Pathology (February 2019) **51(2)**, pp. 177–183

LIPIDS AND CARDIOVASCULAR DISEASE

PCSK9: from biology to clinical applications

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Summary

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a crucial protein governing the circulating levels of low density lipoprotein-cholesterol (LDL-C), by virtue of its pivotal role in the degradation of the LDL receptor (LDLR). In the last 15 years, *in vitro* and *in vivo* studies have allowed our understanding of the physiological role of PCSK9. In the current report, we review the key studies that have established the mode of action of PCSK9, leading to the development of PCSK9 inhibitors for clinical use. Data from clinical trials investigating these therapies clearly and unambiguously demonstrate the safety and efficacy of these new drugs that have the power to dramatically reduce LDL-C and associated cardiovascular diseases.

Key words: PCSK9; LDL receptor; PCSK9 inhibitors; cardiovascular disease.

Abbreviations: apoB, apolipoprotein B; ACS, acute coronary syndrome; ASO, antisense oligonucleotide; CAD, coronary artery disease; CVD, cardiovascular disease; ER, endoplasmic reticulum; FH, familial hypercholesterolaemia; HDL-C, high density lipoprotein cholesterol; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDL, low density lipoprotein; LDL-C, low density lipoprotein cholesterol; LDLR, low density lipoprotein receptor; LNA, locked nucleic acid; mAb, monoclonal antibody; PCSK9, proprotein convertase subtilisin/kexin type 9; siRNA, small interfering ribonucleic acid; SREBP, sterol regulatory element-binding proteins.

Received 11 September, revised 3 October, accepted 3 October 2018
Available online 4 December 2018

INTRODUCTION

Familial hypercholesterolaemia (FH) is an autosomal dominant condition characterised by elevated plasma low density lipoprotein-cholesterol (LDL-C) levels, which are typically above the 95th percentile for age and sex. Molecular defects in the gene encoding the LDL receptor (LDLR) are identified in the vast majority of FH patients.[1,2] Approximately 5% of individuals with an FH phenotype carry mutations in the ligand-binding domain of apolipoprotein B (apoB), the protein component of LDL particle that interacts with the LDLR.[3] Fifteen years ago, genetic defects in PCSK9 were associated with the hypercholesterolaemic phenotype in less than 1% of FH families.[4] The unique mode of action of PCSK9 as an inhibitor of the LDLR has led to the successful development of a novel class of hypocholesterolaemic agents: the PCSK9 inhibitors. Here we review the biology of PCSK9, and the most significant pioneering preclinical studies that have paved the way for the development of PCSK9 inhibitors in clinical trials.

THE BIOLOGY OF PCSK9

A unique protease

PCSK9 is a serine protease of the subtilase family mainly expressed in the liver and to a much lower extent in the intestine, the kidney, and the brain. It was initially thought to be involved in liver regeneration and neuronal differentiation.[5] PCSK9 is synthesised as a 72 kDa zymogen in the endoplasmic reticulum (ER). It undergoes autocatalytic intramolecular processing at the $FAQ_{152}\Downarrow SIP$ site to form a 63 kDa mature enzyme found in the endoplasmic reticulum (ER) and the Golgi apparatus.[6,7] The signal peptide (residues 1–30) and the prodomain (residues 31–152) of PCSK9, precede a catalytic domain (residues 153–425) that contains the canonical N_{186}, H_{226} and S_{386} catalytic triad as well as the oxyanion hole N_{317} residue, followed by a C-terminal domain (residues 425–692). Unlike other proprotein convertases that cleave either after basic amino acids and are related to Kexin (e.g., furin) or after hydrophobic residues such as the Site 1 protease that is related to Pyrolysin, PCSK9 is the only known proprotein convertase of this family that cleaves after non-basic amino acids and thereby is related to proteinase K. The only known substrate for PCSK9 is pro-PCSK9 itself.[8]

In 2003, Abifadel and colleagues identified the $S_{127}R$ and $F_{216}L$ missense mutations in the gene encoding PCSK9 in two French families with FH, previously described as not carrying a mutation in the *LDLR* or *APOB* genes.[4] The PCSK9-$D_{374}Y$ missense mutation was subsequently reported in FH patients from Utah, Norway, and the United Kingdom.[9,10] These mutations were later shown to be 'gain of function' (GOF) mutations. The prevalence of *PCSK9* GOF mutations is very low ($\approx$1%) compared with defects in *LDLR* ($\approx$90%) and *APOB* ($\approx$5%). The risk of coronary artery disease (CAD) associated with the PCSK9-$D_{374}Y$ variant is sharply increased, and exceeds the average risk associated with mutations in the *LDLR* gene.[11,12]

Large cohort studies have been undertaken to address the role of common *PCSK9* sequence variations in lipid metabolism and CAD risk. In 2005, a causative association was established between two relatively common 'loss-of-function' (LOF) mutations in *PCSK9* and low plasma LDL-C levels.[13] The individuals carrying these mutations (PCSK9-$C_{679}X$ or PCSK9-$Y_{142}X$) exhibited LDL-C levels of 2.6 ± 1.1 mmol/L, compared with 3.6 ± 1.1 mmol/L for non-

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: https://doi.org/10.1016/j.pathol.2018.10.012

carriers, which was accompanied by an astonishing 88% reduction in global coronary heart disease risk.[14] No other safety concerns were identified in this patient population. Likewise, persons of European descent carrying the *PCSK9*-$R_{46}L$ LOF mutation exhibited LDL-C levels of 3.0 ± 0.9 mmol/L, compared with 3.5 ± 1.0 mmol/L for non-carriers, which was accompanied by a 47% reduction in global coronary heart disease risk.[14] As a result of these landmark observations, PCSK9 has become a very attractive drug target and the subject of intensive research.

A regulator of LDLR abundance at the cell surface

Besides FH genetic studies, the first evidence of a role for PCSK9 in cholesterol metabolism was the downregulation of PCSK9 hepatic expression observed in cholesterol fed mice.[15] *PCSK9* gene expression was upregulated in mice overexpressing SREBP, a transcription factor activated by low levels of intracellular cholesterol, and in cultured hepatocytes depleted of cholesterol by statin treatment.[16,17] Definitive evidence of a direct role for PCSK9 in lipoprotein metabolism was provided by a series of studies showing that adenoviral-mediated overexpression of PCSK9 promotes the accumulation of LDL in the plasma of control mice but not in that of LDLR deficient animals.[18,19] This was associated with decreased hepatic LDLR levels in mice overexpressing PCSK9.

The molecular mechanism by which PCSK9 modulates LDLR expression is not transcriptional.[17,20] The overexpression of PCSK9 in HepG2 cells accelerates the intracellular degradation of the mature LDLR via a non-proteasomal mechanism.[20,21] PCSK9 is in fact a secreted protein that binds to the LDLR and is subsequently internalised by the receptor, which in turn enhances its degradation in endo/lysosomal vesicles (Fig. 1).[22,23] Thus, exogenous PCSK9 decreases cell surface LDLR expression and PCSK9 is internalised in a manner almost totally dependent upon the LDLR itself *in vitro*. PCSK9 acts as a secreted protein *in vivo*, since secreted PCSK9 derived from a PCSK9 transgenic mouse is able to decrease hepatic LDLR expression in a parabiosed recipient wild-type animal, thereby increasing its plasma LDL levels.[22]

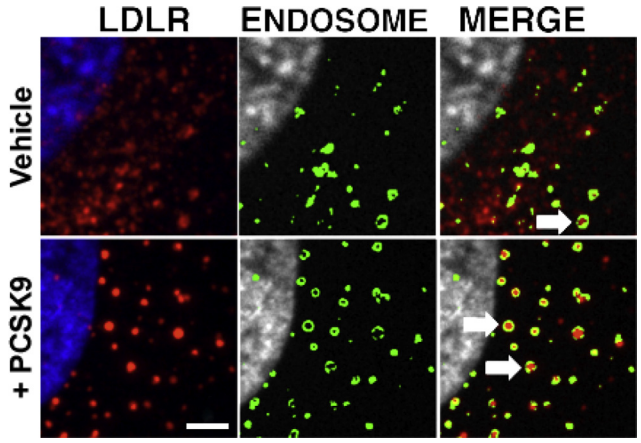

Fig. 1 PCSK9 targets the LDLR toward endo-lysosomal degradation. Human dermal fibroblasts were grown in serum depleted culture conditions in the presence of 10 mg/mL mevastatin to maximally upregulate LDLR expression. Recombinant PCSK9 (300 ng/mL) or vehicle control was added to the culture medium for 10 min. Fibroblasts were then fixed, permeabilised and visualised by confocal microscopy using fluorescent antibodies for the LDLR [clone C7 at 4 mg/mL (in red)], and Rab5 [PA3-915 at 1 mg/mL (in green)] a specific marker of endolysosomes. Nuclei were counterstained with DAPI (in blue). Arrows indicate the colocalisation of LDLR within endo-lysosomes. Scale bar = 1 mm.

The cleavage of the prodomain is required for PCSK9 maturation and secretion.[23,24] This was demonstrated by experiments where the prodomain and the catalytically inactive 62 kDa PCSK9 moiety were co-expressed, allowing the exit of a non-covalently bound PCSK9/prodomain complex from the endoplasmic reticulum to the Golgi complex and along the secretory pathway, which ultimately promoted LDLR degradation.[25–28] After cleavage, the prodomain forms hydrogen bonds with key amino acids of the catalytic domain, thereby preventing access of other potential substrates to the catalytic pocket of PCSK9.[29,30] Therefore, the ability of PCSK9 to promote LDLR degradation is independent of its catalytic activity, indicating that PCSK9 functions as a chaperone, a mode of action that is unique among serine protease.

The region where secreted PCSK9 interacts with the extracellular domain of the LDLR is located in the first epidermal growth factor-like repeat homology domain (EGFA) of the LDLR.[30,31] At the plasma membrane (i.e., at neutral pH), the catalytic domain of PCSK9 interacts with the EGFA domain of the receptor. After endocytosis (i.e., at the acidic pH of endosomes), the affinity between the receptor and PCSK9 is much higher than that observed at neutral pH.[29,32] The prodomain of PCSK9 establishes additional salt bridges with the receptor, and as a result PCSK9 locks the LDLR in an extended (or open) conformation. The failure of the receptor to adopt a closed conformation in the endosome precludes normal recycling to the plasma membrane and targets the LDLR for lysosomal degradation (Fig. 2).[29–33]

Despite these advances, the exact trafficking of the PCSK9/LDLR complex has not been fully elucidated. The PCSK9/LDLR complex can reach the lysosome via the extracellular endosome-lysosome route or alternatively via the Golgi-lysosome pathway that requires a direct interaction between the LDLR and PCSK9 intracellularly.[34,35] The description of one LDLR mutant insensitive to PCSK9 induced degradation via the extracellular but not via the intracellular route illustrates major differences between both pathways in terms of trafficking dynamics.[36] The extracellular pathway appears to be favoured in the liver, since grp94, an endoplasmic reticulum (ER) resident protein expressed in this tissue, prevents PCSK9 from interacting with the LDLR. In addition, grp74 prevents a direct interaction between *PCSK9* LOF variants that are retained in the ER.[37]

Towards the development of PCSK9 inhibitors

Studies of individuals with *PCSK9* LOF variants were pivotal for the efficacy and safety aspects in the development of PCSK9 inhibitors. Mendelian randomisation studies have recently corroborated the causal relationship between PCSK9, LDL-C and cardiovascular disease (CVD) risk, by demonstrating that LOF variants in the *PCSK9* gene have a similar effect on CVD risk per unit change in LDL-C as LOF variants in 3-hydroxy-3-methylglutaryl–coenzyme A reductase (HMGCR) gene.[38–41] The relatively high prevalence of *PCSK9* LOF mutations in the population indicates that such mutations are compatible with normal health. Thus, individuals with *PCSK9* nonsense mutations have similar body mass index, diabetes prevalence and hypertension prevalence compared to those without mutations.

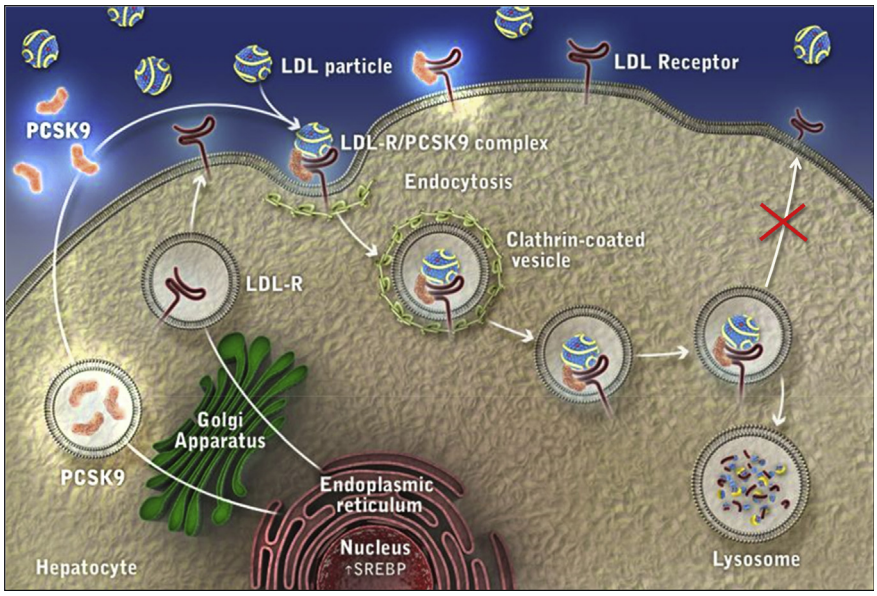

Fig. 2 Schematic representation of PCSK9 mode of action. When internalised by the LDLR, PCSK9 prevents normal recycling of the receptor back to the cell surface (X). The LDLR is routed to the lysosome and degraded.

Most important was the identification of individuals with LOF mutations in both *PCSK9* alleles. For instance, a compound heterozygous woman inherited the $Y_{142}X$ mutation from her mother and the c290_292 delGCC mutation from her father. The latter mutation deletes R_{97} and prevents autocatalytic cleavage and secretion of PCSK9.[42] Her plasma PCSK9 was undetectable and her LDL-C level was 0.36 mmol/L. She was healthy, fertile, normotensive, and college-educated, with normal liver and renal functions. Likewise, a handful of homozygous cases of *PCSK9*-$R_{46}L$ LOF have been reported. Compared with wild-type and heterozygotes, who display LDL-C levels of 3.75 ± 0.03 mmol/L and 3.31 ± 0.07 mmol/L, respectively, homozygotes have LDL-C levels of 3.08 ± 0.54 mmol/L.[43] In line with these observations, a Zimbabwean woman attending an antenatal clinic was found to be homozygous for the *PCSK9*-$C_{679}X$ LOF mutation.[44] She had an LDL-C level of 0.4 mmol/L postpartum. Wild-type and *PCSK9*-$C_{679}X$ heterozygous women had LDL-C levels of 2.2 ± 0.7 mmol/L and 1.6 ± 0.5 mmol/L, respectively. Overall, a complete or near-complete absence of PCSK9 resulting in very low levels of LDL-C does not appear deleterious in humans and is compatible with normal health.

Given the mode of action of PCSK9 as a circulating inhibitor of the LDLR, as well as the apparent healthy profile of individuals with reduced or absent PCSK9 function, PCSK9 rapidly gained status as a very clean drug target to lower LDL-C in humans. This was further demonstrated in preclinical studies and clinical trials.

CLINICAL IMPLICATIONS

Preclinical studies

PCSK9-knockout mice have approximately 2.8-fold higher hepatic LDLR expression and 42–48% lower total plasma cholesterol and lower apoB levels than wild-type animals.[45] These mice are particularly sensitive to statins, and their LDL-C fractional catabolic rate is markedly increased compared with wild-type mice. Similar features are observed in hepatocyte-specific conditional PCSK9-knockout mice, which have 27% lower plasma cholesterol than controls.[46] Compared with their respective controls, LDL-C is reduced by 80% and 60% in the plasma of complete PCSK9-knockout and hepatocyte-specific PCSK9-knockout mice, respectively. The lipoprotein profiles of LDLR-knockout mice and of PCSK9/LDLR double-knockout mice are nearly identical, further demonstrating that PCSK9 modulates circulating cholesterol levels exclusively via the LDLR.[46] Importantly, PCSK9 deletion in mice reduces the incidence of atherosclerosis in an LDLR-dependent manner. PCSK9-knockout mice fed a Western-style diet are protected from cholesterol ester deposition in the aorta on the C57BL/6 (74% reduction) as well as on the apoE-knockout (39% reduction) backgrounds, but not on the LDLR-knockout background.[47]

On these bases, several drug development strategies have been tested to pharmacologically inhibit PCSK9. They primarily include gene silencing with small interfering RNAs (siRNAs) or antisense oligonucleotides (ASOs), as well as monoclonal antibodies (mAbs). The administration of a *PCSK9* ASO inhibitor for 6 weeks to mice fed a high-fat diet resulted in a 2-fold increase in hepatic LDLR expression as well as 38% and 50% reductions in LDL-C and apoB-100 levels, respectively.[48] Likewise, reduction of PCSK9 mRNA in mice by a locked nucleic acid ASO (LNA ASO) resulted in a 2.5- to 3-fold increase in hepatic LDLR expression.[49] In monkeys, an LNA ASO targeting PCSK9 decreased circulating PCSK9 by 85%, plasma LDL-C by 50%, and apoB levels by 35%.[50] Modifying the LNA-ASO sequence to be specific for human PCSK9 demonstrated similar LDL-C reductions while having longer-lasting effects. *PCSK9* gene silencing in monkeys has also been achieved using siRNA, which reduced total cholesterol levels by up to 60% 3 days post-injection.[51]

In mice, a single intravenous infusion of a mAb specific to PCSK9 reduced circulating non-HDL-C levels in a significant and dose-dependent manner. In monkeys, a dose of 10 mg/kg of a mAb that structurally mimics the EGFA domain of the LDLR reduced plasma LDL-C levels by up to 80%

over 10 days.[52] A mAb with a greater affinity for PCSK9 lowered LDL-C by 45% at the 1 mg/kg dose in healthy rhesus monkeys.[53] In combination with simvastatin, this mAb lowered LDL-C by 40% in a group of monkeys with the metabolic syndrome.[53] A mAb directed against the LDLR-EGFA binding domain of PCSK9 dose-dependently reduced LDL-C in humanised mice and non-human primates.[54] The effects were similar when the animals were fed a high-fat diet, with a 64% reduction of LDL-C observed on day 3 post-injection.[55] When administered with simvastatin, this mAb produced an additional 65% reduction in LDL-C levels.[55] When modified to escape degradation, this mAb was found to be as potent as its precursor in mice and monkeys, with a 2.8-fold extended duration of maximum efficacy.[56] A comprehensive view of the important preclinical studies mentioned hereabove is given in Table 1.

Clinical trials

Similar approaches to evaluate PCSK9 inhibition in humans have been tested. Initially the most promising approach was the use of fully human monoclonal antibodies to PCSK9. A single injection can lower PCSK9 levels by up to 100% for more than a week. Phase I/II clinical trials have been conducted by Amgen (compound AMG 145, known today as evolocumab or Repatha)[57–61] and by Sanofi/Regeneron (compound SAR236553/REGN727 known today as alirocumab or Praluent).[62–65] A humanised anti-PCSK9 antibody (compound PF-4950615/RN-316, known as bococizumab) was developed by Pfizer-Rinat.[66] All phase I demonstrated safety and efficacy. In phase II, a reduction of 60–70% of LDL-C was confirmed, especially when subcutaneous injections were performed every 2 weeks. No significant side effects were observed, except possible local reaction to the injection site. Phase I and II clinical trials have been reviewed in detail elsewhere.[67,68] Three large phase III programs with the new PCSK9 antibodies were subsequently undertaken: the FOURIER program with evolocumab,[69] the ODYSSEY program with alirocumab,[70] and the bococizumab program (SPIRE 1 and 2).[66] The main objective of these studies was to evaluate the effect of PCSK9 inhibition on the occurrence of cardiovascular events (composite endpoint of coronary heart death, non-fatal myocardial infarction, fatal and non-fatal stroke, unstable angina requiring hospitalisation) in patients with acute coronary syndrome (ACS). These trials have unequivocally shown that PCSK9 inhibitors robustly and safely lower LDL-C levels regardless of background lipid-lowering therapy and prevent CVD (Table 2).[40,71,72] However, the SPIRE program was terminated, as anti-drug antibodies developed in a significant proportion of patients, likely because bococizumab is a humanised and not a fully human mAb like evolocumab and alirocumab.[73]

In addition to the two fully human monoclonal antibodies alirocumab and evolocumab, which sequester PCSK9 in the circulation and have been approved by regulating bodies and are now prescribed to patients in many countries, other approaches to PCSK9 inhibition, such as the siRNA inclisiran, are in late-stage clinical development.[74,75] These novel approaches to PCSK9 inhibition could have significant benefits compared to PCSK9-inhibiting mAbs, such as 2-yearly administration and lower costs of therapy.

CONCLUSION

The development of PCSK9 inhibitors underlines the advantages of a genetic-driven approach to identify novel drug targets. Discovered 15 years ago as the third gene causing FH,[4] PCSK9 has become a very promising drug target. The elucidation of its original mode of action as a secreted factor able to bind the LDLR and subsequently target it for intracellular degradation, as well as the absence of health issues associated with reduced or absent PCSK9 function, have led to the successful development of these inhibitors that dramatically lower circulating LDL-C levels but most importantly prevent cardiovascular events.

Table 1 Preclinical studies with PCSK9 inhibitors

Study	Drug	Experiment model	Dose	Efficacy (up to)
Graham *et al.*[48]	ASO	High fat fed WT mice	50 mg/kg weekly	↑ 2-fold LDLR ↓ 38% LDL-C
Frank-Kamenetsky *et al.*[51]	siRNA	WT mice and rats	5 mg/kg, 7.5 mg/kg	↓ 50–70% PCSK9 mRNA ↓ 60% TC
Chan *et al.*[52]	mAb	WT mice	10 mg/kg	↑ 2-fold LDLR ↓ 36% TC
		Cynomolgus macaques	10 mg/kg	↓ 80% LDL-C
Gupta *et al.*[49]	LNA ASO	WT mice	5–40 mg/kg single dose or 5 mg/kg weekly doses	↓ 60% PCSK9 mRNA ↑ 2.5- to 3-fold LDLR
Ni *et al.*[54]	mAb	Humanised mice	4–30 mg/kg	↓ 40% LDL-C
		Rhesus macaques	3 mg/kg	↓ 40% LDL-C
Liang *et al.*[55]	mAb	Cynomolgus macaques	10 mg/kg	↓ 70% LDL-C ↓ 39% TC
		Hypercholesterolaemic cynomolgus macaques	3 mg/kg injection + simvastatin	↓ 65% LDL-C
Lindholm *et al.*[50]	LNA ASO	Cynomolgus macaques	20 mg/kg initial dose + 5 mg/kg 4 weekly doses	↓ 85% PCSK9 mRNA and protein ↓ 50% LDL-C
Chaparro-Riggers *et al.*[56]	mAb	Mice	10 mg/kg	↓ ≥30% TC
		Cynomolgus macaques	1.5 mg/kg	↓ 50% LDL-C
Zhang *et al.*[53]	mAb	Humanised mice	Multiple doses	↓ 70% LDL-C
		Healthy rhesus macaques	1 mg/kg	↓ 45% LDL-C
		Metabolic syndrome rhesus macaques	3 mg/kg + simvastatin	↓ 40% LDL-C

ASO, antisense oligonucleotide; LDLR, low density lipoprotein receptor; LDL-C, low density lipoprotein-cholesterol; LNA, locked nucleic acid; mAb, monoclonal antibody; mRNA, messenger ribonucleic acid; TC, total cholesterol; WT, wild-type.

Table 2 Clinical trials

Drug name	Patients enrolled	Trial design	Dosage	Main findings				Reference
				Change of LDL-C	Cardiovascular events	Proportion of patients with LDL-C <70 mg/dL	Side effects	
Bococizumab	27,438 patients at high CVD risk	ST + mAb vs ST + placebo	150 Q2W	↓ 59% vs ST + placebo	ST + mAb → 1.72% ST + placebo → 1.88%	–	ST + mAb → 64% ST + placebo → 61%	Ridker *et al.*[72]
Alirocumab	2341 patients at high CVD risk	ST + mAb vs ST + placebo	150 mg Q2W	↓ 62% vs ST + placebo	ST + mAb → 1.7% ST + placebo → 3.3%	ST + mAb → 79% ST + placebo → 8%	ST + mAb → 81% ST + placebo → 82%	Robinson *et al.*[70]
Evolocumab	4465 patients	ST + mAb vs ST + placebo	140 mg Q2W or 420 mg QM	↓ 61% vs ST + placebo	ST + mAb → 0.95% ST + placebo → 2.18%	ST + mAb → 74% ST + placebo → 4%	ST + mAb → 69% ST + placebo → 65%	Sabatine *et al.*[69]
Evolocumab	27,564 patients with ASCVD	ST + mAb vs ST + placebo	140 mg Q2W or 420 mg QM	↓ 59% vs ST + placebo	ST + mAb → 7.9% ST + placebo → 9.9%	ST + mAb → 87% ST + placebo → 18%	ST + mAb → 77% ST + placebo → 77%	Sabatine *et al.*[71]
Inclisiran	501 patients	ST + SiRNA vs ST + placebo	Single dose (200–500 mg) or two doses (100–300 mg)	Single dose: ↓ 28–42% Two doses: ↓ 35–53%	–	ST + SiRNA → 66%	ST + SiRNA → 76% ST + placebo → 76%	Ray *et al.*[75]

CVD, cardiovascular disease; mAb, monoclonal antibody; Q2W, every two weeks; QM, every month; ST, standard therapy (maximal tolerated dose of statin ± ezetimibe).

Acknowledgements: GL is a laureate of the French national project CHOPIN (CHolesterol Personalized Innovation) granted by the Agence Nationale de la Recherche (ANR-16-RHUS-0007).

Conflicts of interest and sources of funding: GL has received research funding and honoraria from Sanofi, Regeneron, Amgen, Affiris, Pfizer, and Nyrada Inc, related to the development of PCSK9 inhibitors.

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