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Short communication

Lipoprotein compartmentalisation as a regulator of PCSK9 activity

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1. The discovery of PCSK9

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a protein whose function was only first eluded to in 2003 and has since found its way as a new treatment target within the clinic. It is one of the most striking examples of a rapid translation from a genetic-based discovery to an approved therapeutic. By studying families with familial hypercholesterolemia (FH), Boileau and colleagues uncovered a rare gain of function mutation in the PCSK9 gene. FH affects 1 in 250 individuals. Monogenic mutations in the low-density lipoprotein receptor (LDLR, common FH1) or in the apolipoprotein B gene (APOB, rare FH2) constituted the known genetic architecture of FH at the time. However, at least a third of FH patients had neither a mutation in LDLR nor APOB [1]. Boileau and colleagues highlighted the central role that PCSK9 may play in the regulation of low-density lipoprotein-cholesterol (LDL-C) levels [2]. In parallel, Seidah *et al* characterised PCSK9, then termed neural apoptosis-regulated convertase-1 (NARC-1), revealing a role in the developing neural system, alongside a high level of expression within the liver and intestine, the two major sites of lipoprotein synthesis and degradation within the body [3]. These early works paved the way for establishing PCSK9 as a central regulator of the LDLR and novel treatment target.

2. A paradigm shift in LDL biology

The mechanism of action of PCSK9 and its therapeutic targeting has been reviewed in-depth elsewhere (Fig. 1) [4]. Before the discovery of PCSK9, the paradigm of LDL biology stated that the LDLR, upon internalisation complexed with LDL, undergoes a classical endocytic receptor-recycling pathway. Upon acidification within the endosome, LDL dissociates from the LDLR. The LDL particle is degraded in lysosomes, while the LDLR recycles back to the cell membrane (Fig. 1). PCSK9, however, interacts extracellularly with the LDLR. When the LDLR is complexed with PCSK9 and internalised, acidification within the endosomal compartment, rather than disrupting the protein interaction, strengthens the association between PCSK9 and the LDLR, ultimately leading to their degradation within the lysosome (Fig. 1). Thus, patients with gain of function mutations in PCSK9 have a major defect of recycling of the LDLR resulting in hypercholesterolemia. Conversely, therapeutically blocking PCSK9 function markedly lowers LDL-C. Thus far, two monoclonal antibodies blocking the interaction of PCSK9 with the epidermal growth factor precursor homology domain A (EGF-A) of the LDLR (Alirocumab and Evolocumab) were approved. An RNA interference-based therapy to inhibit the cellular production of PCSK9 is currently in the latter stages of clinical trial (Inclisiran, ORION-4) (Fig. 1).

3. Circulating PCSK9

The circulating concentration of PCSK9 varies 100-fold (22–2988 ng/ml), but variations in PCSK9 levels explain only approximately 7% of LDL-C differences across individuals [5]. Similarly, PCSK9 concentrations proved to be a weak predictor of cardiovascular risk in epidemiological studies [4]. The disparity between the therapeutic efficacy of PCSK9 inhibitors and the poor performance of PCSK9 levels as a biomarker is suggestive of a regulatory network acting upon PCSK9 activity, and that the measure of total PCSK9 concentration may provide little functional information. Among the known endogenous regulators

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of PCSK9 protein level and activity are transcriptional regulation, post-translational modification and proteolytic cleavage [4]. Additionally, PCSK9 is thought to reside and circulate bound to specific lipoproteins (Fig. 1). However, although it was first suggested in 2008 [6] that PCSK9 may be carried by lipoproteins, the physiological consequence of this interaction and its relation to disease remains unknown.

4. PCSK9 in preclinical models

Experimental observations in mice have alluded to the fact that a proportion of PCSK9 may be carried by lipoproteins. Caution, however, must be placed upon the extrapolation of findings of PCSK9-lipoprotein associations in mice. Mice show stark differences in cholesterol distribution across lipoprotein fractions when compared to humans. Due to a lack of functional cholesterol ester transfer protein (CETP) [7], the majority (approximately 90%) of circulating cholesterol in mice is in association with high-density lipoprotein (HDL), rather than LDL as is observed in humans. This difference in the lipid composition of lipoproteins across species could drive differences in the interaction between PCSK9 and lipoproteins in the circulation, particularly if the interaction is reliant upon a lipid-binding moiety. HDL particles also typically bind a greater number of amphipathic proteins than LDL, partly due to differences in phospholipid packing. Nevertheless, multiple studies have characterised the association between PCSK9 and lipoproteins in mice. Using plasma of mice overexpressing the human form of PCSK9, PCSK9 co-eluted within LDL and HDL fractions after fast protein liquid chromatography (FPLC) separation of lipoproteins (Table 1) [6]. A subsequent study, also in mice overexpressing the human form of PCSK9, using both FPLC and OptiPrep™-based density ultracentrifugation, concluded that approximately 30% of PCSK9 may associate with LDL, while the remainder was observed within the ApoB-depleted fractions, which importantly contain HDL (Table 1) [8]. Interestingly this study primarily observed monomeric PCSK9 within the LDL fraction, whereas PCSK9 within ApoB-depleted serum was multimeric, an understudied feature of PCSK9 that has been proposed to correlate with its LDLR-degrading capabilities [6,8].

5. PCSK9 in patients with hypercholesterolemia

Lipoprotein apheresis is a therapeutic option in patients with extremely high levels of atherogenic lipoproteins, including lipoprotein (a) [Lp(a)]. The Lp(a) particle is an LDL-like particle with a distinguishing glycoprotein, apolipoprotein(a) [Apo(a)], which is attached to ApoB-100. Lipoprotein apheresis has been one of the most commonly used techniques to suggest that PCSK9 is partially carried by LDL in the human circulation (Table 1) [9–13]. In all but one of the previous five apheresis studies that determined PCSK9 levels, PCSK9 was consistently removed by approximately 50% upon the removal of the majority of LDL, and also Lp(a), suggestive of a significant proportion of PCSK9 to be carried by these ApoB-containing lipoproteins in the circulation (Table 1). Lipoprotein apheresis commonly relies upon poly-anionic matrices in the removal of large, relatively cationic, structures in the circulation, that includes ApoB-containing lipoproteins. However, due to the non-specificity of the technique, it is now appreciated that specific subpopulations of HDL, specifically those enriched in apolipoprotein-E (ApoE), are also removed upon apheresis [14]. Secondly, and perhaps more disconcerting is the observation that apheresis conducted upon ApoB-depleted plasma was able to remove PCSK9 to a large extent [10]. Thirdly, apheresis is performed after administration of heparin, which may result in differences in PCSK9-lipoprotein associations. Finally, lipoprotein apheresis is conducted upon patients with severe hyperlipidaemia. For example, investigations in patients with extremely high levels of Lp(a) led to the observation that PCSK9 may also associate with Lp(a), however the extrapolation of these findings from patients with a very abnormal lipoprotein profile to the general population must be done with caution (Table 1) [15,16].

6. PCSK9 in normolipidemic individuals

Few studies have attempted to determine the extent of the physical association between PCSK9 and lipoproteins in normolipidemic individuals. It was observed that PCSK9 can bind to LDL in normolipidemic individuals, and that the proximal N-terminal region of PCSK9 was required for this interaction (Table 1) [17]. In continuation of this work, it was elegantly determined that an amphipathic α-helical region, a structural feature found in many apolipoproteins, within the N-

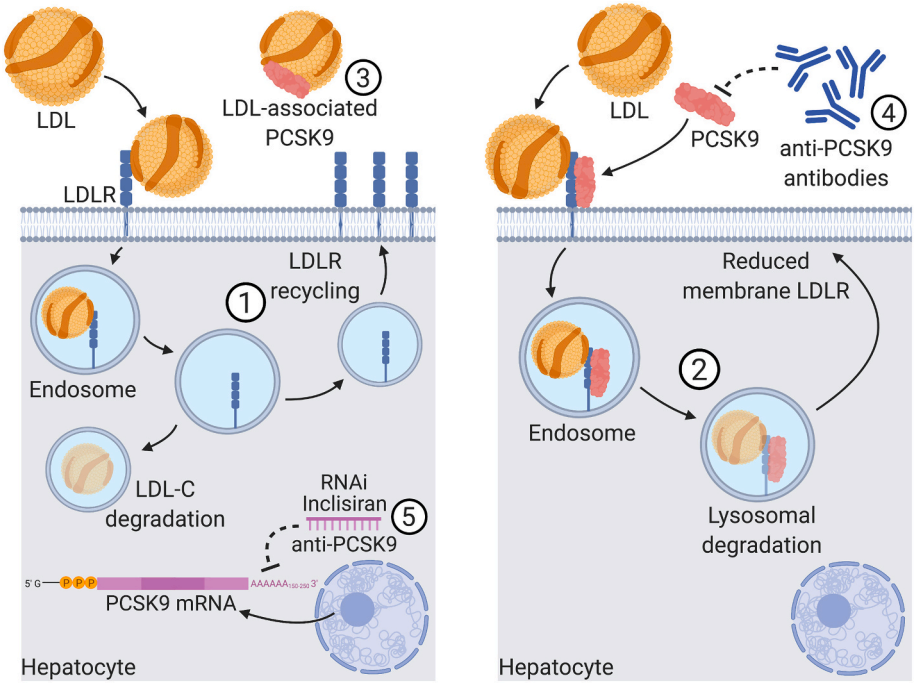

Fig. 1. PCSK9 mechanism of action and therapeutic targeting. LDLR-mediated LDL-C uptake by the hepatocyte follows a classical endocytic, receptor recycling pathway, thereby enabling a replenishment of LDLR at the membrane to further take up LDL-C (1). However, in the presence of PCSK9 binding to the extracellular region of the LDLR, the LDLR is instead directed towards lysosomal degradation, ultimately reducing the cell-surface level of the LDLR and increasing circulating LDL-C levels (2). A proportion of circulating PCSK9 is thought to associate with LDL, where it is suggested that LDL sequesters PCSK9 and prevents its action upon the LDLR (3). Two therapeutic avenues have been explored to inhibit PCSK9, through the antibody-based targeting of, extracellular, circulating PCSK9 (4) or through the RNAi targeting of PCSK9 production within the cell by Inclisiran (5). Created with BioRender.com.

Table 1
Publications reporting an interaction between PCSK9 and lipoproteins.

Mouse studies

Model	Publication	*n* number	Lipoprotein isolation method	PCSK9-lipoprotein association		
				LDL	Lp(a)	HDL
hPCSK9 transgenic mice	Fan *et al,* 2008 [6]	3 (pooled)	• FPLC	+	ND	+
	Tavori *et al*, 2013 [8]	21 (pooled)	• OptiPrep™ gradients • FPLC	+	ND	–

Human Studies

Cohort	Publication	*n* number	Lipoprotein isolation method	% Removal			
				LDL	Lp(a)	HDL	PCSK9
FH	Cameron *et al*, 2012 [9]	4	• Lipoprotein apheresis	−63%	ND	ND	0%
	Tavori *et al*, 2013 [10]	6		−77%	−79%	−17%	−52%
	Duell *et al*, 2014 [11]	4		−59%	ND	ND	−48%
	Hori *et al*, 2015 [12]	18		−76%	−77%	−16%	−52%
	Julius *et al*, 2015 [13]	40		−71%	ND	−10%	−51%
				PCSK9-lipoprotein association			
Very high Lp(a)	Tavori *et al*, 2016 [15]	41	• OptiPrep™ gradients • Modified ELISAs	Preferential association of PCSK9 with Lp(a) over LDL			
	Viney *et al*, 2018 [16]	61	• Modified ELISAs	Raw RLU suggest majority of PCSK9 is HDL associated			
Normolipidemic	Kosenko *et al*, 2013 [17]	5 (pooled)	• OptiPrep™ gradients • FPLC	42% of PCSK9 present in LDL fraction			
				% Removal			
				LDL	Lp(a)	HDL	PCSK9
	Burnap *et al*, 2020 [19]	10	• ApoB immunodepletion	−100%	−100%	−20%	−19%
			• HDL immunodepletion	−50%	−50%	−100%	−93%
			• OptiPrep™ gradients	LDL fraction not devoid of HDL			
			• Modified ELISAs	Majority of PCSK9 is HDL associated			

FPLC and OptiPrep™ ultracentrifugation methodologies in mice overexpressing the human form of PCSK9 provided the first evidence that PCSK9 may associate with LDL and HDL. The use of lipoprotein apheresis, a technique aimed to remove atherogenic ApoB-containing lipoproteins in hyperlipidemic individuals, revealed a large removal of PCSK9 upon the removal of LDL and Lp(a). Studies in individuals with very high Lp(a) levels suggest that PCSK9 may also associate with Lp(a). Finally, of the studies conducted upon healthy, normolipidemic individuals it has been revealed that PCSK9 associates with LDL and HDL. The percentage of PCSK9 per lipoprotein fraction is given when quantitative methodologies have been used. Studies in mice only determined whether PCSK9 could (+) or could not (−) bind the lipoprotein of interest. FPLC, fast protein liquid chromatography. FH, familial hypercholesterolemia. ND, not determined. RLU, relative light units.

terminus of PCSK9 is the molecular feature critical for PCSK9 binding to LDL. Interestingly, this binding was diminished if FH-associated mutations were present in PCSK9 [18]. These findings, alongside the observation that LDL may inhibit PCSK9-mediated LDLR degradation [17], gave the first inclination that PCSK9-lipoprotein association may be important in the development of disease.

We have recently obtained evidence that HDL may act as the primary carrier of PCSK9 in healthy individuals [19]. Similar to the study by Viney *et al* in hyperlipidemic patients [16], we utilised alirocumab to capture plasma PCSK9 in normolipidemic individuals and then detection antibodies specific for either ApoB, Apo(a) and apolipoprotein-A1 (ApoA1) to determine relative levels of PCSK9-lipoprotein association [16]. ApoA1 is the major apolipoprotein of HDL. We observed an approximate 10-fold and 100-fold greater signal for PCSK9-ApoA1 than observed for PCSK9-ApoB and PCSK9-Apo(a), respectively (Table 1) [16]. However, these enzyme-linked immunosorbent assays (ELISAs) rely on the assumption that the affinity of alirocumab is sufficient to capture PCSK9 regardless of its association with different lipoproteins. In addition, we utilised specific lipoprotein immuno-depletion technologies to determine the extent of HDL-PCSK9 interaction. Immunodepletion technologies offer the ability to isolate specific lipoproteins of interest, while preserving structural integrity, crucial in the determination of PCSK9 binding. In contrast, PCSK9 was shown to be absent in lipoproteins isolated by ultracentrifugation, a technique known to disrupt protein-lipoprotein interactions [20]. Lipoprotein immunodepletion, alongside targeted proteomics, OptiPrep™ and ELISA validation, suggested that, although PCSK9 does indeed associate with LDL, a larger proportion may be carried by HDL in normolipidemic individuals (Table 1) [19]. Despite several lines of evidence, further investigations are needed to establish whether there is a direct interaction of PCSK9 with HDL or whether PCSK9 co-purifies with other proteins. For example, a recent study demonstrated that PCSK9 also binds to major histocompatibility complex class I protein (MHC I) [21].

7. Conclusions

Dependent upon isolation technologies utilised and participants studied, PCSK9 has been reported to associate with differing lipoprotein species. Taken together, the studies so far suggest that PCSK9 can be carried primarily by LDL and HDL (Graphical abstract). Many questions remain unanswered, *i.e.* what regulates and promotes the association between PCSK9 and a given lipoprotein? It is currently unknown whether the amphipathic helical region within PCSK9 responsible for the interaction with LDL may also be responsible for the association with HDL. The ability to bind multiple lipoprotein species and the potential functional consequence of these interactions is an area of PCSK9 biology that warrants further exploration (Graphical abstract), particularly in the context of therapeutic targeting of PCSK9. Further understanding the functional relationship that may exist between lipoproteins and PCSK9 could contribute to a better understanding of the inverse relationship between LDL-C and HDL-C and facilitate the assessment of future therapeutics.

Declaration of Competing Interest

S.A.B and M.M are named inventors on a patent application filed by King's College London for PCSK9 measurements.

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