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PCSK9: From Basic Science Discoveries to Clinical Trials

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Abstract

Unknown fifteen years ago, proprotein convertase subtilisin/kexin type 9 (PCSK9) is now common parlance amongst scientists and clinicians interested in prevention and treatment of atherosclerotic cardiovascular disease. What makes this story so special is not its recent discovery nor the fact that it uncovered previously unknown biology, but rather that these important scientific insights have been translated into an effective medical therapy in record time. Indeed, the translation of this discovery to novel therapeutic serves as one of the best examples of how genetic insights can be leveraged into intelligent target drug discovery. The PCSK9 saga is unfolding quickly but is far from complete. Here, we review major scientific understandings as they relate to the role of PCSK9 in lipoprotein metabolism and atherosclerotic cardiovascular disease and the impact that therapies designed to inhibit its action are having in the clinical setting.

Keywords

Proprotein convertase subtilisin/kexin type 9 (PCSK9); PCSK9 inhibitors; low-density lipoprotein (LDL); LDL receptor (LDLR); familial hypercholesterolemia (FH); atherosclerotic cardiovascular disease; dyslipidemia; clinical trials; clinical management guidelines

Introduction

It has only been fifteen years since proprotein convertase subtilis/kexin type 9 (PCSK9) was identified as an important regulator of low-density lipoprotein (LDL) metabolism. As its name suggests, PCSK9 is the ninth member of the proprotein convertase family, a group of serine proteases that are characterized by their ability to hydrolyze peptide bonds in their cognate substrates for activation [1]. Initial clues were provided by a French family with familial hypercholesterolemia in 2003 [2]. Abifadel *et al*. linked gain-of-function (GOF) mutations in *PCSK9* with autosomal dominant hypercholesterolemia and ultimately uncovered a key new player in lipid metabolism. This seminal discovery led to a series of investigations that demonstrated that loss-of-function (LOF) mutations in *PCSK9* associate with life-long low cholesterol levels and marked reductions in the risk of atherosclerotic

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cardiovascular disease (ASCVD) [3-6]. The very rare individuals with homozygous LOF mutations in *PCSK9* (and no circulating protein) demonstrated extremely LDL-cholesterol (LDL-C) [≈15 mg/dL], normal health and reproductive capacity, and no evidence of neurological or cognitive dysfunction [5, 7]. This complementary set of observations has been leveraged into the most important therapy for the treatment of hypercholesterolemia and ASCVD since the introduction of the statins over thirty years ago. Indeed, the so-called PCSK9 inhibitors, fully human monoclonal antibodies that bind PCSK9, reduce LDL-C by approximately 60% and risk of myocardial infarction (MI) and stroke by approximately 20% after over two years of treatment [8]. Remarkably, these agents antagonizing PCSK9 action were approved by regulatory agencies spanning the globe only a decade after its discovery. While the scientific and medical communities have swiftly uncovered many facets of PCSK9 biology, there is still much to learn. Here we survey the most salient aspects of PCSK9 biology and therapeutic modulation as it relates to lipoprotein metabolism, atherosclerosis, and prevention of atherosclerotic cardiovascular events.

A new player in cholesterol homeostasis

Much of the excitement surrounding the discovery of PCSK9 relates to revelations in lipoprotein metabolism, necessitating a reworking of models previously held for decades. In the pre-PCSK9 era, it was thought that all regulatory systems of cholesterol homeostasis were strictly intracellular [9], with the role played by extracellular proteins limited to modulation of packaging, processing, and clearance of plasma lipoproteins. Remarkably, PCSK9 impacts lipoprotein metabolism both within (prior to secretion) and outside (after secretion into the circulation) of the cell [10]. Plasma cholesterol is mostly manufactured, exported, and eventually recaptured by hepatocytes. Cholesterol synthesis is a complex, multistep, and highly regulated pathway, and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA-R) is its key rate-limiting enzyme. Statins antagonize the activity of HMG CoA-R, reduce hepatic cholesterol synthesis, and up-regulate the transcription of the LDL receptor (*LDLR)* gene via a sensing mechanism operated by the sterol regulatory element binding protein (SREBP) pathway [11]. Each cell in the body must maintain membrane cholesterol at a critical concentration in order to ensure proper membrane function. It is thus evident that the cell uses a series of regulatory and counter- regulatory steps to respond to increases and decreases in membrane cholesterol straying from the critical value range. These include synthetic, assembly, secretory, and re-uptake activities. The lipid cargo, mostly triglycerides and cholesterol, is packaged within apolipoprotein B (apoB)-containing very low-density lipoproteins (VLDL), the intravascular precursors of LDL, which primarily transport triglycerides from the liver to peripheral tissues, with cholesterol packaged to enhance stability.

The mechanism by which LDLR internalize LDL was described by Goldstein and Brown in the early 1970s [12], leading to one of the most exciting series of discoveries in the history of medicine and the cataloguing of a critical aspect of cellular life, the sensing and regulation of membrane cholesterol levels. Receptor mediated endocytosis is facilitated by the binding of apoB on the LDL particle to the LDLR and coordinated by an adaptor protein (LDLRAP) that positions LDLR on the sinusoidal side of the polarized hepatocyte, clustered in coated pits [13]. The LDL/LDLR complex then gets internalized within coated vesicles and expands

to join the endosomal compartment, where it eventually merges with the lysosome. The pH gradient in the descent toward the lysosome induces dissociation between receptor and cargo. In the lysosome, the LDL particle is digested and the cholesterol and triglycerides are de-esterified for transport into the cytosol, where they can take on myriad fates. On the other hand, the LDLR is recycled back to the hepatocyte surface to participate for many more rounds of LDL binding and endocytosis [14, 15]. LDLR recycles every 10 minutes with a life span of 20 hours. This process allows a single LDLR to internalize hundreds of LDL particles during its lifespan. For decades, it was assumed that a generic ubiquitin-related sorting of altered molecules eventually terminated this recycling process. The discovery of PCSK9 heralded a new era of understanding; this low abundance circulating protein binds cell-surface LDLR on coated pits and triggers the internalization of the receptor. The interaction between PCSK9 and LDLR locks the receptor in its “open” conformation in the endosome and routes the ligand-receptor pair to the lysosomal compartment for degradation, thus inhibiting the LDLR recycling that follows internalization of ligands such apoB on LDL and apoE on remnants [16-19]. In other words, the normal recycling loop is short-circuited as PCSK9 disables the LDLR from escaping lysosomal digestion, thereby reducing cell surface receptor density and resulting in raised plasma LDL-C.

It must be noted that up to half of plasma PCSK9 is associated with the LDL particle, for a frequency of one PCSK9 molecule for every 500–1000 LDL particles [20, 21]. This introduces the intriguing possibility that the carefully orchestrated cellular regulation of cholesterol concentration is ultimately under the control of a stochastic extracellular system, where every few hundred encounters with canonical LDL, the LDLR meets its fate by interacting with a PCSK9-carrying LDL that terminates its life cycle (Figure 1).

PCSK9 biology

Structure

PCSK9 is synthesized predominantly in the liver as a 75 kDa proprotein. Based on its protein structure [22-24], removal of the signal peptide (amino acids 1-30) produces a secreted heterodimer protein with three domains:

- A pro-domain (amino acids 31-152), which undergoes autocatalytic cleavage but continues to associate with the rest of the protein.
- A catalytic domain (amino acids 153-454), which contains a proteolytic active site (catalytic triad amino acids 186, 226, and 386) inactivated by the associated pro-domain. The proteolytic active site is only required for the autocatalytic cleavage, has no other known targets, and is not related to the LDLR reducing activity of PCSK9 [25, 26]. The LDLR degradation capacity of PCSK9 is based on a protein-protein interaction between the epidermal growth factor-like A domain of the LDLR and amino acids 367-380 in the catalytic domain region of PCSK9 independent of the proteolytic active site.
- A C-terminal domain (residues 455-692), which consists of three similar modules. Most of the N-terminal residues and some of the C-terminal residues are not visible in the proposed crystal structures due to poor electron density.

LDLR degradation and PCSK9 kinetics

The kinetics of PCSK9 binding to cell surface LDLR exhibit Kd values that range from 90 to 840 nM at neutral pH. Its affinity for the LDLR is increased by two orders of magnitude at lower pH with Kd values ranging from 1 to 8 nM [22, 24, 27, 28]. The increased affinity at acidic pH facilitates PCSK9 capture of the LDLR in the late endosome and ensures that the PCSK9-LDLR complex will be targeted to the lysosome for degradation [29]. PCSK9 binding to LDLR occurs in two phases:

- Rapid-phase binding - accounts for one-third of overall equilibrium binding and is characterized by a binding half-time of 5-10 minutes and half-time dissociation of 20 minutes [30].
- Slow-phase binding - accounts for two-thirds of overall equilibrium binding and is characterized by a binding half-time of ~1.5 hours and half-time dissociation of ~5 hours [30].

Although PCSK9-LDLR binding, internalization, and lysosomal shuttling occurs within 2-3 hours from initial contact [31], PCSK9-mediated degradation of LDLR *in vitro* is only evident 12-24 hours after adding PCSK9 to cultured cells [32, 33]. In mice, PCSK9 remains intact in the liver for at least 4 hours after its LDLR-mediated internalization [20]. Therapeutic PCSK9 inhibition in humans only significantly reduces LDL-C levels after 2-3 days from start of therapy[34]. Based on these observations, it is clear that there is a delay between the first PCSK9 interaction with the LDLR and the eventual loss of LDLR. In contemplating this apparent paradox, there are several possibilities to consider. First, the PCSK9-LDLR interaction may not lead to immediate shuttling of both proteins to the lysosome for degradation and may require additional steps and interactions. Alternatively, intracellular LDLR concentrations may be in vast excess relative to cell-surface LDLR density [35, 36], so that the initial elimination of cell-surface LDLR by PCSK9 is rapidly replenished until intracellular stores are also depleted.

Upon synthesis, the PCSK9 proprotein is directed to the endoplasmic reticulum by a signal sequence, subsequently removed by a signal peptidase. The proprotein form then undergoes autocatalytic cleavage, forming a heterodimer (62+13 kDa). This form is then transported to the Golgi via a COPII complex involving Sec24a [37], leading to PCSK9 secretion into the blood stream. Even though PCSK9 and LDLR co-exist within the secretory pathway of hepatocytes and their co-localization was suggested to result in reduced LDLR levels, it was later demonstrated that the interaction does lead to LDLR degradation [38,39 40]. Binding of PCSK9 to GRP94, an ER-resident protein expressed in hepatocytes, protects LDLR from degradation by preventing early binding of PCSK9 to LDLR within the ER [38]. Several LOF mutations in PCSK9 are known to cause impaired processing (e.g., S386A) [41], trafficking (e.g. R46L) [42], or secretion (e.g. S462P) [43] of PCSK9, leading to low plasma PCSK9 levels and consequent hypocholesterolemia. Interestingly, some of the GOF mutations of PCSK9 causing hyperlipidemia are also not secreted (e.g. S127R, D129G) [42]. It is possible that these mutations are able to interact with LDLR in the secretory pathway leading the complex to degradation, bypassing GRP94 protection in the ER.

Regulation of PCSK9 levels and function

As for all plasma proteins, PCSK9 levels represent the balance between production and clearance. Human PCSK9 is expressed in multiple tissues, with liver, small intestine, and kidney the major sources of its plasma levels [44]. The human protein shares substantial homology with its murine counterpart, with 76.6% identity [45]. The gene is found on chromosome 1 in humans and chromosome 4 in mice. PCSK9 is regulated by the SREBP through a sterol-regulatory element (SRE) motif in the promoter region [46, 47]. In addition, an Sp1 motif also controls transcriptional regulation through the SREBP pathway [48]. SREBP regulation of cholesterol synthesis, LDLR, and PCSK9 results in an apparently paradoxical scenario where depletion of intracellular cholesterol levels, leads to the simultaneous up-regulation of both LDLR and PCSK9 expression [48, 49]. This SRE-mediated up-regulation of PCSK9 attenuates the LDL-C lowering effect of medications such as statins and ezetimibe. The PCSK9 promoter also contains a hepatic nucleic factor 1 motif (between the SRE and Sp1 sites), which likely functions as a liver-specific regulatory sequence [50-52]. Metabolic studies in humans have shown that the production rate of PCSK9 is ~20 μg/kg/day with a plasma pool size of ~1000 μg [53]. Thus, given a blood pool of 5 liters, the average plasma concentration of PCSK9 is ~200 ng/ml in an average adult, and the plasma pool turns over very fast, by over 2 pools a day [53]. For comparison, the production rate of VLDL-apoB is 1000-fold higher (~20 mg/kg/day) [53].

At a high level, it would appear that the primary role of PCSK9 is to carry out a suicide mission that ultimately leads to the demise of the LDLR. However, careful study of PCSK9 physiology shows a less threatening picture. PCSK9 is simply another ligand for the LDLR (just like apoB and apoE) and uses the LDLR to exit the plasma compartment. However, if PCSK9 uses LDLR as the main clearance route of elimination and at the same time it causes degradation of LDLR, then a reciprocal regulation between these two proteins controls plasma PCSK9 levels, hepatic LDLR expression, and plasma LDL-C levels [20, 54], rendering the evaluation of these inextricably linked processes at any given time in the steady state extremely difficult, as changes in production or efficiency in any of the two proteins will have consequences on the other and on LDL-C levels. In mice, plasma PCSK9 levels are highly regulated by LDLR expression [20, 55]. In mice, complete removal of the LDLR results in a substantial increase in the plasma half-life of PCSK9 [56] whereas overexpression of hepatic LDLR results in increased clearance of PCSK9 [20]. Humans with homozygous or heterozygous familial hypercholesterolemia (FH) manifest higher levels of plasma PCSK9 compared to non-FH controls [54]. Strangely, LDLR mutations in humans have a larger effect on LDL-C than on PCSK9 concentrations, while the opposite happens in mice [57]. One possible explanation is the human LDLR mutations are classified as either receptor-defective or receptor-negative (<2% of normal LDL internalization ability), with receptor-defective (2-25% of normal LDL internalization ability) mutations being far more common [58, 59]. Thus, the most human LDLR mutants have some residual activity toward binding both LDL and PCSK9, while studies mice are done in the context of a complete absence of the LDLR.

Plasma PCSK9 can be found in two main forms, an intact heterodimer (62+13 kDa), which is often considered the (more) active form (stronger binding to and degradation of LDLR), and a furin-cleaved heterodimer (55+13 kDa) [60], which binds the LDLR less avidly (two-

fold reduced affinity) [61], and is thus considered the less active form[61-63]. In contrast, intracellular PCSK9 is only found in its proprotein form (75 kDa) or as an intact heterodimer ready to be secreted [64]. These findings suggest that the cleavage of PCSK9 by furin occurs outside the cell, by interaction of PCSK9 with either membrane-bound or circulating furin. Although PCSK9 and furin co-exist in the Golgi, there is no clear evidence that furin is able to cleave PCSK9 intracellularly [60]. Another direct regulator of PCSK9 function in plasma is the LDL particle itself [65, 66] It has been shown that PCSK9 associates with LDL with a K_d in the range of 160 to 320 nM [67, 68]. PCSK9 association with LDL is thought to occur via apoB and requires the presence of the PCSK9 pro-domain [67]. We also found that plasma PCSK9 associates with Lp(a), although it does not bind to other apoB-containing lipoproteins such as VLDL or chylomicrons [69]. The *in vivo* relevance of PCSK9 association with LDL and Lp(a) was first shown in patients undergoing lipoprotein apheresis, where together with a ~70% reduction in LDL levels, plasma PCSK9 levels were also reduced by over 50% [70]. Nevertheless, the physiologic role of PCSK9 binding to lipoproteins is not clear. *In vivo* data suggest that the PCSK9 species associated with LDL is primarily the intact heterodimer form, whereas the non-LDL-bound (free) PCSK9 is mainly found in the furin-cleaved conformation [70]. Our *in vivo* and *in vitro* data further suggest that LDL "protects" PCSK9 from furin cleavage and that LDL-bound PCSK9 has a two-fold stronger binding affinity for the LDLR compared with non-LDL-bound PCSK9 [71]. These observations support the notion that LDL-bound PCSK9 is the more functional form of this protein. Furthermore, the LDL-PCSK9 interaction has therapeutic potential as its modulation may lead to increased proteolytic cleavage and reduced plasma PCSK9 activity. Compartmentalization of PCSK9 and its effects on PCSK9 activity are summarized in Figure 2.

On the other hand, it was shown that adding LDL to recombinant PCSK9 *in vitro* leads to reduced affinity of PCSK9 for the LDLR, likely due to competition of LDL and PCSK9 for the LDLR [67, 71]. Thus, it is possible that LDL-bound PCSK9 is a physiologically less functional fraction of plasma PCSK9 [72]. The importance of defining and elucidating the factors that govern the partitioning of plasma PCSK9 and understanding the physiological role of the LDL-bound PCSK9 cannot be overstated. Experimental and translational studies will ultimately determine whether this partitioning in general, and the LDL-PCSK9 interaction in particular, can be exploited for therapeutic gains.

Receptors and other partner proteins

Immediately following the discovery of LDLR-mediated degradation by PCSK9, it was found that this protein interacts with other proteins in both hepatic and extra-hepatic tissues. Similar to its interaction with the LDLR, PCSK9 may engage other members of the LDLR family, such as the VLDL receptor [10], apoE receptor 2 (apoER2) [10], and LDLR-related protein 1 (LRP1) [73]. However, it is possible that PCSK9 interaction with these receptors does not lead to their degradation, or at least not in all tissues [74, 75], and that, perhaps, its interaction with LRP1 and apoER2 leads to downstream signaling instead.

The surface scavenger receptor CD36 [76] and the tetraspanin receptor CD81 [77] were also proposed as possible targets of PCSK9. The nature of these interactions remains unknown, as these proteins do not share homology with the LDLR. CD36 is a scavenger receptor that

also plays a role in muscle lipid utilization, adipose energy storage, and hepatic triglyceride storage and secretion. Thus, systemic modulation of its levels by PCSK9 may have implications on basic human physiology. CD81 plays a role in the regulation of cell development, activation, growth and motility and also serves as an important receptor for hepatitis C virus entry to the cell.

PCSK9 has also been reported to interact with proteins other than receptors, as it reduces levels of beta-secretase 1 [78], an aspartic-acid protease responsible for the proteolytic processing of the amyloid precursor protein, and endothelial sodium channel [79], a membrane-bound ion channel that plays a major role in determining extracellular fluid osmolality. Amyloid-like protein 2 [80] and Annexin A2 [81] may affect the formation and processing of the PCSK9-LDLR complex though interaction with the C-terminal domain of PCSK9. In addition, it was shown that the adrenal cells are insensitive to LDLR-mediated degradation by PCSK9 independently of Annexin A2 levels [20].

Heparan sulfate proteoglycans (HSPG) play an important role in hepatic metabolism through several mechanisms including endocytosis of lipoproteins [82]. It was recently suggested that HSPG lining the hepatocyte surface capture PCSK9 bound to the LDLR and heparin mimetics appear to have PCSK9 inhibitor activity [82]. In order to understand the potential role of HSPG on PCSK9, it remains to be determined whether HSPG knock down *in vivo* (e.g through hepatic Ndst1 knockout [83]) affects PCSK9-mediated LDLR degradation, PCSK9 turnover, and/or PCSK9 levels.

PCSK9 as a biomarker to predict ASCVD risk

PCSK9 and Atherosclerosis Imaging

Since PCSK9 is a circulating protein, it could have direct effects on the plaque beyond its ability to regulate hepatic LDLR levels. Several lines of investigation have also explored the relationship between plasma PCSK9 and subclinical atherosclerosis. Chan *et al.* performed carotid intima-media wall thickness (CIMT) measurements in 295 asymptomatic subjects and found a significant and direct relationship between PCSK9 levels and carotid thickness [84]. These findings corroborated earlier studies on the correlation between PCSK9 and CIMT [85-87]. However, a sub-analysis of the FATE (Firefighters and Their Endothelium) study found no relationship between PCSK9 levels and measures of subclinical atherosclerosis (CIMT and flow-mediated dilation) in 1,527 middle-aged men free of vascular disease [88]. The utility of serial CIMT as a means to evaluate the association of serum PCSK9 with progression of carotid plaque is limited. However, Xie *et al.* performed serial studies in 643 Chinese healthy participants from the general population as part of the Chinese Multi-provincial Cohort Study [89] and found a statistically significant relationship between plasma PCSK9 concentration and progression of atherosclerosis as reflected by total plaque area, independent of plasma LDL-C concentration.

The coronary artery calcium (CAC) score has also been utilized as a means to investigate the relationship of PCSK9 to coronary atherosclerosis. Alonso *et al.* evaluated 161 genetically confirmed FH patients who underwent CAC scoring [90] and found that serum PCSK9 concentrations independently predicted the extent of CAC. Importantly, after full

adjustment, only PCSK9 and apolipoprotein(a) [apo(a)] remained predictive of CAC in this cohort of asymptomatic FH patients. Similarly, Zhao *et al.* evaluated the association of plasma PCSK9 concentrations and CAC in 403 untreated patients presenting with chest pain and found that PCSK9 was independently associated with CAC [91].

The relationship of circulating PCSK9 concentration to atherosclerosis has also been explored in the context of invasive diagnostic imaging as well. Cheng *et al.* utilized intravascular ultrasound (IVUS) virtual histology to evaluate the association of serum PCSK9 levels and necrotic core within coronary atherosclerotic lesions in subjects with known CAD confirmed at the time of angiography [92]. The results demonstrated a direct linear relationship between plasma PCSK9 and the necrotic core fraction in coronary plaque. Importantly, this endpoint remained significant in all subgroups, independently of LDL-C levels and use of statins. These observational studies cannot provide insight into the directionality of the association; thus, many questions remain. Can plasma PCSK9 influence atherogenesis through direct effect on endothelial cells or by direct transit into the subendothelial space? Does PCSK9 production within the plaque ultimately influence plasma levels? Or, is the association of these two factors mediated through other pathways entirely?

Epidemiologic studies

Beyond evaluations of the relationship between PCSK9 and subclinical atherosclerosis, a number of clinical investigations have explored PCSK9 as a biomarker of atherosclerotic risk in both primary and secondary prevention. Four major studies in primary prevention examined this issue. In the FATE study, 1,527 middle aged firefighters who were free of vascular disease at baseline were followed longitudinally for a mean of 7.2 ± 1.7 years [88]. While plasma PCSK9 concentration correlated with LDL-C, insulin, and triglyceride levels, it did not correlate with cardiovascular events. As a sub-analysis of the Women's Health Study, Ridker *et al.* performed a nested case–control evaluation from a prospective cohort of 28,000 initially healthy American women over the age of 45 years old and not on statin therapy [93]. Plasma PCSK9 was measured at baseline among 358 cases (MI, ischemic stroke, CV death) and 358 controls matched for age, smoking, and hormone replacement therapy (women who remained free of cardiovascular disease during 17 years of follow-up). While there was a modest, positive association between PCSK9 level and apoB and triglycerides, no difference was seen in median PCSK9 concentrations in cases vs. controls (304.4 ng/mL vs. 299.7 ng/mL). Moreover, baseline apoB levels predicted incident cardiovascular events but baseline PCSK9 levels did not. Leander *et al.* prospectively evaluated 4,232 apparently healthy 60-year-old men and women living in Stockholm County to investigate the correlation between PCSK9 and future cardiovascular events (composite primary outcome of fatal or non-fatal MI, angina, chronic ischemic heart disease, sudden cardiac death, and fatal or non-fatal ischemic stroke) [94]. During the 15 years of follow-up, the cumulative incidence of the primary outcome was 13%. Consistent with other studies, they noted a modest relationship between plasma PCSK9 and LDL-C ($r=0.18$, $p<0.0001$) and triglyceride levels ($r=0.12$, $p<0.0001$). Unlike the Ridker study, they observed a significant direct relationship between quartiles of PCSK9 concentration and incident cardiovascular events. However, incorporation of plasma PCSK9 levels into their clinical risk prediction model did not lead

to significant improvement in discrimination or net reclassification, metrics that relate to clinical utility. A provocative sub-analysis suggested that subjects with discordant PCSK9 and LDL-C levels (i.e., high PCSK9 – low LDL-C) had the highest future hazard of the primary outcome, even compared to those with high PCSK9 – high LDL-C [94]. More recently, Laugsand *et al.* evaluated the utility of PCSK9 as a circulating biomarker for prediction of incident MI in a nested case–control evaluation from a prospective cohort of a general population sample in Norway (1,488 cases vs. 3,1819 controls, 11.1 years of follow-up) [95]. Risk of MI was 47% higher in those subjects in the highest quartile of PCSK9 relative to those in the lowest quartile after adjustment for age and sex. However, when adjusting for LDL-C, the relationship no longer remained significant.

Results from secondary prevention studies are equally non-definitive. Werner *et al.* prospectively tested whether fasting serum PCSK9 concentration predict cardiovascular events in 504 patients with stable coronary artery disease on background statin therapy with well-controlled LDL-C levels [96]. Although serum PCSK9 levels predicted atherosclerotic events the association was lost when adjusting for fasting triglyceride levels. Similarly, Li *et al.* followed 616 Chinese subjects with stable coronary artery disease for 17 months to assess the relationship between PCSK9 levels and atherosclerotic cardiovascular disease [97]. An association was found between PCSK9 concentrations and the severity of coronary artery disease by SYNTAX score. Additionally, at 17 months, cardiovascular event rates were higher in those with higher PCSK9 levels, though this relationship was noted only amongst those subjects who did not receive coronary revascularization. Finally, Gencer *et al.* evaluated this issue in an acute coronary syndrome (ACS) cohort. They assayed plasma PCSK9 in 2,030 individuals presenting with ACS and undergoing coronary angiography [98]. Plasma PCSK9 levels correlated with measures of inflammation, lipid-lowering therapy, and the clinical onset of ACS, but did not predict mortality at one year.

Based on the above observational trials in primary and secondary prevention, no firm conclusions regarding plasma measures of PCSK9 as a predictor of future cardiovascular events can be drawn. On that basis, a number of systematic reviews and meta-analysis have been performed in the hopes of shedding further light on this relationship [99-101]. In three meta-analyses, when PCSK9 concentration was considered as a categorical value, the highest category of PCSK9 was associated with cardiovascular outcomes. However, when treated as a continuous variable, disparate results emerge. Part of the challenge in evaluating this issue has to do with differences in study design, clinical outcomes, and measurement methods for PCSK9. Based on the available data, clinical measurement of plasma PCSK9 for CVD risk prediction or for prognostic assessment is not recommended.

As discussed earlier in this review, experimental evidence demonstrates that PCSK9 is compartmentalized within the plasma, with approximately 40% of PCSK9 bound to LDL and lipoprotein(a) [Lp(a)] particles, and the remainder not associated with apoB-lipoproteins [20, 21, 70, 102]. Given the stoichiometry between LDL and PCSK9 in plasma, approximately only 1 LDL particle in every 500-1000 carries at least one molecule of PCSK9. If, as we speculate, LDL-bound PCSK9 is the biologically more active from, future analyses should evaluate the separate association of LDL-bound and free PCSK9 with atherosclerotic events. We are currently developing a practical and reproducible assay to quantify PCSK9 in its two

major compartments, which may elevate the status of plasma PCSK9 as a viable clinical biomarker.

Why would PCSK9 predict CVD independently of LDL?

While experimental work supports a critical role of PCSK9 in atherosclerosis, the value of circulating PCSK9 as a biomarker for ASCVD risk assessment in patients remains unclear. Since there is an inextricable link between PCSK9 and LDL-C, one would not necessarily expect plasma PCSK9 concentrations to predict risk above and beyond LDL-C unless PCSK9 has pleiotropic vascular effects.

Mendelian randomization analyses have demonstrated a common theme – genetic variants that are associated with lower LDL-C (including variants in *PCSK9*) also associate with a lower risk of ASCVD [103, 104]. Furthermore, the magnitude of genetically mediated LDL-C reduction relates linearly to the magnitude of ASCVD risk reduction, irrespective of the gene under study [103, 105, 106]. The results of these Mendelian randomization analyses are strikingly similar to those seen in randomized controlled clinical trials testing statins, ezetimibe, and, more recently, PCSK9 inhibitors [107]. The major difference in genetically vs. pharmacologically mediated LDL-C lowering relates to a larger magnitude of risk reduction observed in the genetic analyses, given the lifetime exposure of lower LDL-C. If PCSK9, and its pharmacologic inhibition, manifest clinically important pleiotropic effects on atherosclerotic events beyond LDL-C lowering, there should be differences in the magnitude of CVD risk reduction in analyses of *PCSK9* variants and in the randomized controlled trials testing PCSK9 inhibitors compared with other gene variants and drugs that target LDL-C lowering. However, the perfectly consistent linear relationship between magnitude of LDL-C reduction and magnitude of ASCVD risk reduction [108], irrespective of gene or drug, implies the theoretical pleiotropic effects of PCSK9 are likely not clinically significant.

Therapeutic approaches to inhibit PCSK9 and lower plasma lipids

LDL causes atherosclerosis

The importance of atherogenic lipoproteins as the central actors in the development of ASCVD is now readily accepted [109]. The development and clinical testing of the statins with corresponding significant reductions in atherosclerotic events has been one of the great triumphs of medicine in the 20th century and has provided the key line of evidence in support of the “cholesterol hypothesis.” The Cholesterol Treatment Trialists’ Collaborators (CTTC) produced an authoritative meta-analysis that included >170,000 participants in 26 randomized controlled trials testing statins. The bottom line: reducing LDL-C by 39 mg/dL yielded a 22% reduction in the risk of major vascular events and 10% reduction in all-cause mortality over 5 years and independently of baseline LDL-C [108]. Despite the outstanding efficacy of the statins, we must recognize that two thirds of the expected ASCVD events in statin-treated patients cannot be prevented. In addition, additional LDL-C cholesterol lowering interventions are needed for patients who cannot tolerate statin therapy or fail to attain adequate LDL-C lowering.

The IMPROVE-IT (Improved Reduction of Outcomes: Vytorin Efficacy International Trial) study demonstrated that the addition of ezetimibe, a cholesterol absorption inhibitor, on top of simvastatin to patients right after an acute coronary event provided a statistically significant, though clinically modest additional 2% absolute risk reduction in major adverse cardiovascular events, without a change in mortality [110]. Interestingly, this incremental event reduction is precisely what the CTTC meta-analysis regression line predicts, e.g., the magnitude of LDL-C reduction is directly and linearly related to the magnitude of event reduction. The results of IMPROVE-IT suggest that statins are not unique in their ability to reduce ASCVD events, and that LDL-C lowering is the reason for improved outcomes. The results of this trial also undid the notion of statin exceptionalism, provided the basis to pursue development of additional LDL-C lowering agents, and ushered in the era of PCSK9 inhibitors [111].

The action of PCSK9 on LDLR can be antagonized in different ways, to include monoclonal antibodies (mAbs), small interfering RNAs (siRNA), antisense oligonucleotides (ASO), adnectins, mimetic peptides, and vaccination strategies. While small molecule inhibitors typically are the favored first approach, their development has been challenging given the flat–surface interaction between PCSK9 and LDLR [112]. Thus far, targeting plasma (extracellular) PCSK9 with mAbs is the farthest along with two different fully human mAbs approved for clinical use by regulatory agencies around the world.

Therapeutic monoclonal antibodies

Since 2012, many clinical trials have been performed to evaluate the LDL-C lowering efficacy of anti-PCSK9 monoclonal antibodies in subjects with different levels of CVD risk, alone or combination therapy (statin or ezetimibe), in statin-intolerant patients, and in both heterozygous (HeFH) and homozygous (HoFH) familial hypercholesterolemia. The mAbs have consistently demonstrated remarkable efficacy in reducing LDL-C (≈50% as monotherapy and ≈70% reduction in combination with a statin) with a good short-term safety and tolerability profile [113]. Thus far, three mAbs to PCSK9 (PCSK9 inhibitors) have been tested, with two of them (alirocumab and evolocumab) approved by the U.S. FDA for the management of patients with either FH or ASCVD who require additional LDL-C lowering as an adjunct to diet and maximally tolerated statin therapy. Alirocumab and evolocumab are fully human antibodies and have been in the US market now for nearly 3 years, whereas the third (bococizumab, now abandoned)) was a humanized antibody which retains ~3% of murine protein sequence and for this reason induced immune responses limiting its effectiveness (neutralizing antibodies, discussed below) [114]. It is interesting to note that the FDA approved the two fully human mAbs (alirocumab and evolocumab) on the basis of their LDL-C lowering efficacy, prior to the results of randomized controlled cardiovascular outcome trials.

As discussed earlier, the measurement of total plasma PCSK9 concentration is not likely to be useful in risk prediction models. However, we recently proposed that measurement of total plasma PCSK9 levels in patients on PCSK9 inhibitor therapy may become useful as a diagnostic tool [115]. We demonstrated that patients treated with PCSK9 mAb exhibit an ~7-fold increase in total plasma PCSK9 levels relative to pretreatment levels. The change in

total plasma PCSK9 levels is likely due to delayed clearance of the antibody-PCSK9 complex from the circulation and/or due to an increase in hepatic PCSK9 production, though it is not clear at present which of these mechanisms is quantitatively more important. From a clinical perspective, the change in plasma PCSK9 levels can be used to confirm adherence to therapy and/or optimal injection technique in patients that do not show the expected LDL-C lowering response to PCSK9 inhibitor therapy.

Silencing RNA

Whereas mAbs targeting PCSK9 only antagonize plasma PCSK9, siRNA interferes with its intracellular production, e.g., the translation of PCSK9 mRNA to protein. The siRNA selectively and catalytically silences the translation of their complementary target mRNA in a sequence-specific manner through the formation of effector RNA induced silencing complexes [116, 117]. A phase I trial of the siRNA against PCSK9 named inclisiran, revealed similar LDL-C lowering efficacy as the PCSK9 mAbs [118]. Interestingly, while the mAbs are dosed every two weeks (or once a month at a larger dose), inclisiran has a more durable effect with sustained lowering of LDL-C by an average of 53% and up to 81% at day 180, with a time-adjusted mean of greater than 50% through day 270 after a single injection. The phase II trial of inclisiran, ORION-1 (Trial to Evaluate the Effect of ALN-PCS_{SC} Treatment on Low Density Lipoprotein Cholesterol) enrolled 501 subjects at high ASCVD risk with hypercholesterolemia despite maximally tolerated statin therapy [119]. Subjects randomized to inclisiran sustained dose-dependent reductions in PCSK9 and LDL-C levels. LDL-C was reduced to the greatest extent (53%) in those who received the highest dose (two 300-mg injections) regimen of inclisiran. Changes in other plasma lipids and lipoproteins (including Lp(a) levels) with inclisiran were also similar to those seen with the anti-PCSK9 monoclonal antibodies.

Inclisiran distinguishes itself from mAbs to PCSK9 in several major ways. First, its extended duration of action may hold significant advantages. Should this sustained LDL-C lowering efficacy be realized, leveraging this therapeutic approach to PCSK9 inhibition may also help overcome issues related to medication adherence. Second, whereas the mAbs block only plasma PCSK9, the siRNA approach also reduces hepatocellular levels of PCSK9. Whether this intracellular approach has additional effects remains to be determined. Third, the lowering of plasma PCSK9 with siRNA truly reflects reduced levels of the circulating protein, which is similar to that seem in some PCSK9 LOF, whereas mAbs cause a significant accumulation of PCSK9 bound to the antibody [115]. Fourth, the siRNA approach does not affect extrahepatic production of PCSK9 [120], and thus the concentration of PCSK9 in the atheroma will be higher than for subjects treated with mAbs. Phase III studies with inclisiran are planned.

Vaccination

Vaccination represents an orthogonal approach to PCSK9 inhibition. A vaccine, AT04A, is in development as an agent to induce an antibody response to PCSK9 [121]. In one study, APOE*3Leiden/CETP mice vaccinated with AT04A developed elevated and persistent levels of antibody against PCSK9, with marked reductions in plasma total (-53%, $p<0.001$) and LDL-C levels compared with controls [121]. Additionally, biochemical measures of

inflammation were significantly reduced in vaccinated animals. Total atherosclerotic lesion area was reduced by 64% (p=0.004). Interestingly, antibody concentrations remained high at the end of the study, which may translate to continued reductions in atherogenic lipoprotein concentrations for some time afterwards, resulting in a long-lasting effect. A phase I study with AT04A is ongoing (NCT02508896).

More recently, another group has developed a virus like particle – PCSK9 (PCSK9Qβ-003) vaccine and tested it in both Balb/c mice and LDLR+/− mice [122]. Vaccination resulted in significant reductions in total cholesterol and plasma PCSK9 expression. Additionally, the injected animals were found to have significant up-regulation of hepatic *LDLR*, SREBP-2, hepatocyte nuclear factor 1α, and HMG CoA-R. Positive developments in this area may lead to a viable approach to immunize humans against PCSK9, hindering the development of hypercholesterolemia and atherosclerosis. If the vaccination approach pans out, it could prove more affordable than mAbs, especially because the vaccine would be given once per year, versus once or twice a month. In addition, the vaccination strategy would have the advantage of use in younger patients, and the possibility of truly preventing atheroma formation rather than stabilizing preexisting plaques. The value of life-long exposure to low cholesterol has been proven over and over in natural randomization studies [103, 104].

Beyond these approaches to therapeutic antagonism of PCSK9, other novel methods are being pursued. Recently, a novel targetable pocket in the catalytic subunit of PCSK9 was identified. This structural identification may allow development of oral small molecule inhibitors to antagonize the action of PCSK9 [123, 124]. There is also interest in pursuing *in vivo* based editing of PCSK9 using CRISPR/Cas9 [125], another approach that could have an application in younger subjects for true primary prevention purposes.

PCSK9 inhibition: Effects on other lipid parameters

Lipoprotein(a)

Lp(a) is an atherogenic LDL-like particle with its apoB covalently bound to apo(a) by a disulfide bond. Its plasma levels are largely genetically determined [126]. Observational and genetic epidemiology data provide compelling evidence that Lp(a) has a causal role in atherosclerosis [127]. The regulation of its production and clearance is poorly understood, and no effective targeted therapies exist. Interestingly, PCSK9 inhibitors have demonstrated unexpected reductions in Lp(a), on the order of 25-30% [128, 129]. While the potent reduction in LDL-C achieved by PCSK9 inhibition is mediated through its profound effect on LDLR preservation, the mechanism by which they lower Lp(a) is unknown. Some suggest that the Lp(a) reduction achieved with PCSK9 inhibition is also secondary to the profound increase in LDLR expression, though that notion poses substantial challenges, as: 1) Lp(a) is not an avid ligand for the LDLR [130]; 2) Lp(a) metabolism in FH subjects is similar to that non-FH subjects [131]; 3) Lp(a) levels do not change with statin treatment, which also upregulates LDLR [132]; 4) PCSK9 inhibition in two subjects with homozygous LDLR-null FH lowered Lp(a) but failed to reduce LDL-C levels [133]; 5) loss-of-function PCSK9 mutation carriers do not demonstrate significantly different Lp(a) levels compared to controls [3, 134, 135]; and 6) epidemiological studies do not consistently demonstrate a correlation between plasma PCSK9 and Lp(a) concentrations [84, 90, 93, 94, 136]. On average, the lowering effect of PCSK9

inhibitors on Lp(a) is about half of that on LDL-C. The modest correlation between LDL-C and Lp(a) lowering with PCSK9 inhibition is tempered by significant discordance in the reduction of these two lipid fractions in approximately 40% of treated individuals, who show a robust LDL-C with minimal Lp(a) response [137]. This observation suggests that PCSK9 inhibition activates alternative mechanisms beyond the LDLR and that additional factors ultimately determine the degree to which Lp(a) levels are reduced. It is likely that Lp(a) binds to and is cleared by the LDLR, at least to some extent, as its lowering by PCSK9 inhibitors is inversely related to plasma LDL-C levels [130]. Additionally, Lp(a) clearance may also be determined by the length of the apo(a) isoform. It is possible that some Lp(a) isoforms may be cleared through LRP1 [138] or CD36 [139], two receptors that are also influenced by PCSK9 inhibition [73, 76]. Other data suggest that PCSK9 does not affect Lp(a) catabolism, but rather enhances apo(a) secretion and Lp(a) assembly through unknown mechanisms [140]. Potential mechanisms underlying the impact of PCSK9 inhibition on plasma Lp(a) concentration are summarized in Figure 3.

Triglyceride-Rich Lipoproteins

Studies suggest a role for PCSK9 in triglyceride-rich lipoprotein (TRL) metabolism through LDLR-mediated clearance and possibly through an effect on hepatic and intestinal apoB-lipoprotein production [66, 141, 142]. Animal models have shown both LDLR-dependent and independent roles for PCSK9 on TRL metabolism in the liver [143, 144] and small intestine [145, 146], while individuals with the GOF PCSK9 mutation S127R have a three-fold elevation in apoB100 production rates compared with noncarriers [147]. However, the effect of PCSK9 inhibition on plasma triglyceride levels is not clear, as most studies only show a modest reduction that does not always reach statistical significance [148-152]. Although the absolute changes in triglyceride levels are similar to what is seen with statin therapy (average reduction of ~15%), the effect of PCSK9 inhibition on triglyceride levels is dwarfed by its impact on LDL-C reduction. Metabolic studies in humans have failed to demonstrate an effect of PCSK9 inhibition on VLDL-apoB and VLDL-triglyceride production rate but show an increased fractional catabolic rate for both VLDL-apoB and VLDL-triglycerides, suggesting that PCSK9 does not impact TRL production but only its clearance [153]. PCSK9 inhibition also did not influence post-prandial triglyceride or apoB48 levels, consistent with the notion that PCSK9 is not directly involved in TRL production in the small intestine in humans [153].

High-Density Lipoprotein

Studies in mice support a direct relationship between high-density lipoprotein (HDL)-cholesterol (HDL-C) levels, as deletion of the PCSK9 gene [154], injection of PCSK9 blocking antibodies [155], or administration of antisense oligonucleotides against PCSK9 [156] all led to reductions in HDL-C levels by 30%-50%. In contrast, PCSK9 transgenic animals exhibit a mild increase in HDL-C levels [20]. In contrast, clinical trials with PCSK9 inhibitors demonstrate a modest (<10%) increase in HDL-C and apoAI levels [157]. The absolutes changes in HDL-C levels are similar to what is observed after treatment with statins [158]. A critical difference from humans is that HDL in mice is rich in apoE, which makes it a good target for LDLR-mediated clearance. Thus, the effect of PCSK9 on HDL-C metabolism in mice is directly linked to the effect of PCSK9 on the LDLR. To date, there is no clear

evidence that PCSK9 directly effects HDL production or clearance in humans. One possibility is that the rapid clearance of LDL due to PCSK9 impairs the CETP-mediated cholesterol exchange between HDL and LDL, leading to modestly increased HDL-C levels.

PCSK9 inhibition: Clinical Outcome studies

Cardiovascular outcomes

Prior to the results of the first dedicated randomized controlled cardiovascular outcome trial with PCSK9 inhibition, there were post-hoc analyses of the alirocumab and evolocumab clinical trial programs that suggested that these drugs may be of significant benefit for cardiovascular event reduction. The OSLER (Open-Label Study of Long-Term Evaluation Against LDL Cholesterol) trial examined the long-term effects of evolocumab as an extension of the open-label, randomized controlled OSLER 1 and 2 trials and included 4,465 patients [159]. The majority of patients (~80%) had other cardiovascular risk factors including hypertension, diabetes, metabolic syndrome, current cigarette use, or family history of premature coronary artery disease or of inherited hypercholesterolemia. The baseline LDL-C of 120 mg/dL was reduced by 61% to a mean of 48 mg/dL. In this post-hoc analysis, the incidence of cardiovascular events (death, MI, unstable angina requiring hospitalization, coronary revascularization, stroke, transient ischemic attack, and heart failure requiring hospitalization) occurred in 1% of the evolocumab group versus 2% of the standard therapy group (HR 0.47, $p = 0.0003$).

A similar investigation of the long-term safety, tolerability, and efficacy of alirocumab vs. placebo was conducted in patients at high cardiovascular risk from the ODYSSEY LONG TERM Study of 2,341 subjects. LDL-C levels were reduced by 61% in the alirocumab group to a mean of 48 mg/dL compared to an increase of 0.8% in the placebo group. The post-hoc analysis of cardiovascular events (composite of death from coronary heart disease, nonfatal MI, fatal or nonfatal ischemic stroke, or unstable angina requiring hospitalization) again demonstrated much lower rates with alirocumab than with placebo (1.7% vs. 3.3%, HR 0.52, $p = 0.02$) [160]. It is important to realize that these studies contained few events and were not adequately powered to address cardiovascular outcomes. Regardless, many predicted that the PCSK9 mAbs would yield extraordinary outcomes in the dedicated randomized controlled trials given the magnitude and consistency of suggested cardiovascular benefit from these two post-hoc analyses.

In the meantime, Ference *et al.* performed a Mendelian randomization analysis in an attempt to predict the results of the randomized controlled cardiovascular outcomes trials [104]. The investigators used data from 112,772 individuals from 14 studies, with 14,120 cardiovascular events and 10,635 cases of diabetes, to categorize individuals based on inheritance of the number of LDL–lowering alleles, either variants in the genes encoding PCSK9 and/or HMG CoA reductase (HMGCR), the target of statins. Long-term exposure to either *PCSK9* variants or *HMGCR* variants was associated with a remarkably similar reduction in risk of cardiovascular events per unit reduction in LDL cholesterol (by 19% per 10 mg/dl decrease in LDL-C). In combination, the effects of these variants were additive.

Moreover, a detailed atherosclerosis imaging study reported on the impact of evolocumab on coronary plaque utilizing IVUS. Prior IVUS trials demonstrated statistically significant reductions in percent atheroma volume in individuals who were started on high-intensity statin therapy and achieved LDL-C levels <70 mg/dL [161]. It was not clear if further lowering of LDL-C with PCSK9 inhibition might have more profound effects on plaque regression. The GLAGOV (Global Assessment of Plaque Regression With a PCSK9 Antibody as Measured by Intravascular Ultrasound) trial evaluated changes in coronary atherosclerosis using serial IVUS evaluations in subjects taking statins or statins plus evolocumab [152]. Patients with angiographic coronary artery disease on baseline statin therapy were randomized to monthly evolocumab (n=484) or placebo (n=484). A greater decrease in percent atheroma volume (1% difference) at 76 weeks was seen among those who received evolocumab. Furthermore, more patients on evolocumab than on statin experienced plaque regression (64.3% vs. 47.3%; p<0.001). Several questions remained after GLAGOV; (1) will incremental plaque regression be associated with incremental cardiovascular event reduction as the prior statin monotherapy trials demonstrated, (2) if lowering LDL-C to unprecedented levels only regresses atheroma volume by 1%, have we reached the limits of what can be achieved by dramatic LDL-C lowering, and more fundamentally, (3) if attaining extreme hypocholesterolemia does not dramatically alter plaque, what other orthogonal approaches to ASCVD risk reduction need to be considered?

The FOURIER trial was the first of the randomized controlled cardiovascular outcomes trials with a PCSK9 inhibitor, evolocumab [107]. The investigators randomized 27,564 patients with established ASCVD on optimized statin therapy to either evolocumab or placebo and monitored the rate of major cardiovascular events (cardiovascular death, MI, stroke, hospitalization for unstable angina, or coronary revascularization in the primary outcome measure). At 48 weeks, evolocumab therapy was associated with a 59% reduction in LDL-C from a median baseline of 92 mg/dL to 30 mg/dL. At a median follow-up of 26 months, evolocumab was associated with an absolute 1.5% reduction in the primary outcome, driven primarily by reductions in nonfatal MI, stroke, and revascularization. Effects of evolocumab were consistent regardless of baseline LDL-C level or intensity of background statin use. Evolocumab therapy, with its associated intense LDL-C reduction, did not decrease mortality, though the event curves between the evolocumab and placebo groups were still diverging at the time of trial termination. While there was no overall or cardiovascular-specific mortality benefit with evolocumab, death from cardiovascular disease was remarkably low (< 2%) in both groups. Interestingly, the 7-year IMPROVE-IT trial of ezetimibe added to statin therapy also did not show a reduction in mortality [110]. Other than a modest 2% incidence in injection-site reactions (which led to no excess drug discontinuations vs. placebo), there was no increase in key adverse events including new-onset diabetes or neurocognitive effects in patients receiving evolocumab despite the dramatic LDL-C reduction.

Did FOURIER underperform or were the post-hoc analyses that preceded it simply way off? The landmark analysis presented in FOURIER demonstrated event reductions similar to those reported in years 0–2 of prior statin trials [162]. An enthusiastic interpretation suggests that longer duration of therapy should be associated with greater reduction in major adverse cardiovascular events and perhaps a corresponding mortality reduction. Given the significant

cost of PCSK9 inhibitors and lack of established mortality benefit, their routine addition to standard-of-care statin therapy in patients with established ASCVD has thus far been reserved for those perceived to be at particularly high risk for cardiovascular events.

Subsequent analyses from FOURIER have been encouraging. One investigation assessed the efficacy and safety of evolocumab according to degree of LDL-C reduction at one month [163]. The primary composite outcome declined steadily as LDL-C levels decreased, with no association between LDL-C level and adverse events. A similar reduction was observed in the key secondary endpoint, with 2,669 subjects in the lowest LDL-C category (<20 mg/dL) at 4 weeks experiencing the lowest rate for cardiovascular death, or MI (adjusted hazard ratio 0.69, 95% CI 0.56-0.85, P=0.0001) compared to the group with highest LDL-C (>100 mg/dL). Exploratory analyses in a subgroup of 504 patients with an LDL-C <10 mg/dL showed even further reduction in cardiovascular events with no increase in safety events. While it remains to be seen if prolonged treatment with a PCSK9 inhibitor with profound LDL-C lowering results in mortality benefit, at the very least this therapeutic approach and iatrogenic extreme hypocholesterolemia appear to be safe. In another FOURIER analysis that evaluated 22,351 subjects with a history of prior MI, those with recent MI, multiple prior MIs, and residual multi-vessel coronary artery disease had 34-90% greater risk for vascular events and enjoyed the greatest benefit from PCSK9 inhibition (absolute risk reduction of 2.6-3.4% over three years). [164]. Given expense and barriers to access, this data may help to define appropriate allocation of PCSK9 inhibitors to those with established, high-risk ASCVD. Lastly, the FOURIER investigators evaluated the impact of evolocumab in patients with peripheral arterial disease (PAD) [165]. This analysis of 3,642 patients with PAD demonstrated that evolocumab significantly reduced the primary composite endpoint. Evolocumab reduced the primary endpoint consistently in patients both with and without PAD, but the drop was numerically greater in the PAD patients. In patients with PAD the absolute risk reduction on evolocumab versus placebo was 3.5% (vs. 1.5% in the main FOURIER analysis) yielding an attractive number-needed-to-treat (NNT=29) over 2.5 years. In the no-PAD group, the absolute risk reduction was only 1.4%, for an NNT of 72. Interestingly, evolocumab also reduced the risk of major adverse limb events in all patients compared with placebo (HR 0.58; 95% CI 0.38-0.88), with consistent effects for those with and without PAD.

Bococizumab is the other PCSK9 mAb that has been evaluated in a randomized controlled cardiovascular outcome trials. In the SPIRE (Studies of PCSK9 Inhibition and the Reduction of vascular Events) trials, participants were randomly assigned to receive either bococizumab 150 mg subcutaneously every 2 weeks or placebo. The SPIRE program included six parallel, multinational studies designed to assess the variability and durability of the LDL-C-lowering efficacy of bococizumab and two dedicated cardiovascular outcome trials [114, 166]. The lipid-lowering trials included 4,300 patients on background statin therapy who were followed up for 1 year. At 12 weeks, bococizumab treatment was associated with a reduction in LDL-C levels of 55% relative to placebo. However, treatment with bococizumab often led to the development of antidrug antibodies and specific neutralizing antibodies, which attenuated the LDL-C-lowering response (48% and 29% of patients at 1 year, respectively) [166]. In those who developed higher neutralizing antibody titers (upper tertile and top decile, respectively) in response to bococizumab, LDL-C concentration

decreased by only 31% and 12% from baseline. Not surprisingly, given its immunogenicity, bococizumab was also associated with significantly higher rates of injection-site reactions than placebo (10.4% versus 1.3%; $P < 0.001$).

The two dedicated CVOT in the SPIRE program included a total of 27,438 patients at high risk of ASCVD (93% receiving background statin therapy) and who had either a previous cardiovascular event or history of diabetes mellitus, chronic kidney disease, PAD, or familial hypercholesterolemia (high-risk, primary prevention cohort) [167]. The primary end point included nonfatal MI, nonfatal stroke, hospitalization for unstable angina requiring urgent revascularization, and cardiovascular death. The studies had a very short duration due the decision of the manufacturer to abandon the development of the drug. In the combined trials, those receiving bococizumab achieved the anticipated 56% mean reduction in LDL-C levels [114]. In the lower-risk subject SPIRE-1 trial (baseline LDL-C ≥70 mg/dl; median follow-up 7 months), bococizumab did not reduce the incidence of the primary composite end point compared with placebo. In contrast, in the higher-risk subject SPIRE-2 trial (baseline LDL-C ≥100 mg/dl; median follow-up 12 months), the rate of major cardiovascular events was significantly lowered by bococizumab (HR 0.79, 95% CI 0.65–0.97, $P = 0.02$). When the SPIRE-1 and SPIRE-2 data were pooled, the incidence of the composite primary end point was not significantly different between groups (median follow-up 10 months), although patients with the largest percent reduction in LDL-C levels had a 25% decrease in events (HR 0.75, 95% CI 0.61–0.92, $P = 0.006$), and those who had longer treatment duration (mean 13.6 months) had a 17% reduction in events (HR 0.83, 95% CI 0.70–0.98, $P = 0.03$).

Most recently, the ODYSSEY OUTCOMES trial was presented at the American College of Cardiology Meeting (Presented by Dr. Philippe Steg at the American College of Cardiology Annual Scientific Session (ACC 2018), Orlando, FL, March 10, 2018.). ODYSSEY OUTCOMES enrolled 18,924 subjects within 1-12 months after an index ACS after a run-in phase of 2-16 weeks on high intensity statin therapy. Individuals who demonstrated LDL-C ≥70 mg/dL (or non-HDL-C ≥100 mg/dL or ApoB ≥80 mg/dL) despite high-intensity therapy were randomized to alirocumab every 2 weeks or placebo. Alirocumab was titrated between 75 and 50 mg to target an LDL-C between 25-50 mg/dL, but above 15 mg/dL. The primary outcome was a composite endpoint including coronary heart disease death, MI, ischemic stroke, or unstable angina. Treatment with alirocumab (on-treatment analysis) was associated with a 54.7% reduction in LDL-C and an absolute risk reduction in the primary endpoint of 1.6%. Of note, alirocumab was discontinued in almost 8% of the treatment group due to two consecutive LDL-C measurements below the prespecified threshold of 15 mg/dL. Several of the secondary outcomes favored treatment with alirocumab, though total mortality is to be considered only nominally significant given that the two proximal endpoints in the hierarchical analysis did not reach statistical significance. Nevertheless, the signal suggesting a reduction in total mortality is reassuring given that the opposite was noted in the FOURIER trial.

The three dedicated cardiovascular outcomes trials with therapeutic monoclonal antibodies targeting PCSK9 have taught us much about what can be expected from the provision of therapeutic monoclonal antibodies to high risk patients. First, the type of antibody used to inhibit PCSK9 matters greatly. Humanized antibodies against PCSK9 are immunogenic and

are associated with injection-site reactions, the development of neutralizing antibodies, and an attenuated LDL-C lowering effect. Second, consistent with the 'lower is better for longer' hypothesis, clinical benefits with bococizumab were greater and significant only for those patients who achieved and sustained large reductions in LDL-C. Third, while FOURIER pushed this hypothesis to 'lowest-is-best', the overall cardiovascular risk reduction afforded by the 60% reduction in LDL-C with evolocumab was modest. Fourth, and not surprisingly, the magnitude of absolute benefit is greatest amongst the highest risk patients and maximized with longer exposure to drug.

Even when considering the PCSK9 monoclonal antibodies in the most positive light, questions remain with regards to the cost-effectiveness of this therapeutic approach. Extrapolation of the clinical trial results reveals a NNT of 74 for 2 years of treatment in FOURIER and NNT of 64 over the duration of ODYSSEY Outcomes. Is this tenable for a drug that is priced at ~$14,000 per year, or almost $1,000,000 to prevent one event? This question is at the center of a debate amongst all stakeholders and remains unresolved.

Safety outcomes

In early 2014, the FDA directed developers of PCSK9 inhibitors to monitor neurocognitive adverse effects given concerns over putative cognitive impairment due to extreme LDL-C lowering. EBBINGHAUS (Evaluating PCSK9 Binding Antibody influence on Cognitive Health in High Cardiovascular Risk Subjects), a substudy of FOURIER, evaluated longitudinal neurocognitive changes in patients receiving a combination of evolocumab plus statin vs. statin alone [168]. Before that, a Mendelian randomization study evaluated the association between low LDL-C and risk of dementia using genetic variation in *HMGCR* and *PCSK9* as instrumental variables [127], and found no increased risk of dementia, Parkinson's disease, or epilepsy. More recently an analysis from REGARDS (The Reasons for Geographic and Racial Differences in Stroke) study recapitulated these findings. The investigators evaluated the association between PCSK9 LOF variants and neurocognitive impairment and decline among participants in REGARDS in study participants with (n=241) and without (n=10,454) C697X or Y142X LOF variants of PCSK9, using a comprehensive battery of neurocognitive tests. They found no differences in neurocognitive decline between the two groups, again suggesting that life-long exposure to low LDL-C is not associated with cognitive dysfunction [169].

In EBBINGHAUS, a total of 1,204 patients with both baseline and follow-up cognitive testing were evaluated [159]. Patient baseline characteristics were consistent with those enrolled in FOURIER, with a mean age of 63 years, 72% male and 20% with a prior stroke. Cognitive function was assessed using a standardized, well-validated computer tablet-based testing platform (Cambridge Neuropsychological Test Automated Battery, or CANTAB), which evaluates spatial working memory strategy index of executive function (primary endpoint), as well as other memory assessments, including survey of everyday cognition and investigator-initiated reports of neurocognitive adverse effects. Over a median follow-up of 19.8 months, there was no difference between patients in the evolocumab or placebo treatment groups, with respect to either primary or secondary endpoints. There was also no

evidence to suggest differences in cognitive tests in patients attaining very low LDL cholesterol levels, including those with levels <25 mg/dL.

More recently, a group of investigators assessed the incidence of adverse neurocognitive adverse events amongst participants from fourteen phase II and III trials testing alirocumab [170]. Neurocognitive events were reported by 22 (0.9%) alirocumab-treated patients vs. 9 (0.7%) with placebo in placebo-controlled trials [hazard ratio (HR) 1.24, 95% confidence interval (CI) 0.57–2.68] and 10 (1.2%) with alirocumab vs. 8 (1.3%) with ezetimibe in ezetimibe-controlled trials (HR 0.81, 95% CI 0.32–2.08). Rates of neurocognitive events were similar in patients with LDL-C levels <25 mg/dL (n = 5/839; 0.6%; 0.5/100 patient-years) vs. ≥25 mg/dL (n = 26/2501; 1.0%; 0.8/100 patient-years).

Of course, the last word has not been spoken on this issue. What are the consequences of longer-term therapeutic reductions in LDL-C? Besides the informative genetic analyses, it is important to bear in mind that these PCSK9 mAbs do not cross the blood brain barrier and thus are unlikely to be associated with direct adverse effects on the central nervous system, whose lipid homeostasis is under separate regulation from the systemic one.

Another theoretical complication of PCSK9 inhibitor induced hypocholesterolemia is diabetes. The concern of drug induced diabetes stems from multiple lines of evidence that demonstrate that statins are associated with a modest risk of new-onset hyperglycemia, especially in patients susceptible to develop diabetes by standard markers [171-173]. While this observation has been fairly consistent across trials, the mechanisms at play have not been elucidated. Clinical trials of PCSK9 inhibitors did not show a signal for new-onset diabetes, though these studies are of short duration [174, 175]. Two Mendelian randomization analyses provide insight on this issue as well. In one, LOF variants of *PCSK9* and *HMGCR* were associated with increased risk of diabetes (11% and 13%, respectively) [104]. In another, using data from more than 550,000 individuals and 51,623 type 2 diabetics, long-term exposure to LOF variants of *PCSK9* associated with lower LDL-C and higher fasting glucose concentration and waist-to-hip ratio, and increased risk of type 2 diabetes (odds ratio 1.29, 95% CI 1.11 to 1.50) [176]. Both of these Mendelian randomization studies therefore suggest that, like statins, long-term treatment with a PCSK9 inhibitor may predispose vulnerable patients to increased risk of type 2 diabetes. Again, the mechanism that underpins these observations is unknown, though if this relationship is real, it may be related to upregulation of the LDLR with increased lipid uptake by the pancreatic beta-cell. Although the FOURIER trial did not demonstrate an excess of diabetes in those treated with evolocumab [107], the median duration of therapy was just over two years. It remains to be seen whether the Mendelian randomization analyses or the clinical trials with PCSK9 inhibitors correctly forecast the association between PCSK9 efficiency and diabetes.

Cost, cost-effectiveness, and barriers to access

The issue that has been at the center of the PCSK9 inhibitor debate relates to their cost and cost-effectiveness. With a wholesale acquisition cost of ~$14,000 and only modest reductions in non-fatal atherosclerotic events, stakeholders have wrestled with appropriate allocation and regulations to restrict and target access to these therapies. From the time that

PCSK9 mAbs received regulatory approval, payers have placed hurdles in front of patients and providers. A recent analysis identified five notable features of the prior authorization (PA) requirements for PCSK9 inhibitors, including: 1) a requirement to submit medical records along with PA forms; 2) requirements for data that may be challenging for providers to access (eg, adherence measures typically calculated from pharmacy claims, off-treatment LDL-C levels that may not be available); 3) restriction of approval to specialty prescribers (cardiologists, endocrinologists, lipidologists); 4) requirement for genetic testing even though the same insurers will not cover the cost of testing; and 5) requirement to try multiple lipid-lowering regimens [177].

Of course, the restricted access to PCSK9 inhibitors is a complicated issue. Several cost-effectiveness analyses with these agents have been performed and demonstrate disparate results based on different modeling assumptions, though they all found that these drugs are not cost-effective at their current price [178-180]. Clearly on the basis of these analyses, the economic proposition of the therapeutic mAbs is not viable, unless only the highest risk patients are allocated to therapy. It remains to be seen whether other approaches to PCSK9 inhibition associated with longer duration of effect, such as siRNA and vaccination, may be more cost-effective.

Such barriers to access can be insurmountable for the many providers that do not have the resources to challenge an unjustified denial. In that regard, we recently presented the concept of the “PCSK9 Inhibitor Clinic”, a new model that streamlines and coordinates care delivery in an effort to improve approval/access to therapy [181]. Now, with over 300 patients on therapy, our rate of approval for the PCSK9 mAbs exceeds 97%. This success is due to a combination of appropriate patient selection and a comprehensive, efficient, and consistent approach to the PA and appeal processes. While this model is successful, it may not be scalable, especially in community practices. Clearly, larger efforts led by the American Heart Association and the American College of Cardiology are necessary to engender policy change at the national level.

Conclusion

The discovery of PCSK9 has ushered in an exciting new era for cholesterol management and CVD risk reduction. Fundamental biological insights have provided a far clearer understanding of lipoprotein metabolism. Based on these understandings, we are no longer limited to the previously held view that cholesterol homeostasis is an intracellular affair, but rather appreciate a model whereby a secreted plasma protein, whose action can be easily inhibited, renders dominant control over lipoprotein metabolism. These findings have been translated into newly approved therapies, PCSK9 mAbs that dramatically reduce LDL-C and incrementally reduce atherosclerotic cardiovascular events, with additional therapeutic antagonists of PCSK9 likely on the way. The excitement surrounding the science and unprecedented efficiency in drug development is tempered by the practical realities of cost-effectiveness and barriers to access - issues that must be resolved if the PCSK9 story is to reach its full potential. Given the prospect of PCSK9 modulators to dramatically alter approaches to cardiovascular disease prevention, all stakeholders (scientists, clinicians, guideline committees, payers, and patient advocacy groups) must work together to ensure

that therapies targeting PCSK9 are appropriately evaluated, endorsed, allocated, and reimbursed.

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Abbreviations

PCSK9	proprotein convertase subtilisin/kexin type 9
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
FH	familial hypercholesterolemia
HeFH	heterozygous familial hypercholesterolemia
HoFH	homozygous familial hypercholesterolemia
GOF	gain of function
LOF	loss of function
ASCVD	atherosclerotic cardiovascular disease
MI	myocardial infarction
SRE	sterol-regulatory element
SREBP	sterol regulatory element binding protein
apoB	apolipoprotein B
VLDL	very low-density lipoproteins
LDLRAP	low-density lipoprotein receptor adaptor protein
apoER2	apoE receptor 2
LRP1	LDLR-related protein 1
HSPG	Heparan sulfate proteoglycans
CIMT	carotid intima-media wall thickness
FATE	Firefighters and Their Endothelium
CAC	coronary artery calcium
apo(a)	apolipoprotein(a)

IVUS	intravascular ultrasound
ACS	acute coronary syndrome
Lp(a)	lipoprotein(a)
CTTC	Cholesterol Treatment Trialists' Collaborators
IMPROVE-IT	Improved Reduction of Outcomes: Vytorin Efficacy International Trial
mAbs	monoclonal antibodies
siRNA	small interfering RNAs
ASO	antisense oligonucleotides
ORION-1	Trial to Evaluate the Effect of ALN-PCS_{SC} Treatment on Low Density Lipoprotein Cholesterol
TRL	triglyceride-rich lipoprotein
HDL-C	high-density lipoprotein-cholesterol
OSLER	Open-Label Study of Long-Term Evaluation Against LDL Cholesterol
HMGCR	HMG CoA reductase
GLAGOV	Global Assessment of Plaque Regression With a PCSK9 Antibody as Measured by Intravascular Ultrasound
PAD	peripheral arterial disease
SPIRE	Studies of PCSK9 Inhibition and the Reduction of vascular Events
EBBINGHAUS	Evaluating PCSK9 Binding Antibody influence on Cognitive Health in High Cardiovascular Risk Subjects
REGARDS	The Reasons for Geographic and Racial Differences in Stroke
PA	prior authorization

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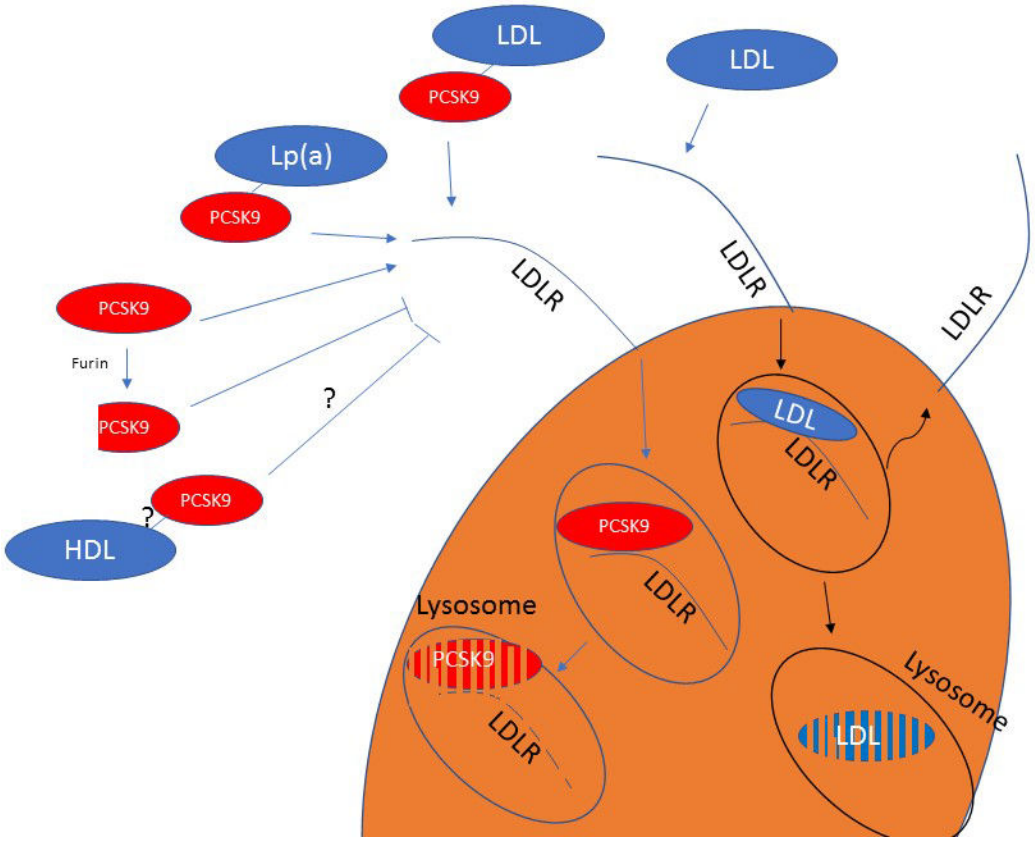

Figure 1. Stochastic interaction between the LDLR and LDL-bound PCSK9 terminate the receptor life

Before the discovery of PCSK9 it was understood that the LDLR can recycle hundreds of time in its ~20-hours life span. Since PCSK9 is found on one in every 500-1000 LDL particle, one can envision a scenario where one in every 500 encounters, an LDLR binds to an LDL particle harboring a molecule of PCSK9. Such a stochastic interaction will then lead to the degradation of the receptor rather to its recycling, explaining, at least in part, why LDLR recycle hundreds of times.

LDLR = low-density lipoprotein receptor; LDL = low-density lipoprotein; PCSK9 = proprotein convertase subtilisin/kexin type 9

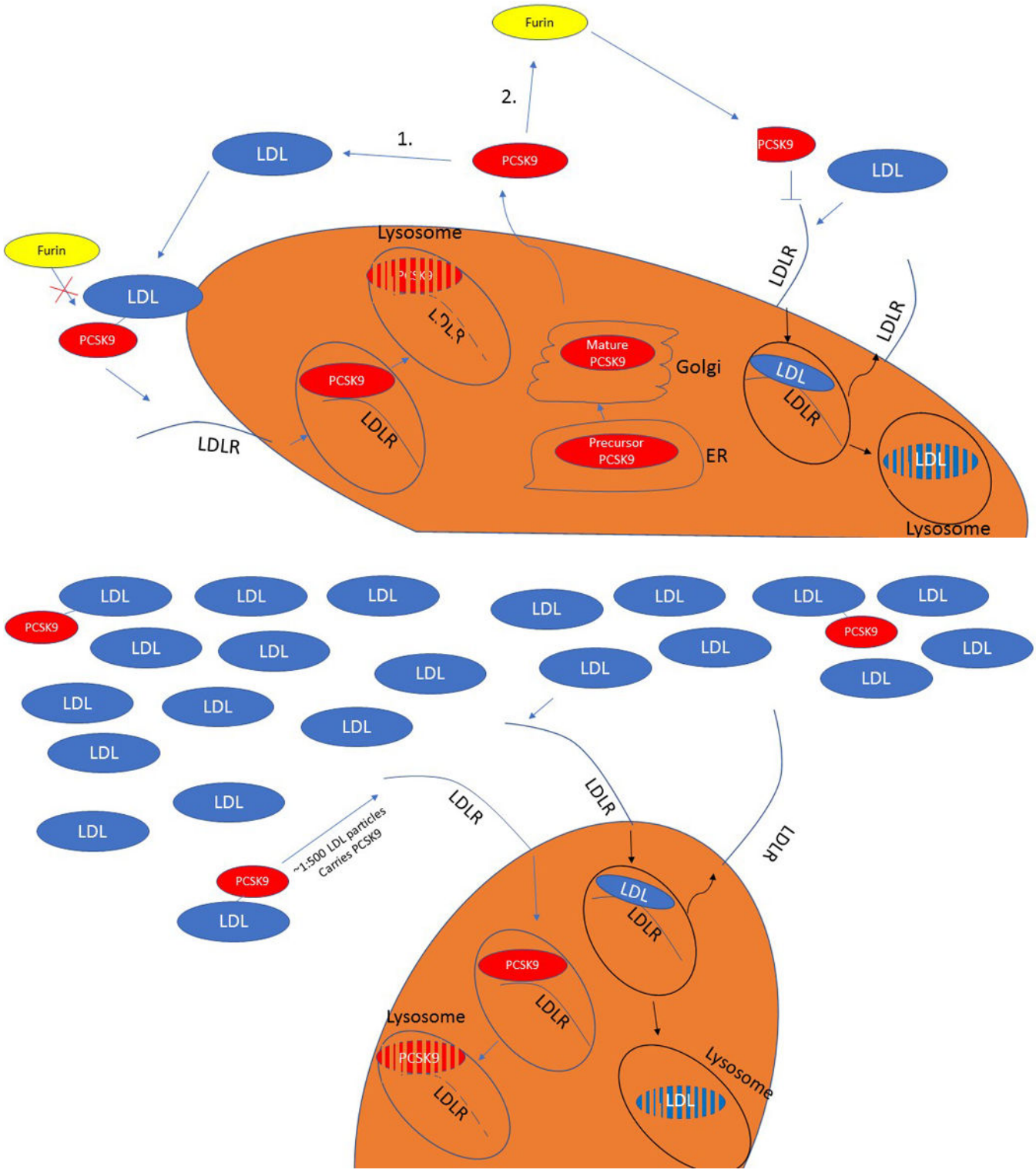

Figure 2. PCSK9 compartmentalization and function in plasma

(A) PCSK9 is found in plasma in primarily two monomeric forms; an active form representing the full-length plasma protein and an inactive/less-active shorter fragment, which is a cleavage product of the full length protein by the protease furin. The active

PCSK9 is found predominantly on LDL and Lp(a) particles, but not on VLDL or chylomicron remnants. In contrast, the furin-cleaved PCSK9 is not found in association with these apoB-lipoproteins. While it is not clear whether PCSK9 (active or furin cleaved) is found in association with HDL, it was suggested the HDL can inhibit PCSK9 function. (B) PCSK9 is secreted as an active form representing the full-length plasma protein. Upon secretion, PCSK9 can take on one of two fates, which ultimately determines its function. *(i)* PCSK9 can interact with an LDL particle, which protects PCSK9 from being cleaved by furin and leaves the protein bound to the particle in its active form, or alternatively, *(ii)* PCSK9 can interact with furin, which leads to the formation of a shorter fragment of PCSK9 that exhibits at least two-fold lower affinity to LDLR with limited ability/inability to degrade it.

PCSK9 = proprotein convertase subtilisin/kexin type 9; LDL = low-density lipoprotein; Lp(a) = lipoprotein(a); VLDL = very low-density lipoprotein; apoB = apolipoprotein B; HDL = high-density lipoprotein; LDLR = low-density lipoprotein receptor

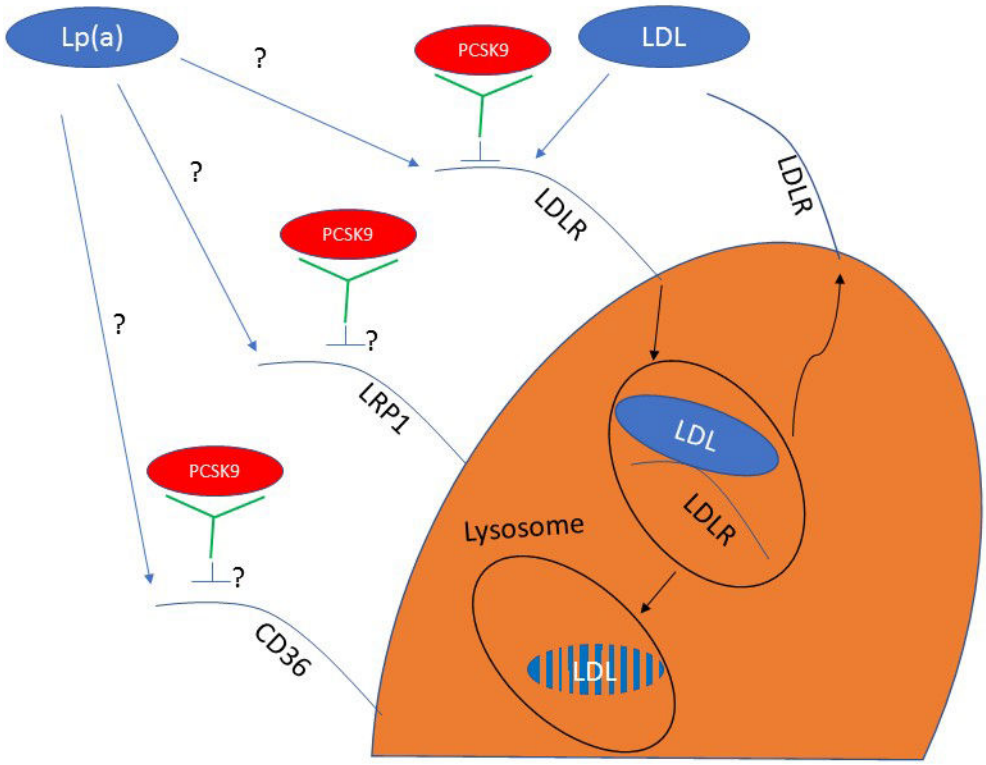

Figure 3. Possible effects of PCSK9 inhibition on Lp(a) metabolism

(i) Therapeutic PCSK9 inhibition prevents PCSK9 interaction with the LDLR, therefore facilitating continued recycling of the receptor and efficient clearance of Lp(a) particles; (*ii* and *iii*) PCSK9 inhibition affects LRP1 and CD36 levels or function, which results in increased Lp(a) clearance through these receptors; *(iv)* PCSK9 directly regulates apo(a) secretion, and the inhibition of PCSK9 prevents this process.

PCSK9 = proprotein convertase subtilisin/kexin type 9; Lp(a) = lipoprotein(a); LRP1 = low-density lipoprotein receptor-related protein 1; CD36 = cluster of differentiation 36; apo(a) = apolipoprotein(a)