater median level of PCSK9 than those without
557 notable cardiovascular events [151]. Importantly, in 682 patients with no history of antiplatelet
558 therapy, plasma PCSK9 levels were remarkably correlated with 11-dehydro-thromboxane B_2 (11-
559 dh-TxB_2, indicative of platelet activation) [151]. This study suggests that PCSK9 may participate
560 in platelet activation during AF. Consistently, plasma and vascular PCSK9 participated in clot
561 formation and thrombosis through activation of platelets and recruitment of leukocytes, seemingly
562 independent of LDLc [152]. Hypothetically, PCSK9 may contribute to AF-associated IS due to
563 the formation of blood clots, thus mandating further investigation.

564 On the other hand, it can be argued that if PCSK9 induces platelets activation, signs for
565 bleeding should appear in mitral regurgitation (MR) or rhythm-control therapy (RCT) [153].
566 Besides, antiplatelets may not be the drug of choice to prevent AF-associated IS, thereby refuting
567 the implication of PCSK9 in platelets activation. In a similar study, 88 AF patients are enrolled to
568 evaluate the correlation between PCSK9 levels and soluble P-selectin (a marker of platelet
569 activation), TxB2 formation, and platelet aggregation, as well as oxidized LDL (oxLDL),
570 isoprostanes, H_2O_2, and oxidative stress markers [154]. AF patients with higher PCSK9 levels
571 exhibit remarkably concentrated oxidative stress and platelet activation. Moreover, co-
572 immunoprecipitation reveals formation of a complex in platelets between PCSK9 and the CD36
573 receptor, contributing to NOX2 activation and thereby, platelet activation. Of note, this effect was

574 further accentuated in the presence of LDL [154]. In a cohort of 907 AF patients, LPS, PCSK9,
575 and Nox2 were measured and evaluated for cardiovascular events [155]. Study findings solidified
576 a tight correlation between PCSK9 and LPS levels. However, the consumption of antiplatelet
577 drugs, wine, olive oil, and a Mediterranean diet is associated to a reduction in PCSK9. Also,
578 patients with higher levels of PCSK9 and LPS show a greater risk of cardiovascular events [155].
579 Altogether, these observations denote that PCSK9 in pathologically implicated in AF and its
580 associated comorbidities. However, additional basic studies are required to uncap this phenomenon
581 from molecular mechanism perspectives.

582

583 ***Heart failure***

584 Accumulating evidence suggests that PCSK9 is associated with heart failure (HF)
585 development [156, 157]. Mice with liver-specific *PCSK9* knockout display compromised exercise
586 resistance and aberrant echocardiographic findings, indicative of HFpEF (HF with preserved
587 ejection fraction) [158]. Moreover, these mice exhibited impaired cardiac metabolism and
588 mitochondrial dysfunction as well as upregulated *CD36* and *LDLR*, leading to accumulation of
589 intracellular lipids [158]. Similar findings were obtained in mice with a double-knockout
590 *PCSK9*/*LDLR* thus excluding the possible involvement of upregulated LDLR in the etiology of
591 HFpEF induced by PCSK9 deficiency [158]. Besides, patients bearing the R46L genetic variant
592 of *PCSK9* exhibited enhanced LV mass but similar HFpEF risk [158]. Although these findings
593 suggest a potential risk for PCSK9 inhibition associated with HFpEF, an observational study
594 involving 2174 patients with chronic HF revealed a positive linear correlation between PCSK9
595 levels and overall mortality risk that resisted multivariable analyses, suggesting the benefit of

PCSK9 inhibition in HF outcome [12]. Hence a partial and systemic inhibition of PCSK9 in patients with HF might elicit other effects that complete hepatic inhibition of PCSK9.

PCSK9 mutation and polymorphisms: impact on CVD

Polymorphism is frequently reported with *PCSK9* gene thus influencing the development of CVD. In this regard, a study on 218 patients and 125 controls show that c.61_63dupCTG (L10) allele (rs72555377) contributes to reduction in LDLc levels and CHD risk likely through direct impacts on atherogenesis [159]. Studies with a larger group of participants may further lighten up the link between L10 allele and pathophysiology of CHD and atherogenesis. Moreover, in a Tunisian cohort study, examining the impact of rs505151 (E670G) polymorphisms on IS and CAD risks has revealed a higher frequency of E670G allele in CAD compared to non-CAD participants [160]. CAD patients carrying the E670G mutation display higher levels of LDLc and plasma cholesterol as well as a higher risk of severe CAD than non-E670G carriers [160]. In sharp contrast, prior cell-based assays suggest that E670G variant induces LOF in PCSK9 due to the inability of FAM20C in recognition and phosphorylation of Ser668 in S668-X-E670 motif [161]. Depending on ethnicity, the effect of E670G variant on LDLc levels may be variable [162-164]. Thus, the E670G has been regarded as a gain-of-function (GOF) variant due to its association with greater levels of LDLc in Chinese descendants [164] and less effects on LDLc levels in Caucasians [162]. In Europeans, the rs505151 polymorphism is correlated with enhanced levels of LDLc in men but not women [163]. In the UK, the E670G variant does not show any remarkable correlation with lipid levels in plasma and cardiovascular risk in men [165], a negative finding that is recapitulated in ethnic Chinese living in Taiwan [166]. In an independent case-control study, the impact of rs1122608 and rs11206510 polymorphisms on 290 CHD patients, 193 non-CHD, and 330 normal

controls are investigated, and the χ^2-test is applied to compare allele frequencies and distribution of genotypes [167]. Two meta-analyses of rs11206510 and rs1122608 polymorphisms have revealed that either of single nucleotide polymorphisms (SNPs) are correlated with CHD in Caucasians but not Asians [167]. Hence, this study suggests that genetic heterogeneity determines CHD susceptibility among these populations. On the contrary, there is a growing contemporary understanding that certain mutations in *PCSK9* gene can reduce the risk of CHD and plasma levels of LDLc. In this regard, exploring the correlation between *R46L* missense and *C679X* and *Y142X* nonsense mutations in *PCSK9* gene and serum LDLc in 1086 whites and 478 African-Americans show that white *L46* carriers exhibit lower LDLc compared to noncarriers of the same race [168]. African-American *Y142X* or *C679X* carriers display lower LDLc compared to noncarriers of the same ethnic background [168]. These observations indicate that *Y142X*, *C679X*, and *L46* mutations in *PCSK9* gene lower LDLc, thereby averting CHD risk. Likewise, a meta-analysis of 1 randomized trial and 8 observational cohorts has revealed a tight correlation between LOF variants of *PCSK9* and reduced LDLc in blacks (35 mg/dL) and whites (13 mg/dL) [169]. Moreover, scrutiny of 3363 black individuals suggests that *PCSK9* nonsense mutations cause 28% and 88% reduction in LDLc and CHD risk, respectively [170]. Examining 9524 white participants also suggests presence of a genomic variation in *PCSK9* contributing to 15% and 47% reduction in LDLc and CHD risk [170]. Furthermore, a large study enrolling 14120 cardiovascular and 10635 diabetic patients uncovers that *PCSK9* and *HMGCR* variants independently reduce cardiovascular events and diabetes risk per unit reduction in LDLc levels [171]. Altogether, these results underscore the notion that LOF variants of *PCSK9* are linked to reduced LDLc and lower CHD risk. Future and larger scale studies should tell whether detection of *PCSK9* variants may be advantageously introduced into CVD prevention campaigns.

PCSK9 in other diseases

Cancer/metastasis

PCSK9 is implicated in cancer progression and its (*PCSK9*) deficiency has been shown to avert the metastasis of cutaneous melanoma to the liver [172-174]. Also, *PCSK9* GOF and LOF variants increased and decreased breast cancer incidence, respectively [175]. Based on these data, *PCSK9* expression level could be utilized as a potential biomarker for prognosis of several malignancies including gastric, pancreas, kidney, hepatocellular carcinoma, and breast cancers [176, 177]. Metabolic syndrome, obesity, and hyperglycemia negatively impact breast cancer patients. Conversely, immune checkpoint inhibitors (ICIs) have garnered increasing interest as a cancer therapy in recent years. However, ICIs such as ipilimumab are associated with evident cardiotoxicity [178]. In an experimental study, both cardiomyocytes and triple-negative breast cancer cells were co-cultured and exposed to ipilimumab under different levels of glucose (modeling hyperglycemia, hypoglycemia, or normoglycemia) [178]. The study revealed that hyperglycemia increased ipilimumab-induced cardiotoxicity and enhanced the survival of breast cancer cells through activation of NLRP3 inflammasome, which triggered cytokine secretion and local inflammation [178]. Furthermore, other evidence suggests that NLRP3 inflammasome signaling (via IL-1β) can increase PCSK9 secretion. In turn, PCSK9 can directly activate NLRP3 inflammasome, further increasing cytokine secretion, local inflammation, and cardiotoxicity. Thus, it can be hypothesized that elevated PCSK9 levels may exacerbate ipilimumab-induced cardiotoxicity in breast cancer patients [179]. Early reports have shown that PCSK9 is highly expressed in the thymus and spleen, thereby regulating immune functions [13, 180]. In addition, LDLR binds to T cell receptors (TCR) and may stimulate anticancer activity of cytotoxic T

665 lymphocytes [181]. Hence, it is proposed that immune checkpoint inhibitors could be
666 advantageous in combination with PCSK9 inhibition and immunotherapy [182-184].

667 Of note, the M2 domain in Cys/His-rich region of PCSK9 can interact with the N-terminus
668 of major histocompatibility class I (MHC-I) molecules thus favoring their endosomal/lysosomal
669 degradation, which reduces antigenicity of malignant cells [185, 186]. Also, furin protease-cleaved
670 PCSK9 containing AA219-692 can compete with full-length (non-cleaved) PCSK9 for binding to
671 MHC-I in both human and mice [187-189]. These findings suggest that peptidomimetics that
672 competitively disrupt the interaction between PCSK9-M2 domain and MHC-I molecules may
673 improve the outcome of cancer immunotherapy. In favor of this hypothesis, *PCSK9*-deficient
674 cancer cells can be rejected by the immune system in preclinical experiments [185]. Moreover, the
675 synergy of *PCSK9*-deficiency with administration of immune checkpoint inhibitors such as
676 *PDCD1*/PD-1 mAb can block tumor growth [190]. Since immune responses are involved in
677 successful chemotherapeutic regimens, it may be useful to combine *PCSK9* inhibition with
678 cytotoxic antineoplastics [191]. Indeed, a combinational therapy of PCSK9 mAb, ezetimibe,
679 statins, and folfirinox (a chemotherapeutic drug for pancreatic cancer treatment) is under
680 investigation in pancreatic adenocarcinoma patients in a phase-I clinical trial
681 (https://clinicaltrials.gov/ct2/show/NCT04862260) [192].

682 Hepatic metastases of colorectal cancers display two patterns of histopathological growth.
683 First, metastases following the desmoplastic pattern attain blood supply via VEGF-mediated
684 angiogenesis. Second, metastases following the replacement pattern utilize vessel co-option
685 mechanism in which they hijack the existing vasculature for blood supply, meaning that they are
686 constitutively resistant against anti-angiogenic therapy [193]. Importantly, circulatory PCSK9
687 reinforces the vessel co-option mechanism in such metastases, implying that combined inhibition of

VEGF and PCSK9 may overcome therapy resistance [194]. Despite all these crucial findings, the research on cancer PCSK9 is still ongoing, and there remains a long way before translating the current state of knowledge into meaningful therapeutic translation.

Viral infection

Nine serine proteases including S1P and furin are known to facilitate the entry of enveloped viruses into host cells and the spread of viral infection [195]. Several pieces of evidence have implicated a link between PCSK9 and infections evoked by Dengue virus (DENV) or SARS-CoV-2, the causal agent of COVID-19. DENV is a positive single-stranded RNA virus (+ssRNA) from the *Flaviviridae* family, which is passed onto humans through *Aedes* mosquitoes, infecting over 400 million populations and causing 25000 mortalities worldwide annually [196-198]. In hepatocytes, DENV infection induces *PCSK9* expression [199] to reduce cell surface LDLRs, resulting in upregulated cholesterol synthesis through activation of SREBP2 pathway [200]. It is conceivable that DENV hijacks PCSK9 for viral packaging. Intriguingly, ensuing DENV infection, cholesterol accumulating in the ER significantly reduces antiviral IFN response in hepatocytes by inhibiting STING signaling [199]. In DENV-infected patients, concomitant elevation of PCSK9, plasma leakage, and viremia occurs [199]. In a preclinical model, PCSK9 inhibition by means of specific mAb alirocumab elevates plasma LDLR levels and decreases DENV viremia [199]. Hence, these findings denote that PCSK9 inhibition could help to control DENV by enhancing antiviral IFN response. However, this hypothesis requires further exploration in clinical studies.

In experimental models, statins upregulate the expression of *ACE2*/ACE2 through which SARS-CoV-2 trespasses into cells [201, 202]. In addition, statins remarkably improve COVID-19 prognosis in elderly patients [203-205]. Mechanistically, statins refine endothelial function, attenuate blood coagulation, and suppress inflammation through cholesterol-dependent, as well as -independent mechanisms [206]. In contrast, CORONADO study has shown that statin therapy is markedly linked to higher mortality of COVID-19 patients with type 2 diabetes, suggesting a controversial impact of statins on COVID-19 mortality [207]. In the same vein, PCSK9 inhibitors might be beneficial for COVID-19 patients, likely by boosting IFN levels [208]. This is particularly relevant for patients with LOF variants in TLR3 and IRF7 in IFN immunity [209]. However, this hypothesis requires careful clinical validation. On theoretical grounds, PCSK9 inhibition together with statins might ameliorate other viral infections such as human cytomegalovirus (HCMV), which is particularly infectious when LRP1 is absent and cellular cholesterol levels are high, through a mechanism reminiscent of DENV [210, 211]. In sharp contrast, PCSK9 has been suggested to confer protection against infection by hepatitis C virus (HCV) [212], suggesting that PCSK9 inhibition should not be regarded as a potential universal remedy against viral infection. However, additional studies are required to explore the implication of PCSK9 in other types of viral infections.

Sepsis

Sepsis is an extreme and dysregulated body response to infections, leading to organ dysfunction, organ failure and mortality [213-215]. In sepsis, PCSK9 upregulation elicits degradation of hepatocyte LDLRs and adipocyte VLDLRs thus restraining the uptake and clearance of pathogenic lipids such as LPS [216]. On the other hand, PCSK9 rectifies cholesterol metabolism and

732 macrophage function of TLRs, thereby augmenting proinflammatory responses. Contrarily,
733 PCSK9 may adversely affect host immune responses [216]. Nonetheless, implication of PCSK9
734 in sepsis pathogenesis has not been adequately explored. Hepatocyte uptake of
735 pathogenic/bacterial LPS resembles a vital step for systematic clearance of LPS. As such, PCSK9-
736 mediated excessive degradation of LDLRs on hepatocytes may potentiate bacterial infection and
737 sepsis [105]. In a mouse model of sepsis, PCSK9 blockade using pharmaceutical inhibitors
738 suppresses inflammation and improves survival [105]. Likewise, in the case of sepsis, *PCSK9* LOF
739 human variants better the survival and retard inflammation *in vivo*. Collectively, these data suggest
740 that forcible inhibition of PCSK9 may serve to combat inflammation, pathogenic LPS, and septic
741 outcomes. Yet, in a controversial report, administration of PCSK9 mAb to mice before and/or after
742 LPS administration has failed to protect against LPS endotoxemia and mortality [217]. By the
743 same token, *PCSK9* ablation does not provide protection against LPS toxicity and death in mice
744 [217], thus demanding for additional in-depth studies.

745

746 **Secondary hyperlipidemia, diabetes, psoriasis, and chronic renal failure**

747 Acquired hyperlipidemia, also known as secondary hyperlipidemia, refers to an abnormal
748 increase in blood lipid levels, including TG and cholesterol. This condition can arise from multiple
749 factors such as certain medication, diabetes, renal failure, alcohol intake, and other lifestyle factors
750 [218, 219]. Accumulating evidence suggests that PCSK9 plays a vital role in cholesterol
751 homeostasis and pathogenesis/development of secondary hyperlipidemia [220-222]. As mentioned
752 earlier, mechanistically, PCSK9 mediates secondary hyperlipidemia by increasing LDL plasma
753 levels [5, 223]. PCSK9 binds to LDLR on hepatocytes plasma membrane, leading to LDLR
754 degradation (detailed mechanism mentioned early), which reduces the number of LDLRs available

for removal/clearance of LDLc from the bloodstream, ultimately, contributing to the secondary hyperlipidemia [218].

Additionally, mutations that enhances PCSK9 activity are associated with hypercholesterolemia. Given that these genetic traits can be passed down through generations, they are predominantly categorized as the primary hyperlipidemia and not necessarily the secondary type [224]. However, PCSK9 implication in diabetes-mediated hyperlipidemia exemplifies its role in the secondary hyperlipidemia. In a cohort of 115 diabetic patients, PCSK9 circulatory levels were measured using a sandwich ELISA technique and it was found that PCSK9 concentrations in the plasma were correlated with LDLc and total cholesterol levels [225]. Only treatment with micronized fenofibrate (200 mg/day) decreased plasma PCSK9 levels by 8.5% and alleviated hyperlipidemia [225]. These findings suggest that through largely unrecognized mechanisms diabetes is associated with increased plasma levels of PCSK9, therefore, leading to the induction or exacerbation of secondary hyperlipidemia. Unfortunately, the current literature has not fully elaborated on the underlying mechanisms and additional experimental studies are required to identify molecular links between diabetes and increased PCSK9 production and plasma levels. Understanding these links may enable better management of diabetes-associated secondary hyperlipidemia and its complications. Furthermore, anti-diabetic medications may also influence PCSK9 levels and their correlation with secondary hyperlipidemia. For instance, in patients with type 1 diabetes, insulin injection has been shown to upregulate *PCSK9* expression by activating transcription factors like HNF1α and SREBP1c, indicating that insulin dose and poor glycemic control are potentially linked with increased production and PCSK9 levels in type 1 diabetic patients [54, 55, 226, 227], thus predisposing them to subsequent complications such as the secondary hyperlipidemia.

Psoriasis is a pro-inflammatory condition causing itchy rashes on the skin mainly on elbows, knees, scalp, and trunk [228]. However, its link with CVD still remains elusive. In two separate cohorts of human psoriasis and *in vitro* mouse models, PCSK9 levels were 20% higher than control, thereby increasing plasma levels of total cholesterol and LDLc in these patients, ultimately, leading to atherosclerosis and impaired endothelial function [229]. These findings denote that PCSK9 levels are elevated in psoriasis patients through largely unknown mechanisms thus predisposing these patients to secondary hyperlipidemia and early/advanced atherosclerosis. Hence, the remaining gaps require further exploration by future studies.

Chronic kidney disease (CKD), also known as chronic renal failure, is linked with the secondary hyperlipidemia in several ways [230, 231]. For exmple, CKD is associated with aberrant function of lipid metabolism, leading to elevated TG levels and decreased HDL, which contributes to secondary hyperlipidemia and increase the risk of CVD. Additionally, CKD often leads to secondary hyperparathyroidism and excessive secretion of parathyroid hormone (PTH), which induces calcium deposition in the liver and adipose tissues, further affecting lipid metabolism, and contributing to the secondary hyperlipidemia [232, 233]. Moreover, the role of PCSK9 in connecting CKD to secondary hyperlipidemia cannot be dismissed. Two independent cohort studies have measured plasma levels of PCSK9 in various subtypes of CKD patients, revealing elevated concentrations of PCSK9 in those with nephrotic syndrome and peritoneal dialysis, both associated with increase proteinuria [234]. Furthermore, the administration of PCSK9 inhibitors has been shown to be safe and effective for improving lipid control and renal function in CKD patients [235]. Collectively, these data suggest that increased PCSK9 levels are one of the mechanisms linking CKD to secondary hyperlipidemia. However, the current literature has not

adequately explored the mechanisms underlying elevated PCSK9 levels during CKD. Therefore, further experimental and basic studies are warranted to address these remaining gaps.

Pediatric populations and hypercholesterolemia/hyperlipidemia

Familial hypercholesterolemia is a type of dyslipidemia, classified under the broader category of hyperlipidemia, affecting millions of people globally [236]. In this condition, LDLc levels rise significantly, resulting in a 10-fold increase in the risk of CVD and premature atherosclerosis [236]. For children and adolescents diagnosed with familial hypercholesterolemia, early interventions such as lifestyle alteration and taking statin medications are recommended [237-240]. However, some patients may not achieve adequate LDLc reduction with statins, possibly due to drug intolerance or severe genetic susceptibility to dyslipidemia, necessitating alternative strategies for lowering LDLc. In the HAUSER-RCT randomized controlled trial, which lasted 24 weeks, 157 pediatric patients with familial hypercholesterolemia were recruited, and treatment with Evolocumab proved to be both effective and safe [241]. While the cornerstone of treating pediatric familial hypercholesterolemia relies on lifestyle modifications and the use of tolerated ezetimibe and statins or a combination of both, targeting PCSK9 with established inhibitors (e.g., Evolocumab) could offer an alternative therapeutic option for children with persistent high LDLc who are intolerant to statins. However, it is essential to confirm the long-term safety of PCSK9 inhibitors, as current data in this regard is limited. This highlights the need for additional clinical trials with extended follow-up periods to better assess the safety of this approach.

Pharmacological and therapeutic targeting of extracellular PCSK9

Monoclonal antibodies

Application of mAb is the leading avenue for targeting extracellular PCSK9 [242]. Use of PCSK9 inhibitors has displayed some promising results in the clinical management of cardiovascular risk factors such as dyslipidemia and hypercholesterolemia, in particularly in those patients with statin intolerance [243, 244]. In a retrospective study, the efficacy of PCSK9-neutralizing mAb evolocumab either alone or in combination with rosuvastatin (a statin) and ezetimibe (an inhibitor of gut cholesterol absorption) is examined in CHD patients [245]. Within 24 weeks, rosuvastatin monotherapy reduces plasma LDLc levels by 34%, whereas the rosuvastatin/ezetimibe and rosuvastatin/ezetimibe/evolocumab combination therapies reduce LDLc levels by 52% and 73%, respectively [245]. Given that this study mainly included Chinese CHD patients, confirmatory approaches engaging other ethnicities are required. Nonetheless, these findings would still favor the superior efficacy of evolocumab therapy in combination with conventional statin therapy in lowering LDLc levels in CHD patients. Given that statins are the major therapeutics to curb LDLc levels in CHD patients [246], patients might consider a combination of lipid-reducing drugs with fixed doses [247]. Retrospective analyses involving 137 CHD patients with chronic or acute coronary syndrome indicate a notable drop in LDLc from a fixed-dose rosuvastatin-ezetimibe combinational treatment in CHD patients [247]. On the other hand, the reduction of LDLc reduces the risk of atherosclerotic heart disease [248]. However, even on statin and other small molecule lipid agents, achieving clinical LDL levels below 2 mmol/L remains challenging as certain patients exhibit much higher cholesterol levels despite high-dose statin administration [249]. Hence, co-treatment with PCSK9 inhibitors may assist these patients to obtain lower cholesterol levels. In this regard, 235 patients (with 150 successful completions)

with atherosclerotic heart disease are prescribed evolocumab [249]. LDL levels are reduced by 60% after one year of evolocumab treatment, and 82% of patients achieve LDL levels of < 2 mmol/L. Reduced cholesterol levels at 3, 6, and 12 months confirm the persistent efficacy of such treatment [249]. Of note, affordability of treatment costs and fear of repeated injections are among the main factors deterring patients from compliance in evolocumab therapy [249]. Altogether, this study confirms therapeutic utility of evolocumab in atherosclerotic heart disease and suggests that mitigating the cost and improving patient education should help to lift the barriers against this type of treatment. To this end, alternative therapeutics such as PCSK9-targeting mAbs (evolocumab or alirocumab) might have a role in transplantation recipients [250]. The impact of evolocumab and alirocumab is investigated on transplanted heart patients with a median survival of 5.5 years from transplantation and a median of 1.6 years of PCSK9 inhibitor treatment [250]. Results have shown substantial attenuation of TG, total cholesterol, non-HDL-cholesterol, and total HDL/cholesterol ratio, along with a mild augmentation of HDL cholesterol (HDLc). Moreover, 33 patients exhibit stable thickness of coronary plaques upon long-term PCSK9 neutralization [250]. These data support favorable effects of evolocumab and alirocumab in patients receiving heart transplantation owing to diminished cholesterol levels and stabilization of arterial intimal hyperplasia with negligible side effects. However, affordability and access of PCSK9 inhibitors remain challenging. **Table 1** summarizes available clinical trials, in particular, large-scale double-blind RCT, using mAbs targeting PCSK9 in hypercholesterolemia and cardiovascular patients. In addition to IgG antibodies, mono-domain antibodies are under development for targeting PCSK9 [6]. In a pre-clinical study, 4 high-affinity mono-domain antibodies are developed that target C-terminal domain of PCSK9 (Cys/His rich) and prevent LDLR degradation in human/mouse hepatocytes and cell lines. These mono-domain antibodies achieve a 32-44% decrease in plasma LDLc and

1.8-fold increase in liver LDLR levels [251]. Mono (single)-domain antibodies are reminiscent to mAbs in terms of antigen-binding but are cheaper and easier to manufacture [251]. For instance, LY3015014, a humanized IgG4 mAb, targets the catalytic domain of PCSK9 and efficiently lowers LDLc level in hypercholesterolemic patients who poorly respond to statin therapy [252]. Adnectins are proteins resembling the 10^{th} domain of fibronectin type III that can be engineered to acquire considerable specificity and high affinity toward their relevant targets. Functionally, they resemble antibody variable domains albeit with different primary sequences with a single mono-domain in the absence of disulfide bonds [253]. The adnectin BMS-962476 binds PCSK9 with high affinity and specificity [254]. In transgenic mice overexpressing human *PCSK9*, administration of BMS-962476 remarkably attenuates circulatory LDLc and PCSK9 levels. Moreover, BMS-962476 administration to cynomolgus monkeys significantly attenuates levels of free PCSK9 (99%) and LDLc (55%) [254]. These pre-clinical data suggest that BMS-962476 could be a promising PCSK9 inhibitor although clinical trials for this inhibitor have been unexpectedly ceased.

Vaccines

Since antibodies have relatively short half-lives *in vivo* and require frequent injections, peptide-based vaccines are designed to provoke the generation of anti-PCSK9 antibodies by the immune system with high affinity and specificity for long-term management of LDLc [255]. In a pre-clinical study, administration of peptide (PCSK9)-based vaccines to rats and $LDLR^{+/-}$ or wild-type mice generates anti-PCSK9 antibodies with high affinity and specificity, leading to a 50% reduction in LDLc in all animals. The immunity mediated by these vaccines lasted for one year in mice [255]. These findings suggest that peptide-based vaccines may be safe and potent means for specific targeting of extracellular PCSK9 and sustained management of LDLc,

hypercholesterolemia, and CVD. AT04A is another peptide-based anti-PCSK9 vaccine that alleviates atherosclerosis and CHD in preclinical mouse models [256]. In atherosclerotic mice, AT04A vaccine produces high and persistent levels of anti-PCSK9 antibodies that overtly reduce plasma levels of LDLc, total cholesterol, and inflammatory factors [256]. Recently, nanoliposomes with negative charges are developed as a delivery system for PCSK9 vaccines [257]. In one study, the peptide IFPT (immunogenic fused PCSK9-tetanus) is coated on nanoliposomes to create a vaccine that is inoculated into atherosclerotic C57BL/6 mice. Four cycles of vaccination generate high titers of IgG antibodies that block extracellular PCSK9 and reduce LDLc (by 42%). Moreover, immunostimulatory IFN-γ-producing and immunosuppressive IL-10-producing splenocytes are decreased and increased, respectively [257]. These results suggest that nanoliposome-delivered IFPT vaccine could mediate long-lasting therapeutic effects against severe atherosclerosis. In a similar study, 100nm nanoparticles produced by self-assembly of BSA (bovine serum albumin) are charged with PCSK9 haptens (multicopy of PCSK9 sequences) to create a nanovaccine [258]. This PCSK9 hapten nano-vaccine triggers higher levels of PCSK9 antibodies compared with those induced by conventional PCSK9 vaccines [258]. In another independent study, a peptide vaccine, made up of short peptides conjugated to a carrier KLH protein is designed and inoculated into male *ApoE* $^{-/-}$ mice three times in biweekly intervals [259]. Using this protocol, high levels of anti-PCSK9 antibodies remain at peak levels for 24 weeks, thus reducing total cholesterol, VLDL, and chylomicron levels, while upregulating LDLR in the liver [259].

Virus-like particle (VLP) vaccines have also been designed to induce anti-PCSK9 antibodies [260]. In macaques and mice, administration of bacteriophage VLP vaccines induces anti-PCSK9 IgG antibodies thus reducing LDLc, TG, phospholipids, total and free cholesterol

levels [260]. In addition, screening appropriate anti-PCSK9 VLP vaccines has identified the PCSK9Qβ-003 vaccine, which induces high levels of anti-PCSK9 antibodies, reduced levels of total cholesterol and PCSK9 in plasma, and upregulated expression of liver *LDLR* in *LDLR*$^{+/-}$ and wild type Balb/c mice [261]. Currently, VLP vaccines are under development by pharmaceutical companies [6]. In addition, 5-weekly vaccination of recombinant HSP25 (rHSP25) to ovariectomized *ApoE* $^{-/-}$ mice alleviates atherogenesis and cholesterol levels in plasma, and upregulates liver expression of *LDLR* [262]. However, postmenopausal E2 therapy upregulates *PCSK9* expression and its promoter activity [262]. These observations indicate that rHSP25 vaccination, unlike E2 therapy, reduces cholesterol levels in plasma without modulating *PCSK9* expression. Therefore, rHSP25 vaccination may be an excellent therapeutic option for atherogenesis management in postmenopausal women. It is well perceived that increased level of HSP27 can modulate atherosclerosis, as subcutaneous injection of HSP27 or inducing its overexpression in *ApoE*$^{-/-}$ mice attenuates plaque and plasma cholesterol levels and alleviates atherosclerotic lesions and inflammation [263]. Immune HSP27 complex (antigen-antibody) upregulates *LDLR* expression and its promoter activity through activation of NF-κB, and attenuates PCSK9 in hepatocytes, as well as significantly suppresses inflammation by yet unspecified mechanism (**Fig. 5**) [263]. Hence, HSP27 vaccination represents a plausible approach to lower cholesterol and PCSK9 levels via upregulation of *LDLR*, leading to prevention of atherogenesis and inflammation. Overall, these observations urge the conduction of clinical trials to evaluate the applicability of these vaccines on human CVD models. Also, the persistent nature of the response to vaccinations would require careful and rigorous long-term safety evaluation.

Peptides and peptidomimetics

Peptides mimicking the EGF-A domain of LDLR, C-terminal and catalytic domains, and N-terminal prodomain of PCSK9 have been designed [264-268]. A mutation in the EGF-A domain of LDLR (His 306→Tyr) strengthens PCSK9-LDLR binding thus contributing to familial hypercholesterolemia [264]. The mutated LDLR exhibits relatively high-affinity interaction with PCSK9 due to formation of a hydrogen bond between Tyr 306 in LDLR and Asp 374 in PCSK9 [264]. According to these observations, addition of sub-fragments of mutated LDLR-Tyr 306 to HepG2 cells with GOF or wild type *PCSK9* expression blocks the binding of extracellular PCSK9 to cell surface LDLRs [264]. These *in vitro* data support the notion that sub-fragments of mutated LDLR in EGF-A domain could be utilized as LDLR mimetics and pharmaceutical inhibitors of extracellular PCSK9. In addition, screening peptide libraries using phage display technology has revealed a 13AA linear peptide (Pep2-8) functioning as a small inhibitor of PCSK9 [269]. Pep2-8 exhibits robust binding affinity to PCSK9 (but not to other proprotein convertases) and competitively inhibits the binding of LDLR-EGF-A to PCSK9, thereby restoring cell surface LDLRs in HepG2 cells [269]. Hence, Pep2-8 might be considered as an LDLR-EGF-A mimetic. Interestingly, peptides mimicking LDLR interactors might be employed for blocking PCSK9 as well. Thus, LRPAP1, a protein that interacts with type A repeats domain in the N-terminal region of LDLR [265], blocks the interaction of PCSK9 with ApoER2, VLDLR, and also to some extent LDLR in mice [265]. Therefore, it might be possible to develop LRPAP1 mimetics that block PCSK9-LDLR interaction. Another group of mimetic peptides are PCSK9 mimics that bind to LDLR to block its interaction with extracellular PCSK9. Thus, peptides corresponding to AA365–384 and AA323–358 of PCSK9 are identified to bind with high affinity to LDLR-EGF-A [270]. Accordingly, a PCSK9-derived 365–384 peptide (containing a disulfide bond) binds to LDLR present on the surface of cultured HepG2 and HuH7 cells [270]. Moreover, peptides derived from

960 the prodomain of PCSK9 (AA31-60, AA31-40, AA91-120) enhance LDLR levels in Huh7 and
961 HepG2 cells *in vitro* [267]. Similarly, a short PCSK9 prodomain-derived peptide (AA31-37)
962 results in significantly enhanced LDLR activity [267]. These cell-based data echo findings in
963 which a long fragment of the PCSK9 prodomain (AA31-152) is fused with the Fc (fragment
964 crystallizable) domain of human IgG1 to yield a chimeric protein. In HepG2 and HEK293 cells
965 Fc-PCSK9-prosegment binds to PCSK9 and remarkably blocks its interaction with LDLR [271].

966 Also, β-strand peptidomimetics have been shown as promising small molecules capable of
967 disrupting PCSK9-LDLR1 interaction [272]. Given that a β-strand conformation composed by the
968 Val328-Cys329-Asn330-Asp331 domain in LDLR and the Cyst78-Phe379-Val380-Ser381
969 domain in PCSK9 plays a role in PCSK9-LDLR interaction, an imidazole-based small β-strand
970 mimic has been developed. This small molecule successfully inhibits the interaction [272] but has
971 not been evaluated *in vivo*, in suitable preclinical models. Polydatin is a stilbenoid glucoside that
972 can be employed for the treatment of metabolic disorders including diabetes [273].
973 Mechanistically, polydatin downregulates and upregulates protein levels of PCSK9 and LDLR,
974 respectively, in HepG2 cells and leptin receptor-deficient *db*/*db* mice. Furthermore, polydatin
975 disrupts PCSK9-LDLR interaction by forming hydrogen bonds with PCSK9 [273]. Interestingly,
976 endogenous AnxA2 binds PCSK9 [23]. Also, a complex of AnxA2 and P11 (a.k.a S100A10) averts
977 PCSK9-mediated LDLR degradation in HepG2, HuH7, and hamster ovarian cells [23].
978 Immunocytochemistry studies have revealed cell surface co-localization of AnxA2 and PCSK9
979 and a competition between AnxA2 and LDLR for binding to PCSK9, suggesting AnxA2 acts as
980 an endogenous inhibitor of PCSK9 [23]. This approach should help to open a novel avenue to
981 generate peptidomimetics resembling the AnxA2 N-terminal Arg-Arg-Thr-Lys-Lys repeat that
982 bind to the C-terminal Cys-His-rich domain of PCSK9. However, there are other candidate

peptides as well. Thus, P5 peptide derived from lupin inhibits PCSK9-LDLR interaction in a dose-dependent manner. P5 treatment of HepG2 cells remarkably boosts the uptake of extracellular LDL [274]. Taken together, efforts are being engaged to generate drugs capable of disrupting the pathogenic interaction between PCSK9 and LDLR. However, convincing evidence pleading in favor of superior clinical activity (as compared to PCSK9-specific mAbs) remains elusive at this stage.

Emerging therapeutic compounds

A number of natural compounds have been documented to modulate *PCSK9* expression directly or indirectly via regulation of its transcription factors, thereby leading to LDLc clearance from the plasma. For instance, deoxythymidine derived from the marine invertebrate *Acanthaster planci* suppresses promoter activity of *PCSK9* in HepG2 cells thus reducing total cholesterol and LDL without evident hepatotoxicity in mice [275]. The *Penthorum Chinese*-derived compound "(7′*E*,8*S*)-2′,4,8-trihydroxy-3-methoxy-2,4′-epoxy-8,5′-neolign-7′-en-7-one" robustly enhances hepatic cholesterol uptake through suppression of *PCSK9* expression and upregulation of LDLR in a manner dependent upon SREBP2 [276]. Moreover, icaritin derived from *Capsella bursa-pastoris* inhibits intracellular expression of PCSK9 through suppression of *SREBF2* and *HNF1α* activities [277]. Moreover, icaritin reduces extracellular levels of PCSK9, hence stabilizing LDLR in HepG2 cells [277]. **Table 2** lists putative therapeutic compounds modulating *PCSK9* expression and protein levels. In a novel approach, an antagonist of PCSK9 named the "Flag-PCSK9$_{PH}$" is designed to disturb interaction between PCSK9 and LDLR in HepG2 cells [278]. Mechanistically, Flag-PCSK9$_{PH}$ is a shortened derivative of human wild-type PCSK9 tightly bound to cell surface LDLR at a neutral pH prior to dissociation from LDLR in endolysosomes at acidic pH, thus

preventing LDLR degradation [278]. **Fig 6** summarizes current available knowledge on natural PCSK9 inhibitors targeting different stages of PCSK9 biological process, e.g., expression, maturation, release, and interaction with LDLR [279].

Genome editing techniques: targeting both intracellular and extracellular PCSK9

CRISPR-Cas9 technology and CRISPR base editing

Due to the emergence of genome-editing technologies, targeted DNA sequences can be permanently inserted, modified, or removed. The CRISPR-Cas9 system is a cutting-edge genome-editing technique with promising efficacy (on-target gene editing) and safety (non-off-target gene editing) for gene therapy [280]. The advent of CRISPR-Cas technology has revolutionized clinical treatment of genetic diseases [281, 282], with genome editing now moving towards the treatment of CVD [283, 284]. Indeed silencing PCSK9 using CRISPR-based editing delivered to hepatocytes either in a lipid nanoparticle formulation [285] or modified adenoviral vectors is extremely efficient and long-lasting in reducing PCSK9 levels by >99% and LDLc level by >55% [286]. Human clinical trials have begun to explore this technology and to test its safety and durability in familial hypercholesterolemia (FH) patients (NCT05398029). In one study, CRISPR-Cas9 technology is applied to edit *PCSK9* gene in chimeric mice bearing human hepatocytes, which provided on-target editing of *PCSK9* gene (with nearly 50% efficacy), thereby reducing PCSK9 levels in plasma [287]. In mice, CRISPR-Cas9 technology is also exploited to induce LOF mutations in *PCSK9* gene in an attempt to lower plasma LDLc levels [288]. Technically, an adenovirus is utilized to express Cas9 protein and a CRISPR RNA was synthesized to target *PCSK9* gene in mouse livers. This allows for >50% on-target mutagenesis of *PCSK9* gene with

minimal off-target mutagenesis, resulting in significant reduction of plasma PCSK9 and LDLc levels, as well upregulation of LDLR protein [288]. One of the major drawbacks of CRISPR-Cas9 technique refers to the absence of effective and safe delivery strategies *in vivo*. Although adeno-associated virus (AAV)-based delivery of CRISPR-Cas9 offers promising efficacy, its immunogenicity compromises the *in vivo* application, especially if repeated administrations are required [289]. In an independent study, new lipid-like nanoparticles are developed to deliver a single-guide CRISPR RNA and *Cas9* mRNA into C57BL/6 mice livers for targeting *PCSK9* [290]. Moreover, limiting the duration of *Cas9* expression has been proposed to diminish Cas9 immunogenicity [287]. Therefore, a self-cleavable CRISPR-Cas9 is engineered to restrict *Cas9* expression. In this system, Cas9 cuts not only genomic locus of *PCSK9* gene but also self-eliminates *Cas9* gene in the AAV vector [287]. This engineered system exhibits reduced off-target activity (up to 20-fold) and *Cas9* expression (up to 60%) *in vivo*, nevertheless, resulting in remarkable PCSK9 and LDLc reduction in plasma [287]. Of note, "CRISPR base editing" or "base editing" is a new generation of CRISPR-Cas9 technology that introduces permanent alteration and point mutation in genes without the formation of DNA double strand breaks and more prone to off-target defects [291, 292]. Most pathological conditions are derived from single-nucleotide polymorphism (SNP) mutations. Hence, only a single-nucleotide modulation would be sufficient to reverse such SNP mutation [293]. CRISPR base editors have been developed in two major categories including adenine base editors (ABEs) and cytidine base editors (CBEs), mediating the conversion of adenine to guanine and cytidine to thymine, respectively [294]. Prior to *in vivo* application of base editors to patients in clinic, editing durability needs demonstration in nonhuman primates. In cynomolgus monkeys, delivery of CRISPR base editors into the liver using lipid nanoparticles significantly and precisely nullifies *PCSK9* thus reducing plasma PCSK9 and

LDLc levels by 90 and 60%, respectively, which remain stable for over 8 months after a single-dose treatment [295]. Likewise, lipid nanoparticle delivery of ABEs to the liver elicits 67 and 34% editing in mice and macaques, thereby reducing plasma PCSK9 and LDLc by 32 and 14% in macaques and 95 and 58% in mice, respectively, without off-target mutations [296]. Despite the safety of ABEs, humoral immune response is detected following ABEs treatment, contributing to the reduced editing efficacy in macaques [296]. Hence, CRISPR-based edition of *PCSK9* gene might permanently reduce LDLc levels in patients to warrant life-long cardiovascular benefits [297]. Nonetheless, the clinical application of CRISPR base editors would require overcoming ethical issues, enduring unwanted effects, and potential off-target alterations in the genome [298]. Collectively, these data suggest that advances in CRISPR-Cas9 gene therapy may yield satisfactory efficacy and safety in gene-editing of human *PCSK9*. However, the clinical applicability of this system is still debatable and requires further research.

Oligonucleotide-based therapeutics: siRNAs

Oligonucleotide therapeutics have been emerged in recent years for silencing un-druggable targets [299]. Oligonucleotides represent small synthetic molecules that do not permanently modify host genomes, implying likely reversibility of these effects. In addition, oligonucleotides can be designed to be highly specific, for instance for silencing the transcripts of mutated genes only with minimal off-target effects [300]. Oligonucleotide-based approaches can be used to generate antisense RNAs, siRNAs (small interfering RNAs), as well oligonucleotides that influence splicing of pre-mRNA to mature mRNAs [6]. siRNAs are noncoding RNAs governing post-transcriptional gene silencing [301]. In an experimental study, siRNAs are developed to target *PCSK9* transcripts specifically in the liver of rats, mice, non-human primates, and humans. As a

result, siRNA-mediated *PCSK9* silencing downregulates *PCSK9* expression up to 50-70% in rats and mice, resulting in a 60% reduction in plasma LDLc [302]. Also, in mice bearing human *PCSK9,* siRNA induces 70% silencing of *PCSK9* transcripts and remarkably reduces PCSK9 protein levels in plasma. Similarly, in non-human primates, siRNA-mediated *PCSK9* silencing reduces plasma PCSK9, LDLc, and ApoB levels [302]. These effects last for 3 weeks, suggesting that targeting *PCSK9* transcripts using siRNAs could be a potential approach for prevention or treatment of CVD. Also, siRNAs can target upstream regulators of *PCSK9*. For example, SCAP plays a key role in regulation of lipid metabolism, mediates the activation of SREBP1 and SREBP2, and has been proposed as a therapeutic target in the treatment of dyslipidemia [303]. In moue livers, siRNA-mediated silencing of *SCAP* mRNA attenuates SREBP gene expression, lipogenesis, and plasma PCSK9 and LDLc levels in a concentration-dependent manner [304]. Similarly, in rhesus monkeys, administration of *SCAP*-targeting siRNA reduces plasma LDLc, TG, and PCSK9 by 50, 50, and 75%, respectively [304]. These data indicate that siRNA-induced silencing of *SCAP* mRNA confers significant lowering of circulating LDLc and PCSK9 in primate and rodent models.

In a randomized phase I clinical study, a PCSK9-targeting siRNA (ALN-PCS) is developed and administered to 32 healthy volunteers [305]. Pharmacokinetic properties, tolerability, and safety of ALN-PCS, as well as its pharmacodynamic impacts are regarded as clinical endpoints. ALN-PCS treatment (0.400 mg/kg) yields 70% and 40% reduction in plasma PCSK9 and LDLc levels, respectively, compared to placebo ($p<0{\cdot}0001$) [305]. Also, in a phase I clinical trial, a highly durable type of ALN-PCS siRNA known as inclisiran (ALN-PCSsc) is supervised on healthy volunteers and its pharmacodynamic, side-effects, and safety parameters are evaluated [306]. The results reveal presence of moderate adverse effects such as diarrhea, back pain,

1097 headache, nasopharyngitis, musculoskeletal pain, and coughing. A single-dose of 300 mg
1098 attenuates PCSK9 levels up to 74.5% within 84 days and multiple doses of 100 mg reduce LDLc
1099 levels up to 50.6% [306]. In conclusion, inclisiran-mediated inhibition of *PCSK9* mRNA does not
1100 pose serious adverse effects and provides a long-lasting decline in plasma LDLc levels up to 6
1101 months. In addition, in ORION-1 trial, a phase II trial, it is demonstrated that inclisiran treatment
1102 of 501 participants with different dosing regimens significantly reduces LDLc levels [307]. In a
1103 single dose regimen, LDLc, non-HDLc, and ApoB levels decline within 210 days and the second
1104 dose further lowers these lipids [307]. These findings are validated in two Phase II trials (Orion-
1105 10 and -11) showing that LDL cholesterol is reduced by ~50% when inclisiran is administered
1106 subcutaneously every 6 months [308]. Until recently, inclisiran significantly attenuates circulatory
1107 LDLc and major adverse cardiac events in 3655 patients with atherosclerotic CVD, heterozygous
1108 familial hypercholesterolemia, and statin-therapy tolerance in ORION Phase III randomized
1109 clinical trial with 18 months follow-up [309]. However, inclisiran did not reduce fatal or non-fatal
1110 MI and stroke [309]. Despite the promise of inclisiran in CVD protection, these findings await
1111 clinical trials with longer follow-up and broader CVD outcomes. Besides, due to the longer
1112 functional half-life of inclisiran, only two or one administration per year would be adequate. Both
1113 in the United States and Europe, inclisiran has been approved for patients with heterozygous
1114 familial hypercholesterolemia [298]. Hence inclisiran provides prolonged and significant
1115 reduction in LDLc and other atherogenic lipoproteins by targeting *PCSK9* mRNA (**Fig. 5**).

1116

1117 ***Antisense oligonucleotides***

1118 Antisense oligonucleotides (ASO) are small DNA or RNA fragments that block mRNA or
1119 mediate its decay likely with the aid of ribonuclease H (RNAseH) enzyme [310]. As high

concentrations of ASO are relatively nontoxic, they have been broadly employed in clinical trials [311]. However, a case of acute kidney injury has been reported in a healthy female volunteer after subcutaneous administration of a bridged/locked nucleic acid (LNA)-ASO (SPC5001 compound) targeting *PCSK9* mRNA (three weekly doses) [312]. A few days following receiving the last dose, the kidney biopsy exposes indications of multifocal necrosis in tubules and urine microscopy indicates enhanced levels of granular casts, white blood cells, and creatine, as well as minimal hematuria thus raising doubt on the clinical applicability of this drug. However, a conservative treatment reduces creatinine levels in the serum and alleviates the symptom [312]. Of note, SPC5001 clinical trials are ceased due to kidney side effects. Another independent study has revealed two *PCSK9*-targeting LNA-ASO that sustainably attenuate plasma LDLc and PCSK9 levels by reducing *PCSK9* mRNA up to 85% in non-human primates (four weekly doses, each 20mg/kg) [313]. Also, LNA-ASO compounds do not exert any toxicological or adverse effects. These data suggest that LNA-ASO targeting *PCSK9* mRNA can offer a safe and viable therapeutic strategy for reduction in plasma LDLc [313]. In addition, transfection of LNA-ASO into human hepatic HuH7 and HepG2 and murine pancreatic β-TC3 cells (known to highly express *PCSK9*) remarkably decreases PCSK9 protein and mRNA levels and boosts LDLR levels [314]. In *in vivo* mice, LNA-ASO administration downregulates *PCSK9* mRNA by 60%, which lasts for over 16 days and robustly enhances liver LDLR levels up to three folds without posing hepatotoxicity [314]. Likewise, another study has shown that LNA ASO diminishes liver *PCSK9* mRNA and plasma PCSK9 and LDLc levels in a dose-dependent pattern in atherogenic C57BL/6J mice (twice a week for 6 weeks) [315]. In a recent study, an ASO has been developed for targeting *PCSK9* mRNA in the liver. Its subcutaneous administration exhibits robust potency in mice with *PCSK9* overexpression, an 18 days half-life in monkey livers, and plasma reduction of PCSK9 in humans

[316]. Of note, its oral administration provides greater liver bioavailability than subcutaneous administration in other animal species including dogs and rats and again diminishes LDLc levels in monkeys [316]. These observations point to the possibility of using orally-deliverable ASO for targeting liver *PCSK9* mRNA. In an attempt to treat hypercholesterolemia, AZD8233, an ASO compound has been developed [317]. Its subcutaneous administration to 73 healthy males at doses ranging from 4 to 120 mg/kg has shown that up to 60 mg of AZD8233 does not raise concerns regarding the prolongation of QT interval [317].

Splice-switching oligonucleotides

Splice-switching oligonucleotides (SSO) are synthetic, antisense, and short nucleic acids that can bind to and block/disrupt pre-mRNA splicing [318]. In an experimental study, an SSO has been developed to inactivate *PCSK9* mRNA by modulating its normal splicing and producing an inactive variant in mice and HepG2 and Huh7 cells [319]. As a result, the SSO exhibits remarkable efficiency and specificity in targeting *PCSK9* mRNA, thereby enhancing protein levels of LDLR [319]. These experimental findings suggest that SSO could be used for reducing PCSK9 expression. However, to the best of our knowledge, there are little recent studies examining such this possibility.

Backbone optimization and GalNAc targeted delivery

Despite undisputable promises of ASO and siRNAs in human disease management, their capacity to evoke immune responses in concomitant with their unfavorable pharmacokinetics have casted major doubt on their wide clinical applications. However, in large measure, these drawbacks

could be reconciled by the application of N-acetylgalactosamine (GalNAc) using targeted drug delivery technologies [320]. Development of GalNAc-siRNA conjugates for targeted delivery to the liver may represent one of the foremost of these cutting-edge technologies [321]. Functionally, *tris*-GalNAc binds to the highly expressed lectins on hepatocytes, ASGPR thus facilitating siRNA endocytosis, leading to robust and selective inhibition of target RNAs *in vivo*. Indeed, GalNAc-siRNA conjugates obviate the liver delivery setback of siRNAs. In this sense, multiple clinical trials of GalNAc-siRNA conjugates (e.g., two phase III) are currently ongoing by pharmaceutical companies [321]. This evolving technology in assistance with breakthroughs in oligonucleotide stability can pave the way for safe, efficacious, specific, and enduring intervention of previously thought undruggable genes such as *PCSK9*, often requiring 2 dose repeats per year.

Disadvantages of PCSK9 for therapeutic targeting in the context of diseases

It is essential to determine if pharmaceutical or genetic inhibition of PCSK9 is a safe strategy for clinical use [322, 323]. For instance, in zebrafish embryos, *PCSK9* knockdown induced neuronal disorganization in the central nervous system, and impaired the development of hind and midbrain areas, thus resulting in embryonic death [324]. This study suggests that non-specific targeting of PCSK9 could adversely affect the central nervous system. However, in mice, *PCSK9* whole-body knockout increased liver LDLRs and LDL clearance, and decreased plasma cholesterol without imparting neurological defects [325]. Nonetheless, *PCSK9* knockout upregulates the expression of *CD36*, encoding cell surface scavenger receptor CD36, which mediates long-chain fatty acids transportation. This may explain why, in the context of a high-fat diet, genetic *PCSK9* deficiency increases liver and kidney fat deposition [326, 327] and increases steatosis and lipid droplets in hepatocytes and kidney epithelial cells [328]. Moreover, *PCSK9*

deficiency induced renal ER stress, fibrosis, and inflammation in high-fat diet mice [327]. While these genetic findings suggest lipotoxicity of PCSK9 targeting, antibody-mediated neutralization of PCSK9 do not elicit such effects. Rather, evolocumab (anti-PCSK9 mAb) treatment reversed high-fat diet toxic effects on wild-type mice. Hence, a molecular complex comprising the evolocumab-PCSK9-CD36 is assembled in endosome-like vacuoles, resulting in a diminution of cell surface CD36 and protection of mice kidneys against lipotoxicity [327]. If reconfirmed in human, PCSK9-neutralizing mAbs could be employed for the alleviation of high-fat diet-induced kidney injuries. Finally, PCSK9 might serve as a diagnostic biomarker of spina bifida aperta in rats and humans [329]. Overall, the safety concern of PCSK9 targeting is still relevant and additional studies should be undertaken to end the debate.

Concluding remarks and future perspectives

Given the pathogenic role of PCSK9 in depleting surface-located LDLR on hepatocytes, ultimately resulting in an atherogenic plasma LDLc elevation, inhibition of PCSK9 by various strategies lowers LDL levels, with considerable implications for the treatment of hyperlipidemia, hypercholesterolemia, atherosclerosis, and CVD. Quantifying plasma levels of PCSK9 using advanced technologies such as proteomics and ELISA assays holds significant biochemical and metabolic importance at both diagnostic and prognostic levels, especially when making therapeutic decisions or assessing available options. Unfortunately, these crucial points have not been thoroughly addressed in the studies mentioned earlier. However, when plasma levels of PCSK9 significantly exceed the normal range, it indicates a pathological state related to the specific disease, thereby increasing the risk of subsequent events such as hyperlipidemia, especially if this condition is not already present. Consequently, these measurements are essential for therapeutic

decision-making in diseases where PCSK9 targeting is either under clinical trial evaluation or the only available treatment option. In addition, PCSK9 may mediate pro-inflammatory effects that occur independently of its interaction with LDLR. In this context, FDA-approved mAbs (as discussed above), as well as natural PCSK9 inhibitors (**Fig. 6**), may help to mitigate cardiovascular risk factors such as hyperlipidemia, familial hypercholesterolemia, and provide a new conceptual treatment option to statin-intolerant patients. Although targeting PCSK9 may diminish cardiovascular events and mortality, future large-scale investigation might further solidify this point. Besides, mAbs efficiently block the binding of PCSK9 to LDLR. However, their exorbitant cost and obligatorily parenteral administration hinder their proper and widespread implementation in clinics. Hence, novel approaches such as antisense oligonucleotides and gene editing techniques might fill the current gaps. Nonetheless, considering the difficulties in galenic formulations and the complexity of transcriptional regulation, 'soft' genetic approaches such as the siRNAs and antisense oligonucleotides are unlikely to achieve robust long-term suppression of *PCSK9* expression in the liver. Off-target mutagenesis by CRISPR-Cas9 technology and the immunogenicity of Cas9 [289] may compromise the application of this technology, pending their resolution by enhancing the fidelity of the system and by rendering Cas9 expression transient. Although delivery of peptide-vaccines using nanoliposomes seems to be encouraging, potential risks associated with application of nanoliposomes in clinical context should be taken into account [330]. Moreover, peptides, peptidomimetics or small molecules designed to break pathogenic PCSK9-LDLR interaction may affect PCSK9 interactions with other receptors including VLDLR and ApoER2, hence generating off-target (side) effects. Hence further investment into basic, pharmacological, and clinical research is warranted to current knowledge on PCSK9 and to target this molecule for optimal management of human diseases including hypercholesterolemia,

atherosclerosis, and CVD. Of note, the fast-raising development and validation of clinical efficacy of therapies that target PCSK9 following its role in LDL metabolism represents a major achievement. Application of the newer approaches described here may provide further inroads to reduce atherosclerotic risk, now a major worldwide health challenge. Beyond the scientific advances, efforts to combat clinical inertia, and to facilitate access of the novel therapeutics to all vulnerable populations remain a collective challenge to combat cardiovascular disease globally.

Conflict of interest statement

GK has been holding research contracts with Daiichi Sankyo, Eleor, Kaleido, Lytix Pharma, PharmaMar, Osasuna Therapeutics, Samsara Therapeutics, Sanofi, Tollys, and Vascage. GK has been consulting for Reithera. GK is on the Board of Directors of the Bristol Myers Squibb Foundation France. GK is a scientific co-founder of everImmune, Osasuna Therapeutics, Samsara Therapeutics and Therafast Bio. GK is the inventor of patents covering therapeutic targeting of aging, cancer, cystic fibrosis and metabolic disorders. Other authors have no potential conflict of interest to declare. Dr. Libby is an unpaid consultant to, or involved in clinical trials for Amgen, AstraZeneca, Baim Institute, Beren Therapeutics, Esperion Therapeutics, Genentech, Kancera, Kowa Pharmaceuticals, Medimmune, Merck, Moderna, Novo Nordisk, Novartis, Pfizer, and Sanofi-Regeneron. Dr. Libby is a member of the scientific advisory board for Amgen, Caristo Diagnostics, Cartesian Therapeutics, CSL Behring, DalCor Pharmaceuticals, Dewpoint Therapeutics, Eulicid Bioimaging, Kancera, Kowa Pharmaceuticals, Olatec Therapeutics, Medimmune, Novartis, PlaqueTec, TenSixteen Bio, Soley Thereapeutics, and XBiotech, Inc. Dr. Libby's laboratory has received research funding in the last 2 years from Novartis, Novo Nordisk and Genentech. Dr. Libby is on the Board of Directors of XBiotech, Inc. Dr. Libby has a financial

interest in Xbiotech, a company developing therapeutic human antibodies, in TenSixteen Bio, a company targeting somatic mosaicism and clonal hematopoiesis of indeterminate potential (CHIP) to discover and develop novel therapeutics to treat age-related diseases, and in Soley Therapeutics, a biotechnology company that is combining artificial intelligence with molecular and cellular response detection for discovering and developing new drugs, currently focusing on cancer therapeutics. Dr. Libby's interests were reviewed and are managed by Brigham and Women's Hospital and Mass General Brigham in accordance with their conflict-of-interest policies.

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Data Availability Statement

No original data were utilized for this work necessitating data repository. The corresponding authors are responsible to respond to any reasonable request regarding this publication.

Authorship contributions

AA provided the initial draft of the manuscript and DP, MM, ID, NS, GL, PL, GK, and JR significantly contributed to the revision, edition, and scientific evaluation of the manuscript.

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2196 **Table 1. Clinical trials of anti-PCSK9 monoclonal antibodies.**

Antibody	Study	Description of Study	Refs
Evolocumab	GAUSS-2 Phase III	Monthly or biweekly administration effectively reduces plasma LDLc (37-39 compared to ezetimibe) in a randomized clinical trial of 307 statin-intolerant patients with hypercholesterolemia	[331]
Evolocumab	MENDEL-2 Phase III	Monthly or biweekly administration reduces plasma LDLc (38-40% compared to ezetimibe) in a randomized clinical trial of 614 patients as a favorable monotherapy	[332]
Evolocumab	RUTHERFORD-2 Phase III	Monthly or biweekly administration remarkably reduces plasma LDLc (59.2%-61.3%) in a randomized clinical trial of 331 patients with heterozygous familial hypercholesterolemia	[333]
Evolocumab	DESCARTES Phase III	Monthly administration remarkably attenuates plasma LDLc (57.5%) in a randomized clinical trial of 901 patients with high levels of LDLc	[334]
Evolocumab	LAPLACE-2 Phase III	Monthly or biweekly administration remarkably decreases plasma LDLc (68.3-76% compared to high statin) in a randomized clinical trial of 2067 elderly patients with hypercholesterolemia or dyslipidemia	[335]
Evolocumab	TESLA-B Phase III	Monthly administration reduces plasma LDLc (30.9%) in a randomized clinical trial of 49 patients with heterozygous familial hypercholesterolemia	[333]
Evolocumab	OSLER-2 Phase III	Monthly administration markedly decreases plasma LDLc (52.3%) in a randomized clinical trial of 1104 patients with dyslipidemia or hypercholesterolemia	[336]
Evolocumab	FOURIER Phase III	Alleviated cardiovascular events after 26 months of administration and reduced plasma LDLc (59%) in a randomized clinical trial of 27000 participants with established atherosclerosis	[133]
Alirocumab	ODYSSEY MONO Phase III	Upon biweekly administration reduces plasma LDLc (32%) in a randomized clinical trial of 103 patients with hypercholesterolemia	[337]
Alirocumab	ODYSSEY ALTERNATIVE Phase III	Upon biweekly administration attenuates plasma LDLc (52.2%) in a randomized clinical trial of 314 statin-intolerant patients with hypercholesterolemia	[338]
Alirocumab	ODYSSEY FH Phase III	Upon biweekly administration attenuates plasma LDLc (49%) in a randomized clinical trial of 800 statin-intolerant patients with heterozygous familial hypercholesterolemia	[339]
Alirocumab	ODYSSEY COMBO II Phase III	Upon biweekly administration attenuates plasma LDLc (30%) in a randomized clinical trial of 720 patients with hypercholesterolemia	[340]
Alirocumab	ODYSSEY CHOICE II Phase III	Upon monthly administration attenuates plasma LDLc (51.7%) in a randomized clinical trial of 200 patients with heterozygous familial/primary hypercholesterolemia	[341]
Alirocumab	ODYSSEY LONG TERM	Upon biweekly administration significantly reduces plasma LDLc (61.3%) in a randomized clinical trial 2341 patients with a high risk of CVD and heterozygous familial hypercholesterolemia	[342]

Tafolecimab	Phase I	60 Chinese patients with hypercholesterolemia and 58 healthy individuals received a single dose of tafolecimab, which significantly lowered plasma LDLc levels in healthy and patient groups with moderate adverse events	[343]

Table 2. Therapeutic compounds modulating *PCSK9* expression levels.

Compounds	Mechanism of Action	Refs
Berberine	Downregulates *PCSK9* expression by reducing SREBP2 and HNF1α levels, increases liver LDLR and reduces plasma LDLc in C57BL/6 mice and HepG2 cells	[49, 344, 345]
Curcumin	Polyphenolic compound elevates LDL uptake via suppression of liver *PCSK9* expression in human Huh7 and HepG2 cells	[346]
Fenofibrate	Activates PPARα, and suppresses promoter activity and expression of *PCSK9*, attenuating PCSK9 protein in human hepatocytes	[347]
	Slightly reduces plasma levels of PCSK9 in type 2 diabetic patients	[225]
Gemfibrozil	Downregulates *PCSK9* expression in human HepG2 cells	[348]
Glitazones	Blocks liver secretion of PCSK9 by attenuating PPARγ activity	[349]
N–3 polyunsaturated fatty acids	Reduces PCSK9 levels in plasma in pre- and postmenopausal females	[350]
	Downregulates liver-specific expression of *SREBP2* and *PCSK9*, thereby, reducing plasma levels of PCSK9 and LDLc	[351, 352]
Quercetin	Downregulates liver expression of *PCSK9* and upregulates *LDLR* expression, thereby, enhancing hepatic uptake of LDL in human Huh7 cells	[353]
Quercetin-3-glucoside	Upregulates *LDLR* expression and blocks hepatic secretion of PCSK9 via downregulation of sortilin expression to enhance liver uptake of LDL in animal models	[353]

Abbreviations: PPARα, peroxisome proliferator-activated receptor alpha; PPARγ, peroxisome proliferator-activated receptor gamma

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- **Figure Legends**

Figure 1: Primary biological structure and processing of PCSK9.

The ER releases immature preproprotein PCSK9 which contains a signal peptide (SP). Subsequent sequential cleavage steps produce proprotein which is ultimately cleaved into propeptide and mature PCSK9 enzyme.

Figure 2: LDLR-LDL and PCSK9-LDLR interactions and internalization.

In the absence of PCSK9, LDL binds with LDLR prior to internalization by clathrin-coated endosomes, leading to the fusion with lysosomes and degradation of LDL with recycling of LDLR. In the presence of PCSK9, the complex of PCSK9-LDLR is internalized, where LDLR is degraded within endolysosomes, resulting in LDL accumulation in the plasma and increased risk of CVD.

Figure 3: Lipoproteins, lipids, and cardiac lipotoxicity.

Both albumin-bound fatty acids (FA) and free FA, as well as those from lipoproteins, can be released from capillaries to enter cardiomyocytes, where they ignite cardiac lipotoxicity as a result of excessive accumulation of FA. However, physiological levels of FA are essential for cardiac contractile function and energy metabolism.

Abbreviation: ACSL1, acyl-CoA synthetase long-chain family member 1; CPT-1 and 2, carnitine palmitoyltransferase I and 2; DAG, diacylglycerols, FATP1, fatty acid transport protein 1; MAG, monoacylglycerols; TAG, triacylglycerol.

Figure 4: PCSK9, increased risk of atherosclerosis and CVD.

Increased plasma PCSK9 levels foster LDLR degradation within the liver, which in turn, boosts circulatory LDL levels, leading to onset and progression of atherosclerotic lesions and other CVD.

Figure 5: Mechanisms of HSP27, siRNA, and vaccines

Immune complex of HSP27 and NF-κB activation trigger upregulation of LDLR at the gene expression level through unknown mechanism(s). After the entrance of siRNA into hepatocytes, RISC complex binds to the guide strand of siRNA and degrades complementary *PCSK9* mRNA, thereby, preventing its protein expression. Also, delivery of vaccines into body cells will induce formation of anti-PCSK9 antibodies which will bind to extracellular PCSK9 and limit their functions on cell-surface LDLRs in the liver.

Abbreviation: RISC, RNA-induced silencing complex.

Figure 6: Natural PCSK9 inhibitors.

A panel of natural PCSK9 inhibitors targeting PCSK9 at different stages of PCSK9 processing including expression, maturation, release, and LDLR interaction.