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Apolipoprotein(a) Secretion is Modulated by Sortilin, Proprotein Convertase Subtilisin/Kexin Type 9, and Microsomal Triglyceride Transfer Protein

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Abstract

Elevated plasma lipoprotein(a) (Lp(a)) levels are a causal risk factor for cardiovascular disease (CVD), but development of specific Lp(a) lowering therapeutics has been hindered by insufficient understanding of Lp(a) biology. For example, the location of the noncovalent interaction that precedes the extracellular disulfide linkage between apolipoprotein(a) (apo(a)) and apolipoprotein B-100 (apoB-100) in Lp(a) biosynthesis is unclear. In this study we modulated known intracellular regulators of apoB-100 production and then assessed apo(a) secretion from human HepG2 cells expressing 17-kringle (17K) apo(a) isoform variants using pulse-chase analysis. Treating 17K-expressing HepG2 cells with proprotein convertase subtilisin-kexin type 9 (PCSK9) significantly increased apo(a) secretion. Treating the same cell line with Lomitapide, a microsomal triglyceride transfer protein (MTP) inhibitor, significantly decreased apo(a) secretion. Overexpression of human sortilin variants (F404Y and K302E) significantly increased apo(a) secretion relative to wild-type. Our findings suggest a role for sortilin, PCSK9, and MTP in modulating Lp(a) levels through effects on apo(a) secretion, possibly through impacting the intracellular bioavailability of apoB-100.

Keywords

Cardiovascular Disease, Lipoprotein(a), apolipoprotein(a), apolipoprotein B-100, sortilin, proprotein convertase subtilisin-kexin type 9, Lomitapide, microsomal triglyceride transfer protein

Lay Summary

Lipoprotein(a) (Lp(a)) is a circulating lipoprotein that contributes to the development of cardiovascular disease. The structure of lipoprotein(a) consists of a low-density lipoprotein-like core that is covalently attached to apolipoprotein(a). Unfortunately, no pharmacological therapies designed to specifically lower Lp(a) currently exist, which reflects a lack of fundamental understanding of the mechanisms regulating Lp(a) production and catabolism. In this study, we modulated known regulators of apoB-100 secretion and subsequently analyzed apo(a) secretion to determine if a non-covalent interaction exists between apo(a) and apoB-100 in Lp(a) production.

Co-Authorship Statement

All of the data in this thesis was collected by Justin Clark. Dr. Michael Boffa and Dr. Marlys Koschinsky contributed to the general supervision of work, including the generation of appropriate experimental designs and editing of this thesis.

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List of Abbreviations

AA – Amino Acid

Apo(a) – Apolipoprotein(a)

Apo B-100 – Apolipoprotein B100

CAD – Coronary Artery Disease

CVD – Cardiovascular Disease

ε-ACA – Epsilon-Aminocaproic Acid

ER – Endoplasmic Reticulum

ERAD – Endoplasmic-Reticulum Associated Protein Degradation

FCR – Fractional Catabolic Rate

FH – Familial Hypercholesterolemia

GWAS – Genome-Wide Association Studies

Hek 293 – Human Embryonic Kidney 293 Cells

HepG2 – Human Hepatocellular Carcinoma Cells

HMG CoA-R – Hydroxy-3-Methylglutaryl Coenzyme A Reductase

LDL – Low Density Lipoprotein

LDL-C – Low Density Lipoprotein Cholesterol

LDLR – Low Density Lipoprotein Receptor

Lp(a) – Lipoprotein(a)

MI – Myocardial Infarction

MTP – Microsomal Triglyceride Transfer Protein

oxPL – Oxidized Phospholipid

PCSK9 – Proprotein Convertase Subtilisin/Kexin Type 9

ROS – Reactive Oxygen Species

sLBS – Strong Lysine Binding Site

SNP – Single Nucleotide Polymorphism

SREBP – Sterol-Regulatory Element-Binding Protein

TGN – Trans Golgi Network

VLDL – Very Low Density Lipoprotein

VPS10 – Vacuolar Protein Sorting 10

WT – Wild-Type

wLBS – Weak Lysine Binding Site

17K – 17-Kringle

Chapter 1

1 Introduction

1.1 Cardiovascular Disease: Significance & Pathogenesis

Cardiovascular disease (CVD) remains the leading cause of death in the developed world [1]. CVD is a general term used to encompass all diseases of the blood vessels and heart. The most common CVD, accounting for the highest levels of morbidity and mortality, is coronary artery disease (CAD) [2]. CAD is a progressive disease caused by atherosclerosis, which is defined by the presence of fatty plaques in the walls of large-and medium-sized arteries [2]. Although much research has been undertaken to fully elucidate the pathogenesis of CAD, the steps involved in the disease process are not yet completely understood [3]. It is believed that an initial insult to the blood vessel induces structural changes to the intercellular tight junctions between the single layer of endothelial cells that line the arteries, resulting in increased vessel permeability [3]. Shear-stress, smoking, and dyslipidemias are all examples of proatherogenic stimuli that can cause endothelial dysfunction and trigger the development of CAD. Although each cause of endothelial dysfunction appears quite distinct, further investigation has shown that they all share the common ability to activate enzymatic systems and cause reactive oxygen species (ROS) overproduction [4]. This creates an oxidative stress environment within the endothelial cells. ROS overproduction incites an inflammatory process by inducing the NF-κB pathway and the expression of NF-κB dependent genes [4]. VCAM-1, ICAM-1, E-selectins, and cytokines such as TNF-α and IL-1, are all examples of NF-κB dependent genes that are expressed in response to ROS production. The expression of these NF-κB dependent genes alters the phenotype of endothelial cells which subsequently results in an abnormal flux of LDL-cholesterol (LDL-C) and leukocytes into the vessel's intima layer [5]. Movement of inflammatory cells and cholesterol into the intimal layer of the vessel represent the initial steps in atherosclerosis pathogenesis, and precipitate the formation of local inflammation and fatty streak development [5].

Monocytes are one example of a leukocyte which migrates into the vessel wall during times of increased endothelial permeability. As the monocyte moves from the blood to the vessel intima, it differentiates into a phagocytic macrophage. These macrophages then begin to ingest oxidized LDL particles through phagocytosis, coat-pitted endocytosis, pinocytosis, and scavenger receptors [6]. Over time, these macrophages eventually become foam cells, and are referred to as such because of their lipid-filled, foam-like appearances. Continued uptake of oxidized lipids by the foam cells eventually results in lysosomal destabilization, ROS production, and the release of proinflammatory cytokines such as IL-1β and IL-18 [5]. This ROS production and cytokine release acts in a positive-feedback manner to further induce endothelial permeability, leading to greater accumulation of leukocytes and lipoproteins in the vessel wall. As a result, the fatty plaque within the vessel intima continues to grow in size, which leads to vessel narrowing and disrupted blood supply to downstream tissues.

Continued LDL-C uptake, as well as local intimal inflammation, causes the mechanisms regulating foam cell cholesterol homeostasis to become overwhelmed [5]. The end result of this homeostatic dysregulation is endoplasmic reticulum (ER) stress pathway-mediated apoptosis and necrosis [7]. Foam cell apoptosis and necrosis leads to increased release of cytokines, which acts both to further propagate the inflammatory response within the lesion's necrotic core, and to induce smooth muscle cell (SMC) migration from the tunica media to the tunica intima [6]. As SMC's migrate into the tunica intima, they undergo a phenotypic switch characterized by proliferation and decreased expression of contractile markers, and form a fibrous cap around the growing atherosclerotic fatty plaque to stabilize it [8]. As the fatty plaque continues to grow and develop in coronary arteries over several decades, it can eventually begin to reduce downstream blood flow to the cardiomyocytes of the heart, which presents clinically as stable angina [9]. Angina is not considered immediately life-threatening as the blood supply to the heart muscle is only reduced and not completely occluded. However, without intervention, the lesion can continue to grow, and the stability of the fibrous cap can become compromised through matrix metalloproteinase mediated degradation [10]. Continued necrotic core growth and fibrous cap instability can eventually result in increased risk of plaque rupture. Upon plaque

rupture, there is immediate thrombosis formation when plaque contents come into contact with the flowing blood. If the thrombosis completely occludes the coronary arteries, a myocardial infarction will ensue, whereby the muscles of the heart become ischemic and potentially necrotic if oxygen-rich blood cannot be re-supplied to the cardiomyocytes in a timely manner.

1.2 Lipoprotein(a) and Apolipoprotein(a)

1.2.1 Beyond LDL-cholesterol

The “Lipid Hypothesis” proposes that elevated levels of LDL-C contribute to the development of atherosclerosis. This hypothesis emerged as a result of early studies conducted by Nikolai Anitschkow. Anitschkow fed rabbits high cholesterol diets and observed that the extent of atherosclerosis formation was proportional to the absolute amount of and length of exposure to plasma cholesterol [11]. The “Lipid Hypothesis” has resulted in the clinical lowering of LDL-C in primary prevention settings to prevent CVD onset, and in secondary prevention to reduce recurrent event rates. Decades of evidence from various clinical trials involving more than 170,000 individuals have shown that the use of statin therapy to lower LDL-C results in reduced cardiovascular event rates [12]. However, recent randomized clinical trials, such as the Scandinavian Simvastatin Survival Study, have shown that approximately 20% of patients reaching goal LDL-C levels, still experience cardiovascular events [13]. These results suggest that the pathogenesis of atherosclerosis may be more complex than originally believed, and this data has pushed researchers in the cardiovascular field to uncover the existence of other risk factors which contribute to heart disease in patients who attain optimal LDL-C levels. The focus has shifted to relationships between HDL-cholesterol levels, elevated triglycerides, and comorbidities such as diabetes and inflammation with cardiovascular risk. Interestingly, the 9612 participant JUPITER trial found that among individuals treated with potent statin therapy, lipoprotein(a) (Lp(a)) was a significant determinant of residual CV risk [14].

1.2.2 Lp(a) as a Causal Risk Factor for CVD

Lp(a) was first described by Käre Berg in 1963 as a lipoprotein particle that shared many similarities with LDL. Like LDL, Lp(a) is a lipid-rich particle that contains a cholesteryl

ester core, a surface monolayer of phospholipids, and a single associated apolipoprotein B-100 (apoB-100) moiety [15]. The covalent attachment of a surface polymorphic glycoprotein called apolipoprotein(a) (apo(a)) to apoB-100 is the only component that clearly distinguishes Lp(a) from LDL [16]. Due to its structural similarities with LDL, many researchers began to hypothesize that Lp(a) might also contribute to CAD. Berg's group was able to determine that plasma Lp(a) levels were higher in myocardial infarction survivors than in control patients [15]. Many other early studies showed an association between elevated plasma Lp(a) and CAD, but these studies were criticized either because the findings could not be repeated, or because they were retrospective in design and thus could not distinguish whether or not Lp(a) was a causative risk factor for CAD [17].

The Physician Health Study published in the early 1990's concluded that there was no evidence for Lp(a) levels contributing to risk of future MI [18]. In this study, plasma samples were taken from 195 participants that subsequently developed angina, and from 195 age and sex-matched controls. Lp(a) plasma levels were measured by a commercially available nephelometric method, and ultimately the results suggested that Lp(a) levels should not be used as a screening tool to assess future cardiovascular risk. These findings decreased enthusiasm for Lp(a) research for more than 15 years. However, when this study was repeated over a decade later using a highly standardized reference-based ELISA method, it was found that individuals with Lp(a) levels in the top 80th percentile had a 2-fold increased risk for developing CAD [19]. In further support of this observation, several prospective studies utilizing new technologies and modern genetic approaches have also identified Lp(a) as an independent and causal risk factor for CAD and have sparked renewed interest in the field of Lp(a) research. Specifically, Mendelian randomization studies have allowed us to definitively establish a causal relationship between elevated plasma Lp(a) levels (over 50 mg/dL) and increased risk (2-fold) for CAD [20]. It has also been identified that roughly 20% of the global population has Lp(a) levels in excess of 50mg/dL, which represents a significant proportion of individuals at elevated risk for future cardiovascular events [21]. Although Lp(a) is now known to be an independent and causal risk factor for CAD, the exact mechanisms of how it contributes to the disease have not yet been fully elucidated.

1.2.3 Structure of Lp(a) and Apo(a)

The structure of Lp(a) shares many similarities with LDL in terms of its protein and lipid composition. Like LDL, the lipid core of Lp(a) is rich in cholesteryl esters and triglycerides, while the surface consists of a monolayer of phospholipids and some unesterified free cholesterol (Fig. 1.1) [22]. What makes Lp(a) unique from LDL is that it has a polymorphic apo(a) glycoprotein covalently attached to the apoB-100 component via a single disulfide bridge (Fig.1.1) [23]. Lp(a) is found circulating exclusively in the plasma of humans, Old World monkeys and apes [24, 25]. This is due to the fact that the *LPA* gene on chromosome 6q26-27, which encodes apo(a), is found and expressed only in these species [26]. Sequencing work performed by Eaton and colleagues has revealed that the *LPA* gene has likely emerged from a duplication of the plasminogen gene [27]. It appears that the two genes diverged during primate evolution about 40 million years ago [26].

Plasminogen is an inactive zymogen that can be cleaved by tissue plasminogen activator (tPA) to the active enzyme plasmin at sites of clot formation. Plasmin is an important player in the fibrinolytic system as it is responsible for degrading fibrin clots. Plasminogen is composed of a C-terminal serine protease domain, and, like other components of the fibrinolytic and coagulation cascades, contains five kringle domains (KI, KII, KIII, KIV, and KV) [28]. Kringles are so-named because of their tri-looped polypeptide structure, which has been said to be similar in appearance to a Danish pastry. These tri-looped polypeptide kringles are stabilized by three invariant internal disulfide bridges [29]. The *PLG* and *LPA* genes share roughly 85% sequence identity in both the untranslated and coding regions, and so it is no surprise that these two genes give rise to proteins that share many structural similarities [26]. The C-terminal serine protease domain found in plasminogen is also present in apo(a) but is functionally inactive in the latter [30]. Apo(a) also contains kringle domains that are somewhat similar in composition to plasminogen's, but due to deletion events, apo(a) lacks the plasminogen KI, KII and KIII domains [31]. Instead, apo(a) consists of 10 different plasminogen-like KIV domains ($KIV_{1\text{-}10}$) and a single plasminogen-like KV domain (Fig. 1.2). These repeated KIV domains can be divided into 10 subgroups based on differences in amino acid sequence, and likely arose as a result of gene expansion and differentiation [31]. Each of the 10 KIV domains are

found in only one copy, with the exception of KIV_2, which can have 3 to over 30 identical tandem repeats (Fig. 1.2) [32]. Differences in the number of tandem repeats in KIV_2 is attributable to allelic variation in the *LPA* gene and gives rise to apo(a) size heterogeneity between individuals [33].

The kringle domains of apo(a) are linked by 24-36 amino acids, which are rich in threonine, serine, and proline [34]. These linker regions are subjected to extensive *O*-linked glycosylation within the Golgi [35]. Each apo(a) KIV domain contains at least one possible site for addition of *N*-linked glycans, which appear to be important for proper folding and secretion of the protein [36]. Apo(a) is also similar to plasminogen in that some of its KIV domains contains amino acid sequences that coordinate lysine binding. Within each lysine binding site (LBS), there are cationic residues that interact with the C-terminus of lysine, and anionic residues that interact with the N-terminus of lysine [37]. The bottom of the LBS pocket is lined by hydrophobic residues that help stabilize the aliphatic lysine backbone [37]. Specifically, $KIV_{5\text{-}8}$ of apo(a) have each been identified as weak lysine binding sites (wLBS), whereas KIV_{10} has been identified as a strong lysine binding site (sLBS) [37]. These differences in lysine binding ability are due to differences in the amino acid composition of each kringle. It is also of note that KIV_9 possesses a free cysteine residue (specifically Cys^{4057}) that has been shown to facilitate the covalent linkage between apo(a) and apoB-100 [38].

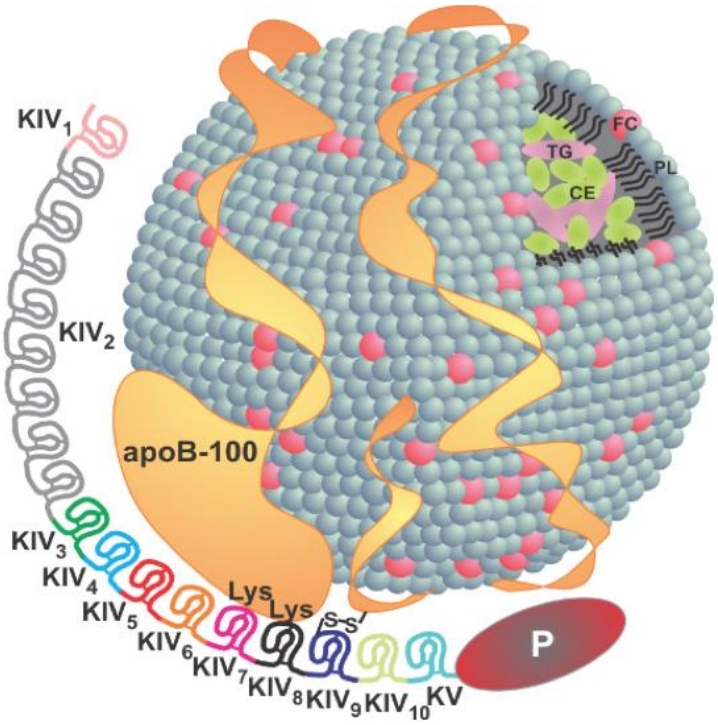


Figure 1.1: ***Structural components of Lipoprotein(a).*** Lipoprotein(a) (Lp(a)) can be distinguished from LDL by the presence of a covalently bound glycoprotein called apolipoprotein(a) (apo(a)). Like LDL, Lp(a) has a triglyceride (TG) and cholesteryl ester (CE) rich core that is surrounded by free cholesterol (FC) and a surface monolayer of phospholipids (PL). Lp(a) is also similar to LDL in that it contains a single associated molecule of apolipoprotein B-100 (apoB-100). Apo(a) consists of multiple repeated kringle IV (KIV) domains, a single kringle V (KV) domain and an inactive protease domain (P). There are 10 distinct subtypes of KIV ($KIV_{1\text{-}10}$) which can be differentiated based on amino acid sequence. Each KIV domain is found in only one copy, with the exception of KIV_2, which can be found in variable numbers of tandem repeats. The variable repeats in KIV_2 give rise to Lp(a) isoform size heterogeneity. Weak lysine binding sites in KIV_7 and KIV_8 mediate noncovalent interactions with specific lysine residues (Lys) in apoB-100. A free cysteine in the C-terminal region of the apoB-100 moiety covalently attaches to a free cysteine in KIV_9 of apo(a) to produce a molecule of Lp(a). Adapted from [39].

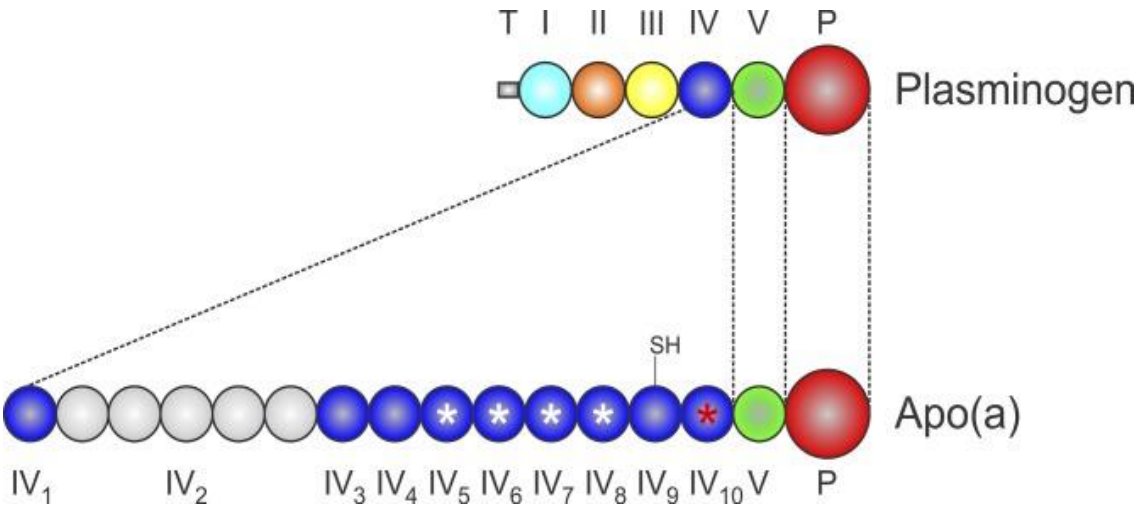


Figure 1.2: ***Structural similarities between apo(a) and plasminogen*****.** Apo(a) shares considerable homology with plasminogen. Like plasminogen, apo(a) is composed of multiple kringle domains. Apo(a) has 10 subtypes of kringle IV (KIV) and each subtype, with the exception of KIV_2, is found in only one copy. KIV_2 gives rise to the considerable size heterogeneity of apo(a) between individuals as it can be found in anywhere from 3 to over 30 identical tandem repeats (an isoform with 5 repeats is shown above). KIV_{5-8} have been identified to contain weak lysine binding sites (white stars), and KIV_{10} has been identified to contain a strong lysine binding site (red star). KIV_9 contains a free cysteine residue (Cys^{4057}) which is responsible for forming the covalent bond between apo(a) and apoB-100. Apo(a) also contains a single KV domain and an inactive serine protease (P). Apo(a) lacks the tail domain, KI, KII, and KIII found in plasminogen. Adapted from [40].

1.2.4 Proposed Mechanisms of Lp(a) Pathophysiology

As mentioned in section 1.2.2, recent Mendelian randomization studies have identified Lp(a) as a causal risk factor for CAD [20]. In fact, Lp(a) is now identified as the single most prevalent inherited risk factor for CVD [21]. Although genetic studies have identified Lp(a) as a causal risk factor, the mechanisms by which Lp(a) contributes to the disease process have not been fully elucidated. The structural similarities of Lp(a) to both plasminogen and LDL has led to the speculation that Lp(a) can contribute to both atherogenesis and thrombosis. It has been established that during periods of endothelial dysfunction, LDL can accumulate and be retained in the subendothelial space [41]. The process of atherogenesis begins when this retained LDL is oxidized, which subsequently leads to cytokine release, endothelial cell activation, monocyte recruitment, and the initiation of a pro-inflammatory response [42]. Because Lp(a) contains an LDL-like component, it was believed that Lp(a) can contribute to atherogenesis in a similar way and induce this pro-inflammatory state within the subendothelial space. Indeed, it has been shown that Lp(a) can readily move into the vessel intima layer, be taken up by resident macrophages, and induce inflammation [43]. However, it is also believed that the apo(a) component of Lp(a) makes it a distinct risk factor from LDL. Homology between apo(a) and plasminogen led many to postulate that Lp(a) may have some impact on thrombosis or fibrinolysis. Importantly, a fluorescence-based *in vitro* technique showed that Lp(a) was able to significantly reduce the conversion of plasminogen to plasmin, thus implicating Lp(a) as a prothrombotic molecule [30]. Therefore, the aforementioned studies suggest that the Lp(a) particle may be able to uniquely contribute to the pathogenesis of CVD, both through a pro-inflammatory / proatherogenic arm, and a prothrombotic arm.

1.2.4.1 The Pro-Inflammatory & Proatherogenic Arm:

One of the major ways Lp(a) is thought to contribute to the pathogenesis of CAD is through its role as an oxidized phospholipid (oxPL) carrier, particularly because of the well-known pro-inflammatory and pro-atherogenic properties that oxPLs possess. It was originally believed that the site of apo(a) oxPL addition was within the KV domain. However, subsequent studies showed the presence of oxPLs on apo(a) variants that lacked the KV domain [44]. Leibundgut's findings, coupled with more recent *in vitro* work, have

determined that the apo(a) KIV_{10} domain is the site of oxPL addition, as disruption of the lysine binding site within this domain results in the inability of apo(a) to bind to oxPLs [45]. It has been shown through *in vitro* and *in vivo* work that oxPLs preferentially bind covalently to the apo(a) KIV_{10} domain of Lp(a) [46]. In fact, transfer studies have shown that oxidized LDL particles will donate their oxPLs to Lp(a) in a time-and temperature-dependent manner [46]. OxPLs appear to influence atherogenesis by initiating robust immune responses that attract leukocytes to the site of vascular lesions. Several studies have demonstrated the ability of oxPLs in apo(a) to activate pro-inflammatory pathways and stimulate the release of various cytokines [47]. Oxidation of apo(a) has been associated with increased release of IL-8, a cytokine responsible for leukocyte recruitment [45]. Furthermore, *in vitro* and *in vivo* analysis has identified Lp(a) as a leukocyte chemoattractant through its ability to stimulate the release of monocyte chemoattractant protein-1 (Mac-1) [48]. Mac-1 is an important player in inflammatory responses by increasing the migration and infiltration of monocytes to sites of injury [48]. Although the mechanism by which Lp(a) modulates the release of Mac-1 has not yet been elucidated, it is plausible to speculate that the oxPLs of apo(a) play some role in this ability to stimulate chemotaxis.

In terms of changes in vascular endothelium, Lp(a), through its apo(a) KIV_{10} strong LBS, has been shown *in vitro* to induce a RhoA/Rho-kinase signaling cascade; this, in turn, results in stress fiber formation, and ultimately causes endothelial cell contraction and increased vessel permeability [49]. Studies in human umbilical vein endothelial cells (HUVECs) showed that apo(a) can further increase vascular permeability by causing disruptions in VE-cadherin/β-catenin complexes in a SRC dependent process [49]. Apo(a) has also been shown to alter vascular endothelium by inducing expression of E-selectin [50] and ICAM-1 [51], which are important leukocyte adhesion molecules that initiate the translocation of monocytes from the vessel lumen to the vascular intima.

Apo(a) has also been shown to modulate endothelial cell and SMC migration. Migration assays showed that apo(a) induced strong RhoA and integrin $\alpha_V\beta_3$-dependent SMC chemorepulsion [52]. SMC chemoattraction is a common feature of the pathogenesis of atherosclerosis as these cells act to increase plaque stability. Therefore, by acting as a SMC

chemorepellent, it is believed that apo(a) subsequently decreases plaque stability and increases vulnerability to rupture [52]. Furthermore, the oxPLs on Lp(a) have been shown to induce apoptosis in endoplasmic-reticulum stressed macrophages. This effect was mediated through CD36 and TLR2 signaling, and led to plaque necrosis and subsequent plaque vulnerability [53] (Fig. 1.3).

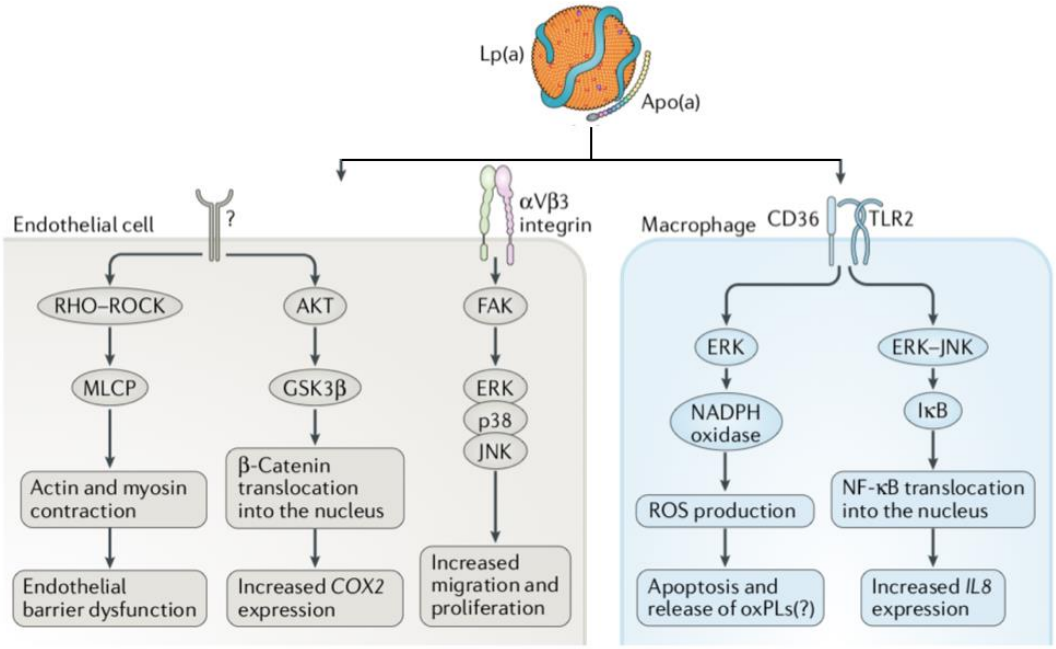


Figure 1.3: ***The proatherogenic effects of Lp(a) and apo(a) on macrophages and endothelial cells.*** The apo(a) KIV_{10} domain possesses a sLBS site capable of covalently binding oxPLs. The oxPLs on apo(a) can stimulate macrophages to release the pro-inflammatory IL-8 chemokine. Apo(a) mediated IL-8 production and release from macrophages appears to be dependent on TLR binding and NF-κβ signaling. The oxPL component of apo(a) can also trigger apoptosis in ER-stressed macrophages. The apoptotic event is dependent on signaling through TLR2 and CD36, and contributes greatly to plaque necrosis and vulnerability. Apo(a), through its oxPL component, can also induce cytoskeletal rearrangements through RhoA/Rho-kinase signaling, which subsequently increases endothelial permeability. Furthermore, apo(a) oxPLs are able to stimulate endothelial COX2 production through AKT-mediated nuclear translocation of β-catenin. COX2 is believed to play an important role in the formation and maintenance of atherosclerotic plaques. The endothelial receptors responsible for Lp(a)-mediated cytoskeletal rearrangements and COX2 expression have not yet been elucidated. Finally, the oxPL component of apo(a) can induce proliferation and migration of both endothelial cells and smooth muscle cells through integrin αVβ3 signaling and FAK-mediated MAPK pathway activation. FAK, Focal adhesion kinase; MLCP; myosin-light-chain phosphatase; NF-κβ, Nuclear factor-κβ; ROS, Reactive oxygen species; TLR2, toll-like receptor 2. Adapted from [47].

1.2.4.2 The Prothrombotic Arm:

As mentioned, apo(a) and plasminogen share extensive sequence homology. This has led many researchers to postulate a role for Lp(a) as a prothrombotic factor in CVD. This idea arose specifically because the protease domain of apo(a) retains the ability to bind to lysine-containing substrates but lacks any enzymatic activity [40]. Apo(a) has not been shown to have any inhibitory effect on fibrin clot break down in the presence of fully activated plasmin [54]. However, several *in vitro* studies have clearly indicated that apo(a) and Lp(a) are able to inhibit tPA-mediated plasminogen activation and thus subsequently inhibit tPA-mediated clot lysis [55, 56]. Furthermore, apo(a) has been shown to reduce the plasmin-mediated positive feedback loop that results in the conversion of Glu^{1}-plasminogen to Lys^{77}-plasminogen [57]. Lys^{77}-plasminogen lacks the tail domain, which in turn makes it a better tPA substrate and enhances plasminogen activation. Therefore, although apo(a) does not appear to inhibit the activity of active plasmin, it has been shown to attenuate both tPA-mediated plasminogen activation and the positive-feedback loop of plasmin-mediated plasminogen activation, thus implicating Lp(a) as an anti-fibrinolytic factor. Sequences within KIV_{5-8}, the sLBS in KIV_{10}, and the KV domain appear to be essential to the inhibition of plasminogen activation [58]. The impact of each domain to the inhibition of plasminogen activation has not yet been identified. However, it can be predicted that the complex 3-dimensional structure of apo(a) is responsible for making contacts with plasminogen, fibrin, and potentially tPA, and thus it is possible that this complex protein structure accounts for the antifibrinolytic properties of Lp(a). In fact, *in vitro* studies using a 12-kringle apo(a) isoform have demonstrated that apo(a) can interact with fibrin, tPA and plasminogen [30].

In vitro and animal model studies have also provided evidence that apo(a) and Lp(a) may enhance atherothrombotic platelet aggregation. Specifically, one study has indicated that apo(a) is able to enhance platelet aggregation and granule release mediated through the thrombin receptor-activating hexapeptide SFLLRN [59]. Lp(a) also appears to contribute to atherothrombosis through its ability to bind to tissue factor pathway inhibitor (TFPI). TFPI suppresses coagulation through its ability to bind to Factor Xa and subsequently the tissue factor/Factor VIIa complex [60]. Therefore, by binding to TFPI Lp(a) is able to

suppress the anti-coagulant activities of this inhibitor. In summary, *in vitro* and animal model studies have provided evidence that the prothrombotic arm of Lp(a) pathogenesis appears to involve both the attenuation of plasminogen activation, as well as atherothrombotic platelet aggregation.

1.2.5 Determinants of Plasma Lp(a) Concentrations

Plasma levels of Lp(a) can vary over 1000-times between individuals [61]. Circulating levels of Lp(a) appear to be largely dictated by production and secretion rather than by catabolism. Early studies using radiolabeled tracers showed that no relationship existed between plasma Lp(a) levels and fractional catabolic rate (FCR), but very strong correlations existed between plasma Lp(a) levels and production rates [62].

As mentioned, the KIV_2 domain of apo(a) can be found in anywhere from 3 to over 30 identical tandem repeats and this gives rise to the considerable apo(a) size heterogeneity between individuals. It is this size heterogeneity, which is dictated by the *LPA* allele size, that is the main determinant of plasma Lp(a) concentrations [62]. *In vitro* studies in baboon hepatocytes have shown that smaller apo(a) isoforms are secreted more efficiently than larger ones. This supports the inverse relationship that has been reported in the population between apo(a) isoform size and plasma Lp(a) concentrations [63]. This is of clinical interest because it suggests that individuals with smaller apo(a) isoform sizes will have higher plasma concentrations of Lp(a), which, as mentioned previously, is now recognized as a causal risk factor for CVD. To support this idea, a large meta-analysis of 58,000 phenotyped subjects revealed that individuals with less than 22 KIV_2 repeats had a 2.08-fold increased relative risk of CAD than individuals with more than 22 KIV_2 repeats [64].

It was originally hypothesized that smaller apo(a) isoform sizes were secreted more efficiently than larger isoforms due to increased rates of protein folding. It was later shown that the rate of protein folding in the ER is identical for all apo(a) isoforms [63]. However, it was determined that the rate of post-translational maturation of apo(a) through carbohydrate chain addition differs between different apo(a) isoform sizes. Pulse-chase studies in baboon hepatocytes have revealed that both an immature, hypoglycosylated form of apo(a), and a mature, fully glycosylated form of apo(a) can be isolated from cell lysates

[65]. Importantly, only the mature, fully glycosylated form is secreted. Each KIV domain has one potential site of *N*-linked glycosylation, whereas the linker regions between KIV domains have up to 6 potential *O*-linked glycosylation sites [66]. For this reason, apo(a) alleles with higher numbers of KIV_2 repeats can have upwards of 300 *O*-linked and 50 *N*-linked carbohydrate chains [63]. The aforementioned pulse-chase studies in baboon hepatocytes also showed that only the immature form of apo(a) was detected in the first 30 minutes of chase. The mature, fully glycosylated form of apo(a) could not be detected until 30-60 minutes of chase time. This suggests that the maturation of apo(a) through carbohydrate chain additions may be a rate limiting step in Lp(a) production and account for plasma Lp(a) concentration differences between different apo(a) isoform sizes [65]. Interestingly, another study conducted in baboon hepatocytes showed that the use of tunicamysin, an inhibitor of *N*-linked glycosylation, as well as castanospermine, an inhibitor of *N*-linked glucose trimming, both completely prevented apo(a) maturation and secretion [63]. Therefore, it can be concluded that both the addition and the trimming of these *N*-linked glucose molecules is essential for apo(a) to move through the secretory pathway. In summary, it appears that larger apo(a) isoform sizes have increased retention times in the ER which leads to increased degradation via the ERAD pathway and presents clinically as decreased levels of plasma Lp(a) [67].

Although this inverse trend does appear to exist in most cases, the relationship between *LPA* allele size and Lp(a) levels can still vary quite extensively. In fact, the copy number variations in KIV_2 have been found to only account for about 75% of the variation in Lp(a) plasma concentrations [68]. Several genetic studies have been able to identify sequence variations within the *LPA* gene, that are causally associated with Lp(a) levels. For example, a rs1853021 single nucleotide polymorphism (SNP) in the 5'-flanking region of the *LPA* gene has been shown to introduce an alternative start codon, which leads to 60% lower transcriptional activity and decreased levels of plasma Lp(a) [69]. The Precocious Coronary Artery Disease study identified two more SNPs, rs3798220 and rs10455872, which are located in the protease-like domain and the long intron of KIV_7, respectively [70]. Although the mechanism is unknown, these SNPs were both found to be causally related to increased plasma Lp(a) levels and risk of coronary disease. Taken together, it has been determined that apo(a) isoform size and specific variants within the *LPA* gene account

for up to 90% of the differences in plasma Lp(a) concentrations observed between individuals [21].

1.2.6 Lp(a) Metabolism

Although ample amounts of literature exist describing the sequence of the *LPA* gene and the physical structure of Lp(a), there still remain many unanswered questions regarding how and where Lp(a) is assembled, secreted and cleared from the circulation. These gaps in knowledge have significantly hindered the developed of therapeutics to specifically lower elevated plasma Lp(a) levels.

1.2.6.1 Lp(a) Biosynthesis and Secretion

The *LPA* gene, which encodes apo(a), is located on chromosome 6q26-27 and is most highly expressed in the liver [71]. Lower amounts of apo(a) mRNA have also been identified in the brain, lung, testes, and adrenal and pituitary glands [72]. Although apo(a) is expressed in a variety of tissues, an elegant liver transplant study was able to show that only apo(a) derived from hepatic tissues is incorporated into the Lp(a) particle. In this study, it was noted that the recipient's apo(a) isoform size on their circulating Lp(a) changes to match that of the donor following liver transplantation [71].

The exact site of Lp(a) particle formation remains elusive. As mentioned previously, apo(a) is transcribed and then translated into the ER where it matures and moves through the golgi and secretory pathway. From here, it is still not well understood how and where this newly translated apo(a) protein associates with an apoB-100 containing lipoprotein to form Lp(a). Until recently, it was believed that newly synthesized apo(a) was freely secreted and would assemble extracellularly with circulating LDL to form Lp(a) [73]. This idea was supported by *in vitro* work in hepatocytes, where apo(a) appeared to assemble with LDL either in the media or on the cell surface [74]. This notion of extracellularly Lp(a) assembly was further supported when circulating Lp(a) complexes were isolated from apo(a) transgenic mice following injection of human LDL-apoB-100 [75]. The extracellular assembly dogma has been challenged by *in vivo* stable isotope studies in humans. Frischmann and colleagues were able to show that the production rates of apo(a) and apoB-100 in Lp(a) were very similar and took this as evidence of intracellular Lp(a) assembly [76]. However, it is

important to remember that an intracellular covalent apo(a) and apoB-100 containing Lp(a) complex has never been directly observed in any *in vivo* or *in vitro* work. The only evidence of an intracellular covalent complex came from *in vitro* work in human hepatic cells where a non-physiologically relevant apo(a) isoform size was used and expressed at non-physiologically relevant levels [77].

The origin of the LDL component of Lp(a) is not well understood. Some studies have shown that the FCR of apo(a) differs from that of LDL-apoB100, which might suggest that apo(a) is exchanged between different apoB-100 containing lipoproteins [78]. However, this could also be evidence of apo(a) and apoB-100 uptake and degradation or recycling to form new Lp(a) particles. One study showed evidence of apo(a) being secreted on a triglyceride-rich very low-density lipoprotein (VLDL) particles [79]. However, the general consensus in the field is that Lp(a) does not arise from lipolysis of apo(a)-containing VLDL particles [80]. The aforementioned metabolic labelling work by Frieschmann and colleagues also showed that the secretion kinetics of apoB-100 that is in complex with apo(a) is different from the secretion kinetics of apoB-100 in other lipoprotein particles. Therefore, it seems plausible that there might exist a unique pool of apoB-100 containing lipoproteins that are secreted from hepatocytes and destined for Lp(a) assembly [76].

Although very few studies have isolated intracellular covalent Lp(a) complexes [77], the kinetic studies showing similarities between apoB-100 and apo(a) secretion and production rates may serve as evidence of the existence of an intracellular non-covalent interaction between these two lipoproteins. These kinetic studies, coupled with the inability to isolate covalent Lp(a) complexes intracellularly, laid the foundation for the development of a two-step hypothesis for Lp(a) assembly (Fig. 1.4). The first step of Lp(a) assembly is believed to involve an initial non-covalent interaction between apoB-100 and apo(a). The rationale for this step developed from *in vitro* studies which showed that disruptions in the weak lysine binding sites of apo(a) resulted in significant decreases in Lp(a) assembly [81]. Importantly, covalent complexes could still be isolated, just at much lower levels than control [76]. As mentioned previously, apo(a) KIV_{5-8} each possess weak lysine binding sites, and so it was initially thought that all of these domains were important for this first step in Lp(a) assembly. However, it was later determined that only the weak lysine binding

sites in KIV_7 and KIV_8 are required for this non-covalent interaction [82]. Specific mutations to both the apo(a) KIV_7 and KIV_8 lysine binding sites resulted in a 13-fold decrease in binding affinity for apoB-100 and a 75% reduction in Lp(a) particle formation [82]. It was also determined that Lys^{680} and Lys^{690} in apoB-100 mediate this strong non-covalent interaction with the KIV_7 and KIV_8 domains of apo(a) [82]. Because these studies show that mutations in the weak lysine binding sites in KIV_7 and KIV_8 result in decreased efficiency of Lp(a) particle assembly, it has been hypothesized that this first step in Lp(a) production is likely important to properly orient apo(a) and apoB-100 for step two. Although the specific domains and residues involved with this step in Lp(a) formation are well known, whether or not this interaction occurs inside the cell remains elusive [83]. Interestingly, it has recently been shown that specific LDL-lowering therapeutics designed to inhibit apoB-100 maturation can also have effects on apo(a) and Lp(a) production and secretion [84]. This has been interpreted as indirect evidence of an intracellular interaction between apo(a) and apoB-100 and will be discussed in more detail below.

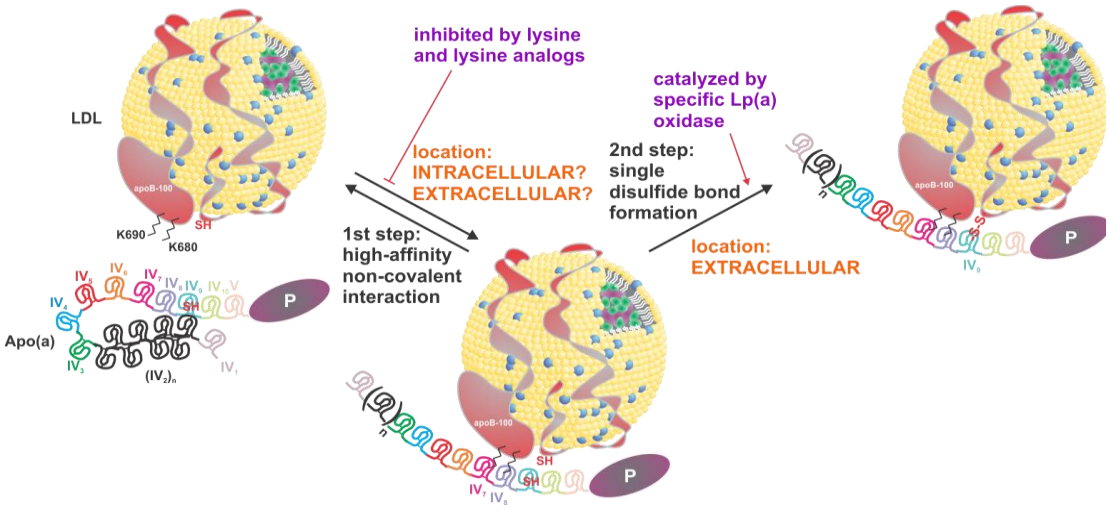


Figure 1.4: ***Schematic representation of the postulated two-step mechanism of Lp(a) assembly.*** Lp(a) biosynthesis takes place in hepatocytes. Assembly of Lp(a) involves the association of apo(a) with an LDL-like lipid particle of unknown origin. The first step of Lp(a) assembly is believed to involve a non-covalent interaction between Lys^{680} and Lys^{690} in apoB-100 and the KIV_7 and KIV_8 wLBS of apo(a). Disruption of the wLBS in KIV_7 and KIV_8 through lysine or lysine analogs results in decreased efficiency of Lp(a) particle assembly. It is not yet known whether the first step in Lp(a) biosynthesis is an intracellular or extracellular event. The second step is thought to occur on the cell surface or in the extracellular environment and involve the disulfide covalent linkage of a free cysteine in the C-terminus of apoB-100 (possibly Cys^{4326} or Cys^{3734}) with Cys^{4057} in KIV_9 of apo(a). A yet unknown extracellular enzyme with specific oxidase capacity appears to accelerate the covalent linkage between apo(a) and apoB-100.

The second step of Lp(a) assembly involves a disulfide covalent interaction between apo(a) and apoB-100 (Fig. 1.4). As mentioned, very few studies have been able to isolate covalent Lp(a) particles from cell lysates, and so it is generally accepted that this step occurs extracellularly. The idea that covalent assembly of Lp(a) occurs outside of the cell is strengthened by studies conducted in a cultured cell model that demonstrated a specific oxidase activity in the cell medium which helps facilitate the covalent linkage between apo(a) and apoB-100 [85]. The identity of this oxidase enzyme and how it interacts with the assembling Lp(a) particle is not known. It has been reported that the free Cys^{4057} in KIV_9 is responsible for single disulfide covalent interaction with a cysteine in the C-terminus of apoB-100 [38]. However, there is conflicting evidence in the literature as to which cysteine in apoB-100 is involved in covalent linkage with apo(a), although the majority of evidence suggests a role for either Cys^{4326} or Cys^{3734} [86, 87].

In summary, Lp(a) assembly is thought to occur via a two-step process: an initial non-covalent interaction dependent on the weak lysine binding sites of apo(a) KIV_{7-8} followed by a single disulfide covalent linkage between free cysteines in KIV_9 of apo(a) and the amino-terminus of apoB-100. The exact site of particle formation remains elusive. Whether or not the first step occurs inside the cell remains to be determined, but if a non-covalent interaction between apo(a) and apoB-100 is present intracellularly, then disruptions in the secretion of apoB-100 would be expected to impact the secretion of apo(a) and vice versa. Better understanding the molecular mechanisms involved in the biosynthesis and secretion of Lp(a) will likely provide insight into potential therapeutic targets for drugs targeted to specifically lower elevated levels of circulating Lp(a).

1.2.6.2 LDL-lowering Therapies & Insight into Lp(a) Assembly

As mentioned, it has recently been observed that certain pharmacological therapies designed to lower plasma LDL-C levels, also appear to have effects on plasma Lp(a) levels. Mipomersen is an antisense oligonucleotide directed against apoB-100 mRNA that promotes the degradation of target mRNA by RNase H1 [88]. The idea to inhibit apoB-100 secretion was motivated by studies of familial hypobetalipoproteinia (FHBL), an inherited condition characterized by low levels of circulating apoB-100/LDL and decreased arterial wall stiffness [89, 90]. By acting to decrease the production and secretion

of apoB-100, it was thought that mipomersen might be able to mimic some of the protective effects observed in FHBL patients. Early clinical trials showed that mipomersen could lower plasma apoB-100 and LDL-C levels by 50% and 35%, respectively [91]. Interestingly, a recent study involving healthy volunteers showed that mipomersen is also effective in lowering Lp(a) levels by 21% [92]. Importantly, changes in Lp(a) production rates were strong predictors of Lp(a) reduction in some individuals [91]. Therefore, mipomersen, a drug which affects the production rates of apoB-100, also appears to affect the production rate of Lp(a).

Another LDL-lowering therapy that targets apoB-100 production and affects plasma Lp(a) levels is lomitapide, a microsomal triglyceride transfer protein (MTP) inhibitor. MTP localizes to the ER where its main role is to load neutral lipids, i.e. triglycerols and cholesterol esters, to assembling apoB-containing lipoproteins [93]. The role of MTP in lipoprotein assembly will be discussed in more detail below. Interest in inhibiting MTP to treat elevated plasma cholesterol spurred from the discovery that genetic mutations in the MTP gene led to abetalipoproteinemia [94]. Lomitapide was one of the first MTP inhibitors to be discovered, and early phase 1 clinical trials in modestly hypercholesterolemic patients showed that a daily oral dose of 100 mg could lower LDL-C by up to 85% [95]. Interestingly, a 26-week phase 3 clinical trial consisting of 23 familial hypercholesterolemia patients receiving either 40 mg per day lomitapide or control showed not only a 40% reduction in LDL-C levels, but also a 13% reduction in plasma Lp(a) levels [95].

Unfortunately, both mipomersen and lomitapide have quite extensive side-effect profiles, and thus their use has been restricted to familial hypercholesterolemia (FH) patients or other high-risk patients that cannot meet cholesterol goals on statins, cholesterol absorption inhibitors, or proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors [95]. Both mipomersen and lomitapide inhibit apoB-100 containing lipoprotein secretion, which has in many cases been shown to result in increased hepatic fat content and increased serum liver transaminase levels [95]. Although these therapeutics may not serve as realistic treatment options for individuals with elevated Lp(a) levels because of their side-effect profiles, they can be utilized *in vitro* to gain insight into the mechanisms of Lp(a)

biosynthesis and secretion. Because lomitapide and mipomersen both function to decrease apoB-100 production and secretion and also subsequently lower Lp(a) levels by an unknown mechanism, they could serve to help identify whether or not an intracellular non-covalent interaction between apo(a) and apoB-100 exists in Lp(a) assembly. If a non-covalent interaction between apo(a) and apoB-100 does exist, then inhibiting apoB-100 mRNA translation with mipomersen, or inhibiting apoB-100 protein lipidation with lomitapide might result in decreased apo(a) secretion and thus explain the observed decreases in Lp(a) production rates associated with these two therapeutics. Assessments of apo(a) secretion rates in the presence and absence of mipomersen and lomitapide would help further support or refute this hypothesis.

1.2.6.3 Lp(a) Catabolism

Plasma Lp(a) clearance occurs almost exclusively in the liver, with minor amounts being catabolized through the kidney [96]. It has been determined that the apo(a) component of Lp(a) is the primary ligand for uptake, as co-injection of excess apo(a) with Lp(a) into mice strongly inhibits plasma Lp(a) clearance [96]. Although a specific Lp(a) uptake receptor has not yet been identified, multiple receptors with other defined ligands have shown to bind to and facilitate the clearance of Lp(a). The LDL receptor (LDLR) [96], VLDL receptor (VLDLR) [97], scavenger receptor class B member 1 [98], and the plasminogen receptor family [99] have all been described as having roles in Lp(a) catabolism. It seems likely that all of these receptors probably play some role in Lp(a) clearance, however the relative contribution of each is not yet known. Interestingly, the statin class of drugs, which lower plasma LDL-C levels through increasing the number of LDLR's at the cell surface, do not seem reduce plasma Lp(a) levels in most cases [100]. Therefore, it could be possible that under normal physiological conditions, each of the aforementioned receptor types play only minor roles in Lp(a) catabolism, and that it is the collective efforts of many different receptors that facilitate adequate Lp(a) clearance.

1.3 Proprotein Convertase Subtilisin/Kexin Type 9

1.3.1 Discovery & Defining Features

In 2003, PCSK9 was discovered and identified as the ninth member of the proprotein convertase family of proteases [101]. Since the 1960's it has been recognized that many hormones and enzymes are first secreted as zymogens, or inactive precursors, that require proteolytic cleavage to become active [102]. For this reason, it was hypothesized that specific enzymes might be responsible for regulating these proteolytic conversions. In the 1990's 8 enzymatic proteins, termed proprotein convertases (PC), were discovered to be responsible for catalytic processing and activation of various hormones, metalloproteases, growth factors, receptors, and surface glycoproteins [103].

The PCSK9 protein is a unique member of the PC family due to its atypical zymogen activation activity [101]. Transcription and translation of the PCSK9 gene results in the production of a 692 amino acid (AA) serine protease [104]. Within the ER, the newly synthesized PCSK9 protein undergoes cleavage of its signal peptide (AA 1-30) [101]. In order to exit the ER, this pro-PCSK9 (AA 31-692) must also cleave itself to release its prosegment (AA 32-152), and in doing so becomes the mature PCSK9 enzyme (AA 153-692) [101]. However, this self-cleaved prosegment becomes noncovalently bound to the substrate binding active site of the mature PCSK9 enzyme, which effectively inhibits any and all enzymatic activity [101]. In this way, PCSK9 is quite different from other members of the PC family because it displays no zymogen activation activities [103]. Although PCSK9 lacks zymogen activation activity, the protein has been observed to bind to specific target proteins and direct them towards intracellular degradation [103].

1.3.2 The LDL-Receptor & PCSK9

The PCSK9 gene is most highly expressed in the liver and is located on chromosome 1p32, a region that has been linked with FH [103]. For these reasons, it was hypothesized that PCSK9 may have some role in cholesterol metabolism. In 2003, Abifadel and colleagues discovered that specific gain-of-function mutations in the PCSK9 gene led to hypercholesterolemia [105]. It was at this point that PCSK9 was recognized to have a role

in lipid metabolism and became the third FH-associated locus, alongside the LDL-R gene and the apoB-100 gene [103].

Cholesterol is a key membrane component of all cells in the human body. Cholesterol can be obtained from the diet and transported in the circulation within chylomicrons or it can be synthesized *de novo*. *De novo* synthesis of cholesterol occurs in liver hepatocytes and is dependent on the activity of the rate-limiting hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA-R) enzyme [106]. The cholesterol produced from this pathway is packaged into triglyceride-rich VLDL particles and moves through the circulation to supply cellular demands. After VLDL particles are stripped of their triglyceride components by lipoprotein lipase, the cholesterol-rich remnant particle is remodelled by the liver to form LDL [107]. The LDL particle supplies cholesterol to all cells of the body through an interaction mediated through the LDL-R.

During periods of low intracellular cholesterol, cells of the body utilize a sensing mechanism involving the sterol-regulatory element-binding protein (SREBP), which facilitates increased expression of the LDL-R [108]. It should be noted here that the statin class of drugs act to inhibit HMG CoA-R. This causes decreases in intracellular cholesterol levels and results in SREBP-mediated increases in LDL-R levels and subsequent LDL-C plasma clearance [109]. The interaction between the apoB-100 component of LDL with the LDL-R at the cell surface triggers a receptor-mediated endocytosis event [110]. The endocytic vesicle containing the LDL/LDL-R complex moves towards the endosome and eventually fuses with the lysosome [110]. It is the low pH environment of the lysosome that facilitates the release of cholesterol and triglycerides from particle to the cytosol for cellular use [110]. This low pH environment also causes the dissociation of the LDL-R from the LDL particle, which can then be recycled back to the cell membrane in order to bind to and uptake more LDL [110].

The discovery of PCSK9 gave insight into another pathway for cholesterol regulation. PCSK9 is a soluble protein that circulates at low abundance and can bind to the epidermal growth factor-A (EGF-A) domain of the LDL-R [111]. Upon binding to the LDL-R at the cell surface, PCSK9 induces endocytosis and the PCSK9/LDL-R complex moves towards

the lysosome, much like the LDL/LDL-R complex pathway [110]. However, in the presence of PCSK9, the LDL-R is not able to dissociate from the complex at low pH to be recycled to the cell membrane [112]. Instead, through a yet unknown mechanism, the binding of the PCSK9/LDL-R complex becomes stronger at low pH, and the LDL-R is shuttled to the lysosome for degradation [113]. Therefore, by promoting LDL-R degradation, PCSK9 acts to inhibit cellular cholesterol uptake, which subsequently increases plasma LDL-C levels.

Recent evidence suggests that PCSK9 can also affect plasma LDL-C levels by augmenting apoB-100 secretion. Sun and colleagues overexpressed human PCSK9 in three separate mouse models and were able to consistently demonstrate significant increases in plasma apoB levels in an LDL-R independent fashion [114]. In this same study, co-immunoprecipitation (co-IP) showed that direct interactions between apoB and PCSK9 exist in cell lysates [114]. Furthermore, pulse-chase studies conducted in C57BL/6 LDL-$R^{-/-}$ mouse primary hepatocytes demonstrated that PCSK9 expression significantly increased both the secretion and intracellular accumulation of apoB-100 and apoB-48 [114]. Therefore, it appears that PCSK9 also has an impact on apoB-containing lipoprotein production through altering its secretion.

1.3.3 PCSK9 Inhibitors

Shortly after the discovery of PCSK9, it was determined that individuals with loss-of-function mutations in the PCSK9 gene had lifelong decreases in plasma LDL-C levels and significant reductions in the incidence of atherosclerosis [115]. It was at this point that pharmaceutical companies began to race to develop drugs that mimicked this PCSK9 loss-of-function phenotype. The most promising and successful PCSK9 inhibiting drugs manufactured to date are evolocumab and alirocumab, both of which are fully humanized monoclonal antibodies that bind to PCSK9 and block its interactions with the LDL-R [116]. By inhibiting the PCSK9-mediated degradation of the LDL-R, these monoclonal antibodies have proven to be effective in reducing plasma LDL-C levels by about 60%, and risk of MI and stroke by about 20% after a 2-year treatment regimen [116].

Interestingly, PCSK9 inhibitors have consistently been shown to also reduce Lp(a) levels by up to 30% [117]. Although the mechanism underlying these reductions is not yet understood, some have postulated that these decreases are due to PCSK9 inhibitor-mediated LDL-R up-regulation and subsequent increased Lp(a) catabolism. However, many are skeptical of this explanation. Lp(a) has been shown to be cleared through the LDL-R, but as mentioned earlier, this receptor likely only plays a minor role in Lp(a) catabolism [118]. In support of the idea that PCSK9 inhibition reduces plasma Lp(a) levels via a mechanism independent of the LDL-R, statins, which also augment LDL-C clearance by increasing LDL-R levels, have in many cases not proven to be effective in lowering Lp(a) levels [119]. These findings suggest that inhibition of PCSK9 may have additional effects beyond the LDL-R, which may account for the observed decreases in plasma Lp(a).

1.3.4 PCSK9 & Lp(a) Metabolism

The aforementioned plasma Lp(a) reductions mediated by PCSK9 inhibitors have motivated researchers in the field to understand how or if PCSK9 regulates Lp(a) metabolism. Work in our lab has shown that the addition of PCSK9 to culture medium inhibits Lp(a) internalization in HepG2 cells [120]. Although the LDL-R is not regarded as a major route for Lp(a) clearance, the Lp(a) internalization inhibition mentioned above did appear to be LDL-R dependent [120]. The effects of PCSK9-mediated Lp(a) internalization inhibition could not be repeated in fibroblasts from FH patients lacking the LDL-R [120]. It could be possible that treatment of cells with high concentrations of exogenous PCSK9 can reduce LDL-R levels enough to observe decreases in Lp(a) internalization versus control. Furthermore, all of the PCSK9 inhibitor studies that have demonstrated decreased Lp(a) levels involve study subjects that are also receiving optimal statin therapy [121]. This combination of treatments significantly increases the levels of LDL-R, and concomitantly significantly decreases its LDL ligand. These conditions decrease any potential competition between Lp(a) and LDL for the LDL-R, which may allow the LDL-R to become a more significant Lp(a) clearance receptor, and account for the observed plasma Lp(a) lowering in the PCSK9 inhibitor studies.

Interestingly, recent evidence suggests that PCSK9 does not significantly impact Lp(a) catabolism but rather enhances Lp(a) production and secretion. Villard and colleagues used

primary human hepatocytes to show that treatment of cells with increasing concentrations of PCSK9 resulted in a 3-fold increase in apo(a) secretion [122]. Moreover, this increase was completely reversed with alirocumab treatment [122]. Therefore, rather than decreasing Lp(a) uptake, it appears that decreases in plasma Lp(a) levels observed in PCSK9 inhibitor-treated individuals is mediated through decreased Lp(a) secretion. However, it has previously been demonstrated that PCSK9 cannot associate with apo(a) [120]. If this holds to be true, then the question of how PCSK9 increases apo(a) secretion can be raised. Intriguingly, *in vitro* studies have demonstrated that PCSK9 treatment does in fact increase apoB-100 secretion [122]. Therefore, it could be postulated that if an intracellular non-covalent interaction existed between apoB-100 and apo(a), then PCSK9-mediated increases in apoB-100 secretion may indirectly account for the observed increases in apo(a) secretion. Continued assessments of the secretion and intracellular accumulation patterns of both apo(a) and apoB-100 in the presence or absence of PCSK9 may help provide further mechanistic understandings of the steps involved in Lp(a) production. Furthermore, these studies would help elucidate how PCSK9 inhibition by alirocumab and evolocumab specifically lowers plasma Lp(a).

1.4 Microsomal Triglyceride Transfer Protein

1.4.1 Structure & Function

MTP is a heterodimeric protein complex that resides in the ER [123]. The smaller 55-kDa "P" subunit has been identified as ubiquitously expressed ER protein disulfide isomerase (PDI) [124]. Non-covalently bound to the P subunit is the larger 97-kDa "M" subunit [124]. The binding of the M subunit to the P subunit physically obstructs its active site, and thus MTP does not retain the ability to catalyze disulfide bonds [125]. The M subunit possesses the unique ability to transfer neutral lipids (triglycerides and cholesterol esters) to specific targets [126]. Interestingly, the P subunit does not have any capacity to transfer lipids, and it has been demonstrated that various missense mutations to the P subunit do not impact MTP's ability to transfer lipids [126]. However, addition of agents that disrupt the heterodimer complex results in aggregation of the M subunit and complete loss of lipid transfer activity, suggesting that the P subunit has a structural stability role [127].

Abetalipoproteinemia is a condition characterized by the inability to absorb dietary fats and fat-soluble vitamins, and is coupled with the complete lack of plasma apoB-containing lipoproteins. MTP was recognized as an important regulator of lipoprotein synthesis when it was discovered that individuals with abetalipoproteinemia had specific loss-of-function mutations in MTP-encoding genes. The liver and intestine are the major sites of MTP expression. Importantly, these organs are also responsible for the production of apoB-containing VLDL and chylomicron particles, respectively. The M subunit of MTP contains three major structural domains: a N-terminal β-barrel domain, a middle α-helical domain, and a C-terminal β-sheet [93]. The N-terminal B-barrel is responsible for interaction with the N-terminus of apoB, the middle α-helical domain interacts with both apoB and the P subunit, while the C-terminal β-sheet is crucial for the lipid binding and lipid transfer activities of MTP [93].

1.4.2 MTP & ApoB-containing Lipoprotein Assembly

MTP was recognized as an important lipid transfer molecule after it was discovered that specific missense mutations in the M subunit lipid transfer domain precipitated the complete lack of circulating apoB in abetalipoproteinemia patients. The dependency of apoB production and secretion on MTP activity was further demonstrated in an elegantly designed *in vitro* study. ApoB and MTP were co-expressed in non-hepatic cells that do not naturally express these proteins [128]. In the absence of MTP, apoB was synthesized but was then subsequently degraded and not secreted [128]. In contrast, when apoB and MTP were coexpressed in this cell model, apoB was both synthesized and secreted [128].

The binding of MTP to apoB is hypothesized to serve two functions [129]. Firstly, it is believed that MTP binding is critical for the prevention of apoB degradation [129]. It is known that newly synthesized apoB undergoes dislocation from the ER membrane and is subsequently degraded by proteasomes if it is not assembled into a lipoprotein [130]. Researchers have postulated that by binding to nascent apoB, MTP acts to prevent its dislocation from the ER membrane, thereby inhibiting its proteosomal degradation [129]. Secondly, it is believed that MTP plays a crucial role in primordial lipoprotein assembly through its lipid transfer activities. MTP-mediated lipidation of apoB-peptides renders

them secretion-competent. Although this process is still not well understood, it is assumed that free MTP in the ER binds to nascent apoB and then proceeds to extract lipids from the ER membrane and transfer them to apoB [129]. It has also been hypothesized that rather than transferring lipids to apoB from the ER membrane, MTP associates with a lipid droplet in the ER lumen, which it then donates to nascent apoB [129]. This droplet would become a lipid core for which the nascent apoB could encircle [129]. Regardless of the mechanism, it is clear that the physical binding and transfer of neutral lipids between apoB and MTP in the ER is essential for the production and secretion of apoB-containing lipoproteins.

1.4.3 Implications with Lp(a)

As discussed previously, MTP has become a lipid lowering target to treat diseases characterized by high circulating levels of apoB-containing lipoproteins [131]. However, because of MTP's important role as a regulator of lipoprotein secretion, studies have shown that individuals taking MTP inhibiting medications, such as lomitapide, experience increased hepatic fat retention and increased plasma liver transaminase levels [132]. These findings have limited lomitapide use to specific cases where other treatment alternatives have proven to be ineffective. Despite its side-effect profile, lomitapide has sparked interest and conversation amongst researchers due to its ability to lower plasma Lp(a) levels. As mentioned, clinical trials have shown that lomitapide can both lower plasma LDL by up to 40% and plasma Lp(a) by up to 13% [93]. However, the mechanism by which lomitapide lowers plasma Lp(a) remains unknown.

It has been hypothesized by some groups that apo(a) is secreted freely on its own and later interacts with circulating LDL or LDL at hepatocyte cell surfaces to form Lp(a) [73]. If this dogma is correct, then lomitapide-mediated inhibition of apoB-containing lipoprotein production would lower Lp(a) levels as a consequence of lower levels of circulating LDL being available for free apo(a) to bind to. However, previously mentioned kinetic studies have shown that apo(a) and apoB-100 production rates are quite similar, and that there might be a unique pool of apoB-100 destined for Lp(a) assembly [76]. These studies have suggested the idea that the initial steps in Lp(a) production may involve a non-covalent intracellular interaction between apo(a) and apoB-100. *In vitro* analysis of apo(a) and apoB-100 secretion in cells treated with lomitapide may help to elucidate the assembly and

production of Lp(a). Unlike apoB-100, apo(a) does not undergo lipidation in the ER, and thus its production does not appear to be MTP-dependent. Therefore, decreased secretion of apo(a) in response to lomitapide treatment may be interpreted as indirect evidence of an apo(a)/apoB-100 intracellular interaction that is critical for apo(a) secretion. Conversely, if apo(a) production does not involve intracellular interactions with apoB-100, then lomitapide treatment would not be expected to impact apo(a) secretion.

1.5 Sortilin

1.5.1 Genome-Wide Association Studies

Atherosclerosis is a multifactorial disease that involves the contribution of genetic and environmental risk factors. Although atherosclerosis has been studied for over a century, CVD still remains the leading cause of death in Western countries. This reflects the complexity of the disease process and emphasizes the need for recognition of novel risk factors and genetic mutations that contribute to the pathophysiology of CVD. Genome-wide association (GWA) studies are observational studies that are used to identify genetic associations on an epidemiological basis, typically by analyzing single nucleotide polymorphisms (SNPs) [133]. These genetic studies provide identification of risk alleles associated with complex diseases, like neurodegenerative and cardiovascular diseases [134]. GWA studies of large populations with wide ethnicity and age demographics have been conducted to identify specific genes or loci associated with elevated plasma LDL-cholesterol (LDL-C) phenotypes. These studies have served to identify numerous SNP variants throughout the human genome that are strongly associated with LDL-C levels. Not surprising, the vast proportion of these SNPs were found to occur in the genes encoding PCSK9, apoB, the LDL-R, and other protein players known to be causally involved in plasma cholesterol regulation. Interestingly, the 1p13 chromosomal region was identified as being more strongly associated with LDL-C levels than any other locus in the human genome. Despite not having any known role in lipid metabolism, this locus has been independently associated with CAD and MI. Importantly, a noncoding rs12740374 variant in the 1p13 locus has been identified as the causal SNP responsible for modulating plasma LDL-C levels and MI risk [134]. In depth analysis has revealed that individuals with the minor allele variant at this locus have significantly elevated expression of the sortilin-

encoding *SORT1* gene. In fact, compared to major allele homozygotes, individuals homozygous for the minor allele variant have 12-fold higher *SORT1* expression levels. Interestingly, these elevated expression levels occurred exclusively in the liver. Further examination has shown that rs12740374 alters the binding of the liver specific C/EBP transcription factor; the major allele disrupts C/EBP binding, which has been demonstrated to decrease *SORT1* expression [134]. After determining that a causal relationship exists between rs12740374 and LDL-C levels, this same research group used an adeno-associated virus approach to specifically overexpress *SORT1* in mouse hepatocytes. *In vivo* analysis showed that *SORT1* overexpression resulted in significantly decreased total plasma cholesterol levels [134]. Therefore, due to its interaction with C/EBP and increased expression of *SORT1*, the minor allele was assumed to be cardioprotective. However, as will be discussed below, there is much controversy surrounding the true role of sortilin in lipid metabolism.

1.5.2 Structure & Function

Sortilin is a multi-ligand sorting receptor involved in the intracellular transport of proteins such as brain-derived neurotrophic factor, Glut4, and lipoprotein lipase [135]. Sortilin is characterized by an extracellular vacuolar protein sorting 10 (VPS10) domain and thus belongs to the VPS10 domain family of receptors [135]. The characteristic VPS10 ligand binding domain is homologous to the yeast VPS10P sorting receptor and is present in all receptors within this family. The four other members of this receptor family are SorLA, sorCS1, sorCS2 and sorCS3. The VPS10 domain, responsible for ligand binding, is 700 amino acids in length and forms a ten-bladed β-propeller physical structure [136]. The sortilin receptor also consists of a transmembrane domain, as well as a sorting and internalization motif-containing cytoplasmic tail [137].

Sortilin is a membrane-embedded receptor that is present in small amounts on the cell surface, but mainly localizes to the membrane of the trans-Golgi network (TGN) [138]. The TGN is a major center for trafficking and recycling of various different proteins, which is consistent with the hypothesized role of sortilin as an intracellular sorting receptor [138]. Depending on the molecule and the metabolic demands of the cell, proteins within the TGN can be sorted for secretion, packaged within endosomes, or targeted to the lysosome for

degradation [138]. To prevent premature ligand binding, sortilin is first synthesized as a propeptide [139]. Upon entering the TGN, sortilin is subsequently cleaved by furin, which enables it to become the mature receptor with full ligand binding capacity [139]. There are three proposed trafficking pathways for sortilin and the VPS10 family of receptors (Fig. 1.5): (i) The complex of sortilin and its ligand can move through the constitutive secretory pathway from the TGN towards the cell membrane. Depending on the properties of the protein, the ligand can either become a plasma membrane-embedded receptor or be secreted to the extracellular environment [140, 141]. (ii) Plasma membrane-embedded sortilin can bind to extracellular ligands and facilitate clathrin-dependent internalization. The internalized complex moves from the cell membrane to endosomes [137], where it can then be trafficked towards the TGN for ligand recycling [142], or towards the lysosome for ligand degradation [143]. (iii) Sortilin and its protein ligand can be exported from the TGN and secreted together into the extracellular environment within secretory granules or exosomes [144, 145]. In each case, the pathway taken is dependent on the binding of specific adaptor proteins to the trafficking motifs within sortilin's cytoplasmic tail [137].

Sortilin is most highly expressed in the brain and spinal cord, and was first identified as a neurotransmitter receptor and important regulator of neuronal survival and degradation [146]. It has been hypothesized that sortilin and other members of the VPS10 family contribute to neuronal death and survivability by regulating the intracellular transport of neurotransmitters and neurotrophic factors towards or away from lysosomal degradation (123). Furthermore, sortilin and its relatives have been implicated in the pathogenesis of Alzheimer's disease after studies showed that improper processing of amyloid precursors by sortilin-related receptors facilitates the Aβ amyloid accumulation characteristic of the disease [146]. Lower levels of sortilin expression have also been observed in the kidneys, heart, liver, adipose tissue and skeletal muscle [147, 148]. As previously mentioned, another known ligand for sortilin is Glut4, the insulin-regulated glucose transporter [149]. Adipocytes and skeletal muscles cells store Glut4 intracellularly within Glut4 storage vesicles (GSVs). During periods of elevated plasma glucose levels, insulin mediated signalling regulates the shuttling of GSVs towards the cell membrane, which subsequently results in increased cellular glucose uptake [149]. Importantly, *in vitro* studies have

demonstrated that sortilin is a key component of GSVs that regulates the trafficking of Glut4 to the cell surface in response to insulin signaling [149]. These findings have suggested a role for sortilin as a regulator of insulin-mediated glucose uptake, and potentially implicate the sorting receptor in the pathogenesis of diabetes mellitus.

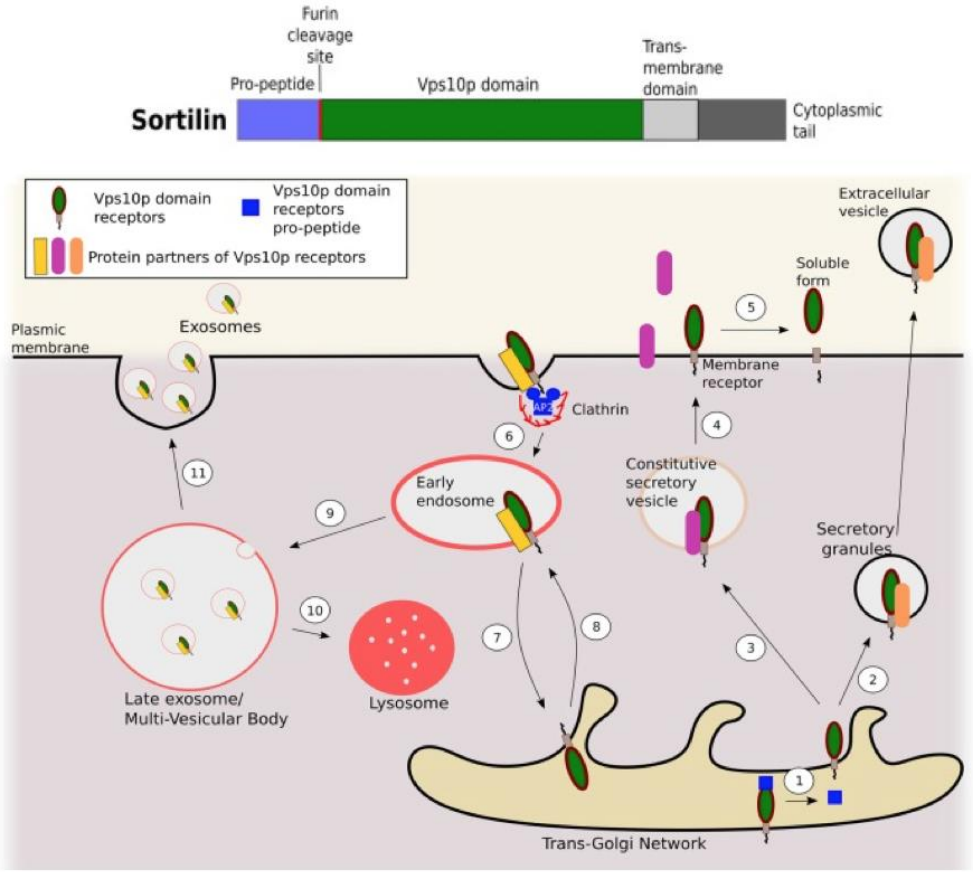


Figure 1.5: ***The proposed intracellular trafficking pathways of VPS10 family receptors.*** Trafficking of VPS10 domain receptors begins with cleavage of the propeptide in the TGN (1) and is thought to occur through at least 3 pathways: **(i)** Sortilin and its protein ligand can move through secretory granules (2) and the complex can be secreted together within extracellular vesicles. **(ii)** Sortilin and its ligand can move through constitutive secretory vesicles (3) towards the plasma membrane (4). Depending on the properties of the ligand, it can become an embedded plasma membrane receptor or be secreted to the extracellular environment. Sortilin can also become an embedded plasma membrane receptor, or can be cleaved by metalloproteases and γ-secretases to become a soluble protein (5). **(iii)** As a plasma membrane receptor, sortilin can bind to extracellular protein ligands and facilitate their internalization through AP-2/clathrin dependent endocytosis (6). Sortilin can direct the internalized protein towards the TGN for recycling (7,8) or towards the multi-vesicular body (9) and lysosome (10) for degradation. In each case, the pathway taken is dependent on the binding of specific adaptor proteins to the trafficking motifs within sortilin's cytoplasmic tail. Adapted from [150].

1.5.3 Sortilin's Enigmatic Role in LDL Metabolism

Several genome-wide studies conducted over the past 10 years have identified sortilin as an important regulator of lipid metabolism. Confusingly, numerous experimental approaches have demonstrated an association between sortilin knockdown and sortilin overexpression with increased plasma LDL-C levels. Although it is clear that sortilin modulates LDL metabolism, the direction of the relationship between sortilin expression and plasma LDL levels remains highly controversial.

Adeno-associated virus (AAV) expression of the *SORT1* gene in humanized mice livers by Musunuru and colleagues resulted in a significant decrease in plasma LDL-C levels [134]. These reductions in LDL-C levels were attributable to decreased secretion of the LDL precursor molecule, VLDL [134]. In further support of these findings, AAV-mediated *SORT1* liver overexpression in C57BL/6 mice fed high-fat diets resulted in markedly reduced plasma triglyceride and apoB-100 levels [151]. Importantly, the reductions in LDL-C observed in these studies appears to involve autophagy-dependent lysosomal degradation of apoB-100 [152]. It has been shown that autophagy formation inhibitors diminish the decreases in triglyceride and apoB-100 secretion associated with sortilin overexpression [152]. Furthermore, specific mutations within the cytoplasmic tail domain of sortilin inhibits its ability to induce the autophagic flux of apoB-100 [152]. It has also been shown, using surface plasma resonance studies, that high affinity binding exists between sortilin and apoB-100 [153, 154]. Taken together, the inverse correlation observed between *SORT1* expression and LDL-C levels in the aforementioned rodent studies appears to be dependent on high affinity binding between sortilin and apoB-100, followed by degradation of VLDL through the autophagy-lysosomal pathway.

The overexpression models that associated sortilin with increased apoB-100 secretion led to the hypothesis that sortilin knockout or knockdown would increase levels of plasma LDL-C and VLDL secretion. However, Kjolby and colleagues found that sortilin-deficient mice had significantly reduced levels of plasma LDL-C [155]. Furthermore, this decrease in plasma LDL-C observed in *SORT1* knockout mice was coupled with lower levels of VLDL secretion [155]. Importantly, this group also used an adenovirus system to specifically overexpress sortilin in mice hepatocytes. However, unlike Musunuru's group,

Kjolby and colleagues observed significant increased lipoprotein secretion and plasma LDL-C levels in response to hepatic sortilin overexpression [155]. Immunofluorescent detection of apoB in *SORT1*$^{-/-}$ and *SORT1*$^{+/+}$ mouse hepatocytes was also performed. In the sortilin-deficient mice, apoB immunostaining demonstrated dispersed staining throughout the cell, characteristic of ER localization [155]. Conversely, adenovirus-mediated sortilin overexpression in mice hepatocytes was associated with apoB accumulation in the trans golgi [155]. Therefore, Kjolby and colleagues concluded that sortilin acts to increase VLDL secretion and plasma LDL-C concentrations by way of an intracellular association between apoB and sortilin within the golgi, which facilitates the movement of apoB-containing lipoproteins through the secretory pathway.

The exact role of sortilin in lipid metabolism remains controversial. The relationship between sortilin, VLDL secretion, and LDL-C levels appears to be quite complex, and it has been postulated that the directionality of the relationship can be influenced by intracellular and extracellular metabolic conditions, as well as the experimental model used. For example, Kjolby's group used mice that had a double knockout of both the LDL-R and sortilin and demonstrated a direct relationship between *SORT1* expression and plasma cholesterol levels [155]. Interestingly, Linsel-Nitschke and colleagues demonstrated an inverse relationship between sortilin and LDL-C that appeared to involve LDL clearance [156]. This group reported that sortilin overexpression in human embryonic kidney 293 (Hek 293) cells resulted in elevated uptake of LDL particles, potentially through a mechanism involving modulation of the LDL-R [156]. Therefore, it could be possible that sortilin plays a role in both apoB-containing lipoprotein catabolism and secretion. If this were the case, then Kjolby's LDL-R knockout mouse model may have highlighted sortilin's role as a regulator of apoB-100 secretion, while masking its role in LDL clearance. It could also be possible that the extracellular lipid environment alters the directionality of sortilin's relationship with plasma cholesterol levels. For example, recent evidence suggests that apoB-100 secretion from *SORT1*$^{-/-}$ mice differs if the mouse is on a control diet or a high-fat diet [157]. In line with this idea, it has also been hypothesized that the use of specific mouse models may be limited in their ability to demonstrate the true nature of the relationship between sortilin and LDL-C. For example, the aforementioned studies conducted by Musunuru and colleagues utilized humanized apoB-100-expressing

mice to more closely mimic the human plasma lipid profile. However, it is possible that the artificial lipid profile of humanized mice alters the normal physiological metabolism of lipoproteins. The expression of human apoB-100 in mice may have stressed the intrinsic lipoprotein biosynthesis pathways which subsequently led to sortilin targeting of apoB-100 to the lysosome rather than towards secretion.

In summary, although GWA studies have implicated the *SORT1*-encoding locus as a regulator of plasma LDL-C levels and risk factor for CVD, sortilin's role in lipoprotein metabolism has not yet been fully elucidated. Preliminary cellular and animal model studies have demonstrated conflicting results in regard to the direction of the relationship between sortilin and plasma LDL-C levels. These contradictory data suggest that sortilin's role in the regulation of lipid metabolism may not be unidirectional. It appears that the relationship between sortilin and plasma LDL levels may be influenced by the extracellular environment, hepatic lipid content, metabolic requirements, and intracellular stress.

1.5.4 Sortilin & Lp(a)

Nearly 90% of the variation in Lp(a) levels observed between individuals is due to genetic differences in the apo(a)-encoding *LPA* gene [157]. Therefore, roughly 10% of plasma Lp(a) variation can be attributable to variations elsewhere in the genome. In order to identify *LPA*-independent genetic effects on Lp(a) levels, Zekavat and colleagues have recently performed deep-coverage whole genome sequencing (WGS) in 8392 individuals of European and African descent [158]. As mentioned, rs12740374 *SORT1* variants have been causally associated with LDL-C levels. Interestingly, Zekavat and colleagues also found an association between rs12740374 with plasma Lp(a) levels [158]. This plasma Lp(a) modulation was determined to be independent of changes in LDL-C, suggesting that sortilin is a novel receptor for Lp(a) metabolism [158].

Previous work in our lab conducted by Gemin and colleagues (unpublished data) has demonstrated that sortilin overexpression enhances apo(a) secretion from human hepatoma cell lines stably expressing apo(a). Conversely, sortilin knockdown, as well as mutation of sortilin's cytoplasmic targeting sequences, was shown to decrease apo(a) secretion. Intriguingly, co-IP studies demonstrated that sortilin is unable to directly associate with

with apo(a). Furthermore, mutation of the wLBS in KIV_7 and KIV_8, postulated to be important for the initial non-covalent interactions with apoB-100, prevented sortilin from affecting apo(a) secretion. As mentioned previously, however, high affinity binding has been observed between sortilin and apoB-100 [153, 154]. Taken together, this evidence suggests that a noncovalent intracellular interaction exists between apo(a) and apoB-100, and that sortilin increases apo(a) secretion indirectly through increasing apoB-100 secretion. However, this theory is challenged by previously mentioned *in vivo* and *in vitro* work that suggests an inverse relationship exists between *SORT1* expression and LDL-C levels. For example, Musunuru's group showed that AAV sortilin expression in mouse livers resulted in decreased apoB-100 secretion [134]. If sortilin decreases apoB-100 secretion and cannot directly associate with apo(a), it would be expected that *SORT1* overexpression would indirectly facilitate decreased apo(a) secretion. However, it is important to note that mice do not express *LPA*. Furthermore, for unknown reasons, all of the hepatocyte cell lines previously described do not endogenously produce Lp(a). If an intracellular interaction exists between apo(a) and apoB-100, this interaction could potentially alter sortilin's physical association with apoB-100, and thus have an impact on apoB-100 secretion. The lack of apo(a) expression and Lp(a) production in commonly-used animal models and established cell lines is another limiting factor in the study of sortilin and its modulation of lipoprotein metabolism. To further understand the relationship between sortilin and apoB-containing lipoprotein metabolism, it will be important to assess apoB-100 secretion from cells in the presence and absence of apo(a) coexpression.

1.6 Rationale, Objectives & Hypotheses

It is now known that elevated plasma Lp(a) concentrations represent the single most prevalent inherited risk factor for CVD [21]. However, the absence of available clinical therapeutics to lower plasma Lp(a) concentrations reflects the lack of fundamental understanding of the cellular pathways regulating Lp(a) metabolism. Since it is believed that anabolism rather than catabolism is the primary determinant of plasma Lp(a) concentrations, it is imperative that the production and secretion of this lipoprotein be understood. It has been suggested that the formation of Lp(a) occurs in a two-step process. The first step likely involves a weak, non-covalent interaction between apoB-100 and the wLBS in KIV_7 and KIV_8 of apo(a), and precedes the second step which involves the covalent linkage of a free cysteine in apoB-100 with a free cysteine in KIV_9 of apo(a). Although the majority of evidence suggests that the covalent step of Lp(a) formation occurs in the extracellular environment, the location of non-covalent association between apo(a) and apoB-100 remains enigmatic. The primary objective of this study was to use an *in vitro* model to asses if a noncovalent intracellular interaction exists between apo(a) and apoB-100 in Lp(a) assembly. *We hypothesize that if an intracellular non-covalent interaction exists between apo(a) and apoB-100, then proteins involved in regulating apoB-100 secretion will also modulate apo(a) secretion and Lp(a) production.* To address this hypothesis, three specific aims have been developed:

The first aim is to determine the mechanism by which sortilin modulates the secretion of apo(a). Previous work in our lab has shown that sortilin cannot directly associate with apo(a) but can significantly increase its secretion. Therefore, it has been postulated that apo(a) binds to apoB-100 in the cell, and sortilin-mediated increases in apoB-100 secretion indirectly increase apo(a) secretion. However, *in vivo* sortilin overexpression studies in mice hepatocytes lacking apo(a) expression have been associated with decreased apoB-100 secretion. If apo(a) and apoB-100 interact intracellularly, then we hypothesize that cells coexpressing apo(a) and apoB-100 may have different apoB-100 secretion patterns in response to sortilin overexpression than cells lacking apo(a) expression. To further our understanding of sortilin's role in Lp(a) metabolism, we collaborated with Dr. Robert Hegele and performed deep sequencing of the *SORT1* locus in a large cohort of individuals

with elevated Lp(a) levels. We identified 7 unique *SORT1* polymorphisms in 9 individuals that had Lp(a) levels in the top 5th percentile of the general population. Importantly, these amino acid substitutions all occurred within sortilin's VPS10 ligand binding domain. We hypothesize that by transfecting these human sortilin polymorphisms into HepG2 cells and then assessing apo(a) secretion, we will gain further insight into the mechanistic role of sortilin in Lp(a) metabolism.

The second aim is to determine the role of PCSK9 in Lp(a) production. Alirocumab and evolocumab are PCSK9 inhibitors used to treat elevated LDL-cholesterol but have also been shown to decrease Lp(a) levels by 20% to 30%. Although the main mechanism of action for PCSK9 inhibitors is to decrease LDL-receptor degradation, the LDL-receptor is not thought to be significantly involved in Lp(a) metabolism under normal physiological conditions. These findings provide evidence of a unique role for PCSK9 in Lp(a) metabolism. If PCSK9 has a role in modulating Lp(a) biosynthesis, we hypothesize that treating HepG2 human hepatoma cells with purified PCSK9 may induce changes in the secretion of apo(a), potentially through alterations in apoB-100 secretion.

The third aim is to determine if MTP plays a role in Lp(a) biosynthesis and secretion. Lomitapide is an MTP-inhibitor used to treat elevated levels of plasma LDL cholesterol. Interestingly, lomitapide treatment has also been shown to significantly reduce plasma Lp(a). The transfer of lipids from MTP to apoB-100 in the ER is essential for the assembly and secretion of all apoB-containing lipoproteins. If a noncovalent intracellular interaction exists between apo(a) and apoB100 during Lp(a) biosynthesis, we hypothesize that MTP inhibition with lomitapide will affect apo(a) secretion, potentially through modulation of apoB-100 secretion.

Chapter 2

2 Methods

2.1 Cell Culture

All cell lines were grown at 37°C with 95% humidity and 5% CO_2. Human hepatocellular carcinoma (HepG2) cells were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12; Gibco) supplemented with 10% Fetal Bovine Serum (FBS; VWR).

Wild-type (WT) HepG2 cells and HepG2 cells stably expressing a 17-Kringle (17K) apo(a) isoform were utilized in this study. Construction of the 17K apo(a) plasmid (17K-pRK5) was performed as previously described [159]. The 17K apo(a) isoform is a physiologically relevant apo(a) variant that possesses KIV_1, $KIV_{3\text{-}10}$, KV, the inactive protease and 8 KIV_2 domain repeats. The stably expressing 17K apo(a) HepG2 cell line was constructed as follows: HepG2 cells were seeded at 75% confluency and transfected using MegaTran 1.0 transfection reagent (Origene) according to manufacturer's protocol. Cells were transfected with 1μg of 17K apo(a) plasmid and 0.2μg of a neomycin resistance-encoding plasmid. After 24 hours of transfection, cells were allowed to recover in fresh medium for 24 hours. Media was then replaced again and supplemented with 0.4mg/μl Geneticin® selective antibiotic (G418 sulfate; Thermo Fisher Scientific). Successful transfection was evaluated using dilution cloning and western blot analysis.

2.2 Construction of Sortilin Expression Plasmids

The WT human sortilin expression system used in this study was generously provided by Dr. Nabil Seidah (McGill University). This pcDNA 3.1C/Myc-His vector contains the human sortilin cDNA with a C-terminal Myc-His tag. In collaboration with Dr. Robert Hegele (Robarts Research Institute) and with informed consent, deep sequencing of the *SORT1* locus was undertaken in 1740 patients with dramatically elevated Lp(a) levels [160, 161]. Seven sortilin amino-acid substitution variants were found to be associated with Lp(a) levels in excess of the top 5^{th} percentile of the general population: I124V, K205N, K302E, F404Y, E444Q, E447G, and V650M. Each missense mutation was constructed

using the Q5® Site-Directed Mutagenesis Kit (NEB) according to manufacturer's protocol. In each case, the WT sortilin pcDNA 3.1C/Myc-His expression plasmid was used as a template. The primers and PCR conditions used for mutagenesis are shown in Table 2.1 and Table 2.2, respectively. Each mutation was verified by DNA sequencing using the Sort_Seq_2 primer (5'-TCCACGTTTCAACAGATCAAG-3') at the Robarts Research Institute sequencing facility.

Table 2.1. ***Primers used for generation of human sortilin polymorphic variants.*** Only sense strands are shown. In each case, the mutated nucleotide is bolded, and the mutated codon is underlined. Each primer was constructed by Integrated DNA technologies.

Mutant	Sequence (Sense Strand)
Sort-I124V	5’-CACTGGGGTC**G**TTCTAGTCTTG-3’
Sort-K205N	5’-ATTTTGCGAA**T**AATTTTGTGCAAAC-3’
Sort-K302E	5’-TATTGGTGTG**G**AAATCTACTCATTTG-3’
Sort-F404Y	5’-GAGACGGACT**A**TACCAACGTG-3’
Sort-E444Q	5’-GAGGAAGCCT**C**AAAACAGTGAATGTG-3’
Sort-E447G	5’-GAAAACAGT**GG**ATGTGATGCTACAGC-3’
Sort-V650M	5’-CAAGTCATCC**AT**GTGTCAGAATGG-3’

Table 2.2. ***PCR exponential amplification for human sortilin mutagenesis using Bio-Rad T100 Thermal Cycler.***

Step	Temperature (°C)	Time
Initial Denaturation	98	30 sec
25 Cycles	98	10 sec
	66 (annealing)	10-30 sec
	72	200 sec
Final Extension	72	120 sec
Hold	4-10	-

2.3 Transient Transfections

For human sortilin pulse-chase analysis experiments, 17K apo(a)-expressing HepG2 cells (17K HepG2 cells) were seeded into 6-well tissue culture plates (Thermo Fisher Scientific) and grown to 70% confluency. Fresh medium was given to cells 4 hours prior to transfection. Cells were then either transfected with 1μg of empty-vector pcDNA 4A/Myc-His, WT sortilin pcDNA 3.1C/Myc-His, Sort-I124V pcDNA 3.1C/Myc-His, Sort-K205N pcDNA 3.1C/Myc-His, Sort K302E pcDNA 3.1C/Myc-His, Sort-F404Y pcDNA 3.1C/Myc-His, Sort-E444Q pcDNA 3.1C/Myc-His, Sort-E447G pcDNA 3.1C/Myc-His or Sort-V650M pcDNA 3.1C/Myc-His using linear polyethylenimine (PEI; Sigma) according to manufacturer's protocol. Transfection was allowed to proceed overnight, and then cells were given fresh medium for 24 hours prior to the start of the experiment.

For specified pulse-chase experiments involving PCSK9 or lomitapide treatment, a HepG2 cell line transiently expressing a 17K apo(a) isoform lacking the wLBSs in KIV_7 and KIV_8 ($17K_{\Delta 7,8}$ HepG2 cells) was used. WT HepG2 cells were grown to 70% confluency in 6-well tissue culture plates. Fresh medium was given to cells 4 hours prior to transfection. Cells were transfected with 1μg of $17K_{\Delta 7,8}$ apo(a)-pRK5 using linear PEI according to manufacturer's protocol. Construction of the $17K_{\Delta 7,8}$ apo(a)-pRK5 expression plasmid was performed as previously described [159]. Transfection was allowed to proceed overnight, and then cells were given fresh medium for 24 hours prior to the start of the experiment.

2.4 Construction and Purification of PCSK9

The PCSK9-pcDNA 4C vector was constructed and stably expressed in human embryonic kidney 293 (HEK293) cells as previously described [160]. Stably-expressing cells were seeded into triple flasks and grown in Opti-MEM (Life Technologies). Conditioned medium was then harvested every three days and supplemented with polymethylsulfonyl fluoride (PMSF) to a final concentration of 1mM. To purify PCSK9, the harvested medium was applied to a nickel-Sepharose excel column (GE Healthcare) as previously described [160]. Eluted protein fractions (4 column volumes) were then dialyzed extensively against PCSK9 storage buffer (25mM HEPES pH 7.9 containing 0.1mM $CaCl_2$, 150mM NaCl, and 10% glycerol). After dialysis, samples were concentrated using PEG 20000 (Sigma)

followed by further dialysis against PCSK9 storage buffer. Purified protein concentrations were assessed using the bicinchonicic acid assay (BCA assay; Pierce). SDS-PAGE was followed by silver stain analysis to assess protein purity. The harvested protein was run at a concentration of 25ng/ul next to 25ng/ul of a PCSK9 standard. The purified PCSK9 protein was then aliquoted and stored at -80°C until use.

2.5 Pulse-Chase Analysis

For the sortilin overexpression studies, apo(a) or apoB-100 secretion was analyzed from 17K HepG2 cells and WT HepG2 cells using pulse-chase studies. Cells were grown in DMEM/F12 supplemented with 10% FBS. The HepG2 cells were seeded into 6-well plates at 3 x 10^5 cells/well and allowed to attach overnight prior to transfection. At a confluency of 70%, cells were transfected with either 1μg of empty-vector pcDNA 4A/Myc-His, WT sortilin pcDNA 3.1C/Myc-His, Sort-I124V pcDNA 3.1C/Myc-His, Sort-K205N pcDNA 3.1C/Myc-His, Sort K302E pcDNA 3.1C/Myc-His, Sort-F404Y pcDNA 3.1C/Myc-His, Sort-E444Q pcDNA 3.1C/Myc-His, Sort-E447G pcDNA 3.1C/Myc-His or Sort-V650M pcDNA 3.1C/Myc-His. Following overnight transfection, cells were given fresh medium and allowed to recover for 24 hours before labelling.

For the PCSK9 and lomitapide (Sigma) treatment studies, apo(a) and apoB-100 secretion were analyzed from HepG2 cells expressing 17K apo(a) or the $17K_{\Delta 7,8}$ apo(a) variant using pulse-chase studies. For analysis of 17K apo(a) and apoB-100 secretion, 17K HepG2 cells were seeded in 6-well plates at 3 x 10^5 cells/well, allowed to attach overnight, and then grown for 48 hours in DMEM/F12 supplemented with 10% FBS prior to the start of the experiment. For analysis of $17K_{\Delta 7,8}$ apo(a) and apoB-100 secretion, WT HepG2 cells were seeded into 6-well plates at 3 x 10^5 cells/well and allowed to attach overnight prior to transfection. Cells were then transfected with 1μg of the $17K_{\Delta 7,8}$ apo(a)-pRK5 expression plasmid. Following overnight transfection, cells were given fresh DMEM/F12 supplemented with 10% FBS and allowed to recover for 24 hours prior to the start of the experiment.

On the morning of the experiment, the cells were given fresh DMEM/F-12 containing 10% FBS and 0.4 mM oleic acid complexed to 1% BSA (w/v) for 4 hours prior to labelling. For

the PCSK9 and lomitapide treatment studies, the DMEM/F-12 containing 10% FBS and 0.4mM oleic acid was also supplemented with either PCSK9 or lomitapide. Following the 4-hour incubation, cells were washed with 1 mL of phosphate buffered saline (PBS) and then 1mL of cysteine and methionine free Dulbecco's Modified Eagle Medium (DMEM; Gibco), without serum, was added for 1 hour. After 1 hour, cells were labelled with 200 μCi/well [^{35}S]-cysteine/[^{35}S]-methionine (Perkin Elmer Life Sciences) for 1 hour. Cells were then washed once with 1 mL of PBS and incubated in 500 μL serum-containing DMEM/F-12 supplemented with 0.4 mM oleic acid for 0, 30, 60, 120, or 360 minutes. For the PCSK9 and lomitapide treatment studies, the chase medium was also supplemented with either PCSK9 or lomitapide. At each of the aforementioned chase times, conditioned medium (500 μL) was collected and supplemented with 1% protease inhibitor cocktail (Sigma). Cells were then washed once with ice-cold PBS containing 50 mM epsilon-aminocaproic acid (ε-ACA) and subsequently lysed with ice-cold radioimmunoprecipitation buffer (RIPA; 50 mM Tris pH 7.4, 20mM EDTA, 150 mM NaCl, 0.1% (w/v) SDS, and 1% (w/v) sodium deoxycholate) supplemented with 1% protease inhibitor. Cell lysates and media samples collected at the aforementioned chase times were then stored on ice prior to clarification by centrifugation for 6 minutes at 15,000 rpm to remove cellular debris.

Following centrifugation, samples were pre-cleared with 30 μL of gelatin-agarose (Sigma) for 3 hours at 4°C with gentle shaking. Samples were then centrifuged for 2 minutes at 5000 rpm to pellet the gelatin-agarose and supernatants were exposed to saturating quantities (1:500) of anti-apo(a) monoclonal (α1-4; made in-house using r-apo(a) as the antigen) or anti-apoB-100 polyclonal (Millipore) primary antibody overnight at 4°C with gentle shaking. After overnight incubation with primary antibody, 50 μL of protein G-agarose beads (Invitrogen) was added to samples for 3 hours at 4°C with gentle shaking. Media and lysate samples were then centrifuged for 1 minute at 3000 rpm. Each pellet was washed 3 times with 500 μL ice-cold RIPA buffer supplemented with 1% protease inhibitor. In each case, the pellet was gently resuspended in RIPA buffer prior to centrifugation for 1 minute at 3000 rpm. After the third wash, pellets were resuspended in 2x SDS sample buffer (250 mM Tris pH 6.8, 4% (w/v) SDS, 40% (v/v) glycerol, and

0.001% (w/v) bromophenol blue), supplemented with 7 µL of 100 mM dithiothreitol (DTT), briefly centrifuged, boiled for 7 minutes and then pulse-centrifuged again.

Samples were then subjected to SDS-PAGE on 6% polyacrylamide gels. Following electrophoresis, gels were fixed in 100 mL of fixing solution (methanol:glacial acetic acid:H_2O, 40:10:50) with gentle shaking for 25 minutes. Gels were then rinsed briefly with milli-Q H_2O and then incubated in 100 mL of Amplify solution (GE Healthcare) with gentle shaking for 30 minutes. Next, the gels were subjected to 100 mL of 1M sodium salicylate containing 3 drops of 100% glycerol with gentle shaking for 30 minutes. Following the washing steps, the gels were dried using a gel dryer (BioRad Model 583) on the 'Page' setting for 1 hour and 15 minutes at 80°C. After the gels had dried, they were exposed to a phosphor K screen in the dark at room temperature for 96 hours and then screens were imaged using the Molecular Dynamics Storm 820 phosphoimager (Amersham). Resultant bands were quantified by densitometry using the ImageJ 1.49v software. The mature and immature forms of intracellular apo(a) were each quantified separately and then summed.

2.6 Western Blotting

To analyze sortilin expression, cell lysates were subjected to SDS-PAGE on 10% polyacrylamide gels. All samples were prepared as 50 µL aliquots, re-suspended in 12.5 µL 4X SDS sample buffer and supplemented with 5 µL of 100 mM DTT. Samples were then boiled for 7 minutes at 100 degrees Celsius and pulse-centrifuged before being subjected to SDS-PAGE for 1.5 hours at 120 volts. The gels were transferred in ice cold transfer buffer (25mM Tris pH 7.8, 1.92 M glycine, 10% (v/v) Methanol) for 1.5 hours at 120 volts onto PVDF membranes (Millipore) and then blocked in 15 mL of 50 mM Tris pH7.6 containing 150 mM NaCl, 0.2% (v/v) Tween-20 (TBST) supplemented with 5% (w/v) powdered non-fat milk for 1 hour at room temperature while gently shaking. Following blocking, membranes were then incubated with either goat-anti-human sortilin (R&D Systems) or mouse-anti-human β-actin (Novus Biologicals) overnight in the same blocking buffer at 4 degrees Celsius while gently shaking. After overnight incubation, the membranes were washed three times in TBST for 10 minutes each while gently shaking.

The membranes were then incubated for 1 hour at room temperature while gently shaking with either sheep-anti-mouse secondary antibody (GE Healthcare) or mouse-anti-goat secondary antibody (Santa Cruz). The membranes were then washed again three times in TBST for 10 minutes each at room temperature while gently shaking. Resultant bands were visualized with SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific) using a FluorChem Q Imager (Alpha Innotech) and densitometric quantification of resulting bands was performed using Alpha View software (Alpha Innotech).

2.7 Statistical Analysis

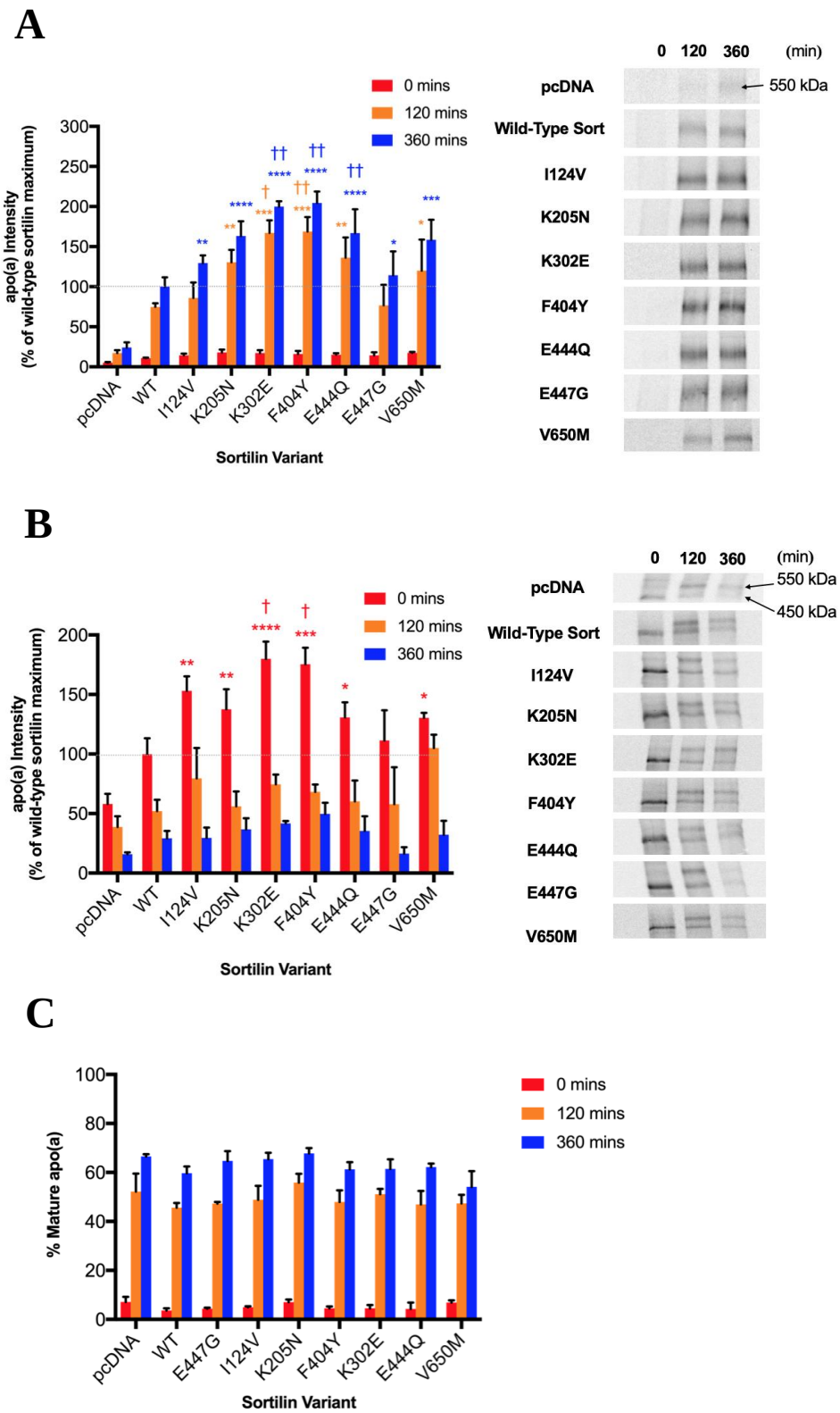
All data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed with the GraphPad Prism version 7 software program. For the human sortilin polymorphic variant pulse-chase studies, differences between samples were characterized using one-way analysis of variance (ANOVA) with a Tukey's multiple comparisons test (*$p<0.05$). The apoB-100 pulse-chase studies performed in WT HepG2 cells and 17K HepG2 cells in response to WT sortilin overexpression were evaluated for differences using a two-tailed Student t-test assuming unequal variances (*$p<0.05$). For the lomitapide treatment studies, apo(a) data were analyzed using one-way ANOVA with a Tukey's multiple comparisons test (*$p<0.05$), while apoB-100 data were analyzed using a two-tailed Student t-test assuming unequal variances (*$p<0.05$). Apo(a) and apoB-100 pulse-chase data for the PCSK9 treatment studies were analyzed using a two-tailed Student t-test assuming unequal variances (*$p<0.05$).

Chapter 3

3 Results

3.1 Human sortilin polymorphic variants increase apo(a) intracellular abundance and apo(a) secretion from HepG2 cells

A causal relationship between circulating Lp(a) levels and the *SORT1* rs12740374 variant has been recently been established through deep-coverage WGS. Previous work in our laboratory has demonstrated that sortilin overexpression stimulates apo(a) secretion and increases apo(a) intracellular abundance in 17K HepG2 cells. To help further understand sortilin's role in Lp(a) metabolism, we collaborated with Dr. Robert Hegele and performed deep sequencing of the *SORT1* locus in a large cohort of individuals with elevated Lp(a) levels. Intriguingly, we identified 7 unique *SORT1* polymorphisms that were present in individuals with Lp(a) levels in the top 5^{th} percentile of the general population. The I124V, K205N, K302E, F404Y, E444Q, and E447G amino acid substitutions were found to occur in sortilin's ten-bladed β-propeller ligand binding domain. The V650M variant, which is the only variant found outside of the β-propeller domain, occurs within a highly conserved 10CC module found adjacent to the transmembrane domain [162]. However, the mechanism by which these specific variants increase plasma Lp(a) had not been evaluated at the cellular level. Here, we transiently transfected 17K HepG2 cells with expression vectors containing WT sortilin, empty vector control (pcDNA), sort-I124V, sort-K205N, sort-K302E, sort-F404Y, sort-E444Q, sort-E447G or sort-V650M and then analyzed apo(a) secretion using pulse-chase studies (Fig. 3.1 A-B). Western blot analysis of cellular lysates revealed that each sortilin mutant was expressed at similar levels (data not shown).


A
0 mins
120 mins
360 mins
apo(a) Intensity
(% of wild-type sortilin maximum)
Sortilin Variant
pcDNA
WT
I124V
K205N
K302E
F404Y
E444Q
E447G
V650M
0
120
360
(min)
550 kDa
Wild-Type Sort
B
450 kDa
C
% Mature apo(a)

D

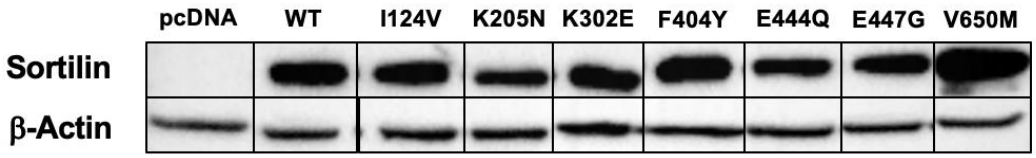

pcDNA
WT
I124V
K205N
K302E
F404Y
E444Q
E447G
V650M
Sortilin
β-Actin

Figure 3.1: ***Effect of human sortilin polymorphisms on apo(a) secretion, apo(a) intracellular abundance, and rate of apo(a) maturation*****.** Pulse-chase analysis of HepG2 cells stably expressing the 17K apo(a) isoform and transiently expressing either wild-type sortilin (WT), sortilin I124V, sortilin K205N, sortilin K302E, sortilin F404Y, sortilin E444Q, sortilin E447G, sortilin V650M, or the corresponding empty vector control (pcDNA). Cells were starved in Cys/Met free media for one hour, pulse labelled with ^{35}S-Cys/Met for one hour, and subsequently incubated for 0 minutes, 120 minutes and 360 minutes in unlabelled complete medium. Cell media (**A**) and lysates (**B**) were collected from cells transfected with wild-type sortilin, empty vector pcDNA and each of the 7 mutants at the indicated times of chase. Samples were then subjected to apo(a) immunoprecipitation, and then analyzed by 6% SDS-PAGE and fluorography. The top band (~550kDa) in the fluorograms represents a mature, fully glycosylated form of apo(a), while the bottom band (~450kDa) represents an immature, hypo-glycosylated form of apo(a). Densitometric band analysis was conducted using the ImageLab 5.2 software, normalized to the maximum observed density for WT sortilin, and plotted as a function of time. (**C**) Percentage of mature apo(a) formed was calculated according to the formula: % Mature apo(a) = [mature apo(a)] / [(mature apo(a)) + (immature apo(a))] × 100. (**D**) Western blot analysis of sortilin expression in HepG2 cells ectopically expressing empty-vector control (pcDNA), wild-type (WT) sortilin, Sort-I124V, Sort-K205N, Sort-K302E, Sort-F404Y, Sort-E444Q, Sort-E447G, or Sort-V650M. The presented data correspond to the mean ± SEM of at least 3 independent experiments. Significance compared to empty vector is represented by asterisks where *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$. Significance compared to wild-type sortilin is represented by daggers, where $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$. Significance was determined using one-way ANOVA.

The representative fluorograms in Fig. 3.1 A show that mature apo(a) (550 kDa) could not be observed in the medium at 0 minutes, but was detectable at 120 minutes and 360 minutes. As demonstrated in Fig. 3.1 A, an apparent trend exists between sortilin overexpression and increased apo(a) secretion. Although no statistical significance was observed, transfecting 17K apo(a)-expressing HepG2 cells with wild-type sortilin resulted in a 4.4-fold and a 4.2-fold increase in apo(a) secretion at 120 minutes and 360 minutes, respectively, versus empty vector control (pcDNA). At the chase time of 120 minutes, each human sortilin polymorphic variant, with the exception of the sort-I124 ($p=0.280$) and sort-E447G ($p=0.548$) variants, were able to significantly increase apo(a) secretion versus pcDNA (Fig. 3.1 A). Apo(a) secretion was most greatly augmented at t=120 minutes by the sort-K302E and sort-F404Y variants. The K302E variant significantly increased apo(a) secretion in comparison to both empty vector control ($p=0.004$) and wild-type sortilin (Fig 3.1 A). The F404Y variant also significantly increased apo(a) secretion versus the empty vector control ($p=0.0002$) and wild-type sortilin ($p=0.01$). When compared to the pcDNA control, overexpression of each of the 7 human sortilin polymorphic variants significantly increased apo(a) secretion at the 360-minute chase time. The K302E, F404Y, and E444Q variants each resulted in significantly increased apo(a) secretion ($p=0.0019$; $p=0.0012$; $p=0.0062$, respectively) compared to wild-type sortilin overexpression at the corresponding time.

Within the cell, apo(a) can be observed as both an immature (450 kDa) and mature (550 kDa) form (Fig. 3.1 B). The immature form represents newly synthesized, hypo-glycosylated apo(a) in the ER. The mature form has undergone addition of *N*-linked and *O*-linked glycans in the ER and golgi compartments, respectively, and represents the secreted form of apo(a) [35]. As can be observed in the representative fluorogram, the different maturation states of apo(a) can be observed as two separate bands. The lower band can be observed at all 3 chase times, and represents the immature and incompletely glycosylated form of apo(a). The top band represents the mature, fully glycosylated, secreted form of apo(a), and is identical in in mobility to the apo(a) detected from conditioned medium samples (Fig. 3.1 A). The mature form of apo(a) was not detectable in cell lysates until the 120-minute chase time, which corresponds to the appearance of apo(a) in the conditioned medium.

As demonstrated in Fig. 3.1 B, an apparent trend exists between sortilin overexpression and increased apo(a) intracellular abundance. Although not statistically different, wild-type sortilin overexpression resulted in a 1.7-fold and a 1.4-fold increase in intracellular apo(a) abundance at the chase times of 0 minutes and 120 minutes, respectively, versus control pcDNA. At time zero of the chase period, each of the 7 human sortilin polymorphic variants, with the exception of sort-E447G, significantly increased apo(a) intracellular abundance compared to the empty vector (Fig. 3.1 B). Apo(a) intracellular abundance was most greatly augmented by sort-K302E and sort-F404Y, which were the variants also associated with the greatest increases in apo(a) secretion. Sort-K302E significantly increased apo(a) intracellular abundance when compared to both pcDNA and wild-type sortilin (Fig. 3.1 B). Similarly, sort-F404Y significantly increased apo(a) intracellular abundance versus both pcDNA and wild-type sortilin. No significant differences in apo(a) intracellular abundance were observed between pcDNA, wild-type sortilin, or each of the 7 sortilin variants at both the 120 and 360-minute chase points. For each of the 7 sortilin variants, quantitative analysis of the cellular lysate fluorograms showed that there were no significant differences in the rate of conversion from immature to mature apo(a) when compared to pcDNA or wild-type sortilin (Fig. 3.1 C).

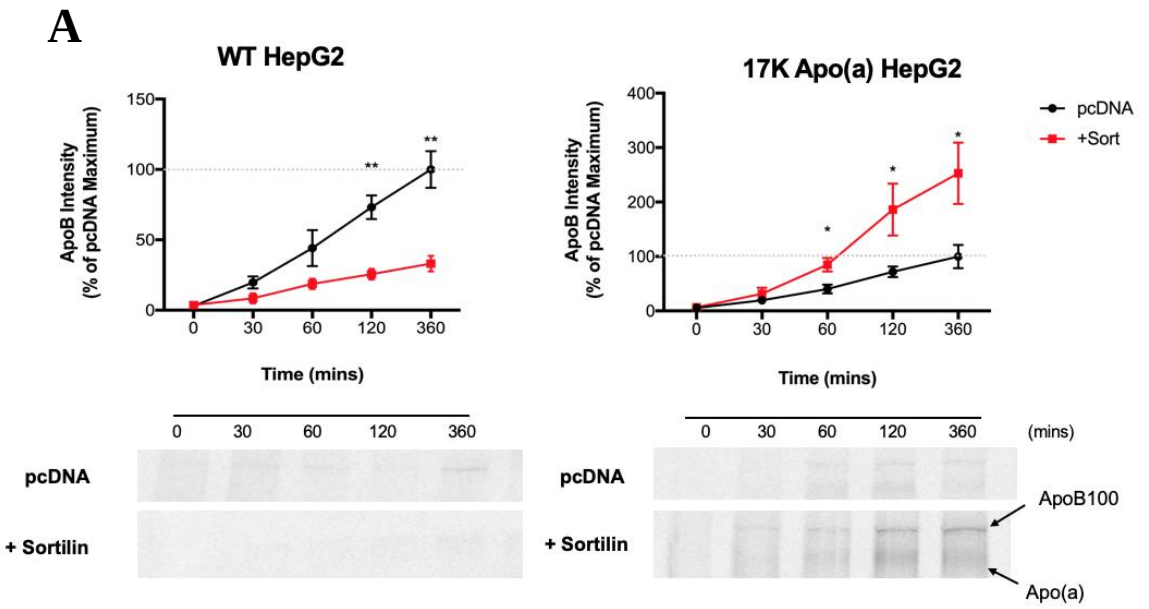
3.2 Sortilin overexpression in HepG2 cells modulates apoB-100 secretion and intracellular abundance

The nature of the relationship between sortilin expression and apoB-100 secretion has proven to be quite complex. Interestingly, previous co-IP work in our lab demonstrated that sortilin can physically associate with apoB-100 but not apo(a), and that sortilin's effects on apo(a) metabolism were diminished in apo(a) variants lacking the wLBS in $KIV_{7,8}$. Taken together, it appears that sortilin indirectly increases apo(a) secretion, potentially through a mechanism involving augmentation of apoB-100 secretion and an intracellular interaction between apo(a) and apoB-100. To test the validity of this hypothesis, we used our stably expressing 17K apo(a) HepG2 cells transiently transfected with wild-type sortilin. We then used pulse-chase studies to analyze apoB-100 secretion and intracellular abundance. As previously mentioned, the direction of the relationship between sortilin expression and apoB-100 secretion remains very controversial. Our hypothesis that sortilin indirectly increases apo(a) secretion through augmenting apoB-100 secretion has been challenged by *in vivo* rodent studies, which have demonstrated that sortilin overexpression in hepatocytes is associated with decreased apoB-100 secretion. However, mouse hepatocytes do not express apo(a), and it could be possible that apo(a) and apoB-100 coexpression impacts the nature of the relationship between sortilin overexpression and apoB-100 secretion. To assess this hypothesis, we also utilized wild-type HepG2 cells, which do not express apo(a). We transiently transfected these cells with wild-type sortilin or empty vector control (pcDNA), and then assessed apoB-100 secretion and intracellular abundance using pulse-chase studies.

In line with the aforementioned rodent hepatocyte findings, Fig. 3.2 A (left) demonstrated that sortilin overexpression in WT HepG2 cells resulted in significantly decreased apoB-100 secretion. The representative fluorogram shows that apoB-100 could be initially visualized in small amounts in the conditioned medium of WT HepG2 cells transfected with empty vector starting at the 30-minute chase time and then gradually accumulated over time. ApoB-100 could also be observed in trace amounts starting at 30 minutes in the conditioned medium of WT HepG2 cells transiently transfected with sortilin. However, WT HepG2 cells transfected with sortilin secreted significantly less apoB-100 at 120

minutes and 360 minutes versus WT HepG2 cells transfected with pcDNA control. Intriguingly, Fig. 3.2 A (right) demonstrates that sortilin overexpression in HepG2 cells stably expressing 17K apo(a) resulted in significantly increased apoB-100 secretion. The representative fluorograms for the 17K apo(a) HepG2 cells display a double banding pattern. The top band is consistent with apoB-100 mobility, while the bottom band is consistent with mature apo(a) mobility (~550 kDa), as observed in Fig 3.1 A and B. In 17K HepG2 cells transfected with pcDNA or sortilin, apoB-100 could be observed in small amounts in cultured medium as early as 30 minutes of chase. However, 17K cells transfected with sortilin demonstrated significantly increased secretion of apoB-100 at 60 minutes, 120 minutes and 360 minutes versus 17K HepG2 cells transfected with pcDNA control. Although the apo(a) band could not be completely resolved in each replicate, qualitative analysis demonstrated that sortilin overexpression was associated with increased apo(a) secretion, similar to what was observed in Fig. 3.1 A.

As demonstrated in Fig. 3.2 B (left), sortilin overexpression in WT HepG2 cells resulted in significantly decreased intracellular abundance of apoB-100 at 0 minutes, 30 minutes and 60 minutes of chase versus pcDNA control. Conversely, sortilin transfection increased apoB-100 intracellular abundance in 17K HepG2 cells (Fig. 3.2 B, right). At 0 minutes and 30 minutes of chase, sortilin transfection significantly increased apoB-100 intracellular abundance versus pcDNA control. Therefore, it appears that sortilin overexpression facilitates increased apoB-100 secretion and intracellular abundance in HepG2 cells stably expressing 17K apo(a). However, in the absence of apo(a) coexpression (WT HepG2 cells), our data indicate that sortilin overexpression results in decreased apoB-100 secretion and intracellular abundance.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17K Apo(a) HepG2
ApoB Intensity (% of pcDNA Maximum)
Time (mins)
pcDNA
+Sort
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(mins)
pcDNA
+ Sortilin
ApoB100
Apo(a)

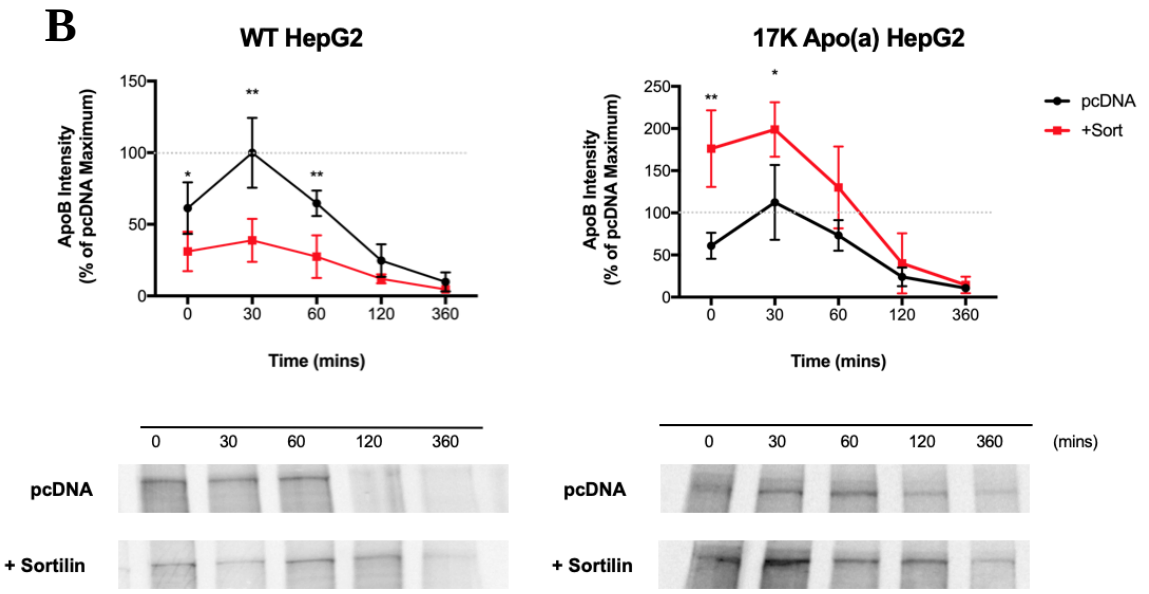

B
WT HepG2
17K Apo(a) HepG2
ApoB Intensity (% of pcDNA Maximum)
Time (mins)
pcDNA
+Sort
0 30 60 120 360
(mins)
pcDNA
+ Sortilin

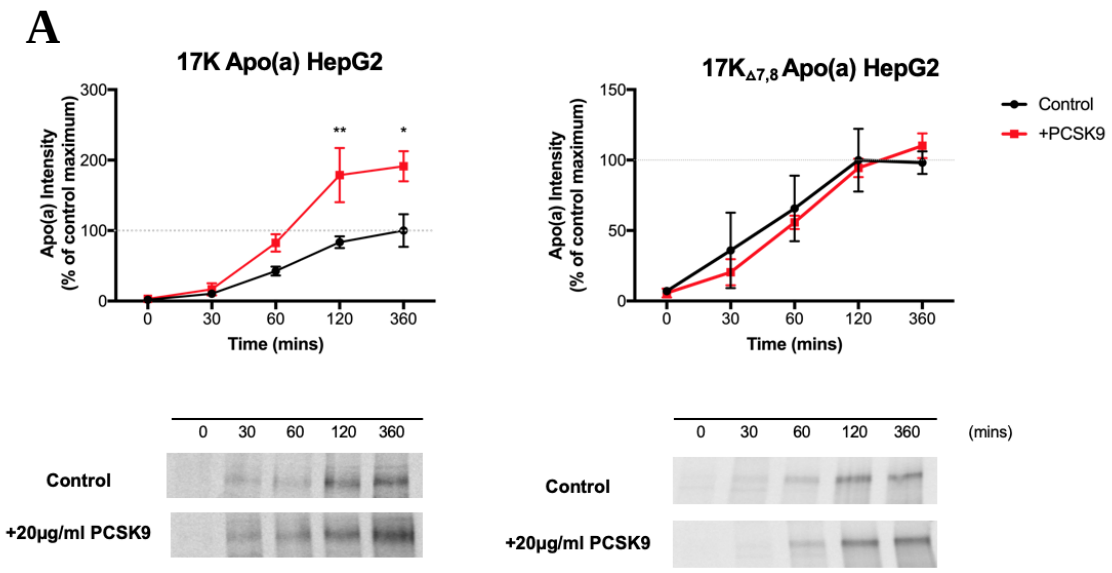
Figure 3.2: ***Effect of sortilin overexpression on apoB-100 secretion and intracellular abundance in the presence or absence of apo(a) coexpression.*** Pulse-chase analysis of HepG2 cells stably expressing the 17K apo(a) isoform and wild-type HepG2 cells lacking apo(a) expression. Cells were transiently transfected with wild-type sortilin or the corresponding empty vector control (pcDNA). Cells were pre-incubated for 4 hours in complete media containing 0.4mM BSA-conjugated oleic acid. Cells were then starved in -Cys/Met free media for one hour, pulse labelled with ^{35}S-Cys/Met for one hour, and subsequently incubated for 0 minutes, 30 minutes, 60 minutes, 120 minutes and 360 minutes in unlabelled complete media containing 0.4mM BSA-conjugated oleic acid. Cell Lysate samples were collected from cells transfected with wild-type sortilin or empty vector pcDNA at the indicated times of chase. Samples were then subjected to apoB immunoprecipitation, and analyzed by 6% SDS-PAGE and fluorography. (**A**) Media samples collected at the indicated times of chase. The lower band (~550 kDa) represents a mature, fully glycosylated form of apo(a), while the upper sharp band represents apo-B100. (**B**) Lysate samples collected at the indicated times of chase. The visible band represents mature apo-B100. Densitometric band analysis was conducted using ImageJ software, normalized to the maximum observed density for pcDNA, and plotted as a function of time. The presented data corresponds to the means ± SEM of 3 or 4 independent experiments. Significance compared to empty vector pcDNA is represented by asterisks where *$p<0.05$, and **$p<0.01$.

3.3 PCSK9-mediated modulation of apo(a) secretion from HepG2 cells is dependent on $KIV_{7,8}$ weak lysine binding sites

As mentioned, inhibition of PCSK9 by Alirocumab and Evolocumab has been associated with decreased plasma Lp(a) levels. Furthermore, PCSK9 has recently been implicated as a regulator of apoB-100 metabolism after PCSK9 knockout in mice resulted in significantly decreased apoB-100 secretion [163]. For these reasons, we postulated that PCSK9 may modulate Lp(a) biosynthesis and secretion. To test this hypothesis, we treated 17K HepG2 cells with PCSK9 or storage buffer, and then analyzed apo(a) secretion and intracellular abundance with pulse-chase studies (Fig. 3.3 A; left). As demonstrated by the representative fluorogram, PCSK9 or storage buffer treatment resulted in apo(a) accumulation in the conditioned medium beginning at the 30-minute chase time. However, compared to treatment with storage buffer control, 17K HepG2 cells treated with PCSK9 had significantly increased apo(a) secretion at the 120-minute and 360-minute chase times. Analysis of 17K HepG2 cell lysates demonstrated that PCSK9 treatment resulted in increased intracellular abundance of apo(a). Similar to Fig. 3.1 B, apo(a) can be observed as both an immature and mature form within cell lysates (Fig. 3.3 B). Treatment of 17K HepG2 cells with PCSK9 resulted in significantly increased intracellular abundance of apo(a) at the 0-minute and 30-minute chase times compared to treatment with storage buffer control (Fig. 3.3 B; left).

To determine if the PCSK9-mediated increases in apo(a) secretion are dependent on interactions with apoB-100, we utilized HepG2 cells expressing the $17K_{\Delta 7,8}$ apo(a) variant. It has previously been demonstrated that the wLBS in $KIV_{7,8}$ is important for facilitating noncovalent interactions with apoB-100 [81]. Therefore, if the observed effects of PCSK9 on apo(a) secretion are mediated indirectly through modulation of apoB-100 metabolism, disruption of the wLBS in apo(a) should elucidate this. As demonstrated in Fig. 3.3 A (right), treatment of cells expressing the $17K_{\Delta 7,8}$ apo(a) variant with PCSK9 did not affect $17K_{\Delta 7,8}$ apo(a) secretion versus control. In line with the fluorographs observed for the 17K HepG2 cells (Fig. 3.3; left), PCSK9 or storage buffer treatment of the $17K_{\Delta 7,8}$ apo(a) HepG2 cells resulted in $17K_{\Delta 7,8}$ apo(a) accumulation in conditioned medium beginning at

the 30-minute chase time. However, unlike the 17K HepG2 cells, PCSK9 treatment of the $17K_{\Delta 7,8}$ apo(a) HepG2 cells did not significantly increase $17K_{\Delta 7,8}$ apo(a) accumulation in the conditioned medium versus control (Fig. 3.3 A; right). $17K_{\Delta 7,8}$ apo(a) HepG2 cellular lysates were also assessed to determine the effects of PCSK9 on $17K_{\Delta 7,8}$ apo(a) intracellular abundance. Again, similar to Fig. 3.1 B, $17K_{\Delta 7,8}$ apo(a) can be observed as both an immature hypo-glycosylated form (upper band), and a higher fully glycosylated form (higher band). As demonstrated in the representative fluorogram (Fig. 3.3 B; right) $17K_{\Delta 7,8}$ apo(a) was visible in its immature form starting at time 0, and then as both a mature and immature form from 30-360 minutes. However, densitometric analysis of cellular lysates demonstrated that PCSK9 treatment did not significantly affect the intracellular abundance of $17K_{\Delta 7,8}$ apo(a) versus control (Fig. 3.3 B; right).


A
17K Apo(a) HepG2
Apo(a) Intensity (% of control maximum)
Time (mins)
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Control
+PCSK9
17K$_{\Delta7,8}$ Apo(a) HepG2
Control
+20µg/ml PCSK9
(mins)

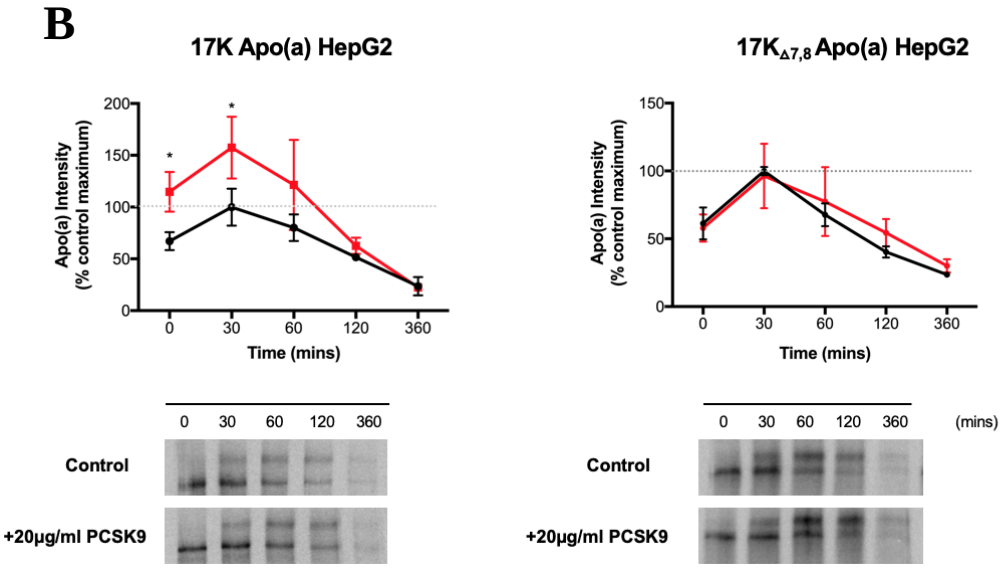

B
17K Apo(a) HepG2
Apo(a) Intensity (% control maximum)
Time (mins)
0 30 60 120 360
Control
+PCSK9
17K$_{\Delta7,8}$ Apo(a) HepG2
Control
+20µg/ml PCSK9
(mins)

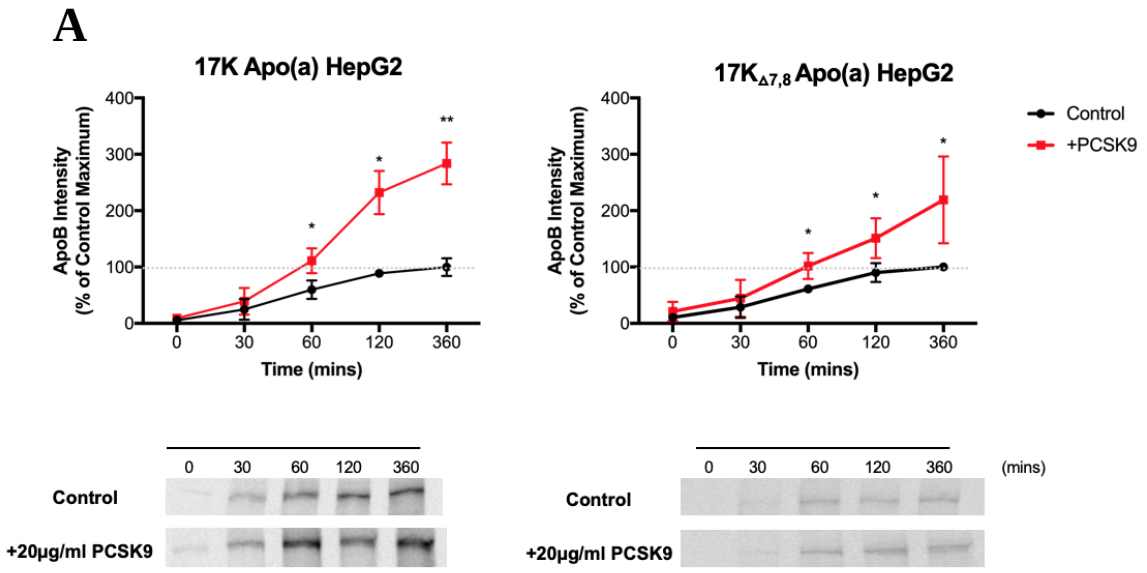
Figure 3.3: ***Treating HepG2 cells with PCSK9 increases wild-type apo(a) secretion and intracellular abundance but does not affect secretion and intracellular abundance of an apo(a) variant lacking the weak lysine binding sites in KIV$_7$ and KIV$_8$.*** Pulse-chase analysis of HepG2 cells stably expressing 17K apo(a) and HepG2 cells transiently expressing △7,8 LBS apo(a) following treatment with 20μg/mL of PCSK9 or control. Cells were first pre-incubated for 4 hours in complete media containing 0.4mM BSA-conjugated oleic acid in the presence or absence of 20μg/mL PCSK9. Cells were then starved in Cys/Met free media for one hour, pulse labelled with ^{35}S-Cys/Met for one hour, and subsequently incubated for 0 minutes, 30 minutes, 60 minutes 120 minutes and 360 minutes in unlabelled complete medium containing 0.4mM BSA-conjugated oleic acid in the presence or absence of 20μg/mL PCSK9. Conditioned medium and cellular lysate samples were collected from cells treated with or without PCSK9 at the indicated times of chase. Samples were then subjected to apo(a) immunoprecipitation, and then analyzed by 6% SDS-PAGE and fluorography. The lower band (~450kDa) represents an immature hypo-glycosylated form of apo(a), while the higher band (~550kDa) represents a mature, fully glycosylated form of apo(a). (**A**) Media samples of 17K apo(a) HepG2 cells (left) and 17K$_{\Delta 7,8}$ apo(a) HepG2 cells (right) treated with or without PCSK9 at the indicated times of chase. (**B**) Lysate samples of 17K apo(a) HepG2 cells (left) and 17K$_{\Delta 7,8}$ apo(a) HepG2 cells (right) treated with or without PCSK9 at the indicated times of chase. Densitometric band analysis was conducted using ImageJ software, normalized to the maximum observed density for control, and plotted as a function of time. The presented data correspond to the means ± SEM of 3 or 4 independent experiments. Significance compared to control is represented by asterisks where *$p<0.05$, and **$p<0.01$.

3.4 PCSK9 treatment increases apoB-100 secretion from HepG2 cells independent of interactions with apo(a)

As demonstrated in Fig. 3.3, PCSK9-mediated increases in apo(a) secretion were dependent on the wLBS in $KIV_{7,8}$, which are the domains shown to be involved in facilitating noncovalent interactions with apoB-100 [81]. To gain further insight into how PCSK9 treatment increases apo(a) secretion, we repeated the experiment in the same cell types but then subjected our samples to apoB-100 immunoprecipitation. Figure 3.4 (A) illustrates that treatment of HepG2 cells exogenously with PCSK9 increases apoB-100 secretion. PCSK9 treatment of 17K HepG2 cells significantly increased apoB-100 secretion at the 60-minute and 120-minute chase times compared control (Fig. 3.4 A; left). ApoB-100 secretion was maximally increased from 17K HepG2 cells by PCSK9 at the 360-minute chase time (Fig. 3.4 A; left). To determine if the same pattern of apoB-100 secretion existed in cells expressing an apo(a) variant unable to noncovalently interact with apoB-100, we repeated the experiment in $17K_{\Delta 7,8}$ apo(a)-transfected HepG2 cells. As demonstrated in Fig. 3.4 A (right), apoB-100 secretion was significantly increased in $17K_{\Delta 7,8}$ apo(a)-transfected HepG2 cells after 60-minutes, 120-minutes, and 360-minutes of chase versus control. Comparisons of the maximal apoB-100 secretion intensity (t=360 minutes) in the 17K HepG2 cells and $17K_{\Delta 7,8}$ HepG2 cells demonstrate that PCSK9 was able to augment apoB-100 secretion to a greater degree in the 17K HepG2 cells (Fig. 3.4 A). Therefore, the presence of the wLBS in $KIV_{7,8}$ of apo(a) appeared to affect the relationship between PCSK9 treatment and apoB-100 secretion.

Cellular lysates were also analyzed to determine if the wLBS in $KIV_{7,8}$ can affect the intracellular abundance of apoB-100 in response to PCSK9 treatment (Fig. 3.4 B). In both the 17K HepG2 cells treated with or without PCSK9 and the $17K_{\Delta 7,8}$ HepG2 cells treated with or without PCSK9, apoB-100 could be visualized in the representative fluorograms from 0 minutes of chase to 360 minutes of chase (Fig. 3.4 B). PCSK9 treatment of 17K HepG2 cells significantly increased the intracellular abundance of apoB-100 at the 0-minute and 30-minute chase times versus control (Fig. 3.4 B; left). The intracellular abundance of apoB-100 was also significantly increased by PCSK9 treatment in the $17K_{\Delta 7,8}$ HepG2 cells at the 0-minute and 30-minute chase times versus control (Fig. 3.4 B; right).

Interestingly, when comparing PCSK9 treatment in 17K HepG2 cells and $17K_{\Delta 7,8}$ HepG2 cells, the intracellular abundance of apoB-100 appeared to be maintained at higher levels throughout the chase in cells expressing the apo(a) variant lacking the wLBS in $KIV_{7,8}$. At the 60-minute chase time, the intracellular abundance of apoB-100 in $17K_{\Delta 7,8}$ HepG2 cells treated with PCSK9 appeared to be much greater than that control, although this effect was not significant (Fig. 3.4 B; right). Conversely, apoB-100 intracellular abundance in 17K HepG2 cells treated with PCSK9 trended much more closely to the intracellular abundance of 17K HepG2 cells treated with control (Fig. 3.4 B; left). In summary, PCSK9 treatment increased apoB-100 secretion and intracellular abundance regardless of the apo(a) species being expressed, however a trend existed between PCSK9 and prolonged apoB-100 intracellular abundance in HepG2 cells expressing the apo(a) variant lacking the wLBS in $KIV_{7,8}$.


A
17K Apo(a) HepG2
17K$_{\Delta 7,8}$ Apo(a) HepG2
ApoB Intensity (% of Control Maximum)
Time (mins)
Control
+PCSK9
0 30 60 120 360
(mins)
Control
+20µg/ml PCSK9

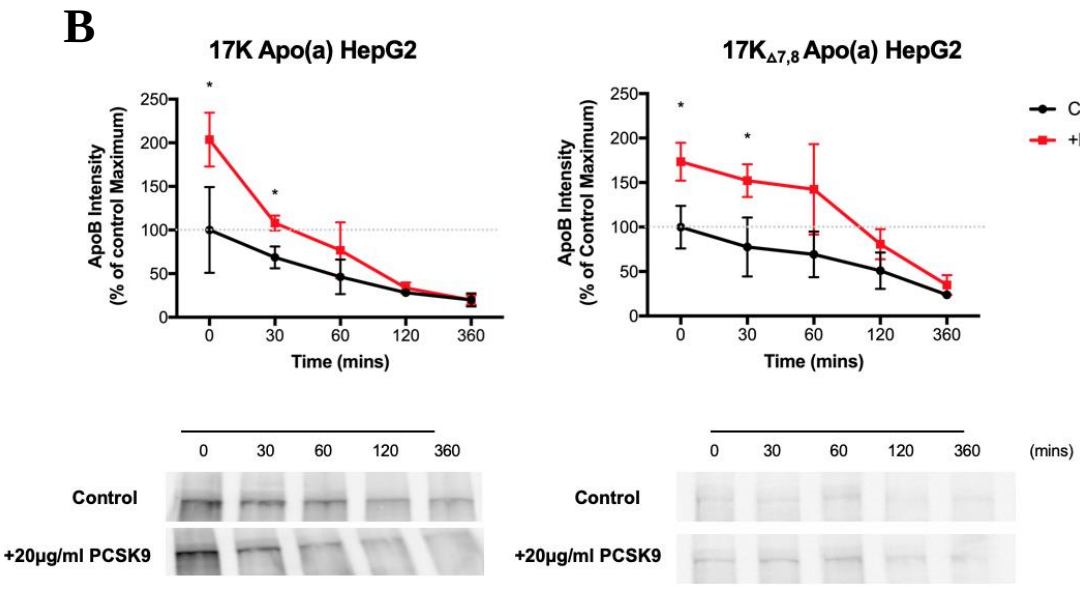

B
17K Apo(a) HepG2
17K$_{\Delta 7,8}$ Apo(a) HepG2
ApoB Intensity (% of control Maximum)
ApoB Intensity (% of Control Maximum)
Time (mins)
Control
+PCSK9
0 30 60 120 360
(mins)
Control
+20µg/ml PCSK9

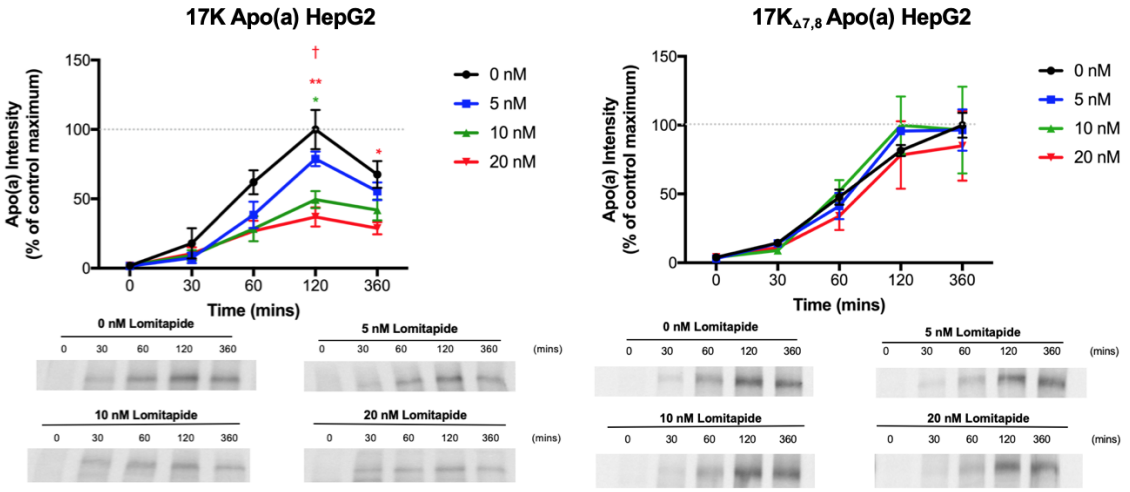
Figure 3.4: ***PCSK9 treatment increases apoB-100 secretion and intracellular abundance in HepG2 cells independent of the apo(a) species being coexpressed.*** Pulse-chase analysis of HepG2 cells stably expressing 17K apo(a) and HepG2 cells transiently expressing △7,8 LBS apo(a) following treatment with 20μg/mL of PCSK9 or control. Cells were pre-incubated for 4 hours in complete medium containing 0.4mM BSA-conjugated oleic acid in the presence or absence of 20μg/mL PCSK9. Cells were then starved in Cys/Met free medium for one hour, pulse labelled with ^{35}S-Cys/Met for one hour, and subsequently chased for 0 minutes, 30 minutes, 60 minutes 120 minutes and 360 minutes in unlabelled complete media containing 0.4mM BSA-conjugated oleic acid in the presence or absence of 20μg/mL PCSK9. Conditioned medium and cellular lysate samples were collected from cells treated with PCSK9 or control at the indicated times of chase. Samples were then subjected to apoB-100 immunoprecipitation, and then analyzed by 6% SDS-PAGE and fluorography. (**A**) Media samples of 17K apo(a) HepG2 cells (left) and 17K$_{\Delta 7,8}$ apo(a) HepG2 cells (right) treated with or without PCSK9 at the indicated times of chase. (**B**) Lysate samples of 17K apo(a) HepG2 cells (left) and 17K$_{\Delta 7,8}$ apo(a) HepG2 cells (right) treated with or without PCSK9 at the indicated times of chase. Densitometric band analysis was conducted using ImageJ software, normalized to the maximum observed density for control, and plotted as a function of time. The presented data correspond to the mean ± SEM of 3 or 4 independent experiments. Significance compared to control is represented by asterisks where *$p<0.05$, and **$p<0.01$.

3.5 Lomitapide treatment inhibits apo(a) secretion from HepG2 cells in a dose-dependent manner

The transfer of lipids onto apo-B100 through the actions of MTP is essential for the biosynthesis and secretion of VLDL particles. Since VLDL is considered the precursor molecule for LDL, therapeutics that target MTP, such as lomitapide, have been designed to lower plasma LDL-C levels. Interestingly, lomitapide treatment has also been observed to lower Lp(a) levels through an unknown mechanism. We treated HepG2 cells with lomitapide and performed pulse-chase studies to analyze how inhibition of MTP affects apo(a) secretion (Fig. 3.5 A). Furthermore, we utilized HepG2 cells expressing the wild-type 17K apo(a) isoform and cells expressing the 17K$_{\Delta 7,8}$ apo(a) to determine if an intracellular interaction between apo(a) and apoB-100 impacts lomitapide's ability to modulate apo(a) metabolism. The representative fluorograms show that apo(a) was detectable in conditioned medium of both 17K and 17K$_{\Delta 7,8}$ HepG2 cells and across all treatment groups from 30-360 minutes of chase (Fig. 3.5 A), which is in line with the secretion patterns of apo(a) observed in Fig. 3.3. As demonstrated in Fig. 3.5 A (left; blue line), treatment of 17K HepG2 cells with 5 nM of lomitapide did not significantly affect apo(a) secretion. However, compared to control, a trend of decreased apo(a) secretion in response to 5 nM of lomitapide treatment was evident. Analysis of 17K HepG2 cells treated with 10 nM of lomitapide showed that apo(a) secretion was significantly decreased at the 120-minute chase time compared to control (Fig. 3.5 A; left, green line). Treatment of 17K HepG2 cells with 10 nM of lomitapide did not significantly affect apo(a) secretion at the 0, 30, 60- and 360-minute chase times versus control. However, a trend between 10 nM lomitapide treatment and decreased apo(a) secretion at those time points could again be observed. As demonstrated in Fig. 3.5 A (left; red line), treatment of 17K HepG2 cells with 20 nM of lomitapide significantly decreased apo(a) secretion at the 360-minute chase time compared to 0 nM control. Apo(a) secretion from 17K HepG2 cells was maximally inhibited by 20 nM of lomitapide at the 120-minute chase time versus control. Furthermore, at the 120-minute chase time, 20 nM of lomitapide significantly decreased apo(a) secretion when compared to 5 nM of lomitapide treatment. Next, we assessed secretion of the 17K$_{\Delta 7,8}$ apo(a) variant to determine if lomitapide's ability to decrease apo(a) secretion was dependent on the wLBS in apo(a). As demonstrated in Fig. 3.5 A (right), treatment of

17K$_{\Delta 7,8}$ HepG2 cells with 5 nM, 10 nM, or 20 nM of lomitapide did not significantly affect apo(a) secretion into conditioned medium when compared to control.

A


17K Apo(a) HepG2
17K$_{\Delta 7,8}$ Apo(a) HepG2
Apo(a) Intensity (% of control maximum)
Time (mins)
0 nM
5 nM
10 nM
20 nM
0 nM Lomitapide
5 nM Lomitapide
10 nM Lomitapide
20 nM Lomitapide
(mins)

B

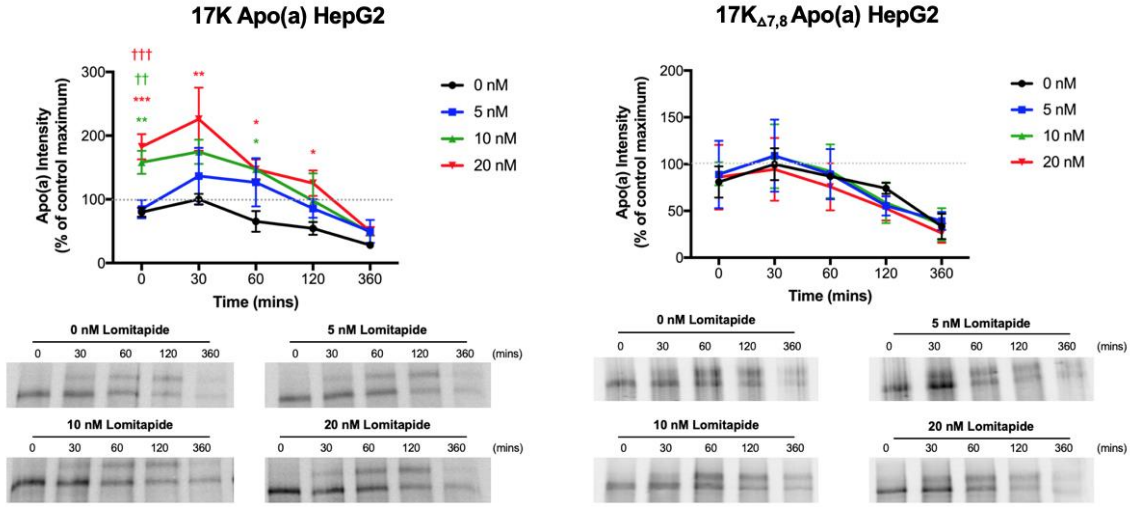

17K Apo(a) HepG2
17K$_{\Delta 7,8}$ Apo(a) HepG2
Apo(a) Intensity (% of control maximum)
Time (mins)
0 nM
5 nM
10 nM
20 nM
0 nM Lomitapide
5 nM Lomitapide
10 nM Lomitapide
20 nM Lomitapide
(mins)

Figure 3.5: ***Treating HepG2 cells with lomitapide affects apo(a) metabolism differently dependent on the presence or absence of the weak lysine binding sites in KIV$_7$ and KIV$_8$.*** Pulse-chase analysis of HepG2 cells stably expressing 17K apo(a) and HepG2 cells transiently expressing 17K$_{\triangle 7,8}$ apo(a) treated with 5nM, 10nM or 20nM of lomitapide compared to control. Cells were pre-incubated for 4 hours in complete medium containing 0.4mM BSA-conjugated oleic acid and 0nM, 5nM, 10nM or 20nM of lomitapide. Cells were then starved in Cys/Met free medium for one hour, pulse labelled with ^{35}S-Cys/Met for one hour, and subsequently chased for 0 minutes, 30 minutes, 60 minutes 120 minutes and 360 minutes. For the chase period, cells were incubated in unlabelled complete medium containing 0.4mM BSA-conjugated oleic acid and either 0nM, 5nM, 10nM or 20nM of lomitapide; conditioned medium and lysate samples were collected from cells at the indicated times of chase. Samples were then subjected to apo(a) pull-down immunoprecipitation, and then analyzed by 6% SDS-PAGE and fluorography. The lower band (~450kDa) represents an immature hypo-glycosylated form of apo(a), while the higher band (~550kDa) represents a mature, fully glycosylated form of apo(a). (**A**) Media samples of 17K apo(a) HepG2 cells treated with 0nM, 5nM, 10nM or 20nM of lomitapide. (**B**) Lysate samples of 17K apo(a) HepG2 cells treated with 0nM, 5nM, 10nM or 20nM of lomitapide. Densitometric band analysis was conducted using the ImageJ software, normalized to the maximum observed density for 0nM lomitapide treatment, and plotted as a function of time. The presented data corresponds to the means ± SEM of 3 or 4 independent experiments. Significance compared to control is represented by asterisks where *$p<0.05$, and **$p<0.01$. Significance compared to 5nM of lomitapide is represented by daggers where $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, and $^{\dagger\dagger\dagger}p<0.001$. The coloured lines representing the different treatment concentrations are indicated on the figure.

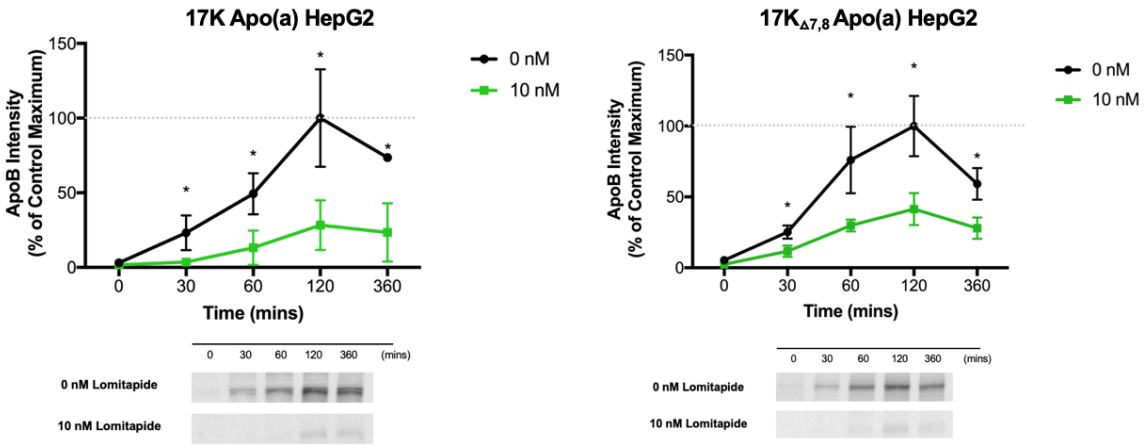
Cell lysates were also analyzed to determine if lomitapide could impact the intracellular abundance of apo(a). The representative fluorograms (Fig. 3.5 B) of cell lysates across all of the treatment groups demonstrate that apo(a) can be observed in both its immature form (~450kDa) from 0-360 minutes, and its mature form (~550kDa) from 30-360 minutes, similar to what was shown in Fig. 3.3. As demonstrated in Fig. 3.5 B (left; blue line), apo(a) intracellular abundance in 17K HepG2 cells was not significantly affected by 5 nM of lomitapide treatment compared to control. Although statistical significance was not attained, there was an observable trend between 5 nM of lomitapide treatment and increased apo(a) intracellular abundance when compared to control. Treatment of 17K HepG2 cells with 10 nM of lomitapide resulted in significantly increased intracellular abundance of apo(a) at the time zero and the 60-minute chase time versus control (Fig. 3.5 B; left, green line). Treatment with 10 nM of lomitapide was also able to significantly increase the intracellular abundance of apo(a) in 17K HepG2 cells when compared to 5 nM lomitapide treatment at time zero of the chase period (Fig. 3.5 B; left, green line). Treatment of 17K HepG2 cells with 20 nM of lomitapide maximally increased the intracellular abundance of apo(a). As demonstrated in Fig. 3.5 B (left; red line), treatment of 17K HepG2 cells with 20 nM significantly increased the intracellular abundance of apo(a) at the 0-minute, 30-minute, 60-minute and 120-minute chase times versus control. Treating 17K HepG2 cells with 20 nM of lomitapide was also able to significantly increase the intracellular abundance of apo(a) when compared to treatment with 5 nM of lomitapide at time zero of the chase period (Fig. 3.5 B; left). The intracellular abundance of the $17K_{\Delta 7,8}$ apo(a) variant was also analyzed in $17K_{\Delta 7,8}$ HepG2 cells in response to lomitapide treatment. Intriguingly, treatment of $17K_{\Delta 7,8}$ HepG2 cells with 5 nM, 10 nM and 20 nM of lomitapide did not significantly affect the intracellular abundance of $17K_{\Delta 7,8}$ apo(a) when compared to control (Fig. 3.5 B; right).

3.6 Lomitapide treatment decreases apoB-100 secretion and intracellular abundance in HepG2 cells

As demonstrated in Fig. 3.5, the ability of lomitapide to modulate apo(a) metabolism appears to be dependent on the presence of the wLBS in $KIV_{7,8}$. Because the wLBS in $KIV_{7,8}$ has been shown to be involved in noncovalent interactions with apoB-100, we treated 17K HepG2 cells and $17K_{\Delta 7,8}$ HepG2 cells with lomitapide and then analyzed apoB-100 secretion and intracellular abundance to gain further insight into how lomitapide regulates apo(a) metabolism. As demonstrated in Fig. 3.6 A (left), treatment of 17K HepG2 cells with 10 nM of lomitapide significantly reduced the secretion of apoB-100 at the 30-minute, 60-minute, 120-minute and 360-minute chase points versus control. Similarly, as shown in Fig. 3.7 A (right), treatment of $17K_{\Delta 7,8}$ HepG2 cells with 10 nM lomitapide also significantly reduced the secretion of apoB-100 at the 30-minute, 60-minute, 120-minute and 360-minute chase times versus the no treatment control.

Cell lysates were analyzed to determine if the wLBS in $KIV_{7,8}$ of apo(a) alters the relationship between lomitapide treatment and apoB-100 intracellular abundance. As demonstrated in Fig. 3.6 B (left), treatment of 17K HepG2 cells with 10 nM of lomitapide significantly decreased the intracellular abundance of apo-B100 at the 0-minute and 30-minute chase times versus the no treatment control. Similarly, treatment of the $17K_{\Delta 7,8}$ HepG2 cells with 10 nM of lomitapide significantly decreased the intracellular abundance of apoB-100 at the 0-minute, 30-minute, and 60-minute chase times versus the no treatment control (Fig. 3.6 B; right). In summary, lomitapide treatment was able to decrease the secretion and intracellular abundance of apoB-100 independent of the presence or absence of a wLBS in apo(a).

A


17K Apo(a) HepG2
17K$_{\Delta 7,8}$ Apo(a) HepG2
ApoB Intensity (% of Control Maximum)
Time (mins)
0 nM
10 nM
0 nM Lomitapide
10 nM Lomitapide
(mins)

B

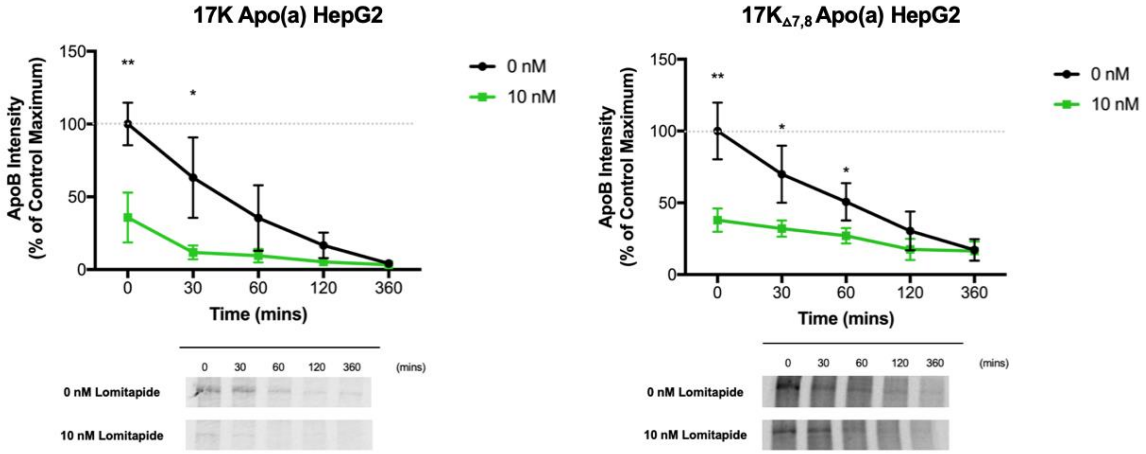

17K Apo(a) HepG2
17K$_{\Delta 7,8}$ Apo(a) HepG2
ApoB Intensity (% of Control Maximum)
Time (mins)
0 nM
10 nM
0 nM Lomitapide
10 nM Lomitapide
(mins)

Figure 3.6: ***Lomitapide treatment decreases apoB-100 secretion and intracellular abundance in HepG2 cells independent of apo(a) coexpression.*** Pulse-chase analysis of HepG2 cells stably expressing 17K apo(a) and HepG2 cells transiently expressing $17K_{\triangle 7,8}$ apo(a) in the presence or absence of 10nM of lomitapide. Cells were pre-incubated for 4 hours in complete medium containing 0.4mM BSA-conjugated oleic acid in the presence or absence of 10nM lomitapide. Cells were then starved in Cys/Met free medium for one hour, pulse labelled with ^{35}S-Cys/Met for one hour, and subsequently chased for 0 minutes, 30 minutes, 60 minutes 120 minutes and 360 minutes. Cells were chased in unlabelled complete medium containing 0.4mM BSA-conjugated oleic acid in the presence or absence of 10nM lomitapide. Conditioned medium and lysate samples were collected from cells treated with either 0nM or 10nM of lomitapide at the indicated times of chase. Samples were then subjected to apoB-100 immunoprecipitation, and then analyzed by 6% SDS-PAGE and fluorography. (**A**) Media samples of 17K apo(a) HepG2 cells incubated in the presence or absence of 10nM lomitapide. (**B**) Lysate samples of 17K apo(a) HepG2 cells incubated in the presence or absence of 10nM lomitapide. Densitometric band analysis was conducted using the ImageJ software, normalized to the maximum observed density for no lomitapide treatment, and plotted as a function of time. The presented data correspond to the mean ± SEM of 3 or 4 independent experiments. Significance compared to control (0nM lomitapide) is represented by asterisks where *$p<0.05$, and **$p<0.01$.

Chapter 4

4 Discussion

Elevated levels of plasma Lp(a) have recently been recognized as the single most prevalent inherited risk factor for CVD [21]. Importantly, it has been identified that approximately 20% of the global population have Lp(a) levels in excess of 50 mg/dL, which is a plasma Lp(a) concentration that has been associated with a 2-fold increased risk of myocardial infarction [21, 70] Although elevated Lp(a) levels are now recognized as a causal risk factor for CAD, there are currently no clinically approved pharmacological therapies available to specifically lower Lp(a) levels [20]. The absence of specific Lp(a)-lowering therapies strongly reflects the limited understanding of Lp(a) biology, including how the particle is assembled and secreted from liver hepatocytes.

It had generally been accepted that Lp(a) formation occurs exclusively extracellularly and involved freely secreted apo(a) interacting covalently with circulating LDL. However, this idea has recently been challenged by *in viv*o human kinetic studies, which have shown that the production rate of the apoB-100 component of Lp(a) and the production rate of apo(a) are quite similar [76]. Furthermore, this work has also demonstrated that the production rate of apoB-100 in Lp(a) appears to be very different from the production rate of other particles containing apoB-100, such as VLDL. Taken together, these studies suggest that the secretion of apo(a) is closely coupled to the secretion of a unique pool of Lp(a)-specific apoB-100, and that Lp(a) particle assembly may in part occur intracellularly.

Although an intracellular Lp(a) assembly dogma was initially challenged by the inability to isolate covalently linked apo(a) and apoB-100 from cell lysates, a noncovalent interaction between apo(a) and apoB-100 within the secretory pathway cannot be ruled out. As mentioned, Lp(a) has been hypothesized to assemble in two-steps. The second step of Lp(a) assembly is believed to involve the covalent interaction between apo(a) and the apoB-100 component of an LDL-like particle. This step of Lp(a) assembly appears to occur exclusively extracellularly, both because very few studies have isolated covalently linked apo(a) and apoB-100 complexes from cell lysates [77], and because a unique oxidase capacity of the extracellular environment seems to promote covalent Lp(a) assembly [85].

The first step of Lp(a) assembly has been hypothesized to involve noncovalent interactions between the wLBS in $KIV_{7,8}$ of apo(a) with specific lysine residues in apoB-100, and likely serves to properly orient the molecules for the subsequent formation of the covalent bond [81]. Importantly, it is not yet known whether this noncovalent interaction between apo(a) and apoB-100 occurs intracellularly. However, as mentioned, the *in vivo* human kinetic studies have demonstrated that the production rates of apo(a) and Lp(a)-specific apoB-100 are quite similar, and thus it would seem plausible that step-one of Lp(a) formation occurs intracellularly. Because production is the primary determinant of plasma Lp(a) levels [62], further understanding the processes of Lp(a) assembly and secretion, such as whether apo(a) and apoB-100 interact within liver hepatocytes, is critical information necessary to aid in the development of specific Lp(a)-lowering pharmaceutical therapies.

4.1 Human sortilin polymorphisms increase apo(a) secretion

GWA studies, rodent studies, and *in vitro* work have all implicated sortilin as a modulator of LDL-C. Although it has been consistently demonstrated that sortilin overexpression and knockdown are associated with altered levels of apoB-100 secretion or LDL clearance, the direction of the relationship between sortilin expression and plasma cholesterol levels remains controversial. For example, while one group has demonstrated that sortilin knockout in mice is associated with decreased plasma cholesterol levels [155], another group has shown that sortilin overexpression in HEK 293 cells induces increased cholesterol uptake [156]. These disparities highlight the complex nature of the relationship between sortilin and plasma cholesterol levels.

More recent genomic studies have also identified specific variants in the *SORT1*-encoding 1p13 locus as being causally associated with altered plasma Lp(a) levels [158]. In line with this finding, previous unpublished work from our lab (Gemin, M.) has demonstrated that sortilin overexpression is associated with increased apo(a) secretion from HepG2 cells, while sortilin knockdown, and mutation of sortilin's cytoplasmic targeting sequences, are associated with decreased apo(a) secretion. Interestingly, co-IP work in our lab has also shown that sortilin can bind directly to apoB-100, but cannot interact with apo(a), which suggests that sortilin modulates apo(a) secretion indirectly, potentially through intracellular

interactions with apoB-100. In order to further understand the role of sortilin in apo(a) secretion, and to gain additional insight into Lp(a) assembly and secretion, we collaborated with Dr. Robert Hegele at Robarts Research Institute and performed deep sequencing of the *SORT1* locus in 1740 patients with dramatically elevated Lp(a) levels. Importantly, we identified 7 unique *SORT1* polymorphisms within 9 individuals that had Lp(a) levels in the top 5^{th} percentile of the general population. It was later determined that the I124V, K205N, K302E, F404Y, E444Q, and E447G amino acid substitution variants occurred specifically within sortilin's ten-bladed β-propeller ligand binding domain. The V650M variant, which is the only variant found outside of the β-propeller domain, occurs within a highly conserved 10CC module. The 10CC module is found adjacent to the transmembrane domain, consists of 10 cysteine residues, and has also been shown to participate in ligand binding [162]. Overall, these single amino acid substitutions result in conserved replacements (where one amino acid is changed to another with similar properties; e.g. I124V), loss of charge replacements (e.g. E444Q), and charge change replacements (where a positive charge is changed for a negative charge or vice versa; e.g. K302E). In each case, all of these substitutions could potentially alter the interaction between the sortilin receptor and its ligand.

To gain insight into how these specific *SORT1* polymorphisms contribute to elevated plasma Lp(a) levels, we transiently transfected expression plasmids containing the 7 sortilin variants individually into HepG2 cells expressing a physiologically relevant 17-kringle apo(a) isoform and then studied apo(a) secretion using pulse-chase analysis. Overall, transfection of HepG2 cells with either wild-type sortilin or one of the 7 human sortilin polymorphic variants increased apo(a) secretion relative to empty-vector control (Fig. 3.1). These results are supported by work completed in mouse hepatocytes, which demonstrated that sortilin overexpression promoted ligand localization towards the golgi and secretory pathway [155]. These findings are also in line with previous work from our lab, and potentially further implicate sortilin as an intracellular receptor that augments apo(a) secretion. Importantly, the I124V, K205N, K302E, F404Y, E444Q, E447G and V650M sortilin variants significantly increased apo(a) secretion compared to empty-vector control (Fig. 3.1). Furthermore, the K302E, F404Y, and E444Q human sortilin

polymorphisms significantly increased apo(a) secretion versus wild-type sortilin control. Therefore, the elevated plasma Lp(a) levels observed in individuals possessing these sortilin variants may be attributable to increased apo(a) secretion. Because individuals that possessed these amino acid substitutions had Lp(a) levels within the top 5th percentile of the general population, it was hypothesized that each variant would increase apo(a) secretion relative to wild-type control. Unexpectantly, transfection of HepG2 cells with the I124V, K205N, E447G and V650M sortilin variants did not significantly impact apo(a) secretion compared to wild-type control. One limitation to this study was that we did not have access to the specific apo(a) isoform sizes expressed within the individuals that had deep sequencing of their *SORT1* locus performed. In our study, we utilized a 17K apo(a) isoform, which has 8 repeated KIV_2 domains. However, it is important to remember that KIV_2 ranges from 3 to over 30 identical tandem repeats in the human population [32]. Thus, due to high allelic heterogeneity, it is unlikely that those individuals identified as having elevated Lp(a) levels and either the I124V, K205N, E447G or the V650M sortilin variant, also possessed the 17K apo(a) isoform used in this study. Therefore, it could be possible that different apo(a) isoform sizes impact sortilin's interactions with apoB-100, which thus affects sortilin's ability to indirectly augment apo(a) secretion. This explanation could account for why the I124V, K205N, E447G and V650M polymorphisms did not increase the secretion of 17K apo(a) in our study. It is also important to recall that sortilin can localize to the plasma membrane and function as an endocytic cell surface receptor. For example, sortilin-mediated endocytosis of Progranulin is believed to play a key role in the pathogenesis of fronto-temporal lobar degeneration [164]. In this study, we did not analyze apo(a) internalization. Therefore, it could be possible that the elevated plasma Lp(a) levels observed in individuals with the I124V, K205N, E447G or V650M sortilin variants are attributable to decreased Lp(a) uptake.

Previous work from our lab has also demonstrated that sortilin increases the intracellular abundance of apo(a). Work completed in this thesis confirms that finding, as a general trend between sortilin overexpression and apo(a) intracellular abundance was observed (Fig. 3.1). The I124V, K205N, K302E, F404Y, E444Q, and V650M amino acid substitutions all significantly increased the intracellular abundance of apo(a) relative to empty-vector control. Moreover, the K302E and F404Y variants, which significantly

increased apo(a) secretion relative to wild-type sortilin, also significantly increased apo(a) intracellular abundance relative to wild-type control. This data further supports a role for sortilin as a regulator of apo(a) secretion through decreasing apo(a) intracellular degradation. Unexpectantly, the E447G variant was able to increase apo(a) secretion, without increasing its intracellular abundance. Importantly, the glutamic acid to glycine substitution results in a loss of charge replacement. It could be possible that this net loss of negative charge within the β-propeller domain of sortilin impacts the interaction between the receptor and its ligand. This specific substitution at position 447 may augment sortilin's ability to bind to apoB-100 enough to increase apo(a) secretion through constitutive secretory granules, but not enough to prevent dissociation of apoB-100 and apo(a) at the lysosome. The inability of this variant to prevent apo(a) degradation might also help explain why E447G overexpression was unable to increase apo(a) secretion to the level of the K302E, F404Y, and E444Q variants. A similar unexpected finding was the observation that the E444Q variant was able to significantly increase apo(a) secretion relative to wild-type sortilin but was unable to increase apo(a) intracellular abundance relative to wild-type sortilin. Importantly, similar to the E447G substitution, the E444Q substitution results in a loss of negative charge replacement. Therefore, it appears that these specific loss of negative charge substitutions result in increased apo(a) secretion but are unable to prevent intracellular apo(a) degradation. Based on this finding, it could be predicted that the observed increase in apo(a) secretion by the loss of charge variants would not be sustainable beyond 360 minutes due to decreased intracellular apo(a) bioavailability secondary to increased intracellular degradation.

Apo(a) is first synthesized into the ER in an immature hypo-glycosylated form. As apo(a) is translated into the ER, *N*-linked glycosylation is occurring within the KIV domains, which appears to be crucial for proper protein folding [66]. Within the medial to trans golgi, apo(a) reaches its mature form after *O*-linked glycans are added to the linker regions between kringle domains [66]. Sortilin is first synthesized as a propeptide in order to prevent premature ligand binding [139]. As the sortilin propeptide enters the TGN, furin cleavage releases the prodomain, which enables the mature receptor to obtain full ligand binding capacity. Therefore, the TGN is the subcellular location at which sortilin obtains

full ligand binding capacity, and the location where apo(a) receives *O*-linked glycosylation and transitions to its 550 kDa mature form. Based on our hypothesis, sortilin is in some way interacting with the mature form of apo(a) in the TGN, and this association acts to both prevent apo(a) degradation, as well as to facilitate apo(a) secretion. Based on the idea that sortilin is interacting with and preventing the degradation of apo(a), it might thus be expected that the ratio of mature apo(a) to immature apo(a) would be increased in cells transfected with wild-type sortilin, and cells transfected with the various sortilin variants that augmented apo(a) intracellular abundance. Unexpectantly, analysis of differences in apo(a) maturation between the various sortilin mutants and wild-type sortilin or empty-vector control revealed no significant differences in the ratio or rate of transition from the immature hypo-glycosylated form to the mature fully glycosylated form (Fig. 3.1). In retrospect, one specific limitation to this study was that the first two apo(a) chase times were at 0 and 120 minutes. In comparison to empty-vector control, it could be possible that wild-type sortilin and the sortilin variants increased the relative ratio of mature apo(a) to immature apo(a) by associating with and preventing the intracellular degradation of mature apo(a) between 0-minutes and 120-minutes of chase. However, because sortilin overexpression was also associated with increased apo(a) secretion, the ratio of mature apo(a) to immature apo(a) might have been reduced by the 120-minute chase time as a result of augmented secretion of mature apo(a). Conversely, the lack of difference in apo(a) maturation rates could be due to sortilin binding to and stabilizing the immature form of apo(a). As mentioned, apo(a) glycosylation begins in the ER as *N*-linked glycans are added to the newly translated protein. However, the addition of *O*-linked glycans, which accounts for the bulk of the mass of apo(a) maturation, is a late golgi event [164]. Therefore, it is likely that immature apo(a) is present within the late golgi, which as mentioned, is the subcellular location where sortilin obtains its ligand binding capacity. Importantly, *in vitro* studies have demonstrated that the immature hypo-glycosylated form of apo(a) is able to associate with apoB-100 [164]. Therefore, it could be possible that immature apo(a) associates with apoB-100 within the late golgi, at which point that complex could also interact with sortilin. If sortilin is able to associate and prevent the degradation of immature apo(a), and then remain complexed with mature apo(a), this might explain why differences

in the ratio of mature apo(a) to immature apo(a) were not observed in cells transfected with wild-type sortilin or a human sortilin variant.

In summary, we have demonstrated that overexpression of the I124V, K205N, K302E, F404Y, E444Q, E447G and V650M human sortilin polymorphisms significantly increases apo(a) secretion versus empty-vector control. Furthermore, with the exception of the E447G variant, each sortilin polymorphism significantly increased the intracellular abundance of apo(a) versus empty-vector control. These findings suggest that the elevated Lp(a) levels observed in individuals with either the I124V, K205N, K302E, F404Y, E444Q, E447G or V650M sortilin polymorphisms may in part be due to decreased intracellular apo(a) degradation and increased apo(a) secretion. Previous work in our lab has demonstrated that sortilin can physically associate with apoB-100, but cannot interact with apo(a). Moreover, sortilin did not affect the secretion of apo(a) lacking the wLBS in $KIV_{7,8}$, which is believed to be important for mediating noncovalent interactions with apoB-100. Although sortilin cannot physically associate with apo(a), this study provides further evidence that sortilin and human sortilin polymorphic variants are able to regulate apo(a) secretion. Therefore, it appears that apo(a) metabolism is modulated by sortilin indirectly, potentially through intracellular noncovalent interactions with apoB-100.

4.2 Sortilin increases apoB-100 secretion only when apo(a) is coexpressed

We have demonstrated in this thesis that a relationship exists between sortilin overexpression and increased apo(a) secretion. As mentioned, it has previously been shown in our lab that mutation of the wLBS in KIV_7 and KIV_8, thought to be important for the initial non-covalent interactions with apoB-100, prevents sortilin from affecting apo(a) secretion (Gemin, M., unpublished data). This finding, coupled with the fact that high affinity binding between apoB-100 and sortilin has been documented [153 154], suggests that a noncovalent intracellular interaction exists between apoB-100 and apo(a), and that sortilin increases apo(a) secretion indirectly by increasing apoB-100 secretion. However, this theory is challenged by a previous study in mice, which demonstrated that sortilin overexpression decreased apoB-100 secretion through increasing apoB-100 lysosomal degradation [134]. If sortilin directs apoB-100 towards the lysosome for degradation, and

if apo(a) and apoB-100 noncovalently interact within the cell, it could be expected that sortilin would decrease the secretion of apo(a) by facilitating its trafficking to the lysosome indirectly through an interaction with apoB-100. Notwithstanding, it is important to acknowledge that the aforementioned apoB-100 secretion studies were performed in mice, which is an animal model that does not express apo(a). Therefore, we hypothesized that if apo(a) and apoB-100 interact intracellularly, it could be possible that this association affects the nature of the physical interaction between sortilin and apoB-100, and alters the sortilin-mediated trafficking patterns of apoB-100.

To test this hypothesis, we performed pulse-chase analysis to study apoB-100 secretion in response to sortilin overexpression from wild-type HepG2 cells (which do not express apo(a)) and from HepG2 cells stably expressing a physiologically-relevant 17-kringle apo(a) isoform. In line with findings from Musunuru's group [134], sortilin overexpression in our wild-type HepG2 cells resulted in both decreased apoB-100 secretion, and decreased apoB-100 intracellular abundance (Fig. 3.2). This pattern of metabolism is characteristic of increased intracellular degradation, and suggests that sortilin overexpression mediates the trafficking of apoB-100 towards the lysosome in wild-type HepG2 cells. Interestingly, the opposite trend was observed in the 17K apo(a) HepG2 cells. Sortilin overexpression in 17K apo(a) HepG2 cells increased both the secretion and intracellular abundance of apoB-100 (Fig. 3.2). This pattern of metabolism is similar to what was observed for apo(a) secretion, and suggests that sortilin is acting to increase the secretion of apoB-100 by preventing its intracellular degradation in these cells.

These findings provide strong, albeit indirect, evidence of an intracellular interaction between apo(a) and apoB-100. The only variable modulated between the studies analyzing apoB-100 secretion in response to sortilin overexpression in the wild-type HepG2 cells, and the studies analyzing apoB-100 secretion in response to sortilin overexpression in the 17K apo(a) HepG2 cells, was the coexpression of apo(a). Intriguingly, the presence of intracellular apo(a) was sufficient to completely reverse the nature of the relationship between sortilin overexpression and apoB-100 secretion. Although the exact mechanisms have not been elucidated, it could be possible that the binding of apo(a) to apoB-100 within the cell alters the physical interaction between apoB-100 and sortilin. By modifying how

apoB-100 associates with sortilin, the presence of apo(a) may in turn alter the intracellular trafficking patterns of apoB-100. This idea that apoB-100 metabolism can be altered through its physical interaction with apo(a) is also supported through studies of Lp(a) catabolism. The LDL-R is the main receptor responsible for clearance of plasma LDL. Despite the numerous structural similarities between Lp(a) and LDL, only a small proportion of plasma Lp(a) is cleared through the LDL-R under normal physiological conditions [118]. This observation has led to the hypothesis that the association between apo(a) and apoB-100 in Lp(a) physically disrupts the typical interactions between apoB-100 and the LDL-R. The finding that sortilin overexpression mediates increased apoB-100 secretion and intracellular abundance also helps support our hypothesis that sortilin increases apo(a) secretion indirectly through increasing apoB-100 secretion (Fig. 3.1). Because sortilin cannot physically associate with apo(a) (Gemin, M., unpublished data), and because apoB-100 and apo(a) display the same patterns of secretion and intracellular abundance in 17K apo(a) cells, it seems likely that an intracellular interaction between apo(a) and apoB-100 can account for the observed sortilin-mediated increases in apo(a) secretion and intracellular abundance.

In summary, the nature of the relationship between sortilin and apoB-100 secretion remains complex and controversial. While some groups have observed a direct relationship between sortilin expression and apoB-100 secretion [155], others have observed an inverse relationship [156]. For this reason, it has been postulated that the directionality of the relationship between sortilin expression and apoB-100 secretion can be influenced by both intracellular and extracellular metabolic conditions, as well as the specific experimental model used. In this study, we demonstrate that the coexpression of apo(a), potentially through intracellular interactions with apoB-100, influences the direction of the relationship between sortilin overexpression and apoB-100 secretion. Therefore, it appears that the presence of intracellular apo(a) is another factor which could help explain some of the disparate findings in the field regarding sortilin and apoB-100 metabolism.

4.3 PCSK9 increases the secretion and intracellular abundance of both apo(a) and apoB-100

PCSK9 is unique from other members of the proprotein convertase family in that it possesses no zymogen activation activities [103]. Although PCSK9 has no apparent enzymatic activities, it has been shown to be an important transport and trafficking receptor [103]. PCSK9 is best known for its role in LDL metabolism. Under normal circumstances, the binding of LDL to the LDL-R triggers clathrin-dependent endocytosis [110]. As the LDL/LDL-R complex moves from the endosome to the lysosome, the low pH environment of the lysosome causes the dissociation of the LDL-R from LDL [110]. At this point, the LDL-R can then be recycled back to the plasma membrane to facilitate further uptake of LDL. Like LDL, PCSK9 can also bind to the LDL-R at the cell surface and trigger endocytosis. However, it has been demonstrated that the binding of PCSK9 to the LDL-R in the low pH environment of the lysosome prevents the dissociation and recycling of the LDL-R to the cell surface [112]. As a consequence, the binding of PCSK9 to the LDL-R impairs cholesterol clearance from the plasma by inducing increased lysosomal degradation of the LDL-R [113]. Interestingly, a role for PCSK9 in the regulation of apoB-containing lipoprotein assembly and secretion has also been recently demonstrated. Pulse-chase studies conducted in LDL-$R^{-/-}$ mouse hepatocytes showed that PCSK9 expression augments both the intracellular abundance and secretion of apoB-100 [114]. Therefore, it appears that PCSK9 promotes increased plasma cholesterol levels by decreasing LDL catabolism and by increasing apoB-containing lipoprotein anabolism.

Because of its ability to augment plasma cholesterol levels, PCSK9 has become a popular LDL-lowering therapeutic target. The two most promising inhibitors of PCSK9, evolocumab and alirocumab, have been demonstrated to effectively lower plasma LDL-C levels by up to 60% [116]. Intriguingly, evolocumab and alirocumab have also been shown to lower plasma Lp(a) levels by approximately 30% through a currently unknown mechanism [117]. It has been postulated that the decreased plasma Lp(a) levels induced by evolocumab and alirocumab treatment are due to increased Lp(a) catabolism through the LDL-R, which is similar to how plasma LDL levels are believed to be lowered by this drug class. However, this theory is challenged by work that has shown that the LDL-R does not

play a significant role in the clearance of Lp(a) under normal physiological conditions [118]. In further opposition, the statin class of drugs, which also augments levels of the LDL-R at the cell surface, does not appear to be effective in lowering Lp(a) [14]. These findings suggest that evolocumab and alirocumab lower plasma Lp(a) levels through a mechanism independent of the LDL-R. In support of this idea, it has recently been shown that treatment of human hepatocytes with PCSK9 results in a 3-fold decrease in apo(a) secretion [122]. Importantly, this effect was reversed by alirocumab treatment [122]. However, this finding is seemingly contradicted by work from our lab, which has shown an inability for PCSK9 to directly associate with apo(a) [160]. This finding, coupled with work that has demonstrated a role for PCSK9 in augmenting apoB-100 secretion, led us to postulate that the observed PCSK9-induced increases in apo(a) secretion are occurring indirectly through intracellular noncovalent interactions with apoB-100.

In order to both gain further mechanistic understanding of Lp(a) assembly and secretion, and identify a role for PCSK9 in Lp(a) metabolism, we treated 17K apo(a) HepG2 cells and $17K_{\Delta 7,8}$ apo(a) HepG2 cells (an apo(a) variant that lacks the wLBS in $KIV_{7,8}$) with PCSK9 and then analyzed apo(a) and apoB-100 secretion using pulse-chase studies. In support of the findings from Villard's group [122], work in this thesis has demonstrated that PCSK9 treatment significantly increases the secretion of apo(a) from 17K apo(a) HepG2 cells relative to control (Fig. 3.3). Furthermore, treatment of 17K apo(a) HepG2 cells with PCSK9 also significantly increased the intracellular abundance of apo(a), suggesting a role for PCSK9 in preventing apo(a) degradation. This thesis also demonstrates that PCSK9 treatment increases the secretion of apoB-100 versus control (Fig. 3.4), which further supports the findings of Sun's group [114]. Similar to what was observed for apo(a), PCSK9 treatment of HepG2 cells also increased the intracellular abundance of apoB-100, which again provides evidence of a role for PCSK9 in preventing apoB-100 degradation.

Intriguingly, PCSK9 treatment did not affect apo(a) secretion or intracellular abundance relative to control in HepG2 cells expressing the $17K_{\Delta 7,8}$ apo(a) variant (Fig. 3.3). However, PCSK9 treatment was still able to augment apoB-100 secretion and intracellular abundance in these cells. As mentioned previously, the wLBS in $KIV_{7,8}$ has been shown to facilitate

noncovalent interactions with lysine residues in apoB-100. However, it remains unknown whether or not this noncovalent interaction occurs intracellularly. Because mutation of the wLBS in $KIV_{7,8}$ in this study diminished the ability of PCSK9 to regulate apo(a) metabolism, we believe that these findings provide evidence that an intracellular noncovalent interaction exists between apo(a) and apoB-100. Previously, it has been demonstrated that PCSK9 is able to tightly associate with apoB-100 [120]. This finding, coupled with the fact that PCSK9 appeared to modulate apoB-100 secretion in a manner independent of the apo(a) variant expressed, suggests that PCSK9 regulates apoB-100 secretion through direct physical interactions. On the other hand, PCSK9 was only able to regulate the secretion and intracellular abundance of apo(a) that possessed the wLBS in $KIV_{7,8}$. Therefore, it seems likely that apo(a), through its wLBS, noncovalently interacts with apoB-100 within the cell, and is indirectly being trafficked by PCSK9 due to its association with apoB-100. In this sense, apo(a) secretion and intracellular abundance appears to be increased by PCSK9 through a "piggyback" effect with apoB-100. In further support of this idea that an intracellular interaction is occurring between apo(a) and apoB-100, we observed that apoB-100 secretion was augmented to a greater extent in the 17K apo(a) HepG2 cells than the $17K_{\Delta 7,8}$ apo(a) HepG2 cells (Fig. 3.4). If apo(a) is interacting with apoB-100 within the cell in such a way that the physical interaction between apoB-100 and PCSK9 is modified, this could potentially increase the secretion of apoB-100 mediated through PCSK9 treatment.

It is important to note that both sortilin overexpression and PCSK9 treatment increased the secretion and intracellular abundance of apoB-100 and apo(a) in a similar manner, which might suggest that some redundancy exists between the two receptor pathways. However, it has also been demonstrated by Gustafsen's group that sortilin is able to associate with PCSK9 and act as a trafficking receptor to augment its secretion [165]. As might be expected, co-localization studies showed extensive interactions between sortilin and PCSK9 in the TGN, which is the subcellular location where sortilin gains its ligand binding capacity [152]. Based on the findings from the aforementioned study, it appears that sortilin acts as a chaperone protein that regulates PCSK9 movement through the secretory pathway. As previously mentioned, co-IP studies have demonstrated that both PCSK9 [114] and

sortilin (Gemin, M., unpublished data) are able to directly associate with apoB-100. Therefore, it could be possible that a sortilin/PCSK9/apoB-100/apo(a) complex forms in the TGN and moves through the secretory pathway during Lp(a) biosynthesis. Because PCSK9 has been shown to facilitate pH-dependent interactions with its ligands, like the LDL-R, its presence could be important for the appropriate loading and unloading of the complex within specific subcellular locations. On the other hand, because of its ability to bind specific adaptor proteins and facilitate a role as a transport protein, sortilin's presence is likely essential for properly transporting the complex through the secretory pathway. It would be interesting to study the secretion of apo(a) and apoB-100 in response to PCSK9 treatment from sortilin knockout cells. If sortilin is in fact responsible for properly shuttling the sortilin/PCSK9/apo(a)/apoB-100 complex through the secretory pathway, it might be expected that PCSK9 treatment in this cell line would not be able to mediate the increased secretion of apo(a) and apoB-100 observed in this study.

In summary, we provide further evidence in this thesis to support a role for PCSK9 in augmenting apo(a) and apoB-100 secretion. Importantly, it appears that PCSK9 is able to increase the secretion of apo(a) indirectly through increasing the secretion of apoB-100. Our work demonstrated that PCSK9 could only modulate the metabolism of apo(a) isoforms possessing the wLBS in $KIV_{7,8}$, but could modulate the secretion of apoB-100 independently of which apo(a) variant was expressed. Because the PCSK9-mediated increases in apo(a) secretion appear to be dependent on the wLBS in $KIV_{7,8}$, this work further supports the existence of an intracellular interaction between apo(a) and apoB-100 in Lp(a) biosynthesis.

4.4 MTP inhibition decreases the secretion of both apo(a) and apoB-100

MTP is an ER resident protein that is responsible for regulating the assembly of all apoB-containing lipoproteins. Specific mutations within the M subunit lipid transfer domain of MTP are phenotypically associated with abetalipoproteinemia, which is a disease characterized by the complete absence of circulating apoB-containing lipoproteins [94]. It has been hypothesized that the binding of MTP to apoB in the ER serves two functions [129]. Firstly, it is thought that the binding of MTP to apoB serves to protect apoB from

degradation. Importantly, when newly synthesized apoB becomes dislocated from the ER membrane, it subsequently undergoes proteosomal degradation [130]. Therefore, by binding to apoB and preventing its dislocation from the ER membrane, it is thought that MTP protects newly synthesized apoB from degradation. Secondly, the lipid transfer activity of MTP is thought to render apoB secretion competent [129]. The exact mechanism behind how MTP transfers lipids to apoB, as well as where these lipids originate from, is still not well understood [129]. Regardless of the mechanism, MTP-mediated lipidation within the ER appears to be crucial in facilitating the secretion of apoB.

Similar to PCSK9, MTP's ability to regulate the secretion of apoB-containing lipoproteins has made it an attractive LDL-cholesterol lowering therapeutic target. Lomitapide is one such MTP inhibitor that has proven to be effective in lowering plasma LDL by up to 40% [93]. However, because MTP plays such a large role in apoB-containing lipoprotein assembly and secretion, lomitapide treatment is often associated with increased hepatic fat retention and plasma liver transaminase levels, and is thus restricted to use in high-risk homozygous FH patients where other therapies have proven ineffective [93]. Intriguingly, lomitapide treatment has also been shown to lower plasma Lp(a) levels by up to 13% through a yet unknown mechanism [93].

As mentioned previously, it has been postulated by some groups that apo(a) is freely secreted from liver hepatocytes and later associates with membrane-bound or circulating LDL for form the Lp(a) particle [74, 166]. Based on this idea, the lomitapide-mediated lowering of plasma Lp(a) would be due to decreased secretion of the apoB-containing VLDL particle, which is the precursor to LDL. By lowering the secretion of VLDL, less LDL would be available in the plasma for apo(a) to associate with, and thus fewer Lp(a) particles would be assembled. However, the extracellular assembly model for Lp(a) has been challenged by more recent kinetic studies, which have shown that the secretion rates of apo(a) and apoB-100 are quite similar, and that there appears to be a unique apoB-100 pool destined specifically for Lp(a) assembly [76]. Based on these findings, it has been postulated that instead of free apo(a) interacting with circulating LDL, an LDL-like particle is synthesized *de novo* in liver hepatocytes and secreted together with apo(a). Although these kinetic studies have shown that the apo(a) and apoB-100 components of Lp(a) are

secreted at similar rates, whether or not these two proteins interact intracellularly has not been directly observed. By studying the secretion of both apo(a) and apoB-100 in response to lomitapide treatment *in vitro*, we hoped to gain insight into Lp(a) assembly and the mechanism behind how lomitapide treatment reduces plasma Lp(a) levels.

In order to both gain further mechanistic understandings of Lp(a) assembly, and discern how lomitapide treatment results in decreased plasma Lp(a) levels, we treated 17K apo(a) HepG2 cells and $17K_{\Delta 7,8}$ apo(a) HepG2 cells (an apo(a) variant that lacks the wLBS in $KIV_{7,8}$) with lomitapide and then analyzed apo(a) and apoB-100 secretion using pulse-chase studies. Work in this thesis demonstrates that lomitapide treatment significantly decreases the secretion and intracellular abundance of apoB-100 in HepG2 cells (Fig. 3.6). These findings are in line with the work of Jamil's group, who also showed that lomitapide treatment significantly reduced apoB-100 secretion after a 30-minute lag interval [167]. Combined, this work supports a role for lomitapide in lowering plasma LDL through decreasing the secretion of apoB-containing lipoproteins. Importantly, our work also demonstrates that lomitapide treatment decreases apoB-100 secretion and intracellular abundance independent of the apo(a) species expressed (Fig. 3.6).

Interestingly, we also show here that lomitapide significantly decreases the secretion of 17K apo(a) from HepG2 cells (Fig. 3.5). Based on the hypothesis that freely secreted apo(a) interacts with circulating LDL to form Lp(a), it would not be expected that lomitapide treatment would impact apo(a) secretion. We postulated that apo(a) secretion was decreased by lomitapide treatment indirectly through intracellular interactions with apoB-100 and decreased apoB-100 secretion. However, we could not neglect the possibility that MTP physically associates with apo(a) in the ER and plays a more direct role in apo(a) metabolism. To help decipher the mechanisms involved in lomitapide-mediated regulation of apo(a) secretion, we used HepG2 cells expressing $17K_{\Delta 7,8}$ apo(a), which again lacks the wLBS in $KIV_{7,8}$ thought to be involved in noncovalent interactions with apoB-100. Importantly, lomitapide treatment was unable to affect the secretion or intracellular abundance of the apo(a) variant lacking the wLBS in $KIV_{7,8}$ (Fig. 3.5). Therefore, similar to how sortilin and PCSK9 modulate apo(a) metabolism, it appears that lomitapide decreases apo(a) secretion indirectly. By inhibiting the lipidation of apoB-100, which

subsequently increases its proteosomal degradation, lomitapide likely reduces the amount of apoB-100 available to associate with apo(a). As the amount of apoB-100 available to associate with apo(a) decreases, the movement of apo(a) through the secretory pathway is presumably impaired. As mentioned previously, co-IP studies have demonstrated the both sortilin (Gemin, M., unpublished data) and PCSK9 [114] can physically interact with apoB-100, but cannot associate with apo(a). Therefore, apo(a) secretion is likely unable to benefit from the indirect increases in secretion mediated through the effects of PCSK9 and sortilin, and instead partially accumulates somewhere upstream in the secretory pathway. This theory is supported by our finding that lomitapide treatment significantly decreased the intracellular abundance of apoB-100 (Fig. 3.6), but significantly increased the intracellular abundance of 17K apo(a) (Fig. 3.5).

In summary, this work provides evidence to support a role for MTP in Lp(a) metabolism through regulating the secretion of apoB-100. Furthermore, because lomitapide was able to decrease the secretion of 17K apo(a), but had no effect on $17K_{\Delta 7,8}$ apo(a) secretion, these findings also provide evidence of an intracellular interaction between apo(a) and apoB-100. The production of apo(a) is not thought to involve interactions with MTP, as apo(a) does not undergo lipidation [168]. Therefore, it appears that by decreasing the intracellular abundance of apoB-100 and consequentially reducing the intracellular interactions between apo(a) and apoB-100, lomitapide treatment indirectly impedes apo(a) secretion. If Lp(a) assembly did not involve an intracellular interaction between apo(a) and apoB-100, it would seem unlikely that lomitapide-mediated modulation of apoB-100 secretion would have any impact on apo(a) secretion. Taken together, work in this thesis also provides a possible explanation as to how lomitapide treatment reduces plasma Lp(a) levels. Rather than reducing the amount of circulating LDL available for free apo(a) to associate with, our work suggests that lomitapide treatment directly inhibits the secretion of the Lp(a) particle, which appears to assemble, at least partially, in liver hepatocytes.

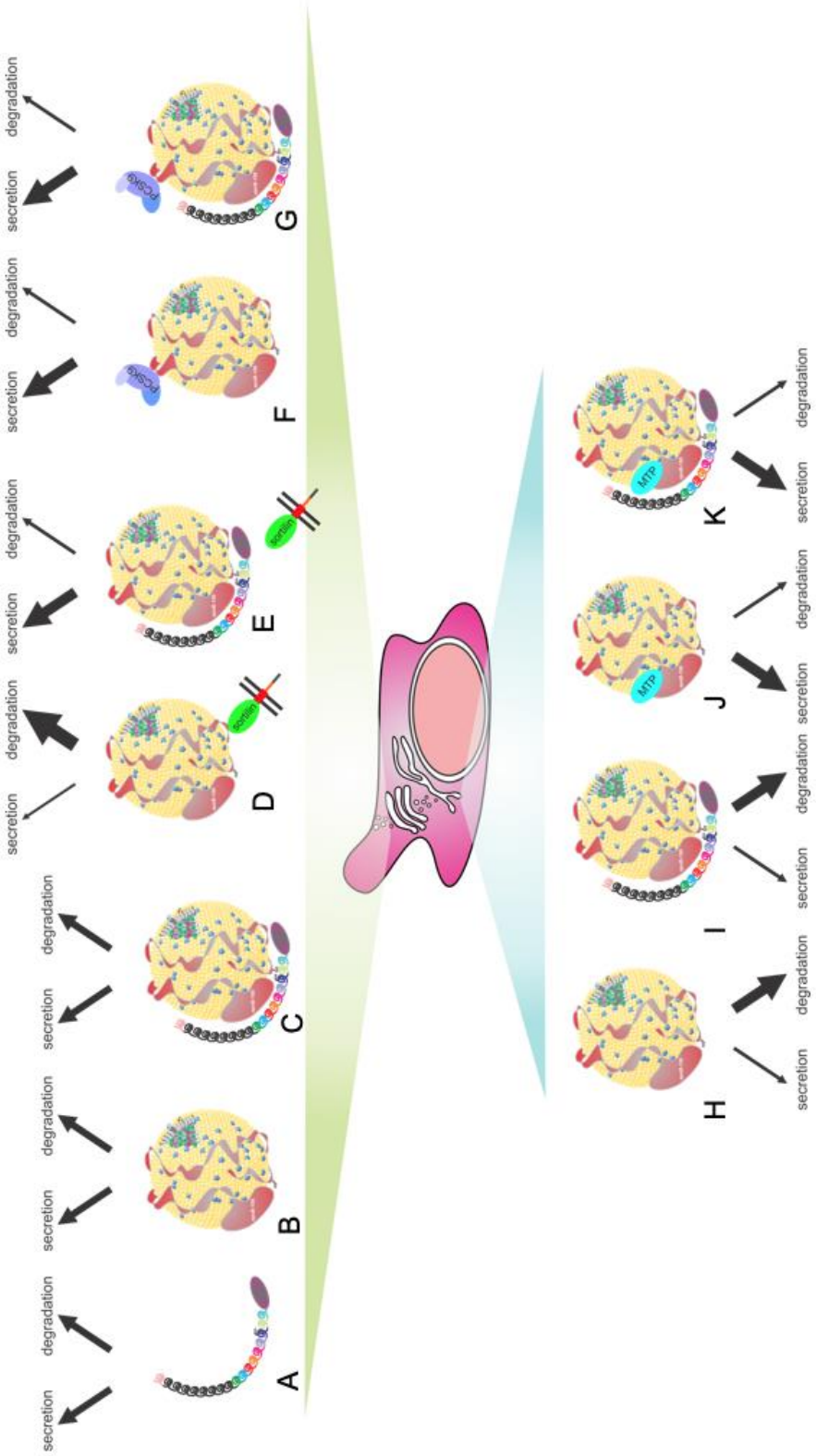

A
B
C
D
E
F
G
H
I
J
K
secretion
degradation
sortilin
PCSK9
MTP

Figure 4.1: ***A model of the role of sortilin, PCSK9 and MTP on apoB-100 containing lipoprotein secretion.*** Under normal conditions, a delicate balance between hepatic secretion and intracellular degradation of apo(a), Lp(a), and other apoB-100-containing lipoproteins is achieved (**A, B, C**). In the presence of sortilin and absence of apo(a), apoB-containing lipoprotein degradation is increased (**D**). However, through a yet unknown mechanism, the presence of sortilin results in decreased degradation and increased secretion of apoB-containing lipoproteins that are interacting with apo(a) (**E**). Because sortilin does not obtain full ligand binding capacity until reaching the late golgi, the interactions between sortilin and apoB-containing lipoproteins likely occurs within the cis or trans golgi compartments (green arrow). PCSK9 has been shown to directly interact with apoB-100, protecting it from lysosomal degradation, and facilitating its secretion (**F**). Despite evidence demonstrating an inability for PCSK9 to directly interact with apo(a), treatment of HepG2 cells with PCSK9 results in augmented secretion of apo(a) (**G**). It would seem plausible that this PCSK9-mediated increase in apo(a) secretion occurs as a result of non-covalent intracellular interactions between apo(a) and apoB-100. Within the ER (blue arrow), improperly lipidated lipoproteins are targeted for proteasomal degradation (**H, I**). MTP localizes to the ER membrane where it prevents apoB-100 membrane dislocation and participates in primordial lipoprotein lipid loading and assembly. By preventing the dislocation of apoB-100 from the ER membrane, MTP protects apoB-100 from degradation, which subsequently results in increased apoB-containing lipoprotein secretion (**J**). MTP also appears to have a role in facilitating apo(a)-containing lipoprotein secretion (**K**), as inhibition of MTP with lomitapide results in decreased apo(a) secretion. However, this effect of MTP inhibition on apo(a) secretion was attenuated in apo(a) variants lacking the wLBS in $KIV_{7\text{-}8}$. Therefore, it seems plausible that apo(a) and apoB-100 interact intracellularly, and MTP increases apo(a) secretion indirectly through augmenting the bioavailability and secretion of apoB-100.

4.5 Study limitations and future directions

To determine whether the observed effects of PCSK9 and lomitapide treatment on 17K apo(a) secretion were due to a noncovalent intracellular interaction with apoB-100, an 17K apo(a) variant lacking the wLBS in $KIV_{7\text{-}8}$ ($17K_{\Delta 7,8}$ apo(a)) was utilized. In our study, we measured wild-type 17K apo(a) secretion in response to lomitapide and PCSK9 treatment from HepG2 cells stably expressing 17K apo(a). However, due to time constraints, one limitation to this study was that $17K_{\Delta 7,8}$ apo(a) secretion was measured from HepG2 cells transiently expressing $17K_{\Delta 7,8}$ apo(a). Future experiments conducted to examine the effects of lomitapide and PCSK9 treatment on wild-type 17K apo(a) secretion and $17K_{\Delta 7,8}$ apo(a) secretion should utilize either HepG2 cells stably expressing both wild-type 17K apo(a) or $17K_{\Delta 7,8}$ apo(a), or HepG2 cells transiently expressing both wild-type 17K apo(a) or $17K_{\Delta 7,8}$ apo(a).

Finally, in this study we measured apo(a) secretion from HepG2 cells in response to either wild-type sortilin, Sort-I124V, Sort-K205N, Sort-K302E, Sort-F404Y, Sort-E444Q, Sort-E447G, or Sort-V650M overexpression relative to empty-vector control. Although Western Blot analysis of cellular lysates demonstrated comparable expression levels of wild-type sortilin with the Sort-I124V, Sort-K205N, Sort-K302E, Sort-F404Y, Sort-E444Q, and Sort-E447G polymorphic variants, it did appear that the Sort-V650M variant was expressed at slightly higher levels. In future studies, sortilin overexpression should be normalized to β-actin expression. Subsequently, apo(a) secretion levels could then be adjusted relative to the measured amount of sortilin overexpression.

4.6 Summary and Conclusions

Elevated levels of plasma lipoprotein(a) (Lp(a)) are the most prevalent inherited risk factor for CVD, but development of specific Lp(a) lowering therapeutics has been hindered by a lack of fundamental understanding of the lipoprotein's biology. Lp(a) is believed to be synthesized in two steps. The first step involves noncovalent associations between the wLBS in $KIV_{7,8}$ and lysine residues in apoB-100, and likely serves to properly orient the apolipoproteins for step two. Step two of assembly involves a stronger covalent linkage between a free cysteine in KIV_9 of apo(a) with a free cysteine in the C-terminus of apoB-

100. Because very few apo(a)/apoB-100 covalent complexes have ever been isolated from cell lysates, it is generally accepted that step two occurs extracellularly. However, the location of the step one noncovalent association remains enigmatic. To determine if a noncovalent intracellular interaction exists between apo(a) and apoB-100 in Lp(a) assembly and secretion, we modulated known cellular regulators of apoB-100 metabolism, and then analyzed the secretion of both apo(a) and apoB-100. In this thesis, we have shown that the presence of intracellular apo(a) can modulate the secretion patterns of apoB-100 in response to sortilin overexpression. Furthermore, we have shown that the PCSK9 and MTP-mediated regulation of apo(a) secretion is dependent on the wLBS in $KIV_{7,8}$, which is believed to be important in facilitating noncovalent interactions with apoB-100. Taken together, work in this thesis suggests a role for sortilin, MTP, and PCSK9 in apo(a) secretion, likely through modulating the bioavailability of apoB-100. Furthermore, our work provides indirect evidence of a noncovalent intracellular interaction between apo(a) and apoB-100 in Lp(a) assembly.

References

[1] A. Timmis *et al*, "European Society of Cardiology: Cardiovascular Disease Statistics 2017," *European Heart Journal*, vol. 39, *(7)*, pp. 508-579, 2018.

[2] J. Frostegård, "Immunity, atherosclerosis and cardiovascular disease," *BMC Medicine*, vol. 11, *(1)*, pp. 117-117, 2013.

[3] A. L. Pyle and P. P. Young, "Atheromas Feel the Pressure," *American Journal of Pathology, the*, vol. 177, *(1)*, pp. 4-9, 2010.

[4] U. Förstermann, N. Xia and H. Li, "Roles of Vascular Oxidative Stress and Nitric Oxide in the Pathogenesis of Atherosclerosis," *Circulation Research*, vol. 120, *(4)*, pp. 713-735, 2017.

[5] D. P. Ramji and T. S. Davies, "Cytokines in atherosclerosis: Key players in all stages of disease and promising therapeutic targets," *Cytokine and Growth Factor Reviews*, vol. 26, *(6)*, pp. 673-685, 2015.

[6] A. J. Lusis, "Atherosclerosis," *Nature*, vol. 407, *(6801)*, pp. 233-241, 2000.

[7] A. X. Zhou and I. Tabas, "The UPR in atherosclerosis," *Seminars in Immunopathology*, vol. 35, *(3)*, pp. 321-332, 2013.

[8] S. S. M. Rensen, P. A. Doevendans and G. J. J. M. van Eys, "Regulation and characteristics of vascular smooth muscle cell phenotypic diversity," *Netherlands Heart Journal*, vol. 15 (3), *(3)*, pp. 100-8, 2007.

[9] R. Ferrari *et al*, "Expert consensus document: A 'diamond' approach to personalized treatment of angina," *Nature Reviews. Cardiology*, vol. 15, *(2)*, pp. 120-132, 2017;2018.

[10] P. Lu *et al*, "Extracellular matrix degradation and remodeling in development and disease," *Cold Spring Harbor Perspectives in Biology*, vol. 3, *(12)*, pp. a005058-a005058, 2011.

[11] N. Anitschkow, “Über die Veränderungen der Kaninchenaorta bei experimenteller Cholesterinsteatose. Beitr,” *Pathololigical Anatomy and Histology*, vol. 56, pp. 379–404, 1913.

[12] Cholesterol Treatment Trialists' (CTT) Collaboration, Cholesterol Treatment Trialists’ (CTT) Collaboration and Cholesterol Treatment Trialists' (CTT) Collaboration, "Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170 000 participants in 26 randomised trials," *Lancet, the*, vol. 376, *(9753)*, pp. 1670-1681, 2010.

[13] Scandinavian Simvastatin Survival Study Group, "Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S)," *The Lancet*, vol. 344, *(8934)*, pp. 1383-1389, 1994.

[14] A. V. Khera *et al*, "Lipoprotein(a) Concentrations, Rosuvastatin Therapy, and Residual Vascular Risk: An Analysis From the JUPITER Trial (Justification for

the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin)," *Circulation,* vol. 129, *(6),* pp. 635-642, 2014.

[15] K. Berg, "A New Serum Type System in Man — The Lp System," *Vox Sanguinis,* vol. 10, *(5),* pp. 513-527, 1965.

[16] M. Koschinsky, M. Boffa, "Lipoprotein(a) as a therapeutic target in cardiovascular disease," *Expert Opinion on Therapeutic Targets*, vol. 18, (7), pp. 747-757, 2014

[17] B. G. Nordestgaard and A. Langsted, "Lipoprotein (a) as a cause of cardiovascular disease: insights from epidemiology, genetics, and biology," *Journal of Lipid Research,* vol. 57, *(11),* pp. 1953-1975, 2016.

[18] P. M. Ridker, C. H. Hennekens and M. J. Stampfer, "A Prospective Study of Lipoprotein(a) and the Risk of Myocardial Infarction," *JAMA, vol.* 270, (18), pp. 2195–2199, 1993

[19] N. Rifai *et al*, "Apolipoprotein(a) Size and Lipoprotein(a) Concentration and Future Risk of Angina Pectoris with Evidence of Severe Coronary Atherosclerosis in Men: The Physicians' Health Study," *Clinical Chemistry*, vol. 50, (8), pp. 1364-1371, 2004.

[20] P.R. Kamstrup *et al*, "Genetically elevated lipoprotein(a) and increased risk of myocardial infarction," *JAMA*, vol. 301, (22) 2331-2339, 2009.

[21] F. Kronenberg and G. Utermann, "Lipoprotein(a): resurrected by genetics," *Journal of Internal Medicine,* vol. 273, *(1),* pp. 6-30, 2013.

[22] C. Ehnholm *et al*, "Protein and carbohydrate composition of Lp(a)lipoprotein from human plasma," *Biochemistry*, vol. 11, *(17),* pp. 3229-3232, 1972.

[23] J. W. Gaubatz *et al*, "Human plasma lipoprotein [a]. Structural properties," *Journal of Biological Chemistry,* vol. 258, *(7),* pp. 4582, 1983.

[24] C. Doucet *et al*, "Chimpanzee lipoprotein(A): Relationship between apolipoprotein(A) isoform size and the density profile of lipoprotein(A) in animals with different heterozygous apo(A) phenotypes," *Journal of Medical Primatology*, vol. 27, *(1),* pp. 21-27, 1998.

[25] R. M. Lawn, K. Schwartz and L. Patthy, "Convergent Evolution of Apolipoprotein(a) in Primates and Hedgehog," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, *(22),* pp. 11992-11997, 1997.

[26] R. M. Lawn *et al*, "cDNA sequence of human apolipoprotein(a) is homologous to plasminogen," *Nature,* vol. 330, *(6144),* pp. 132-137, 1987.

[27] D. L. Eaton *et al*, "Partial Amino Acid Sequence of Apolipoprotein(a) Shows that It is Homologous to Plasminogen," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 84, *(10),* pp. 3224-3228, 1987.

[28] A. Rydzewski and F. J. Castellino, "The Effector Roles of Kringle 1 and Kringle 2 in the Enzymatic Properties of Recombinant Tissue-Type Plasminogen Activator as Revealed by Generation of Recombinant Molecules Containing Each Kringle

Linked to the Protease Domain," *Archives of Biochemistry and Biophysics,* vol. 300, *(1),* pp. 472-482, 1993.

[29] D. A. Walz, D. Hewett-Emmett and W. H. Seegers, "Amino Acid Sequence of Human Prothrombin Fragments 1 and 2," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 74, *(5),* pp. 1969-1972, 1977.

[30] M. A. Hancock *et al,* "Inhibition of plasminogen activation by lipoprotein(a): critical domains in apolipoprotein(a) and mechanism of inhibition on fibrin and degraded fibrin surfaces," *The Journal of Biological Chemistry,* vol. 278, *(26),* pp. 23260-23269, 2003.

[31] J. Guevara J *et al,* "A structural assessment of the apo[a] protein of human lipoprotein[a]," *Proteins,* vol. 12, *(2),* pp. 188-199, 1992.

[32] van der Hoek, Y. Y *et al,* "The apolipoprotein(a) kringle IV repeats which differ from the major repeat kringle are present in variably-sized isoforms," *Human Molecular Genetics,* vol. 2, *(4),* pp. 361-366, 1993.

[33] M. L. Koschinsky *et al,* "Apolipoprotein(a) size heterogeneity is related to variable number of repeat sequences in its mRNA," *Biochemistry,* vol. 29, *(3),* pp. 640-644, 1990.

[34] G. M. Fless, M. E. ZumMallen and A. M. Scanu, "Physicochemical properties of apolipoprotein(a) and lipoprotein(a-) derived from the dissociation of human plasma lipoprotein (a)," *Journal of Biological Chemistry,* vol. 261, *(19),* pp. 8712, 1986.

[35] A. M. Scanu and C. Edelstein, "Learning about the structure and biology of human lipoprotein [a] through dissection by enzymes of the elastase family: facts and speculations," *Journal of Lipid Research,* vol. 38, *(11),* pp. 2193, 1997.

[36] D. K. Bonen *et al,* "Inhibition of N-linked glycosylation results in retention of intracellular apo[a] in hepatoma cells, although nonglycosylated and immature forms of apolipoprotein[a] are competent to associate with apolipoprotein B-100 in vitro," *Journal of Lipid Research,* vol. 39, *(8),* pp. 1629, 1998.

[37] E. Angles-Cano and G. Rojas, “Apolipoprotein(a): structure-function relationship at the lysine-binding site and plasminogen activator cleavage site.” *Biol Chem,* vol. 383, (1) pp. 93-99, 2002.

[38] M. L. Koschinsky *et al,* "Identification of the cysteine residue in apolipoprotein(a) that mediates extracellular coupling with apolipoprotein B-100," *Journal of Biological Chemistry,* vol. 268, *(26),* pp. 19819, 1993.

[39] M. L. Koschinsky and S. M. Marcovina, "Structure-function relationships in apolipoprotein(a): insights into lipoprotein(a) assembly and pathogenicity," *Current Opinion in Lipidology,* vol. 15, *(2),* pp. 167-174, 2004.

[40] M. B. Boffa and M. L. Koschinsky, "Lipoprotein (a): truly a direct prothrombotic factor in cardiovascular disease?" *Journal of Lipid Research,* vol. 57, *(5),* pp. 745-757, 2016.

[41] D. C. Schwenke and T. E. Carew, "Initiation of atherosclerotic lesions in cholesterol-fed rabbits. II. Selective retention of LDL vs. selective increases in LDL permeability in susceptible sites of arteries," *Arteriosclerosis: An Official Journal of the American Heart Association, Inc,* vol. 9, *(6),* pp. 908-918, 1989.

[42] S. SenBanerjee *et al*, "KLF2 Is a novel transcriptional regulator of endothelial proinflammatory activation," *The Journal of Experimental Medicine,* vol. 199, *(10),* pp. 1305-1315, 2004.

[43] B. G. Nordestgaard and L. B. Nielsen, "Atherosclerosis and arterial influx of lipoproteins," *Current Opinion in Lipidology,* vol. 5, *(4),* pp. 252-257, 1994.

[44] G. Leibundgut *et al*, "Determinants of binding of oxidized phospholipids on apolipoprotein (a) and lipoprotein (a)," *Journal of Lipid Research,* vol. 54, *(10),* pp. 2815-2830, 2013.

[45] C. A. Scipione *et al*, "Mechanistic insights into Lp(a)-induced IL-8 expression: a role for oxidized phospholipid modification of apo(a)," *Journal of Lipid Research,* vol. 56, *(12),* pp. 2273-2285, 2015.

[46] C. Bergmark *et al*, "A novel function of lipoprotein [a] as a preferential carrier of oxidized phospholipids in human plasma," *Journal of Lipid Research,* vol. 49, *(10),* pp. 2230-2239, 2008.

[47] M. B. Boffa and M. L. Koschinsky, "Oxidized phospholipids as a unifying theory for lipoprotein(a) and cardiovascular disease," *Nature Reviews. Cardiology,* vol. 16, *(5),* pp. 305-318, 2019.

[48] S. N. Sotiriou *et al*, "Lipoprotein(a) in atherosclerotic plaques recruits inflammatory cells through interaction with Mac-1 integrin," *FASEB J,* vol. 20, (3), pp. 559-561, 2006.

[49] T. Cho, Y. Jung and M. L. Koschinsky, "Apolipoprotein(a), through Its Strong Lysine-binding Site in KIV10, Mediates Increased Endothelial Cell Contraction and Permeability via a Rho/Rho Kinase/MYPT1-dependent Pathway," *Journal of Biological Chemistry,* vol. 283, *(45),* pp. 30503-30512, 2008.

[50] S. Allen *et al*, "Expression of adhesion molecules by lp(a): A potential novel mechanism for its atherogenicity," *FASEB J, vol.* 12, pp. 1765–1776, 1998.

[51] S. Takami *et al*, "Lipoprotein(a) enhances the expression of intercellular adhesion molecule-1 in cultured human umbilical vein endothelial cells," *Circulation,* vol. 97, *(8),* pp. 721-728, 1998.

[52] K. Riches *et al*, "Apolipoprotein(a) acts as a chemorepellent to human vascular smooth muscle cells via integrin αVβ3 and RhoA/ROCK-mediated mechanisms," *International Journal of Biochemistry and Cell Biology,* vol. 45, *(8),* pp. 1776-1783, 2013.

[53] T. A. Seimon *et al*, "Atherogenic Lipids and Lipoproteins Trigger CD36-TLR2-Dependent Apoptosis in Macrophages Undergoing Endoplasmic Reticulum Stress," *Cell Metabolism,* vol. 12, *(5),* pp. 467-482, 2010.

[54] W. Sangrar *et al*, "Antifibrinolytic effect of recombinant apolipoprotein(a) in vitro is primarily due to attenuation of tPA-mediated Glu-plasminogen activation," *Biochemistry,* vol. 34, *(15),* pp. 5151-5157, 1995.

[55] J. Loscalzo *et al*, "Lipoprotein(a), fibrin binding, and plasminogen activation," *Arteriosclerosis: An Official Journal of the American Heart Association, Inc,* vol. 10, *(2),* pp. 240-245, 1990.

[56] J. Knapp and W. Herrmann, "In vitro inhibition of fibrinolysis by apolipoprotein(a) and lipoprotein(a) is size- and concentration-dependent," *Clinical Chemistry and Laboratory Medicine (CCLM),* vol. 42, *(9),* pp. 1013-1019, 2004.

[57] N. T. Feric *et al*, "Apolipoprotein(a) inhibits the conversion of Glu-plasminogen to Lys-plasminogen: a novel mechanism for lipoprotein(a)-mediated inhibition of plasminogen activation," *J Thromb Haemost,* vol. 6, (12), pp. 2113-2120, 2008.

[58] M. N. Rahman *et al*, "Comparative analyses of the lysine binding site properties of apolipoprotein(a) kringle IV types 7 and 10," *Biochemistry,* vol. 41, *(4),* pp. 1149-1155, 2002.

[59] M. L. Rand *et al*, "Apolipoprotein(a) Enhances Platelet Responses to the Thrombin Receptor-Activating Peptide SFLLRN," *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 18, *(9),* pp. 1393-1399, 1998.

[60] J. Broze G J *et al*, "The lipoprotein-associated coagulation inhibitor that inhibits the factor VII-tissue factor complex also inhibits factor Xa: insight into its possible mechanism of action," *Blood,* vol. 71, *(2),* pp. 335, 1988.

[61] D. C. Crawford *et al*, "LPA and PLG sequence variation and kringle IV-2 copy number in two populations," *Human heredity*, vol. *66,* (4), pp. 199–209, 2008.

[62] D. J. Rader *et al*, "Variation in lipoprotein(a) concentrations among individuals with the same apolipoprotein (a) isoform is determined by the rate of lipoprotein(a) production," *J Clin Invest*, vol. 91, (2) pp. 443-447, 1993.

[63] A. L. White, B. Guerra and R. E. Lanford, "Influence of Allelic Variation on Apolipoprotein(a) Folding in the Endoplasmic Reticulum," *Journal of Biological Chemistry,* vol. 272, *(8),* pp. 5048-5055, 1997.

[64] S. Erqou *et al*, "Apolipoprotein(a) isoforms and the risk of vascular disease: systematic review of 40 studies involving 58,000 participants," *J Am Coll Cardiol,* vol. 55, (19), pp. 2160-2167, 2010.

[65] A. L. White, D. L. Rainwater and R. E. Lanford, "Intracellular maturation of apolipoprotein[a] and assembly of lipoprotein[a] in primary baboon hepatocytes," *Journal of Lipid Research,* vol. 34, *(3),* pp. 509, 1993.

[66] McLean JW, Tomlinson JE, Kuang WJ, Eaton DL, Chen EY, Fless GM, et al. cDNA sequence of human apolipoprotein(a) is homologous to plasminogen. Nature. 1987;330(6144):132-7.

[67] A. L. White *et al*, "Presecretory degradation of apolipoprotein [a] is mediated by the proteasome pathway," *Journal of Lipid Research,* vol. 40, *(2),* pp. 275, 1999.

[68] H. G. Kraft *et al*, “Frequency distributions of apolipoprotein(a) kringle IV repeat alleles and their effects on lipoprotein(a) levels in Caucasian, Asian, and African populations: the distribution of null alleles is non-random,” *European Journal of Human Genetics*, vol. *4*, pp. 74–87, 1996.

[69] K. Schmidt *et al*, "Genetics of the Lp(a) apo(a) system in an autochthonous Black African population from the Gabon," *European Journal of Human Genetics*, vol. 14, *(2)*, pp. 190-201, 2006.

[70] K. Schmidt *et al*, "Structure, function, and genetics of lipoprotein (a)," *Journal of Lipid Research*, vol. 57, *(8)*, pp. 1339-1359, 2016.

[71] H. G. Kraft *et al*, "Changes of genetic apolipoprotein phenotypes caused by liver transplantation. Implications for apolipoprotein synthesis," *The Journal of Clinical Investigation*, vol. 83, *(1)*, pp. 137-142, 1989.

[72] J. E. Tomlinson, J. W. McLean and R. M. Lawn, "Rhesus monkey apolipoprotein(a). Sequence, evolution, and sites of synthesis," *Journal of Biological Chemistry*, vol. 264, *(10)*, pp. 5957, 1989.

[73] S. Frank, S. Durovic and G. M. Kostner, "The assembly of lipoprotein Lp(a)," *European Journal of Clinical Investigation*, vol. 26, *(2)*, pp. 109-114, 1996.

[74] A. L. White and R. E. Lanford, "Cell surface assembly of lipoprotein(a) in primary cultures of baboon hepatocytes," *Journal of Biological Chemistry*, vol. 269, *(46)*, pp. 28716, 1994.

[75] M. F. Linton *et al*, "Transgenic mice expressing high plasma concentrations of human apolipoprotein B100 and lipoprotein(a)," *The Journal of Clinical Investigation*, vol. 92, *(6)*, pp. 3029-3037, 1993.

[76] M. E. Frischmann *et al*, "In vivo stable-isotope kinetic study suggests intracellular assembly of lipoprotein(a)," *Atherosclerosis*, vol. 225, *(2)*, pp. 322-327, 2012.

[77] D. K. Bonen *et al*, "Expression of a recombinant apolipoprotein(a) in HepG2 cells. Evidence for intracellular assembly of lipoprotein(a)," *The Journal of Biological Chemistry*, vol. 272, *(9)*, pp. 5659-5667, 1997.

[78] M. R. Diffenderfer *et al*, "Distinct metabolism of apolipoproteins (a) and B-100 within plasma lipoprotein(a)," *Metabolism*, vol. 65, *(4)*, pp. 381-390, 2016.

[79] J. L. Jenner *et al*, "The metabolism of apolipoproteins (a) and B-100 within plasma lipoprotein (a) in human beings," *Metabolism*, vol. 54, *(3)*, pp. 361-369, 2005.

[80] G. Reyes-Soffer, H. N. Ginsberg and R. Ramakrishnan, "The metabolism of lipoprotein (a): an ever-evolving story," *Journal of Lipid Research*, vol. 58, *(9)*, pp. 1756-1764, 2017.

[81] B. R. Gabel *et al*, "Lipoprotein(a) Assembly: Quantitative Assessment of the Role of Apo(a) Kringle IV Types 2-10 in Particle Formation," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 16, *(12)*, pp. 1559-1567, 1996.

[82] L. Becker *et al*, "Quantitative evaluation of the contribution of weak lysine-binding sites present within apolipoprotein(a) kringle IV types 6-8 to lipoprotein(a) assembly," *The Journal of Biological Chemistry,* vol. 279, *(4),* pp. 2679-2688, 2004.

[83] L. Becker *et al*, "Identification of a Critical Lysine Residue in Apolipoprotein B-100 That Mediates Noncovalent Interaction with Apolipoprotein(a)," *Journal of Biological Chemistry,* vol. 276, *(39),* pp. 36155-36162, 2001.

[84] E. Merki *et al*, "Antisense oligonucleotide directed to human apolipoprotein B-100 reduces lipoprotein(a) levels and oxidized phospholipids on human apolipoprotein B-100 particles in lipoprotein(a) transgenic mice," *Circulation,* vol. 118, *(7),* pp. 743-753, 2008.

[85] L. Becker, M. E. Nesheim and M. L. Koschinsky, "Catalysis of covalent Lp(a) assembly: evidence for an extracellular enzyme activity that enhances disulfide bond formation," *Biochemistry,* vol. 45, *(32),* pp. 9919-9928, 2006.

[86] J. Guevara J *et al*, "Interaction of apolipoprotein[a] with apolipoproteinB-100 Cys3734 region in lipoprotein[a] is confirmed immunochemically," *Journal of Protein Chemistry,* vol. 15, *(1),* pp. 17-25, 1996.

[87] B. Gabel *et al*, "Carboxyl-terminal truncation of apolipoproteinB-100 inhibits lipoprotein(a) particle formation," *FEBS Letters,* vol. 350, *(1),* pp. 77-81, 1994.

[88] G. S. Thomas *et al*, "Mipomersen, an apolipoprotein B synthesis inhibitor, reduces atherogenic lipoproteins in patients with severe hypercholesterolemia at high cardiovascular risk: a randomized, double-blind, placebo-controlled trial," *Journal of the American College of Cardiology,* vol. 62, *(23),* pp. 2178, 2013.

[89] G. Schonfeld, X. Lin and P. Yue, "Familial hypobetalipoproteinemia: genetics and metabolism," *Cellular and Molecular Life Sciences,* vol. 62, *(12),* pp. 1372-1378, 2005.

[90] R. R. Sankatsing *et al*, "Hepatic and cardiovascular consequences of familial hypobetalipoproteinemia," *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 25, *(9),* pp. 1979-1984, 2005.

[91] J. J. P. Kastelein *et al*, "Potent reduction of apolipoprotein B and low-density lipoprotein cholesterol by short-term administration of an antisense inhibitor of apolipoprotein B," *Circulation,* vol. 114, *(16),* pp. 1729-1735, 2006.

[92] R. Nandakumar *et al*, "Effects of mipomersen, an apolipoprotein B100 antisense, on lipoprotein (a) metabolism in healthy subjects," *Journal of Lipid Research,* vol. 59, *(12),* pp. 2397-2402, 2018.

[93] M. M. Hussain *et al*, "Multiple functions of microsomal triglyceride transfer protein," *Nutrition & Metabolism,* vol. 9, *(1),* pp. 14-14, 2012.

[94] A.D. Marais, J. C. Firth and D. J. Blom, “Homozygous familial hypercholesterolemia and its management,” *Seminars in Vascular Medicine*, 4, (1), 43–50, 2004

[95] D. J. Rader and J. J. P. Kastelein, "Lomitapide and Mipomersen Two First-in-Class Drugs for Reducing Low-Density Lipoprotein Cholesterol in Patients with Homozygous Familial Hypercholesterolemia," *Circulation,* vol. 129, *(9),* pp. 1022-1032, 2014.

[96] S. L. Hofmann *et al*, "Overexpression of human low-density lipoprotein receptors leads to accelerated catabolism of Lp(a) lipoprotein in transgenic mice," *The Journal of Clinical Investigation,* vol. 85, *(5),* pp. 1542-1547, 1990.

[97] K. M. Argraves *et al*, "The atherogenic lipoprotein Lp(a) is internalized and degraded in a process mediated by the VLDL receptor," *The Journal of Clinical Investigation,* vol. 100, *(9),* pp. 2170-2181, 1997.

[98] X. Yang *et al*, "Scavenger receptor-BI is a receptor for lipoprotein(a)," *Journal of Lipid Research,* vol. 54, *(9),* pp. 2450-2457, 2013.

[99] M. Sharma *et al*, "Recycling of Apolipoprotein(a) After PlgRKT-Mediated Endocytosis of Lipoprotein(a)," *Circulation Research,* vol. 120, *(7),* pp. 1091-1102, 2017.

[100] G. M. Kostner *et al*, "HMG CoA reductase inhibitors lower LDL cholesterol without reducing Lp(a) levels," *Circulation,* vol. 80, *(5),* pp. 1313-1319, 1989.

[101] N. G. Seidah *et al*, "The Secretory Proprotein Convertase Neural Apoptosis-Regulated Convertase 1 (NARC-1): Liver Regeneration and Neuronal Differentiation," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 100, *(3),* pp. 928-933, 2003.

[102] D. F. Steiner *et al*, "Insulin Biosynthesis: Evidence for a Precursor," *Science,* vol. 157, *(3789),* pp. 697-700, 1967.

[103] N. G. Seidah *et al*, "PCSK9: A Key Modulator of Cardiovascular Health," *Circulation Research,* vol. 114, *(6),* pp. 1022-1036, 2014.

[104] N. G. Seidah and A. Prat, "The biology and therapeutic targeting of the proprotein convertases," *Nature Reviews. Drug Discovery,* vol. 11, *(5),* pp. 367-383, 2012.

[105] P. Moulin *et al*, "Mutations in PCSK9 cause autosomal dominant hypercholesterolemia," *Nature Genetics,* vol. 34, *(2),* pp. 154-156, 2003.

[106] T. F. Osborne *et al*, "Operator constitutive mutation of 3-hydroxy-3-methylglutaryl coenzyme A reductase promoter abolishes protein binding to sterol regulatory element," *Journal of Biological Chemistry,* vol. 263, *(7),* pp. 3380, 1988.

[107] T. Kita *et al*, "Delayed Clearance of Very Low Density and Intermediate Density Lipoproteins with Enhanced Conversion to Low Density Lipoprotein in WHHL Rabbits," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 79, *(18),* pp. 5693-5697, 1982.

[108] M. D. Shapiro, H. Tavori and S. Fazio, "PCSK9: From Basic Science Discoveries to Clinical Trials," *Circulation Research,* vol. 122, *(10),* pp. 1420-1438, 2018.

[109] K. N. Maxwell *et al*, "Novel putative SREBP and LXR target genes identified by microarray analysis in liver of cholesterol-fed mice," *Journal of Lipid Research,* vol. 44, *(11),* pp. 2109-2119, 2003.

[110] S. Huang *et al*, "Mechanism of LDL binding and release probed by structure-based mutagenesis of the LDL receptor," *Journal of Lipid Research,* vol. 51, *(2),* pp. 297-308, 2010.

[111] D. Zhang *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," *Journal of Biological Chemistry*, vol. 282, *(25),* pp. 18602-18612, 2007.

[112] N. Nassoury *et al*, "The cellular trafficking of the secretory proprotein convertase PCSK9 and its dependence on the LDLR," *Traffic (Copenhagen, Denmark),* vol. 8, *(6),* pp. 718-732, 2007.

[113] M. N. Mansour *et al*, "Structural and biophysical studies of PCSK9 and its mutants linked to familial hypercholesterolemia," *Nature Structural & Molecular Biology,* vol. 14, *(5),* pp. 413-419, 2007.

[114] H. Sun *et al*, "Proprotein Convertase Subtilisin/Kexin Type 9 Interacts with Apolipoprotein B and Prevents Its Intracellular Degradation, Irrespective of the Low-Density Lipoprotein Receptor," *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 32, *(7),* pp. 1585-1595, 2012.

[115] J. C. Cohen *et al*, "Sequence Variations in PCSK9, Low LDL, and Protection against Coronary Heart Disease," *The New England Journal of Medicine,* vol. 354, *(12),* pp. 1264-1272, 2006.

[116] M. S. Sabatine *et al*, "Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease," *The New England Journal of Medicine,* vol. 376, *(18),* pp. 1713-1722, 2017.

[117] Raal, Frederick J., MB BCh, MMed, PhD *et al*, "Reduction in Lipoprotein(a) With PCSK9 Monoclonal Antibody Evolocumab (AMG 145)," *JACC (Journal of the American College of Cardiology),* vol. 63, *(13),* pp. 1278-1288, 2014.

[118] D. J. Rader *et al*, "The low-density lipoprotein receptor is not required for normal catabolism of Lp(a) in humans," *The Journal of Clinical Investigation,* vol. 95, *(3),* pp. 1403-1408, 1995.

[119] A. V. Khera *et al*, "Lipoprotein(a) Concentrations, Rosuvastatin Therapy, and Residual Vascular Risk: An Analysis From the JUPITER Trial (Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin)," *Circulation,* vol. 129, *(6),* pp. 635-642, 2014.

[120] T. Kosenko *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," *The Journal of Biological Chemistry*, vol. 288, *(12),* pp. 8279-8288, 2013.

[121] N. R. Desai *et al*, "AMG145, a Monoclonal Antibody Against Proprotein Convertase Subtilisin Kexin Type 9, Significantly Reduces Lipoprotein(a) in Hypercholesterolemic Patients Receiving Statin Therapy: An Analysis From the LDL-C Assessment With Proprotein Convertase Subtilisin Kexin Type 9 Monoclonal Antibody Inhibition Combined With Statin Therapy (LAPLACE)–Thrombolysis in Myocardial Infarction (TIMI) 57 Trial," *Circulation,* vol. 128, *(9),* pp. 962-969, 2013.

[122] E. F. Villard *et al*, "PCSK9 Modulates the Secretion But Not the Cellular Uptake of Lipoprotein(a) Ex Vivo: An Effect Blunted by Alirocumab," *JACC. Basic to Translational Science,* vol. 1, *(6),* pp. 419, 2016.

[123] J. R. Wetterau and D. B. Zilversmit, "A triglyceride and cholesteryl ester transfer protein associated with liver microsomes," *Journal of Biological Chemistry,* vol. 259, *(17),* pp. 10863, 1984.

[124] J. R. Wettereau and D. B. Zilversmit, "Purification and characterization of microsomal triglyceride and cholesteryl ester transfer protein from bovine liver microsomes," *Chemistry and Physics of Lipids,* vol. 38, *(1),* pp. 205-222, 1985.

[125] J. R. Wetterau *et al*, "Protein disulfide isomerase is a component of the microsomal triglyceride transfer protein complex," *Journal of Biological Chemistry,* vol. 265, *(17),* pp. 9801, 1990.

[126] L. Wang, D. G. Fast and A. D. Attie, "The Enzymatic and Non-enzymatic Roles of Protein-disulfide Isomerase in Apolipoprotein B Secretion," *Journal of Biological Chemistry,* vol. 272, *(44),* pp. 27644-27651, 1997.

[127] J. R. Wetterau *et al*, "Protein disulfide isomerase appears necessary to maintain the catalytically active structure of the microsomal triglyceride transfer protein," *Biochemistry,* vol. 30, *(40),* pp. 9728-9735, 1991.

[128] D. A. Gordon *et al*, "Inhibition of the Microsomal Triglyceride Transfer Protein Blocks the First Step of Apolipoprotein B Lipoprotein Assembly but Not the Addition of Bulk Core Lipids in the Second Step," *Journal of Biological Chemistry,* vol. 271, *(51),* pp. 33047-33053, 1996.

[129] J. P. Segrest *et al*, "Structure of apolipoprotein B-100 in low density lipoproteins," *Journal of Lipid Research,* vol. 42, *(9),* pp. 1346, 2001.

[130] S. Liang *et al*, "The amino-terminal domain of apolipoprotein B does not undergo retrograde translocation from the endoplasmic reticulum to the cytosol. Proteasomal degradation of nascent apolipoprotein B begins at the carboxyl terminus of the protein, while apolipoprotein B is still in its original translocon," *The Journal of Biological Chemistry,* vol. 275, *(41),* pp. 32003-32010, 2000.

[131] M. Rizzo M and A. S. Wierzbicki, “New lipid modulating drugs: the role of microsomal transport protein inhibitors,” *Current Pharmaceutical Design,* vol. 17, (*9*), pp. 943-949, 2011.

[132] M. Cuchel *et al*, "Inhibition of Microsomal Triglyceride Transfer Protein in Familial Hypercholesterolemia," *The New England Journal of Medicine*, vol. 356, *(2)*, pp. 148-156, 2007.

[133] T. Zeller, S. Blankenberg and P. Diemert, "Genomewide association studies in cardiovascular disease--an update 2011," *Clinical Chemistry*, vol. 58, *(1)*, pp. 92-103, 2012.

[134] K. V. Sachs *et al*, "From noncoding variant to phenotype via SORT1 at the 1p13 cholesterol locus," *Nature*, vol. 466, *(7307)*, pp. 714-719, 2010.

[135] C. M. Petersen *et al*, "Molecular Identification of a Novel Candidate Sorting Receptor Purified from Human Brain by Receptor-associated Protein Affinity Chromatography," *Journal of Biological Chemistry*, vol. 272, *(6)*, pp. 3599-3605, 1997.

[136] M. K. Grøftehauge *et al*, "Ligands bind to Sortilin in the tunnel of a ten-bladed β-propeller domain," *Nature Structural & Molecular Biology*, vol. 16, *(1)*, pp. 96-98, 2009.

[137] D. Kasper *et al*, "The sortilin cytoplasmic tail conveys Golgi-endosome transport and binds the VHS domain of the GGA2 sorting protein," *The EMBO Journal*, vol. 20, *(9)*, pp. 2180-2190, 2001.

[138] L. T. Pallesen and C. B. Vaegter, "Sortilin and SorLA Regulate Neuronal Sorting of Trophic and Dementia-Linked Proteins," *Molecular Neurobiology*, vol. 45, *(2)*, pp. 379-387, 2012.

[139] C. M. Petersen *et al*, "Propeptide cleavage conditions sortilin/neurotensin receptor-3 for ligand binding," *The EMBO Journal*, vol. 18, *(3)*, pp. 595-604, 1999.

[140] G. Hermey *et al*, "Tumour necrosis factor alpha-converting enzyme mediates ectodomain shedding of Vps10p-domain receptor family members," *The Biochemical Journal*, vol. 395, *(2)*, pp. 285-293, 2006.

[141] S. F. Evans *et al*, "Neuronal brain-derived neurotrophic factor is synthesized in excess, with levels regulated by sortilin-mediated trafficking and lysosomal degradation," *The Journal of Biological Chemistry*, vol. 286, *(34)*, pp. 29556-29567, 2011.

[142] M. N. J. Seaman, "Identification of a novel conserved sorting motif required for retromer-mediated endosome-to-TGN retrieval," *Journal of Cell Science*, vol. 120, *(Pt 14)*, pp. 2378-2389, 2007.

[143] R. Tanimoto *et al*, "The perlecan-interacting growth factor progranulin regulates ubiquitination, sorting, and lysosomal degradation of sortilin," *Matrix Biology*, vol. 64, pp. 27-39, 2017.

[144] M. Yang *et al*, "Precursor of brain-derived neurotrophic factor (proBDNF) forms a complex with Huntingtin-associated protein-1 (HAP1) and sortilin that modulates proBDNF trafficking, degradation, and processing," *The Journal of Biological Chemistry*, vol. 286, *(18)*, pp. 16272-16284, 2011.

[145] C. M. Wilson *et al*, "Sortilin mediates the release and transfer of exosomes in concert with two tyrosine kinase receptors," *Journal of Cell Science,* vol. 127, *(Pt 18),* pp. 3983-3997, 2014.

[146] C. Reitz *et al*, "SORCS1 alters amyloid precursor protein processing and variants may increase Alzheimer's disease risk," *Annals of Neurology,* vol. 69, *(1),* pp. 47-64, 2011.

[147] I. Hermans-Borgmeyer *et al*, "Expression of the 100-kDa neurotensin receptor sortilin during mouse embryonal development," *Molecular Brain Research,* vol. 65, *(2),* pp. 216-219, 1999.

[148] S. Boggild *et al*, "Spatiotemporal patterns of sortilin and SorCS2 localization during organ development," *BMC Cell Biology,* vol. 17, *(8),* pp. 8, 2016.

[149] J. Shi and K. V. Kandror, "The Luminal Vps10p Domain of Sortilin Plays the Predominant Role in Targeting to Insulin-responsive Glut4-containing Vesicles," *Journal of Biological Chemistry,* vol. 282, *(12),* pp. 9008-9016, 2007.

[150] H. Talbot *et al*, "Regulatory Roles of Sortilin and SorLA in Immune-Related Processes," *Frontiers in Pharmacology,* vol. 9, 2019.

[151] D. Ai *et al*, "Activation of ER stress and mTORC1 suppresses hepatic sortilin-1 levels in obese mice," *The Journal of Clinical Investigation,* vol. 122, *(5),* pp. 1677-1687, 2012.

[152] J. Amengual *et al*, "Autophagy Is Required for Sortilin-Mediated Degradation of Apolipoprotein B100," *Circulation Research,* vol. 122, *(4),* pp. 568-582, 2018.

[153] A. Strong *et al*, "Hepatic sortilin regulates both apolipoprotein B secretion and LDL catabolism," *The Journal of Clinical Investigation,* vol. 122, *(8),* pp. 2807-2816, 2012.

[154] C. E. Sparks *et al*, "Phosphatidylinositol (3,4,5)-trisphosphate binds to sortilin and competes with neurotensin: Implications for very low-density lipoprotein binding," *Biochemical and Biophysical Research Communications,* vol. 479, *(3),* pp. 551-556, 2016.

[155] M. Kjolby *et al*, "Sort1, Encoded by the Cardiovascular Risk Locus 1p13.3, Is a Regulator of Hepatic Lipoprotein Export," *Cell Metabolism,* vol. 12, *(3),* pp. 213-223, 2010.

[156] P. Linsel-Nitschke *et al*, "Genetic variation at chromosome 1p13.3 affects sortilin mRNA expression, cellular LDL-uptake and serum LDL levels which translates to the risk of coronary artery disease," *Atherosclerosis,* vol. 208, *(1),* pp. 183-189, 2009;2010.

[157] D. M. Conlon, "Role of sortilin in lipid metabolism," *Current Opinion in Lipidology,* vol. 30, *(3),* pp. 198-204, 2019.

[158] S. M. Zekavat *et al*, "Deep coverage whole genome sequences and plasma lipoprotein(a) in individuals of European and African ancestries," *Nature Communications,* vol. 9, *(1),* pp. 2606-14, 2018.

[159] B. R. Gabel and M. L. Koschinsky, "Analysis of the proteolytic activity of a recombinant form of apolipoprotein(a)," *Biochemistry,* vol. 34, *(48),* pp. 15777-15784, 1995.

[160] R. Romagnuolo *et al*, "Lipoprotein(a) catabolism is regulated by proprotein convertase subtilisin/kexin type 9 through the low-density lipoprotein receptor," *The Journal of Biological Chemistry,* vol. 290, (*18*), 11649-11662, 2015.

[161] A. M. Scanu and C. Edelstein, "Learning about the structure and biology of human lipoprotein [a] through dissection by enzymes of the elastase family: facts and speculations," *Journal of Lipid Research,* vol. 38, *(11),* pp. 2193, 1997.

[162] U. B. Westergaard *et al*, "Functional Organization of the Sortilin Vps10p Domain," *Journal of Biological Chemistry,* vol. 279, *(48),* pp. 50221-50229, 2004.

[163] A. M. Scanu and C. Edelstein, "Learning about the structure and biology of human lipoprotein [a] through dissection by enzymes of the elastase family: facts and speculations," *Journal of Lipid Research,* vol. 38, *(11),* pp. 2193, 1997.

[164] F. Hu *et al*, "Sortilin-Mediated Endocytosis Determines Levels of the Frontotemporal Dementia Protein, Progranulin," *Neuron,* vol. 68, *(4),* pp. 654-667, 2010.

[165] C. Gustafsen *et al*, "The Hypercholesterolemia-Risk Gene SORT1 Facilitates PCSK9 Secretion," *Cell Metabolism,* vol. 19, *(2),* pp. 310-318, 2014.

[166] G. Chiesa *et al*, "Reconstitution of lipoprotein(a) by infusion of human low-density lipoprotein into transgenic mice expressing human apolipoprotein(a)," *Journal of Biological Chemistry,* vol. 267, *(34),* pp. 24369-24374, 1992.

[167] H. Jamil *et al*, "An Inhibitor of the Microsomal Triglyceride Transfer Protein Inhibits apoB Secretion from HepG2 Cells," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 93, *(21),* pp. 11991-11995, 1996.

[168] M. L. Koschinsky *et al*, "Apolipoprotein(a): Expression and characterization of a recombinant form of the protein in mammalian cells," *Biochemistry,* vol. 30, *(20),* pp. 5044-5051, 1991.

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