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PCSK9 Mutations in Familial Hypercholesterolemia: from a Groundbreaking Discovery to Anti-PCSK9 Therapies

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Abstract
Purpose of Review In 2003, Abifadel et al. (*Nat. Genet.* 34:154–156, 2003) identified *PCSK9*, encoding proprotein convertase subtilisin/kexin type 9, as the third causal gene for autosomal dominant hypercholesterolemia. This review focuses on the main steps from this major breakthrough in familial hypercholesterolemia (FH) to the latest clinical trials with the anti-PCSK9 antibodies.
Recent Findings The year 2015 was remarkable in cardiovascular disease through the field of cholesterol. Nearly 30 years after the discovery of statins, a new class of effective lipid-lowering drugs has emerged: the anti-PCSK9 antibodies. The discovery of the first gain-of-function mutations of *PCSK9* in FH rapidly became the center of interest of researchers worldwide. Preclinical and clinical studies launched by pharmaceutical companies led to the first three anti-PCSK9 antibodies, two of which (evolocumab and alirocumab) reduce LDL cholesterol levels by 50–60% and received FDA and European Medicines Agency approvals in 2015 on top of statin therapy. Recently, results of the Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Subjects With Elevated Risk (FOURIER) trial, the outcome trial of evolocumab over 2.2 years, showed a reduction of 15–20% in the risk of major cardiovascular outcomes in high-risk patients receiving statin therapy. Results of ODYSSEY OUTCOMES trial, evaluating the effect of alirocumab in 18,000 patients with established CVD are also eagerly awaited in 2018.
Summary The evolution of research on PCSK9, starting from the discovery of the first set of mutations in *PCSK9* in FH in 2003, is an amazing example of successful translational research. It shows how rigorous and powered genetic analyses can lead to the discovery of a new class of lipid-lowering drugs that give hope in fighting high cholesterol levels and their cardiovascular complications.

Keywords PCSK9 · Familial hypercholesterolemia · Cardiovascular disease · Clinical trials · Anti-PCSK9 antibodies

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Introduction

Cardiovascular diseases (CVDs) are major causes of mortality in the developed countries. Finding new drugs that lower LDL cholesterol (LDL-C) levels and reduce related CVD is extremely challenging. In 2003, Abifadel et al. discovered that gain-of-function mutations of proprotein convertase subtilisin/kexin type 9 (*PCSK9*) cause familial hypercholesterolemia (FH), thus revealing for the first time that PCSK9 was linked to cholesterol and its cardiovascular complications [1••].

The pioneering discovery of *PCSK9* as a cause of FH inspired several teams and pharmaceutical companies to

develop therapeutic agents targeting PCSK9. The efforts led to the first phase III clinical trials of a new promising lipid-lowering class: the anti-PCSK9 monoclonal antibodies (mAbs). This review highlights the various steps of the PCSK9 story starting from the discovery of PCSK9's implication in cholesterol metabolism to the successful anti-PCSK9 clinical trials and recent results of the first cardiovascular outcomes trial that were eagerly awaited.

The recruitment and genetic analyses of families with autosomal dominant hypercholesterolemia (ADH) through the French research network for hypercholesterolemia revealed the existence of genes associated with the disease, other than the two classically known genes encoding the LDL receptor (*LDLR*) and apolipoprotein B (*APOB*). A linkage analysis strategy in one of these families allowed the localization of the third gene responsible for ADH on the short arm of chromosome 1p32 in a multiplex French family [2], which was subsequently confirmed in a large family from Utah [3]. Rigorous and extensive positional cloning and sequencing approaches were performed in the genomic region at 1p32. Indeed, at the time, the human genome sequence was still far from being available and the regional physical map was also incomplete. Furthermore, to increase the power of the strategy, families who did not carry mutations in *LDLR* and *APOB* genes were identified and linked to this locus. The approach led to a better definition of the boundaries of the mapped chromosomal region and to the identification of the third gene implicated in FH [1••]. Among the genes at the locus, which were only found in patented databases at first, was the gene encoding neural apoptosis-regulated convertase type 1 (NARC1). This protein characterized by our collaborator in Montreal, Nabil Seidah, as the ninth member of the proprotein convertase family, is highly expressed in the liver, gut, kidney, and nervous system [4]. The high expression in the liver and its genetic localization in the candidate region at 1p32 led us to sequence *NARC1* gene, which we named *PCSK9* a few months later [1••], after consultation with the HUGO Gene Nomenclature Committee. Through sequencing of *NARC1*/*PCSK9* in patients and family members, we identified two mutations, p.Ser127Arg and p.Phe216Leu, in three families with ADH in September 2002 (publication in Abifadel et al. in 2003). This seminal result was crucial in discovering the clinical implication of PCSK9 in familial hypercholesterolemia and its cardiovascular complications [1••].

PCSK9 Gain-of-Function Mutations

The first mutation p.Ser127Arg was identified in two large multiplex families with several cases of hypercholesterolemia, tendon xanthomas, myocardial infarction, and stroke. These families, one from Nantes and the other from Dijon, seem to share a common ancestor as shown by the segregation of the same haplotype with the mutation in both families [1••]. A second nonsynonymous mutation in exon 4 of *PCSK9* (p.Phe216Leu) was identified in a family from Lille in which the proband died from myocardial infarction at the age of 49 with an LDL-C level of 356 mg/dL [1••]. The excellent segregation of the mutations in the families and their absence in numerous normocholesterolemic controls, together with the wealth of genetic, mapping, and sequencing data for this genomic region (notably negative sequencing results of more than 50 candidate genes), proved with very strong genetic evidence that *PCSK9* was the third causal gene for familial hypercholesterolemia [1••].

After the publication of the first gain-of-function (GOF) mutations of *PCSK9* in *Nature Genetics* in 2003 [1••], a third mutation of *PCSK9*, p.Asp374Tyr, was identified in the kindred from Utah that had confirmed the mapping to 1p of the gene (see above) [5]. This mutation is also associated with a severe phenotype and was found in three Norwegian families [6] and in three English families [7]. In vitro studies showed that the two GOF mutations, p.Ser127Arg and p.Asp374Tyr, resulted in a 23% decreased level of cell surface LDL receptors and a 38% decreased level of internalization of LDL particles compared with wild-type PCSK9 [8]. In animal models, human mutant PCSK9 (p.Ser127Arg and p.Phe216Leu) overexpressed in the liver of mice leads to hypercholesterolemia due to a dramatic decrease of hepatic LDL receptor levels through a post-transcriptional mechanism [9]. Overexpression of wild-type PCSK9 in mice also caused a twofold increase in plasma total cholesterol and a fivefold increase in non-HDL cholesterol with no effect on LDLR messenger RNA (mRNA) levels [9].

In vivo kinetics of apo B-100-containing lipoproteins conducted in two French subjects carrying the p.Ser127Arg mutation in *PCSK9* showed that this mutation dramatically increased the production rate of apo B-100 (threefold) compared with controls or *LDLR*-mutated patients and led to a higher direct overproduction of VLDL (twofold), intermediate-density lipoprotein (IDL) (threefold), and LDL (fivefold) [10]. Other *PCSK9* hypercholesterolemic mutations have been identified. These GOF mutations and their impact on the LDL receptor are summarized in Fig. 1 [11–18].

More recently, *PCSK9* GOF mutations were compiled from 12 centers in eight countries [19]. The patients carried 16 different missense mutations. Similar to FH and familial dysbetalipoproteinemia, 44% of patients had a history of CVD. Coronary artery disease was the most prevalent manifestation (33%) with an average age of onset of 49.4 ± 13.8 years. Although lipid-lowering therapy (primarily statins) improved lipid profiles, a substantial proportion failed to achieve guideline LDL-C levels [19].

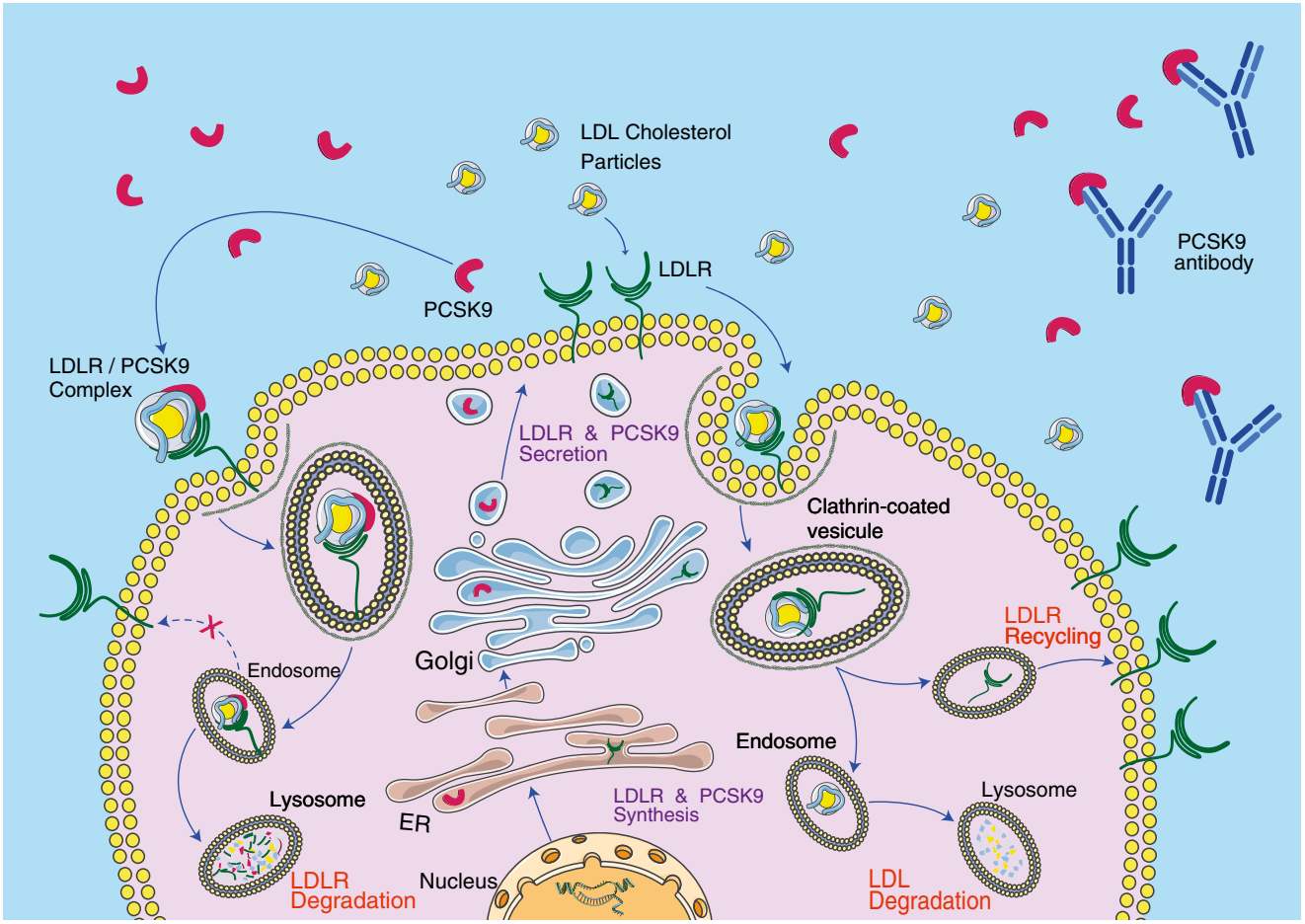

Fig. 1 Impact of PCSK9 on LDL receptor, *PCSK9* main mutations, and mechanism of action of inhibitors. The PCSK9 structure is characterized by a signal sequence, a prodomain, and a catalytic domain, followed by a C-terminal region. PCSK9 is synthesized as an inactive proenzyme of 75 kDa that undergoes autocatalytic cleavage in the endoplasmic reticulum (ER) which produces an approximately 60-kDa catalytic fragment. Autocatalytic cleavage of the zymogen in the ER is essential for transport from this compartment and for secretion [11]. PCSK9 degrades LDL receptor (LDLR) independently of its catalytic activity by involving mainly extracellular and possibly intracellular pathways. Secreted PCSK9 binds to the epidermal growth factor domain A (EGF-A) of the LDL receptor at the cell surface, and the PCSK9/LDL receptor complex will be internalized into endosomal/lysosomal compartments [12]. PCSK9-LDL receptor affinity is increased in the endosome due to higher acidity [13]. Failure to release PCSK9 may hinder receptor recycling and reduce the cell surface abundance of LDL receptor [14]. The LDL receptor would then be rerouted from the endosome to the lysosome where it is degraded [12]. Thus, PCSK9 functions as a chaperone to prevent LDL receptor recycling and/or target LDL receptors for lysosomal degradation. The 60-kDa mature and secreted form is cleaved by other proprotein convertases, particularly furin and/or proprotein convertase PC5/6A into an inactivated or less efficient fragment of 53 kDa [11]. *PCSK9 gain-of-function mutations*: the gain-of-function mutations p.Ser127Arg and p.Phe216Leu were identified in French families [1••], and the p.Asp374Tyr was identified in a kindred from Utah [5]. The p.Leu108Arg, the p.Asp35Tyr, and the p.Arg218Ser were also identified in French families [15]. The p.Ser127Arg variant interferes with autocatalytic cleavage, which is crucial for secretion from the cell. The p.Arg218Ser, p.Phe216Leu, and p.Asp374Tyr mutations result in total (p.Arg218Ser) or partial loss of the furin/PC5/6A processing of PCSK9, which increases the stability of PCSK9 [15, 16]. The p.Asp374Tyr variant binds LDL receptor 25 times more tightly than does wild-type PCSK9 at neutral pH, remains in a high-affinity complex at acidic pH, and is approximately tenfold more active in reducing LDL receptor levels than the wild-type protein. Other gain-of-function mutations have been identified: the p.Asp374His in Portuguese and French patients, the p.Asp129Gly in a family originating from New Zealand and a patient from the UK, and the p.Arg215His in two families from Norway. More recently, the p.Glu32Lys was reported in Japanese patients with hypercholesterolemia [13–16]. *PCSK9 loss-of-function mutations*: no protein was detected with the p.Tyr142Ter mutation. Some mutants associated with hypocholesterolemia either remain in the ER (p.Cys679Ter and the p.Gly106Arg mutations) or do not sort to endosomes (p.Leu253Phe and p.Gln554Glu), resulting in loss of function [13–16]. *PCSK9 monoclonal antibodies*: immediately after an injection of anti-PCSK9 antibody, the antibody binds to free circulating PCSK9 and reduces its plasma concentration. This results in a reduction of the degradation of LDLR in the lysosomal compartments. Thus, the number of LDLR on the cell surface increases, and in turn, more LDL particles bound to LDLR. LDL-C concentration is subsequently reduced. As PCSK9 antibody gets metabolized and its concentration lowered with time, plasma PCSK9 concentration increases and bound to LDLR, leading to its degradation in the lysosomal compartment. Subsequently, LDL-C concentrations increase. This effect of PCSK9 inhibition on LDL-C has been confirmed in clinical studies [17, 18]

PCSK9 Loss-of-Function Mutations

This breakthrough discovery of Abifadel et al. revealed a new player in cholesterol metabolism and its diseases, which paved the way for other teams to identify additional mutations in the *PCSK9* in not only hypercholesterolemic but also hypocholesterolemic subjects. When a new gene is discovered in hypercholesterolemia, searching for mutations associated with low LDL-C levels is the logical next step since it is well known that some mutations in the *APOB* gene are associated with hypercholesterolemia while others with hypocholesterolemia. The search for mutations in *PCSK9* in subjects with low plasma levels of LDL-C (LDL-C < 58 mg/dL) from the Dallas Heart Study, a multiethnic population of Dallas County, Texas, and the Atherosclerosis Risk in Communities (ARIC) study led to the identification of two nonsense mutations, p.Tyr142Ter and p.Cys679Ter, in 2.6% of the African-American subjects in 2005 [20]. These nonsense mutations were associated with a 28% reduction in mean LDL-C level and an 88% reduction in the risk of coronary heart disease (CHD). In the USA, one in every 50 African-American subjects has a nonsense mutation in *PCSK9*. These mutations were also found in 3.7% of African women from Zimbabwe and associated with a 27% reduction in LDL-C levels [20, 21]. However, another variant, p.Arg46Leu, was found in Caucasian subjects from the ARIC study and was associated with a 15% reduction in LDL-C levels and a 47% reduction in the risk of CHD [22, 23]. Several large studies have also shown the reduction in risk of ischemic heart disease or the reduction of the risk of early-onset myocardial infarction in the p.Arg46Leu carriers [24, 25]. Furthermore, three probands with very low LDL-C levels (15 mg/dL) and no immunodetectable circulating PCSK9 due to homozygous or compound heterozygous loss-of-function mutations were reported. The natural human *knockouts* were healthy and fertile adults [20, 26, 27]. Thus, hypocholesterolemia due to a deficiency of PCSK9 appears to be benign, with no complication. In animal studies, livers of knockout mice lacking PCSK9 ($Pcsk9^{-/-}$) display increased LDL receptors (but not mRNA) that leads to a decrease in plasma cholesterol levels of 4% compared to wild-type litter mates [28].

Regulation of *PCSK9* Gene Expression

Transcriptional regulation of the *PCSK9* gene has been evaluated over the past decade. Studies showed that *PCSK9* is upregulated by SREBP-1a and SREBP-2, LXR agonist, and insulin, but downregulated by dietary cholesterol, glucagon, ethinylestradiol, chenodeoxycholic acid, and the bile acid-activated farnesoid X receptor (FXR) [29–31]. Several cholesterol-lowering drugs induce changes in *PCSK9* expression. Statins give rise to an upregulation of *PCSK9* in HepG2 cells and in human primary hepatocytes through the increased expression of SREBP-2 [32]. Administration of statins to $Pcsk9^{-/-}$ mice produced an exaggerated increase in liver LDL receptors and an enhanced LDL clearance from plasma [28]. In humans, it has been shown that statins increased serum levels of PCSK9 [33]. Furthermore, when added to statin, ezetimibe further increased PCSK9 levels [34]. Fibrates increase plasma PCSK9 levels in most studies [35–38] while berberine, a natural hypocholesterolemic compound, suppresses *PCSK9* expression [39].

Serum PCSK9 levels measured by ELISA seem to be directly correlated with serum LDL-C and total cholesterol levels [40]. Factors regulating *PCSK9* expression and circulating levels have been previously reviewed [16]. Interestingly, it has been very recently shown that serum PCSK9 concentration is associated with future risk of CVD even after adjustment for established CVD risk factors [41]. Further studies are needed to confirm this observation. Furthermore, PCSK9 interacts with annexin A2 [42] and resistin [43]. In fact, in HepG2 cells, resistin increased *PCSK9* mRNA and protein expression by 40 and 30%, respectively, possibly via SREBP-2 upregulation or a post-transcriptional stabilization of the PCSK9 protein [43]. Annexin A2 interacts at the cell surface with the carboxyl-terminal cysteine and histidine-rich domain of PCSK9, inhibiting its LDLR-degrading activity [42]. Other interactors are possible but still unknown. It is noteworthy that a role of PCSK9 in lipoprotein assembly was also described. PCSK9 markedly increases intestinal triglyceride-rich apolipoprotein B lipoproteins (TRL apo B) production through mechanisms mediated in part by transcriptional effects on apo B, microsomal triglyceride transfer protein (MTP), and lipogenic genes and in part by post-transcriptional effects on the LDLR and MTP [44]. Furthermore, studies showed significant associations between serum PCSK9 levels and the levels of circulating TRL markers in a broad spectrum of the population [45, 46].

A New Therapeutic Class: the PCSK9 Inhibitors

PCSK9 is currently the most studied drug target for treating dyslipidemia. Several methods are being explored to inhibit PCSK9 function and reduce its plasma level (reviewed in [47]). These approaches target either extracellular PCSK9, such as mAbs or vaccines and small protein inhibitors (peptides/adnectins) [48, 49], or intracellular PCSK9, such as antisense oligonucleotides (ASOs) and small interfering RNA (siRNA) [50–52]. Indeed, antibodies (exogenous monoclonal or vaccine-derived) selectively bind to a specific epitope of circulating PCSK9 leading to its sequestration and

small protein inhibitors (peptides/adnectins) prevent the extracellular interaction between PCSK9 and LDLR. ASO and siRNA target intracellular PCSK9 by gene silencing techniques leading to a reduction in its production. However, the most successful strategy to date that has undergone clinical development is mAbs that target PCSK9. By binding to PCSK9 and preventing its interaction with the LDL receptor, mAbs lead to an increase in the number of functioning LDL receptors at cell surface and, thus, an increase in LDL uptake by hepatocytes. The mechanism of action of this new therapeutic class results in a reduction in plasma LDL-C concentrations [53].

Several mAbs targeting PCSK9 have been developed; however, only two are still extensively studied in clinical trials involving a variety of patient populations. Indeed, alirocumab and evolocumab, two fully human anti-PCSK9 antibodies, are being evaluated in the PROFICIO and the ODYSSEY clinical trial programs. Bococizumab (RN316/PF-04950615; Pfizer), a humanized antibody evaluated in the large SPIRE program [54], has been withdrawn because of the development of antidrug-neutralizing antibodies that reduce its efficacy over time [55••]. This outcome is reasonably attributable to the residual mouse sequence in the monoclonal antibody, while alirocumab and evolocumab sequences are fully human.

Monoclonal Antibodies Targeting PCSK9 and Their Related Clinical Studies

In 2015, both alirocumab (Praluent®, Sanofi-Aventis/Regeneron) and evolocumab (Repatha®, Amgen) received Food and Drug Administration (FDA) and European Medicines Agency (EMA) approvals [56]. As mentioned on their package inserts, both drugs are "indicated as an adjunct to diet and maximally tolerated statin therapy for the treatment of adults with heterozygous familial hypercholesterolemia or clinical atherosclerotic CVD, who require additional lowering of low-density lipoprotein-cholesterol." Evolocumab has an additional indication for homozygous FH patients [57, 58]. However, homozygous patients with LDL receptor-negative activity do not respond to anti-PCSK9 cholesterol-lowering effect [59••]. The recommended doses for the anti-PCSK9 injectable treatments are 75 mg for alirocumab administered subcutaneously (SC) every 2 weeks (Q2W) with the possibility of an uptitration to 150 mg every 2 weeks for additional LDL-C-lowering effect. As for evolocumab, the recommended dose is 140 mg SC Q2W or 420 mg SC Q4W (every month) [57, 58].

Indeed, following the encouraging results of phase I and II trials for alirocumab and evolocumab, long-term and large-scale phase III programs were launched to evaluate their safety and efficacy in reducing cardiovascular events. The trials evaluating alirocumab or evolocumab, ranging from 8 to 78 weeks, have demonstrated consistent reductions in LDL-C of 40–60% across different types of patients and background therapies. Both antibodies are effective as monotherapy as well as in addition to statin therapy. They are also effective in patients with heterozygous FH and in those having a difficulty to tolerate statin therapy [60]. Both antibodies are safe and well tolerated, and interestingly, they reduce lipoprotein(a) (Lp(a)) plasma levels of up to 30% [61, 62].

The phase III clinical trials and their impact on LDL-C and Lp(a) levels are summarized in Table 1. This table includes the following: (1) monotherapy trials using evolocumab (MENDEL-2) and alirocumab (ODYSSEY MONO) in patients with hypercholesterolemia and moderate cardiovascular risk [63, 64], (2) trials investigating the concomitant use of PCSK9 inhibitors with statin therapy in high cardiovascular risk patients (COMBO I, COMBO II, ODYSSEY OPTIONS I, ODYSSEY OPTIONS II, LAPLACE-2, and DESCARTES) [65–70], (3) trials studying the efficacy and tolerability of PCSK9 inhibitors in patients with statin intolerance (ODYSSEY ALTERNATIVE, CHOICE II, GAUSS-2, and GAUSS-3) [71–74], and (4) treatment of FH patients who are poorly managed with current available treatment (the RUTHERFORD-2 study evaluated evolocumab in heterozygous FH (HeFH) while TESLA Part B and TAUSSIG were conducted in homozygous FH (HoFH)) [59, 75, 76]. Alirocumab was also tested in HeFH in the ODYSSEY FH I and FH II studies and HIGH FH study, as well as in HeFH patients, carriers of *PCSK9* gain-of-function mutation [19, 77, 78]. These trials showed that more than 60% of patients achieved LDL-C < 70 mg/dL or risk-based LDL-C targets in high cardiovascular risk patients with inadequately controlled LDL-C levels, in patients with statin intolerance, and in HeFH patients.

Cardiovascular Benefits of PCSK9 Inhibitors: Long-Term Clinical Trials

Long-term clinical studies of PCSK9 inhibitors are crucial in order to validate the durability of the LDL-C-lowering effect observed in previous studies. Those studies are also necessary to prove the long-term safety and mainly the cardiovascular benefit of PCSK9 inhibitors. Evolocumab was evaluated in OSLER-2, whereas alirocumab was evaluated in ODYSSEY LONG TERM.

Results from OSLER-2 and ODYSSEY LONG TERM trials are very promising regarding the reduction in cardiovascular events. In the OSLER-2 study, treatment with evolocumab was associated with a significantly lower rate of cardiovascular events at 1 year compared with standard therapy (0.95 and 2.18%, respectively; hazard ratio, 0.47; 95% confidence interval, 0.28–0.78; $P = 0.003$). Most adverse events (AEs) occurred with similar frequency in the two groups (69.2% in the evolocumab group and 64.8% in the standard therapy group) [79]. Similarly, in the ODYSSEY

Table 1 Implications and indications of lipid-lowering effect of the two anti-PCSK9 antibodies (alirocumab and evolocumab) that have received the FDA and EMA approvals. The percentage of reduction of the LDL-C and Lp(a) levels is given in different phase III studies

Population type	Alirocumab				Evolocumab			
	Trial name	Dosing (mg)	LDL cholesterol reduction	Lp(a) reduction	Trial name	Dosing (mg)	LDL cholesterol reduction	Lp(a) reduction
Monotherapy	ODYSSEY MONO	75 Q2W	32%†	4.4%†	MENDEL-2	140 Q2W 420 Q4W	57.1%‡ and 39.3%† 54.8%‡ and 37.6%†	20.4%‡ and 20.4%† 17.8%‡ and 15.8%†
In combination with statin therapy	ODYSSEY COMBO I	75 Q2W	45.9%‡	14.6%‡	LAPLACE-2	140 Q2W	68.2–76.3%‡ and 39.6–47.2%†	22.4–36.5%‡ and 29.2–32.6%†
	ODYSSEY COMBO II	75 Q2W	29.8%†	21.7%†		420 Q4W	55–70.5%‡ and 38.9–41.1%†	19.8–32.2%‡ and 27.4–34.9%†
	ODYSSEY OPTIONS I	75 Q2W	44.1 and 54.0%*	23.6–30.8%§	DESCARTES	420 Q4W	48.5–61.6%‡	9.7–29.3%‡
	ODYSSEY OPTIONS II	75 Q2W	50.6 and 36.3%**	22.7–27.9%§				
Statin-intolerant patients	ODYSSEY ALTERNATIVE	75 Q2W	30.4%†	18.7%†	GAUSS-2	140 Q2W 420 Q4W	38.1%† 37.6%†	25.3%† 27.9%†
	ODYSSEY CHOICE II	75 Q2W 150 Q4W	53.5%§ 51.7%§	21.8%§ 15.5%§	GAUSS-3	420 Q4W	52.8%§	21.1§
Familial hypercholesterolemia	ODYSSEY FH I and FH II (HeFH)	75 Q2W	51.4%‡ 57.9%‡	17.7%‡ 20.3%‡	RUTHERFORD-2 (HeFH)	140 Q2W 420 Q4W	59.2%‡ 61.3%‡	31.6%‡ 28.2%‡
	ODYSSEY HIGH FH	150 Q2W	45.7§	23.5§	TAUSSIG	420 Q4W	23.3%§	11.9%§
	Hopkins et al. PCSK9 GOFm	150 Q2W	73%§	43.3%§	TESLA Part B (HoFH)	420 Q4W	30.9%‡	11.8%‡
Long-term trials	ODYSSEY LONG TERM	150 Q2W	62%‡	25.6%‡	OSLER-2	420 Q4W	61%‡	25.5%‡
					FOURIER	140 Q2W 420 Q4W	59%‡	26.9%§

HeFH heterozygous familial hypercholesterolemia, *HoFH* homozygous familial hypercholesterolemia, *GOFm* gain-of-function mutations, *Q2W* biweekly dose, *QM* monthly dose, *NA* not analyzed; ODYSSEY MONO, a study of the efficacy and safety of alirocumab versus ezetimibe in patients with hypercholesterolemia (n = 103); ODYSSEY COMBO I, a study of the efficacy and safety of alirocumab versus placebo on top of lipid-modifying therapy in patients with high cardiovascular risk and hypercholesterolemia (n = 311); ODYSSEY COMBO II, a study of the efficacy and safety of alirocumab versus ezetimibe on top of statin in high cardiovascular risk patients with hypercholesterolemia (n = 720); ODYSSEY OPTIONS I, a study of the efficacy and safety of alirocumab in combination with other lipid-modifying treatments (n = 355); ODYSSEY OPTIONS II, a study of alirocumab added on to rosuvastatin versus other lipid-modifying treatments (n = 305); ODYSSEY ALTERNATIVE, a study of alirocumab in patients with primary hypercholesterolemia and moderate, high, or very high cardiovascular risk, who are intolerant to statins (n = 314); ODYSSEY CHOICE II, a phase III study to evaluate alirocumab in patients with hypercholesterolemia not treated with a statin (n = 233); ODYSSEY FH I, a study of the efficacy and safety of alirocumab versus placebo on top of lipid-modifying therapy in patients with heterozygous familial hypercholesterolemia not adequately controlled with their lipid-modifying therapy (n = 486); ODYSSEY FH II, a study of alirocumab in patients with heterozygous familial hypercholesterolemia (HeFH) who are not adequately controlled with their lipid-modifying therapy (n = 249); ODYSSEY HIGH FH, a study of the efficacy and safety of alirocumab versus placebo on top of lipid-modifying therapy in patients with heterozygous familial hypercholesterolemia (n = 107); ODYSSEY LONG TERM, a study of the long-term safety and tolerability of alirocumab versus placebo on top of lipid-modifying therapy in high cardiovascular risk patients with hypercholesterolemia (n = 2341). The results of the LDL-C and the Lp(a) reductions for all these latter studies are given after 24 weeks of alirocumab treatment except for that of Hopkins et al. where results are given at 8 weeks of treatment. MENDEL-2, a trial of the monoclonal antibody against PCSK9 to reduce elevated LDL-C in subjects currently not receiving drug therapy for easing lipid levels (n = 614); LAPLACE-2, the Low-density Lipoprotein Cholesterol Assessment with PCSK9 Monoclonal Antibody Inhibition Combined with Statin Therapy-2 trial (n = 2067); DESCARTES, Durable Effect of PCSK9 Antibody Compared with Placebo Study (n = 901; study duration, 52 weeks); GAUSS-2, the Goal Achievement after utilizing an anti-PCSK9 antibody in Statin Intolerant Subjects trial (n = 307); GAUSS-3, Goal Achievement After Utilizing an Anti-PCSK9 Antibody in Statin Intolerant Subjects-3 (n = 218); RUTHERFORD-2, Reduction of LDL-C with PCSK9 Inhibition in Heterozygous Familial Hypercholesterolemia Disorder Study-2 (n = 331); TAUSSIG, Trial Assessing Long Term Use of PCSK9 Inhibition in Subjects With Genetic LDL Disorders (n = 106); TESLA Part B, Trial Evaluating PCSK9 Antibody in Subjects with LDL Receptor Abnormalities (n = 49); OSLER-2, Open Label Study of Long Term Evaluation against LDL-C Trial-2 (n = 4465). The results of the LDL-C and the Lp(a) reductions for all these latter studies are given after 12 weeks of evolocumab treatment. FOURIER, Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Subjects With Elevated Risk (n = 27,564)

†Versus ezetimibe

‡Versus placebo

§Versus baseline

*Among atorvastatin 20- and 40-mg regimens, respectively, add-on alirocumab reduced LDL-C levels at week 24 by 44.1 and 54.0%; add-on ezetimibe, 20.5 and 22.6%; doubling of atorvastatin dose, 5.0 and 4.8%; and switching atorvastatin 40 mg to rosuvastatin 40 mg, 21.4%

**In the baseline rosuvastatin 10 mg group, LDL-C reductions were observed with add-on alirocumab (− 50.6%) versus ezetimibe (− 14.4%) and double-dose rosuvastatin (− 16.3%). In the baseline rosuvastatin 20 mg group, LDL-C reduction with add-on alirocumab was − 36.3% compared with − 11.0% with ezetimibe and − 15.9% with double-dose rosuvastatin

LONG TERM study, in a post hoc analysis, alirocumab reduced the rate of major CVD events compared to placebo (1.7 vs. 3.3%; hazard ratio, 0.52; 95% confidence interval, 0.31 to 0.90; nominal $P = 0.02$) in patients with hyperlipidemia on maximally tolerated statins who were at high risk of CHD. The percentage of patients with any AE was similar in the two study groups (81.0% with alirocumab and 82.5% with placebo) [80]. Although promising, these results require confirmation in a larger powered trial.

2017 and 2018 are two interesting years for long-term cardiovascular outcome studies for both PCSK9 mAbs. Recently, results of the FOURIER (Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Subjects With Elevated Risk) trial, the first outcome trial of a PCSK9 monoclonal antibody therapy, were published [81••]. It is a randomized, double-blind, placebo-controlled trial involving 27,564 patients with atherosclerotic CVD and LDL-C levels of 70 mg/dL or higher who were receiving statin therapy. Evolocumab reduced LDL-C levels of 59% as compared with placebo. After a median follow-up of 2.2 years, evolocumab treatment significantly reduced the risk of the primary end point, a composite of cardiovascular death, myocardial infarction, stroke, hospitalization for unstable angina, or coronary revascularization (1344 patients [9.8%] vs. 1563 patients [11.3%]; hazard ratio, 0.85; 95% confidence interval [CI], 0.79 to 0.92; $P < 0.001$), and the key secondary end point, a composite of cardiovascular death, myocardial infarction, or stroke (816 [5.9%] vs. 1013 [7.4%]; hazard ratio, 0.80; 95% CI, 0.73 to 0.88; $P < 0.001$) relative to placebo [81••]. Importantly, there was no difference in treatment-emergent AEs except for increased injection site reactions in the evolocumab group (2.1 vs. 1.6%) [81••]. This reduction in cardiovascular events may be related to the regression in atherosclerotic plaque development by evolocumab. Indeed, the GLAGOV study had demonstrated that despite history of prior statin treatment, evolocumab lead, after 76 weeks of treatment, to a 0.95% decrease in percent atheroma volume (PAV) compared with an increase of 0.05% in placebo among patients with angiographic coronary disease [82].

Results of long-term cardiovascular outcomes for alirocumab are being evaluated in the ODYSSEY OUTCOMES study in 18,000 patients on maximally tolerated statin therapy and are eagerly awaited in 2018 [83••].

The impact of anti-PCSK9 mAbs was also investigated in several meta-analyses. Interestingly, a recently published meta-analysis including 24 randomized clinical trials comprising 10,159 patients has showed that treatment with PCSK9 antibodies reduced all-cause mortality (OR, 0.45 [CI, 0.23 to 0.86]; $P = 0.015$) and cardiovascular mortality (OR, 0.50 [CI, 0.23 to 1.10]; $P = 0.084$). The rate of myocardial infarction was significantly reduced with the use of PCSK9 antibodies (OR, 0.49 [CI, 0.26 to 0.93]; $P = 0.030$) with no increment in serious adverse event [84]. A more recent pooled analysis of 10 ODYSSEY trials showed that for every 39 mg/dL lower achieved LDL-C with alirocumab, the risk of major adverse cardiovascular events (MACEs) appeared to be 24% lower (adjusted hazard ratio, 0.76; 95% confidence interval, 0.63–0.91; $P = 0.0025$). Percent reductions in LDL-C from baseline were inversely correlated with MACE rates (hazard ratio, 0.71; 95% confidence interval, 0.57–0.89, per additional 50% reduction from baseline; $P = 0.003$) [85]. These findings are consistent with previous clinical studies evaluating statins, where a reduction in LDL-C levels of 39 mg/dL (1 mmol/L) was associated with a 23% risk reduction in major coronary events [86]. In another meta-analysis, PCSK9 inhibitors reduced the incidence of all-cause mortality (OR, 0.43 [95% CI, 0.22–0.82]; $P = 0.01$) but was associated with an increased incidence of neurocognitive AEs (OR, 2.34 [95% CI, 1.11–4.93]; $P = 0.02$) when compared with placebo [87]. However, this increment in neurocognitive AEs was not observed in another meta-analysis of 25 randomized clinical trials encompassing 12,200 patients where alirocumab was associated with reduced rates of death (relative risk, 0.43 [95% CI, 0.19 to 0.96]; $P = 0.04$). In this study, both evolocumab and alirocumab were safe and well tolerated [88]. In fact, these drugs have been proven to be safe and well tolerated in phase I, II, and III trials with no clinically significant safety issues, the most commonly reported AEs being nasopharyngitis, injection site pain, headache, skin, and burning sensation. To have conclusive responses concerning neurocognitive events, the EBBINGHAUS study was designed. It aims to assess the effect of evolocumab on cognitive function in approximately 1972 participants with clinically evident CVD. Results of this study showed that over a median follow-up of 19 months, the mean (±SD) change from baseline over time in the raw score for the spatial working memory strategy index of executive function (primary end point) was -0.21 ± 2.62 in the evolocumab group and -0.29 ± 2.81 in the placebo group ($P < 0.001$ for non-inferiority; $P = 0.85$ for superiority). There were no significant between-group differences in the secondary end points of scores for working memory, episodic memory, or psychomotor speed. There was also no evidence to suggest differences in cognitive tests in patients attaining very low LDL-C levels, including those with levels < 25 mg/dL or 0.65 mmol/L [89••]. It is important to point out that LDL-C < 25 mg/dL levels have been noted in a significant number of patients. Caution should be taken because long-term side effects of very low levels of LDL-C are still unknown. This concern was addressed in a recent study which pooled and analyzed data from 14 trials in order to determine the safety of alirocumab in patients with at least two consecutive LDL-C values < 25 or < 15 mg/dL in the ODYSSEY program, with follow-up as long as 104 weeks. Similar rates of AEs occurred in patients achieving LDL-C < 25 and < 15 mg/dL (72.7 and 71.7%, respectively), compared with 76.6% in those who did not achieve LDL-C < 25 mg/dL. Neurological and neurocognitive events were

similar among the three groups, although cataract incidence appeared to be increased in the group achieving LDL-C levels < 25 mg/dL [90].

Bococizumab, the third anti-PCSK9 mAb, was evaluated in the SPIRE program, in two parallel, multinational trials where 27,438 patients were randomly assigned to receive bococizumab 150 mg subcutaneously Q2W or placebo. In the lower-risk, shorter-duration trial with a median follow-up of 7 months, major cardiovascular events occurred in 173 patients each in the bococizumab group and the placebo group (hazard ratio, 0.99; 95% CI, 0.80 to 1.22; $P = 0.94$). In the higher-risk, longer-duration trial (the median follow-up was 12 months), major cardiovascular events occurred in 179 and 224 patients, respectively (hazard ratio, 0.79; 95% CI, 0.65 to 0.97; $P = 0.02$) [91]. However, the trials were stopped early after Pfizer elected to discontinue the development of bococizumab owing in part to the development of high rates of antidrug antibodies. Indeed, the investigators have shown that in six multinational trials evaluating bococizumab, high-titer antidrug antibodies developed in a substantial proportion of the patients who received bococizumab, which markedly diminished the magnitude and durability of the reduction in LDL-C levels. In addition, among patients with no antidrug antibodies, there was wide variability in the reduction in LDL-C levels at both 12 and 52 weeks. It is important to note as well that injection site reactions were significantly more common in the bococizumab group than in the placebo group (10.4 vs. 1.3%, $P < 0.001$) [55••].

Several studies have demonstrated that statin therapy increases the risk of diabetes [92, 93]. Recently, it has been shown that PCSK9 variants associated with lower LDL-C were also associated with circulating higher fasting glucose concentration, bodyweight, and waist-to-hip ratio, and an increased risk of type 2 diabetes [94]. Another study has also shown that variants in PCSK9 had approximately the same effect as variants in HMGCR on the risk of cardiovascular events and diabetes per unit decrease in the LDL-C level [95]. Therefore, safety outcomes concerning the onset of diabetes in PCSK9 inhibition trials should be carefully assessed. In the FOURIER trial, there was no significant difference between the study groups with regard to new-onset diabetes [81••]. Moreover, there was no evidence of an effect of alirocumab on transition to new-onset diabetes in 3448 individuals without diabetes at baseline with a follow-up period of 6–18 months, compared to either placebo or ezetimibe. The hazard ratio (HR; 95% confidence interval) for diabetes-related treatment-emergent adverse event in alirocumab was 0.64 (0.36–1.14) vs. placebo and 0.55 (0.22–1.41) vs. ezetimibe [96]. Longer follow-up with a larger number of individuals is needed to conclusively rule out an effect. More recently, in a meta-analysis of 14 studies of alirocumab, no difference in rates of treatment-emergent adverse events related to diabetes mellitus was observed when controlling for baseline characteristics predictive of achieving LDL-C < 25 mg/dL [90]. Ongoing clinical studies evaluating evolocumab (NCT02662569) and alirocumab (ODYSSEY DM-Dyslipidemia (NCT02642159), ODYSSEY DM-Insulin (NCT02585778)) in diabetic subjects and in subjects with hyperlipidemia or mixed dyslipidemia will hopefully provide more evidence regarding this issue.

Other Modalities to Lower PCSK9

Beside mAbs, several strategies to inhibit or lower PCSK9 levels have been investigated, such as gene silencing by RNA interference. Inclisiran (ALN-PCSsc) is a long-acting RNA interference (RNAi) therapeutic agent that inhibits the synthesis of PCSK9 and is taken up specifically by hepatocytes. In the single-dose phase, inclisiran doses of 300 mg or more reduced the PCSK9 level (up to a least-squares mean reduction of 74.5% from baseline to day 84), and doses of 100 mg or more reduced the LDL-C level (up to a least-squares mean reduction of 50.6% from baseline). Reductions in the levels of PCSK9 and LDL-C were maintained at day 180 for doses of 300 mg or more. All multiple-dose regimens reduced the levels of PCSK9 (up to a least-squares mean reduction of 83.8% from baseline to day 84) and LDL-C (up to a least-squares mean reduction of 59.7% from baseline to day 84). No serious AEs were observed [97]. Results of the phase II study were recently published by Ray et al. [98••]. Patients at high risk of CVD who had elevated LDL-C levels were randomly assigned to receive a single dose of placebo or 200, 300, or 500 mg of inclisiran or two doses (at days 1 and 90) of placebo or 100, 200, or 300 mg of inclisiran. The two-dose 300-mg inclisiran regimen produced the greatest reduction in LDL-C levels: 48% of the patients had an LDL-C level below 50 mg/dL (1.3 mmol/dL) at day 180. At day 240, PCSK9 and LDL-C levels remained significantly lower than at baseline in association with all inclisiran regimens. During 210 days of exposure to inclisiran, the rates of serious AEs were 11% among patients receiving the drug and 8% among patients receiving placebo [98].

Another strategy that has been used to inhibit PCSK9 is the antisense technology. SPC5001, a 14-mer oligonucleotide with locked nucleic acid modifications, was developed and tested in a phase I clinical trial. However, treatment was associated with injection site reactions and one patient under the highest dose experienced acute tubular necrosis [51]. Thus, clinical development of SPC5001 was terminated. In order to develop more potent and less toxic antisense molecules, Yamamoto et al. developed a series of antisense oligonucleotides modified with bridged nucleic acids that are under investigation [99].

To provide an alternative strategy for PCSK9 inhibition that allows long-term LDL-C management, a peptide-based anti-PCSK9 vaccination approach was studied [100]. In a

recent study by Landlinger et al. [101], the AT04A anti-PCSK9 vaccine was evaluated for its therapeutic potential in ameliorating or even preventing CHD in the atherogenic APOE*3Leiden-CETP mouse model. It induced high and persistent antibody levels against PCSK9, causing a significant reduction in plasma total cholesterol (− 53%, $P < 0.001$) and LDL-C compared with controls. It also resulted in a decrease in atherosclerotic lesion area (− 64%, $P = 0.004$) and aortic inflammation as well as in more lesion-free aortic segments (+ 119%, $P = 0.026$), compared with control. AT04A is currently being tested in a phase I clinical trial [101].

PCSK9-binding adnectins are another strategy to inhibit PCSK9. Adnectins are based on the 10th type III domain (10Fn3) of human fibronectin, whose variable loops can be efficiently engineered to introduce surfaces that bind therapeutic targets with high affinity and specificity. Safety, tolerability, and efficacy of adnectin BMS-962476, an ~ 11-kDa polypeptide conjugated to polyethylene glycol, were evaluated in a first-in-man study [49]. BMS-962476 was well tolerated when administered to healthy subjects, and AEs were similar to placebo. Maximal dose-related reductions of LDL-C were up to 48%, and doses > 0.3 mg/kg reduced free PCSK9 over 90%. This study showed that BMS-962476 rapidly reduces free PCSK9 and LDL-C, is well tolerated, and has no notable safety signals [49].

High-efficiency genome editing to disrupt the *PCSK9* gene was also evaluated. In a recent study, a base editor comprising CRISPR-Cas9 was delivered into the livers of adult mice in order to assess whether it could introduce site-specific nonsense mutations into the *Pcsk9* gene. In adult mice, this resulted in substantially reduced plasma PCSK9 protein levels (> 50%), as well as reduced plasma cholesterol levels (≈ 30%). There was no evidence of off-target mutagenesis [102].

Conclusion

The most significant discovery in the field of atheroma-related CVD in the past three decades was the identification of gain-of-function mutations in PCSK9 associated with familial hypercholesterolemia. Following many years of stagnation in the development of new efficient lipid-lowering molecules, this discovery led to the emergence of anti-PCSK9 therapeutic class. It is a new hope for many types of populations, especially FH patients, statin-intolerant patients, and patients with high LDL-C levels despite maximally tolerated statin therapy.

Results of FOURIER, the first cardiovascular outcome trial of a PCSK9 inhibitor, were keenly awaited. The reduction of 15–20% in the risk of major cardiovascular outcomes by evolocumab in high-risk patients receiving statin therapy over only 2.2 years of follow-up is very promising. Based on the absolute reduction in LDL-C achieved in FOURIER, the cardiovascular benefit matches results from the major statin trials very closely at 2 years [103]. Therefore, FOURIER probably might have induced a greater risk reduction if a longer follow-up period had been investigated. The distinctively low LDL-C levels achieved by patients receiving evolocumab were well tolerated, with no increase in diabetes, cataracts, or neurocognitive changes [81••]. Although reassuring, long-term follow-up is needed to confirm these findings. Most importantly, these findings could affect clinical practice and future treatment guidelines will need to define whom to treat with PCSK9 inhibitor therapy. However, concerns about cost-effectiveness have arisen. Indeed, at approximately $14,000 per patient per year in the USA, PCSK9 mAbs are far more expensive than statin therapy.

Results of ODYSSEY OUTCOMES trial, evaluating the effect of alirocumab in 18,000 patients with established CVD, are also eagerly awaited in 2018. Perhaps, by following patients for more than 3 years, a greater cardiovascular outcome reduction will give more arguments to healthcare providers for reimbursement considerations.

Future PCSK9 targeting therapies such as RNA interference therapy may hold promising results regarding a more sustainable reduction in LDL-C levels and perhaps a more potent consequence on cardiovascular outcomes. Indeed, LDL-C concentration was lower than 1.3 mmol/L at 180 days in almost half of the patients. The ORION-4 cardiovascular outcome trial will assess the cardiovascular benefits of two injections per year using inclisiran. If the outcome is positive than lipid management of patients at high risk of CVD, it could be substantially changed.

The evolution of research on PCSK9, starting from the discovery in 2003 of the first mutation of *PCSK9* in familial hypercholesterolemia [1••], is an amazing example of successful translational research showing how rigorous and powered genetic analyses can lead to a new class of lipid-lowering drugs that gives hope in fighting high cholesterol levels and their cardiovascular complications.

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Compliance with Ethical Standards

Conflict of Interest Marianne Abifadel and Catherine Boileau are consultants (advisory board or lecturers or research studies) for Amgen and Sanofi-Regeneron.

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

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