Exploring Alternate Conformations of PCSK9: Effects on LDL, LDL Receptor Binding, and Inflammation

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Abstract:

Proprotein convertase subtilisin/kexin type-9 (PCSK9) is a central regulator of circulating levels of low-density lipoprotein-cholesterol (LDL-C) and related cardiovascular disease (CVD) risk. PCSK9 is a soluble secreted protein expressed predominantly in the liver, where it mediates lysosomal degradation of cell surface LDL receptor (LDLR), the primary conduit for LDL-C plasma clearance. Cell culture and mouse *in vivo* studies support the notion that PCSK9 mediates pro-inflammatory signaling, and therapeutic monoclonal antibodies (*e.g.,* alirocumab) that bind PCSK9 also exert anti-inflammatory properties in addition to LDL-C lowering. Plasma PCSK9 exists in intact (full-length) and truncated forms. A significant number of the former are LDL-bound. An N-terminal intrinsically disordered region (IDR) within the PCSK9 prodomain (aa 31-52) – an area rich in negatively charged amino acids – is critical in this association and has also been shown to exert a negative allosteric effect on PCSK9-LDLR binding affinity. Herein, we showed that mutagenesis to change amino acid residues within this region leads to a PCSK9 variant with altered LDL and LDLR association. Specifically, inserting a disordered Gly-Ser-Gly-Gly (GSGG) linker to replace acidic Ser47-Glu-Glu-Asp increased LDLR- dependent uptake to the same extent as a complete deletion of the IDR, whereas individual amino acid replacements within the IDR showed similar LDL and LDLR binding as wild-type (WT)- PCSK9. The major truncated PCSK9 variant found in human plasma is a proteolytic product of furin (f-PCSK9), which is considered inert because it cannot bind LDLR. However, f-PCSK9 interaction with native LDL (nLDL) and oxidatively modified LDL (oxLDL) was increased by 1.5-fold and ~10-fold, respectively, compared with intact PCSK9. F-PCSK9 also showed preferential binding to artificial liposomes mimicking the outer lipid membrane of LDL. The endocytic uptake of f-PCSK9 in HEK293 cells transiently overexpressing the scavenger receptors CD36 or LOX1 was increased compared to intact PCSK9. OxLDL co-incubation increased f-PCSK9 uptake in M1-polarized THP-1 human macrophages; pro-inflammatory cytokines

such as IL-1β and TNF α significantly increased, as determined by qPCR. F-PCSK9 was increased in the serum of LPS-treated female mice 24 hours post-treatment. The therapeutic anti-PCSK9 monoclonal antibody alirocumab blocked furin proteolysis of PCSK9. These results suggest that the f-PCSK9 may function as an inflammatory signaling molecule and mediate oxLDL interactions with scavenger receptors in immune cells.

Overall, these findings suggest a new paradigm in PCSK9 biology whereby furin proteolysis reveals novel immune functions and could aid in designing future PCSK9 inhibitors directed at different subspecies of PCSK9.

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Contributions of Collaborators

Some of the data presented in this dissertation were obtained in collaboration with other research groups, and members of the Lagace lab performed some of the experiments. It is essential to acknowledge these contributions.

LPS treatment was performed in Dr. Erin Mulvihill's lab by Natasha Trzaskaski at the University of Ottawa Heart Institute.

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qPCR primer design for IL-1β, CD36, LOX1, and TNF-α was generated by Kaitlyn Beehler, a former lab technician in the Lagace lab.

Data in all the figures except for Figures 11B and 15, which Dr. Lagace performed, were generated by me but with extensive guidance in the experimentation design and project direction from Dr. Thomas Lagace.

Contents

Nonstandard Abbreviations and Acronyms Used

aa	Amino acid
AcCoA	Acetyl coenzyme A
ACE2	Angiotensin-converting enzyme 2
ADH	Autosomal dominant hypercholesterolemia
[apo (a)]	apolipoprotein (a)
Apo A /B / C /E	Apolipoprotein A / B / C
Apo B48 / ApoB100	Apolipoprotein B48 or B100
ARH	Autosomal recessive hypercholesterolemia
AS	Atherosclerosis
bHLH-Zip	Basic helix-loop-helix leucine zipper domain
β-Me	Beta mercaptoethanol
BSA	Bovine serum albumin
CD36	Cluster of differentiation 36
CETP	Cholesteryl Ester Transferase Protein
CVD	Cardiovascular Disease
DAMPs	Damage-associated molecular patterns
DMEM	Dulbecco Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EGF-A	Epidermal growth factor-like repeat A
ER	Endoplasmic reticulum.
FBS	Fetal bovine serum
FH	Familial hypercholesterolemia
F-PCSK9	Furin-cleaved PCSK9
HBS-C	HEPES-buffered saline with calcium
HDL	High-density lipoprotein
HEK293	Human embryonic kidney immortalized cell line
Hepa1c1c7	Human hepatoma immortalized liver cell line

HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HNF1α	Hepatocyte Nuclear factor 1 alpha
HuH7	Human Hepatoma immortalized cell line
HSPG	Heparin-sulfate proteoglycans
IDL	Intermediate density lipoprotein
IDR	Intrinsically disordered region
INSIG	Insulin-induced gene 1
ITS	Insulin - transferrin - selenium
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein - cholesterol
LDLR	Low-density lipoprotein receptor
LOX1	Lectin-like oxidized LDL receptor 1
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
NCLPDS	Newborn calf lipoprotein-deficient serum
OxLDL	Oxidized LDL
OxPL	Oxidized Phospholipid
PACE 4	Paired basic amino acid-cleaving enzyme 4
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline.
PC	Proprotein convertase
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin/ kexin type-9
PRRs	Pattern recognition receptors
PMA	Phorbo 12-myristate 13-acetate
RNA	Ribonucleic acid
RT-qPCR	Real-Time quantitative PCR
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
S1P / S2P	Site 1 protease / Site 2 protease
SCAP	SREBP cleavage activating protein

SDS-PAGE	Sodium dodecyl-sulfate - polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
SR	Scavenger receptor
TAG	Triacylglycerol
TBS-C	Tris-buffered saline with calcium
TFR	Transferrin receptor
THP-1	Tohoku hospital pediatrics 1
PMA	Phorbol 12-myristate-13-acetate
TRL	Triglyceride-rich lipoproteins
WT	wild type

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Chapter 1: Introduction

Elevated plasma low-density lipoprotein cholesterol (LDL-C) is a prevalent risk factor in cardiovascular complications due in part to cholesterol overaccumulation in macrophages and smooth muscle cells in the arterial plaque [1, 2], and these complications are a persistent burden on the healthcare system worldwide [3]. Atherosclerosis is a major underlying factor leading to cardiovascular complications [4] and is characterized by luminal narrowing resulting from functional alteration in the vascular wall [5]. It is well-established that vascular inflammation fuels the progression of atherosclerosis [6, 7]. Damage to the endothelium with infiltration and modification of cholesterol-rich lipoproteins (mainly LDL-C) play a pivotal role [8], as well as the interplay between immune cells and chemical compounds within the vascular wall [4]. However, an elevated level of circulating LDL-C is critical to the development of atherosclerosis [1].

Proprotein convertase subtilisin/kexin type 9 (PCSK9), first identified and linked primarily through gain-of-function (GOF) *PCSK9* mutations to familial hypercholesterolemia (FH) in 2003, is a member of the proprotein convertases subtilisin/kexin family [9]. PCSK9 is a soluble secreted protein expressed predominantly in the liver, with lower expression in the intestine, brain, kidney, and vascular endothelial cells [10-12]. The newly discovered protein was initially named neural apoptosis-regulated convertase-1 (NARC-1) partly because of its perceived role in the differentiation of cortical neurons and in regulating neuronal apoptosis [10, 12]. The name proprotein convertase subtilisin/kexin type-9 was later adopted by the HGNC (Hugo gene nomenclature committee). Mechanistic studies have now established that PCSK9 is a central regulator of the circulating level of LDL-C [11, 13, 14] through its ability to bind and mediate the cellular degradation of LDL receptor (LDLR). LDLR is a cell surface protein that inhibits the production of LDL through the rapid removal of IDL (intermediate density lipoprotein) from the circulation and also clears LDL-C through receptor-mediated endocytosis [1, 11, 14, 15]. PCSK9

forms a protein-protein interaction with the LDLR at the cell surface, and upon endocytosis, bound PCSK9 redirects LDLR to the lysosome for degradation (*i.e.*, extracellular pathway) [11, 14], thereby disrupting the natural recycling of the LDLR to the cell surface for more LDL-C clearance [1], and ultimately leading to elevated plasma LDL-C, and atherosclerosis development and progression. There is also evidence that PCSK9 can bind to nascent LDLR and redirect it to lysosomes from within the secretory pathway (*i.e.*, intracellular pathway) [16]. However, clinical trials showing extensive LDL-C lowering (up to 70%) through inhibition of circulating PCSK9 using injectable monoclonal blocking antibodies indicate that the extracellular pathway is the major mode of PCSK9-mediated LDLR degradation in humans [17].

Plasma human and mouse PCSK9 exist in full-length (intact) and truncated forms [18, 19]. Approximately 25% of PCSK9 in human circulation is processed by the enzyme furin, which, along with PCSK9, is a member of the proprotein convertase (PC) family of serine proteases. Furin-mediated proteolysis excises an N-terminal region of the catalytic domain (aa 153 -218) of PCSK9 that overlaps the binding site to the LDLR epidermal growth factor-like (EGF)-A domain [19, 20]. Thus, furin-cleaved PCSK9 (f-PCSK9) does not bind LDLR and is considered inert [20]. However, limited proteolysis by PC enzymes is a finely regulated mechanism that can also generate biologically active proteins or molecules at the right subcellular level and context. Interestingly, PCSK9 was initially thought to be mainly involved only in FH through LDLR regulation [9]; however, recent evidence indicates other multidimensional novel functions of PCSK9. For example, animal and cell culture studies support that PCSK9 is involved in LDLR- independent mechanisms in foam cell formation, endothelial dysfunction, increased inflammatory cytokines storm, platelet activation, and redox system regulation [21-25]. Of note, it remains unclear which of the subspecies of PCSK9 is involved in these multidimensional novel roles. Although expensive [26], conventional modulation of PCSK9 through therapeutic injectable monoclonal

antibodies (mAbs) effectively blocks the PCSK9/LDLR interaction at the cell surface and lowers LDL-C plasma levels [27]. Importantly, PCSK9-inhibitory mAbs (PCSK9i-mAbs) also display anti-inflammatory, anti-thrombotic, and immunomodulatory properties independent of LDL-C reduction [23, 28, 29], suggesting a new paradigm in PCSK9 biology.

The study described herein explores the alternate confirmations of PCSK9 and the functional role of these confirmations in the context of PCSK9 biology.

1.1.1 The Proprotein Convertase Family (PCs)

The human genome contains over 560 proteases classified into five distinct groups based on their catalytic mechanism and subdivided further into families due to similarities in their amino acid sequence and three-dimensional geometry [30, 31]. The serine proteases are one of these groups – so named 'serine' because of the unique role of serine at their active site – a vast group comprising one-third of all proteolytic enzymes [32]. In humans, the most abundant are the chymotrypsin- trypsin-like serine proteases due to the diverse and significant number of physiological processes they catalyze [32]. However, the human PCs related to bacteria subtilisin and yeast kexin are a small 9-member serine endoprotease family critical in maintaining cellular function by activating various cellular processes through limited proteolysis [31-37]. Limited proteolysis involves functional alteration resulting from posttranslational processing through the endoproteolytic cleavage of precursor proteins into their active cognate states, usually at the intended target site [31, 38]. The first seven members represent a closely related family called the core PCs recognizing and cleaving substrate proteins with paired basic arginine residues in the consensus sequence RxxR – namely PCSK1 (proprotein convertase subtilisin/kexin 1), PCSK2, furin, PCSK4, PCSK5, PCSK6 (also called Paired basic amino acid-cleaving enzyme 4 [PACE4]), and PCSK7 [32]. The other two, SKI-1/SIP (subtilisin/kexin isoenzyme-1/site-1 protease)

and PCSK9 (proprotein convertase subtilisin/kexin type 9), are most closely related to pyrolysin and proteinase K, with substrate recognition consensus site and aspects of their domain architecture being distinctive from the first seven members [32, 37].

1.1.2 Structure and Biochemistry of the PCs

The early perception of protease-mediated reactions was thought to be nonspecific. However, proteolytic processing is a finely regulated physiological process vital for biological regulation [31, 38]. Proteases themselves often require proteolytic processing to acquire catalytic activity, as they are synthesized as inactive proenzymes. This ensures that proteolytic activity only occurs at the intended biological target sites [31]. PCs follow a similar pattern as they rely on autocatalytic activation for their biological function [31, 35]. Structurally, the PCs are synthesized as a multi- segmented proenzyme consisting of *i*) a signal peptide, which helps by translocating the proenzyme to the endoplasmic reticulum (ER) lumen; *ii*) a prodomain that acts as an intramolecular chaperone, and is involved in regulation, activation, and maturation by aiding in enzyme protein folding; *iii*) a catalytic domain that has a highly conserved catalytic triad consisting of aspartate, histidine, and serine residues; *iv*) a regulatory P domain; and *v*) a C-terminal domain unique to each PC in terms of length and the cytosolic domain [31, 32, 35, 39]. Among the PCs, furin is the most characterized. Because of its domain homology to other members of the PC family, with some distinct differences to SKI-1/SIP and PCSK9 in terms of domain architecture, it forms the basis for all PC characterization [36, 39]. Furthermore, besides the highly conserved catalytic domain, furin also has a conserved P domain necessary for pH regulation, calcium requirement, and protease activity [39]. The cytosolic domain of furin controls sorting and cellular localization to the trans-Golgi, cell surface, and endosomes [32, 39]. Subcellular localization enables furin and other PCs to interact with substrates in different cellular compartments as a component of their proteolytic functions.

For proteolytic activation, furin undergoes autocatalytic cleavage using its consensus rules of Arg-X-Lys/Arg-Arg↓. To prevent protease activity before its target subcellular localization, the prodomain is retained after the first autocleavage, which evidence suggests occurs at a neutral pH. Upon reaching its target destination, a final autocleavage occurs at acidic pH within the prodomain, which completely exposes the active site/catalytic domain and ultimately activates the protease [36, 40]. Except for PC2, all other PCs follow a similar maturation and activation pattern [41]; however, to date, no secondary activating proteolysis event within its prodomain has been identified for PCSK9.

1.1.3 Role of the PCs in Health and Disease

The secretory pathway contains many secreted proteins that are initially synthesized as zymogens, and through limited proteolysis, these zymogens are activated to produce biologically active proteins [42, 43]. Although the PC processing site is embedded within the zymogen backbone, their subcellular localization within the constitutive route or regulated secretory pathway and widespread expression at the systemic level positions PC enzymes as essential components of cellular homeostasis in health and disease [31, 32]. For example, PC1 is implicated in obesity [44- 47]. Despite its restrictive subcellular localization [48], it also processes proinsulin to active mature insulin [49].

PC2 is also implicated in obesity and the functional maturation of various neuropeptides [49, 50]. Not only the first PC to be identified and the most characterized, furin’s ubiquitous nature [39] places this PC member at the crossroads of many different pathways, such as atherosclerosis progression through its role in inflammation [51, 52], embryogenesis, Alzheimer's disease, viral infection, bacterial toxin activation, and several cancers [39, 53-57]. Unlike the ubiquitous furin, PC4 is primarily expressed in the testicular germ cells, and this tissue-specific expression suggests a role in fertilization through sperm maturation and capacitation [58-60]. Conversely, PCSK5 functional alteration is linked to multiple

developmental anomalies [61, 62], cancer, and HDL-C modulation [53, 63]. Also known as PACE4, PC6 could be likened to furin, as it shares similar chromosomal localization in humans and mice [64]. Evidence suggests it is involved in various cancers, tumor progression, mediation of submandibular gland development [64, 65], and a possible role in iron homeostasis [66]. PC7 is also implicated in iron homeostasis [67] and is suggested to have a role in regulating specific cognitive performance in mice and humans [68].

Although the coexpression of PC7, furin, PC5, and PC6 show some form of functional redundancy, evidence suggests that they each have unique physiological functions [69-71]. This is particularly true of SKI-1/S1P and PCSK9 in lipid metabolism [69]. SKI-1/S1P has been shown to be a critical regulator of cholesterol and fatty acid metabolism through the proteolytic activation of sterol regulatory element-binding proteins 1 and 2 (SREBPs) [69, 72-74]. Recent work also indicates that SARS-CoV2 cell-to-cell induced fusion is facilitated through the proteolytic activation of SREBP2 by SKI-1/S1P [75]. In contrast, PCSK9 regulates cholesterol homeostasis and SARS- CoV2-induced fusion through mediating LDLR and ACE2 degradation [69, 75]. It was reported that SKI-1/S1P could be essential for other viral production, such as Hepatitis C, Nairobi sheep disease virus, and Crimean-Congo hemorrhagic fever virus [76-78]. A role in developing bone by SKI-1/S1P has also been suggested through the nucleation of mineral crystals [79]. Of note, the role of PC1 to PC8 (SKI-1/S1P) in cellular homeostasis in health and disease state is by proteolytically processing the various substrates involved in cellular homeostasis. Conversely, the PCSK9 mechanism of action is non-proteolytic. Therefore, PCSK9 might have evolved to suit its current mechanism of action by limiting its proteolytic activity to its own auto-catalytic processing.

1.2 PCSK9: A unique PC member

Proprotein convertase subtilisin/kexin type-9 (PCSK9) is the ninth member of the PC family, and work

done during its early days suggests a potential role in liver regeneration and cortical neuron differentiation [10, 80]. However, PCSK9 is best known for inducing the lysosomal degradation of the LDLR, a key player in cholesterol homeostasis [11, 13, 69]. This has led to a paradigm shift in our understanding of cholesterol homeostasis, which has shown a direct role of extracellular protein in cholesterol regulation [15, 81]. Emerging evidence indicates that not all PCSK9 functional activity involves induced degradation; regulation of downstream signaling has also been suggested [82]. Specifically, PCSK9 is involved in the immune regulation of infections and tumors [83], regulation of cellular proliferation through the NODAL signaling pathway [84], and a role in systemic immune activation as evidence indicates a liner correlation between elevated circulating PCSK9 and bacterial and viral infections [85-87].

Although our knowledge of PCSK9 biology has grown beyond regulating cholesterol homeostasis, this remains the best-characterized aspect of its biological function through LDLR regulation [9, 11, 13, 14, 82]. The LDLR is a member of a structurally and functionally related protein family of seven cell surface receptors [88, 89]. Like the role of LDLR, their functions involve the internalization of lipoproteins, exotoxins, and lipid-carrier complexes [89]. Mounting evidence suggests PCSK9 is involved in regulating members of this family in a non-proteolytic manner as a binding chaperone [90]. It was reported that PCSK9 induced the degradation of the very low-density lipoprotein receptor (VLDLR) and apolipoprotein (apo) E receptor 2 (ApoER2) [91, 92], along with a possible role in the modulation of triglyceride-rich lipoprotein metabolism [93]. Induced degradation of VLDLR and ApoER2 by PCSK9 might be explained in part because of a high degree of homology in the epidermal growth factor (EGF) domain present in these receptors [94], including the presence of an EGF-A domain, the primary domain by which PCSK9 interacts with the LDLR [14]. Other members of the LDLR family include LRP1 (LDLR-related protein 1), LRP1b, LRP2, and LRP4 [88].

Communications between adjacent cells are essential for efficient cellular functions and are critical to

cell growth and differentiation. Dysregulation or breakdown of these pathways is linked to various human disorders. Notably, LRP1, a vital player in the regulation of Notch signaling, interacts with PCSK9 [95, 96], suggesting a possible role of PCSK9 in cell-to-cell communication. The suggestion that PCSK9 regulates VLDLR, ApoER2, and LRP1 only adds to the scope of yet unknown PCSK9 biology [92, 93, 97]. An inverse correlation between PCSK9 and endotoxin clearance has been noted [98]. Interestingly, this was connected to the induced degradation of LDLR by PCSK9, resulting in negative pathogenic lipid uptake and clearance within the LDL particles [99]. Beyond the LDLR and its family members, there are indications of a possible role of PCSK9 in regulating high-density lipoprotein (HDL) [100]. This action of PCSK9 might be a product of a feedback mechanism, as it was also reported that HDL upregulates PCSK9 activities [101]. An association between PCSK9 and lipoprotein (a) [Lp(a)] has also been established [102, 103].

Furthermore, evidence of the role of PCSK9 beyond its regulation of LDLR family members is emerging. For example, mechanistic studies indicate that PCSK9 forms a protein-protein interaction with the tumor cell surface major histocompatibility protein class 1 (MHC1) protein, disrupting the ability of MHC1 to recycle to the cell surface and rerouting it to the lysosome for degradation [104], which ultimately reduces the intratumoral infiltration of cytotoxic T cells [105]. Interestingly, it was shown that attenuating the biological function of PCSK9 through either PCSK9 gene deletion or antibody inhibition prevents this protein-protein interaction and the growth of cancer cells in mice [104]. Almeida et al. [106] also pointed out this as a good strategy in cancer treatment. The role of PCSK9 in cancer might be multi-dimensional as it is also involved in T cell reprogramming [104, 107]. T cells function prominently in adaptive immune responses, such as against pathogens, allergens, and tumors [108]. Reprogramming of T cells by PCSK9 action would significantly affect their immune function [107]. It is tempting to speculate that the role of PCSK9 in cancer is not exhaustive yet, as it may also play a role in stimulating

inflammatory cytokines, which in turn could promote the growth of cancer cells [21]. It is worth noting that inflammation is initially the immune system's response to wounds and infections, with the ultimate target of resolving them. Many interplays occur between the pro-resolving molecules, such as immune cells and chemical compounds within the microenvironment, and due to these interplays, protracted inflammation might result [109]. This might partly explain why the progression of atherosclerosis is fueled by inflammation within the vascular wall [6, 7, 110]. Interestingly, atherosclerosis is a chronic inflammatory disease [111], and several studies have demonstrated the involvement of PCSK9 in every stage of atherosclerosis progression [8, 21-23].

1.2.1 Structure

Structurally, PCSK9 is nearly homologous to the other members of the PC family, but it appears to lack a regulatory P-domain [112]. Its domain structure includes a signal peptide (aa [amino acid] 1-30), a pro-domain (aa 31-152), a subtilisin-like catalytic domain (aa 153 – 452), and a C-terminal domain (aa 453 – 692) (**Figure 1a**) [112-114]. Like any other protein whose structural feature is critical to its biological functions, each domain of PCSK9 plays a central yet distinct role in its biochemical process. For example, the signal peptide acts as a guide while promoting the translocation of the zymogen into the ER lumen [115]. Changes in the amino acid sequence within this region have been shown to affect PCSK9 biology [116]. As in the other PCs, PCSK9's prodomain plays the role of a chaperone and promotes maturation through proper protein folding [31]. Of note, between Gln31 and Thr60 within the PCSK9 prodomain is a region reminiscent of a disordered state and which is unobserved in all x-ray crystal structures of PCSK9 (>30 in total) deposited in the Protein Data Base [113] (**Figure 1**). The prodomain also incorporates two α- helices and four antiparallel β sheets; while the former functions as a cover for the β-sheets, the latter helps extend the prodomain and the catalytic domain interface, possibly

through electrostatic and hydrophobic interactions [112-114]. Notably, the PCSK9 prodomain contains a so-called β0 strand (aa 61-75) that interacts with adjacent β-sheets through hydrogen bonds. This feature, unique to PCSK9 among the PC family, might have evolved to stabilize or otherwise regulate the prodomain/catalytic domain interface [113, 114]. The subtilisin-like catalytic domain encompasses a highly conserved catalytic triad – Asp186, His226, and Ser386 - which differs slightly from other PCs in terms of positioning [114]. The catalytic domain contains three disulfide bonds and two α helices, forming its noncovalent interaction interface with the prodomain with hydrogen bonds possibly acting as backbone [113, 114]. A C-terminal cysteine-histidine rich (CHR) domain comprises three tightly packed subdomain modules, designated as M1, M2, and M3, arranged in a pseudo-three-fold axis [113], with each subdomain forming a six-stranded β- propeller [113, 114]. Evidence suggests highly conserved cysteine residues but relatively little sequence identity across the three subdomains [114].

1.2.2 Processing and secretion

PCSK9 processing and secretion follows a similar pattern to other PCs: after the co-translational cleavage of its signal peptide, the nascent formed protein undergoes autocatalytic intramolecular processing in the ER at VFAQ↓SIP, a prerequisite for exiting the ER [13, 69]. Unlike other members of the PC family, in which the complete removal of the prodomain through a second but slower cleavage to attain full maturity and enzymatic activation, after the initial autocatalytic cleavage, PCSK9 is secreted into the secretory pathway/extracellular space with the structure of the full prodomain intact. Thus, the PCSK9 prodomain (~16 kDa) remains noncovalently attached to the larger (~60 kDa) Cat/C-term segment of the protein [13] (**Figure 1**), preventing substrate access to the active site. As such, the mature secreted form of PCSK9 is catalytically inert. Notably, furin-mediated proteolysis of PCSK9 has been shown to promote prodomain dissociation [19]; however, this would not result in PCSK9 enzymatic

activity as it removes the active site Asp186 residue.

PCSK9 exists in multiple forms in the extracellular milieu because it is the target of posttranslational modifications (PTMs), such as sulfation (Tyr 38), phosphorylation (Ser 47 and 688), proteolytic cleavage, and N-linked glycosylation. These biological modifications have been shown to impact PCSK9's function positively or negatively [10, 19, 20, 117-119].

1.2.3 Plasma PCSK9

As previously mentioned, PCSK9 in the extracellular milieu exists in multiple forms resulting from PTMs [10, 19, 117-119]. In human and mouse plasma, evidence indicates that there are two major forms of circulating PCSK9: intact and furin-cleaved (f-) PCSK9; the latter is a product of limited proteolysis, mainly by furin [18-20, 120, 121]. F-PCSK9 was initially characterized through its generation when PCSK9 was co-overexpressed in cells with furin, with the cleavage site localized to the sequence RFHR218$^{\downarrow}$QA within the catalytic domain [120]. The truncated form of PCSK9 was reduced by >80% in the serum of *Furin*$^{-/-}$ mice, confirming that furin is the major activity, with other PCs likely also contributing [20]. As this site lies within the primary LDLR binding interface, f-PCSK9 cannot bind to LDLR. Indeed, several GOF mutations in PCSK9 (R218S, F216L) have been identified in FH patients that alter the cleavage site, so these PCSK9 variants are likely more active in binding/degrading LDLRs due to decreased furin proteolysis [19]. A transiently transfected HEK293 with a recombinant PCSK9 lacking the N-terminal region of the catalytic domain excised by furin showed impaired secretion despite having a similar processing level to the intact PCSK9 [120], supporting that the segment cleaved off by furin is critical to PCSK9 secretion. Furin is a transmembrane protein that cycles between the plasma membrane and the trans-Golgi, so its enzymatic cleavage of PCSK9 could occur within the secretory pathway, extracellularly, or a combination of both.

Approximately 15-30% of circulating plasma PCSK9 in humans is in a truncated (cleaved) form

consistent with furin proteolysis [19]. Evidence suggests that LDLR is the primary conduit of plasma PCSK9 clearance [11, 81]; however, as noted above, f-PCSK9 does not bind to LDLR due mainly to the excised N-terminal portion of the catalytic domain [19]. Although the evidence of plasma f-PCSK9 is well established [18, 19, 120, 121], plasma clearance of this subspecies is still poorly understood.

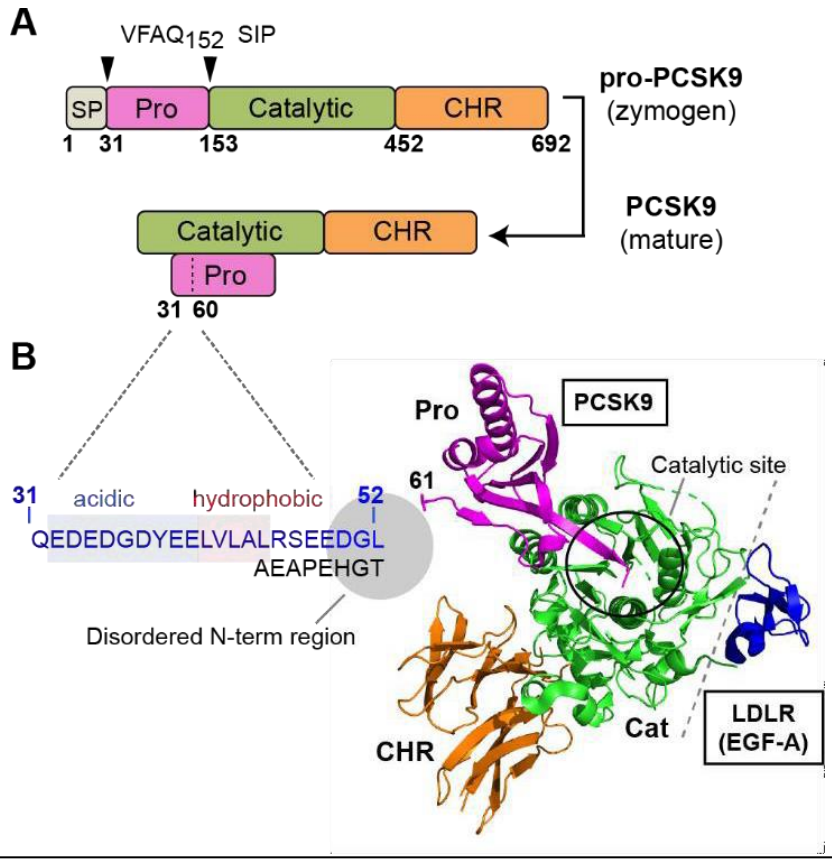

Figure 1: Overview of PCSK9 structure (A) pro-PCSK9 (zymogen form) with the signal peptide, pro-domain, catalytic domain, and C-term along with the processed mature PCSK9 (cleaved) with the prodomain non-covalently attached to the catalytic domain. The location of the self-cleavage site in the pro- PCSK9 is shown, and the amino acid sequence of the intrinsically disordered N-terminal region (IDR) in the processed mature. The acidic residues are highlighted in blue. (B) The crystal structure of PCSK9, along with the EGF-A domain of the LDLR and the point of interaction interface between LDLR and PCSK9. The circle position within the crystal structure represents the catalytic triad, and the gray area represents the IDR. Protein structure manipulated from the original PCSK9 crystal model by **Kwon et al., 2008.**

1.2.4 PCSK9-mediated LDLR degradative pathways

In accordance with its tightly bound prodomain, PCSK9's inherent protease activity is not required to mediate LDLR degradation in the endocytic pathway. Instead, secreted PCSK9 acts as a binding chaperone. Typically, upon endocytosis, the LDLR releases its bound ligand LDL within the acidic environment of early endosomes, and the receptor is sorted for recycling to the cell surface. However, PCSK9 becomes more tightly bound to the EGF-A domain of LDLR at acidic pH, and this persistent binding of PCSK9 reroutes the receptor to lysosomes, where both PCSK9 and LDLR are degraded (Figure 2). The LDLR EGF-A domain binding site in PCSK9 is a large and relatively flat binding interface within the catalytic domain spanning from the N-terminal Ser153 to Ser381 [122]. It is within this region that therapeutic PCSK9i-mAbs bind to PCSK9 and prevent its interaction with cell surface LDLR [123].

The nonproteolytic mechanism of action of PCSK9 is unique among PCs [14]; however, this is not unique within the serine protease family. For example, the hepatocyte growth factor/scatter factor (HGF/SF) also performs its function by binding its target protein rather than a proteolytic mechanism [124]. PCSK9's cleavage consensus motif (non-basic) is another unique aspect of this protease, and this might explain why PCSK9 may not cleave any substrate other than itself [32]. Interestingly, a recent preprint by Pandya et al. [125] suggested SARS-CoV2 (severe acute respiratory syndrome coronavirus 2) as a possible substrate of PCSK9; thus, it cannot be ruled out that PCSK9's inherent protease activity is activated under certain circumstances, even if not required for its role in LDLR degradation. The PCSK9 prodomain extends into the catalytic pocket, shielding further substrate from accessing this catalytic (active) site [113, 114] with no clear target loops for a secondary proteolysis event that could cause prodomain dissociation [113]. Presumably, the prodomain would need to dissociate for PCSK9 proteolytic function on SARS- CoV-2 or other substrates.

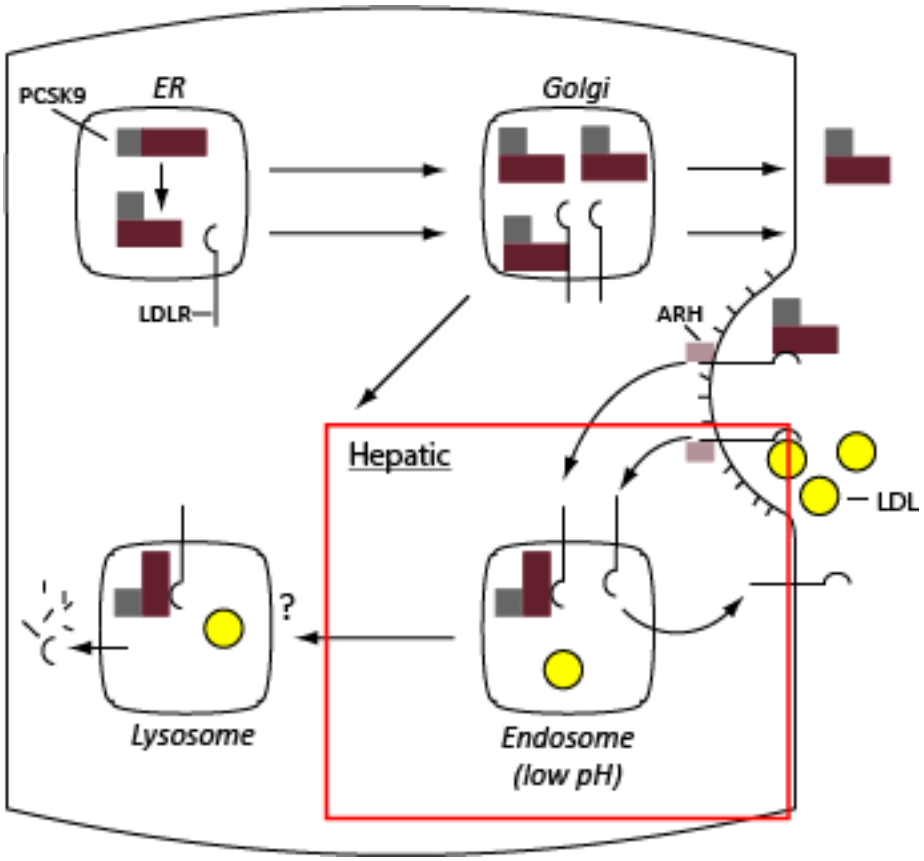

Figure 2: Schematics of PCSK9-LDLR degradation pathway. There are two PCSK9- mediated LDLR degradative pathways: i) intracellular: PCSK9 binds LDLR at the secretory compartment and chaperones the LDLR from the trans-Golgi to the lysosome for degradation, and ii) extracellular: secreted mature PCSK9 can also bind the cell surface LDLR, and upon internalization, PCSK9 bound LDLR within the acidic early endosome (depicted in red square) remain tightly associated, disrupting the LDLR recycling and rerouting both the receptor and PCSK9 to the lysosome for degradation.

1.3 PCSK9 and Hypercholesterolemia

Plasma atherogenic cholesterol concentration is primarily a product of the circulating cholesterol levels transported within apoB100-containing lipoproteins in the form of LDL and, to some extent, VLDL, IDL (intermediate-density lipoproteins), and Lp(a) [1, 8, 126-128]. This is determined by several factors within the context of the following classification – primary (genetic), secondary (intake of drugs or high saturated fat), or multifactorial (mostly hyperlipidemia) [1, 128, 129]. Of the three, the primary and secondary combined account for over 80% of hypercholesterolemia – a markedly elevated plasma atherogenic cholesterol concentration [129, 130]. FH is one of the most common genetic disorders, affecting ~1:250 people globally. FH is mainly inherited in an autosomal co-dominant form, with the majority of these cases (85-90%) due to inactivating mutations in *LDLR*. More than 2300 different FH-associated *LDLR* mutations have been identified to date. Less common are mutations in *APOB* (~5% of FH cases) encoding apoB100, a structural protein of the LDL particles and ligand of LDLR. Most rarely, FH can be caused by GOF mutations in *PCSK9* (<1%) [9, 131-136]. Evidence has also identified some sporadic variants causing FH, such as mutations in LDLR-adaptor protein (*LDLRAP*1), apolipoprotein E (*APOE*), lysosomal acid lipase (*LIPA*), signal-transducing adaptor protein family 1 (*STAP1*), and Patatin-like phospholipase-domain-containing family (*PNPLA5*) [131, 134].

1.3.1 PCSK9 in FH

Although GOF mutations in *PCSK9* account for <1% of FH cases, it is the second locus implicated in FH through the post-transcriptional regulation of the LDLR [9, 137]. Hepatic LDLR is primarily responsible for regulating and clearing LDL-C from the circulation by binding the apoB100 moiety on LDL and mediating the endocytic uptake and release of this lipoprotein in early sorting endosomes [1, 81]. Upon internalization, the LDLR releases itself from this complex within the acidic environment of the

endosomes and recycles to the cell surface to continue the uptake process [81]. Disruption of this LDLR function, either due to the mutation in *LDLR*, LDLR's inability to recycle to the cell surface, or mutation in *APOB* to disrupt the LDL-LDLR interaction, would result in FH. Circulating PCSK9 can also bind to LDLR at the cell surface through the LDLR EGF-A domain [11, 14]. Upon internalization, the PCSK9-LDLR complex is routed to the lysosome for degradation. As a result, LDLR recycling is interrupted in the presence of bound PCSK9, thus decreasing the protein level of LDLR on the cell surface [11, 14]. Thus, GOF PCSK9 mutations that increase LDLR degradation are causative for FH [11, 138-140]. Conversely, evidence indicates that loss-of-function (LOF) mutations in PCSK9 are associated with life-long reductions in plasma circulating cholesterol and are protective against CVD [141-143].

1.4 PCSK9 and Lipoproteins

Along with nucleic acid, proteins, and carbohydrates, lipids are the other main human organic molecules essential for biological function. Due to their hydrophobic nature, lipids require packaging and transportation within human circulation in lipoproteins, a concept that first originated from the realization that plasma lipids were bound to protein [144]. Structurally, lipoproteins are complex macromolecules with a central hydrophobic core of non-polar lipids, primarily triglycerides and cholesterol esters, surrounded by a hydrophilic membrane, mainly free cholesterol and phospholipids and specialized proteins called apolipoproteins which consist of a single polypeptide chain [145]. Evidence supports that apolipoprotein, relative to the lipid content, determines the functional role of these complexes, contributing to the complex stability, acting as a ligand for cell surface receptors, and as a cofactor for metabolism [146]. Factors such as metabolic, feeding, and hormonal states constantly influence the re-modulation of plasma lipoproteins in their lipid-to-protein ratios, shaping their eventual biological function, which also extends to the transport of bacterial endotoxin from invasion sites to the liver [145, 147, 148].

Interestingly, an association between lipoprotein synthesis and PCSK9 has been established [81, 149]. Based on size, density, and protein composition, lipoproteins are classified into seven classes: chylomicrons, chylomicron remnants, VLDL, IDL, LDL, HDL, and Lp (a) [145], and some suggestions indicate the level of interactions with PCSK9 varies from one class to another [81, 149].

1.4.1 Chylomicrons.

With about 99% of its constituent being lipid, chylomicrons are the largest lipoproteins in human circulation, with apoB48 as the primary protein component. However, they also contain apo A-I, A-II, and A-IV and acquire apo E and C from HDL after they have been secreted [146, 150]. Chylomicrons arise solely from the intestine and are primarily responsible for transporting dietary fat and fat-soluble vitamins in the circulation. Although evidence suggests that the primary determinants of chylomicron production/size are the fed/fasted state, the rate of dietary fat absorption, and the type and amount of fat absorbed, their production is also highly regulated through either paracrine or systemic factors [150-152]. Interestingly, the intestine also represents the second largest source of circulating PCSK9 [10, 12]. It was recently demonstrated that PCSK9 stimulates chylomicron production by upregulating apoB48 expression at the transcriptional and posttranscriptional levels and the lipid content of chylomicrons [153, 154]. Although apoB48 is less than 2% of chylomicron mass, it is critical for chylomicron assembly and metabolism [146, 150]. The suggestion that PCSK9 is involved in the mRNA and protein expression level of these significant components of chylomicron indicates yet another critical role of PCSK9 in cellular homeostasis [153]. It is worth noting that overproduction or poor clearance of chylomicrons, leading to increased residence time within the circulation, is implicated in postprandial hypertriglyceridemia, an independent risk factor for atherosclerosis [155]. Furthermore, reducing the production of chylomicrons could represent a potential target for reducing the initiation or progression

of atherosclerosis [152-154].

1.4.2 Chylomicron remnants

As previously stated, chylomicrons are synthesized in the intestine [150] and enter the circulation as carriers of dietary triglycerides, which are hydrolyzed by lipoprotein lipase (LPL) into monoglyceride and free fatty acid (FFA) that are then absorbed by the extrahepatic tissues or cells for immediate use or storage [156-158]. The removal of triglyceride by the various cells or tissues through the actions of LPL results in chylomicron remnants, which are enriched in fat-soluble vitamins and acquire the exchangeable apolipoprotein apoE [145, 156]. It has been demonstrated that the inactivation of LPL or its cofactors results in the build-up of chylomicrons and triglycerides in circulation [156-158]. In healthy humans, chylomicron remnants are rapidly removed from the circulation by the liver through the apoE ligand via LDLR and LRP1 [145, 156, 159]. Interestingly, since PCSK9 induces LDLR degradation [11, 92], it could increase the residence time of the chylomicron remnants within the circulation.

1.4.3 Very Low-density lipoprotein (VLDL)

Within human circulation are two established lipoprotein pathways: exogenous and endogenous. While exogenous substances originate in the intestine with dietary lipids, endogenous substances begin in the liver by packaging and secretion of triglyceride-rich VLDL particles [145]. With apoB100 as its core structural protein [160], VLDL also contains apo C-I, II, III, and E [157, 161]. Like intestine-derived chylomicrons, liver-derived VLDL transports lipids to extrahepatic tissues for immediate use or storage after hydrolysis by LPL [157]. Of note, the non-exchangeable apolipoprotein in VLDL is apoB100, indicating its critical structural role in the packaging/secretion of VLDL [162], partly explaining why *APOB* is the second locus implicated in FH [139]. ApoB100 secretion from the liver is highly regulated

at the posttranslational level, with poorly lipidated protein being degraded [163]. Interestingly, PCSK9 is suggested to be involved in this regulation by inhibiting intracellular apoB100 degradation and enhancing its secretion [139, 160].

1.4.4 Intermediate-density lipoprotein (IDL)

Also known as VLDL remnant, IDL complexes are enriched in cholesterol primarily because of the removal of triglycerides, the core lipid from the VLDL hydrolyzed by LPL for cell and tissue utilization [145]. As it is produced from a VLDL precursor, IDL contains apoB100 as its core structural protein and acquires the exchangeable LDLR and LRP1 ligand apoE [145, 162]. Although IDLs are proatherogenic due to the enriched cholesterol content, they are short-lived as they are rapidly endocytosed through receptor-mediated interactions or metabolized further into LDL [145].

1.4.5 Low-density lipoprotein

With about 75% lipid and 25% protein, primarily apoB100, LDL particles are the primary cholesterol carrier in human circulation [164]. Among the different circulating lipoproteins, LDLs are the only known particle to undergo structural transition close to body temperature, although the physiological relevance remains unclear [165]. Structurally, LDL is a highly heterogeneous particle with two major compartments: the neutral lipid core, which consists mainly of cholesterol esters and, to a lesser extent, triglycerides, and an outer amphipathic monolayer that contains free cholesterol and phospholipid, with an embedded single copy of apoB100 around the roughly spherical structure [165, 166]. Of the 75% lipid, ~30% are outer membrane phospholipids, primarily phosphatidylcholine (PC) (65%) and sphingomyelin (SM) (25%) [167]. Furthermore, LDL particles can be subdivided into large buoyant, intermediate, and small dense sub-species [168] that are distinct in their molecular characteristics such

as receptor affinity, atherogenic behavior, and susceptibility to modification [165].

1.4.6 LDL-PCSK9 binding

Current evidence supports that PCSK9 can form a protein-protein interaction with apoB100 of LDL particles [169-171]. Although a previous study had suggested that PCSK9 in human serum is likely not associated with any specific lipoprotein [172], further evidence indicated that harsh conditions for lipoprotein isolation, such as high-salt ultracentrifugation were the likely reason as this method results in the dissociation of PCSK9 from LDL [171]. Detailed studies using density gradient separation from our group and others have shown that PCSK9 was indeed contained in the LDL fraction isolated from human plasma [169, 171]. Through mechanistic studies, our group demonstrated that PCSK9 binds to LDL in a specific and saturable manner with a measured affinity (KD) of ~350nM, which is similar to that of the LDLR-PCSK9 binding interaction [169]. We have also determined using mapping studies that the LDL-PCSK9 binding interaction requires the IDR in the N-terminus of the PCSK9 prodomain [169, 173]. This disordered region of PCSK9 is enriched in acidic residues, and we and others have previously identified it as an allosteric autoinhibitory domain to the PCSK9-LDLR binding interaction [169, 174]. As previously stated, LDL particles can be subdivided into large buoyant, intermediate, and small dense sub-species [168] with distinct molecular characteristics, such as receptor affinity, atherogenic behavior, and susceptibility to modification [165]. As PCSK9 forms a protein-protein interaction with apoB100 of LDL particles [182-184], it is logical to assume that the various subspecies of LDL or oxidative modifications might affect PCSK9 binding. It is also unclear how post-translational modifications of PCSK9 affect its binding interaction with LDL. Tavori et al. [175] provided evidence that f-PCSK9 is not associated with apoB100, and LDL:PCSK9 interaction might protect against furin-induced proteolysis of PCSK9. The former may be explained by the finding that the prodomain of PCSK9 readily

dissociates from f-PCSK9 and that the prodomain has been shown to be critical to PCSK9-LDL association [169, 173].

1.4.7 High-density lipoprotein

HDL particles are heterogeneous groups of particles ranging between 8-10 nm in mean size and density of 1.063 – 1.21 g/ml that have cholesteryl ester and triglycerides as their core lipid surrounded by an amphipathic layer of free cholesterol, phospholipids, and apolipoproteins [176- 178]. Unlike LDL, which is derived from the catabolism of circulating VLDL in humans through the action of LPL, HDL synthesis occurs both in the intestine and the liver, and evidence suggests that the origin determines the apolipoproteins it carries. While liver-derived HDL contains A-type and C-type apolipoproteins, intestine-derived HDL contains only A-type [179, 180]. In humans, regardless of origin, HDLs are natural delivery carriers of lipids, proteins, vitamins, hormones, and even micro-RNAs to distal organs, and evidence indicates that about 87 different proteins are contained within each HDL particle [177]. This large number of proteins explains, in part, why HDL is the most protein-enriched lipoprotein of all the circulating lipoproteins and perhaps also antiatherogenic, anti-inflammatory, and immune-induced response effects that have been attributed to HDL. The role of HDL in reverse cholesterol transport is the most well-characterized aspect of its biological functions, in which excess cholesterol from peripheral tissues is exported to HDL or pre-HDL acceptors (mainly lipid-poor apoA-I) and transported through the plasma compartment back to the liver for eventual intestinal excretion [176-178, 180].

Based on a co-immunoprecipitation analysis, Burnap et al. [181] had suggested that HDL, not LDL, is the primary lipoprotein carrier of PCSK9 in human circulation. Furthermore, PCSK9-induced LDLR degradation and other PCSK9 functionalities were suggested to be potentiated by its association with apoA-1 of HDL [101]. However, more recently, Dafnis et al. [100] showed that PCSK9 is only minimally

associated with HDL in human plasma (~6% of total PCSK9) but that PCSK9 may indirectly attenuate the anti-atherogenic properties of HDL in endothelial cell activation.

1.4.8 Lipoprotein (a) [Lp(a)]

First described in 1963 as having LDL-like properties, Lp(a) is a unique lipoprotein only found in humans, old-world monkeys, and hedgehogs [145, 167, 182, 183]. Structurally, Lp(a) is a variant of LDL with an additional protein called apolipoprotein (a) [apo(a)] covalently bound to the apoB100 through a disulfide bridge [167, 182, 184]. Of note, apo(a) has been likened to plasminogen, and there are indications that it may have evolved either through duplication, deletion, gene conversion, and even point mutations [184], fueling speculation that Lp(a) might have a role in wound healing and tissue repair [182, 184]. Elevated plasma Lp(a) levels are an independent risk factor of AS progression, and it has been demonstrated that Lp(a) binds and transports oxidized phospholipid (oxPL) in human circulation, which has been shown to induce an immune response, initiate proinflammatory signaling, and contribute to plaque vulnerability [185].

PCSK9i-mAb therapies consistently lower Lp(a) levels by 20-30% in clinical trials [186], an effect that is not altered due to the lack of LDLRs in homozygous FH [187]. Kinetic analysis in humans shows that PCSK9i-mAb therapy mainly decreases Lp(a) production with a lower effect on Lp(a) catabolism [188]. Furthermore, evidence suggests PCSK9 interacts with Lp(a) by modulating its secretion and not by modulating its cellular uptake via LDLR, which binds poorly to Lp(a) [183, 189]. Even though more than 30% of circulating PCSK9 are LDL-bound [169, 171], PCSK9 was found to be preferentially associated with Lp(a) compared to LDL particles in subjects with high Lp(a) levels; thus, the mechanism of PCSK9 binding may be identical between these closely-related lipoprotein particles [190]. This perhaps gives clarity to prior studies suggesting a positive correlation between plasma PCSK9 and Lp(a)

concentration in type 2 diabetic mellitus (T_2DM), as Lp(a) and PCSK9 are shown to be elevated in these populations [191, 192].

1.5 PCSK9 and vascular inflammation

Continuous accumulation of modified LDL within the arterial walls due to persistently elevated plasma cholesterol is an initiating event in atherosclerosis development and progression [8]; however, chronic vascular inflammation is also considered an independent critical factor [6, 7]. Vascular inflammation is a response triggered by damage to the vasculature, accompanied by infiltration, modification, and retention of LDL within the arterial walls and accelerated by elevated plasma LDL-C [8]. The role of PCSK9 in this process in the context of elevated plasma cholesterol is well-established. Independent of the LDLR, PCSK9 has also been implicated in the TLR4 (toll-like receptor 4)/NF-KB (nuclear factor kappa-light B cells) proinflammation pathway [193]. Interestingly, TLR4 is involved in both local and systemic immune responses, and PCSK9 overexpression has been shown to upregulate TLR4 [193], although the underlying mechanism remains unknown. One aspect of the immune response is the secretion of pro-resolving molecules such as proinflammatory cytokines [109]. It is tempting to speculate that TLR4 upregulation by PCSK9 may enhance the secretion of proinflammatory cytokines as part of the immune response. Local PCSK9 expression was reportedly increased in atherosclerotic lesions [194], which could further fuel the release of more proinflammatory cytokines.

Although the liver is the major source of circulating PCSK9, it is also expressed in various cell types within atherosclerotic lesions, including macrophages (albeit at a lower level), vascular smooth muscle cells, and endothelial cells, and thus could achieve high local concentrations and interact with cellular processes within the artery wall [23, 195]. Interestingly, endothelial cells are critical in regulating vascular tone and maintaining vascular homeostasis, such as smooth muscle proliferation and

inflammation [196]. While the role of PCSK9 in endothelial dysfunction might be initially related to hypercholesterolemia associated with LDLR degradation driven mainly by liver-derived circulating PCSK9 since inflammation has been shown to upregulate both systemic and local PCSK9 expression [87, 193], this could potentially exacerbate chronic inflammation via LDLR-independent mechanisms. LDL particles are prone to oxidation in the circulation due to the imbalance between reactive oxygen species production (ROS) and radical scavenger production (antioxidant defense system) [197], leading to the production of ox(oxidized)-LDL. A significant source of oxLDL is from the injured arterial walls through inflammatory pathways involving ROS generation [198]. Interestingly, PCSK9 is suggested to amplify ROS in vascular cells [199, 200]. Similarly, oxLDL upregulates ROS production, which further mediates oxLDL production in a positive feedback loop [201-204]. Although speculative, PCSK9 and oxLDL could provide crosstalk in proinflammatory processes. OxLDL belongs to native molecules known as "danger-associated molecular patterns" (DAMPs) that are induced by inflammation and then bind to signaling scavenger receptors and amplify the immune response to inflammation, tissue injury, and oxidative stress [205, 206]. As such, the uptake and generation of oxLDL in the endothelium further exacerbates chronic inflammation and drives atherosclerosis progression.

Elevated plasma PCSK9 and oxLDL are also implicated in platelet activation [207, 208]. While oxLDL-induced platelet activation is suggested to be CD36 mediated [207], elevated plasma PCSK9 was linearly correlated with 11-dehydro-thromboxane B2, a molecule released in response to activated platelets [208], suggesting a possible role of PCSK9 in platelet activation [25]. Upon recruitment to the vascular wall, activated platelets promote vascular inflammation through the secretion of various chemokines [209-211].

Furthermore, as previously stated, inflammation upregulates the expression of systemic and local PCSK9 [193, 194, 212]. Interestingly, furin is upregulated in an inflammatory state; reciprocally, its

bioactivities also upregulate factors that further enhance vascular inflammation, suggesting a feedforward mechanism [51, 52, 213]. Due to the enhanced activity of furin during local or systemic-induced inflammation, it logically follows that the f-PCSK9 protein level would also be upregulated; however, the functional consequence of this is unknown. Although furin proteolysis inactivates the LDLR-binding function of PCSK9 [20], it remains unexplored whether f-PCSK9 is also inert in terms of other PCSK9 interactions.

1.5.1 Scavenger receptors

First described and with the name coined primarily because of their inability to bind n(native)-LDL [214], scavenger receptors (SRs) belong to a large superfamily of cell-surface receptor proteins that are functionally and structurally diverse [215-217]. This family shares no structural resemblance; however, reports suggest they recognize unmodified proteins, modified molecules such as DAMPs, and several microbial structures such as PAMPs (pathogen-associated molecular pattern), LPS (lipopolysaccharide), LTA (lipoteichoic acid), etc. [214], an indication suggesting mutual ligands among this family. Recognition of these various structures, in turn, elicits an innate immune response, which explains why SRs are also called pattern recognition receptors (PRR) [214-217]. Based on their domain structure, they are grouped into ten classes (A-J), and members within a class share sequence similarity and function. However, they generally function through phagocytosis, endocytosis, adhesion, and signaling mechanisms [216]. Although SRs are expressed mainly by myeloid cells, recent work has demonstrated crosstalk between SRs and PCSK9 [204, 217].

1.5.2 Cluster of Differentiation 36 (CD36)

CD36 belongs to a superfamily known as the scavenger receptor class B (SR B), which consists of three

principal members: the SR-B1, SR-B2, and CD36 (SR-B3) [218-220]. CD36 is a cell surface multiligand receptor expressed in many cells and tissues, such as hepatocytes, macrophages, intestine, adipose tissue, skeletal muscle, monocytes, platelets, and heart [218-221]. This may explain the involvement of CD36 in roles such as mediating fatty acid transport and metabolism, facilitating oxLDL uptake into macrophages, cholesterol efflux, and the selective delivery of lipoprotein cholesteryl esters [218, 219]. Therefore, this receptor is a critical player in lipid homeostasis. Emerging evidence suggests an interaction with caveolin [220], possibly aiding in cellular fatty acid uptake as CD36 recycles between the cell surface and the endosome [220]. Not only does CD36 bind and internalize oxLDL in macrophages, contributing to foam cell formation because of cholesterol and other lipid accumulation and exacerbating chronic vascular inflammation, CD36 is also a receptor for unmodified phosphatidyl serine (PS), and the modified PS (oxPS) expressed on apoptotic cell surfaces [222]. CD36 also exacerbates chronic vascular inflammation through other roles, such as in platelet activation [148].

It has been suggested that CD36 forms a protein-protein interaction with PCSK9 at the cell surface, and upon endocytosis, this results in the lysosomal degradation of the CD36, ultimately affecting the cellular uptake of fatty acid [91, 218]. Of note, PCSK9 also induced intracellular CD36 degradation within the secretory pathway [218]. Interestingly, PCSK9 and CD36 are both expressed on macrophages (albeit lower PCSK9 expression) [23, 195, 218-221], suggesting a possible interaction of these two proteins on macrophages. Although this may induce CD36 degradation, the interaction between CD36 and PCSK9 might also lead to downstream signaling [223].

1.5.3 Lectin-Like Oxidized low-density lipoprotein (LOX1)

CD36 is not the only SR interacting with PCSK9; it has also been suggested to interact with LOX1[204]. LOX1 is a class E scavenger receptor (SR E), one of the seven-member families of the Dectin-1 cluster,

belonging to the C-type lectin receptors superfamily [224-226]. LOX1 is also known as oxidized LDL receptor 1 (OLR1) because it is the primary receptor for oxLDL on macrophages, smooth muscle cells, and endothelial cells, among others, although SR class A and CD36 are also receptors for oxLDL on these cells. However, LOX1 can recognize minimally oxidized LDL (mmLDL), while CD36 recognizes only moderate to fully oxidized LDL [226-229]. LOX1 binds oxLDL with high affinity and, noticeably, can also bind a broad spectrum of structurally distinct ligands, such as bacterial products, C-reactive proteins, and advanced glycation end-products (AGEs) with similar affinity [224]. Interestingly, these ligands also upregulate LOX1 protein and gene expression levels and are involved in upregulating LOX1- mediated signaling pathways directly or indirectly [204, 229-233]. This explains why LOX1 expression is generally low across the different cells where it is expressed, and binding to these various ligands would rapidly upregulate its expression level for further binding interactions, resulting in a feed-forward mechanism [227].

Furthermore, LOX1 is implicated in the initiation and progression of atherosclerosis. However, this role is considered multifactorial, mainly through involvement in endothelial dysfunction, monocyte recruitment, platelet activation and aggregation, foam cell formation, and interaction with PCSK9 [201-204, 234, 235]. Indeed, crosstalk between LOX1 and PCSK9 has been reported, leading to enhanced pro-inflammatory signaling and positive feedback on expression levels of both proteins in an inflammatory state [204, 229].

1.6 Regulation of circulating PCSK9

Increased systemic or local expression of PCSK9 in response to different stimuli is suggested to be involved in lipid homeostasis, cardiovascular complications, induced immune response, cellular functions, and signaling pathways [9, 11, 75, 91, 98, 104, 235]. These stimuli are known to be tightly regulated at the transcription and posttranscriptional levels [236].

1.6.1 Transcriptional

PCSK9 is mainly regulated by sterol regulatory element-binding protein-2 (SREBP-2), a membrane-bound transcription factor that regulates multiple genes, including *LDLR*, involved in cholesterol biosynthesis and uptake [15, 81, 237, 238]. Cholesterol, a 27-carbon polycyclic molecule, plays a critical function in the maintenance of the integrity and fluidity of the cell membranes; it is the precursor for steroid hormones, vitamin D, bile acids, and in the formation of caveolae, and the covalent modification of embryonic signaling proteins [72, 196-198]. Due to cholesterol's critical role in cellular function and because it is not an energy source and cannot be broken down by any known enzyme from its lipid core, its biosynthesis and uptake are tightly regulated to maintain homeostatic balance [72, 239]. SREBP isoforms are members of the basic- helix-loop-helix-leucine zipper family of transcription factors that function through the binding to the sterol regulatory elements (SRE) in their promoter regions [72, 238, 240, 241]. While SREBP-2 is mainly involved in cholesterol homeostasis, SREBP-1c is involved in regulating fatty acid, triacylglycerol, and phospholipid, thus working together with SREBP-2 to maintain overall membrane lipid balance.

Following its initial synthesis as an inactive ER-bound protein, SREBP-2 is activated through limited proteolysis [235]. Within the ER, and in response to elevated cellular cholesterol, SREBP- 2 is in complex with SREBP cleavage-activating protein (SCAP) and insulin-induced gene protein (Insig). Upon depletion of cellular cholesterol, Insig detaches from the SREBP:SCAP complex, allowing for its incorporation into transport vesicles and transport to the Golgi where proteolytic cleavage of the precursor membrane-bound SREBP2 occurs, releasing a soluble active transcription factor form that then translocates to the nucleus where it binds to SRE elements and activates its target genes, including *LDLR* and *PCSK9* [81, 238, 240, 242]. This creates a paradoxical situation as PCSK9-mediated LDLR degradation would

counter the cellular need to increase cholesterol levels. Thus, it is likely that PCSK9 activity is also subject to post-translational regulation; however, these mechanisms remain poorly understood. Besides SREBP-2, the PCSK9 promoter contains a functional binding site for hepatocyte nuclear factor-1α (HNF-1α), a liver-enriched transcription factor [81, 105]. As previously mentioned, [10, 12] besides the liver, PCSK9 is also secreted in the intestine, the brain, and the kidney. Interestingly, HNF-1α has also been shown to have some role in these tissues [243]. A synergy between HNF-1α and SREBP-2 in upregulating PCSK9 has also been suggested [105]. There are suggestions that PCSK9 expression could be upregulated under the feeding state by transcription factor E2F2 through binding to the PCSK9 promoter region, and SP1, which is located upstream of SRE, is also suggested to influence PCSK9 expression but independent of the cellular cholesterol level [105].

Furthermore, age and sex have also been suggested to have a role in PCSK9 expression level [244]. The sex difference could be associated with hormonal differences, as it was indicated that the PCSK9 expression level was higher in females [245]. Growth hormones have also been implicated in the post-transcriptional regulation of PCSK9, as well as fasting and refed states [246]. In addition, there are indications that exercise may influence PCSK9 expression levels [247] along with natural plant extracts such as berberine that act through HNF-1α-mediated transcriptional effects [248]. Thus, while PCSK9 and LDLR expressions are partially co-regulated via SREBP-2, there are multiple alternate transcriptional regulators of PCSK9 expression that could alter this balance in certain settings.

1.6.2 Non-transcriptional

Post transcriptionally, miRNA is suggested to have a role in PCSK9 regulation by its target of 3’ UTR of the PCSK9 mRNA [105]. Although evidence remains limited, there are indications that PCSK9 expression is regulated by blood flow rate through the TLR4-MyDD-NF-kB signaling pathway [105]. As

mentioned, PCSK9 can also bind to plasma lipoproteins. Regulation of its LDLR-degrading ability via these interactions could allow PCSK9 activity to be responsive to aspects of intravascular lipoprotein metabolism, such as lipolysis and lipoprotein remodeling during the fasting/feeding cycle.

1.6.3 LDL as a regulator of PCSK9

Current evidence supports that PCSK9 binds to apoB100 of LDL particles [169, 171], with >30% of circulating PCSK9 in normolipidemic humans bound to this LDL protein moiety [169]. A recent mechanistic study in our group supports that LDL binding stabilizes an allosteric interdomain interaction in PCSK9 that inhibits its ability to bind and interact with the LDLR [173]. This study is consistent with previous findings in our lab that succinctly revealed in a cell-based assay that in the presence of a physiological concentration of LDL, PCSK9 displays a significantly decreased ability to degrade the LDLR [169]. We have also identified numerous GOF *PCSK9* mutations associated with FH that abolished the LDL binding interaction *in vitro* [249], further supporting an inhibitory role of LDL binding in PCSK9 regulation. Although unexplored, the LDL-PCSK9 interaction could also regulate PCSK9's role in systemic inflammation, as plasma lipoproteins are critical in innate immunity, such as in the absorption and clearance of cytotoxic bacterial lipids [250-252].

1.7 Objectives and Hypothesis

As mentioned [21-25], knowledge of PCSK9 biology has grown beyond its established role in regulating LDLR protein levels; several bodies of evidence suggest a direct role of PCSK9 in atherosclerosis, inflammation, and other metabolic dysfunction. Of importance, the role of the f-PCSK9 subspecies in an LDLR-independent proinflammatory role is still unclear. Although the therapeutic monoclonal antibody that binds and blocks PCSK9:LDLR binding interaction [27] also shows other beneficial effects, such

as arrest of inflammation progression [28], the underlying mechanism(s) remain unknown. There is also concern about the cost of this therapy [26]; thus, improved knowledge could lead to better and more cost-effective therapies targeting specific aspects of PCSK9 function. The f-PCSK9 physiological function is still unclear, other than the noticeable effect that it is inert in LDLR degradation due to the removal of a portion of the PCSK9 catalytic domain involved in LDLR binding [19]. Over the years, our group has focused on the structure-function aspect of PCSK9 biology to understand the role of subtle structural changes in PCSK9 bioactivities. This thesis examines alternate conformations of PCSK9 to further enhance our understanding of how these slight structural changes affect PCSK9's physiological roles through the following:

1- The impact of alternate conformation of PCSK9 prodomain and how it affects PCSK9 biological function. We hypothesize that critical residues in the prodomain propagate structural changes in the catalytic domain, resulting in a lower LDLR binding affinity. Furthermore, the LDL-PCSK9 association would 'lock in' an auto-inhibited conformation

2- Further examine the functional effect of removing the N-terminal region of the PCSK9 catalytic domain by furin, with a focus on how this removal affects PCSK9 bioactivity in the context of the PCSK9-LDL interaction.

3- Our final objective was to investigate the role of furin proteolytic processing on PCSK9 bioactivities in the context of PCSK9's role in inflammation. We hypothesize that furin proteolysis improves PCSK9-lipoprotein interactions, positively impacting atherosclerosis progression by mediating cell surface interaction of proinflammatory oxLDL.

Chapter 2: Key Residues within the PCSK9 Intrinsically Disordered Region are involved in PCSK9 binding to LDL and LDLR.

2.1 Introduction:

Secreted mature PCSK9 consists of two non-covalently attached segments: the N-terminal prodomain (~14 kDa), which is released by autocatalytic processing in the ER, and a larger segment (~60 kDa) consisting of the catalytic and C-terminal domains (**Figure 3B**). In addition to blocking substrate access to the protease active site, the non-covalently attached prodomain also acts as a chaperone in the maturation/folding of the protein [31, 112]. A large (>500 Å) surface region of the catalytic domain (aa 153-381) contains the primary binding interface to the epidermal growth factor-like repeat A (EGF-A) domain of LDLR [14] (**Figure 1B).** Although completely removed from the binding interface of PCSK9 and the EGF-A domain of the LDLR, it has been shown that deletion of amino acids **Gln31 – Leu52** within an N-terminal intrinsically disordered region (IDR) in the prodomain (herein referred to as Del53-PCSK9) **(Figure 3)** enhanced PCSK9's binding affinity to LDLR >7-fold, possibly through relieving a negative allosteric mechanism in autoinhibition [122]. The N-terminal IDR is enriched in negatively charged amino acids (Asp and Glu) and is highly conserved across vertebrate species [174] **(Figure 3C).** Kosenko et al. [169] reported ~30 -40% of human plasma PCSK9 is LDL bound. Interestingly, this same study from the Lagace lab found that the Del53-PCSK9 variant was unable to bind LDL, indicating an essential role of the N-terminal IDR in the PCSK9-LDL interaction. It has been speculated that the negative allosteric effect of the N-terminal 21 amino acids missing in Del53-PCSK9 is due to an intramolecular electrostatic interaction between the negatively charged residues of this segment with positively charged residues in the catalytic domain [174]. However, due to its location and inherent flexibility **(Figure 3A)**, the N-terminal prodomain IDR in PCSK9 could potentially interact with multiple intramolecular sites in any of the three domains of mature PCSK9 [253].

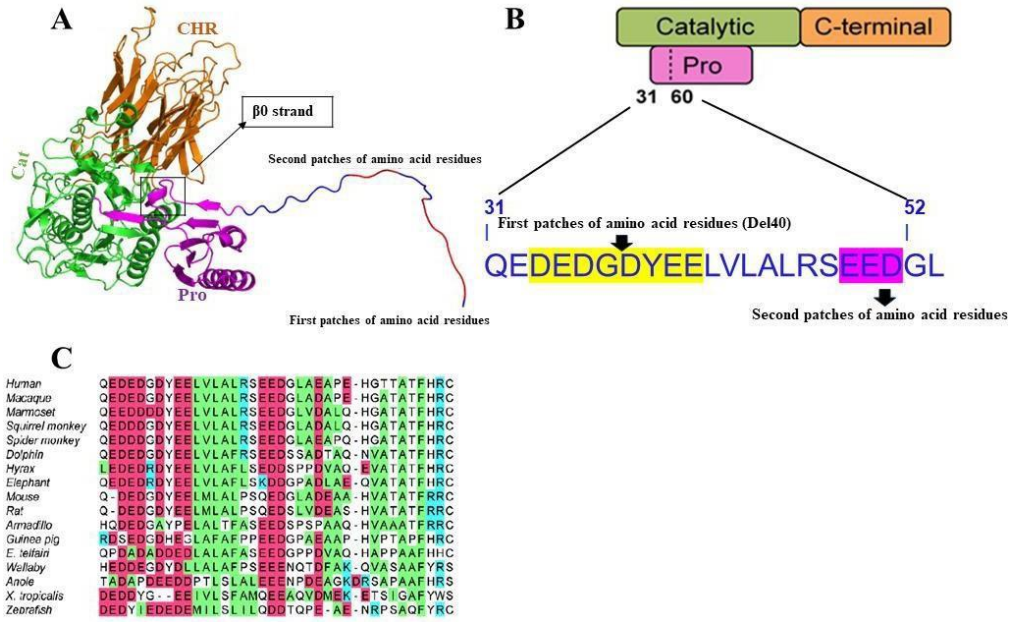

Figure 3: Overview of PCSK9 IDR and the conserved residues (A) The structure of PCSK9 modeled using AlphaFold, showing the three domains and including the PCSK9 IDR, highlighting the first and second patches of amino acids within this segment and the so-called β0 strand. (B) Schematic of the secreted mature PCSK9 (intact) with the prodomain non-covalently attached to the catalytic domain. The amino acid sequence of the disordered N-terminal region (IDR), with particular emphasis on the 21 amino acids (Del53) within, with the first shaded in yellow (Del40) and the second patches of the charged residues shaded in pink. (C) Overview of PCSK9 IDR across species with the highly acidic residues highlighted in red **(Holla Ø, L., et al., 2011).**

As seen in the PCSK9 IDR sequence (**Figures 3A and B**), two patches of negatively charged residues are separated by several hydrophobic residues. Deletion of either of the acidic patches or substitution of individual amino acids to uncharged residues was previously shown to have a lesser effect on autoinhibition of LDLR binding/degradative function than the complete deletion (*i.e.*, Del53-PCSK9), with the conclusion that length and overall negative charge of the IDR are the critical factors in its allosteric role rather than primary amino acid sequence [174]. In support of this notion, work from the Seidah group showed that the N-terminal IDR in PCSK9 could be swapped with an acidic IDR of similar length from

another protein (GPIHBP1: glycosylphosphatidylinositol-anchored high-density lipoprotein–binding protein 1) without loss of the autoinhibitory effect on LDLR binding [254]. By contrast, in terms of LDL binding function, recent work in the Lagace lab showed that deletion of only the first stretch of acidic amino acid residues, encompassing **Asp33 to Glu40** (herein referred to as Del40-PCSK9), completely abolished the ability of PCSK9 to bind to LDL particles *in vitro* [173] as was also seen in previous studies of the larger deletion in Del53-PCSK9 [169].

The Del40-PCSK9 variant with the shorter deletion retains the second acidic stretch encompassing **Glu48 to Asp50,** which could be brought into proximity to the arginine residues in the prodomain and catalytic domain interface to exert an autoinhibitory effect on LDLR binding affinity. Interestingly, this stretch is preceded by Ser47, which can be phosphorylated [255], thus adding to the negative charge. A nearby potential interactor of this second acidic sequence is the so-called β0 strand, a structural feature in the PCSK9 prodomain unique among the PCs, and which has been suggested to account for the firm retention of the PCSK9 prodomain to the rest of the protein [113, 114].

Collectively, the current literature supports that the two acidic sequences in the prodomain IDR both contribute to the complete autoinhibitory effect [174]. This is also supported by preliminary studies in the Lagace lab in which the Del40-PCSK9 variant was found to display only marginally increased LDLR binding compared to WT-PCSK9 (S. Sarkar, unpublished), suggesting that the key LDLR binding effector lies upstream of this shorter deletion. This possibility is supported by the positioning of Glu48 to Asp50 near the β0 strand of the prodomain, which structural evidence suggests stabilizes the prodomain-catalytic domain interface. **Therefore, we hypothesize that critical residues in the prodomain IDR regulate the stability of the prodomain-catalytic domain interface and are involved in autoinhibition of PCSK9 by propagating structural changes that negatively affect the primary binding**

interface with the LDLR EGF-A domain in the catalytic domain. Furthermore, the LDL-PCSK9 association would 'lock in' this auto-inhibited confirmation, thus explaining how LDL binding inhibits PCSK9. Mutating specific key residues would change PCSK9's ability to regulate the LDLR and reinforce our earlier working hypothesis that not all the residues within Gln31 – Leu52 in PCSK9 IDR play an equal role in PCSK9 ability to regulate the LDLR protein level.

2.2 Results:

To further probe the autoinhibitory effect of the prodomain IDR on PCSK9-LDLR binding affinity, we first focused our attention on individual residues in the second acidic stretch (**Glu48- Glu-Asp**) to understand how much of a contribution they each offer. This was done by mutating each of these acidic residues individually to either neutral Ala or positive-charged Arg. We also included in our study Glu48Lys, a natural GOF *PCSK9* [249] mutation associated with FH, further suggesting the importance of the acidic Glu48 to LDLR binding. We transiently expressed these mutant PCSK9 constructs in HEK293 cells and collected and concentrated the conditioned medium containing secreted PCSK9 to assess cell uptake dependent on the cell surface LDLR interaction. In these experiments, an LDLR-encoding plasmid was first transiently expressed in HEK293 cells, followed by 3 h incubation with a medium containing WT or mutant PCSK9 in the presence of chloroquine to prevent lysosomal degradation of internalized PCSK9. Our analysis showed that most of these amino acid substitutions did not affect LDLR-dependent PCSK9 uptake compared to WT-PCSK9 **(Figure 4)**, with the exception of a significant ~75% increased uptake with Glu48Lys, a GOF PCSK9 mutation [249] along with a trend towards increased uptake for the Glu48Arg. This finding is consistent with previous studies finding that GOF *PCSK9* mutations can, in some cases, increase PCSK9's ability to bind and interact with the LDLR [11, 138, 140]. The results in **Figure 4** suggest that the ability of individual acidic residues to regulate the

allosteric inhibition of PCSK9-LDLR binding is limited, consistent with earlier work [174].

We next tested the cumulative effect of altering all 3 of the acidic residues along with Ser47, which, as mentioned above, can be phosphorylated to add to the negative charge of this region. Rather than deleting residues **47-50**, we sought to preserve the relative spacing by replacement of **Ser47- Asp50** with a disordered **Gly-Ser-Gly-Gly** linker (herein referred to as GSGG-PCSK9). There was no significant difference between GSGG-PCSK9 and Del53-PCSK9 in terms of increased LDLR-dependent PCSK9 cellular uptake compared to WT-PCSK9 **(Figure 5)**, as the disordered linker insertion may have disrupted the allosteric autoinhibition that was observed in the PCSK9 prodomain.

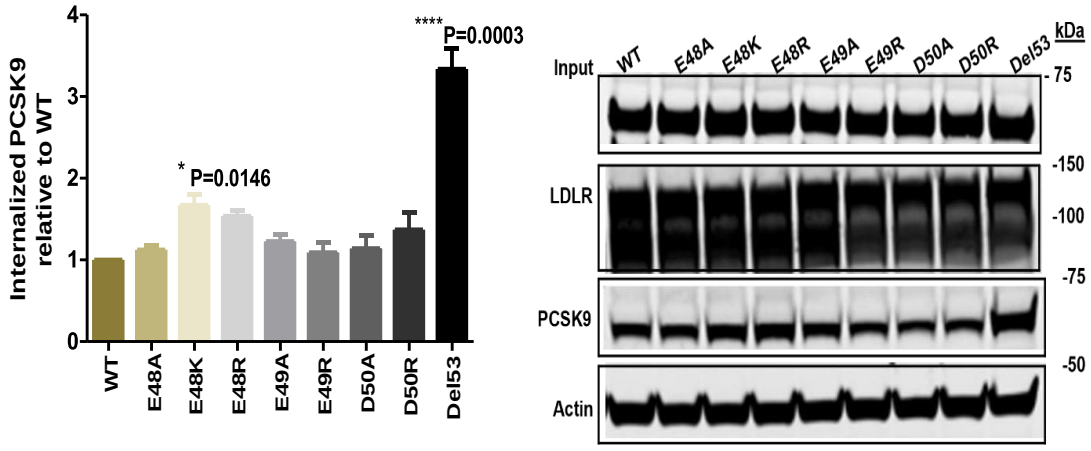

Figure 4: Substituting Glu48 to Asp50 in PCSK9 IDR does not significantly affect LDLR interactions.

Plasmids encoding C-terminally FLAG-tagged full-length PCSK9, either WT–or mutants or Del53–PCSK9, were transiently transfected into HEK293 cells. The conditioned medium containing secreted PCSK9 of these various mutants was harvested and quantified before proceeding to LDLR-dependent PCSK9 cellular uptake. HEK293 cells were transiently transfected with human wild-type (WT)- LDLR. 24 hours post-transfection, cells were treated with the mutants mentioned above obtained from the conditioned medium for 3 hours at 37ºC in the presence of 50 µM chloroquine (CQ); the role of the CQ is to prevent the lysosomal degradation of PCSK9 by raising the lysosomal pH. Following the 3 hours incubation, cells were then lysed. uBCA was done to quantify the amount to be loaded, and proteins were resolved by SDS-PAGE and detected by western blotting using α-actin as a loading control. Quantification shown in the graph represents mean n=3 with standard error, and ****p≤0.0003 according to 1-way ANOVA.

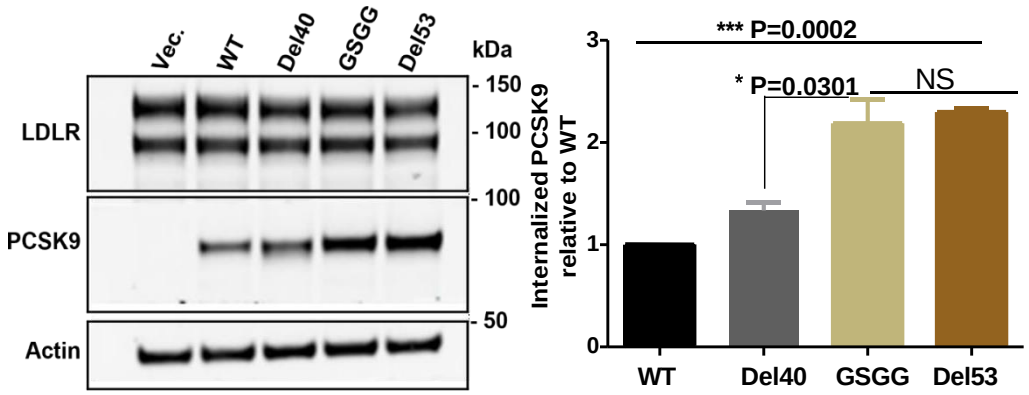

Fig. 5: Substitution of S47EED to GSGG-PCK9 significantly enhanced LDLR binding interactions.

Plasmids encoding C-terminally FLAG-tagged full-length PCSK9, either WT, Del40, GSGG, Del53, or empty vector, were transiently transfected into HEK293 cells. The conditioned medium containing these mutants' secreted PCSK9 was harvested and quantified before proceeding to LDLR-dependent PCSK9 cellular uptake.

Next, HEK293 cells were transiently transfected with human wild-type (WT)- LDLR. After 24 hours, the cells were treated with the conditioned medium or an empty vector and incubated for 3 hours at 37 °C in 50 µM chloroquine (CQ). The CQ was used to prevent the lysosomal degradation of PCSK9 by raising the lysosomal pH. After the incubation, the cells were lysed, and the amount of PCSK9 was quantified using uBCA. The proteins were then resolved by SDS-PAGE and detected by western blotting using α-actin as a loading control. The LDLR-dependent PCSK9 cellular uptake was quantified using the Licor. The quantification shown in the graph represents the mean of n=3 with standard error, and the statistical significance was determined using 1-way ANOVA followed by Dunnett's post test (*** $p < 0.0002$).

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As mentioned earlier, the IDR of PCSK9's prodomain is critical for the PCSK9-LDL interaction, as truncation of this region in Del53-PCSK9 abolishes LDL-PCSK9 binding [169]. Del53-PCSK9 displays both a ~4- to 7-fold enhanced PCSK9-LDLR binding and abolished LDL binding [169, 174, 254]. Key residues within this segment may be critical to PCSK9-LDL interaction. To understand the role of individual residues within this segment, we tested the effect of each on PCSK9's binding interactions with LDL as above for the PCSK9-LDLR interaction after disrupting their molecular nature through site-directed mutagenesis to replace the native negatively-charged amino acid with either a neutral (Ala) or positive-charged (Arg) residue. Other than an Asp50-to-Arg substitution, the other individual substitutions did not significantly affect the binding of PCSK9 to LDL particles **(Figure 6)**. These results suggest that individual residuals may have a subtle effect on PCSK9 structure; however, as in the case of previous studies on the LDLR binding interaction [174], the impact is limited, and a factor of positioning within the IDR and overall negative charge of the segment. In that case, a complete disruption might result in a substantial impact by substituting critical residues or the charged environment. To this end, we tested the effect of the GSGG-PCSK9 variant in which the insertion of a disordered linker for the Ser47-Arg50 sequence disrupts the native amino acid molecular nature of this segment but preserves the relative spacing. Interestingly, there was about ~85% less binding interaction of GSGG-PCSK9 and LDL particles compared to WT-PCSK9 **(Figure 7)**. As previously stated, [169, 173], Del53- and Del40-PCSK9 both abolish LDL-PCSK9 bindings. While GSGG-PCSK9 displayed a significant disruption in LDL binding but did not completely abolish this interaction, this suggests that the first acidic stretch from amino acids 31-40 plays the most critical role, with the second acidic stretch also contributing. Different combinations of double mutations from Ser47 to Asp50 were also attempted [Ser47 to Ala, Glu48 to Lys (SA+EK), Glu48 to Lys, and Asp50 to Ala (EK+DA) to understand better the cumulative effect for residues in the **Ser47-Arg50** sequence on PCSK9-LDL binding. We observed that neither of these sets

of double amino acid mutations showed as strong of a disruptive effect on LDL binding as was seen for GSGG-PCSK9 (**Figure 8**). Of note, EK+DA-PCSK9 had the most disruptive impact on LDL binding compared to SA+EK, suggesting that positioning and the nature of the residues' charges might influence PCSK9's interaction with the LDL. However, although the SA+EK-PCSK9 variant did not have as robust a disruptive LDL binding effect as EK+DA-PCSK9 or GSGG-PCSK9, its impact was still significant compared to WT-PCSK9. The stimulatory effect of these different combinational mutations in PCSK9 in terms of LDLR-dependent cellular uptake mirrored that of the inhibitory effect on LDL binding (**Figure 9**); while the interactions of these double mutants with LDLR were not as strong as GSGG-PCSK9, the stimulatory effect was significant when compared with WT-PCSK9.

Given the above observations, we focused on the GSGG-PCSK9 variant to test if the enhanced LDLR-dependent cellular uptake could translate to increased endogenous LDLR degradation in Hepa1c1c7 cells, a mouse hepatoma cell line with high levels of LDLR expression, especially precultured in sterol-depleting conditions (lipoprotein-deficient serum combined with a statin). As expected, the addition of GSGG-PCSK9 to the culture medium significantly induced the degradation of LDLR in these cells compared to WT-PCSK9 (**Figure 10**); however, Del53-PCSK9 was considerably more potent than GSGG-PCSK9. Notably, there was a >7-fold increase in cell- associated GSGG-PCSK9 prodomain as seen from western blots of whole cell lysates (**Figure 10, lane 4),** suggestive of a possible prodomain dissociation. GSGG-PCSK9 prodomain dissociation could be the likely reason for the reduced GSGG-PCSK9 ability to degrade the LDLR as efficiently as Del53-PCSK9. An effort was made to generate a recombinant PCSK9 prodomain as a single unit to understand better the effect of PCSK9 prodomain dissociation on the LDLR regulation, and this was unsuccessful, as was also reported in the literature.

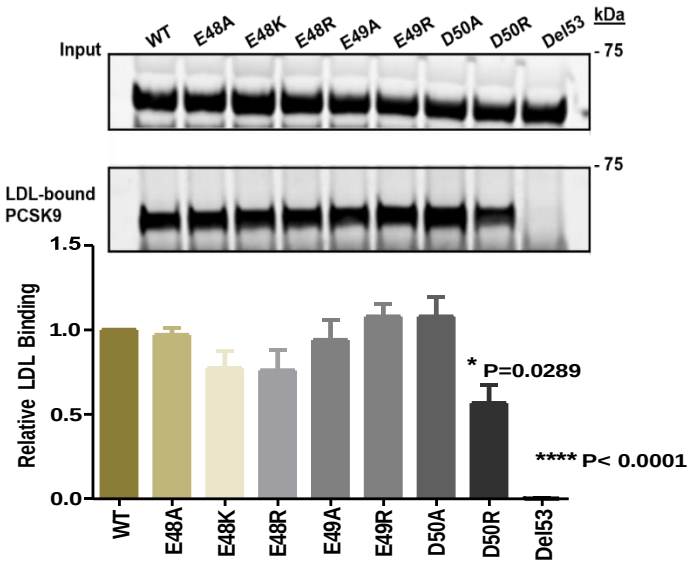


Figure. 6: Key residues in PCSK9 IDR affect LDL Association

Plasmids encoding C-terminally FLAG-tagged full-length human PCSK9, either WT, mutants, or Del53-PCSK9, were transiently transfected into HEK293 cells. The conditioned medium containing secreted PCSK9 was harvested and quantified prior to LDL binding. An equal amount of PCSK9 (~1μg) was incubated with LDL (0.5mg) at 37°C for 1 hour. Binding reactions were then subjected to density gradient ultracentrifugation for 2 hours to separate LDL-bound from the unbound PCSK9. Following ultracentrifugation, an LDL-containing fraction of 0.5 ml was drawn using a 22G needle and then immunoprecipitated with anti-serum 1697 directed against human PCSK9; the relative LDL-bound PCSK9 was determined by western blotting and quantified by Licor. Quantification shown in the graph represents mean n=3 with standard error, and stars indicate statistical differences according to one sample t-test.

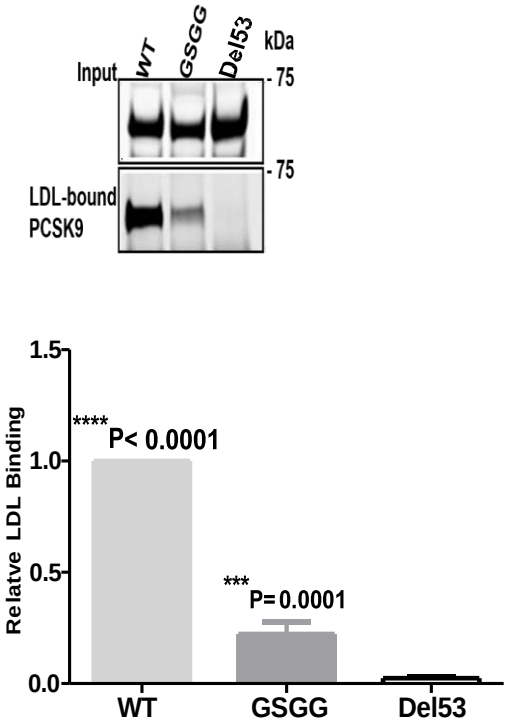


Figure 7: GSGG-PCSK9 Disrupt LDL-PCSK9 binding.

Plasmids encoding C-terminally FLAG-tagged full-length PCSK9, either WT-, GSGG-, or Del53 –PCSK9, were transiently transfected into HEK293 cells. The conditioned medium containing secreted PCSK9 was harvested and quantified prior to LDL binding. An equal amount of PCSK9(~1µg) was incubated with LDL (0.5mg) at 37°C for 1 hour. Binding reactions were then subjected to density gradient ultracentrifugation for 2 hours at 4ºC and 100,000 rmp to separate LDL-bound from the unbound PCSK9. Following ultracentrifugation, an LDL-containing fraction of 0.5 ml was drawn using a 22G needle and immunoprecipitated with anti-serum 1697 directed against human PCSK9. The relative LDL-bound PCSK9 was determined by western blotting and quantified by Licor. The quantification shown in the graph represents the mean of n=3 with standard error, and the statistical significance was determined using 1-way ANOVA followed by Dunnett's post test (**** $p < 0.0001$).

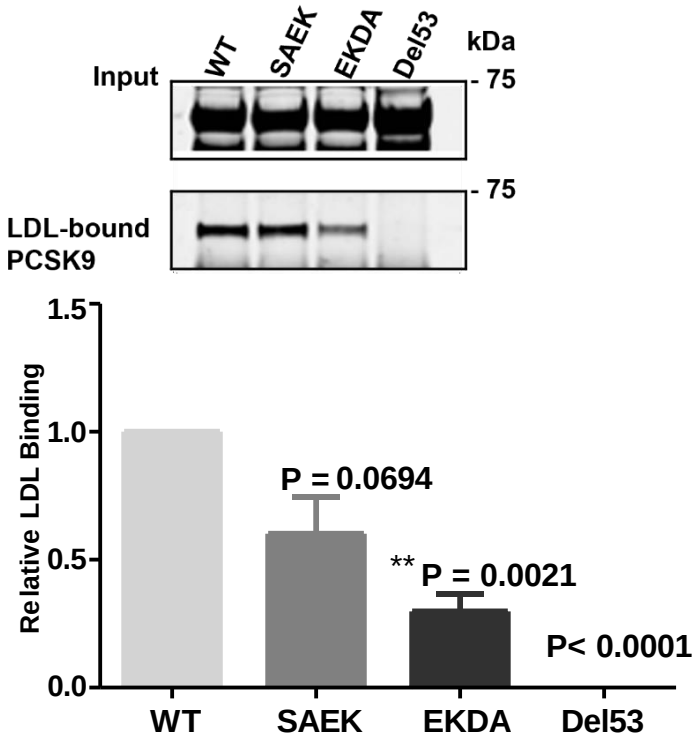


Figure 8: Double substituting key residues in PCSK9 IDR negatively affects LDL binding.

Plasmids encoding C-terminally FLAG-tagged full-length PCSK9, either WT-, SAEK-, EKDA-, or Del53 –PCSK9, were transiently transfected into HEK293 cells. The conditioned medium containing secreted PCSK9 of these mutants was harvested and quantified before proceeding to LDL binding. An equal amount of PCSK9 was incubated with isolated human LDL at 37°C for 1 hour. Binding reactions were then subjected to density gradient ultracentrifugation for 2 hours at 100,000 rmp to separate LDL-bound from the unbound PCSK9. Following ultracentrifugation, an LDL-containing fraction of 0.5 ml was drawn using a 22G needle and then immunoprecipitated with anti-serum 1697 directed against human PCSK9. The relative LDL-bound PCSK9 was determined by western blotting and quantified by Licor. Quantification shown in the graph represents mean n=3 with standard error, and stars indicate statistical differences according to one sample t-test

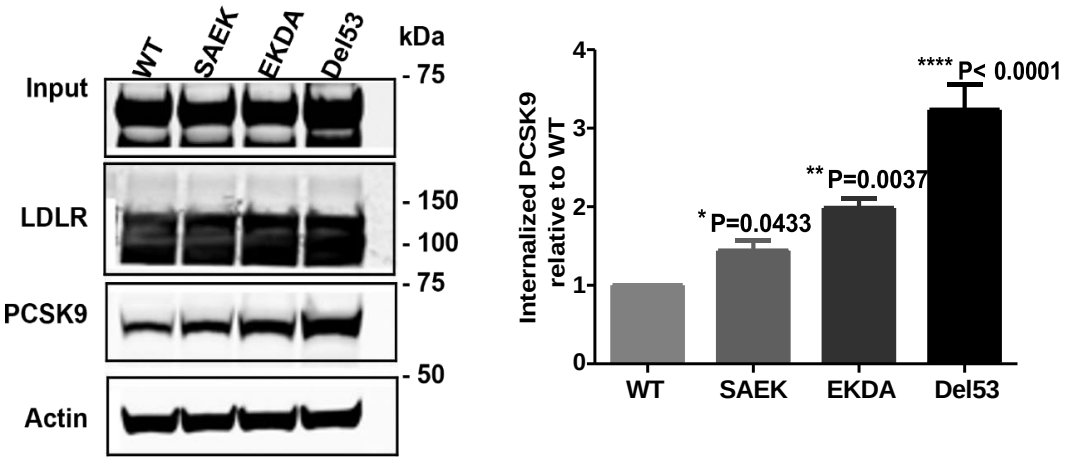


Figure 9: Double substituting key residues in PCSK9 IDR significantly improves LDLR binding.

Plasmids encoding C-terminally FLAG-tagged full-length PCSK9, either WT-, SAEK-, EKDA-, or Del53 –PCSK9, were transiently transfected into HEK293 cells. The conditioned medium containing secreted PCSK9 of these mutants was harvested and quantified before proceeding to LDLR-dependent PCSK9 cellular uptake. HEK293 cells were transiently transfected with human wild-type (WT)-LDLR. 24 hours post- transfection, cells were treated with plasmids encoding C-terminally FLAG-tagged full-length human PCSK9, either WT-PCSK9 or mutants from a conditioned medium for 3 hours at 37ºC in the presence of 50 μM chloroquine (CQ); the role of the CQ is to prevent the lysosomal degradation of PCSK9 by raising the lysosomal pH. Following the 3 hours incubation, cells were then lysed. uBCA was done to quantify the amount to be loaded, and proteins were resolved by SDS-PAGE and detected by western blotting using α-actin as a loading control. Quantification shown in the graph represents mean n=3 with standard error, and stars indicate statistical differences according to one sample t-test.

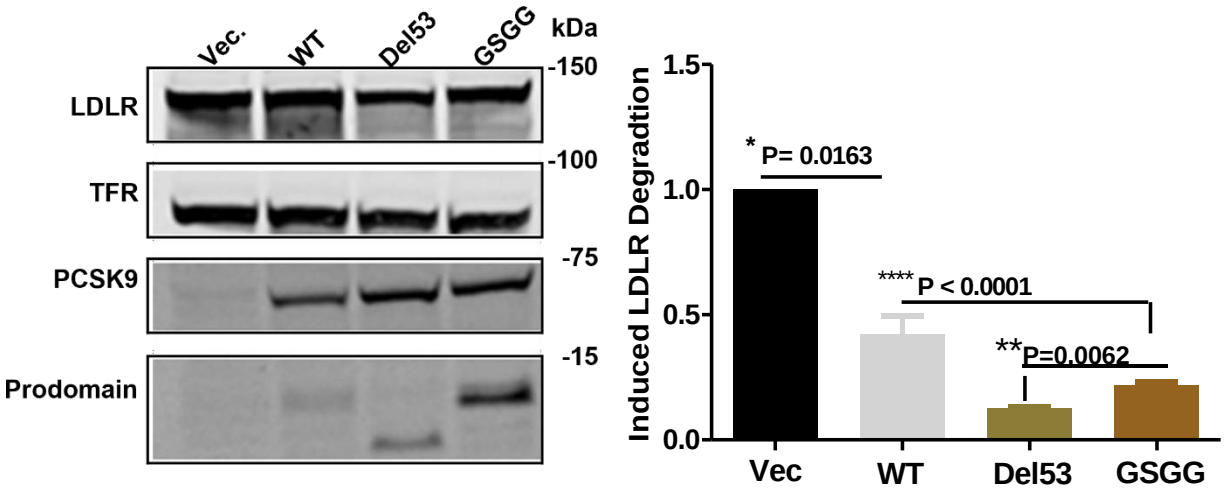


Figure 10: PCSK9 prodomain dissociation significantly reduced LDLR regulation.

Plasmids encoding C-terminally FLAG-tagged full-length PCSK9, either WT-, Del53-, or GSGG-PCSK9, were transiently transfected into HEK293 cells. The conditioned medium containing secreted PCSK9 of these mutants was harvested and quantified before proceeding to induced LDLR degradation assay.

HepaIc1c7 cells were cultured in a sterol-depleting medium, treated with an equal amount of PCSK9 in the conditioned medium containing the previously mentioned PCSK9 mutants above, and incubated for 4 hours at 37ºC. Following 4 hours of incubation, cells were harvested and then lysed; the amount of protein to be loaded was determined by uBCA, and proteins were resolved on SDS-PAGE and detected by western blotting. LDLR levels were normalized with transferrin receptor (*Tfr*). The graph is a representation of 3 independent experimental repeats. The statistical difference between Del53 and GSGG was according to the student t-test.

2.3 Discussion:

The traditional protein structure paradigm dictates that a protein's functions primarily depend on a fixed three-dimensional structure. Advances in structural biology have challenged this concept, suggesting that protein structure can be highly dynamic, and regions within proteins not captured by rigid structural analysis could be critical to the overall function of that protein [256]. One feature consistent with proteins is their structurally heterogeneous nature. Protein features comprise folded regions with variable conformational stabilities, including IDRs without a defined structural identity yet critical in the protein function [256]. All protein-database deposited PCSK9 crystal structures, including full-length PCSK9, C-terminally truncated PCSK9, and PCSK9 in complexes such as with peptide inhibitors, all lack a portion of the N-terminal prodomain of PCSK9 encompassing residues Glu-31 (first residue after the signal sequence) to Thr-60 [257], indicative of a disordered state. One feature familiar with any IDR within a protein, which is also the case with N-terminal IDR of PCSK9 prodomain, is that they are often enriched in charged and polar amino acids with the potential to regulate the protein function through competitive interaction or allosteric modulation [174, 256, 257]. One way to alter this allosteric modulation or competitive interactions is by disrupting the mechanism through which this region affects the protein function. The Del53-PCSK9 variant is reminiscent of the above concept by removing this region enriched in charged and polar amino acids. Indeed, evidence indicates that Del53-PCSK9 is hyperactive in cellular LDLR degradation, with 4 to 7-fold higher activity than WT-PCSK9 [169, 249]. This implies that this IDR of PCSK9 is autoinhibitory to PCSK9 bioactivities in the context of its interaction with the LDLR. It is also possible that all the native amino acids in the prodomain IDR may not contribute equally, with crucial residues having a significant effect. The role of this variant in other novel physiological functions of PCSK9 is still unclear, although it was shown to be critical in PCSK9 and apoB100 (LDL) interaction [169]. It remains to be determined whether the opposite roles of the prodomain IDR in the LDLR and

LDL binding activities of PCSK9 are interconnected mechanistically. For instance, binding to LDL could require, and even stabilize, an intramolecular interaction of the acidic IDR that is also involved in the autoinhibition of LDLR binding. In this manner, LDL association would inhibit PCSK9's ability to bind and mediate degradation of LDLR and explain previous findings that LDL inhibits PCSK9-mediated LDLR degradation in cell culture [169] and that numerous FH-associated GOF *PCSK9* mutations result in decreased or abolished ability to bind to LDL [173, 249].

Other than **Glu48Lys**, individual residues within this region show similar LDLR regulation as WT-PCSK9 (**Figure 4**), a point established earlier by Holla et al. [174], where it was suggested that the enhanced ability of the Del53-PCSK9 to regulate the LDLR could be a product of 'electrostatic steering' mechanism and individual residues likely contributing to the overall effect. On the other hand, individual residues could have an outsize effect based on their position within the IDR and, thus, their relative importance to intramolecular interactions involving the IDR. The GSGG-PCSK9 LDLR interaction at the cell surface supports this speculation (**Figure 5**), as there was no significant difference between it and Del53-PCSK9, containing a deletion of both acidic tracts in the IDR, in the regulation of LDLR, which gives credence to our working hypothesis that key residues could perhaps be the driving force of electrostatic steering and intramolecular interactions of the acidic prodomain IDR. Since the SA+EK-PCSK9 and EK+DA-PCSK9 double mutants of PCSK9 also showed enhanced LDLR regulation but still were not as potent as Del53-PCSK9 and GSGG-PCSK9, it is likely that even two amino acid combinations are not sufficient to exert the full allosteric effect, but that it requires longer sequences of acidic residues and overall positioning with the IDR (**Figure 9**).

Our results using site-mutagenesis analysis of the prodomain IDR emphasize the closely opposing effects of this region on LDL and LDLR binding. Specifically, mutations found to lower LDL binding of PCSK9 consistently showed a corresponding stimulatory effect on LDLR-dependent cell uptake of

PCSK9. This further supports that these two mechanisms are somehow linked by interactions involving the flexible prodomain IDR. Several studies, including from the Lagace lab, have shown that the prodomain IDR within PCSK9 can adopt a transient-helical structure, and this transient form is critical for PCSK9 binding to the LDL but not LDLR [173, 257]. Thus, Del53- and Del40-PCSK9 could abolish the LDL interaction in part due to the disruption of this α-helical structure in addition to the deletion of key interacting residues [173]. Computational molecular dynamics (MD) simulation analysis was used to construct a 3D structural model of PCSK9 that included the α-helical conformation in the prodomain IDR [173]. This provided evidence of a novel interdomain interaction involving the prodomain helix and a region in the C-terminal domain of PCSK9 harboring several FH-associated GOF mutations (R469W, R496W, and F515L) that were subsequently found to be disruptive for the PCSK9-LDL interaction [173]. In later studies, additional FH-associated *PCSK9* GOF mutations in the prodomain were also shown to lower (L108R) or abolish (S127R, D129G) the LDL binding interaction [249]. Although the identified key residues in the prodomain and C-terminal sites are distant from each other, they are located on the same molecular plane, suggesting that the large LDL particle binds to these two sites simultaneously. If so, this multidomain binding mode could affect the global conformation of PCSK9 and negatively impact the LDLR binding interface in the catalytic domain. Another possibility is that only the prodomain site near the critical Ser127 residue binds to apoB100 in LDL, with the other site in the C-terminal domain allowing this access by engaging the prodomain N-terminus in an interdomain interaction. This scenario assumes that the acidic prodomain N-terminus would normally bind at or near the Ser127 residue. Interestingly, this region of the prodomain contains several positive-charged Arg residues shown to facilitate the binding of PCSK9 to negatively-charged heparin-sulfate proteoglycans (HSPGs) on the surface of hepatic cells, which in turn promotes PCSK9-mediated LDLR degradation [258]. Therefore, it is conceivable that binding of clustered Arg residues in the prodomain to cell

surface HSPGs precludes the acidic prodomain IDR from interacting electrostatically with this same Arg cluster. Also of note, a recent study showed that cell surface HSPG binding abrogates the inhibitory effect of LDL on PCSK9's ability to bind cell surface LDLRs [259]. Collectively, the current data and the available literature point to the possibility of a prodomain region at or near the Ser127 residue being a site of both LDL-apoB100 binding and an autoinhibitory interaction of the N-terminal IDR. However, further studies are needed to identify the precise sites in PCSK9 involved in autoinhibition and LDL binding and whether these are indeed the same.

As mentioned earlier, among the different circulating lipoproteins, evidence suggests LDLs are the only known particle that undergoes structural transition close to body temperature [165]. It is unclear if this structural transition influences the PCSK9 interaction with LDL. There was a significantly diminished LDL interaction with SA+EK- and EK+DA-PCSK9 and, most dramatically, with GSGG-PCSK9 (**Figures 7 & 8**). It is unknown whether the prodomain helical structure would be affected by these mutations since the affected residues lie upstream of the identified helical region [257]. Thus, it is possible that the diminished LDL binding of the PCSK9 double mutants (SA+EK, EK+DA) and GSGG-PCSK9 is unrelated to changes in the transient helical structure but that these residues are more directly involved. The latter proposition is complicated by our finding that the prodomain readily dissociated from GSGG-PCSK9 when added to cultured Hepa1c1c7 cells. Thus, the discrepancies between GSGG-PCSK9-induced LDLR degradation and LDLR-dependent uptake could be partly explained. First, the GSGG-PCSK9 displays increased LDLR binding affinity as an intact unit, accounting for robust LDLR- dependent cell uptake. However, upon endocytosis of the GSGG-PCSK9:LDLR complex, the prodomain dissociates in early endosomes, presumably a function of the amino acid changes in the prodomain IDR, and possibly triggered by cellular conditions such as the acidic pH of early endosomes. Furthermore, it was reported that all three PCSK9 domains are tightly folded together to deliver their biological

function [113]. A dissociation **(Figure 10, lane 4)** could have disrupted this tightly folded unit, resulting in a lower ability to retain the firm binding interaction seen at the cell surface between GSGG-PCSK9 and LDLR **(Figure 5)**. This same prodomain dissociation mechanism may also explain the defect in GSGG-PCSK9 binding to LDL **(Figure 7),** given the critical role of the prodomain in this interaction [169, 173].

The charge and the amino acid sequence in the acidic prodomain IDR of PCSK9 are critical factors in regulating LDLR binding affinity [174]. However, as part of the linker, the posttranslational phosphorylation site (Ser47) could hold some key to the enhanced GSGG-PCSK9:LDLR interaction at the cell surface level. Previous mutation analysis of Ser phosphorylation through Ser47Asp mutagenesis did not show any significant differences in LDLR-dependent PCSK9 uptake (Lagace lab, unpublished data); however, in the context of the nearby acidic residues Glu48-Glu-Asp, phosphorylation of Ser47 would increase the negative charge of this region and could provide an additive effect. In support, we found that single mutations in this region, such as the natural GOF mutation E48K [249], had less impact than the complete replacement of this acidic stretch in GSGG-PCSK9. Of note is the proximity of the Ser47-Glu-Glu-Asp region to the β0 strand of the PCSK9 prodomain, which is proposed to be important to the binding interface of the PCSK9 prodomain with the Cat/CHR domain segment [113, 114]. Although speculative, this acidic region in the prodomain IDR may affect LDLR binding affinity and prodomain association through interactions with the β0 strand, and disruption of this interaction via replacement of the Ser47-Arg50 sequence in GSGG-PCSK9 could have the dual effect of increasing LDLR binding affinity by relieving autoinhibition, but also weakening the prodomain/Cat interface and promoting prodomain dissociation. If it could be specifically targeted, the latter effect of prodomain dissociation could represent a novel avenue for PCSK9 inhibition, as this would likely destabilize the protein and prevent its biological interaction with LDLR. It would also be interesting to see if this mechanism of

prodomain dissociation is channeled into other physiological regulations of PCSK9, such as its still poorly understood roles in inflammation and innate immune function [7, 85].

2.4 Materials and Methods.

Media

Medium A: Contained DMEM (4.5 g/L glucose) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin sulfate. Medium B: Contained medium A supplemented with 10% FBS (v/v) Medium C: Contained medium A supplemented with 5% (v/v) NCLPDS. Medium D: Contained α-MEM (1.0 g/L) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin sulfate. Medium E: Contained medium D supplemented with 10% FBS (v/v). Medium F: Contained Medium D supplemented with 5% (v/v) NCLPDS, 10 µM pravastatin, and 50 µM sodium mevalonate. Medium G: Contained RPMI medium supplemented in 10 mM L-Glutamine, 10 mM HEPES (pH 7.4), 50 pM β-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate, 10% FBS (v/v), 1 mM sodium pyruvate and 5 ml Glucose (100 g/L)

Mutagenesis of PCSK9

Using the QuickChange™ site-directed mutagenesis (modified) protocol (Stratagene, La Jolla Ca), point or double-point mutation was introduced into full-length human WT-PCSK9 with a FLAG- tag epitope [249]. The desired modifications were custom-designed with the above template by generating forward and reverse primers according to the QuickChange™ guidelines. Briefly, each 50 ul PCR contained 1X (10X pfuUltra) Phusion High Fidelity buffer, 10 µM dNTPs, 10% DMSO, 5 ul (10 ng/ ul) of template DNA, 2 ul (62.5 ng/ul) of forward and reverse primers and 2.5 unit of pfuUltra DNA polymerase. Thermocycling was initiated using the hot start: 95ºC for 1 minute (1X), followed by 18 cycles of

amplification, each of these consisting of denaturation 95°C for 50 seconds, annealing 60ºC for 50 seconds and extension 68ºC for 8 minutes (1 minute/kb – 7.5kb), with a final extension of 68°C for 7 minutes (1X), and hold for 4ºC.

Conditioned Media

HEK 293 cells were maintained in monolayer cultures (10 cm dish, Corning) at 37ºC and 5% CO2 in medium B, and upon ~80% confluency, each dish was transfected with 3 µg of DNA (either empty vector, WT, or mutant PCSK9) and 10 ul of Polyjet (FroggaBio) as per the manufacturer protocol. 18 h post-transfection, cells were washed 2X with PBS and incubated for 48 h in medium A supplemented with 1X ITS. After 48 h, media was collected and centrifuged at 1000xG for 5 minutes so any floating cells could be pelleted; the supernatant was carefully transferred to a 10- kDa Amicon Ultra-4 centrifugal filter unit and concentrated 10-fold. Concentrated conditioned media were transferred to new tubes, and PCSK9 concentration in condition media was determined by western blot using purified PCSK9 as standard before storing it at - 80ºC for subsequent usage. The cell lysate was prepared by washing cells in ice-cold PBS-CM (1 mM $MgCl_2$ and 0.1 mM $CaCl_2$**),** following 2X washes; lysate was then prepared in Tris Lysis Buffer (50mm Tris-Cl, pH 7.4, 150 mm NaCl, 1% NP-40, 0.5% sodium deoxycholate, 5 mm EDTA, 5 mm EGTA), 1X CompleteC **™** protease inhibitor cocktail, 1X DTT (Dithiothreitol) and 1 mM phenylmethylsulfonyl fluoride. The relative level of processing and secretion of PCSK9 was determined by western blotting.

LDLR-dependent Cellular PCSK9 Uptake

HEK293 cells were cultured in a 6-well plate and maintained in medium B to 80% confluence, after which cells were transiently transfected with 1 µg of either empty vector or WT human LDLR plasmid per well using Polyjet DNA transfection reagent according to the manufacturer instructions. 18 h post-transfection, the cell medium was aspirated off, washed 2X, and changed to medium B with 50 µM chloroquine per well for 30 minutes. After 30 minutes, the cell medium was replaced with a prepared condition medium containing empty vector, WT, or mutant PCSK9 (~0.5 µg) to 1 ml in medium C with 50 µM of chloroquine for 3 h. After the 3 h incubation, cell lysate was prepared and analyzed by western blotting to determine LDLR-dependent PCSK9 uptake.

LDLR Degradation Assays:

Hepa1c1c7 cells (60 mm plates) were maintained in medium E. When cell cultures were 70 – 80 confluency, the medium was replaced with medium F for 20 h, after which either empty vector, WT, or mutants PCSK9 in conditioned medium (~5 µg) were added to the medium and incubated for 4 h at 37ºC. Cell lysate was prepared and analyzed by western blotting to determine the level of LDLR degradation.

LDL isolation

LDL was isolated from human plasma as described [249]. Briefly, blood samples were drawn from fasted and healthy volunteers into commercially made evacuated tubes containing EDTA, and plasma was separated by low-speed centrifugation (2000 xG for 20 minutes at 4ºC). Protease inhibitors and antioxidants (1 mM PMSF, 50 U/ml aprotinin, Complete™ protease inhibitor cocktail, and 20 µM butylated hydroxytoluene) were added to the cleared collected plasma. LDL particle (density 1.019 – 1.065 g/ml) was isolated using the sequential potassium bromide flotation ultracentrifugation as described [249], followed by extensive dialysis against phosphate-buffered saline (PBS) containing 0.25 Mm EDT. Protein

concentrations in lipoprotein preparations were determined using the Lowry assay. LDL obtained was stored away from light by wrapping the tube in foil paper and kept at 4ºC for subsequent usage.

In vitro PCSK9-LDL binding

Binding reactions (1 ml volume), each containing 0.5 mg of LDL and 100 ul of concentrated conditioned media of either empty vector, WT, or mutants PCSK9, 50 ul of 0.5% BSA (w/v) in HBS-C buffer (25 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$) and HBC-S buffer was used to make up the rest of the reaction volume. Reaction complexes were incubated at 37ºC for 1 h. As described, LDL-bound and unbound PCSK9 were separated by Optiprep gradient ultracentrifugation [249]. Briefly, 0.65 ml of 60% Optiprep, 1 ml of reactant complexes, and 1.35 ml of 25 mM HEPES buffer (pH 7.4) are placed in a 3.3 ml Beckman Optiseal tube. After thoroughly mixing and left to stand for 1 – 2 minutes, 0.3 ml of 25 mM HEPES buffer (pH 7.4) was used to overlay the 3 ml. Tubes were centrifuged at 100,000 rmp for 2 h at 4ºC in a TLN100 roto (Beckman Coulter). Following centrifugation, an LDL-containing fraction of 0.5 ml was drawn using a 22G needle and immunoprecipitated with anti-serum 1697 directed against human PCSK9, and relative LDL-bound PCSK9 was determined by western blotting.

Western Blot

Western blot was previously described [249]. Briefly, Protein in cell lysate, conditioned media, or immunoprecipitates were prepared in 1 X Bolt sample buffer (Thermo-Invitrogen) and supplemented with 50 mM dithiothreitol, reaction complexes heated for 5 minutes at 96ºC. Following heating, complexes were loaded for electrophoresis using the 4% - 12% Tris/HEPES- SDS Bolt™ precast gels (Thermo-Invitrogen) for 1 h at 165 Volt. Resolved proteins were transferred to a nitrocellulose membrane (Bio-Rad) and allowed to run overnight in a transfer tank for immunoblotting. Primary antibody

incubation was done at room temperature for 1 h, following 3X washes, secondary infrared dye (IRDye800)-labeled antibodies were used to detect target proteins using the LI-COR Odyssey infrared imaging system (LI-COR Biosciences). Band intensity was quantified using the Odyssey 2.0 software (LI-COR Biosciences).

Antibodies used

The following antibodies were used for the western blotting: 15A6, a monoclonal antibody cloned from mouse hybridoma (gift from UT Southwestern Medical Center, Dallas), recognizes an epitope of the PCSK9 CHR domain. 3143, a rabbit anti-serum, recognizes the C-terminal 14 amino acids of LDLR (gift from UT Southwestern Medical Center, Dallas). 1697, rabbit anti-serum against the full-length human PCSK9 (Biomatik – Cambridge, Canada). Anti-actin mouse monoclonal (Signal). Goat anti-serum apoB (Millipore) and mouse anti-human transferrin receptor (Invitrogen). Secondary IRDye-labeled goat anti-mouse and anti-rabbit IgG antibody (LI-COR Biosciences).

Statistical analysis

All statistical analyses were determined by student t-tests or one-way ANOVA using GraphPad Prism 9 software. All experiments represent a minimum of three independent experimental repeats, and error bars indicate the standard error of the mean (SEM).

Chapter 3: Effect of proteolytic processing on PCSK9-LDL interaction

3.1 Introduction:

PCSK9 undergoes posttranslational modifications (PTMs), including sulfation, phosphorylation, and limited proteolysis. PTMs are known to affect the biological activities of proteins [19, 117, 118]. Not all PTMs in proteins produce a positive outcome; for example, limited proteolysis by some PCs on endothelial lipase results in enzyme inactivation [260]. About 15 to 25% of PCSK9 in human circulation are truncated products due to limited proteolysis, primarily by furin [18, 19, 69]. This action removes a ~7-kDa portion (amino acid 153 – 218) **(Supplemental Figure 1)** of the N-terminus of the catalytic domain of PCSK9 [18]. The excised region overlaps the binding site of PCSK9 and LDLR EGF-A domain [19]; thus, as previously stated, f-PCSK9 is defective for LDLR binding. As with the enhanced LDLR binding/degradative function of Del53-PCSK9 because of the removal of amino acids **Gln31 – Leu52,** the excision of a 7-kDa portion of the catalytic domain of PCSK9 by furin proteolysis could accentuate this variant's bioactivity. Or, as reported by Benjannet et al. [19], it could be rendered inert, as seen in the context of LDLR degradation. Beyond LDLR regulation, PCSK9 has recently been linked to inflammation. For example, very recently, it was reported that IL-6, a marker of inflammation, was reduced in COVID-19 patients upon treatment with PCSK9i-mAbs compared to a placebo [261, 262]. Recent evidence also supports that f-PCSK9 was upregulated along with the intact PCSK9 after myocardial infarction [121], and Kataoka et al. [263] reported that f-PCSK9 concentration predicted future coronary events in a Japanese subject. These findings suggest that f-PCSK9's pathological role is independent of PCSK9-mediated LDLR regulation.

As mentioned earlier, a substantial amount of circulating PCSK9 is LDL-bound. It remains unclear if f-PCSK9 is capable of LDL binding. Hori et al. reported that both intact PCSK9 and f- PCSK9 were depleted from human plasma that had undergone LDL apheresis, a form of dialysis in which LDL

particles are adsorbed to a matrix prior to reintroduction of the partially delipidated plasma back into the patient [264]. While this suggests that f-PCSK9 was removed as a component of the LDL, it is also possible that it bound non-specifically to the matrix material. By contrast, Fazio et al. reported that plasma f-PCSK9 was mainly found in an apoB-free fraction of human plasma and thus not bound to LDL; furthermore, in this same study, they showed evidence that LDL association of intact PCSK9 protected it from furin proteolysis [265]. The failure to detect f- PCSK9 in an LDL fraction from plasma could reflect dissociation of the prodomain, which has been shown to be promoted by furin proteolysis of PCSK9 [19]. This would likely preclude LDL binding of f-PCSK9 since the prodomain has been demonstrated to contain critical determinants for LDL binding by intact PCSK9 [169]. Thus, the conflicting results in terms of f-PCSK9:LDL association in the literature could reflect differences in experimental conditions affecting the persistent binding of the prodomain to the Cat/C-term of f-PCSK9. Although furin proteolysis of PCSK9 is inactivating in terms of its LDLR binding/degradative function, it remains to be determined whether this PTM also abolishes the binding of PCSK9 to LDL. Alternatively, cleavage of PCSK9 by furin could generate a new bioactivity involving LDL association if this binding function remains intact. This possibility is intriguing given the unexplained role of PCSK9 in inflammation, a process known to be promoted by modified forms of LDL. Indeed, if furin proteolysis of PCSK9 reveals a latent binding motif, the interaction of this variant with LDL could be enhanced. **Therefore, we hypothesized that furin proteolysis of PCSK9 accentuates its LDL interaction potential dependent upon retention of the prodomain.**

3.2 Results

To further understand the role of proteolytic cleavage on PCSK9 binding activity on LDL, and since >30% of circulating PCSK9 are LDL bound [169], our first attention was to determine if circulating cleaved PCSK9 is also LDL bound. To do that, pooled plasma samples were obtained from eight fasted

healthy volunteers – 4 males and four females – and the samples were subjected to a layered Optiprep gradient ultracentrifugation with slight modification as described [169]. According to Kosenko et al. [169], this method helps to resolve plasma PCSK9 into three fractions (light, medium, and heavy density), along with clearly visible fractions of LDL-containing zone within these fractions **(Figure 11A)**. As seen previously, the intact PCSK9 Cat/C-term segment (~60 kDa) was readily detected in the LDL bound fraction [169], which also contained the truncated f-PCSK9 Cat/C-term segment (~55 kDa) in a similar proportion as that in the whole plasma **(Figure 11B)**. Notably, there was no discernible decrease in the amount of prodomain found in the LDL fraction compared to that in whole plasma, supporting that the PCSK9 in the LDL fraction, including f-PCSK9, contained the prodomain attached to the rest of the protein. This result clearly indicates that circulating cleaved PCSK9 is also LDL-bound, just like the intact PCSK9.

Direct binding of f-PCSK9 to LDL (native or modified) in an *in vitro* setting has not been reported previously. Since the prodomain is critical to PCSK9 and LDL binding interactions [169], we generated an f-PCSK9 variant with little to no dissociation of the prodomain. This was done by incubating recombinant human furin with purified PCSK9 at room temperature for 6 hours, followed by the addition of a specific furin inhibitor peptide (furin inhibitor 1) to prevent further proteolysis. F-PCSK9 and intact PCSK9 were then incubated with isolated human LDL for 1 hour, and the reaction mixture was subjected to density gradient ultracentrifugation as described [169]. The binding of f-PCSK9 to native LDL (nLDL) was increased >1.5-fold compared to intact PCSK9 **(Figure 11C)**, whereas binding to modified LDL (oxLDL) was increased >10-fold **(Figure 11C)**. Of note, oxLDL is a proinflammatory mediator of atherosclerosis [266], and there is evidence that PCSK9 potentiates oxLDL signaling [267]; thus, the f-PCSK9 variant, considering the strong interaction with oxLDL in **Figure 11C** might be involved in this signaling.

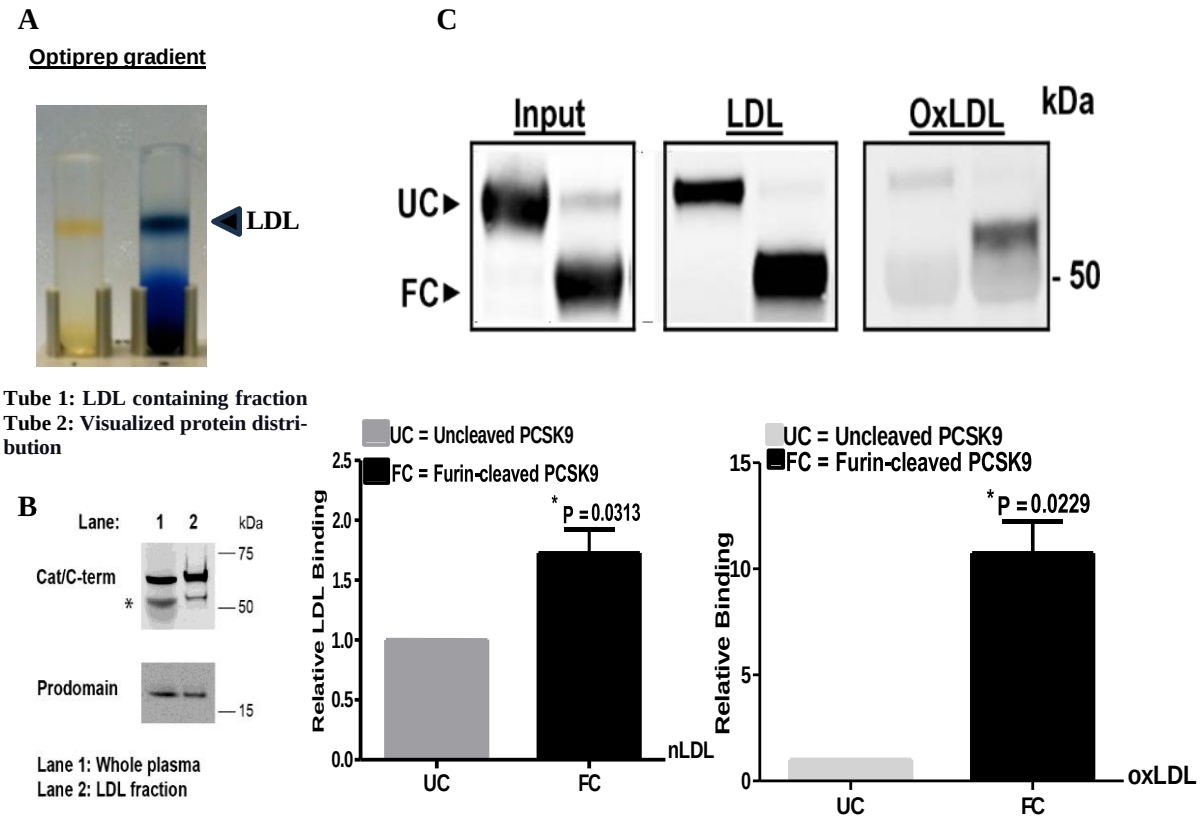


Figure 11: Furin proteolyzed PCSK9 associate with LDL.

A) Optiprep gradient indicating the LDL-bound fraction and the visualization of the protein distribution within the gradient using Coomassie R-250. B) Pooled human plasma showing LDL-bound intact and f- PCSK9 from the gradient ultracentrifugation separation methods. Results show the whole plasma (input, lane 1) and the LDL-bound PCSK9 (lane 2). C) nLDL- and oxLDL -bound PCSK9 (intact and f-PCSK9) and the input (control). ▶ LDL visualization. ⁎denote band consistent with furin-cleaved PCSK9 (f-PCSK9). Quantification shown in the graph represents mean n=3 with standard error, and stars indicate statistical differences according to one sample t-test.

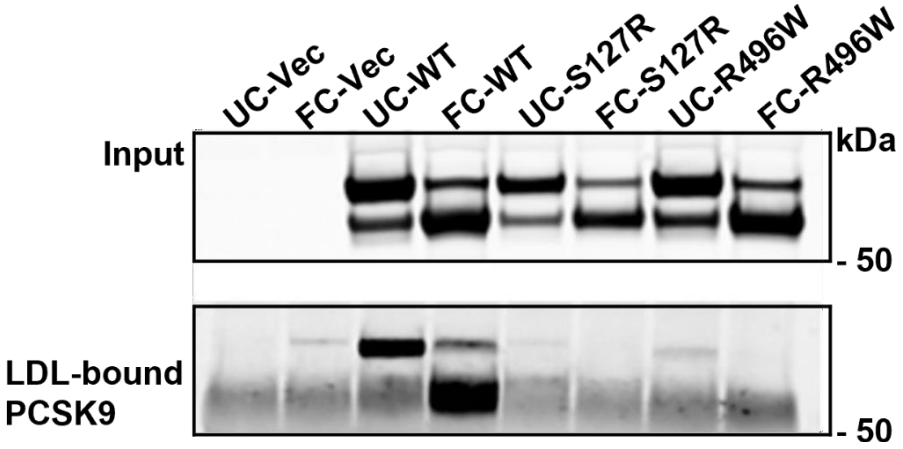


Figure 12: Furin proteolysis does not improve GOF PCSK9 with altered LDL association.

Cleaved PCSK9 was generated in-house. Briefly: Condition medium containing C- terminally FLAG-tagged full-length PCSK9, either WT-, S127R-, or R496W-PCSK9, was incubated with or without recombinant human furin (rhf) at room temperature for 6 hours. Furin inhibitor 1 was added to stop further protease activity, as per the manufacturer. Cleaved (FC) and Uncleaved (UC) PCSK9 variants were incubated with isolated human LDL at 37°C for 1 hour. Binding reactions were then subjected to density gradient ultracentrifugation for 2 hours at 100,000 rmp to separate LDL-bound from unbound. Following ultracentrifugation, an LDL-containing fraction of 0.5 ml was drawn using a 22G needle and immunoprecipitated with anti-serum 1697 directed against human PCSK9. The relative LDL-bound PCSK9 was determined by western blotting and quantified by Licor. The blot shown is a representation of three independent experimental repeats.

Our group previously reported that certain GOF *PCSK9* mutations, including S127R in the prodomain and R496W in the C-terminal domain, abolish LDL binding [173, 249]. Since furin cleavage of PCSK9 improves its LDL binding interaction, as seen in **Figure 11C**, we sought to understand if the furin-cleaved GOF variants were now able to bind to LDL through an alternate mechanism. However, if the mechanism whereby f-PCSK9 binds LDL is the same as for intact PCSK9 but improved, we would expect cleaved GOF PCSK9 variants to remain defective for LDL binding. As suspected, there was no noticeable LDL binding for the intact (uncleaved) or furin- cleaved versions of these GOF-PCSK9 mutants **(Figure 12)**.

The enhanced binding of f-PCSK9 to nLDL and oxLDL could involve an improved membrane association. To test this possibility, f-PCSK9 or intact PCSK9 were incubated with artificial liposomes containing phosphatidylcholine (PC), cholesterol (Chol), and sphingomyelin (SM) in a 60:20:20 molar ratio to reflect levels of these lipids in the outer membrane of LDL particles. As shown in **Figure 13**, the binding of f-PCSK9 to PC:Chol:SM liposomes was significantly enhanced compared to intact PCSK9. Thus, the increased f-PCSK9 and LDL interaction may reflect strong membrane binding.

LDLR is the primary conduit of plasma LDL-C and PCSK9 clearance [1, 268]. There is a >1.5- fold increase in f-PCSK9 binding to LDL **(Figure 11C)**. Since f-PCSK9 lacks a site critical in LDLR association, binding to LDL could facilitate f-PCSK9 clearance as a passive component of LDL-LDLR endocytosis. To test this possibility, f-PCSK9, and intact PCSK9 were preincubated with isolated human LDL, and the complexes were incubated in HEK293 cells transiently transfected with LDLR as described [249]. Interestingly, the enhanced *in vitro* LDL-f-PCSK9 binding did not improve LDLR-dependent uptake of f-PCSK9 **(Figure 14)**, suggesting that this route is not the means of f-PCSK9 plasma clearance.

Also, as stated in the literature, f-PCSK9 is inert in the context of LDLR degradation, so we next turned

our attention to determining if this variant tested above shares similar functional characteristics that are inert in regulating LDLR. As seen in **(Figure 15)**, there was no significant difference between f-PCSK9 and vector control in LDLR degradation. This indicates that the f-PCSK9 we generated here shares similar functional capability as the f-PCSK9 described in the literature.

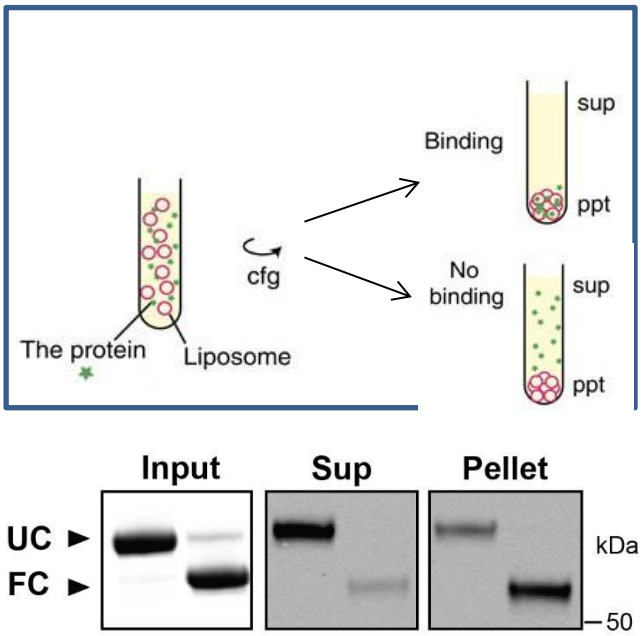


Figure 13: Proteolyzed PCSK9 associate with Liposome.

Liposome preparation was described previously (see methods for liposome preparation). Liposome was incubated with intact (UC) or f-PCSK9 (FC) at 37ºC for 45 minutes. Binding reactions were then subjected to density gradient ultracentrifugation for separation. The pellet was redissolved in low-salt HBS-C at the same volume as the supernatant (Sup). Pellet represents bound PCSK9, while Sup represents unbound PCSK9. Western blot analysis of the supernatant and pellet shows an enhanced liposome/f-PCSK9 association. The blot shown above is a representation of two independent experimental repeats.

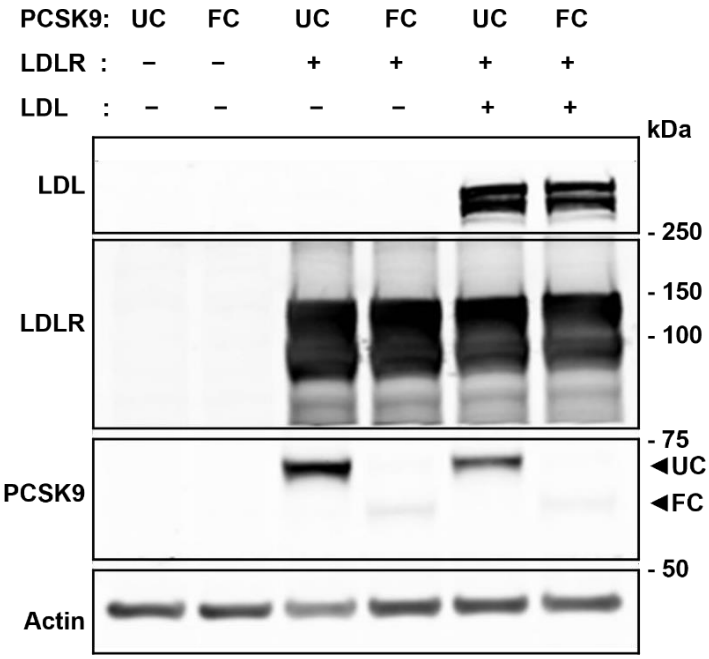


Figure. 14: Proteolyzed PCSK9 is not a passive component of LDL-LDLR endocytosis.

F-PCSK9 generation was described previously. HEK 293 cells were maintained in monolayer cultures (6-well dish) at 37ºC and 5% CO2 in medium B, and upon ~80% confluency, cells were transiently transfected with empty vector plasmid or human LDLR (0.5µg per well) and 1.5 ul of Polyjet (FroggaBio) as per the manufacturer protocol. 24 hours post-transfection, the cells were treated in the presence of 50 µM chloroquine and incubated for 3 hours with intact (UC) or f-PCSK9 (FC) (~3µg), preincubated in the absence (-) or presence (+) of native LDL (1mg) for an hour at 37ºC. Following 3 hours of incubation, cells were then lysed. uBCA was done to quantify the amount of protein to be loaded. Protein was resolved by SDS-PAGE and detected by western blotting using α-actin as a loading control. Protein was resolved by SDS-PAGE and detected by western blotting using α-actin as a loading control. The blot represents three independent experimental repeats.

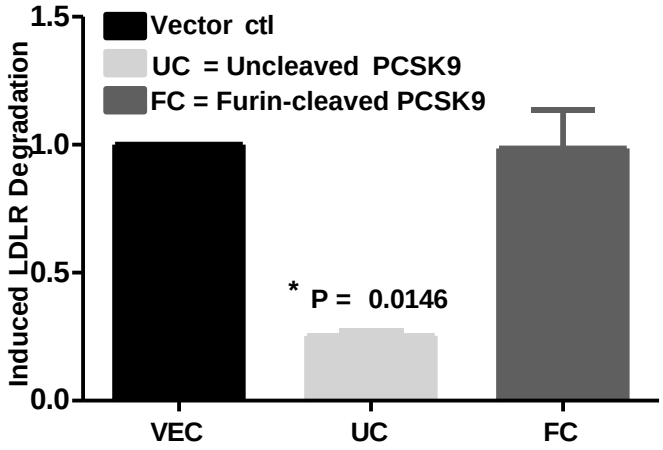


Figure. 15: Proteolyzed PCSK9 is inert in LDLR degradation.

Plasmids encoding vector control, WT-PCSK9, were transiently co-transfected with or without furin in HEK293. Conditioned medium was harvested, and the relative amount of cleaved (f-PCSK9) or un-cleaved (UC) was determined by western blot. HepaIc1c7 cells were cultured in a sterol-depleting medium before treatment with the conditioned medium containing the vector control, UC, or FC, and incubated for 4 hours at 37ºC. Cells were harvested and lysed, and proteins resolved on SDS-PAGE and detected by western blotting. LDLR levels were normalized with transferrin receptor (*Tfr*). Quantification shown in the graph represents mean n=2 with standard error, and stars indicate statistical differences according to one sample t-test

3.3 Discussion:

The results in this chapter suggest that the two subspecies of PCSK9 found in human plasma are LDL-bound. Although earlier reports had suggested a significant portion of the circulating intact PCSK9 is in the LDL fraction [169, 175], it was also reported that f-PCSK9 variants were found in an apoB100 deficient fraction [175], suggesting that this f-PCSK9 variant does not associate with LDL. Herein, we show for the first time that f-PCSK9 associates with and binds LDL. The prodomain is crucial to this binding interaction **(Figure 11)**, which is consistent with previous reports suggesting that the prodomain is critical to LDL and PCSK9 interaction [169, 173]. The discrepancy between our report here and that of Tavori et al. [175] could be due to how much of the prodomain remained bound to the rest of the protein. One would speculate that perhaps the methods or molecules used were harsh. It is also possible that f-PCSK9 generated in different cellular compartments has a differing propensity for prodomain dissociation [32, 39]. Since furin cycles between the trans-Golgi, plasma membrane, and endosomes, it could conceivably cleave PCSK9 at any of these sites with differing effects on prodomain association. Similar to a previous report [19], we found that when plasmid vectors expressing PCSK9 and furin were co-transfected into HEK293 cells, the vast majority of the secreted f-PCSK9 did not contain the prodomain (data not shown). By contrast, when purified recombinant PCSK9 was incubated with furin enzyme, as in the experiments detailed in **Figure 11**, the prodomain stayed largely intact, and LDL and oxLDL binding activity was enhanced. In recent unpublished studies, we found that adding PCSK9 exogenously to HEK293 cells stably overexpressing furin resulted in efficient cleavage of the PCSK9 with >70% retention of the prodomain (data not shown). Thus, it is possible that physiological furin-proteolysis of PCSK9 can occur extracellularly with retention of the prodomain and enhanced ability of the generated f-PCSK9 to bind to native and modified forms of LDL in plasma.

Removal of amino acids 153 to 218 in f-PCSK9 may reveal a latent membrane binding motif in this

variant, as evidenced from the enhanced binding of f-PCSK9 to nLDL, oxLDL, and artificial membrane liposomes **(Figures 11 and 12)**. As mentioned, furin is upregulated in an inflammatory state, suggesting it could also interact with PCSK9 at this stage. As stated earlier, PCSK9 is majorly synthesized by the liver as a 74-kDa zymogen. Among the PCs, PCSK9 is the only convertase that undergoes autocatalytic cleavage once, generating the secreted protein containing the excised prodomain bound non-covalently to the catalytic/C-terminal domain segment [13]. Evidence indicates that the other PCs' second cleavage potentiates their enzymatic potentials [36, 40]. Proteolytic cleavage of PCSK9 by furin might represent a second cleavage, potentially leading to novel physiological function, as seen with the other PCs after the second autocatalytic cleavage. Nature may have designed furin-proteolyzed PCSK9 to function in certain situations based on the encounter level between the two proteins. For example, LDL-bound PCSK9 was suggested to be protected against furin-induced proteolysis [265], perhaps reducing the free PCSK9 that furin can encounter and cleave. Although, the basis for this observation or the physiological relevance of this induced protection was not clarified in the said study. However, LDL-bound PCSK9, when exposed to an in vitro recombinant human furin (rhfurin), resulted in a cleaved PCSK9 variant (result not shown), suggesting that LDL-bound PCSK9 is not protected against induced furin proteolysis in vitro. Several studies indicate that f-PCSK9 is active [120, 265, 269], and LDL- bound PCSK9 potentiates this variant's ability to induce the degradation of the LDLR [265]. However, we did not observe robust LDLR-dependent uptake of f-PCSK9, nor its ability to degrade the LDLR, which is consistent with the notion that this variant is defective for LDLR binding due mainly to the removal of the excised 7-kDa of the N-terminus of the catalytic domain and, therefore, it is inactive **(Figure 14 and 15)** [18-20, 270].

The endocytic uptake of PCSK9 and LDLR and that of PCSK9 and APLP2 (amyloid precursor- like protein 2) complex is one of the primary means of plasma PCSK9 clearance, although the former

(PCSK9 and LDLR) is well characterized. However, this interaction also leads to the degradation of both proteins [14, 271]. As mentioned, f-PCSK9 is inert in LDLR binding, suggesting a different route for this variant's clearance. However, a recent study showed a shorter half-life of f-PCSK9 in a pulse-chase experiment when expressed in HEK293 cells compared to intact PCSK9 [120]. It is possible that this latent binding motif revealed by the cleavage by furin could potentially avail f-PCSK9 with diverse arrays of ligands for endocytic uptake/clearance. OxLDL belongs to a family generally referred to as a "danger-associated molecular pattern" (DAMP) generated in response to inflammation, tissue injury, or oxidative stress [205]. OxLDL interacts with specific receptors, eliciting a pro-inflammatory or innate immune response. Our observation here that f-PCSK9 shows preferential binding to oxLDL **(Figure 11D)** could partly explain why f-PCSK9 levels predict future coronary outcomes, as reported by Kataoka et al. [263]. Although speculative, oxLDL binding could convert f-PCSK9 to a DAMP, or f-PCSK9 could behave as a DAMP directly. Interestingly, this line of thought is supported by an earlier report that found upregulation of intact and f-PCSK9 after MI [121].

Furthermore, our results also indicate that binding of f-PCSK9 to native or modified LDL does not improve LDLR-dependent uptake of this variant as was first thought that this could allow f-PCSK9 to act as a passive component of LDLR:LDL endocytosis; however, this enhanced interaction could influence the uptake of this variant through an alternate lipoprotein, or possibly oxidized lipoprotein receptors.

3.4 Materials and Methods

Plasma collection

Blood samples were drawn in the morning from eight healthy volunteers – 4 males and four females into commercially available evacuated EDTA tubes. Plasma was obtained by low-speed centrifugation (2000 g for 20 minutes at 4ºC), a clear supernatant was obtained, and the 8 samples pooled

together. The pooled sample was used immediately for LDL-bound PCSK9 (intact and f- PCSK9) isolation, and protease inhibitor (1 mM PMSF and 50 unit/liter of aprotinin) was added to the rest and stored at -80ºC.

Optiprep plasma separation of LDL-bound PCSK9

Plasma LDL-bound PCSK9 separation was described previously with slight modification [169]. Briefly, the binding reaction of 3.3 ml total volume containing 0.8 ml of pooled plasma, 2 ml low- salt HBS-C buffer (25 mM HEPES-KOH, pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$), 0.5 ml of 60% Optiprep and placed in a 3.3 ml Beckman Optiseal tube and overlay with 0.3 ml of low-salt HBS- C buffer until the meniscus is below neck of tube. Centrifugation was done at 100,00rpm using Optima TLX with decel "9" for 2.5 h at 4ºC. Following centrifugation, an LDL-containing fraction of 0.5 ml was drawn using a 22G needle and immunoprecipitated with anti-serum 1697 directed against human PCSK9, and relative LDL-bound intact- and f-PCSK9 was determined by western blotting.

Oxidation of LDL

LDL oxidation was described [272] with slight modification. Briefly, 100µg/mL of native LDL was incubated with 5µM of copper sulfate (oxidant) in 1mL of 1X PBS (phosphate buffered saline – pH-7.4) at 37ºC for 24 h. Further oxidation was terminated by adding 50µM of 1mM EDTA (A copper chelator). The oxLDL preparation is stored away from light by wrapping it with foil paper and kept at 4ºC for subsequent usage.

Furin-cleaved PCSK9 preparation

A concentrated condition medium containing C-terminally FLAG-tagged full-length PCSK9 (intact) (1mL) was incubated with recombinant human furin (4.6µg) (R&D System) for six h at room temperature. According to the manufactural instructions, further proteolysis was stopped by adding furin inhibitor 1 (EMD Millipore). The cleaved PCSK9 obtained was either used immediately or stored for future usage at -20ºC. Large-scale furin-cleaved was prepared by transiently transfecting HEK 293 cells maintained in monolayer cultures (5 x 10cm dish, Corning) at 37ºC and 5% CO2 in medium B, and upon ~80% confluency, each dish was co-transfected with 3 µg of C-terminally FLAG-tagged full-length WT-PCSK9 plasmid and either in with 3µg human furin plasmid or without furin and 20ul of Polyjet (FroggaBio) as per the manufacturer protocol. 18 h post-transfection, cells were washed 2X with warm PBS and incubated for 48 h in medium A supplemented with 1X ITS. After 48 h, media was collected and centrifuged at 1000xG for 5 minutes so any floating cells could be pelleted; the supernatant was carefully transferred to a 10-kDa Amicon Ultra-4 centrifugal filter unit and concentrated 10-fold. Concentrated conditioned media were transferred to new tubes, and PCSK9 concentration in condition media was determined by western blot using purified PCSK9 as a standard before storing it at - 20ºC for subsequent usage.

LDLR-dependent PCSK9 uptake in the presence of LDL

HEK 293 cells were maintained in monolayer cultures (6-well dish, Corning) at 37ºC and 5% CO2 in medium B, and upon ~80% confluency, cells were transiently transfected with empty vector plasmid or human LDLR (0.5µg per well) and 1.5 ul of Polyjet (FroggaBio) as per the manufacturer protocol. 24 hours post-transfection, the cells were treated in the presence of 50 µM chloroquine and incubated for 3 hours with intact (UC) or f-PCSK9 (FC) (~3µg), preincubated in the absence (-) or presence (+) of native LDL (1mg) for an hour at 37ºC. Following 3 hours of incubation, cells were then lysed. uBCA was done

to quantify the amount of protein to be loaded. Protein was resolved by SDS-PAGE and detected by western blotting using α-actin as a loading control.

Liposome preparation

Liposome preparation was described [273] with slight modifications. Briefly, 1.6µmole of phosphatidylcholine (PC) (1,2-Dioleoyl-sn-glycero-3-phosphocholine), 0.2µmole of cholesterol (Chol), and 0.2µmole of sphingomyelin (SM) (all from Avanti) were dissolved in a 12X75mm glass tube (Fisher Scientific) containing chloroform along with 1µL of a fluorescent-labeled phospholipid derivative NBD-PE. The mixture was thoroughly mixed before being dried under a gentle stream of Nitrogen gas (N2), after which the tube was placed in a speed vacuum (Speed- vac) for 45 minutes. Following Speed-vac, the air inside the tube was displaced with N2, sealed with a plastic cap, and immediately placed at -20ºC overnight until the next day. The following day, the dried lipid mixture was resuspended in 3mL low-salt HBS-C (25 mM HEPES-KOH, pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$) and vortex for 1 h at room temperature to obtain multilamellar vesicles (MLV), Following the 1 h vortex, extrusion was done by passing the MLV 15X through 50 nm membrane using the liposoFast to make of small unilamellar vesicles (SUV). Extruded liposome was stored away from light at room temperature and used within three days.

Liposome binding assay

Binding reactions (400µL), each containing 15.9µg of either intact- or f-PCSK9, 200 µL extruded liposome, and low-salt HBS-C (25 mM HEPES-KOH, pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$) were incubated for 45 minutes at 37ºC. Following 45 minutes of incubation, ~10% final optiprep containing 300µL from the reactant above, 150µL 60% optiprep, and 550µL low-salt HBS-C and placed in 7X20mm centrifuge tube (Beckman Coulter) and mix by inversion. The mixture was underlay with 700µL low-salt HBS-

C to cushion and overlay with ~600µL low-salt HBS-C. Following 1 h of ultracentrifugation of 39000rmp at 4ºC, the supernatant was removed, and the pellet was redissolved in the same amount with low-salt HBS-C. The relative liposome-PCSK9 binding was determined by western blotting.

See Chapter 2, section 2.4 for LDL binding and LDLR degradation assays.

Chapter 4: Furin proteolytic processing of PCSK9 potentiates its bioactivities.

4.1 Introduction:

PCSK9 is a central regulator of circulating LDL-C levels and related CVD risk, such as atherosclerosis development and progression, by disrupting the natural recycling of the LDLR [11, 13, 14]. PCSK9 binds the LDLR at the cell surface through a protein-protein interaction, leading to the degradation of this complex inside the cell, thereby reducing the number of LDLRs at the cell surface for endocytic uptake of LDL-C [1, 11, 14]. Elevated plasma LDL-C is critical in atherosclerotic plaque initiation and progression [1]. However, it has also been suggested that damage to the endothelium is as vital in atherosclerotic plaque initiation as the infiltration and the subsequent modification in the vascular wall by these lipoproteins [8]. Recent evidence indicates that PCSK9's link to atherosclerotic plaque initiation and progression extends beyond plasma LDL-C and LDLR regulation, with evidence that PCSK9 is implicated directly through foam cell formation, endothelial dysfunction, increased inflammatory cytokines storm, platelet activation, and the regulation of the redox system [21-24].

As mentioned, intact and truncated (f-PCSK9) forms of PCSK9 are found in circulation [18-20, 120, 121]. The latter is a product of limited proteolysis by furin [18-20]. Furin expression is upregulated in an inflammatory state, which activates factors that increase inflammation [51, 52], suggesting a feedforward mechanism. Furin-dependent mechanisms have also been proposed to play a role in atherosclerotic plaque progression [51, 52]. A rapid turnover of intact PCSK9 to f- PCSK9 in response to a systemic or local inflammatory state due to increased furin expression may also be at play in the atherosclerotic plaque microenvironment. This notion is supported by a report suggesting a reduction in atherosclerotic plaque size in patients on PCSK9 inhibitors [274]. This may be attributed to an LDL-C lowering effect and also inhibition of f-PCSK9 generation. Indeed, this may partly explain the other beneficial properties attributed to anti-PCSK9 antibodies that block the protein-protein interaction of PCSK9 and LDLR.

Oxidation of LDL within the arterial wall has been shown to drive plaque progression [275]. OxLDL induces proinflammatory signaling because it is captured by receptors such as the CD36 and LOX1 expressed in endothelial cells and immune cells [276]. We have evidence that f-PCSK9 binds much more efficiently to oxLDL than intact PCSK9 (see **Figure 11C**). OxLDL could bridge f-PCSK9 to these receptors or vice/versa, fueling chronic inflammation. Furin upregulation in this inflammatory state could lead to f-PCSK9 generation in a positive feedback mechanism. This notion is supported by evidence indicating that PCSK9 induces CD36 degradation and crosstalk with LOX1 [204, 229]. The latent membrane binding motif revealed by the limited proteolysis of PCSK9 by furin **(see Chapter 3 Figure 13)** could allow this variant to interface with CD36 or LOX1, which both localize to plasma membrane caveolae/lipid rafts, and oxLDL involvement could enhance this interaction further without necessarily leading to CD36 degradation. This raises the possibility that f-PCSK9 binding these receptors could facilitate its cellular uptake, establishing a clearance channel. **Therefore, we hypothesize that furin proteolysis improves PCSK9/lipoprotein binding interactions and positively impacts atherosclerosis progression by mediating scavenger receptor interaction of proinflammatory oxLDL.**

4.2 Results

Inflammation induces PCSK9 mRNA expression in the liver and targeting it through attenuating its action using PCSK9 inhibitory monoclonal antibodies (PCSK9i-mAbs) alleviate oxidation, inflammation, and atherosclerosis [193, 212], suggesting that factors that upregulate PCSK9 expression would also upregulate the f-PCSK9 variant. A recent report that f-PCSK9 concentration predicts future coronary events [263] reinforces this notion. However, it remains unknown if f- PCSK9 is merely a predictive marker of coronary events or if it plays a causative role. Based on the above, we obtained the serum of LPS-treated mice (1 mg/kg body weight, intraperitoneally) 24 hours post-treatment. LPS treatment

induces inflammation [277], and Feingold et al. [212] had earlier reported the upregulation of PCSK9 mRNA in the livers of LPS-treated mice. With the serum obtained, we immunoprecipitated with anti-serum 1783 directed against mouse PCSK9. As seen below, we confirmed the upregulation of the intact PCSK9 protein and a truncated variant consistent with the furin-proteolyzed PCSK9 variant **(Figure 16)**.

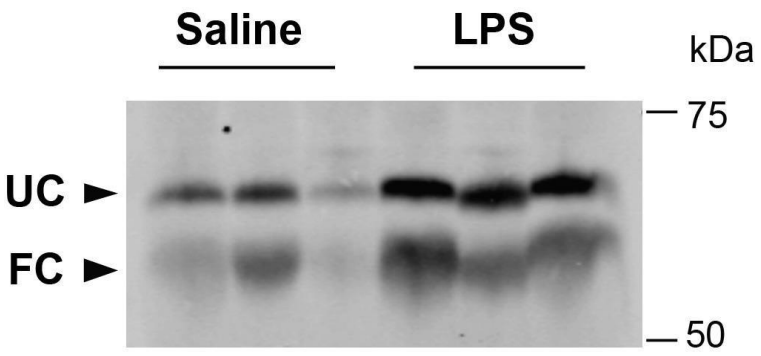


Figure 16 Inflammation enhances f-PCSK9 expression:

Serum of LPS-treated female C57Bl/6 mice was obtained 24 hours post-treatment. Immunoprecipitation (IP) was done on the LPS-treated and saline-treated (control) groups. Protein was resolved on SDS-PAGE and detected by western blotting. N=3 for each group. UC: Uncleaved PCSK9; FC: Furin-cleaved

Recent evidence suggests PCSK9 increases markers of inflammation [193, 266], and as mentioned above, inflammation also upregulates PCSK9 expression, suggesting a feedforward mechanism. We hypothesize that f-PCSK9 could also upregulate markers of inflammation since it is also upregulated in this phase. To test this working hypothesis, THP-1-derived macrophages were polarized into M0 (untreated) and proinflammatory M1 state as described [278], with slight modification; briefly, cells were treated with 100 ng/ml LPS and 20 ng/ml IFN-Ɣ, 24 hours post- treatment, cells were further treated with intact PCSK9 or f-PCSK9 (1 µg/well). Total RNA was isolated using a Trizol reagent and processed for reverse transcription. qRT-PCR analysis was performed on some selected markers of inflammation (IL-1β, CD36, LOX1, TNF α) using the Universal SYBR Green Supermix (Roche) and gene expression

were normalized to HPRT or SRP14. We observed that IL-1β and TNF-α expression were significantly higher in the f-PCSK9 treated cells **(Figure 17A** - D**)**. Of note was a significant increase of LOX1 mRNA expression in f-PCSK9-treated M0 macrophages **(Figure 17C)**, suggesting this variant could be driving M0 to the M1 phenotype. It is known that oxLDL has a role in proinflammatory signaling [276]. To test if f-PCSK9 could be involved, THP-1-derived macrophages were polarized into resting macrophages (M0), proinflammatory (M1), and anti-inflammatory (M2) states and treated with either intact PCSK9 or f-PCSK9 in the presence of oxLDL. As seen **(Figure 18)**, f-PCSK9 was internalized in M1 macrophages at a much greater level than the intact PCSK9, indicating that this variant could be the dominant PCSK9 in this phase. It is possible that this uptake could be upregulated in the presence of cell surface scavenger receptors. We also tested this possibility by transiently transfecting HEK293 cells with plasmids encoding Scavenger receptor class B1 (SCARB1), CD36, or LOX1, and 24 hours post-transfection, the cells were treated with intact or f-PCSK9 in the absence or presence of oxLDL. There was no significant difference between vector control and SCARB1-dependent uptake of intact and f-PCSK9 **(Figure 19)**, whereas uptake of f- PCSK9, but not the intact PCSK9, was increased in cells overexpressing either CD36 or LOX1, and this was further enhanced in the presence of oxLDL (**Figure 20 A and B)**, suggesting a possible role of this variant in inflammatory signaling.

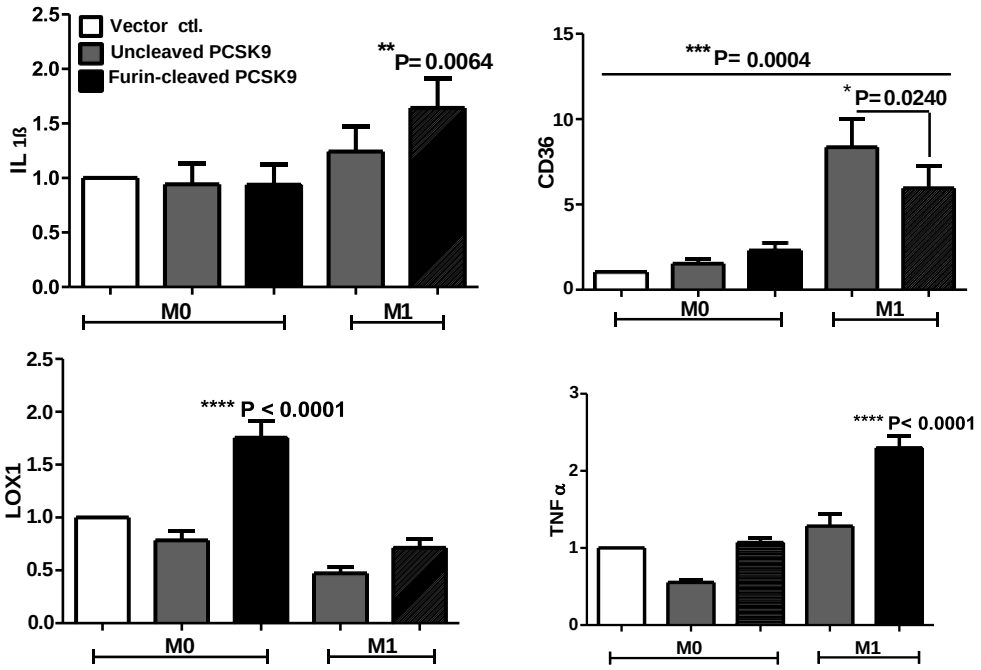


Figure 17: Markers of inflammation are significantly enhanced by f-PCSK9 expression.

THP-1 undifferentiated macrophages were either polarized to M1 (100 ng/mL LPS and 20 ng/mL IFN- Ɣ) or M2 (20 ng/mL IL-4 and 20 ng/mL IL-13) and incubated for 24 hours. Following 24 h incubations, cells were treated with either intact – or f-PCSK9 (~1µg) for a further 24 hours incubation period, after which total RNA was harvested using Trizo reagent according to the manufacturer's instructions. cDNA reactions were prepared from 800 ng of total RNA using the Roche transcription first strand cDNA synthesis kit per the manufacturer's instructions, and a 1:5 dilution was made for qPCR. Using IL-1β, CD36, LOX-1, and TNF-α primers and SYBR green dye, real-time quantitative PCR was carried out, and data normalized to HPRT1 gene expression and fold change relative to control (A) IL-1β, (B) CD36, (C) LOX1, and (D) TNF-α. Quantification shown in the graph represents mean n=3 with standard error, and stars indicate statistical differences according to one sample t-test.

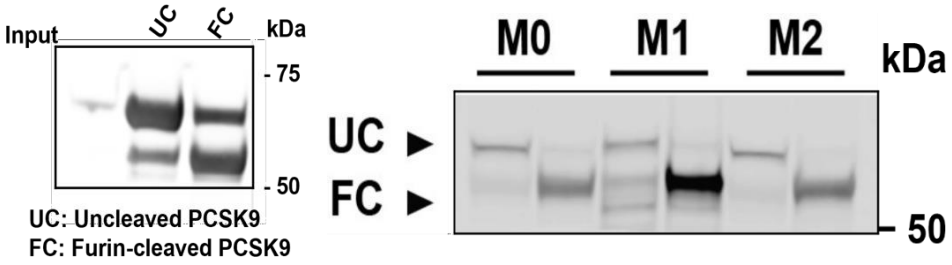


Figure 18: Furin proteolysis of PCSK9 improves its uptake in immune cells.

THP-1 cells were polarized into resting (M0), pro-inflammatory (M1), and anti-inflammatory (M2) macrophages. 24 hours after polarization, cells were treated with intact (UC) or f-PCSK9 (FC) for 3 hours at 37ºC. Cells were lysed, and uBCA was done before it was resolved by SDS-PAGE and detected by western blotting. The blot represents 3 independent experimental repeats**.**

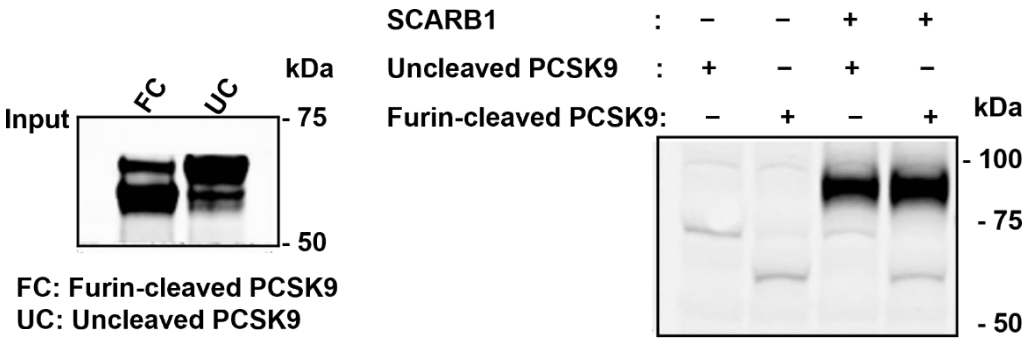


Figure 19: Scavenger receptor class B does not interact with PCSK9.

Input PCSK9 (A) consisting of predominantly uncleaved (intact) (UC) or furin-cleaved (FC) was incubated for 3 hours in HEK293 cells transiently transfected with vector control plasmid or plasmids expressing SCARB1in the presence of chloroquine (CQ); the role of the CQ is to prevent the lysosomal degradation of PCSK9 by raising the lysosomal pH. Following the 3 hours incubation, cells were then lysed. uBCA was done to quantify the amount to be loaded, and proteins were resolved by SDS-PAGE and detected by western blotting using α-actin as a loading control.

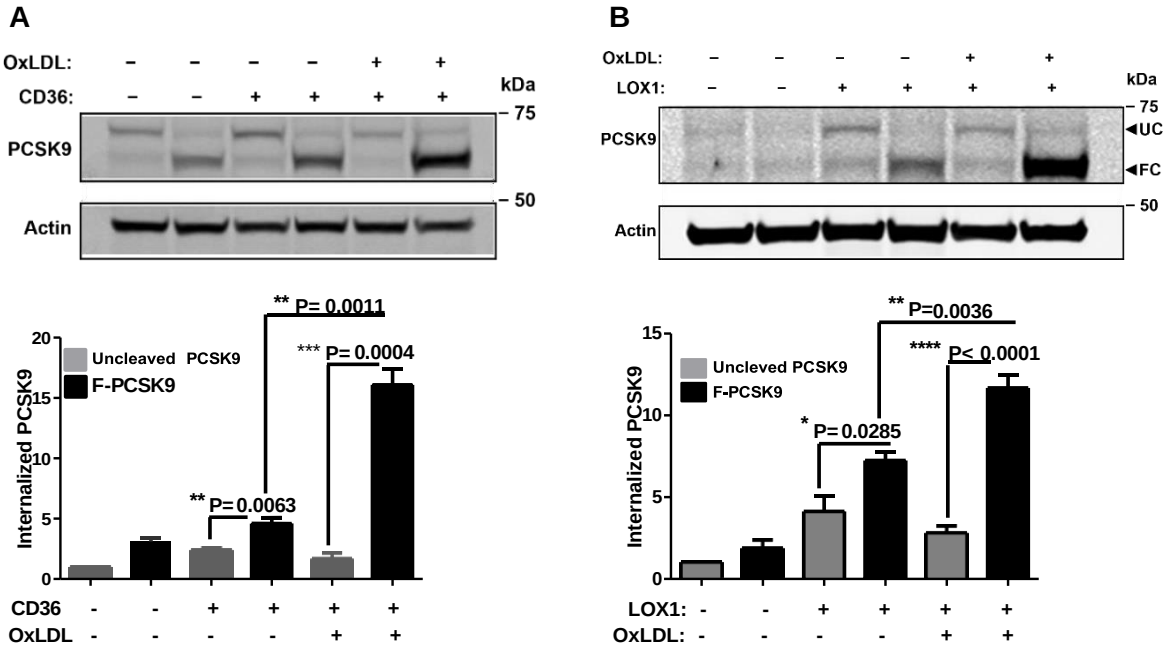


Figure 20: F-PCSK9 interacts with CD36 and LOX-1.

HEK293 cells were transiently transfected with vector control plasmid or plasmids expressing (A) CD36 or (B) LOX1. 24 hours post-transfection, the cells were treated and incubated for 3 hours with uncleaved (UC) or f-PCSK9 (FC) (5 µg), preincubated in the absence (-) or presence (+) of oxLDL (0.5µg/ml) for an hour at 37ºC. Following 3 hours of incubation, cells were then lysed, and uBCA was done to quantify the amount of protein to be loaded. Protein was resolved by SDS-PAGE and detected by western blotting using α-actin as a loading control. Quantification shown in the graph represents mean n=3 with standard error, and stars indicate statistical differences according to one sample t-test

Several clinical studies have shown the beneficial effects of PCSK9i-mAbs [21, 22, 193, 261, 262, 274]; this effect is possibly achieved, in part, by preventing the action of furin on PCSK9, which would ordinarily lead to f-PCSK9 generation. When this possibility was tested by preincubating with an inhibitor, such as alirocumab, furin proteolysis of PCSK9 was blocked, as seen in **Figure 21**.

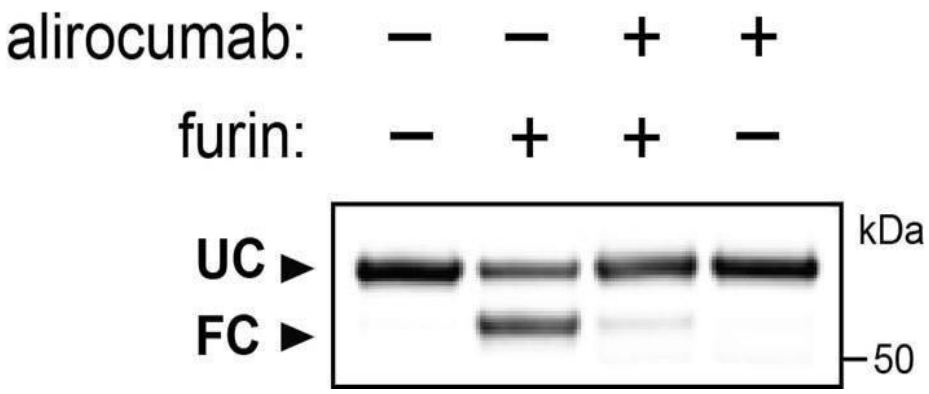


Figure. 21: Alirocumab inhibits furin-induced PCSK9 proteolysis.

C-terminally FLAG-tagged full-length PCSK9 (intact) (1µg) was preincubated for 1 hour at 37ºC in the absence (-) or presence (+) of alirocumab (0.5µg). Following the 1-hour incubation, reactants were further incubated for 6 hours at room temperature in the absence (-) or presence (+) of recombinant human furin (0.44µg). Further proteolysis was stopped by adding 1X Bolt buffer and 1X DTT and boiled for 5 minutes at 96ºC. Protein was resolved on SDS-PAGE and detected by western blotting. Blot is a representation of two independent experimental repeats.

4.3 Discussion:

Consistent with earlier reports that local and systemic inflammation upregulates intact PCSK9, herein, we show that f-PCSK9 was also upregulated along with the intact PCSK9 in LPS-treated mice **(Figure 16)**, suggesting that factors that upregulate intact PCSK9 also upregulate the cleaved variant. As reported by Feingold et al. [212], the upregulation of intact PCSK9 in an inflammatory state is not a product of SREBP activation, with the conclusion that the increased expression is likely through the decrease in the activities of FXR (farnesoid X receptor)/or PPAR-α (peroxisome proliferator-activated receptor α) that negatively regulate PCSK9 mRNA expression and/or increased activity of the transcription factor HNF-1α as a positive regulator. Still, this increase in intact PCSK9 expression may not fully explain the rationale behind the upregulation of the f- PCSK9 variant in this setting. As mentioned earlier, inflammation also upregulates furin expression [51, 52]. Increased furin activity within this inflammatory phase would likely enhance the interaction or contact of furin and intact PCSK9, generating the f-PCSK9 variant. The longer the inflammatory phase, the more f-PCSK9 generation would occur. This suggests that the upregulation of intact PCSK9 acts in synergy with that of furin, leading to the increased plasma levels of the f-PCSK9 variant. In support of this notion, we observed a robust uptake of the f- PCSK9 variant in proinflammatory M1-polarized macrophages, mirroring the acute inflammatory state **(Figure 18)**. It is also possible that other molecular mechanisms might be at play as well.

TNF-α (Tumor necrosis factor -α) and 1L-1-β (Interleukin 1-β) are two families of cytokines implicated in the acute inflammatory phase [279, 280]. Ding et al. [281] reported an increase in PCSK9 secretion by IL-1-β involving the NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) inflammasome. Inflammation and oxLDL are known activators of the NLRP3 inflammasome, and Katsuki et al. [282] reported that exogenous PCSK9 induced mRNA expression of markers of inflammation such as TNF-α, IL1-β, etc. This report also corroborated an earlier account of

the upregulation of markers of inflammation by exogenous PCSK9 [283]. However, these studies did not explore whether this effect was due to intact PCSK9 or f-PCSK9, which may have also been present. Indeed, in our experience, any preparation of PCSK9, whether purified or in conditioned medium, will invariably contain f-PCSK9. As noted, furin is also upregulated in the inflammatory state, so one cannot rule out the possibility of the f- PCSK9 variant contribution in a physiological setting. Herein, we observed an increase in the mRNA of TNF-α and IL-1β in THP-1 macrophages treated with the f-PCSK9 variant **(Figure 17A & D)**. Our working hypothesis was that f-PCSK9 might function as a 'DAMP' independently or in conjunction with other ligands involved in the innate immune response. The increased mRNA of TNF-α and IL-1β observed here supports the f-PCSK9 'DAMP' concept. This line of thought might indeed be the likely scenario as we also observed an upregulation of mRNA of LOX1 in the f-PCSK9 treated M0 macrophages **(Figure 17C)**, suggesting that f-PCSK9 could contribute to driving this phenotype to an acute inflammatory state.

Limited proteolysis by enzymes such as furin can not only inactivate substrate molecules, as in the case of the inability of f-PCSK9 to bind to LDLR but can also, in some cases, generate new bioactivities in substrate molecules [31]. Our data in **Figure 13** (Chapter 3) suggests that removal of ~65 amino acids (Ser153 – Arg218) from the N-terminus of the catalytic domain of PCSK9 by furin [19] reveals a latent membrane binding ability, which could facilitate its recognition by scavenger receptors LOX1 and CD36 **(Figure 20)**, and possibly other ligands associated with Chol/SM rich membranes such as cellular caveolae/lipid rafts and the outer membrane surface of circulating LDL particles. This recognition or binding might be weak, requiring a high concentration of PCSK9 to elicit these interactions *in vitro*. However, it is important to state that PCSK9 levels are upregulated in an inflammatory state, not only systemically (mainly liver-derived) but also locally by various cell types, including vascular endothelial cells at sites of inflammation. Thus, local PCSK9 concentrations may be much higher at sites of vascular

inflammation than those present in plasma in the basal state. Because factors that upregulate inflammatory conditions are constantly enhanced, leading to the worsening of this phase, local PCSK9 concentrations, including f-PCSK9, might also mirror these factors. Although CD36 and LOX1-induced uptake of exogenous f-PCSK9 was enhanced in the presence of oxLDL, it is currently unclear if this is due to oxLDL binding to the scavenger receptor, with f-PCSK9 as a passive component of the oxLDL, or if oxLDL association in some way improves the ability of the f-PCSK9 to interact with CD36 or LOX1. Of note, evidence suggests PCSK9 induces the degradation of CD36, while crosstalk exists between PCSK9 and LOX1 [204, 218, 229]. Although our focus here was f-PCSK9 cellular uptake and possible downstream signaling effects, it would be interesting to see if the f-PCSK9 variant also induces CD36 degradation. Furthermore, PCSK9i- mAbs, such as Alirocumab, are suggested to attenuate arterial wall inflammation [284]. Inhibition of furin-mediated proteolysis by PCSK9i-mAbs at vascular sites of inflammation would alter the generation of local f-PCSK9, significantly reducing the inflammatory signaling pathway. In support of this possibility, the furin-cleavage site in PCSK9 is within the binding interface to the LDLR EGF-A domain, the target site of PCSK9i-mAbs. Indeed, we observed that the PCSK9i alirocumab blocks the cleavage of PCSK9 by furin **(Figure 21).** This is also consistent with an earlier report by Schroeder et al., who were interested in exploring whether therapeutic Abs targeting PCSK9 would have a lesser inactivating effect on PCSK9-mediated LDLR degradation if the furin site was also blocked [270]. It is necessary to state that inhibition of furin-mediated PCSK9 proteolysis might not be the only way these PCSK9i-mAbs reduce inflammation; it might also involve multiple other factors. For example, blocking the PCSK9:LDLR interaction, which is the primary objective of these therapies, significantly reduced plasma LDL-C, and this would reduce the infiltration and modification of LDL-C within the vascular wall [8]. Of note, oxLDL is the primary source of cholesterol accumulation in macrophages [285]. As shown in **Figure 11C**, f-PCSK9 readily interacts with oxLDL. Perhaps this

interaction would enhance the further accumulation of oxLDL in a vicious cycle, ultimately enhancing the formation of foam cells. Inhibiting the generation of f-PCSK9 and thereby lowering the interaction of oxLDL with f-PCSK9 could be another way by which PCSK9i-mAbs arrest inflammation. In conclusion, although inert in LDLR degradation or perhaps other cellular functions, our studies support that proteolytic processing of PCSK9 by furin potentiates its bioactivities in stimulating inflammation.

4.4 Materials and Methods.

Animal Care

C57Bl/6 (WT) female mice were cared for according to the Canadian Council on Animal Care Guidelines, 2020. Mice were maintained and housed in the Animal Care and Veterinary facility at the University of Ottawa Health Institute, and all experiments were approved (animal use protocol HI-2909) by the Institute.

Lipopolysaccharide (LPS) Treatment

LPS from Escherichia coli (Sigma-Aldrich, L4391) was diluted with sterile PBS to a working solution. Either saline or LPS working solution was put into a 0.5 mL syringe with a 30 G needle. Female C57BI/6 mice (6-10 weeks old), previously maintained on a standard laboratory diet, were weighed. Saline or LPS was intraperitoneally injected into the mice as per their body weight (1 mg/kg body weight). Following 24 h post injections, Mice were sacrificed, and blood was collected through cardiac puncture into a sterile tube and left for 20 minutes for the blood sample to clot. Following clotting, the tubes were centrifuged at 12,000 RPM for 10 minutes. The clear supernatant (serum) was collected and stored at -80ºC for subsequent utilization.

RNA Isolation and RT-qPCR

THP-1 cells were maintained in medium G until the cells reached confluency ($1.5x10^6$ cells/mL). To turn THP-1 monocytes into undifferentiated macrophages (M0), the cells were incubated with 100 nM PMA at 37ºC in a CO2 chamber for 72 h. Following 72 h incubation, THP-1 undifferentiated macrophages were either polarized to M1 (100 ng/mL LPS and 20 ng/mL IFN-Ɣ) or M2 (20 ng/mL IL-4 and 20 ng/mL IL-13) and incubated for another 24 h. Following 24 h incubations, cells were treated with

either intact – or f-PCSK9 (~1µg) for a further 24 h incubation period, after which total RNA was harvested using Trizo reagent (Roche Diagnostic) according to the manufacturer's instructions. cDNA reactions were prepared from 800 ng of total RNA using the Roche transcription first strand cDNA synthesis kit per the manufacturer's instructions, and a 1:5 dilution was made for qPCR. Using the primers' sequence of interest and SYBR green dye (Roche Diagnostic), real-time quantitative PCR was carried out, and data normalized to either HPRT1 or SRP14 gene expression and fold change relative to control was calculated.

Cellular PCSK9 uptake in THP-1 cells

THP-1 cell was maintained in medium G until the cell reached confluency ($1.5x10^6$ cell/mL). To turn THP-1 monocytes into undifferentiated macrophages (M0), the cells were incubated with 100 nM PMA at 37ºC in a CO_2 chamber for 72 h. Following 72 h incubation, THP-1 undifferentiated macrophages were either polarized to M1 (100 ng/mL LPS and 20 ng/mL IFN-Ɣ) or M2 (20 ng/mL IL-4 and 20 ng/mL IL-13) and incubated for another 24 h. ~5 µg of either intact or f-PCSK9 was preincubated with 0.5 mg of oxLDL for 1 h at 37ºC. Following 1 h incubation, cells were treated with PCSK9/oxLDL complex for a further 3 h in the presence of 50 µM chloroquine before cell harvest. Relative cellular PCSK9 uptake was determined by western blotting.

Inhibition of Furin-induced Proteolysis Assay

C-terminally FLAG-tagged full-length PCSK9 (intact) (~1µg) was preincubated for 1 hour at 37ºC in the absence (-) or presence (+) of alirocumab (0.5µg). Following the 1-hour incubation, reactants were further incubated for 6 hours at room temperature in the absence (-) or presence (+) of recombinant human furin (0.44µg). Further proteolysis was stopped by adding 1X Bolt buffer and 1X DTT (reducing agent) and

boil for 5 minutes at 96ºC. Protein was resolved on SDS-PAGE and detected by western blotting. See Chapter 3, section 3.4 for f-PCSK9 generation and cellular PCSK9 uptake in HEK293 cells.

Chapter 5: Conclusion and future study directions.

This dissertation sets out to expound on alternate confirmations of PCSK9 and the functional role of these conformations on PCSK9 biology. Specifically, this work sheds light on the structure- function aspects of PCSK9 biology to further enhance our understanding of the role of structural changes in i) the PCSK9-LDL interaction, ii) PCSK9's ability to regulate LDLR, and iii) PCSK9's role in inflammation.

i) PCSK9-LDL interaction: While the molecular site in apoB100 that binds PCSK9 is currently unidentified and would need further studies, below are some key findings from this work in the context of the LDL-PCSK9 interaction. For the first time, we show that LDL binds and associates with human circulating cleaved-PCSK9, presumably with the prodomain attached **(chapter 3)**. Although furin-cleaved GOF PCSK9 mutants such as S127R-PCSK9 and R496W-PCSK9 abolished *in vitro* LDL association, it appears the prodomain of these variants still retain some form of LDL binding (result not shown). A previous study from our group established **Gln31 – Leu52** in PCSK9 prodomain as necessary residues significant for LDL binding, as deleting these amino acids abolishes this function [169]. This suggests that the prodomain might not only be essential in LDL-PCSK9 interaction but could be the critical domain in this interaction or possibly act independently. One way to verify the role of PCSK9 prodomain in LDL interaction in future work would be to generate a PCSK9 prodomain alone (by inputting a stop codon at Q152 through mutagenesis) variant in fusion protein such as glutathione S-transferase (GST)-tag, either in WT-PCSK9 or Del35-PCSK9 background and test LDL binding potency. Of note, our in vitro LDL-f-PCSK9 and oxLDL-f-PCSK9 interaction was enhanced 1.5-fold and 10-fold, respectively, possibly because the excision by furin of aa S153 to R218 may have exposed a latent binding motif in PCSK9, suggesting that the N-segment in PCSK9 catalytic domain is inhibitory to nLDL and

oxLDL binding. Furin-mediated proteolysis and membrane association, as we also report a preferential binding of cleaved PCSK9 with liposomes, could result in global conformational changes in PCSK9 that potentially affect its critical functional properties. One possible way to go about this in future work would be through structural studies such as mapping of this N-segment's functional role by performing alanine scanning to identify essential residues and through modeling using X-ray structure determination of intact- and f-PCSK9 bound to phospholipid-based detergent bicelles to mimic the effect of biological membranes. The rationale is that improved lipid binding of f-PCSK9 would result in crystal formation in the presence of phospholipid-based detergent bicelles, and this can be used to identify membrane-interacting regions of PCSK9. Also, determining how much cleaved circulating PCSK9 is bound to oxLDL would be interesting. As previously stated, cleaved PCSK9 is inert in LDLR degradation; thus, the physiological relevance of LDL-bound cleaved PCSK9 is unclear in the regulation of LDLR. Clarifying the functional role of this variant in future work would enhance our understanding of this species in human circulation.

ii) Regulation of LDLR by PCSK9: PCSK9's ability to regulate LDLR, as seen in **Chapters 2 and 3**, mainly depends on PCSK9 structural integrity. PCSK9 forms a protein-protein interaction with the LDLR. A large body of evidence has established the EGF-A domain of LDLR and the catalytic domain of PCSK9 as the primary binding interface; however, despite being far removed from this interface, the PCSK9 prodomain IDR is a potent allosteric autoinhibitor of LDLR binding. Disruption of the molecular nature of the IDR of PCSK9 by deletion has been shown to enhance LDLR-PCSK9 interaction [169, 174]. Much emphasis is placed on regulating the activities of PCSK9 on LDLR by disrupting the binding interface at the EGF-A domain of LDLR and the catalytic domain of PCSK9. Although expensive, PCSK9i-mAb-based therapeutics are

highly successful in drastically reducing PCSK9-mediated LDLR degradation. An alternate route to regulate LDLR degradation, such as a small molecule PCSK9 inhibitor, could be designed to target the IDR of PCSK9 without necessarily disrupting the LDLR binding activity at the cell surface. Once within the acidic early endosomal compartment in a complex with the LDLR, this small molecule could trigger prodomain dissociation from PCSK9 so the LDLR would be released to recycle back to the cell surface. Our studies with using GSGG-PCSK9 in Chapter 2 provide a basis for the promise of this approach, as this variant with only a short sequence replacement in the prodomain IDR bound effectively to cell surface LDLRs but was less effective at inducing LDLR degradation in hepatic cells due to subsequent prodomain dissociation. As mentioned in Chapter 3, proteolyzed f-PCSK9 is inert in LDLR degradation, and mAb-based therapy such as alirocumab attenuates PCSK9's action on LDLR [286]. Therefore, at first glance, it would appear that furin proteolysis of PCSK9 and the mAb-based treatment accomplish a similar feat – upregulation of LDLR expression. However, limited proteolysis by enzymes such as furin can also generate new bioactivities in substrate molecules, as seen in Chapter 4 of this dissertation. As shown in Figure 21, the PCSK9i-mAb alirocumab blocks the proteolysis of PCSK9 by furin. Thus, this and evolocumab, the other clinically approved PCSK9i-mAb therapy, could have the dual benefit of preventing the PCSK9-LDLR interaction while also preventing the conversion of intact PCSK9 to pro-inflammatory f-PCSK9. However, these mAb therapies are costly, and designing novel PCSK9 inhibitors that consider the characteristics of the different subspecies of PCSK9 would be innovative.

iii) PCSK9's role in inflammation: As demonstrated in Chapter 4, proteolytic processing of PCSK9 potentiates its bioactivities not only towards LDL (native and oxidized) but also scavenger receptors such as CD36 and LOX1 as well as proinflammatory cytokine expressions. Plasma

intact-PCSK9 clearance occurs through binding to LDLR [14], and f-PCSK9 was shown to be reduced in evolocumab-treated patients [121]. However, to our knowledge, no studies have been performed *in vivo* to specifically study the role of f-PCSK9 in inflammation. Thus, it would be essential for future studies to evaluate the effect of proteolytic processing of PCSK9 on inflammation by specifically looking at the LDLR-independent role of PCSK9. One possible way to do this would be through AAV-PCSK9 vectors targeting various tissues. Liver-targeted AAV8- PCSK9 has been shown to increase susceptibility to Western diet-induced atherogenesis in mice and is widely used as a means to generate LDLR-deficient phenotype. Amino acid substitutions of interest could be introduced into the commercially available vector in which an AAV8-hPCSK9- D374Y (hPCSK9$^{\mathbf{DY}}$**)** transgene is driven by a liver-specific APOE/hAAT1 promoter, and through mutagenesis, furin-resistant R218S-PCSK9 (hPCSK9$^{\mathbf{DY/RS}}$), furin optimized RRR218RE (hPCSK9$^{\mathbf{DY/RRRRE}}$) or R496W, a mutant our group previously determined that abolishes LDL binding (hPCSK9$^{\mathbf{DY/RW}}$) could be expressed along with a luciferase (AAV8-Luc) control for any possible effect of AAV8 injection. Both sexes of wild-type mice should be investigated to examine the possibility of hormonal differences with the Western diet regime for 12 weeks to examine parameters of PCSK9 function in the context of intact PCSK9 vs. cleaved PCSK9 with or without LDL association. The use of different variants of PCSK9 would allow for discerning the effects of furin proteolysis and lipoprotein association on PCSK9-driven atherosclerosis development in the context of a high-fat Western diet. These types of *in vivo* experiments will be critical in moving forward toward improved therapeutics targeting PCSK9. They would also inform on the widespread use of AAV-PCSK9 vectors to induce an *Ldlr*-/- phenotype in experimental mice, as the generation of f-PCSK9 in these models could produce independent proinflammatory effects.

Conclusion

Knowledge of PCSK9 biology has grown beyond the regulation of LDLR protein levels. However, exploring alternate conformations of PCSK9 to design novel therapeutics for inhibiting PCSK9 bioactivities is still unexplored. As demonstrated through this dissertation, the removal of ~65 amino acids (Ser153—Arg218) from the N-terminal of the catalytic domain of PCSK9 by furin can elicit downstream signaling function. Disrupting the molecular nature of PCSK9 prodomain IDR can also affect its role in LDLR regulation.

This dissertation has demonstrated a new paradigm in PCSK9 biology, and this will further aid our understanding in designing future PCSK9 inhibitors directed at different conformations of PCSK9.

References

1. Goldstein, J.L. and M.S. Brown, *A century of cholesterol and coronaries: from plaques to genes to statins.* Cell, 2015. **161**(1): p. 161-172.
2. Cagnina, A., et al., *[Atherosclerosis, an inflammatory disease].* Rev Med Liege, 2022. **77**(5-6): p. 302-309.
3. Collaborators, G.B.D.C.o.D., *Global, regional, and national age-sex specific mortality for 264 causes of death, 1980-2016: a systematic analysis for the Global Burden of Disease Study 2016.* Lancet, 2017. **390**(10100): p. 1151-1210.
4. Poller, W.C., M. Nahrendorf, and F.K. Swirski, *Hematopoiesis and Cardiovascular Disease.* Circ Res, 2020. **126**(8): p. 1061-1085.
5. Bentzon, J.F., F. Otsuka, R. Virmani, and E. Falk, *Mechanisms of plaque formation and rupture.* Circ Res, 2014. **114**(12): p. 1852-66.
6. Hansson, G.K., A.K. Robertson, and C. Soderberg-Naucler, *Inflammation and atherosclerosis.* Annu Rev Pathol, 2006. **1**: p. 297-329.
7. Hansson, G.K., *Inflammatory mechanisms in atherosclerosis.* J Thromb Haemost, 2009. **7 Suppl 1**: p. 328-31.
8. Bartels, E.D., C. Christoffersen, M.W. Lindholm, and L.B. Nielsen, *Altered metabolism of LDL in the arterial wall precedes atherosclerosis regression.* Circ Res, 2015. **117**(11): p. 933-42.
9. Abifadel, M., et al., *Mutations in PCSK9 cause autosomal dominant hypercholesterolemia.* Nat Genet, 2003. **34**(2): p. 154-6.
10. Seidah, N.G., et al., *The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation.* Proc Natl Acad Sci

U S A, 2003. **100**(3): p. 928-33.

11. Lagace, T.A., et al., *Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and in livers of parabiotic mice.* J Clin Invest, 2006. **116**(11): p. 2995-3005.
12. Naureckiene, S., et al., *Functional characterization of Narc 1, a novel proteinase related to proteinase K.* Arch Biochem Biophys, 2003. **420**(1): p. 55-67.
13. Benjannet, S., et al., *NARC-1/PCSK9 and its natural mutants: zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol.* J Biol Chem, 2004. **279**(47): p. 48865-75.
14. Zhang, D.W., et al., *Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation.* J Biol Chem, 2007. **282**(25): p. 18602-18612.
15. Brown, M.S. and J.L. Goldstein, *A receptor-mediated pathway for cholesterol homeostasis.* Science, 1986. **232**(4746): p. 34-47.
16. Poirier, S., et al., *Dissection of the endogenous cellular pathways of PCSK9-induced low density lipoprotein receptor degradation: evidence for an intracellular route.* J Biol Chem, 2009. **284**(42): p. 28856-64.
17. Stein, E.A., et al., *Effect of a monoclonal antibody to PCSK9 on LDL cholesterol.* N Engl J Med, 2012. **366**(12): p. 1108-18.
18. Han, B., et al., *Isolation and characterization of the circulating truncated form of PCSK9.* J Lipid Res, 2014. **55**(7): p. 1505-14.
19. Benjannet, S., et al., *The proprotein convertase (PC) PCSK9 is inactivated by furin and/or PC5/6A: functional consequences of natural mutations and post-translational modifications.* J Biol Chem, 2006. **281**(41): p. 30561-72.

20. Essalmani, R., et al., *In vivo evidence that furin from hepatocytes inactivates PCSK9.* J Biol Chem, 2011. **286**(6): p. 4257-63.

21. Yurtseven, E., D. Ural, K. Baysal, and L. Tokgozoglu, *An Update on the Role of PCSK9 in Atherosclerosis.* J Atheroscler Thromb, 2020. **27**(9): p. 909-918.

22. Cheng, J.M., et al., *PCSK9 in relation to coronary plaque inflammation: Results of the ATHEROREMO-IVUS study.* Atherosclerosis, 2016. **248**: p. 117-22.

23. Barale, C., E. Melchionda, A. Morotti, and I. Russo, *PCSK9 Biology and Its Role in Atherothrombosis.* Int J Mol Sci, 2021. **22**(11).

24. Cammisotto, V., et al., *Proprotein Convertase Subtilisin Kexin Type 9 (PCSK9) Beyond Lipids: The Role in Oxidative Stress and Thrombosis.* Antioxidants (Basel), 2022. **11**(3).

25. Qi, Z., et al., *PCSK9 (Proprotein Convertase Subtilisin/Kexin 9) Enhances Platelet Activation, Thrombosis, and Myocardial Infarct Expansion by Binding to Platelet CD36.* Circulation, 2021. **143**(1): p. 45-61.

26. Navar, A.M., et al., *Association of Prior Authorization and Out-of-pocket Costs With Patient Access to PCSK9 Inhibitor Therapy.* JAMA Cardiol, 2017. **2**(11): p. 1217-1225.

27. Ferri, N., et al., *Pharmacological rationale for the very early treatment of acute coronary syndrome with monoclonal antibodies anti-PCSK9.* Pharmacol Res, 2022. **184**: p. 106439.

28. Marfella, R., et al., *Evidence of an anti-inflammatory effect of PCSK9 inhibitors within the human atherosclerotic plaque.* Atherosclerosis, 2023. **378**: p. 117180.

29. Paciullo, F., et al., *PCSK9 at the crossroad of cholesterol metabolism and immune function*

during infections. J Cell Physiol, 2017. **232**(9): p. 2330-2338.

30. Heutinck, K.M., et al., *Serine proteases of the human immune system in health and disease.* Mol Immunol, 2010. **47**(11-12): p. 1943-55.

31. Cendron, L., et al., *Proprotein convertases regulate trafficking and maturation of key proteins within the secretory pathway.* Adv Protein Chem Struct Biol, 2023. **133**: p. 1-54.

32. Artenstein, A.W. and S.M. Opal, *Proprotein convertases in health and disease.* N Engl J Med, 2011. **365**(26): p. 2507-18.

33. Zhou, A., G. Webb, X. Zhu, and D.F. Steiner, *Proteolytic processing in the secretory pathway.* J Biol Chem, 1999. **274**(30): p. 20745-8.

34. Parvaz, N. and Z. Jalali, *Molecular evolution of PCSK family: Analysis of natural selection rate and gene loss.* PLoS One, 2021. **16**(10): p. e0259085.

35. Garten, W., *Characterization of Proprotein Convertases and Their Involvement in Virus Propagation*. 2018, Activation of Viruses by Host Proteases.

36. Dobo, J., A. Kocsis, R. Dani, and P. Gal, *Proprotein Convertases and the Complement System.* Front Immunol, 2022. **13**: p. 958121.

37. Seidah, N.G. and A. Prat, *The proprotein convertases are potential targets in the treatment of dyslipidemia.* J Mol Med (Berl), 2007. **85**(7): p. 685-96.

38. Minina, E.A., P.N. Moschou, and P.V. Bozhkov, *Limited and digestive proteolysis: crosstalk between evolutionary conserved pathways.* New Phytol, 2017. **215**(3): p. 958-964.

39. Thomas, G., *Furin at the cutting edge: from protein traffic to embryogenesis and disease.* Nature Reviews Molecular Cell Biology, 2002. **3(10)**: p. 753 - 766.

40. Osman, E.E.A., A. Rehemtulla, and N. Neamati, *Why All the Fury over Furin?* J Med Chem, 2022. **65**(4): p. 2747-2784.

41. Barrett, A.J., *Proteases.* Curr Protoc Protein Sci, 2001. **Chapter 21**: p. Unit 21 1.

42. Barlowe, C.K. and E.A. Miller, *Secretory protein biogenesis and traffic in the early secretory pathway.* Genetics, 2013. **193**(2): p. 383-410.

43. Rose, M., M. Duhamel, F. Rodet, and M. Salzet, *The Role of Proprotein Convertases in the Regulation of the Function of Immune Cells in the Oncoimmune Response.* Front Immunol, 2021. **12**: p. 667850.

44. Mahmoud, R., V. Kimonis, and M.G. Butler, *Genetics of Obesity in Humans: A Clinical Review.* Int J Mol Sci, 2022. **23**(19).

45. Ramos-Molina, B., M.G. Martin, and I. Lindberg, *PCSK1 Variants and Human Obesity.* Prog Mol Biol Transl Sci, 2016. **140**: p. 47-74.

46. Van Dijck, E., et al., *Rare Heterozygous PCSK1 Variants in Human Obesity: The Contribution of the p.Y181H Variant and a Literature Review.* Genes (Basel), 2022. **13**(10).

47. Stijnen, P., et al., *The association of common variants in PCSK1 with obesity: a HuGE review and meta-analysis.* Am J Epidemiol, 2014. **180**(11): p. 1051-65.

48. Seidah, N.G., et al., *Cloning and primary sequence of a mouse candidate prohormone convertase PC1 homologous to PC2, Furin, and Kex2: distinct chromosomal localization and messenger RNA distribution in brain and pituitary compared to PC2.* Mol Endocrinol, 1991. **5**(1): p. 111-22.

49. O'Rahilly, S., et al., *Brief report: impaired processing of prohormones associated with abnormalities of glucose homeostasis and adrenal function.* N Engl J Med, 1995. **333**(21): p. 1386-90.

50. Miller, R., et al., *Selective roles for the PC2 processing enzyme in the regulation of peptide neurotransmitter levels in brain and peripheral neuroendocrine tissues of PC2 deficient mice.* Neuropeptides, 2003. **37**(3): p. 140-8.

51. Stawowy, P. and E. Fleck, *Proprotein convertases furin and PC5: targeting atherosclerosis and restenosis at multiple levels.* J Mol Med (Berl), 2005. **83**(11): p. 865-75.

52. Turpeinen, H., et al., *Proprotein convertases in human atherosclerotic plaques: the overexpression of FURIN and its substrate cytokines BAFF and APRIL.* Atherosclerosis, 2011. **219**(2): p. 799-806.

53. Seidah, N.G., et al., *The Proprotein Convertases in Hypercholesterolemia and Cardiovascular Diseases: Emphasis on Proprotein Convertase Subtilisin/Kexin 9.* Pharmacol Rev, 2017. **69**(1): p. 33-52.

54. He, Z., A.M. Khatib, and J.W.M. Creemers, *The proprotein convertase furin in cancer: more than an oncogene.* Oncogene, 2022. **41**(9): p. 1252-1262.

55. Zhang, Y., et al., *The emerging role of furin in neurodegenerative and neuropsychiatric diseases.* Transl Neurodegener, 2022. **11**(1): p. 39.

56. Jaaks, P. and M. Bernasconi, *The proprotein convertase furin in tumour progression.* Int J Cancer, 2017. **141**(4): p. 654-663.

57. Thomas, G., F. Couture, and A. Kwiatkowska, *The Path to Therapeutic Furin Inhibitors: From Yeast Pheromones to SARS-CoV-2.* Int J Mol Sci, 2022. **23**(7).

58. Gyamera-Acheampong, C. and M. Mbikay, *Proprotein convertase subtilisin/kexin type 4 in mammalian fertility: a review.* Hum Reprod Update, 2009. **15**(2): p. 237-47.

59. Tardif, S., B. Guyonnet, N. Cormier, and G.A. Cornwall, *Alteration in the*

processing of the ACRBP/sp32 protein and sperm head/acrosome malformations in proprotein convertase 4 (PCSK4) null mice. Mol Hum Reprod, 2012. **18**(6): p. 298-307.

60. Iamsaard, S., et al., *Enzymatic activity of sperm proprotein convertase is important for mammalian fertilization.* J Cell Physiol, 2011. **226**(11): p. 2817-26.

61. Szumska, D., et al., *Pcsk5 is required in the early cranio-cardiac mesoderm for heart development.* BMC Dev Biol, 2017. **17**(1): p. 6.

62. Nakamura, Y., et al., *PCSK5 mutation in a patient with the VACTERL association.* BMC Res Notes, 2015. **8**: p. 228.

63. Iatan, I., et al., *Genetic variation at the proprotein convertase subtilisin/kexin type 5 gene modulates high-density lipoprotein cholesterol levels.* Circ Cardiovasc Genet, 2009. **2**(5): p. 467- 75.

64. Bassi, D.E., H. Mahloogi, and A.J. Klein-Szanto, *The proprotein convertases furin and PACE4 play a significant role in tumor progression.* Mol Carcinog, 2000. **28**(2): p. 63-9.

65. Akamatsu, T., et al., *Salivary gland development: its mediation by a subtilisin-like proprotein convertase, PACE4.* J Med Invest, 2009. **56 Suppl**: p. 241-6.

66. Guillemot, J. and N.G. Seidah, *PACE4 (PCSK6): another proprotein convertase link to iron homeostasis?* Haematologica, 2015. **100**(9): p. e377.

67. Guillemot, J., et al., *Implication of the proprotein convertases in iron homeostasis: proprotein convertase 7 sheds human transferrin receptor 1 and furin activates hepcidin.* Hepatology, 2013. **57**(6): p. 2514-24.

68. Wetsel, W.C., et al., *Disruption of the expression of the proprotein convertase PC7 reduces BDNF production and affects learning and memory in mice.* Proc Natl Acad Sci U

S A, 2013. **110**(43): p. 17362-7.

69. Seidah, N.G., M.S. Sadr, M. Chretien, and M. Mbikay, *The multifaceted proprotein convertases: their unique, redundant, complementary, and opposite functions.* J Biol Chem, 2013. **288**(30): p. 21473-81.

70. Creemers, J.W. and A.M. Khatib, *Knock-out mouse models of proprotein convertases: unique functions or redundancy?* Front Biosci, 2008. **13**: p. 4960-71.

71. Scamuffa, N., et al., *Proprotein convertases: lessons from knockouts.* FASEB J, 2006. **20**(12): p. 1954-63.

72. Brown, M.S. and J.L. Goldstein, *The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor.* Cell, 1997. **89**(3): p. 331-40.

73. Sakai, J., et al., *Molecular identification of the sterol-regulated luminal protease that cleaves SREBPs and controls lipid composition of animal cells.* Mol Cell, 1998. **2**(4): p. 505-14.

74. Sakai, J. and R.B. Rawson, *The sterol regulatory element-binding protein pathway: control of lipid homeostasis through regulated intracellular transport.* Curr Opin Lipidol, 2001. **12**(3): p. 261-6.

75. Essalmani, R., et al., *SKI-1/S1P Facilitates SARS-CoV-2 Spike Induced Cell-to-Cell Fusion via Activation of SREBP-2 and Metalloproteases, Whereas PCSK9 Enhances the Degradation of ACE2.* Viruses, 2023. **15**(2).

76. Blanchet, M., N.G. Seidah, and P. Labonte, *SKI-1/S1P inhibition: a promising surrogate to statins to block hepatitis C virus replication.* Antiviral Res, 2012. **95**(2): p. 159-66.

77. Bost, C., et al., *The proprotein convertase SKI-1/S1P is a critical host factor for*

Nairobi sheep disease virus infectivity. Virus Res, 2023. **329**: p. 199099.

78. Bergeron, E., M.J. Vincent, and S.T. Nichol, *Crimean-Congo hemorrhagic fever virus glycoprotein processing by the endoprotease SKI-1/S1P is critical for virus infectivity.* J Virol, 2007. **81**(23): p. 13271-6.

79. Gorski, J.P., et al., *Potential role of proprotein convertase SKI-1 in the mineralization of primary bone.* Cells Tissues Organs, 2009. **189**(1-4): p. 25-32.

80. Zaid, A., et al., *Proprotein convertase subtilisin/kexin type 9 (PCSK9): hepatocyte-specific low- density lipoprotein receptor degradation and critical role in mouse liver regeneration.* Hepatology, 2008. **48**(2): p. 646-54.

81. Lagace, T.A., *PCSK9 and LDLR degradation: regulatory mechanisms in circulation and in cells.* Curr Opin Lipidol, 2014. **25**(5): p. 387-93.

82. Shapiro, M.D., H. Tavori, and S. Fazio, *PCSK9: From Basic Science Discoveries to Clinical Trials.* Circ Res, 2018. **122**(10): p. 1420-1438.

83. Zhang, X., et al., *Immune Regulation of the Liver Through the PCSK9/CD36 Pathway During Heart Transplant Rejection.* Circulation, 2023. **148**(4): p. 336-353.

84. Roudaut, M., et al., *PCSK9 regulates the NODAL signaling pathway and cellular proliferation in hiPSCs.* Stem Cell Reports, 2021. **16**(12): p. 2958-2972.

85. Zanni, M.V., et al., *Proprotein Convertase Subtilisin/Kexin 9 Levels in Relation to Systemic Immune Activation and Subclinical Coronary Plaque in HIV.* Open Forum Infect Dis, 2017. **4**(4): p. ofx227.

86. Miyazawa, H., et al., *Increased serum PCSK9 concentrations are associated with periodontal infection but do not correlate with LDL cholesterol concentration.* Clin Chim Acta, 2012. **413**(1-2): p. 154-9.

87. Kohli, P., et al., *HIV and Hepatitis C-Coinfected Patients Have Lower Low-Density Lipoprotein Cholesterol Despite Higher Proprotein Convertase Subtilisin Kexin 9 (PCSK9): An Apparent "PCSK9-Lipid Paradox".* J Am Heart Assoc, 2016. **5**(5).

88. May, P., E. Woldt, R.L. Matz, and P. Boucher, *The LDL receptor-related protein (LRP) family: an old family of proteins with new physiological functions.* Ann Med, 2007. **39**(3): p. 219-28.

89. Go, G.W. and A. Mani, *Low-density lipoprotein receptor (LDLR) family orchestrates cholesterol homeostasis.* Yale J Biol Med, 2012. **85**(1): p. 19-28.

90. Seidah, N.G. and D. Garcon, *Expanding Biology of PCSK9: Roles in Atherosclerosis and Beyond.* Curr Atheroscler Rep, 2022. **24**(10): p. 821-830.

91. Roubtsova, A., et al., *Circulating proprotein convertase subtilisin/kexin 9 (PCSK9) regulates VLDLR protein and triglyceride accumulation in visceral adipose tissue.* Arterioscler Thromb Vasc Biol, 2011. **31**(4): p. 785-91.

92. Poirier, S., et al., *The proprotein convertase PCSK9 induces the degradation of low density lipoprotein receptor (LDLR) and its closest family members VLDLR and ApoER2.* J Biol Chem, 2008. **283**(4): p. 2363-72.

93. Dijk, W., C. Le May, and B. Cariou, *Beyond LDL: What Role for PCSK9 in Triglyceride-Rich Lipoprotein Metabolism?* Trends Endocrinol Metab, 2018. **29**(6): p. 420-434.

94. Reddy, S.S., T.E. Connor, E.J. Weeber, and W. Rebeck, *Similarities and differences in structure, expression, and functions of VLDLR and ApoER2.* Mol Neurodegener, 2011. **6**: p. 30.

95. Fu, T., Y. Guan, J. Xu, and Y. Wang, *APP, APLP2 and LRP1 interact with PCSK9 but are*

not required for PCSK9-mediated degradation of the LDLR in vivo. Biochim Biophys Acta Mol Cell Biol Lipids, 2017. **1862**(9): p. 883-889.

96. Bian, W., et al., *Low-density-lipoprotein-receptor-related protein 1 mediates Notch pathway activation.* Dev Cell, 2021. **56**(20): p. 2902-2919.e8.

97. Shan, L., et al., *PCSK9 binds to multiple receptors and can be functionally inhibited by an EGF-A peptide.* Biochem Biophys Res Commun, 2008. **375**(1): p. 69-73.

98. Boyd, J.H., et al., *Increased Plasma PCSK9 Levels Are Associated with Reduced Endotoxin Clearance and the Development of Acute Organ Failures during Sepsis.* J Innate Immun, 2016. **8**(2): p. 211-20.

99. Yuan, Y., et al., *PCSK9: A Potential Therapeutic Target for Sepsis.* J Immunol Res, 2020. **2020**: p. 2687692.

100. Dafnis, I., et al., *PCSK9 is minimally associated with HDL but impairs the anti-atherosclerotic HDL effects on endothelial cell activation.* J Lipid Res, 2022. **63**(10): p. 100272.

101. Burnap, S.A., et al., *PCSK9 Activity Is Potentiated Through HDL Binding.* Circ Res, 2021. **129**(11): p. 1039-1053.

102. Enkhmaa, B., et al., *PCSK9 in African Americans and Caucasians in Relation to Lp(a) Level, Apo(a) Size and Heritability.* J Endocr Soc, 2020. **4**(7): p. bvaa073.

103. Lambert, G., et al., *The complexity of lipoprotein (a) lowering by PCSK9 monoclona antibodies.* Clin Sci (Lond), 2017. **131**(4): p. 261-268.

104. Liu, X., et al., *Inhibition of PCSK9 potentiates immune checkpoint therapy for cancer.* Nature, 2020. **588**(7839): p. 693-698.

105. Xia, X.D., et al., *Regulation of PCSK9 Expression and Function: Mechanisms and Therapeutic Implications.* Front Cardiovasc Med, 2021. **8**: p. 764038.

106. Almeida, C.R., B.H. Ferreira, and I.F. Duarte, *Targeting PCSK9: a promising adjuvant strategy in cancer immunotherapy.* Signal Transduct Target Ther, 2021. **6**(1): p. 111.

107. Kim, Y.U., P. Kee, D. Danila, and B.B. Teng, *A Critical Role of PCSK9 in Mediating IL-17-Producing T Cell Responses in Hyperlipidemia.* Immune Netw, 2019. **19**(6): p. e41.

108. Kumar, B.V., T.J. Connors, and D.L. Farber, *Human T Cell Development, Localization, and Function throughout Life.* Immunity, 2018. **48**(2): p. 202-213.

109. Leslie, M., *Inflammation's stop signals.* Science, 2015. **347**(6217): p. 18-21.

110. Ragusa, R., et al., *PCSK9 and atherosclerosis: Looking beyond LDL regulation.* Eur J Clin Invest, 2021. **51**(4): p. e13459.

111. Hansson, G.K. and A. Hermansson, *The immune system in atherosclerosis.* Nat Immunol, 2011. **12**(3): p. 204-12.

112. Hampton, E.N., et al., *The self-inhibited structure of full-length PCSK9 at 1.9 A reveals structural homology with resistin within the C-terminal domain.* Proc Natl Acad Sci U S A, 2007. **104**(37): p. 14604-9.

113. Cunningham, D., et al., *Structural and biophysical studies of PCSK9 and its mutants linked to familial hypercholesterolemia.* Nat Struct Mol Biol, 2007. **14**(5): p. 413-9.

114. Piper, D.E., et al., *The crystal structure of PCSK9: a regulator of plasma LDL-cholesterol.* Structure, 2007. **15**(5): p. 545-52.

115. Lebeau, P.F., et al., *The Emerging Roles of Intracellular PCSK9 and Their Implications in Endoplasmic Reticulum Stress and Metabolic Diseases.* Metabolites, 2022. **12**(3).

116. Benito-Vicente, A., et al., *Leu22_Leu23 Duplication at the Signal Peptide of PCSK9*

Promotes Intracellular Degradation of LDLr and Autosomal Dominant Hypercholesterolemia. Arterioscler Thromb Vasc Biol, 2022. **42**(7): p. e203-e216.

117. Gauthier, M.S., et al., *Posttranslational modification of proprotein convertase subtilisin/kexin type 9 is differentially regulated in response to distinct cardiometabolic treatments as revealed by targeted proteomics.* J Clin Lipidol, 2018. **12**(4): p. 1027-1038.

118. Ben Djoudi Ouadda, A., et al., *Ser-Phosphorylation of PCSK9 (Proprotein Convertase Subtilisin- Kexin 9) by Fam20C (Family With Sequence Similarity 20, Member C) Kinase Enhances Its Ability to Degrade the LDLR (Low-Density Lipoprotein Receptor).* Arterioscler Thromb Vasc Biol, 2019. **39**(10): p. 1996-2013.

119. Dewpura, T. and J. Mayne, *Analyses of PCSK9 post-translational modifications using time-of- flight mass spectrometry.* Methods Mol Biol, 2011. **768**: p. 167-87.

120. Oleaga, C., et al., *Insights into the kinetics and dynamics of the furin-cleaved form of PCSK9.* J Lipid Res, 2021. **62**: p. 100003.

121. Nakamura, A., et al., *Plasma kinetics of mature PCSK9, furin-cleaved PCSK9, and Lp(a) with or without administration of PCSK9 inhibitors in acute myocardial infarction.* J Cardiol, 2020. **76**(4): p. 395-401.

122. Kwon, H.J., et al., *Molecular basis for LDL receptor recognition by PCSK9.* Proc Natl Acad Sci U S A, 2008. **105**(6): p. 1820-5.

123. Roth, E.M. and M.H. Davidson, *PCSK9 Inhibitors: Mechanism of Action, Efficacy, and Safety.* Rev Cardiovasc Med, 2018. **19**(S1): p. S31-s46.

124. Donate, L.E., et al., *Molecular evolution and domain structure of plasminogen-related growth factors (HGF/SF and HGF1/MSP).* Protein Sci, 1994. **3**(12): p. 2378-94.

125. Pandya, M., et al., *An inimitable proprotein convertase subtilisin kexin-9 (PCSK9)*

cleavage site VFAQ on Spike protein along with furin cleavage site makes SARS-CoV-2 unique. bioRxiv, 2023: p. 2023.05.04.539453.

126. Ference, B.A., et al., *Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel.* Eur Heart J, 2017. **38**(32): p. 2459-2472.

127. Borén, J., et al., *Low-density lipoproteins cause atherosclerotic cardiovascular disease: pathophysiological, genetic, and therapeutic insights: a consensus statement from the European Atherosclerosis Society Consensus Panel.* Eur Heart J, 2020. **41**(24): p. 2313-2330.

128. Civeira, F., M. Arca, A. Cenarro, and R.A. Hegele, *A mechanism-based operational definition and classification of hypercholesterolemia.* J Clin Lipidol, 2022. **16**(6): p. 813-821.

129. Benito-Vicente, A., et al., *Familial Hypercholesterolemia: The Most Frequent Cholesterol Metabolism Disorder Caused Disease.* Int J Mol Sci, 2018. **19**(11).

130. Capurso, A., *[Hypercholesterolemia: a picture of the situation]*. Recenti Prog Med, 1991. **82**(7-8): p. 405-8.

131. Tokgozoglu, L. and M. Kayikcioglu, *Familial Hypercholesterolemia: Global Burden and Approaches.* Curr Cardiol Rep, 2021. **23**(10): p. 151.

132. Vallejo-Vaz, A.J., et al., *Pooling and expanding registries of familial hypercholesterolaemia to assess gaps in care and improve disease management and outcomes: Rationale and design of the global EAS Familial Hypercholesterolaemia Studies Collaboration.* Atheroscler Suppl, 2016. **22**: p. 1-32.

133. Berberich, A.J. and R.A. Hegele, *The complex molecular genetics of*

familial hypercholesterolaemia. Nat Rev Cardiol, 2019. **16**(1): p. 920.

134. Knowles, J.W., et al., *Reducing the burden of disease and death from familial hypercholesterolemia: a call to action.* Am Heart J, 2014. **168**(6): p. 807-11.

135. Schultheis, A.H., *Hypercholesterolemia: prevention, detection and management.* Nurse Pract, 1990. **15**(1): p. 40-6, 51-5-6.

136. Ray, K.K., et al., *World Heart Federation Cholesterol Roadmap 2022.* Glob Heart, 2022. **17**(1): p. 75.

137. Maxwell, K.N. and J.L. Breslow, *Proprotein convertase subtilisin kexin 9: the third locus implicated in autosomal dominant hypercholesterolemia.* Curr Opin Lipidol, 2005. **16**(2): p. 167- 72.

138. Naoumova, R.P., et al., *Severe hypercholesterolemia in four British families with the D374Y mutation in the PCSK9 gene: long-term follow-up and treatment response.* Arterioscler Thromb Vasc Biol, 2005. **25**(12): p. 2654-60.

139. Sun, X.M., et al., *Evidence for effect of mutant PCSK9 on apolipoprotein B secretion as the cause of unusually severe dominant hypercholesterolaemia.* Hum Mol Genet, 2005. **14**(9): p. 1161-9.

140. Leren, T.P., *Mutations in the PCSK9 gene in Norwegian subjects with autosomal dominant hypercholesterolemia.* Clin Genet, 2004. **65**(5): p. 419-22.

141. Zhao, Z., et al., *Molecular characterization of loss-of-function mutations in PCSK9 and identification of a compound heterozygote.* Am J Hum Genet, 2006. **79**(3): p. 514-23.

142. Cohen, J.C., E. Boerwinkle, T.H. Mosley, Jr., and H.H. Hobbs, *Sequence variations in*

PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med, 2006. **354**(12): p. 1264-72.

143. Guo, Q., X. Feng, and Y. Zhou, *PCSK9 Variants in Familial Hypercholesterolemia: A Comprehensive Synopsis.* Front Genet, 2020. **11**: p. 1020.

144. Olson, R.E., *Discovery of the lipoproteins, their role in fat transport and their significance as risk factors.* J Nutr, 1998. **128**(2 Suppl): p. 439s-443s.

145. Feingold, K.R., *Introduction to Lipids and Lipoproteins*, in *Endotext*, K.R. Feingold, et al., Editors. 2000, MDText.com, Inc. Copyright © 2000-2023, MDText.com, Inc.: South Dartmouth (MA).

146. Chapman, M.J. and P. Forgez, *Lipid transport systems: some recent aspects in swine, cattle and trout during development.* Reprod Nutr Dev (1980), 1985. **25**(1b): p. 217-26.

147. McNamara, D.J., *Dietary fatty acids, lipoproteins, and cardiovascular disease.* Adv Food Nutr Res, 1992. **36**: p. 253-351.

148. Kostner, G. and S. Frank, *Lipoproteins: role in health and diseases*. 2012: BoD–Books on Demand.

149. Norata, G.D., et al., *Biology of proprotein convertase subtilisin kexin 9: beyond low-density lipoprotein cholesterol lowering.* Cardiovasc Res, 2016. **112**(1): p. 429-42.

150. Hussain, M.M., et al., *Signposts in the assembly of chylomicrons.* Front Biosci, 2001. **6**: p. D320- 31.

151. Julve, J., J.M. Martín-Campos, J.C. Escolà-Gil, and F. Blanco-Vaca, *Chylomicrons: Advances in biology, pathology, laboratory testing, and therapeutics.* Clin Chim Acta, 2016. **455**: p. 134-48.

152. Dash, S., C. Xiao, C. Morgantini, and G.F. Lewis, *New Insights into the Regulation of*

Chylomicron Production. Annu Rev Nutr, 2015. **35**: p. 265-94.

153. Rashid, S., et al., *Proprotein convertase subtilisin kexin type 9 promotes intestinal overproduction of triglyceride-rich apolipoprotein B lipoproteins through both low-density lipoprotein receptor- dependent and -independent mechanisms.* Circulation, 2014. **130**(5): p. 431-41.

154. Le May, C., et al., *Proprotein convertase subtilisin kexin type 9 null mice are protected from postprandial triglyceridemia.* Arterioscler Thromb Vasc Biol, 2009. **29**(5): p. 684-90.

155. Packard, C.J., J. Boren, and M.R. Taskinen, *Causes and Consequences of Hypertriglyceridemia.* Front Endocrinol (Lausanne), 2020. **11**: p. 252.

156. Cooper, A.D., *Hepatic uptake of chylomicron remnants.* J Lipid Res, 1997. **38**(11): p. 2173-92.

157. Havel, R.J., *Triglyceride-rich lipoproteins and plasma lipid transport.* Arterioscler Thromb Vasc Biol, 2010. **30**(1): p. 9-19.

158. Goldberg, I.J. and M. Merkel, *Lipoprotein lipase: physiology, biochemistry, and molecular biology.* Front Biosci, 2001. **6**: p. D388-405.

159. Huang, Y. and R.W. Mahley, *Apolipoprotein E: structure and function in lipid metabolism, neurobiology, and Alzheimer's diseases.* Neurobiol Dis, 2014. **72 Pt A**: p. 3-12.

160. Sun, H., et al., *Proprotein convertase subtilisin/kexin type 9 interacts with apolipoprotein B and prevents its intracellular degradation, irrespective of the low-density lipoprotein receptor.* Arterioscler Thromb Vasc Biol, 2012. **32**(7): p. 1585-95.

161. Tiwari, S. and S.A. Siddiqi, *Intracellular trafficking and secretion of VLDL.* Arterioscler Thromb Vasc Biol, 2012. **32**(5): p. 1079-86.

162. Behbodikhah, J., et al., *Apolipoprotein B and Cardiovascular Disease: Biomarker and Potential Therapeutic Target.* Metabolites, 2021. **11**(10).

163. Benoist, F. and T. Grand-Perret, *ApoB-100 secretion by HepG2 cells is regulated by the rate of triglyceride biosynthesis but not by intracellular lipid pools.* Arterioscler Thromb Vasc Biol, 1996. **16**(10): p. 1229-35.

164. Goldstein, J.L. and M.S. Brown, *The low-density lipoprotein pathway and its relation to atherosclerosis.* Annu Rev Biochem, 1977. **46**: p. 897-930.

165. Prassl, R. and P. Laggner, *Molecular structure of low density lipoprotein: current status and future challenges.* Eur Biophys J, 2009. **38**(2): p. 145-58.

166. Fernández-Higuero, J.A., et al., *Human LDL structural diversity studied by IR spectroscopy.* PLoS One, 2014. **9**(3): p. e92426.

167. Jackson, R.L., J.D. Morrisett, and A.M. Gotto, Jr., *Lipoprotein structure and metabolism.* Physiol Rev, 1976. **56**(2): p. 259-316.

168. Ivanova, E.A., et al., *Small Dense Low-Density Lipoprotein as Biomarker for Atherosclerotic Diseases.* Oxid Med Cell Longev, 2017. **2017**: p. 1273042.

169. Kosenko, T., et al., *Low density lipoprotein binds to proprotein convertase subtilisin/kexin type-9 (PCSK9) in human plasma and inhibits PCSK9-mediated low density lipoprotein receptor degradation.* J Biol Chem, 2013. **288**(12): p. 8279-8288.

170. Fan, D., et al., *Self-association of human PCSK9 correlates with its LDLR-degrading activity.* Biochemistry, 2008. **47**(6): p. 1631-9.

171. Tavori, H., et al., *Serum proprotein convertase subtilisin/kexin type 9 and cell surface low-density lipoprotein receptor: evidence for a reciprocal regulation.* Circulation, 2013. **127**(24): p. 2403-13.

172. Alborn, W.E., et al., *Serum proprotein convertase subtilisin kexin type 9 is correlated directly with serum LDL cholesterol.* Clin Chem, 2007. **53**(10): p. 1814-9.

173. Sarkar, S.K., et al., *A transient amphipathic helix in the prodomain of PCSK9 facilitates binding to low-density lipoprotein particles.* J Biol Chem, 2020. **295**(8): p. 2285-2298.

174. Holla Ø, L., et al., *Removal of acidic residues of the prodomain of PCSK9 increases its activity towards the LDL receptor.* Biochem Biophys Res Commun, 2011. **406**(2): p. 234-8.

175. Tavori, H., I. Giunzioni, M.F. Linton, and S. Fazio, *Loss of plasma proprotein convertase subtilisin/kexin 9 (PCSK9) after lipoprotein apheresis.* Circ Res, 2013. **113**(12): p. 1290-5.

176. Boes, E., et al., *Genetic-epidemiological evidence on genes associated with HDL cholesterol levels: a systematic in-depth review.* Exp Gerontol, 2009. **44**(3): p. 136-60.

177. Ben-Aicha, S., L. Badimon, and G. Vilahur, *Advances in HDL: Much More than Lipid Transporters.* Int J Mol Sci, 2020. **21**(3).

178. Kontush, A., et al., *Structure of HDL: particle subclasses and molecular components.* Handb Exp Pharmacol, 2015. **224**: p. 3-51.

179. Deng, S., Y. Xu, and L. Zheng, *HDL Structure.* Adv Exp Med Biol, 2022. **1377**: p. 1-11.

180. Lund-Katz, S. and M.C. Phillips, *High density lipoprotein structure-function and role in reverse cholesterol transport.* Subcell Biochem, 2010. **51**: p. 183-227.

181. Burnap, S.A., et al., *High-Density Lipoproteins Are the Main Carriers of PCSK9 in the Circulation.* J Am Coll Cardiol, 2020. **75**(12): p. 1495-1497.

182. Korneva, V.A., T.Y. Kuznetsova, and U. Julius, *Modern Approaches to Lower*

Lipoprotein(a) Concentrations and Consequences for Cardiovascular Diseases. Biomedicines, 2021. **9**(9).

183. Chemello, K., et al., *Lipoprotein(a) Cellular Uptake Ex Vivo and Hepatic Capture In Vivo Is Insensitive to PCSK9 Inhibition With Alirocumab.* JACC Basic Transl Sci, 2020. **5**(6): p. 549-557.

184. Schmidt, K., A. Noureen, F. Kronenberg, and G. Utermann, *Structure, function, and genetics of lipoprotein (a).* J Lipid Res, 2016. **57**(8): p. 1339-59.

185. Tsimikas, S. and J.L. Witztum, *The role of oxidized phospholipids in mediating lipoprotein(a) atherogenicity.* Curr Opin Lipidol, 2008. **19**(4): p. 369-77.

186. Stein, E.A. and F. Raal, *Reduction of low-density lipoprotein cholesterol by monoclonal antibody inhibition of PCSK9.* Annu Rev Med, 2014. **65**: p. 417-31.

187. Stein, E.A., *Low-density lipoprotein cholesterol reduction by inhibition of PCSK9.* Curr Opin Lipidol, 2013. **24**(6): p. 510-7.

188. Watts, G.F., et al., *Controlled study of the effect of proprotein convertase subtilisin-kexin type 9 inhibition with evolocumab on lipoprotein(a) particle kinetics.* Eur Heart J, 2018. **39**(27): p. 2577- 2585.

189. Villard, E.F., et al., *PCSK9 Modulates the Secretion But Not the Cellular Uptake of Lipoprotein(a) Ex Vivo: An Effect Blunted by Alirocumab.* JACC Basic Transl Sci, 2016. **1**(6): p. 419-427.

190. Tavori, H., et al., *PCSK9 Association With Lipoprotein(a).* Circ Res, 2016. **119**(1): p. 29-35.

191. Nekaies, Y., et al., *Plasma proprotein convertase subtilisin/kexin type 9 is associated with Lp(a) in type 2 diabetic patients.* J Diabetes Complications, 2015. **29**(8): p. 1165-70.

192. Momtazi, A.A., et al., *PCSK9 and diabetes: is there a link?* Drug Discov Today, 2017.

22(6): p. 883- 895.

193. Punch, E., et al., *Effects of PCSK9 Targeting: Alleviating Oxidation, Inflammation, and Atherosclerosis.* J Am Heart Assoc, 2022. **11**(3): p. e023328.

194. Tang, Z.H., et al., *New role of PCSK9 in atherosclerotic inflammation promotion involving the TLR4/NF-κB pathway.* Atherosclerosis, 2017. **262**: p. 113-122.

195. Leucker, T.M., et al., *Coronary Endothelial Dysfunction Is Associated With Elevated Serum PCSK9 Levels in People With HIV Independent of Low-Density Lipoprotein Cholesterol.* J Am Heart Assoc, 2018. **7**(19): p. e009996.

196. Park, K.H. and W.J. Park, *Endothelial Dysfunction: Clinical Implications in Cardiovascular Disease and Therapeutic Approaches.* J Korean Med Sci, 2015. **30**(9): p. 1213-25.

197. Poznyak, A.V., et al., *Overview of OxLDL and Its Impact on Cardiovascular Health: Focus on Atherosclerosis*. Front Pharmacol, 2020. **11**: p. 613780.

198. Di Pietro, N., G. Formoso, and A. Pandolfi, *Physiology and pathophysiology of oxLDL uptake by vascular wall cells in atherosclerosis.* Vascul Pharmacol, 2016. **84**: p. 1-7.

199. Jaén, R.I., et al., *Functional Crosstalk between PCSK9 Internalization and Pro-Inflammatory Activation in Human Macrophages: Role of Reactive Oxygen Species Release.* Int J Mol Sci, 2022. **23**(16).

200. Tang, Z., et al., *PCSK9 siRNA suppresses the inflammatory response induced by oxLDL through inhibition of NF-κB activation in THP-1-derived macrophages.* Int J Mol Med, 2012. **30**(4): p. 931- 8.

201. Lu, T., et al., *Reactive oxygen species mediate oxidized low-density lipoprotein-induced inhibition of oct-4 expression and endothelial differentiation of bone marrow stem cells.* Antioxid Redox Signal, 2010. **13**(12): p. 1845-56.

202. Lara-Guzmán, O.J., et al., *Oxidized LDL triggers changes in oxidative stress and inflammatory biomarkers in human macrophages.* Redox Biol, 2018. **15**: p. 1-11.

203. Magwenzi, S., et al., *Oxidized LDL activates blood platelets through CD36/NOX2-mediated inhibition of the cGMP/protein kinase G signaling cascade.* Blood, 2015. **125**(17): p. 2693-703.

204. Ding, Z., et al., *Cross-talk between LOX-1 and PCSK9 in vascular tissues.* Cardiovasc Res, 2015. **107**(4): p. 556-67.

205. Miller, Y.I., et al., *Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity.* Circ Res, 2011. **108**(2): p. 235- 48.

206. Miller, Y.I. and S. Tsimikas, *Oxidation-specific epitopes as targets for biotheranostic applications in humans: biomarkers, molecular imaging and therapeutics.* Curr Opin Lipidol, 2013. **24**(5): p. 426-37.

207. Ma, M., C. Hou, and J. Liu, *Effect of PCSK9 on atherosclerotic cardiovascular diseases and its mechanisms: Focus on immune regulation.* Front Cardiovasc Med, 2023. **10**: p. 1148486.

208. Camera, M., et al., *PCSK9 as a Positive Modulator of Platelet Activation.* J Am Coll Cardiol, 2018. **71**(8): p. 952-954.

209. Nording, H.M., P. Seizer, and H.F. Langer, *Platelets in inflammation and atherogenesis.* Front Immunol, 2015. **6**: p. 98.

210. Bakogiannis, C., M. Sachse, K. Stamatelopoulos, and K. Stellos, *Platelet-derived chemokines in inflammation and atherosclerosis.* Cytokine, 2019. **122**: p. 154157.

211. Cognasse, F., et al., *Platelets as Key Factors in Inflammation: Focus on*

CD40L/CD40. Front Immunol, 2022. **13**: p. 825892.

212. Feingold, K.R., et al., *Inflammation stimulates the expression of PCSK9.* Biochem Biophys Res Commun, 2008. **374**(2): p. 341-4.

213. Ren, K., T. Jiang, X.L. Zheng, and G.J. Zhao, *Proprotein convertase furin/PCSK3 and atherosclerosis: New insights and potential therapeutic targets.* Atherosclerosis, 2017. **262**: p. 163-170.

214. Areschoug, T. and S. Gordon, *Scavenger receptors: role in innate immunity and microbial pathogenesis.* Cell Microbiol, 2009. **11**(8): p. 1160-9.

215. Taban, Q., et al., *Scavenger receptors in host defense: from functional aspects to mode of action.* Cell Commun Signal, 2022. **20**(1): p. 2.

216. Alquraini, A. and J. El Khoury, *Scavenger receptors.* Curr Biol, 2020. **30**(14): p. R790-r795.

217. Prabhudas, M., et al., *Standardizing scavenger receptor nomenclature.* J Immunol, 2014. **192**(5): p. 1997-2006.

218. Demers, A., et al., *PCSK9 Induces CD36 Degradation and Affects Long-Chain Fatty Acid Uptake and Triglyceride Metabolism in Adipocytes and in Mouse Liver.* Arterioscler Thromb Vasc Biol, 2015. **35**(12): p. 2517-25.

219. Wang, W., et al., *Scavenger receptor class B, type 1 facilitates cellular fatty acid uptake.* Biochim Biophys Acta Mol Cell Biol Lipids, 2020. **1865**(2): p. 158554.

220. Glatz, J.F.C., M. Nabben, and J. Luiken, *CD36 (SR-B2) as master regulator of cellular fatty acid homeostasis.* Curr Opin Lipidol, 2022. **33**(2): p. 103-111.

221. Qiao, L., et al., *Transcriptional regulation of fatty acid translocase/CD36 expression by CCAAT/enhancer-binding protein alpha.* J Biol Chem, 2008. **283**(14): p. 8788-95.

222. Silverstein, R.L., *Inflammation, atherosclerosis, and arterial thrombosis: role of the scavenger receptor CD36.* Cleve Clin J Med, 2009. **76 Suppl 2**(Suppl 2): p. S27-30.

223. Fazaeli, M., A. Khoshdel, M. Shafiepour, and M. Rohban, *The influence of subclinical hypothyroidism on serum lipid profile, PCSK9 levels and CD36 expression on monocytes.* Diabetes Metab Syndr, 2019. **13**(1): p. 312-316.

224. Tone, K., M.H.T. Stappers, J.A. Willment, and G.D. Brown, *C-type lectin receptors of the Dectin-1 cluster: Physiological roles and involvement in disease.* Eur J Immunol, 2019. **49**(12): p. 2127- 2133.

225. Zelensky, A.N. and J.E. Gready, *The C-type lectin-like domain superfamily.* Febs j, 2005. **272**(24): p. 6179-217.

226. Twigg, M.W., K. Freestone, S. Homer-Vanniasinkam, and S. Ponnambalam, *The LOX-1 Scavenger Receptor and Its Implications in the Treatment of Vascular Disease.* Cardiol Res Pract, 2012. **2012**: p. 632408.

227. Pirillo, A., G.D. Norata, and A.L. Catapano, *LOX-1, OxLDL, and atherosclerosis.* Mediators Inflamm, 2013. **2013**: p. 152786.

228. Xu, S., et al., *LOX-1 in atherosclerosis: biological functions and pharmacological modifiers.* Cell Mol Life Sci, 2013. **70**(16): p. 2859-72.

229. Ding, Z., et al., *PCSK9 regulates expression of scavenger receptors and ox-LDL uptake in macrophages.* Cardiovasc Res, 2018. **114**(8): p. 1145-1153.

230. Iwashima, Y., et al., *Advanced glycation end products-induced gene expression of scavenger receptors in cultured human monocyte-derived macrophages.* Biochem Biophys Res Commun, 2000. **277**(2): p. 368-80.

231. Jamuna, S., R. Ashokkumar, and S.N. Devaraj, *Amelioration of C-Reactive Protein and*

Lectin Like Oxidized Low Density Lipoprotein Receptor Complex Induced Endothelial Dysfunction by Oligomeric Proanthocyanidins. Appl Biochem Biotechnol, 2023. **195**(4): p. 2664-2686.

232. Jeannin, P., et al., *Complexity and complementarity of outer membrane protein A recognition by cellular and humoral innate immunity receptors.* Immunity, 2005. **22**(5): p. 551-60.

233. Ding, Z., et al., *Regulation of autophagy and apoptosis in response to ox-LDL in vascular smooth muscle cells, and the modulatory effects of the microRNA hsa-let-7 g.* Int J Cardiol, 2013. **168**(2): p. 1378-85.

234. Chen, M., T. Masaki, and T. Sawamura, *LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells: implications in endothelial dysfunction and atherosclerosis.* Pharmacol Ther, 2002. **95**(1): p. 89-100.

235. Wu, C.Y., et al., *PCSK9 siRNA inhibits HUVEC apoptosis induced by ox-LDL via Bcl/Bax-caspase9- caspase3 pathway.* Mol Cell Biochem, 2012. **359**(1-2): p. 347-58.

236. Glerup, S., R. Schulz, U. Laufs, and K.D. Schlüter, *Physiological and therapeutic regulation of PCSK9 activity in cardiovascular disease.* Basic Res Cardiol, 2017. **112**(3): p. 32.

237. Burke, A.C., J.S. Dron, R.A. Hegele, and M.W. Huff, *PCSK9: Regulation and Target for Drug Development for Dyslipidemia.* Annu Rev Pharmacol Toxicol, 2017. **57**: p. 223-244.

238. Jeong, H.J., et al., *Sterol-dependent regulation of proprotein convertase subtilisin/kexin type 9 expression by sterol-regulatory element binding protein-2.* J Lipid Res, 2008. **49**(2): p. 399-409.

239. Cortes, V.A., et al., *Physiological and pathological implications of cholesterol.* Front Biosci (Landmark Ed), 2014. **19**(3): p. 416-28.

240. Eberlé, D., et al., *SREBP transcription factors: master regulators of lipid homeostasis.* Biochimie, 2004. **86**(11): p. 839-48.

241. Hua, X., et al., *SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element.* Proc Natl Acad Sci U S A, 1993. **90**(24): p. 11603-7.

242. Horton, J.D., et al., *Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes.* Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12027-32.

243. Wang, X., et al., *The impact of hepatocyte nuclear factor-1α on liver malignancies and cell stemness with metabolic consequences.* Stem Cell Res Ther, 2019. **10**(1): p. 315.

244. Baass, A., et al., *Plasma PCSK9 Is Associated with Age, Sex, and Multiple Metabolic Markers in a Population-Based Sample of Children and Adolescents.* Clinical Chemistry, 2009. **55**(9): p. 1637- 1645.

245. Ghosh, M., C. Gälman, M. Rudling, and B. Angelin, *Influence of physiological changes in endogenous estrogen on circulating PCSK9 and LDL cholesterol.* J Lipid Res, 2015. **56**(2): p. 463-9.

246. Persson, L., et al., *Circulating proprotein convertase subtilisin kexin type 9 has a diurnal rhythm synchronous with cholesterol synthesis and is reduced by fasting in humans.* Arterioscler Thromb Vasc Biol, 2010. **30**(12): p. 2666-72.

247. Ngo Sock, E.T., N.A. Chapados, and J.M. Lavoie, *LDL receptor and Pcsk9 transcripts are decreased in liver of ovariectomized rats: effects of exercise training.* Horm Metab Res,

2014. **46**(8): p. 550- 5.

248. Cameron, J., et al., *Berberine decreases PCSK9 expression in HepG2 cells.* Atherosclerosis, 2008. **201**(2): p. 266-73.

249. Sarkar, S.K., et al., *Pathogenic gain-of-function mutations in the prodomain and C-terminal domain of PCSK9 inhibit LDL binding.* Front Physiol, 2022. **13**: p. 960272.

250. Walley, K.R., et al., *PCSK9 is a critical regulator of the innate immune response and septic shock outcome.* Sci Transl Med, 2014. **6**(258): p. 258ra143.

251. Azzam, K.M. and M.B. Fessler, *Crosstalk between reverse cholesterol transport and innate immunity.* Trends Endocrinol Metab, 2012. **23**(4): p. 169-78.

252. Vreugdenhil, A.C., et al., *LPS-binding protein circulates in association with apoB-containing lipoproteins and enhances endotoxin-LDL/VLDL interaction.* J Clin Invest, 2001. **107**(2): p. 225-34.

253. Seidah, N.G., *The Elusive Inhibitory Function of the Acidic N-Terminal Segment of the Prodomain of PCSK9: The Plot Thickens.* J Mol Biol, 2019. **431**(5): p. 904-907.

254. Benjannet, S., et al., *Effects of the prosegment and pH on the activity of PCSK9: evidence for additional processing events.* J Biol Chem, 2010. **285**(52): p. 40965-78.

255. Dewpura, T., et al., *PCSK9 is phosphorylated by a Golgi casein kinase-like kinase ex vivo and circulates as a phosphoprotein in humans.* Febs j, 2008. **275**(13): p. 3480-93.

256. Uversky, V.N., *Functional roles of transiently and intrinsically disordered regions within proteins.* Febs j, 2015. **282**(7): p. 1182-9.

257. Ultsch, M., et al., *Identification of a Helical Segment within the Intrinsically Disordered Region of the PCSK9 Prodomain.* J Mol Biol, 2019. **431**(5): p. 885-903.

258. Gustafsen, C., et al., *Heparan sulfate proteoglycans present PCSK9 to the LDL*

receptor. Nat Commun, 2017. **8**(1): p. 503.

259. Galvan, A.M. and J.S. Chorba, *Cell-associated heparin-like molecules modulate the ability of LDL to regulate PCSK9 uptake.* J Lipid Res, 2019. **60**(1): p. 71-84.

260. Gauster, M., A. Hrzenjak, K. Schick, and S. Frank, *Endothelial lipase is inactivated upon cleavage by the members of the proprotein convertase family.* J Lipid Res, 2005. **46**(5): p. 977-87.

261. Navarese, E.P., et al., *PCSK9 Inhibition During the Inflammatory Stage of SARS-CoV-2 Infection.* J Am Coll Cardiol, 2023. **81**(3): p. 224-234.

262. Kubica, J., et al., *IMPACT of PCSK9 inhibition on clinical outcome in patients during the inflammatory stage of the SARS-COV-2 infection: Rationale and protocol of the IMPACT-SIRIO 5 study.* Cardiol J, 2022. **29**(1): p. 140-147.

263. Kataoka, Y., et al., *Circulating Furin-Cleaved Proprotein Convertase Subtilisin/Kexin Type 9 Concentration Predicts Future Coronary Events in Japanese Subjects.* JACC Asia, 2021. **1**(3): p. 360-368.

264. Hori, M., et al., *Removal of plasma mature and furin-cleaved proprotein convertase subtilisin/kexin 9 by low-density lipoprotein-apheresis in familial hypercholesterolemia: development and application of a new assay for PCSK9.* J Clin Endocrinol Metab, 2015. **100**(1): p. E41-9.

265. Fazio, S., et al., *Threshold Effects of Circulating Angiopoietin-Like 3 Levels on Plasma Lipoproteins.* J Clin Endocrinol Metab, 2017. **102**(9): p. 3340-3348.

266. Ding, Z., et al., *PCSK9 and inflammation: role of shear stress, pro-inflammatory cytokines, and LOX-1.* Cardiovasc Res, 2020. **116**(5): p. 908-915.

267. Schlüter, K.D., et al., *Oxidized low-density lipoprotein (oxLDL) affects load-free cell*

shortening of cardiomyocytes in a proprotein convertase subtilisin/kexin 9 (PCSK9)-dependent way. Basic Res Cardiol, 2017. **112**(6): p. 63.

268. Grefhorst, A., M.C. McNutt, T.A. Lagace, and J.D. Horton, *Plasma PCSK9 preferentially reduces liver LDL receptors in mice.* J Lipid Res, 2008. **49**(6): p. 1303-11.

269. Lipari, M.T., et al., *Furin-cleaved proprotein convertase subtilisin/kexin type 9 (PCSK9) is active and modulates low density lipoprotein receptor and serum cholesterol levels.* J Biol Chem, 2012. **287**(52): p. 43482-91.

270. Schroeder, K.M., et al., *Proteolytic cleavage of antigen extends the durability of an anti-PCSK9 monoclonal antibody.* J Lipid Res, 2015. **56**(11): p. 2124-32.

271. DeVay, R.M., D.L. Shelton, and H. Liang, *Characterization of proprotein convertase subtilisin/kexin type 9 (PCSK9) trafficking reveals a novel lysosomal targeting mechanism via amyloid precursor-like protein 2 (APLP2).* J Biol Chem, 2013. **288**(15): p. 10805-18.

272. Ray, K., et al., *Oxidation-sensitive nociception involved in endometriosis-associated pain.* Pain, 2015. **156**(3): p. 528-539.

273. Lombardo, D. and M.A. Kiselev, *Methods of Liposomes Preparation: Formation and Control Factors of Versatile Nanocarriers for Biomedical and Nanomedicine Application.* Pharmaceutics, 2022. **14**(3).

274. Nicholls, S.J., et al., *Assessing the impact of PCSK9 inhibition on coronary plaque phenotype with optical coherence tomography: rationale and design of the randomized, placebo-controlled HUYGENS study.* Cardiovasc Diagn Ther, 2021. **11**(1): p. 120-129.

275. Que, X., et al., *Oxidized phospholipids are proinflammatory and proatherogenic in hypercholesterolaemic mice.* Nature, 2018. **558**(7709): p. 301-306.

276. Moor, K.J., F.J Sheedy and and E.A. Fisher, *Macrophages in atherosclerosis: a dynamic balance.* Nat Rev Immunol, 2013. **13**(10): p. 709-21

277. Raduolovic, K., et al., *Injections of Lipopolysaccharide into Mice to Mimic Entrance of Microbial- derived Products After Intestinal Barrier Breach.* J Vis Exp, 2018(135).

278. Baxter, E.W., et al., *Standardized protocols for differentiation of THP-1 cells to macrophages with distinct M(IFNγ+LPS), M(IL-4) and M(IL-10) phenotypes.* J Immunol Methods, 2020. **478**: p. 112721.

279. Dinarello, C.A., *Interleukin-1 in the pathogenesis and treatment of inflammatory diseases.* Blood, 2011. **117**(14): p. 3720-32.

280. Josephs, S.F., et al., *Unleashing endogenous TNF-alpha as a cancer immunotherapeutic.* Journal of Translational Medicine, 2018. **16**(1): p. 242.

281. Ding, Z., et al., *NLRP3 inflammasome via IL-1β regulates PCSK9 secretion.* Theranostics, 2020. **10**(16): p. 7100-7110.

282. Katsuki, S., et al., *Proprotein Convertase Subtilisin/Kexin 9 (PCSK9) Promotes Macrophage Activation via LDL Receptor-Independent Mechanisms.* Circ Res, 2022. **131**(11): p. 873-889

283. Ricci, C., et al., *PCSK9 induces a pro-inflammatory response in macrophages.* Sci Rep, 2018. **8**(1): p. 2267.

284. Hoogeveen, R.M., et al., *PCSK9 Antibody Alirocumab Attenuates Arterial Wall Inflammation Without Changes in Circulating Inflammatory Markers.* JACC Cardiovasc Imaging, 2019. **12**(12): p. 2571-2573.

285. Luquero, A., L. Badimon, and M. Borrell-Pages, *PCSK9 Functions in*

Atherosclerosis Are Not Limited to Plasmatic LDL-Cholesterol Regulation. Front Cardiovasc Med, 2021. **8**: p. 639727.

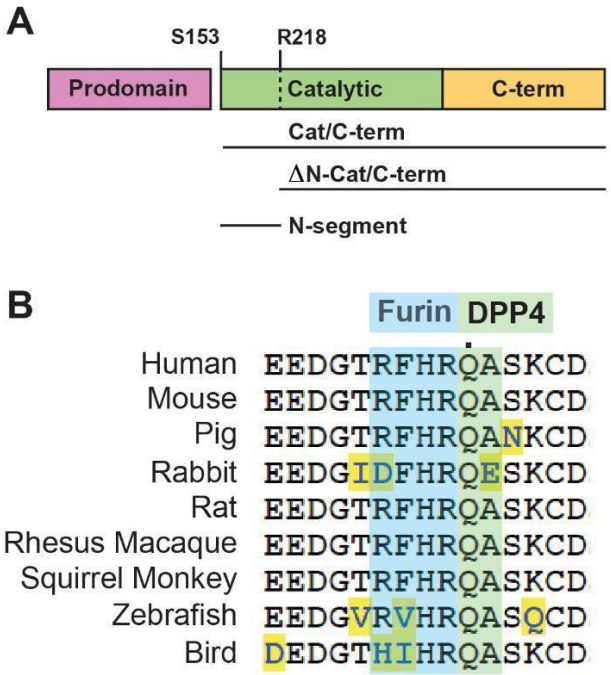
286. Stein, E.A., et al., *Effect of the proprotein convertase subtilisin/kexin 9 monoclonal antibody, AMG 145, in homozygous familial hypercholesterolemia.* Circulation, 2013. **128**(19): p. 2113-20.

Appendix A – Mutagenesis Primers

Sequences of forward (F) and reverse (R) primers used to mutate C-terminally FLAG-tagged wild-type human PCSK9.

Mutant		Primer Sequence (5'→ 3')
E48R	F:	CCTTGCGTTCCcgGGAGGACGGCC
	R:	GGCCGTCCTCCcgGGAACGCAAGG
E49R	F:	TGCGTTCCGAGcgGGACGGCCTGGCC
	R:	GCCAGGCCGTCCcgCTCGGAACGCA
G47SGG	F:	AGCCTTGCGTggCagtGgGGgCGGCCTGGCCG
	R:	GCCAGGCCGcCCcCactGccACGCAAGG
S47A	F:	CTAGCCTTGCGTgCCGAGGAGGACGG
	R:	CCGTCCTCCTCGGcACGCAAGGCTAG
E48KD50A	F:	GCGTTCCaAGGAGGcCGGCCTGG
	R:	CCAGGCCGgCCTCCTtGGAACGC
Q152•	F:	GTCTTTGCCtAGAGCATCCCGTG
	R:	CACGGGATGCTCTaGGCAAAGAC
S47AE48K	F:	CTTGCGTGCCAAGGAGGACGGCC
	R:	GGCCGTCCTCCTTGGCACGCAAG
E49A	F:	GCGTTCCGAGGcGGACGGCCT
	R:	GGCCGTCCgCCTCGGAA
D50R	F:	GTTCCGAGGAGcgCGGCCTGGCCGA
	R:	GCCAGGCCGcgCTCCTCG
E48K	F:	TTGCGTTCCaAGGAGGACGGC
	R:	GGCCGTCCTCCTtGGAACGCAAGGC
E48A	F:	GCCTTGCGTTCCGcGGAGGACGGCC
	R:	GGCCGTCCTCCgCGGAACGCAAGGC
D50A	F:	CAGGAGGACGAGGACcGCGACTACGAGGAGC
	R:	GCTCCTCGTAGTCGCgGTCCTCGTCCTCCTG
R218S	F:	CGCTTCCACAGtCAGGCCAGC
	R:	GCTGGCCTGaCTGTGGAAGCG
S47D	F:	CCTTGCGTgaCGAGGAGGACGGCC
	R:	GGCCGTCCTCCTCGtcACGCAAGG
RRRR^{218}E	F:	GGGACCCGCcgCCgCAGAgAGGCCAGC
	R:	GCTGGCCTcTCTGcGGcgGCGGGTCCC
RRRR^{218}ET	F:	CCCGCcgCCgCAGAgAGaCCAGCAAG
	R:	CTTGCTGtCCTcTCTGcGGcgGCGGG
RRRR^{218}	F:	GACCCGCcgCCgCAGACA
	R:	CCTGTCTGcGGcgGCGGG

Appendix B Supplementary data



Supplementary Figure 1: Schematic Proteolytic processing of PCSK9 A) Secreted mature PCSK9 showing the prodomain, catalytic, and C-terminal domain, the furin-cleavage fragments, and the excised ~7-kDa segment. (B) Evolutionary sequence conservation at furin- and DPP4-cleavage sites

Curriculum Vitae

Ikhuosho Charity Asikhia

EDUCATION

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY
University of Ottawa, 2019 to Present

- Thesis title: Exploring Alternate Conformations of PCSK9: Effects on LDL, LDL Receptor Binding, and Inflammation

COMPLETED COURSEWORK TOWARD MASTER'S DEGREE IN BIOCHEMISTRY,
University of Ottawa, 2018 to 2019

- Thesis title: Exploring Alternate Conformations of PCSK9: Effects on LDL, LDL Receptor Binding, and Inflammation

POSTGRADUATE DIPLOMA IN BIOCHEMISTRY, Nigeria
The Federal University of Technology, 2014 - 2015

SELECTED COURSES (ASSOCIATE IN SCIENCE (A.S.) IN MEDICAL LABORATORY TECHNOLOGY, Monrovia
Mother Patern College of Health Sciences, Stella Maris Polytechnic, 2011 – 2012
Relevant Coursework

Parasitology, Clinical chemistry, Human anatomy and physiology, Hematology, Clinical microbiology and Urinalysis

HIGHER NATIONAL DIPLOMA IN CHEMISTRY/BIOCHEMISTRY
Benue Polytechnic, Ugbokolo, Nigeria, 2006 - 2008

NATIONAL DIPLOMA IN SCIENCE LABORATORY TECHNOLOGY
Federal Polytechnic, Auchi, Nigeria 2002 - 2004

RESEARCH AND PROFESSIONAL EXPERIENCE

Graduate research associate, November 2017 - present
Atherosclerosis, Genetics and Cell Biology Group, University of Ottawa Heart Institute Supervisor: Thomas Lagace Ph.D.

- Extensive Molecular Biology Techniques Expertise
- Highly skilled in Site-Directed Mutagenesis

- Extensive Cell Biology Techniques
- DNA Extraction
- Protein Chemistry
- HPLC - Protein Purification
- Research Planning
- Team Leadership
- Ability to Balance Multiple Priorities

LAB TECHNICIAN

Bio Geo Chemistry Research Group, University of Eastern Finland, Kuopio, 2017 Supervisor: Henri Siljani, Ph.D.

- Cell Biology
- Molecular Biology Techniques
- Analyzed experimental results while maintaining compliance with specifications
- Collaborated with other scientists and fellow technicians on designing experiments and developing protocols

MEDICAL SUPPLY TEAM MEMBER (INTERN), Joensuu, Eastern

Suomen Punainen risti (Finnish Red Cross), September 2016 – April 2017

- Ensure Efficient Medical Supply to the Team through detailed reports outlining progress toward the departmental goal
- Work with the Medical Team to Ensure a Smooth Flow of Operation
- Ensure Operational Standards are always Met
- Ensure the Correct Temperature is Maintained on all Items

BIOLOGY/CHEMISTRY LABORATORY SUPERVISOR, Monrovia, Montserrado

Mother Patern College of Health Sciences, April 2011 – April 2013

- Ensured efficient operation of the Lab through regular audits of laboratory operations to identify areas for improvement
- Work with various Instructors to provide training to Students
- Ensure Operational Standards are always met
- Established quality assurance and quality control processes to ensure the accuracy of results
- Instructor for third-year Chemistry
- Instructor - Nursing (Introductory to Biochemistry for first-year Nursing students).

LAB TECHNICIAN, Kaduna

St. Louis Hospital, September 2009 – August 2010

- Followed safety protocols and procedures to maintain a safe working environment
- CD4 counts
- Routine Chemistry Test
- Routine Microbiology Test
- Lab Management.

LAB ASSISTANT, Benin, Edo State

King's Laboratory, January 2009 – August 2009

- Provided technical support during experiments by troubleshooting any issues that arose
- Performed and documented appropriate quality control records
- Conducted routine laboratory procedures within established guidelines
- Prepared, stored, and transferred samples for testing
- Assisted in preparing laboratory equipment and materials for experiments, including setting up apparatus according to instructions
- Analyzed samples using various techniques such as chromatography, spectroscopy, and microscopy
- Provided supportive activities in the lab operation, samples, and related daily maintenance
- Assisted with organizing data sets into meaningful information for reporting purposes

LAB ASSISTANT, Benin City, Edo State

Excel Medical Diagnostic, February 2005 – July 2006

- Performed data entry into laboratory databases for analysis and storage
- Adhered to all safety protocols in each laboratory area
- Organized sample collections from external sources for further analysis in the lab setting.
- Provided supportive activities in the lab operation, samples, and related daily maintenance.
- Prepared, stored, and transferred samples for testing
- Assisted in preparing laboratory equipment and materials for experiments, including setting up apparatus according to instructions
- Prepared samples for testing following standard protocols and safety procedures

Awards

- International Doctoral Scholarship 2019 - 2021
- CIHR Travel Award 2022 - Best abstract Ph.D. category. Canadian Lipid & Vascular Summit
- Faculty of Medicine (uOttawa) Travel Award 2022
- University of Ottawa Heart Institute Travel Award 2022

Publications

- Asikhia, I.; Chouli, L.; Kosenko, T.; & Lagace T. Proteolysis by furin improves PCSK9's binding interactions with native and oxidized low-density lipoprotein dependent upon prodomain retention. (2024) – Manuscript in Preparation
- Asikhia, I.; Renon, A.; Kosenko, T.; & Lagace T. An acidic N-terminal region in PCSK9 regulates LDL receptor binding affinity and prodomain association. (2024) – Manuscript in Preparation
- Sarkar, S. K., Matyas, A., Asikhia, I., Hu, Z., Golder, M., Beehler, K., Kosenko, T., & Lagace, T. A. (2022). Pathogenic gain-of-function mutations in the prodomain and C-terminal domain of PCSK9 inhibit LDL binding. Frontiers in Physiology, 13, 960272. https://doi.org/10.3389/fphys.2022.960272
- Sarkar, S. K., Foo, A. C. Y., Matyas, A., Asikhia, I., Kosenko, T., Goto, N. K., Vergara-Jaque, A., & Lagace, T. A. (2020). A transient amphipathic helix in the prodomain of PCSK9 facilitates binding to low-density lipoprotein particles. The Journal of Biological Chemistry, 295(8), 2285–2298. https://doi.org/10.1074/jbc.RA119.010221
- Salawu, S. O., Olukemi, B. E., Asikhia, I. C., & Akindahunsi, A. A. (2018). Mineral Elements Bio-Accessibility and Antioxidant Indices of Blanched Basella Rubra at Different Phases of in vitro Gastrointestinal Digestion. Preventive nutrition and food science, 23(1), 22–29. https://doi.org/10.3746/pnf.2018.23.1.22