The Effect of Single Nucleotide Polymorphisms in Apolipoprotein(a) KIV Types 6-9 on Lipoprotein(a) Particle Assembly and Apolipoprotein(a) Secretion In Vitro

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The Effect of Single Nucleotide Polymorphisms in Apolipoprotein(a) KIV Types 6-9 on Lipoprotein(a) Particle Assembly and Apolipoprotein(a) Secretion In Vitro

By

Sera Sayegh

A Thesis
Submitted to the Faculty of Graduate Studies through the Department of Chemistry & Biochemistry in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada

2018

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The Effect of Single Nucleotide Polymorphisms in Apolipoprotein(a) KIV Types 6-9 on Lipoprotein(a) Particle Assembly and Apolipoprotein(a) Secretion In Vitro

by

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May 7, 2018
DECLARATION OF CO-AUTHORSHIP

I hereby declare that this thesis incorporates material that is a result of joint research, as follows:

This thesis incorporates the outcome of joint research in collaboration with Dr. Santica Marcovina, Dr. Corey Scipione, Dr. James Gauld, and Master’s candidate Travis Dewolfe under the co-supervision of Dr. Marlys Koschinsky and Dr. Michael Boffa. The majority of key ideas, experimental contributions, data analysis, and written examinations were performed by the author. 17K apo(a) pRK5 plasmid with mutated SmaI sites used to generate mutant apo(a) variants were generated and provided by Dr. Corey Scipione.

Contribution from Dr. Santica Marcovina was through providing purified anti-apo(a) antibody.

Recombinant-apo(a) stable HEK293 cell lines were provided by Dr. Marlys Koschinsky.

Computational chemistry and molecular dynamic simulations were performed by Dr. James Gauld, Associate Professor at the University of Windsor, and his Master’s student, Travis Dewolfe.

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ABSTRACT

Elevated plasma levels of lipoprotein(a) (Lp(a)) is considered to be the strongest genetic risk factor for cardiovascular diseases (CVDs). Lp(a) is similar in composition to low density lipoprotein (LDL), but is distinguishable by its unique polymorphic glycoprotein, apolipoprotein(a) (apo(a)). Lp(a) plasma levels are primarily determined by allelic variations in the gene that encodes apo(a). Rate of apo(a) secretion from hepatocytes and the efficiency of Lp(a) assembly influences plasma levels. Lp(a) is assembled by a non-covalent interaction between its two components, that precedes formation of a single disulfide bond between apo(a) and apolipoproteinB-100 (apoB-100) of the LDL moiety. Single nucleotide polymorphisms (SNPs) identified in the apo(a) domains that are implicated in this two-step assembly process have been associated with plasma Lp(a) levels. G17R, P52L, S37F, and T23P, have been associated with decreased Lp(a) levels, whereas R18W has been associated with increased levels. The work in this study uses site-directed mutagenesis to introduce the aforementioned SNPs into a physiologically relevant 17K apo(a) isoform to establish potential causation for the observed associative Lp(a) levels followed by in vitro assays of Lp(a) formation. Pulse-chase experiments in transiently transfected human hepatoma cells (HepG2) with mutants and wildtype (WT) 17K apo(a) was used to study the secretion of apo(a) variants and a recombinant Lp(a) assay with purified components was used to study the assembly of Lp(a). Computational chemistry was used to determine conformational changes introduced by the SNPs in their respective apo(a) domains. Interpretation of the presented data suggests that R18W enhances the rate of covalent Lp(a) assembly and P52L decreases efficiency of covalent Lp(a) assembly. S37F, G17R, and T23P, did not present data that correlated to their effects on human Lp(a) plasma concentration.
DEDICATION

I dedicate this work to all of my family and friends for their endless love and support
ACKNOWLEDGEMENTS

First and foremost, I would like to sincerely thank Dr. Marlys Koschinsky for this incredible opportunity by accepting me into her laboratory at the University of Windsor. I am truly grateful for her endless amount of patience, guidance, and knowledge. I would also like to extend a sincere thank you to Dr. Michael Boffa for all of his generous advice, support, and knowledge. This experience and thesis would not have been possible without Dr. Marlys Koschinsky and Dr. Michael Boffa.

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<th>Abbreviations</th>
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<tbody>
<tr>
<td>α5</td>
<td>Anti-apo(a) antibody</td>
</tr>
<tr>
<td>apo(a)</td>
<td>Apolipoprotein(a)</td>
</tr>
<tr>
<td>apoB-100</td>
<td>ApolipoproteinB 100</td>
</tr>
<tr>
<td>apoB-n</td>
<td>Truncated apolipoproteinB-100 where n = percentage</td>
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<tr>
<td>ASO</td>
<td>Antisense oligonucleotide</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BiP</td>
<td>Immunoglobulin heavy chain binding protein</td>
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<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CEBP</td>
<td>CCAAT/enhancer binding proteins</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
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<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CRF</td>
<td>Chronic renal failure</td>
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<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>CXN</td>
<td>Calnexin</td>
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<tr>
<td>DH</td>
<td>DNase hypersensitivity sites</td>
</tr>
<tr>
<td>DHII</td>
<td>DNase II hypersensitivity sites</td>
</tr>
<tr>
<td>DHIII</td>
<td>DNase III hypersensitivity sites</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic reticulum associated degradation</td>
</tr>
<tr>
<td>ESRD</td>
<td>End stage renal disease</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCR</td>
<td>Fractional catabolic rate</td>
</tr>
<tr>
<td>FH</td>
<td>Familial hypercholesterolemia</td>
</tr>
<tr>
<td>FSR</td>
<td>Fractional synthetic rate</td>
</tr>
<tr>
<td>FXR</td>
<td>Farnesoid X Receptor</td>
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<tr>
<td>G17R</td>
<td>17K apo(a) G17R KIV₈</td>
</tr>
<tr>
<td>GA</td>
<td>Golgi apparatus</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney cell line</td>
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</table>
HepG2  Human hepatoma
HNF-1α  Hepatocyte nuclear factor 1 alpha
HNF-4α  Hepatocyte nuclear factor 4 alpha
ICAM-1  Intercellular adhesion molecule-1
IGF-I  Insulin-like growth factor-I
IL  Interleukin
IL-6  Interleukin 6
IL-8  Interleukin 8
KI  Plasminogen(-like) kringle I
KII  Plasminogen(-like) kringle II
KIII  Plasminogen(-like) kringle III
KIV  Plasminogen(-like) kringle IV
KIVn  Plasminogen(-like) kringle IV where n = subtypes (1-10)
KV  Plasminogen(-like) kringle V
LBS  Lysine binding site
LDL  Low density lipoprotein
LDLR  Low density lipoprotein receptor
LF-A1  Lymphocyte function-associated antigen 1
Lp(a)  Lipoprotein(a)
LPA  Lipoprotein(a) gene
LTGF-β  Latent transforming growth factor-beta
M-CSF  Macrophage colony stimulating factor
MD  Molecular dynamics
MEM  Minimum essential medium
MI  Myocardial infarction
MMP  Metallomatrix proteinase
MOE  Molecular Operating Environment
NaCl  Sodium chloride
NAMD  MD simulation software
NEB  New England Biolabs
NMR  Nuclear magnetic resonance
oxPL  Oxidized phospholipid
P52L  17K apo(a) P52L KIV₈
PAI-1  Plasminogen activator inhibitor-1
PBS  Phosphate buffered-saline
PCSK9  Proprotein convertase subtilin kinexin 9
PDB  Protein data bank
PDI  Protein disulfide isomerase
PEI  Polyethylenimine
PMSF  Phenylmethylsulfonyl fluoride
PNR  Pentanucleotide repeat; (TTTTA)n, where n=6-11
PR  Production rate
R18W  17K apo(a) R18W KIV9
RMSD  Root mean squared deviation
RT  Retention time
S37F  17K apo(a) S37F KIV6
SDS  Sodium dodecyl sulfate
SDS-PAGE  SDS-polyacrylamide gel electrophoresis
SLBS  Strong lysine binding site
SNP  single nucleotide polymorphism
T23P  17K apo(a) T23P KIV8
TF  Transcription factor
TGF  Transforming growth factor
THP-1  Human acute monocytic leukemia cell line
t-PA  Tissue plasminogen activator
UGGT  UDP-glucose:glycoprotein glucosyltransferase
UTR  Untranslated region
VCAM-1  Vascular cell adhesion molecule-1
VLDLR  Very low density lipoprotein receptor
VNTR  Variable number tandem repeat
VSMC  Vascular smooth muscle cell
WLBS  Weak lysine binding site
WT  Wild-type
CHAPTER 1

Introduction

1.1 Formation of Atherosclerotic Lesions

Cardiovascular disease (CVD) is the leading cause of mortality globally and presents itself in a majority of the cases as coronary artery disease (CAD) [1, 2]. This disease is a manifestation of the progressive, inflammatory process known as atherosclerosis [3]. Atherosclerosis is characterized by the narrowing and stiffening of the medium and large arteries as a result of plaque build-up in the arterial wall [4]. There are many risk factors associated with this underlying mechanism including metabolic, behavioural, genetic, psychological, socioeconomic status, and gender, illustrating its complexity [5]. Atherogenesis is localized in areas where flow pattern is disturbed as a result of curvature, bifurcations, and vessel branching [6, 7]. Endothelial dysfunction is considered an early marker of atherosclerosis as this initiates the atherosclerotic process and has been observed in both the coronary and peripheral vasculature [8, 9].

Endothelial dysfunction refers to an impairment of modulation of vascular homeostasis which includes the regulation of vascular growth, vascular permeability, and vascular remodeling [8]. The vascular endothelium is a monolayer of cells that acts as a barrier between the inner vessel wall, known as the intima, and the circulating blood that flows through the lumen of the vessel [10]. This monolayer of endothelial cells (ECs) is the major regulator in vascular homeostasis due to its ability to induce phenotypic changes in the vessel wall in response to chemical or biomechanical stimuli [8, 11]. These cells are bound together by tight junctions creating a permeability barrier, that regulates the entry of molecules, ions, and immune
cells into the vessel wall [12]. Morphological changes in response to chemical stimuli (ie. cytokines) or biomechanical stress (ie. shear stress), results in EC activation and the reorganization of the tubulin and actin cytoskeletons causing an increase in permeability into the intimal space [13]. This increase in permeability allows for the passive diffusion of lipoproteins (ie. low-density lipoproteins (LDL)) into the intima where they potentially undergo modification by oxidation or lipolysis, and in turn initiate a pro-inflammatory response [14].

Activated ECs secrete a variety of chemokines, pro-inflammatory cytokines (ie. interleukins (IL)), secretins, transforming growth factors (TGFs), and also increase the expression of pro-inflammatory cell adhesion molecules (ie. intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)) on the luminal side of the vessel that collectively recruit circulating immune cells to the growing atherosclerotic plaque [15, 16]. Circulating monocytes roll across the endothelial surface, a process mediated by selectins, and interact with adhesion molecules expressed on the ECs [16]. This interaction leads to the trans-endothelial migration of the monocytes into the intimal space where they differentiate into macrophages by stimulating factors (ie. macrophage colony stimulating factor (M-CSF)) secreted by activated ECs as well as vascular smooth muscle cells (VSMCs) [16, 17]. These macrophages react to the microenvironment of the intima and engulf the modified lipoproteins present via scavenger receptors, developing into lipid-filled macrophages referred to as foam cells in the atherosclerotic lesion [18, 19]. These foam cells aggregate together to form the atheromatous core of the lesion and continue to engulf modified lipoproteins until they necrotize or undergo apoptosis, developing the lipid-rich necrotic core [20]. Macrophage death further amplifies the inflammatory process by the release of pro-inflammatory elements that recruit additional monocytes to the atherosclerotic lesion and release metalloproteinases (MMPs) that
facilitate plaque destruction by extracellular matrix (ECM) degradation, destabilizing the necrotic core [21].

VSMCs are the major producers of ECM in both healthy and atherosclerotic vessels [22]. The pro-inflammatory microenvironment of the atherosclerotic lesion stimulates VSMC proliferation, migration, and production of proteoglycans and fibronectin in ECM deposition [22, 23]. This ECM allows for efficient entrapment of additional LDL that can quickly become oxidized, promoting lipid uptake by macrophages, and in turn foam cell formation [22]. The proliferating VSMCs in the intima release pro-inflammatory elements, ECM, and in combination with the fibrous elements present in the microenvironment of the atherosclerotic lesion, form a overlaying fibrous cap on the lipid-rich, necrotizing core [22]. A combination of MMPs, growing necrotic core, and a perpetual pro-inflammatory environment, weakens the fibrous cap and increases the risk of atheromatous rupture [24]. This releases pro-thrombotic constituents into the blood potentially leading to catastrophic coronary events such as myocardial infarction (MI) as a result of thrombotic occlusion [24].

Lipid-lowering therapy, such as statin therapy, has been used in prevention of catastrophic coronary events by hindering and reversing the progression of atherosclerosis associated with particular risk factors [25, 26]. Furthermore, changes in lifestyle, such as an increase in physical activity and an increase in antioxidant nutrition has also been shown to retard the progression of atherosclerosis and in turn has been a preventative measure in atherosclerotic CVDs [27, 28]. Despite various ways in which atherosclerosis can be prevented or its progression hindered, some risk factors associated with this mechanism are relatively resistant to the standardized lipid lowering strategies, such as elevated plasma levels of a genetically determined lipoprotein, lipoprotein(a) (Lp(a)) [29].
Figure 1.1: Schematic Progression of Atherosclerosis. Atherosclerosis is a progressive inflammatory process. Under normal conditions, endothelial cells (EC) serve as a barrier between the lumen and intimal space of the artery. Atherogenesis is first initiated by biochemical or biomechanical stimuli causing endothelial dysfunction resulting in an increase in permeability and an upregulation of monocyte adhesion receptors allowing for infiltration of lipids, including Lipoprotein(a) (Lp(a)), and monocyte migration into the intimal space. Due to stimuli present in the microenvironment of the vessel wall, monocytes differentiate into macrophages that engulf modified lipids, forming apoptotic foam cells that contribute to the necrotic core. Stimuli released by multiple activated cells in the growing atheroma, promote vascular smooth muscle cells (VSMC) proliferation and migration to the surface of the atheroma forming the fibrous cap. Matrix-degrading enzymes in combination with the growing atheroma, increases plaque susceptibility to rupture potentially leading to catastrophic thrombotic occlusion. ICAM1, intracellular adhesion molecule 1; VCAM1, vascular cell adhesion molecule 1; MCP1, monocyte chemoattractant protein 1; CCR2, chemokine receptor type 2; IL-6, interleukin 6; IL-1β, interleukin 1 beta, TNF-α, tumor necrosis factor alpha. Modified from reference [30].
1.2 Lipoprotein(a) and Apolipoprotein(a)

1.2.1 Structure of Lipoprotein(a) & Apolipoprotein(a)

Lp(a) was first discovered in 1963 by Kåre Berg and is structurally similar to LDL in that they both possess a lipid-rich core associated with an apolipoprotein B-100 (apoB-100) molecule [31, 32]. A unique glycoprotein, known as apolipoprotein(a) (apo(a)), covalently bound to the apoB-100 molecule of the LDL moiety distinguishes Lp(a) from LDL [33].

Preceding the discovery of apo(a), size heterogeneity of Lp(a) was observed in the population and it was later determined that this remarkable size heterogeneity is attributed to the apo(a) moiety [34]. cDNA sequence analysis revealed a high degree of homology between apo(a) and the human serine protease zymogen, plasminogen [35]. As such, apo(a) bears striking resemblance to plasminogen in its amino acid sequence and structure [36]. Both proteins possess a number of repeating tri-looped motifs, classified as kringles, that are stabilized by three intramolecular, invariant disulfide bonds [37]. Plasminogen possesses five distinct kringles followed by a catalytically-active serine protease domain [35]. Apo(a) possesses ten subtypes of plasminogen-like kringle IV (KIV1-10) domains based on amino acid sequence, each present in a single copy with the exception of KIV2, which may be present in as few as three copies to as many as 30 copies [38-40]. This variable number of tandem repeats (VNTR) of the KIV2 domains is responsible for the size heterogeneity observed within the population. Apo(a) also possesses a single plasminogen-like kringle V (KV) domain and an inactive plasminogen-like protease domain in the C-terminal end [36, 41]. In apo(a) the kringle domains are linked by sequences of 26-36 amino acids long that are rich in serine, threonine, and proline residues [42]. These linker regions are sites of O-linked glycosylation, while the KIV domains each possess N-
linked glycans that are added to arginine residues of the consensus sequence arginine-X-serine/threonine; where X can be any amino acid except proline [42-44].

Apo(a), like plasminogen, also possesses lysine binding sites (LBS) in some of its KIV domains. The LBS consists of anionic, cationic, and hydrophobic amino acid residues that form a cleft that can accommodate lysine and lysine-analogs [45]. Collectively, the LBS stabilizes the positive charges of the functional group of lysine, the negatively-charged carboxylic group, and the aliphatic backbone of lysine, respectively [45]. Weak lysine binding sites (WLBS) are found in each of the KIV5-8 and a strong lysine binding site (SLBS) is found in KIV10, which bears the greatest homology to the KIV domain of plasminogen [35, 46-48]. KIV9 possesses the single free cysteine residue in apo(a) involved in the covalent linkage with the apoB-100 molecule of the LDL moiety [49].
Figure 1.2: Structural Representation of the Lp(a) Particle. Lipoprotein(a) (Lp(a)) is composed of a low density lipoprotein-like (LDL) particle covalently bound to a unique glycoprotein, apolipoprotein(a) (apo(a)), by a single disulfide bond. Like LDL, the lipid component of Lp(a) possesses an associated apolipoproteinB-100 (apoB-100) protein, which is responsible for the disulfide bond between the two protein components of Lp(a), and is similar in composition to LDL in that it possesses a triglyceride (TG) and cholesterol ester (CE) core surrounded by a layer of phospholipids (PL) and free cholesterol (FC). Apo(a) bears striking resemblance to the human zymogen, plasminogen, in amino acid sequence and in the presence of repeating structural motifs known as kringles. Apo(a) possesses 10 distinguishable plasminogen like kringle IV (KIVn; n=1-10) domains, each present in a single copy with the exception of KIV2. The KIV domains are followed by a single plasminogen-like kringle V domain, and an inactive protease domain (P). The variable number of KIV2 domains gives rise to the size heterogeneity observed in the population. Adapted from reference [32].
Figure 1.3: Structural Representation of the Homology Between Apo(a) & Plasminogen.
Plasminogen possesses an N-terminal tail sequence, five distinct kringle domains classified as I through V (KI-V), and a serine protease domain. Apo(a) is composed of 10 plasminogen-like KIV domains, distinguishable from each other by amino acid sequence, followed by a single plasminogen-like KV domain, and an inactive protease domain. KIV\textsubscript{10} exhibits the greatest sequence identity to the KIV domain found in plasminogen and possesses a strong lysine binding site (LBS). The variable number of tandem repeats in the KIV\textsubscript{2} domain may be present in as few as 3 copies to as many as 30 copies and gives rise to the observable size heterogeneity of Lp(a) that exists in the population. KIV\textsubscript{5,8} each possess a weak LBS with KIV\textsubscript{7,8} playing a role in the noncovalent interaction that precedes covalent bond formation between apo(a) and apoB-100 in Lp(a). KIV\textsubscript{9} possesses the single free cysteine residue in apo(a) responsible for the covalent linkage with apoB-100. Adapted from reference [38].
1.2.2 LPA gene: Regulatory Elements & Evolution

*LPA*, the gene that encodes apo(a), is found on human chromosome 6q26-27 next to the gene that encodes plasminogen in a head-to-head orientation [50]. As previously mentioned, apo(a) bears extensive homology to the fibrinolytic proenzyme, plasminogen [35]. Apo(a) differs from plasminogen at the amino-terminal end where it lacks the sequences that code for the KI, KII, and KIII domains of plasminogen, but is similar in the carboxyl-terminal end that codes for KIV, KV, and the protease domain [35]. Moreover, the 5’ untranslated region (UTR), signal peptide regions, and 3’ UTR of apo(a) and plasminogen share almost complete sequence identity [35]. The Lp(a) size heterogeneity that exists within the population due to the variable number of KIV domains has been attributed to allelic differences in the number of tandem repeats of this sequence, and are therefore heritable and expressed co-dominantly [51]. The 3’ end of *LPA*, which codes for KIV\textsubscript{3-10}, KV, and the inactive protease domain, has determined to be invariable within the population [52].

*LPA* was reported to be present in Old World monkeys, humans, and hedgehogs [35, 53, 54]. In hedgehogs, the apo(a) glycoprotein, a product of parallel evolution, possesses variable tandemly-repeated plasminogen-like kringle III domains, with LBS retained in several of these domains, and lacks sequences corresponding to the KIV, KV, and protease domains [53]. Studies comparing the homology of the 3’-UTR between apo(a) and plasminogen predicts that the apo(a) gene, found in the aforementioned subset of primates, and the plasminogen gene arose as a result of gene duplication of a precursor gene that occurred approximately 40 million years ago [53]. However, when comparing the homology of the 5’-UTR between plasminogen and apo(a) genes, this predicts the divergence of these two genes to be approximately 7 million years ago, suggesting that gene conversion occurred after the initial duplication event [55]. It is proposed
that the human apo(a) gene further evolved as a combination of exon deletions that encode the preactivation peptide and KI-III, multiplication of exons that encode KIV, and single-base substitutions, such as the one present in the inactive plasminogen-like protease domain [55, 56]. In both evolutionary cases of apo(a), a seventh unpaired cysteine residue is found in only one of the kringle domains that covalently binds the apo(a) moiety to the apoB-100 molecule of the LDL particle [53, 54].

A pentanucleotide tandem repeat (PNR) polymorphism (TTTTA)$_n$ (n=6-11) located 1373 bp upstream of the signal sequence in $LPA$, was demonstrated to be in linkage disequilibrium with the VNTR and associated with Lp(a) levels. PNRs of 6-8 were associated with VNTRs of KIV$_2$ within 6-31 and PNRs of 9-11 were associated with a decrease in VNTRs of KIV$_2$ fewer than 24, with the most extreme case of 11 PNRs associated with a VNTRs of KIV$_2$ range of 9-14. Interestingly, the small isoforms sizes related to the PNRs of 9-11 demonstrate a decrease in plasma Lp(a) levels contrary to what is expected when the effect of the VNTRs of KIV$_2$ on Lp(a) plasma levels is considered alone; inverse relationship between apo(a) isoform size and plasma Lp(a) levels [56, 57].

Studies using deletion analysis revealed several regulatory regions within $LPA$. A region located -98 to +130 bp of the apo(a) transcription initiation site has been shown to possess enhancer activity by hepatocyte nuclear factor 1α (HNF-1α) binding in a trans-acting manner; a mutation that abolishes the binding of HNF-1α has been shown to decrease apo(a) promoter activity by approximately 90% [58]. Apo(a) is specifically synthesized and expressed in hepatocytes, suggesting the involvement of transcription factors (TFs) with tissue specific distribution, as is seen with HNF-1α enrichment in the liver. However, HNF-1α has been detected in the kidneys, intestines, and spleen, whereas apo(a) has not, suggesting the
involvement of additional regulatory factors associated with apo(a) expression [58, 59].

Furthermore, farnesoid X receptor (FXR), known to play a role in lipoprotein metabolism and activated by bile acids, has been shown to bind to a negative control region located at -826 bp in the LPA promoter and interfere with the hepatocyte nuclear factor 4α (HNF-4α)-mediated transcription [60]. The apo(a) gene also possesses non-specific regulatory elements in the promoter (ie. TATA and CAAT box), hepatocyte-specific regulatory elements (ie. CCAAT/enhancer binding proteins (CEBP)) and lymphocyte function-associated antigen 1 (LF-A1), that could potentially affect LPA gene expression [56]. DNase hypersensitivity (DH) sites are associated with open chromatin conformation and two enhancer DH sites (ie. DHII and DHIII) were found upstream of the apo(a) transcription active site in the intergenic sequence between apo(a) and plasminogen [61]. DH sites have been implicated in gene expression and variation, therefore may potentially serve as a regulatory sites for LPA gene expression [62].

Studies have shown that estrogen, tamoxifen, and insulin-like growth factor-I (IGF-I) exhibit apo(a) lowering affects at the mRNA level, potentially suggesting modulation by transcription or mRNA stability [63, 64]. Contrastingly, growth hormone (GH) has been shown to increase apo(a) mRNA levels [64].

1.2.3 Production, Maturation, and Secretion of Apolipoprotein(a)

As previously mentioned, apo(a) is primarily synthesized and secreted from hepatocytes involving a relatively complex pre-secretory biosynthetic pathway that is yet to be fully determined [59]. There is evidence to suggest that the intracellular movement of apo(a) and post-translational modification efficiency influences the plasma levels of Lp(a) since secretion of this protein from hepatocytes determines plasma Lp(a) levels [65]. Researchers found that apo(a) was first synthesized as a lower molecular weight precursor that matured into a protein of greater
mass [66]. This immature variant of apo(a) represents a hypoglycosylated immature form, whereas the mature form of apo(a) is a heavily glycosylated protein whereby approximately 28% of apo(a)’s final mass is attributable to these added sugar derivatives [67, 68].

Proper completion of post-translational modifications of apo(a) is a requirement for the mature, secretable form of the glycoprotein from hepatocytes [69-71]. Glycosylation, the addition of sugar derivatives to proteins, has been shown to play a role in folding, degradation, and more importantly, to influence the pre-secretory biosynthetic pathway of apo(a) [66, 71-74]. Apo(a) derived from conditioned media and plasma possess O-linked glycans, typically found in the linker regions of apo(a) that are rich in threonine and serine amino acid residues, and N-linked glycans, typically found in at least one site in each KIV domain of the protein [42, 67]. N-linked glycans are important for maintaining protein solubility and providing substrate recognition sites for chaperones that facilitate protein folding in the endoplasmic reticulum (ER), prior to transport to the Golgi apparatus (GA) where further modifications occur [72]. In a previous study, when N-linked glycosylation was inhibited, hypoglycosylated apo(a) aggregated in the ER, thus decreasing the efficiency by which chaperones interacted with apo(a) to facilitate proper folding [73, 75].

Apo(a) has been found to interact with several chaperone proteins that influence apo(a)’s progression through the pre-secretory biosynthetic pathway. Immunoglobulin heavy chain binding protein (BiP), protein disulfide isomerase (PDI), and calnexin (CXN) have all been shown to interact with apo(a) [71, 73, 74]. PDI is a chaperone protein of the ER that catalyzes disulfide bond formation and the rearrangement of incorrect disulfide bonds to aid protein folding [74, 76]. As such, PDI plays a role in the correct folding of apo(a) kringle domains, which each contain three invariant disulfide bonds [74, 76]. Chaperone proteins are generally
considered to be ER quality control regulators. Chaperone proteins have been demonstrated to interact with proteins in the ER, retaining misfolded proteins in this compartment targeting them for continual folding or the endoplasmic reticulum associated degradation (ERAD) pathway [77]. N-linked glycans mediate the interactions between apo(a) and CNX, which recognizes mono-glucosylated N-linked side chains, regulating proper protein folding together with glucose-trimming enzymes and UDP-glucose:glycoprotein glucosyltransferase (UGGT) in a deglycosylation/reglycosylation cycle [74, 78]. Once properly folded, N-linked carbohydrates are processed correctly, apo(a) is shuttled to the GA where it may be processed further by the addition of O-linked glycans to generate a mature form of the protein and destined for secretion by mechanisms that have not yet been identified [67, 79].

In summary, apo(a) is co-translationally synthesized in the ER and begins folding with the aid of several chaperone proteins [71, 73]. Misfolded apo(a) is retained in the ER where it is targeted for ERAD while properly folded and processed apo(a) is destined for further maturation in the secretory pathway [73]. Larger isoform sizes have been found to have greater ER retention times, and therefore lower secretion rates, when compared to smaller isoform sizes, likely due to the additional time required for ER processing [65, 80]. This increase in ER retention time of larger isoforms also increases their susceptibility to ERAD [73]. This demonstrates the complexity of the movement of apo(a) in the pre-secretory biosynthetic pathway and underlies, at least in part, the inverse correlation between apo(a) isoform size and plasma Lp(a) levels that is observed in the population.

1.2.4 Lipoprotein(a) Assembly

It has been shown that Lp(a) assembly is a two-step mechanism that involves a noncovalent interaction between apo(a) and the apoB-100 moiety of the LDL particle that
precedes a single disulfide bond formation [81]. As previously mentioned, KIV5-8 each contain a WLBS; the WLBS in KIV7 and KIV8 have been directly shown to mediate a lysine-dependent noncovalent interaction with lysine residues 690 and 680 in the N-terminal domain of apoB-100 moiety, respectively [47, 82]. The efficiency of the subsequent covalent bond formation is dictated by the affinity of the noncovalent interaction as well as the conformational state of apo(a) [47, 81, 83-85]. *In vitro* studies suggests that the noncovalent interaction between apo(a) and apoB-100 aligns the two moieties such that efficient disulfide bond formation can occur [82].

The aforementioned conformational state of apo(a) pertains to the “closed” conformation that apo(a) adopts with variants containing greater than 1 copy of the KIV2 domain. *In vitro* studies strongly suggest that this “closed” conformation involves the SLBS in KIV10 participating in intramolecular interactions with Amino-terminal sequences of apo(a) in a lysine-independent manner; however an “opened” apo(a) conformation may be achieved by the use of lysine analogues and is characterized by a change in tryptophan fluorescence, increase radius of gyration, alteration in domain stability, and enhanced covalent Lp(a) assembly [84, 86, 87]. Furthermore, the number of KIV2 repeats in these studies has been demonstrated to regulate the extent of inhibition of Lp(a) assembly with which this “closed” conformation institutes possibly by steric hindrance and restricting access of apoB-100 of LDL to the participating cysteine residue in apo(a) KIV9 [84, 86].

It is clear that the free cysteine residue in apo(a) involved in the covalent linkage to apoB-100 is Cys4057 that resides in the KIV9 domain [47, 49, 88]. However, the cysteine residue in apoB-100 that is involved in the covalent linkage with apo(a) that has not been clearly established. ApoB-100 contains 25 cysteine residues, 16 of which are involved in intramolecular
disulfide bonds leaving 9 potential cysteine residues that can participate in the disulfide bond formation with Lp(a) [89]. Of these potential 9 cysteine residues, several studies using different experimental approaches suggest that either Cys3734 or Cys4326 of apoB-100 is involved in this covalent interaction. Studies using experimental techniques where by free sulfhydryl groups are labeled by a fluorescent probe have demonstrated that Cys3734 is labelled in LDL particles but not in Lp(a) particles [90]. Similar results were obtained using immunochemical techniques where immunoreactivity of an antibody specific to the 3730-3746 region of apoB-100 was affected by the presence of apo(a) [91]. Furthermore, molecular modelling of uncomplexed cysteine residues in apoB-100 peptides were subjected to energy minimization and docking with KIV9; this approach demonstrated that the apoB-100 segment spanning 3732-3745 displayed the best fit and largest number of van der Waals contacts with the KIV9 domain of apo(a), thus further implicating apoB-100 Cys3734 in the disulfide bond formation with apo(a) [90].

Other studies have shown that Cys4326 of apoB-100 participates in the intermolecular covalent interaction with apo(a) in Lp(a) particles. In vivo and in vitro studies utilizing a truncated apoB-90 variant in transgenic mice, demonstrated that removal of the last 10% of the C-terminus of apoB-100, of which contains unpaired 4190 and 4326 cysteine residues, was unable to form a Lp(a) particle [92]. Human studies of hypobetalipoproteinemia, in which patients express C-terminally-truncated apoB species, is consistent with this finding as plasma Lp(a) particles of varying densities found in these patients are composed only of full-length apoB-100 [93-95]. In vitro Lp(a) assembly studies using rat hepatoma cells expressing a 6% C-terminal truncated apoB protein (apoB-94), also demonstrated that covalent assembly of Lp(a) was completely inhibited in this truncated version that lacks Cys4326 [96]. Interestingly, C-terminal truncations in apoB have been shown to affect the surface conformation of apoB with
altered lipid core content of the LDL particle suggesting that perhaps these truncations affect the ability of apoB to associate with apo(a). Interestingly, even the noncovalent interactions between apo(a) and the truncated apoB species have not been observed [96-98]. Site-directed mutagenesis experiments in transgenic mice, in which Cys3734 and Cys4326 were converted to serine residues, demonstrated that only Cys4326 was required for covalent Lp(a) assembly [99]. A Cys4326 to glycine substitution in transgenic mice yielded similar results in that apoB-100 completely lacked the capacity to bind apo(a) [100].

It was previously mentioned that the noncovalent interactions between apo(a) and apoB-100, as well as the conformational state of apo(a), can affect the efficiency of covalent assembly [82, 84, 85]. However, the rate of spontaneous covalent bond formation between the two moieties that compromise Lp(a), in combination with barely detectable uncomplexed apo(a) levels in plasma, suggests that this may be an enzyme-catalyzed reaction [84, 101]. In vitro studies in HepG2 cells have demonstrated the potential role of an extracellular enzyme with oxidase activity to catalyze the intermolecular covalent bond between apo(a) and apoB-100 [102]. Furthermore, kinetic data gathered from these studies suggests that the mechanism of catalysis is consistent with a ping-pong reaction [102].

The site of Lp(a) assembly has yet to be clearly established and has given rise to controversy in the field, as evidence in multiple systems and varying experimental conditions support a plasma membrane-associated, intracellular, and/or extracellular site of assembly. Studies in primary baboon hepatocytes have demonstrated that Lp(a) assembly occurs extracellularly on the cell surface [103]. It was proposed that in this system Lp(a) assembly occurs by secreted apo(a) binding back to the cell surface where apoB-containing species may then interact with the cell surface-associated apo(a), inducing a conformational change, and
releasing it as the intact Lp(a) particle [103]. Other studies in the same cell system as well as in human hepatoma (HepG2) cells and transgenic mice, have supported extracellular assembly through indirect methods [49, 66, 75, 104, 105].

Conversely, intracellular Lp(a) assembly has also been proposed by using different experimental techniques. The intracellular assembly model was first proposed due to the observation of apo(a)-apoB-100 complexes in the lysates of human primary hepatocytes [106]. In vitro studies in stably transfected human hepatoma cells expressing an apo(a) minigene that possesses the minimal critical domains of KIV required for association with apoB-100 (signal sequence, 6 non-identical KIV domains, the KV domain, and the protease domain; referred to as 6K), was used to study the intracellular assembly of Lp(a) [107]. Investigators found that incubation of a very effective anti-apo(a) antiserum did not prevent Lp(a) production or accumulation in conditioned medium [107]. In the same study, both immature and mature forms 6K apo(a) were detected complexed to apoB-100 in cell lysates [107]. Furthermore, in vitro studies in HepG2 cells using the previously described 6K and 17K apo(a) isoforms, demonstrated that secretion from hepatoma cells may be linked to elements of cellular triglyceride assembly and secretion due to the increase in apo(a) secretion in response to oleate treatment that was abrogated by the inhibition of acyl-CoA synthase or a microsomal triglyceride transfer protein inhibitor [108]. Lastly, a more recent in vivo study in humans demonstrated that the mean fractional synthetic rate (FSR), plasma retention time (RT), and production rate (PR) of apoB-100 of Lp(a) was similar to that of apo(a), but significantly differed from apoB-100 of LDL and that 92% of apoB-100 associated with Lp(a) is directly synthesized in the liver with the remaining 8% associated with plasma LDL, strongly suggesting intracellular assembly [109].
The possibility of multiple sites of Lp(a) assembly remains, although one may be more prevalently used in vivo.

1.2.5 Pathophysiology and Epidemiology of Lipoprotein(a)

The importance of understanding all of the mechanisms leading to elevated plasma levels of Lp(a) to determine effective ways in which levels can be reduced, reflects the fact that the LPA gene is considered the strongest genetic risk factor for CVDs and that the plasma levels of Lp(a) are largely determined by allelic variations at the LPA locus [110-112]. Furthermore, the fact that plasma levels of Lp(a) are present a few months after birth and remain relatively constant throughout an individual’s lifetime, further underscores the importance of developing targeted therapies for reducing plasma Lp(a) levels since the possibility of it contributing to CVD may occur early in life. A wide range of epidemiological, genome-wide association and Mendelian randomization studies, as well as large meta-analyses have identified Lp(a) as an independent, casual risk factor for CVD [113-120]. Meta-analyses demonstrated a 2-fold risk increase in elevated Lp(a) levels associated with small apo(a) isoform size and coronary heart disease (CHD) and ischemic stroke; no correlation was observed with non-vascular outcomes [118, 119]. This was further supported by a genome-wide association and Mendelian randomization studies whereby genetically-determined Lp(a) levels were causally associated with increased odds of valvular calcification, a process that precedes stenosis, and valvular stenosis in aortic-valve disease [115, 121]. More evidence supporting Lp(a) as a risk factor for CVD was observed in a general Danish population whereby researchers reported an increasing risk of myocardial infarction (MI) with increasing Lp(a) levels, with no evidence of a risk threshold as has been reported in early studies [117]. Although many of these association studies
have observed effects that increased Lp(a) plasma levels has on multiple CVD risk, this relationship is somewhat complicated by heterogeneity between different ethnic groups.

The increase in CVD risk associated with Lp(a) plasma levels at 25 mg/dl correlates with approximately 30% of Caucasians and 60-70% of African Americans who possess Lp(a) levels above this value [113, 119]. Elevated Lp(a) levels in Caucasians and African Americans demonstrated comparable association with CVD incidents. However, in the case of ischemic strokes, the African American population demonstrated a greater association between Lp(a) levels and incidents [122]. A comparative study in African American, Caucasian, and Hispanic populations demonstrated levels oxidized phospholipids (oxPL) associated with apoB-100 were consistent with genetically determined Lp(a) plasma levels and positively correlated with small apo(a) isoform size, suggesting a genetic predisposition elevated levels of oxPL associated with Lp(a) levels in these populations [123].

An earlier epidemiological study in African Blacks, Caucasian, Chinese, and Asian-Indians examined the frequency of Lp(a) plasma distribution and observed that levels are highly skewed to low Lp(a) distributions in Chinese and Caucasian populations and that Lp(a) in the African Blacks demonstrated a broader, Gaussian distribution of levels [37]. A study assessing the frequency of Lp(a) level distribution of 7 different ethnicities observed similar distributions and average Lp(a) values to the aforementioned ethnicities with a consistent trend showing that African Blacks and Asian-Indians exhibit a significantly different distribution when compared to that of the Chinese and Caucasian groups [124]. Furthermore, in several other population studies, the inverse relationship between Lp(a) plasma levels and apo(a) isoform size was demonstrated in all ethnicities under investigation, but varied in the impact of size polymorphism on Lp(a) concentration, expressed allele frequencies, and non-expressed allele frequencies [124, 125]. To
illustrate the extent of the impact with which the size polymorphism varies across ethnicities, the African Lp(a) levels demonstrated between 15-19% dependency on the size polymorphism, whereas, the Chinese populations demonstrated a 77% dependency [124-126]. Moreover, when comparing the same phenotypes observed in African populations and other ethnic groups, African populations exhibit several-fold elevated Lp(a) levels despite the presence of comparable isoform size [124-126]. Lastly, a study conducted between African and Caucasian populations investigating the sequences coding for KIV5-10, observed no common SNPs between the two populations. A R18W SNP found in KIV9 that has been associated with elevated Lp(a) levels, independent of the KIV2 number, has only been identified in African populations [127]. The interracial heterogeneity with respect to the relationship of Lp(a) plasma levels with apo(a) isoform size, apo(a) allele frequencies, and CHD risk, are important and strongly suggest a role of other sequence variations in the LPA gene that contribute to variation in plasma Lp(a) levels.

1.2.6 Postulated Proatherothrombotic Mechanisms of Action of Lipoprotein(a)

It is generally accepted that elevated Lp(a) level is an independent, causal risk factor in CVDs and in vitro and in vivo studies, collectively, have proposed mechanisms by which Lp(a) elicits its pathogenic effects. Lp(a) has been proposed to aid in the initiation and progression of atherosclerosis, as well as to contribute to thrombotic events. An autopsy study in humans quantified the Lp(a) deposited in atherosclerotic lesions and found that there was enrichment in the lesions, primarily associated with the ECM in the arterial wall [128, 129]. Moreover, Lp(a) was shown to co-localize with macrophages and VSMCs in human coronary atheromas [130]. Lp(a) has been shown to promote EC dysfunction and increases EC permeability by rearrangement of the actin cell cytoskeleton, and up-regulation of a vascular inflammatory regulator, prostaglandin E2 [131-133]. In vitro studies have demonstrated Lp(a)’s ability to
induce monocyte chemotactic activity in ECs and interleukin-8 (IL-8) induced expression in Tamm-Horsfall Protein 1 (THP-1) macrophages [134, 135]. Furthermore, in vitro studies using oxidized Lp(a) and comparing it to its native form, demonstrated that oxidized Lp(a) inhibits cellular growth, promotes differentiation, and increases monocyte adhesion in a human macrophage cell line [136]. An immunohistochemical study of xanthomas, an environment that has similar lipid composition and cell components comparable to early lesions in atherosclerosis, demonstrated that only native apoB-100 and apo(a) were found extracellularly while their oxidized LDL counterparts were detected in macrophages [137]. This suggests that oxidized Lp(a) and oxidized LDL may promote foam cell formation [137]. In vitro studies using VSMCs and ECs derived from bovine and human demonstrated Lp(a)’s ability to inhibit plasminogen activation, thereby preventing the activation of latent transforming growth factor-β (LTGF-β) and in turn promoting VSMC proliferation and migration [138, 139]. Importantly, Lp(a) has been demonstrated to bind to ECM proteins abundant in the atherosclerotic lesion such as fibrin, fibronectin, and glycosaminoglycans, suggesting a mechanism for the retention of Lp(a) in the intimal space [140-144].

As previously mentioned, the apo(a) component of the Lp(a) particle bears striking resemblance to the human zymogen, plasminogen, which when activated into plasmin, is the major fibrinolytic protease [35, 145]. Both ECs and platelets express plasminogen receptors and can accelerate the activation of plasminogen to plasmin, thereby promoting fibrinolysis or thrombolysis [145-149]. Part of Lp(a)’s pro-thrombotic properties are a result of the structural similarities between the apo(a) moiety and plasminogen. Consequently, apo(a) can compete with plasminogen for cell-surface binding sites as was demonstrated in in vitro studies using human EC and monocytic cell lines, thereby interfering with pericellular plasminogen activation [150-
Lp(a) can mediate upregulation of plasminogen activator inhibitor-1 (PAI-1) expression in human EC, thereby inhibiting plasmin generation by tissue-type plasminogen activator (t-PA) [153]. Moreover, Lp(a) has been shown to compete with plasminogen and t-PA binding to fibrin, competitively inhibiting plasminogen activation by t-PA, and attenuating clot lysis associated in an *in vitro* assay [143, 154-157]. Collectively, Lp(a) has been shown to disrupt hemostasis and promote thrombosis in *in vitro* systems by impeding the fibrinolytic potential of these systems.

With regard to Lp(a)’s pathogenicity, its role as an oxPL carrier has been of great interest due the implications, particularly the pro-inflammatory and the pro-atherogenic properties, that are associated with oxPLs. The presence of oxPL on Lp(a) in part, has been proposed to contribute to the pathogenicity of Lp(a). Lp(a) has been demonstrated to possess five oxPL species that have been demonstrated to associate with apo(a), at a site in the KIV₁₀ domain, as well as in the lipid phase of LDL [158, 159]. Furthermore, *in vitro* and *in vivo* studies have demonstrated that oxPLs bind preferentially to Lp(a); in transfer studies, oxidized LDL donates its oxPL to Lp(a) in a time and temperature dependent manner [160, 161]. This has been observed in both *in vitro* and in patients who have had post-percutaneous coronary intervention [160, 161]. Studies of angiographically-documented CAD provided evidence that the oxPL to apoB-100 ratio is equivalent to that of the oxPL in Lp(a) levels, suggesting that the oxPL of Lp(a) may to some extent mediate the atherogenic effects of this lipoprotein [162]. Interestingly, oxPL plasma levels were correlated with Lp(a) levels in humans and in transgenic mice, whereby transgenic mice expressing only human apo(a) or human apoB-100 alone exhibited low levels of oxPL [160, 163]. This suggests the necessity of an intact Lp(a) as a carrier for oxPL [160, 163]. Furthermore, patients with elevated levels of Lp(a) demonstrated an increase in the rate of progression of aortic stenosis owing to the corresponding increase of oxPL and its role in
promoting valve mineralization [161, 164]. Taken together, considerable evidence supporting the relationship between Lp(a) levels, associated oxPLs, and potential atherogenicity has accumulated.

Ways for which the oxPL on Lp(a) can mediate its pathogenic effects have been demonstrated experimentally in several systems, including human. The upregulation of IL-8 expression in human macrophage cell lines was determined to be attributable to the oxPL found on apo(a) since disruption of the SLBS in the KIV10 domain and separately, treatment with a phospholipase known to release the oxPLs associated with apo(a), demonstrated a substantial decrease in IL-8 expression by apo(a) [135]. Collectively, the co-localization of oxidized Lp(a) and macrophages in atheromas, stimulation of monocyte differentiation, and the avid uptake of oxidized Lp(a) by human monocyte-macrophages, thus foam cell formation, potentiates the risks posed by oxPL associated Lp(a) in the microenvironment of the atherosclerotic lesion [130, 136, 165, 166]. It is made evident that the ability of Lp(a) and oxPLs to facilitate both thrombosis and atherogenesis respectively, the two concomitantly pose a significant threat for the progression of these two linked disease states.
Figure 1.4: Postulated Mechanisms of Lp(a) Pathogenicity. Many case-studies, genetic studies, and meta-analyses have identified lipoprotein(a) (Lp(a)) as an independent and causal risk factor for cardiovascular diseases (CVD). Lp(a) possesses both pro-atherogenic and pro-thrombotic properties with both major components of the particle contributing to its pathophysiology, independently and in concert as the intact particle. The pro-atherogenic mechanisms in which Lp(a) may potentially play a role in are shown on the left side, whereas the pro-thrombotic mechanisms in which Lp(a) may potentially play a role in are shown on the right side. PL, phospholipid; SMC, smooth muscle cell; EC, endothelial cell; TFPI, tissue factor pathway inhibitor; PAI-1, plasminogen activator inhibitor-1. Adapted from reference [167].
Figure 1.5: Proposed Pathogenic Properties of Lp(a) Mediated by Apo(a). Both the lipid moiety as well as the apolipoprotein(a) (apo(a)) moiety contribute to the overall pathophysiology of lipoprotein(a) (Lp(a)). This image demonstrates the pathogenic properties associated with Lp(a), both pro-atherogenic and pro-thrombotic, mediated by the apo(a) moiety and specifies which domain is potentially responsible for the postulated mechanisms. DANCE, developmental arteries and neural crest epidermal growth factor (EGF)-like; SMC, smooth muscle cells; EC, endothelial cells; IL-8, interleukin 8. Adapted from reference [168].
1.2.7 Lipoprotein(a) Catabolism & Treatment

Lp(a) catabolism is yet another aspect of Lp(a) that is poorly understood. However, for the most part it is generally agreed that Lp(a) synthesis determines plasma protein levels rather than catabolism of the particle [65]. Several pieces of evidence have strongly implicated the role of the kidney in Lp(a) catabolism, which initially excited curiosity in 1987 when 71 patients with chronic renal failure (CRF) demonstrated a 3-fold increase in Lp(a) plasma concentration relative to controls [169-172]. This observation was consistent with other studies of end-stage renal disease (ESRD) and unrelated to the size polymorphism of apo(a) [169-172]. Intriguingly, after renal transplant surgery in patients with ESRD, their Lp(a) plasma levels decreased to levels typically associated with the isoform size polymorphism [171]. Furthermore, studies demonstrated a positive correlation between detected fragments of apo(a) in human plasma and relatively smaller fragments in human urine, suggesting that circulating apo(a) fragments are processed further in the kidneys prior to excretion [173-175]. It was thus proposed that the limiting step of excretion by the kidneys is the initial proteolytic fragmentation of apo(a) in circulation [173-175]. More supporting evidence of intrarenal Lp(a) catabolism was demonstrated with detection of a Lp(a) renal arteriovenous concentration gradient with significantly lower Lp(a) concentration in the renal vein compared to the ascending aorta [172, 176-178]. Additionally, immunohistochemical analysis showed localization of apo(a) in renal tissue [172, 176-178]. Observation of a decrease in the fractional catabolic rate (FCR) of Lp(a) in ESRD patients, and an increase in RT of plasma Lp(a) in ESRD patients also strongly suggested a role for the kidney in Lp(a) catabolism [172, 176-178].

Lp(a) catabolism in the liver was initially hypothesized due to an *in vitro* study in human fibroblasts that demonstrated an interaction between the LDL receptor (LDLR) and Lp(a) that
was further supported by observed elevated plasma Lp(a) levels in patients with familial hypercholesterolaemia (FH); a condition for which patients lack LDLRs [179-184]. An earlier in vitro study, suggested that smaller isoforms of Lp(a) were preferentially cleared by the LDLR, whereas larger isoforms were preferentially cleared by low-density lipoprotein receptor-related protein/α2-macroglobulin receptor (LRP) [185]. Additionally, it was determined that elevated Lp(a) level is an independent risk factor for CVD in patients with FH, with a higher risk in individuals that were LDLR-negative and Lp(a) levels greater than 50 mg/dL, and that elevated Lp(a) levels in these patients demonstrated a gene-dosage effect; homozygous FH patients had higher Lp(a) levels than heterozygous FH patients [186, 187]. In vivo studies in animals demonstrated that overexpression of LDLRs resulted in an increase in Lp(a) clearance and LDLR defective animals contrastingly demonstrated a decrease in Lp(a) clearance [188-190]. Conversely, in vivo studies in FH patients as well as in LDLR knockout transgenic mice model, demonstrated that LDLRs are not necessary for Lp(a) clearance since the associated FCRs did not differ significantly between LDLR defective species and controls [188, 191, 192]. Though the role of LDLR in Lp(a) catabolism has been arguable, other factors may be involved that could potentially explain the variation in extent of the role with which LDLR plays in this metabolic process such as in the case of the effect that proprotein convertase subtilisin/kexin type 9 (PCSK9) may have.

Studies investigating the role of PCSK9 on LDL catabolism have provided some insight into the catabolism of Lp(a) modulated by PCSK9, further implicating the role of the LDLR on Lp(a) degradation. By targeting the LDLR for degradation, PCSK9 serves as a hepatic endogenous regulator for this receptor and mutant forms have been implicated in FH [193-195]. Additionally, a decrease in Lp(a) plasma concentration was observed in normal patients and in
FH patients administered PCSK9 inhibitor alone or in combination with statin therapy, respectively [196-199]. *In vitro* studies suggested that this was at the level of catabolism due to Lp(a) uptake by the LDLR being modulated by PCSK9 in human hepatoma cells and primary fibroblasts [196-199].

Early *in vitro* studies in human hepatoma cells and human fibroblasts potentially identified two different receptor pathways for which Lp(a) can be internalized and subsequently degraded: a calcium-dependent LDLR pathway with high affinity for the intact particle and an efficient plasminogen receptor pathway for free apo(a) [200]. Since no one mechanism has been demonstrated to largely decrease Lp(a) plasma levels and mechanisms that have been suggested exhibit variability under different conditions, it is reasonable to assume that multiple receptors may be involved in Lp(a) catabolism. Other receptors that have been implicated in the catabolism of Lp(a) in *in vitro* studies include the very low-density lipoprotein receptor (VLDLR), megalin/gp330 receptor, and scavenger receptor class B type 1 [185, 201-204]. Moreover, Lp(a) has been demonstrated to be susceptible to proteolysis by polymorphonuclear cells isolated from human peripheral blood through an elastase-induced fragmentation of free-apo(a) and complexed apo(a) in Lp(a), possibly related to the fragments observed in urine [174, 175, 205, 206]. As such, it is also reasonable to believe that other proteins may be involved in Lp(a) catabolism due to the effects observed with PCSK9 and elastases.

Therapy targeting reduction in Lp(a) levels have proven difficult due to the controversy and uncertainty surrounding the metabolism of Lp(a) as well as side-effects that arose with the use of some therapies. Earlier studies in patients demonstrated a 35% decrease in Lp(a) levels when treated with niacin alone or in combination with neomycin; however, some patients exhibited intolerance to the treatment [207-213]. Although treatment with niacin has been
associated with an increased risk for diabetes, it still remains the treatment of choice in cases where cardiovascular risk is greatly increased as a consequence of elevated Lp(a) levels [207-213]. As previously mentioned, inhibitors of PCSK9 administered to human subjects resulted in a dose-dependent decrease of Lp(a) levels by up to 30% [196, 214, 215]. Additionally, a more recent treatment with antisense oligonucleotides (ASO) targeting either apoB-100 or apo(a) mRNA have been demonstrated to reduce plasma levels of Lp(a) in mice and human models [216, 217]. In a two-randomized, double-blind, placebo-controlled study, phase 2 trial study, an ASO designed to be highly and selectively taken up by hepatocytes, demonstrated a significant, dose-dependent decrease in plasma Lp(a) levels in both single-dose and multi-dose that was physiologically tolerable [218].

1.2.8 Lipoprotein(a) Plasma Levels

Lp(a) plasma levels vary significantly within the population, but remain relatively stable within an individuals’ lifetime with the exception drastic changes in hormone levels demonstrated to affect Lp(a) concentration [219]. It was found that in postmenopausal women and in men who have undergone orchidectomy, whereby in both cases there is a decrease in endogenous estrogen and testosterone levels, respectively, both caused an increase in Lp(a) plasma concentrations [220]. Upon exogenous treatment of estrogen in both of the aforementioned cases, there was a decrease in plasma Lp(a) concentration [220]. Another example of hormonal influence on Lp(a) plasma levels was observed in a study whereby Lp(a) concentration was monitored after exogenous GH and IGF-1 treatment therapy in males with idiopathic osteoporosis [221]. Researchers observed an increase in Lp(a) plasma concentration due to GH treatment and a decrease in Lp(a) plasma concentration due to IGF-1 [221].
It was determined that approximately 91% of the variability in Lp(a) plasma concentration observed may be attributed to genetic variation where 70% is attributable to the size polymorphism, suggesting that allelic variations may also have an effect on Lp(a) plasma concentration [110]. The notion that allelic variations have an effect on Lp(a) plasma concentration was further supported by the observation in which an individual homozygous for apo(a) isoform size exhibited differences in Lp(a) expression specific to each allele as a result of sequence polymorphisms [222]. Furthermore, other observable differences in Lp(a) plasma concentration were a result of single nucleotide polymorphisms associated with the previously mentioned DH sites. The DHII site exhibits no base substitutions; however, the DHIII site demonstrates three common base substitutions that affect Lp(a) plasma concentration: A to G at -1230 bp, C to A at -1617 bp, and a G to T at -1712 bp [223]. These base substitutions caused a 70% increase, a 30% decrease, and a 40% decrease in Lp(a) plasma concentrations, respectively [223]. Other cis-acting sequences have been studied in the 5’-flanking region and these polymorphisms include base substitutions of G to A at -773, C to T at +93, and G to A at +121 with respect to the transcription initiation site [224]. These substitutions caused a small silencing effect, positive regulation in expression, and negative regulation in expression, respectively, by gain or loss of binding sites by TF as these sites are very close in proximity to the previously mentioned TFs [224]. Moreover, the C to T substitution at +93 of the transcription initiation site has been proposed to impair translation efficiency rather than transcription by the introduction of an additional ATG start codon with its own in-frame stop codon thereby generating a truncated mutant form of the protein and decreasing Lp(a) levels as a consequence [225].

Null alleles have been observed in the population that are independent of apo(a) isoform size and may be due to mutations that affects the synthesis of the protein or the secretion of it as
was demonstrated in one study using baboon hepatocytes [104, 226]. Single nucleotide polymorphisms (SNP) have also been known to affect plasma levels whereby two-thirds of null alleles were found to be transcript negative and one-third of null alleles were found to be transcript-positive in baboons [227]. In one of the cases of the transcript-positive null alleles, a mutation in the 5’ donor splice site resulted in a 141 bp deletion due to exon skipping that in turn affected the secretion of this protein [227]. Additionally, a SNP within the protease domain of apo(a) resulting in an isoleucine to methionine amino acid substitution at amino acid 4399 resulted in an increase in Lp(a) plasma concentration and severe CAD [228, 229].

Further evidence supporting the hypothesis of apo(a) allele variation and its relation to Lp(a) plasma levels was demonstrated in a population study where Ogorelkova and colleagues sought to discover SNPs in KIV_{6-10} domains of the apo(a) gene [127]. This region was of particular interest to them due to the implications that these domains have on Lp(a) particle assembly; a factor known influence plasma Lp(a) levels [127]. As previously stated, Lp(a) particle assembly is a two-step process involving a noncovalent interaction between apo(a)’s KIV_{7-8} and apoB-100 preceding a single disulfide bond formation involving the free cysteine residue in apo(a)’s KIV_{9} domain and the apoB-100 of the LDL-moiety [47, 88]. Although, the KIV_{6} of apo(a) was once thought to participate in the initial noncovalent step of Lp(a) particle assembly, it’s involvement in this step of Lp(a) particle assembly was disproved [82]. Nonetheless, at the time of Ogorelkova and colleagues’ investigation, the lack of KIV_{6}’s involvement in Lp(a) assembly was not yet discovered. In their study, they performed a systematic SNP analysis of exons in KIV_{5-10} in two African (Black South African and Khoi San) and Caucasian (Tyroleans) populations in an attempt to also discover potential cause for the heterogeneity observed in Lp(a) plasma levels between these two ethnic groups [127]. They
found that S37F in KIV₆ (S37F), G17R in KIV₈ (G17R), and P52L in KIV₈ (P52L) demonstrated a decrease in Lp(a) concentration and R18W in KIV₉ (R18W) demonstrated an increase in Lp(a) concentrations [127]. Statistical analysis was not possible for the P52L mutant since only two subjects possessed this allele [127]. Both G17R and S37F mutants demonstrated a significant decrease in Lp(a) plasma concentration, independent of the KIV₂ VNTR, in a gene-dose effect; homozygotes for both mutants demonstrated a greater decrease in Lp(a) plasma concentration when compared to heterozygotes [127]. The R18W mutant demonstrated a significant increase in Lp(a) plasma levels, independent of the KIV₂ VNTR, in a gene-dose effect; homozygous demonstrated a two-fold increase in Lp(a) plasma concentrations when compared to heterozygotes [127]. Additionally, T23P in KIV₈ (T23P) demonstrated variable effects: significantly lower Lp(a) plasma concentrations were observed in homozygotes and higher Lp(a) plasma concentrations were observed in heterozygotes in the Tyrolean population but the same trend was not observed when the SNP was studied in a Finnish population [127]. Furthermore, this T23P was associated with significantly higher frequencies of apo(a) KIV₂ alleles ranging between 21-25 KIV repeats [127]. The mutations they discovered demonstrated allelic frequencies of greater than 2%, with the exception of P52L (0.8% allelic frequency in Caucasian population), and no SNP was common to both the Caucasian and African population [127]. The S37F and the G17R mutants demonstrated relatively low allelic frequencies, 2.7% and 2.8%, respectively, in the African populations, whereas the R18W mutant demonstrated a 8% allelic frequency in the African population [127]. The T23P demonstrated an allelic frequency in the Tyrolean population of 17%, but Gorelikova and colleagues assumed the observed effects were likely due to chance since they were unable to detect the same trend when they investigated another European-Caucasian population [127]. Table 1.1 summarizes the findings of the study.
conducted by Ogorelkova and colleagues. Considering the five SNPs discovered by Ogorelkova and colleagues have not been studied \textit{in vitro}, with the exception of T23P, we aimed to investigate the effects that these SNPs have on Lp(a) assembly utilizing an \textit{in vitro} system [127, 230].

Table 1.1 SNPs found in KIV\textsubscript{6-9} of apo(a) associated with Lp(a) plasma levels

<table>
<thead>
<tr>
<th>SNP</th>
<th>Amino Acid Change</th>
<th>Kringle</th>
<th>Effect</th>
<th>Allelic Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>C→T</td>
<td>S37F</td>
<td>KIV\textsubscript{6}</td>
<td>↓[Lp(a)] in plasma</td>
<td>2.7%</td>
</tr>
<tr>
<td>G→A</td>
<td>G17R</td>
<td>KIV\textsubscript{8}</td>
<td>↓[Lp(a)] in plasma</td>
<td>2.7%</td>
</tr>
<tr>
<td>A→C</td>
<td>T23P</td>
<td>KIV\textsubscript{8}</td>
<td>↓[Lp(a)] in plasma in homozygotes  ↑[Lp(a)] in plasma in heterozygotes</td>
<td>17%</td>
</tr>
<tr>
<td>C→T</td>
<td>P52L</td>
<td>KIV\textsubscript{8}</td>
<td>↓[Lp(a)] in plasma</td>
<td>0.89%</td>
</tr>
<tr>
<td>C→T</td>
<td>R18W</td>
<td>KIV\textsubscript{9}</td>
<td>↑[Lp(a)] in plasma</td>
<td>8%</td>
</tr>
</tbody>
</table>

\textit{1.2.9 Hypothesis}

To date, accumulated evidence has suggested that allelic variation in the \textit{LPA} gene predominantly determines plasma levels, that Lp(a) assembly takes place as a two-step mechanism that involves multiple kringles within apo(a) interacting noncovalently and covalently with the apoB-100 moiety of the LDL-like particle, and that proper-folding of apo(a) is crucial for apo(a) secretion, with smaller isoforms folding more quickly than larger ones. As such, we hypothesize that the SNPs G17R, T23P, P52L, S37F, and R18W, may affect Lp(a) plasma concentration by affecting the respective kringle structures thereby altering secretion of apo(a) from human hepatoma cells, and/or its ability to assemble with LDL to form Lp(a).

\textit{1.2.10 Objectives}

To investigate the proposed hypotheses, three objectives were developed. The SNPs under consideration were introduced separately into a physiologically relevant, recombinant
apo(a) (r-apo(a)) isoform that possesses 17-kringles (17K) and compared to wild-type 17K (WT) in the following experiments:

I. Pulse-Chase assays in HepG2 cells were performed in order to determine the effects that these SNPs may have on secretion rates of the respective apo(a) variants and in turn, Lp(a) concentrations,

II. A recombinant Lp(a) (r-Lp(a)) assay using purified Lp(a) components of known concentration in HEK293 conditioned media was used to determine the effects of these SNPs on covalent Lp(a) assembly, and

III. Computational chemistry was used to assess the structural differences that these SNPs may impose in their corresponding kringles that could potentially explain the observed differences in the assays described above.
CHAPTER 2
Materials & Methods

2.1 Cell Culture

All cell lines used in the experiments were grown and cultured in humidifiers with the following conditions: 95% relative humidity, 37°C, and 5% CO₂ concentration. Human embryonic kidney (HEK293) cells were cultured in minimum essential medium (MEM; Gibco) supplemented with 5% fetal bovine serum (FBS; Gibco) and 1% antibiotic-antimycotic (Gibco). Human hepatocellular carcinoma (HepG2) cells were obtained from American Type Culture Collection (ATCC) and cultured in MEM supplemented with 10% FBS (ATCC) and 1% antibiotic-antimycotic (Gibco). Both cell types used were cultured and maintained in 100 mm tissue culture plates (Sarstedt).

2.2 Construction, expression, and purification of recombinant 17K apo(a)

The construction of 17K r-apo(a) in the pRK5 expression vector and the stable expression of this plasmid in HEK293 cells has been previously described [231]. Conditioned medium was harvested from the stable cell line and supplemented with the protease inhibitor phenylmethylsulfonyl fluoride (PMSF), to a final concentration of 1 mM. From the conditioned medium, r-apo(a) was purified using Lysine-Sepharose affinity chromatography as previously described [51, 231]. Using the previously determined molar extinction coefficient of 17K r-apo(a) [231], protein concentration was determined spectrophotometrically and presence of the protein was detected using 7% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot and silver staining analysis [86]. 500 ng of the purified recombinant protein was compared with a 500 ng purified standard.
2.3 Generation of 17K r-apo(a) variants: G17R KIV₈, R18W KIV₉, P52L KIV₈, S37F KIV₆, T23P KIV₈

Using the 17K r-apo(a) pRK5 expression vector containing mutated XmaI sites at 4793 base pairs and 7854 base pairs (17KΔΔSmaI pRK5), the expression vector and pBluescript II SK+ were subjected to endonuclease digestion with EcoRV and XmaI (New England Biolabs; NEB) for 1 h at 37°C. The digested fragment of approximately 1896 base pairs from the 17K pRK5 expression vector (encoding apo(a) KIV₆ to KV) and the 2943 base pair fragment of pBlUEScript II SK+ were gel purified and ligated together. The ligation product was subjected to Q5 site-directed mutagenesis (NEB) as per manufacturer’s manual using the following sense and antisense primers to create the 17K variants used in the experiments: P52L KIV₈ sense, 5’-TTA TAC TAT CTA AAT GCT GGC CTG ACC AG-3’, P52L KIV₈ antisense, 5’-TGG GAT CCT CCG GCG AGT-3’, G17R KIV₈ sense, 5’-CCG AGG TGA TCG ACA GAG TTA TCG-3’, G17R KIV₈ antisense, 5’-TAG CAG TCC TGG ACC CCA-3’, R18W KIV₉ sense, 5’-TGG TGA TGG ATG GAG TTA TCG-3’, R18W KIV₉ antisense, 5’-TGG TAG CAA TCC TGG ACC-3’, T23P KIV₈ sense, 5’-TTA TCG AGG CCC ACT CTC CAC-3’, T23P KIV₈ antisense, 5’-CTC TGT CCA TCA CCT CGG-3’. The mutants and their respective primers are outlined in Table 2.1. After confirming the mutations by DNA sequencing services provided by Robarts Research Institute (London, Ontario), the mutant 2943 base pair fragments were excised from the pBluescript SK+ plasmid and ligated back into the digested 17KΔΔSmaI pRK5 expression vector. The S37F KIV₆ mutated fragment from 4793 base pairs to 7854 base pairs was generated by GenScript in the pUC57 plasmid due to difficulties in selectively mutating the site of interest that is owed to the extensive homology in the region of
interest in the KIV<sub>6</sub> subtype other KIV subtypes. The mutant 2943 base pair fragment in the pUC57 plasmid was excised and ligated into the digested 17KΔΔSmaI pRK5 expression vector.

Table 2.1 Primer sequences for construction of 17K r-apo(a) variants<sup>a</sup>

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>P52L</td>
<td>5’ - TTA TAC TAT &lt;CTA&gt; AAT GCT GGC CTG ACC AG – 3’</td>
</tr>
<tr>
<td>G17R</td>
<td>5’ – CCG AGG TGA &lt;TCG&gt; ACA GAG TTA TCG – 3’</td>
</tr>
<tr>
<td>T23P</td>
<td>5’ – TTA TCG AGG &lt;CCC&gt; ACT CTC CAC – 3</td>
</tr>
<tr>
<td>R18W</td>
<td>5’ – TGG TGA TGG &lt;ATG&gt; GAG TTA TCG – 3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Sense primers used to generate the 17K r-apo(a) mutant variants are shown. Mutant nucleotides are boldfaced and mutant codons are underlined. Primers were designed using NEBase Changer v1.2.7 (http://nebasechanger.neb.com/).

2.4 Assessing the expression and secretion of 17K r-apo(a) variants

HepG2 and HEK293 cells were grown to 70% confluency in 6-well tissue culture plates (Sarstedt). The cells were transiently transfected with 1µg of either wildtype 17K pRK5 expression vector, 17KΔΔSmaI pRK5, 17KΔΔSmaI pRK5 P52L KIV<sub>8</sub>, 17KΔΔSmaI pRK5 G17R KIV<sub>8</sub>, 17KΔΔSmaI pRK5 T23P KIV<sub>8</sub>, 17KΔΔSmaI pRK5 R18W KIV<sub>9</sub>, or 17KΔΔSmaI pRK5 S37F KIV<sub>6</sub> using linear polyethylenimine (PEI; Sigma) as per the manufacturer’s manual. Conditioned medium and lysates from each transient transfection were collected and subjected to 7% SDS-PAGE analysis followed by western blot analysis using anti-apo(a) antibody (a5) to confirm the expression and secretion of the respective r-apo(a) mutants (Appendix A.1; Figure A.1).

2.5 Purification and expression of 17K r-apo(a) variants

Mutant expression vectors were co-transfected with a mammalian pCMV6-Neo (Origene) selection vector into HEK293 cells with a 10:1 DNA ratio, respectively, using linear PEI as per the manufacturer’s protocol. Cells were then subjected to Geneticin® selective
antibiotic (G418 sulfate; Thermo Scientific) selection at a concentration of 150 µg/mL 48 h after transfection until viable populations were observed. Media samples from stably-expressing populations were assessed using western blot analysis to confirm transfection of the mutant expression vectors. Conditioned medium from stably expressing cells were harvested and supplemented with PMSF to a final concentration of 1 mM. Mutant variants were then purified by way of Lysine-Sepharose affinity chromatography as previously described for the wildtype (WT) recombinant protein [51, 231]. Western blotting was used for comparative analysis of the purified recombinant mutants (Appendix A.2; Figure A.2).

2.6 Pulse-Chase analysis of r-apo(a) variants

HepG2 cells for pulse-chase experiments were grown to 70% confluency in 100 mm tissue culture plates (Sarstedt). These cells were allowed to attach overnight after which they were co-transfected with either 17K pRK5ΔΔSmaI, 17K pRK5ΔΔSmaI KIV8 T23P, 17K pRK5ΔΔSmaI KIV8 P52L, 17K pRK5ΔΔSmaI KIV8 G17R, 17K pRK5ΔΔSmaI KIV6 S37F, or 17K pRK5ΔΔSmaI KIV9 R18W expression vectors and pRL-TK Renilla Luciferase reporter vector (Promega) using linear PEI as per manufacturer’s protocol with a 10:1 DNA ratio, respectively. Cells were then trypsinized and seeded into a 6-well tissue culture plate (Sarstedt) at 6.5 x 10⁵ cells/well and allowed to recover overnight with fresh medium (MEM supplemented with 10% ATCC FBS and 1% antibiotic-antimycotic) prior to labelling experiments. Cells were then washed with 1 mL of PBS and incubated for 1 h with cysteine- and methionine-free Dulbecco’s Modified Eagle Medium (Cys-/Met-DMEM; GIBCO) supplemented with 1% antibiotic-antimycotic and lacking FBS. The cells were then pulse-labelled using the same media supplemented with 200 µCi/well of [³⁵S]-cysteine/[³⁵S]-methionine labeling solution (Perkin Elmer Life Sciences) for 1 h. At this time, the cells were washed once with 1 mL of PBS and
chased with 1 mL of MEM supplemented with 10% ATCC FBS and 1% antibiotic-antimycotic for 0, 30, 60, 120, 240, and 480 mins. At each of these time points, conditioned medium was collected, stored on ice, and the cells were washed once with 1 mL of cold PBS, and lysed by addition of 500 µL of cold lysis buffer (50 mM of Tris-HCL pH 8.0, 1% w/v NP-40, 0.5% w/v sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.1% w/v SDS, 1 mM PMSF) for 1 minute. Lysates and conditioned medium were centrifuged at 12,100 x g in a tabletop microcentrifuge for 6 minutes to pellet any cellular debris. Supernatants were transferred into fresh microcentrifuge tubes and pre-cleared with 30 µL of gelatin-agarose (Sigma) with gentle agitation for 2 h at 4°C. Samples were then centrifuged at 600 x g in a tabletop microcentrifuge for 2 mins and supernatants were transferred into fresh microcentrifuge tubes. The samples were subjected to immunoprecipitation by incubating the samples with 1 µL of anti-apo(a) antibody (a5) (stock concentration of 1 µg/µL) with gentle agitation overnight at 4°C [232]. The following day, the samples were supplemented with 30 µL of Protein-G agarose beads (Novex) with gentle agitation for 2 h at 4°C. The samples were then centrifuged at 500 x g for 2 mins using a tabletop microcentrifuge and the supernatants were aspirated. The pelleted beads were washed with 500 µL of ice-cold RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 20 mM EDTA, 1% w/v sodium deoxycholate, 0.1% w/v SDS), microcentrifuged at 500 x g for 2 mins, and the supernatants were aspirated. This was repeated two more times for a total of 3 washes. A fourth wash was performed using 500 µL of TE buffer (10 mM Tris pH 7.5, 1 mM EDTA) after which the pellets were re-suspended in 30 µL of 2x SDS sample buffer (250 mM Tris pH 6.8, 4% w/v SDS, 0.001% w/v bromophenol blue, 40% v/v glycerol) and supplemented with 7 µL of 100 mM dithiothreitol (DTT). Samples were then pulse-centrifuged to pellet the beads, boiled for 7 mins, and pulse-centrifuged once more. 30 µL of the immunoprecipitates were subjected to SDS-
PAGE using 7% polyacrylamide gels. Gels were incubated with 100 mL of a fixing solution (methanol:H2O:glacial acetic acid; 40:50:10 ratio) for 20 mins with gentle agitation, after which they were washed with milli-Q H2O, and incubated with 100 mL of amplifying solution (Amersham Biosciences) for another 20 mins with gentle agitation. Gels were then incubated with 100 mL of milli-Q H2O containing 5 drops of 100% glycerol for 10 mins with gentle agitation. Gels were then dried using a BioRad Model 583 drier for 45 mins at 80°C. Using a phosphor K screen (BioRad), the gels were exposed for 72 h prior to imaging the screens using a BioRad Molecular Imager FX phosphoimager. Quantification of the resulting bands was accomplished using densitometric analysis with Alpha View Software (Alpha Innotech). For lysates specifically, the combined density of both the immature and mature forms of the intracellular r-apo(a) were used in generating results (Appendix A.4).

2.7 Transfection efficiency

Transfection efficiency for pulse-chase experiments was assessed using Renilla luciferase assay system (Promega). Quantification of Renilla luciferase activity of transfected cells seeded into a 6-well plate was accomplished as per the manufacturer’s protocol. Densitometric values, determined from lysates and media samples of the mutant and WT apo(a) variants from pulse-chase experiments, were normalized using the corresponding luciferase activity values associated with each sample, to account for differences in cell numbers and transfection efficiencies (Appendix A.4).

2.8 Computational chemistry

All MD models were prepared and analyzed using the Molecular Operating Environment (MOE) program [233]. The NAMD program was used to run all MD simulations.[234] NMR structures of kringle proteins were used as starting templates for all subsequent models. These
include kringle IV type 6 (PDB ID: 1JFN), kringle IV type 8 (PDB ID: 2FEB), and kringle IV type 9.[235, 236] All mutants were obtained using in silico mutagenesis in MOE. The mutations were S37F for kringle IV type 6, T23P, G17R, and P52L for kringle IV type 8, and R18W for kringle IV type 9. Hydrogen atoms were attached using the default protocols found in MOE followed by a spherical solvation around the protein at 6 Å. All structures were minimized using AMBER 12:EHT molecular mechanics forcefield [237, 238].

No restraints were applied to the atoms of the minimized structures except for the wall restraints applied to the spherical water droplet. An annealing simulation at a constant volume and pressure was performed in which the temperature rose from 150 to 300°K over 150 ps. Following annealing, a production run was performed for 20 ns at a temperature of 300°K.

The root mean squared deviation (RMSD) of the lysine binding site of each Kringle in the KIV6 mutant and KIV8 mutant models and the free cysteine residue in the KIV9 mutant were subjected to cluster analysis to generate an average structure from the highest cluster and were further minimized by Amber 12:EHT molecular mechanics forcefield.[237, 238].

2.9 Western blotting for analysis of r-Lp(a) formation

For recombinant r-Lp(a) experiments, media samples were subjected to SDS-PAGE on 5% polyacrylamide 0.75 mm gels. All samples were prepared using 30 µL of each samples supplemented with 30 µL of 2X SDS sample buffer and boiled for 7 minutes. Samples were then pulse-centrifuged before subjecting them to SDS-PAGE at 150V for 4 h. Gels were electrophoretically transferred onto PVDF membranes (Millipore) in ice cold transfer buffer (25 mM Tris pH 7.8, 1.92 M glycine, 10% v/v Methanol) for 2 h at 100V. PVDF membranes were blocked in 15 mL of NET buffer (50 mM Tris, pH 7.6, 6 mM EDTA, 150 mM NaCl, 0.05% v/v
Triton-X100) supplemented with 6% w/v powdered non-fat milk for 1 h at room temperature with gentle agitation. Post-blocking, membranes were submerged in blocking buffer supplemented with mouse-anti-human apo(a) a5 antibody (0.1 µg/mL) [232] for 1 h at room temperature with gentle agitation. Membranes were washed three times with NET buffer for 15 mins each with gentle agitation and subjected to secondary antibody labelling using blocking buffer supplemented with sheep-anti-mouse secondary antibody (GE Healthcare) for 1 h at room temperature with gentle agitation. Membranes were washed three times in NET buffer for 15 mins each with gentle agitation. Using SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific) and a FluoroChem Q Imager (Alpha Innotech), immunoreactive material was visualized and quantified using densitometric techniques with the aid of Alpha View software (Alpha Innotech).

2.10 LDL purification

With written informed consent from a healthy human volunteer who exhibited no detectable Lp(a), blood was collected into BD Vacutainers containing sodium polyanethol sulfonate and acid citrate dextrose. The blood was centrifuged at 2000 x g for 15 min at 4°C and the plasma layer was removed. Using sequential flotation ultracentrifugation (1.02 g/mL < d < 1.068 g/mL) LDL was isolated from the plasma with centrifugation conditions of 45000 x g for 18 h at 4°C. Isolated LDL was extensively dialyzed against buffer A (20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.01% NaN₃, and 0.01% EDTA). The sample was then subjected to gel filtration chromatography using Sepharose CL-4B (Sigma Aldrich) in a 2.5 cm x 80 cm column using buffer A supplemented with 0.1% v/v Tween-20 and 0.1 M proline. Fractions were collected and absorbances at 280 nm were measured. Samples exhibiting absorbances greater than 0.1 were pooled and diluted 3-fold with Milli-Q dH₂O and applied to a DEAE-Sepharose
Fast Flow (Pharmacia) ion exchange 2.5 x 3 cm column. LDL was eluted using an NaCl concentration gradient (50 to 150 mM NaCl in 20 mM Tris-HCl, pH 7.4). LDL-containing fractions were pooled and dialyzed extensively against HEPES-buffered saline (HBS; 20 mM HEPES, pH 7.4, 150 mM NaCl) at 4°C. The concentration of the final product was determined using bicinchoninic acid assay (Pierce) with BCA as a standard as per the manufacture’s protocol. Detection of LDL in the sample was achieved by SDS PAGE using a 5% poly acrylamide gel and silver staining analysis under non-reduced conditions (Appendix A.4; Figure A.3).

2.11 Recombinant Lp(a) Particle Assembly Assays

WT HEK293 cells were grown to 100% confluency in a 100 mm tissue culture plate (Sarstedt) and incubated for 48 h. Conditioned media was removed from the cells and syringe-filtered using a 0.2 µm (Millipore) filter. The filtrate was supplemented with 100 nM of purified LDL and 5 nM of each of the WT 17K r-apo(a) or mutant variants (except for G17R mutant). Samples were incubated in a 37°C bath and 30 µL samples were collected at 0, 2, 4, 6, and 8 h. Samples were each supplemented with 10 µL 4X SDS Sample buffer and boiled for 7 minutes under non-reducing conditions, with exception of the 8 h time point which was assessed under both non-reducing and reducing conditions. Samples were then subjected to SDS PAGE using a 5% poly acrylamide gel followed by western blot analysis as previously described. Using the densitometric values obtained, %Lp(a) formed was calculated by the following equation: %Lp(a) = [Lp(a)]/([Lp(a) + apo(a)]) × 100 [82].
2.12 Statistical methods

For all pulse-chase experiments involving comparisons of variants with wild-type 17K r-apo(a), data sets were compared using two-tailed student’s T-test assuming unequal variances. Statistical significance was assumed at p < 0.05. Unfortunately, in the case of r-Lp(a) assays, there were insufficient number of trials and therefore, statistics could not be completed.
CHAPTER 3

Results

3.1 R-Lp(a) Particle Assembly

Lp(a) particle assembly is a two-step process. The first step involves a noncovalent interaction between apo(a) and apoB-100 such that the two moieties are brought into close proximity preceding disulfide bond formation [41, 81]. WLBS found in each of KIV₇₋₈ have been shown to play a major role in the noncovalent interaction between apo(a) and apoB-100 [82]. Disulfide bond formation occurs between apoB-100 and the free cysteine residue in KIV₉ of apo(a) [49, 88]. As such, we hypothesized that the SNPs in these kringle domains could potentially affect the assembly of these particles at either the first or second step of the assembly process.

To study the impact these SNPs have on covalent Lp(a) particle assembly, conditioned medium collected from HEK293 cells were supplemented with 100 nM of purified human LDL and 5 nM of the following purified recombinant apo(a) mutant variants: S37F, T23P, P52L, and R18W. These samples were incubated at 37°C to allow for the two purified components to form the covalent particle and samples were collected at time 0, 2, 4, 6, and 8 h. The percentage of the assembled particle was calculated as the average of two independent trials using the following formula: \( \%\text{Lp(a)} = \frac{\text{Lp(a)}}{\text{Lp(a)} + \text{apo(a)}} \times 100 \) [82]. The second immunoblot image used for densitometric analysis is provided in Appendix A.6; Figure A.5. Due to insufficient number of trials (n=2), statistical analysis could not be performed on the corresponding data. Tentatively, this data suggests the potential for these variants to affect Lp(a) particle assembly; however, further experiments need to be conducted to confirm these findings.
Figure 3.1: The effect of SNPs in 17K apo(a) on R-Lp(a) particle assembly. In vitro r-Lp(a) particle assembly assays over an 8 hour period were used to study the effects that these mutants have on covalent particle assembly. (A) Representative western blot image of apo(a) variants WT, P52L, S37F, T23P, and R18W purified from stably expressing HEK293 cells coupled with purified LDL to form r-Lp(a). Recombinant Lp(a) was separated from free apo(a) by SDS PAGE on a 5% gel and visualized by immunoblotting and imaging on a Fluorochem Q Imager. (B) Represents the average of two independent experiments. Using densitometric analysis, the percent of r-Lp(a) formed was determined for 17K apo(a) variants (WT (●), P52L (■), S37F (○), T23P (▲), and R18W (△)) with respect to time using the equation \%Lp(a) = [Lp(a)]/[Lp(a) + apo(a)] × 100, which represents the efficiency of Lp(a) particle assembly. The colours correspond to the mutant variant as indicated in the legend.
3.2 Secretion of 17K apo(a) variants from HepG2 cells

Lp(a) plasma concentration relies heavily on the rate of biosynthesis of the particle [65, 192]. Two posttranslational regulatory processes that effect Lp(a) plasma concentration is the rate of extracellular assembly and the rate of secretion of apo(a) from hepatic cells that has shown to be inversely related to the number of KIV2 repeats in apo(a) [80]. Differences in the KIV2 copy number in apo(a) has shown to only account from 30-70% of the variability of Lp(a) concentration the population and for alleles with identical repeats of the KIV2, Lp(a) levels can differ to up to 200-fold suggesting that additional sequence variation may have an effect a Lp(a) level variability [222, 239]. As such, we hypothesized that these SNPs may have an effect on apo(a) secretion from HepG2 cells.

To study the effect that these SNPs have on apo(a) secretion, a $^{35}$S-Met/Cys pulse-chase experiment was performed. HepG2 cells transiently expressing a recombinant 17K apo(a) variant was used as a control for comparative purposes with HepG2 cells transiently expressing the following recombinant 17K mutant apo(a) variants: P52L, S37F, T23P, G17R, and R18W (Figure 3.2A-C). The densitometric data generated for the secretion assays were first normalized to luciferase activity to account for differences in transfection efficiency, then subsequently normalized to the highest WT density observed for comparative purposes. However, it is possible that normalizing to luciferase activity contributed to variability. As such, densitometric data generated from these secretion assays were investigated without normalizing to luciferase activity (Appendix A.5; Figure A.4). In all cases, for both mutant variants and WT variant, 30 mins was the earliest detectable time at which mature apo(a) was secreted into the medium (Figure 3.2A right).
The data in Figure 3.2B suggests that the R18W mutant showed slightly less intracellular apo(a) accumulation from time 0-60 minutes when compared to the WT variant and that the T23P mutant showed greater intracellular apo(a) accumulation when compared to WT at times 0, 120, 240, and 480 mins. This data suggests that G17R showed greater intracellular accumulation when compared to WT at times 30, 240, and 480 mins and P52L mutant showed greater intracellular accumulation of apo(a) when compared to WT at times 240 and 480 mins. These data showed no statistically significant differences for the S37F mutant when compared to the WT r-apo(a). Due to large error bars, it is important to note that further experiments must be conducted in order to confirm the observed trends.

The data in Figure 3.2C suggests that the P52L mutant showed greater media apo(a) accumulation at time 480 min when compared to WT variant and that the G17R mutant showed slightly greater media apo(a) accumulation at times 60 and 120 min when compared to the WT variant. The data suggests that the R18W mutant showed slightly greater media apo(a) accumulation at time 60 min when compared to the WT variant and that the T23P mutant showed slightly greater media apo(a) accumulation at time 60 min when compared to the WT variant. The S37F mutant showed no statistically significant variation in media apo(a) accumulation when compared to the WT variant. As for the lysate data, the large variability observed in these experiments indicates that more independent trials need to be performed. Furthermore, it is important to note that the data generated from fluorograms corresponding to each apo(a) variant investigated were compared directly with one another, despite the likelihood of different phosphoimager exposures, as a result of insufficient area on phosphor K screens to accommodate all 6 fluorograms. As such, it is crucial to repeat these experiments such that differences in phosphoimager exposures are eliminated. Nonetheless, minimizing the effects of
potential variability posed by differences in phosphoimager exposure was accomplished by ensuring the time between exposure and imaging was consistent (staggering) and that a WT sample was always present on each phosphoimager exposure for comparative purposes.
A

B

C
Figure 3.2: The effect of 17K apo(a) mutant variants on apo(a) secretion from HepG2 Cells.
Pulse-chase analysis of HepG2 cells transiently expressing either WT, P52L, S37F, T23P, G17R or R18W. Cells were starved in Cys-/Met- medium for 60 minutes, pulse-labelled for 60 minutes with $^{35}$S-Met/Cys, and subsequently chased in unlabelled media for 0, 30, 60, 120, 240, and 480 minutes. (A) Representative fluorograms of cell lysates (left) and media samples (right) are shown. Each sample was collected at the aforementioned chase time points, immunoprecipitated, and analyzed by 7% SDS PAGE and fluorographic methods. The upper band represents the mature, fully glycosylated forms of apo(a) (m-apo(a)) while the lower band represents immature, hypoglycosylated forms of apo(a) (p-apo(a)). (B) Graphical representation of the resulting densities of the intracellular apo(a) variants (combination of both mature and immature apo(a) forms) at the corresponding chase times. (C) Graphical representation of the resulting densities of secreted apo(a) variants at the corresponding chase times. Densitometric analysis was performed using AlphaView where the mutant variants were normalized to the maximum density observed for the wild type apo(a) variant and plotted as a function of time. Significance compared to wildtype apo(a) are represented by asterisks, where *p<0.05 and n=5 (5 independent experiments). The colours correspond to the different mutant as indicated in the legend.
3.3 Computational Chemistry

As previously mentioned, Lp(a) particle assembly is a two-step process with the first step being a noncovalent interaction between apo(a) and apoB-100 bringing the two moieties into close proximity such that a covalent interaction between the two can occur. KIV$_6$ and KIV$_8$ each possess WLBS; however, the WLBS in the KIV$_8$ has been shown to play a crucial role in the noncovalent interaction of the assembly process, whereas the KIV$_6$ has not [82]. Furthermore, the free cysteine residue in the KIV$_9$ is crucial for the second step of the assembly process, as this is the residue that forms a covalent bond with the apoB-100 of the LDL-like moiety to form Lp(a) [49, 88]. As such, we hypothesize that these SNPs may affect the accessibility of these sequences of interest in their respective kringles.

MD models were prepared and analyzed using MOE software. The structure of the wild-type kringles has been previously reported and using an in silico mutagenesis function on MOE, mutant apo(a) variants were generated (Figure 3.3) [235, 236, 240].

The amino acid residues in blue represent the amino acids involved in the WLBS: Arg$^{46}$, Asp$^{65}$, Glu$^{67}$, Trp$^{71}$, Tyr$^{73}$, Arg$^{80}$, and Trp$^{81}$ and the single amino acid residue in red represents the amino acid that is mutated (Figure 3.3A-D) [241]. In the case of G17R, P52L, T23P, and S37F, when comparing the accessibility of the WLBS in these mutants (right) to that of the wild-type apo(a) (left), it can be assumed that the accessibility to this site has been altered (Figure 3.3A-D). In the case of G17R (right), the WLBS appears to have taken on a slightly more closed conformation when compared to the wild type KIV$_8$ (left) variant (Figure 3.3A). This also appears to be the case for T23P (right) and P52L (right) (Figure 3.3B-C) with P52L showing a greater closed conformation relative to the wild type apo(a) (left) and the other two mutants in the KIV$_8$ domain. The S37F (right) mutation in the KIV$_6$ appeared to have the largest change in
conformation relative to the wild-type (left) and compared to all other mutants (Figure 3.3D). The WLBS in this mutant appears to be more open when compared to the wild-type KIV₆.

Lastly, the R18W mutation (right) in the KIV₉ domain appears to have increased the accessibility to the free cysteine residue used for the covalent interaction with apoB-100 of the LDL-like particle when compared to wild type KIV₉ (left) (Figure 3.3E).
Figure 3.3: Molecular dynamic simulations of wild type and mutant apo(a) variants.
Molecular operating environment (MOE) software was used to produce average occupancy images using the NAMD program to determine structural differences between wild-type apo(a) and the mutant variants under investigation: P52L, S37F, T23P, G17R, or R18W. RMSDs of the lysine binding site of each kringle in the KIV₆ mutant and KIV₈ mutant models and the free cysteine residue in the KIV₉ mutant were subjected to cluster analysis to generate an average structure from the highest cluster and were further minimized by Amber 12:EHT molecular mechanics forcefield. A production run was performed for 20 ns at a temperature of 3000 K. For all MDs, the mutated amino acids are represented by a red residue, amino acid residues of interest are represented in blue (A-D represents amino acids involved in LBS and E represents free cysteine residue involved in covalent assembly), and yellow sticks are indicative of the three invariant disulfide bonds characteristic of kringle motifs. (A) Wild type KIV₈ (left) and G17R mutated KIV₈ (right). (B) Wild type KIV₈ (left) and P52L mutated KIV₈ (right). (C) Wild type KIV₈ (left) and T23P mutated KIV₈ (right). (D) Wild type KIV₆ (left) and S37F mutated KIV₆ (right). (E) Wild type KIV₉ (left) and R18W mutated KIV₉ (right).
CHAPTER 4

Discussion

It is agreed upon and demonstrated by a plethora of accumulated data to date, that Lp(a) plasma levels are primarily determined by allelic variations in the \textit{LPA} gene. Several SNPs have been discovered that affect Lp(a) levels and interracial heterogeneity has been demonstrated with respect to Lp(a) levels in several studies [37, 110-112, 124-126]. In a human study, five SNPs resulting in amino acids changes were shown to have an effect on Lp(a) plasma levels, which include: S37F, G17R, P52L, T23P, and R18W [127]. In the current study we investigated these SNPs using an \textit{in vitro} system in an attempt to understand how the SNPs could potentially affect plasma Lp(a) levels in human patients. It was previously reported that S37F, G17R, and P52L variants, were associated with decreased Lp(a) levels, R18W was associated with increased Lp(a) levels, and T23P was associated with decreased levels of Lp(a) in those homozygous for the respective SNP in one Caucasian population [127].

Other previously described SNPs were found to affect Lp(a) plasma levels by altering recognition sites of TFs [223], decreasing translation efficiency [104, 222], increasing levels with or without a defined mechanism [224, 225, 228, 229], and causing mRNA-positive null alleles [104, 226, 227]. With respect to the mRNA-positive null variants, the allelic variations may affect the folding of the protein or other post-translational processes, leading to the increased intracellular retention of the variant, and decreased efficiency of secretion [226, 227]. Furthermore, the plasma Lp(a) levels associated with the VNTR of the KIV\textsubscript{2} domain demonstrated an inverse correlation of apo(a) size and plasma Lp(a) levels due to increased post-translational processing required for the additional domains, causing a decrease in overall secreted protein [65]. Protein folding takes place in the ER with the aid of chaperones, and it has
been demonstrated that apo(a)’s interactions with chaperones as well as post-translational processing influences its progression through the pre-secretory biosynthetic pathway [66, 71, 73-75, 78]. Taken together with the established expression and secretion of the S37F, G17R, P52L, T23P, and R18W in human hepatoma and kidney cells, these SNPs could potentially affect Lp(a) plasma levels by affecting the processing of the respective apo(a) variants and progression through the pre-secretory biosynthetic pathway, therefore affecting its secretion. However, the variability in secretion that was observed in our analysis of the mutants compared to the WT variant (Figure 3.2) is independent of the VNTR, since all mutations were introduced into the same isoform size of apo(a) (17K).

If accumulation of apo(a) in cell lysates and a consequential decrease of apo(a) in the conditioned medium of the cells was observed, this may suggest that the mutant apo(a) is being retained intracellularly due to the effects of the respective amino acid changes on movement through the pre-secretory biosynthetic pathway and secretion. Conversely, a decrease in intracellular apo(a) accompanied by a consequential accumulation of apo(a) in the conditioned medium, could suggest an increased efficiency in the movement through the pre-secretory biosynthetic pathway and secretion. Additionally, accumulation of the mature form of intracellular apo(a) in combination with a comparable secreted concentration, may suggest that the amino acid change increased the rate of conversion from immature apo(a) to mature apo(a). This is due to the fact that only correctly processed and folded proteins are permitted to progress in the pre-secretory biosynthetic pathway and exit the ER to undergo further maturation in the GA where the mature form of apo(a) is found intracellularly [242].

Unfortunately, much of the data obtained in the pulse-chase experiments (Figure 3.2) did not reach statistical significance, with the exception of some time points, rendering it difficult to
establish connections between the SNPs and their correlation with plasma Lp(a) levels. Nonetheless, a loose interpretation of these data may be proposed, but should not be considered as concrete on the basis of large error and lack of sufficient statistical significance. When compared to the WT apo(a) variant, mutants G17R and T23P demonstrated accumulation of intracellular apo(a), reaching statistical significance in only two and one time points, respectively (Figure 3.2C). S37F mutant appeared to have accumulated intracellularly, however no statistically significance was associated with this observation when compared to WT. The intracellular accumulation of these mutants may represent a conformational change that nominally decreases the accessibility of chaperones to the mutant variants, thus decreasing the efficiency of post-translational processing in the ER, increasing ER residency time, and decreasing the efficiency with which apo(a) progresses through the pre-secretory biosynthetic pathway. Furthermore, when comparing S37F, G17R, and T23P to WT at the 60 min time point (Figure 3.2A), these mutants demonstrate a relatively higher concentration of the immature form of apo(a), perhaps suggesting that the intracellular accumulation of these mutants is caused be a decrease in conversion efficiency within the ER. Conversely, R18W and P52L demonstrated a similar trend to WT in the intracellular processing, reaching statistical significance in only three and two time points, respectively. This may suggest that the affect of R18W and P52L on Lp(a) plasma levels may occur at the level of the Lp(a) particle assembly.

The intracellular data (Figure 3.2B) in combination with the secretion data (Figure 3.2C), render it difficult to determine the cause that these SNPs have on the secretion of apo(a). P52L, G17R, and T23P, all demonstrated increased levels of secreted apo(a) when compared to WT, reaching statistical significance in only 2 time points, 3 time points, and 4 time points, respectively. S37F mutant demonstrated an increase in apo(a) secretion, however this was not
found to be statistically significant. In the case of P52L, the similar trend in intracellular accumulation and an increase in secreted protein in the conditioned medium when compared to WT, may suggest a decrease in residence time in the trans-GA. The increased secretion of the other mutants in the conditioned medium and the corresponding increase in intracellular accumulation suggests that this data is faulty and should be repeated to obtain results that are more precise and relate to one another. R18W demonstrated a decrease in apo(a) secreted in the conditioned medium reaching statistical significance in 3 time points and in combination with the similar trend in intracellular accumulation when compared to WT, suggests that perhaps this mutant causes a prolonged residence time in the trans-GA. Moreover, the increase plasma Lp(a) levels associated with the R18W in combination with the data provided in this study, suggests that the increase in Lp(a) plasma levels may be attributed to the affect that this mutant has on Lp(a) particle assembly. Additionally, in the cases of P52L and R18W, where the data suggests that trans-GA residence time has been affected, the corresponding gel image (Figure 3.2A) should demonstrate an increase or decrease in accumulated mature apo(a), respectively, which was difficult to discern.

The KIV7 and the KIV8 domains play a crucial role in the assembly of Lp(a) in that these two domains mediate the noncovalent interaction between apo(a) and apoB-100 of the LDL-like moiety that precede the covalent bond formation; the affinity of the former interaction dictates the efficiency of the latter [47, 82]. This noncovalent interaction is mediated by the WLBS in the aforementioned domains interacting with specific lysine residues in the apoB-100 moiety, bringing the two moieties into close proximity and proper orientation, thereby allowing for efficient covalent bond formation. As such, if the P52L, G17R, and T23P mutants, found in the KIV8 domain cause a decrease in affinity of the noncovalent interaction between the two
moieties, this may decrease the overall efficiency of particle assembly. Unfortunately, due to unsuccessful attempts in generating G17R stable cell lines and time limitations, purified G17R protein could not be generated and so this mutant was not included in the r-Lp(a) assays. Furthermore, statistics could not be performed on the r-Lp(a) assembly assays due to insufficient number of trials; however, a loose interpretation of the date may be suggested but not taken as concrete. Interestingly, T23P and P52L demonstrated an increase and a decrease in particle assembly, respectively (Figure 3.1B). It is possible that the decrease in Lp(a) assembly in the P52L sample may be attributable to the conformational change induced by the amino acid change, potentially compromising the integrity of the WLBS and in turn the interaction with crucial lysine residues on apoB-100. Conversely, the effect of T23P on Lp(a) assembly demonstrated was not expected of T23P with the same premise that pertains to P52L regarding the conformational change that affects the noncovalent step of Lp(a) assembly. However, the study that identified these SNPs suggested that the T23P associated Lp(a) levels may be due to chance since homozygous individuals demonstrated an decrease in Lp(a) plasma levels and heterozygous individuals demonstrated an increase in Lp(a) plasma levels, and that these trends exhibited heterogeneity across different Caucasian populations, though interracial heterogeneity is not unheard of when plasma Lp(a) levels are being discussed [127]. Nonetheless, if considering the decrease in plasma Lp(a) levels regarding patients homozygous for T23P, an increase in r-Lp(a) assembly (Figure 3.1) and an increase in secreted T23P (Figure 3.2C), yields unexpected results since this would likely increase plasma Lp(a) levels. Furthermore, the T23P mutant in the human study was associated with the KIV₂ VNTR, where KIV₂ repeats ranging from 21-25 were present in significantly higher frequencies [127]. It may be of particular interest to repeat these experiments using an isoform size that takes into consideration the identified
association between the T23P mutant and KIV\textsubscript{2} VNTR. Table 4.1 summarizes the data obtained from this study. Effects that reached statistical significance are denoted with asterisks on the Table.

**Table 4.1 Summary of data generated from apo(a) secretion and r-Lp(a) assembly assays**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>In vivo Effects</th>
<th>Effects on intracellular processing</th>
<th>Effects on apo(a) secretion</th>
<th>Effects on in vitro Lp(a) assembly</th>
</tr>
</thead>
<tbody>
<tr>
<td>S37F in KIV\textsubscript{6}</td>
<td>↓[Lp(a)] in plasma</td>
<td>↓ efficiency</td>
<td>↑ efficiency</td>
<td>↑ efficiency</td>
</tr>
<tr>
<td>G17R in KIV\textsubscript{8}</td>
<td>↓[Lp(a)] in plasma</td>
<td>↓ efficiency*</td>
<td>↑ efficiency*</td>
<td>N/A</td>
</tr>
<tr>
<td>T23P in KIV\textsubscript{8}</td>
<td>↓[Lp(a)] in plasma in homozygotes ↑[Lp(a)] in plasma in heterozygotes</td>
<td>↓ efficiency*</td>
<td>↑ efficiency*</td>
<td>↑ efficiency</td>
</tr>
<tr>
<td>P52L in KIV\textsubscript{8}</td>
<td>↓[Lp(a)] in plasma</td>
<td>No apparent change</td>
<td>↑ efficiency*</td>
<td>↓ efficiency</td>
</tr>
<tr>
<td>R18W in KIV\textsubscript{8}</td>
<td>↑[Lp(a)] in plasma</td>
<td>No apparent change</td>
<td>↓ efficiency*</td>
<td>↑ efficiency</td>
</tr>
</tbody>
</table>

It has been observed that apo(a) typically maintains a “closed” conformational state that is maintained by the interdomain interactions between the KIV\textsubscript{10} domain and the N-terminal domains [84, 86]. This “closed” conformation demonstrated a decrease in Lp(a) assembly efficiency by decreasing the rate of covalent bond formation between the two components of Lp(a), possibly by restricting access to the free cysteine residue in the apo(a) moiety [84, 86, 87]. As such, it is possible to alter the rate of covalent bond formation through conformational changes in apo(a) as a whole. The R18W mutant, located in the KIV\textsubscript{9} domain along with the free cysteine residue involved in the covalent assembly, may enhance Lp(a) particle assembly by increasing the rate of disulfide bond formation through a conformational change that could potentially increase the accessibility of the participating cysteine residue in apo(a) for covalent
bond formation with apoB-100. Interestingly, R18W showed an increase in the rate of Lp(a) formation relative to WT (Figure 3.1B). More support for this hypothesis would be the comparable trend observed between R18W and WT in conversion rates (from immature to mature apo(a); Figure 3.2B) and secretion rates (Figure 3.2C).

The r-Lp(a) covalent assembly assay employed in this study may suggest that these mutations could potentially affect the rate of assembly, but could not be used to distinguish whether the noncovalent or covalent step of Lp(a) particle assembly is affected by these mutants. As such, the mutants can be subjected to other previously reported experiments to determine which step of Lp(a) particle assembly these mutants specifically affect. For example, investigating the noncovalent interaction between apo(a) variants and apoB-100 can be achieved by fluorescently labeling human purified LDL with 5’-(iodoacetamido)fluorescein, which covalently binds to free cysteine residues, thereby eliminating covalent assembly between apo(a) and apoB-100. Fluorescein-labeled LDL of known concentration can then be titrated with known concentrations apo(a) variants. Quenching of fluorescence as a result of the noncovalent interaction between apo(a) and fluorescein-labeled LDL, can be measured and dissociation constant values (K_D), reflective of apo(a)’s affinity for LDL, can be deduced as previously reported [85]. Subsequently, covalent assembly of Lp(a) can be studied by incubating radio-labeled apo(a) variants of known concentration with native purified LDL of known concentration and measuring the formation of the intact particle as a function of time, similar to the experiment that was conducted in this study. However, K_D values from the aforementioned noncovalent Lp(a) assays will need to be determined in order to evaluate the effect that these apo(a) variants have on covalent Lp(a) assembly by investigating changes in the rate constant (k) for disulfide bond formation using previously derived equations as reported by Becker and colleagues [82].
The SNPs under investigation are located in the domains that participate in Lp(a) assembly, with the exception of S37F, which is located in the KIV₆ domain. The data in this present study demonstrated an accumulation of intracellular S37F (Figure 3.2B) and an increased S37F in conditioned media (Figure 3.2C), though it did not reach significance when compared to WT. In the Lp(a) assembly assay, S37F demonstrated an increased rate of assembly. These results appear to be inconsistent considering that in humans, Lp(a) levels associated with this SNP were found to be lower and none of the data presented, in the Lp(a) assay or secretion assays, support a potential mechanism for which S37F can decrease Lp(a) levels, unless intracellular accumulation was considered alone [127]. The computational analysis of S37F (Figure 3.3D), demonstrates a significant conformational change when compared to WT. Because of this conformational change and the fact that this domain is adjacent to the KIV₇ domain, which has been implicated in noncovalent assembly, it is possible that the conformational change imposed by S37F may effect the efficiency with which the KIV₇ domain non-covalently interacts with the corresponding lysine residue of the apoB-100 component of the LDL-like moiety. As such, additional experiments should be conducted on this mutant to determine the mechanism for the observed decrease in Lp(a) plasma levels associated with this SNP [127].

The insufficient number of independent r-Lp(a) assembly assays may be attributed to the difficulties posed by successfully transferring the entire relatively large complexed protein (~828 kDa) during the western blot analysis to obtain a clearer image that would subsequently be used for further analysis. Future experiments may want to consider studying the effects that these mutants have on covalent r-Lp(a) assembly using radiolabelling techniques either in a cell system or using purified components, both of which have been previously described. Briefly,
HepG2 cells would be transiently transfected with the expression vectors corresponding to the apo(a) variants as was described in the secretion assays in this current study, incubated with Cys-/Met-DMEM for 1 h, and then incubated with fresh Cys-/Met-DMEM supplemented with radiolabelled sulfur (\(^{35}\)S). Radiolabelled apo(a) and the radiolabelled, endogenously expressed apoB-100 would accumulate and form covalent Lp(a) particles at different times points [102]. Samples would then be subjected to immunoprecipitation, SDS PAGE using a 4% gel using nonreducing conditions, and imaged using the same sequence, as described in the secretion assays in this current study. This technique would eliminate the difficulties associated with incomplete transfers and in turn provide a clearer image for determining densitometric values. Alternatively, a purified system could also be implemented using radiolabelling [102]. Briefly, cell lines stably expressing the apo(a) variants would be incubated with Cys-/Met-DMEM for 1 h then replaced with fresh Cys-/Met-DMEM supplemented with \(^{35}\)S for a longer period of time. Radiolabelled apo(a) variants would be purified from harvested conditioned medium (CM) and LDL would be purified from blood. Both purified components of known concentration would be added to CM from either HepG2 or HEK293 cells, and allowed to form complexes at different time points. Samples would be subjected to immunoprecipitation, SDS PAGE using a 4% gel under nonreducing conditions, and imaged using the same sequence, as described in the secretion assays in this current study. This too would eliminate incomplete transfers, provide a clearer image, and also mock the purified system used in this study.

Computational chemistry was used to determine any conformational changes in the investigated mutants and their corresponding kringle domains (Figure 3.3). When comparing the free cysteine residue that participates in the covalent assembly of Lp(a) in WT and R18W (Figure 3.3E), the variant exhibits a more protruded free cysteine residue from the overall
domain and what appears to be a less sterically hindered free cysteine residue. This may be used to further support the hypothesis of R18W providing increased accessibility to the free cysteine residue, which could potentially increase the rate of covalent assembly.

When analyzing the amino acid residues that make up the WLBS in the KIV₈ of the mutants T23P, G17R, and P52L (Figure 3.3A-C), it appears the residues that make up the WLBS form tighter pocket or perhaps a less accessible one to lysine residues when compared to WT. As such, the reduced accessibility of the WLBS in these mutants may lower the efficiency of the lysine-dependent noncovalent interaction between apo(a) and the apoB-100 component of the LDL moiety, thereby decreasing efficiency of assembly. In the case of P52L and considering the lower concentration of assembled Lp(a) particle observed with P52L, these data may support the hypothesis that the amino acid change lowers the efficiency of the noncovalent interaction as a result of an induced conformational change that reduces the accessibility of this site (Figure 3.3B). Taking into account the tighter WLBS observed in the computational analysis of T23P (Figure 3.3C) and the role that its corresponding domain has in covalent assembly, the in vitro data associated with this variant did not provide expected results in that the rate of r-Lp(a) assembly was increased relative to WT and could suggest that the proposed hypothesis regarding a tighter WLBS and its effect on the lysine-dependent noncovalent assembly is not accurate [127]. In the case of G17R, the conformational change that could potentially reduce the accessibility of the WLBS (Figure 3.3A) may also support a reduced efficiency associated with Lp(a) assembly; however, data for Lp(a) assembly with this particular mutant could not be determined due. Nonetheless, what is loosely interpreted from the presented data is that these amino acid changes in each of the SNPs under investigation cause a significant conformational change in their respective domains relative to the WT domains. Future computational analysis
such as docking and energy minimization analysis with respect to lysine and mutant domains associated with LBSs, would provide more qualitative and quantitative information regarding the integrity and functionality of the LBS as a result of these mutations.

In addition to the computational analysis of the single kringle domains that possess the mutations, it may be of particular interest to look at the overall structure of apo(a) as a consequence of the amino acid changes caused by the SNPs due to the implications that the conformational status of apo(a) has on covalent Lp(a) assembly and the considerable structural changes that are observed in the single kringle mutants (Figure 3.3) [86]. It was postulated that the “closed” conformation of apo(a) is maintained by interdomain interactions between the KIV\textsubscript{10} domain and the N-terminal domains, which do not include the domains that these SNPs are found in. Nonetheless, the structural changes caused by these SNPs in their corresponding kringles may affect, to some extent, the overall structure of apo(a) and possibly the interdomain forces the dictate the conformational state of apo(a) [84, 86, 87]. Furthermore, molecular modelling as previously described, when determining the potential site for which apoB-100 participates in covalent assembly with the KIV\textsubscript{9} of apo(a), identified the P\textsubscript{3732}SCKLDFREIQIYKK peptide of apoB-100 as the energetically favourable site of attachment with apo(a) [90]. Though the site of covalent assembly with apoB-100 has not been identified with 100% certainty, it would still be interesting to observe if there are any differences in energy minimization and docking when performed using the R18W mutant and comparing it to the WT.

It is known that apo(a) is both N-linked and O-linked glycosylated, which takes place in the ER and GA, respectively [73]. It is also known that each kringle domain of apo(a) possesses 3 invariant disulfide bonds and that these bond formations occur in the ER. As such, the conformational changes caused by the SNPs may affect the efficiency of disulfide bond
formation in its corresponding kringle domain or in the overall structure of the apo(a) variant by restricting or enhancing chaperone accessibility. Additionally, these SNPs may also affect the process of N-linked glycosylation, which is initiated in the ER, and has been implicated in proper protein folding. Furthermore, only properly processed and folded variants are permitted to exit the ER and progress through the pre-secretory pathway for further processing; apo(a) maturation continues in the GA with the addition of O-linked glycosylation and N-linked glycan modification [73]. Moreover, it has been observed that the increased ER retention associated with the larger apo(a) isoforms, also increases the susceptibility of apo(a) to the ERAD pathway [73]. Taken together, it may be of particular interest to study relative ER retention times or ratios of these mutants, potentially identifying possible cause for the variance observed with regard to intracellular accumulation, relative variance in conversion rates, and relative variance in secretion rates. This may be achieved by experiments involving stably expressing HepG2 cells and subjecting them to steady state radiolabeling experiments as previously described [104]. The radiolabeled lysates from each mutant would be subjected to endoglycosidase digestion; endoglycosidase H removes high mannose N-linked sugar and proteins exhibit resistance to this form of digestion in the medial GA [243, 244]. Apo(a) would be immunoprecipitated from these samples and relative mobility of apo(a), compared to a control (ie. lysates treated with PBS), at a particular time point would allow determination of the relative ratio of apo(a) transported from the ER to the GA associated with a particular mutant; these data could then be compared to the ratio obtained of the WT variant.

Identifying changes in folding kinetics by way of pulse-chase experiments as previously described [68, 245], could be implemented on stably expressing HepG2 cell lines to determine whether the conformational changes imposed by these SNPs affect apo(a) folding kinetics.
Briefly, at chase times the lysates would be treated with an alkylating agent (to preserve the partially disulfide bonded apo(a) intermediates), apo(a) would then be immunoprecipitated, and relative mobility of the apo(a) intermediates, as well as immature and mature forms of apo(a) would be observed [68]. This method could be used to determine the efficiency of disulfide bond formation with mutant apo(a) variants compared to WT apo(a). This method could also be used to determine whether increased ER retention, if observed, is attributed to differences in folding kinetics or not. In the cases of previously investigated transcript-positive null alleles of apo(a), ER retention was not due to folding kinetics [68], thus other mechanisms involved in apo(a)’s transport from the ER to the GA may be the cause for increased retention [104], but said mechanisms have yet to be determined.

Decreased intracellular degradation of apo(a) and a corresponding increase in apo(a) secretion has been observed with enhanced the interaction between apo(a) and the ER chaperone protein, CXN, after glycosidase I and II processing [74]. Pulse-chase experiments involving stably expressing HepG2 cells with apo(a) variants, as previously described [74], followed by co-immunoprecipitation of apo(a) and CXN in cell lysates, immunoprecipitation of apo(a) in cultured media, would provide some insight as to whether these mutants have an enhanced or reduced interaction with CXN. This experiment could be applied to other chaperones (ie. BiP and PDI) known to interact with apo(a) in the ER. Enhanced or reduced interaction between apo(a) variants and PDI may be provide cause to increased or decreased folding kinetics of apo(a), if observed, due to the role PDI has in catalyzing disulfide bond formation in the ER [246]. Enhanced or reduced interaction with BiP may provide cause to ER retention of apo(a) variants, increased degradation, or decreased secretion, if observed, due to its role in targeting proteins for ERAD and ER retention of misfolded proteins [247, 248].
Subjecting the apo(a) variants to several other Lp(a) assembly assays would be extremely beneficial in determining direct correlations between the mutant apo(a) variants and their effects on efficiency of Lp(a) assembly. As previously described, titrating fluorescently labelled LDL with the apo(a) variants under investigation and observing the relative change in fluorescence compared to WT apo(a), may be one method used to determine the effect that these mutants may have on the non-covalent interaction between the two components of Lp(a) [90]. To study the effects that these mutants may have on covalent assembly with respect to modifying the extent with which the covalent assembly-inhibitory “closed” conformation of apo(a) imposes, could be determined by previously describe methods [84]. Briefly, purified LDL of known concentration may be incubated with radiolabelled apo(a) of known concentration in conditioned media at a fixed time point and titrated with epsilon-aminocaproic acid. If an increase or decrease in covalent Lp(a) assembly is observed in the mutant apo(a) variants when compared to the WT apo(a), this may suggest that the SNPs imposes an overall conformational change on apo(a) that may either reduce or enhance the intermolecular interactions that dictate the “closed” conformational state of apo(a), respectively. Moreover, generating a KIV_{6-8} truncated form of apo(a) containing S37F, T23P, G17R, or P52L amino acid changes, and subjecting them to titration with apoB675-689 or epsilon-aminocaproic acid, measuring the change in intrinsic fluorescence, and comparing it to WT KIV_{8} as previously described, may also provide more supportive evidence as to whether the amino acid changes in the KIV_{8} caused by these SNPs affect the efficiency of the lysine-dependent, non-covalent interaction [82]. The truncated apo(a) variant containing the S37F amino acid change, which is associated with the KIV_{6} domain, may provide insight as to whether the effect it poses on the conformational integrity of its own resident domain, could consequentially pose an effect on that of the adjacent KIV_{7-8} domains that
have been implicated in noncovalent assembly. Other experiments may be implemented to provide insight into the kinetics of noncovalent and covalent assembly; however, the proposed ones utilizing the full length apo(a) protein would provide a more physiologically relevant system since the conformational state of the overall protein effects Lp(a) assembly.

As previously mentioned, Ogorelkova and colleagues sought to identify SNPs specifically in the KIV_{6-9} domain of apo(a) due to their functional implications in Lp(a) assembly. This was accomplished by screening for SNPs in the exons of these respective domains only. Notably, the study did account for differences in the VNTR of KIV\textsubscript{2} to determine whether the observed effects of Lp(a) plasma concentrations associated with these SNPs were independent of the observed effects associated with the VNTR of KIV\textsubscript{2}. This was achieved by measuring the average Lp(a) concentration associated with apo(a) alleles (expected values), with respect to isoform size in the studied population, and estimated differences between the measured Lp(a) plasma concentrations associated with these mutants and the expected value [127]. Moreover, despite the functional implications of these domains in Lp(a) assembly, with the exception of the KIV\textsubscript{6} domain’s lack of involvement in Lp(a) assembly, it is important to mention that the association of these mutants with Lp(a) plasma concentrations could possibly be due to allelic associations with unidentified mutations in other parts of the apo(a) gene, which was minimally considered in this study. Indeed, linkage disequilibrium phenomena have been observed in \textit{LPA}. For example, the C to T polymorphism found +93 of the transcription initiation site has been found to be in linkage disequilibrium with apo(a) alleles possessing 24-34 kringle domains and with 9 PNRs [249]. As such, neither the interpretation of the data presented nor the study conducted by Ogorelkova and colleagues can conclusively assert that the Lp(a) plasma levels associated with these mutants are due to their potential effects on Lp(a) particle assembly.
In conclusion, the interpretation of the data presented in this study should only be taken as speculation considering all samples did not reach statistical significance (secretion assays), the lack of sufficient number of trials in order to complete statistical analysis (r-Lp(a) assembly assays), incomplete data sets (G17R mutant in r-Lp(a) assay), and a clear lack of precision (large error bars in data sets). Nonetheless, the molecular modelling in combination with the limited presented data allowed for some preliminary speculation of causative mechanisms for the investigated SNPs and its respective effects on Lp(a) plasma concentration using an in vitro system. The data presented suggests that the observed increase and decrease Lp(a) plasma levels in humans associated with R18W and P52L mutants, are due to enhanced and reduced efficiency of Lp(a) assembly, respectively. G17R mutant demonstrated intracellular accumulation and increased secretion, when taken together could not explain the decrease in human Lp(a) plasma concentration associated with this mutant. Similarly, S37F mutant demonstrated intracellular accumulation, increased secretion, and an enhanced efficiency of Lp(a) assembly, when taken together could not be used to explain the decrease in human Lp(a) plasma concentration associated with this mutant. Lastly, T23P demonstrated an increase in intracellular accumulation, increase in secretion, and an enhanced efficiency in Lp(a) assembly, when taken together could not be used to explain the decrease in Lp(a) plasma concentration in individuals homozygous for this variant. As previously mentioned, these assays should be repeated with a more meticulous execution in combination with other experiments that were discussed to properly and reliably identify the mechanistic details affecting human Lp(a) plasma levels caused by these mutants.
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APPENDIX

SUPPLEMENTAL DATA

A.1 Expression & secretion of mutant variants in HEK293 and HepG2 cells

After confirming that the single nucleotide polymorphisms corresponding to the mutant apo(a) variants G17R, R18W, S37F, P52L, and T23P, were successfully achieved via DNA sequencing services provided by Robarts Research Institute, the successful insertion of the mutant fragments into the 17KΔΔSmaI pRK5 expression vector needed to be confirmed. This was achieved by transient transfection of the full length mutant expression vectors into HEK293 and HepG2 cells and comparing it to the WT expression vector and a purified 17K apo(a) standard.

Figure A.1: Expression and secretion of mutant variants in HEK293 and HepG2 Cells.
HEK293 cells and HepG2 cells were transiently transfected with expression vectors coding for apo(a) variants investigation as described in Chapter 2.4. Conditioned medium and cell lysates at 8 h were immunoblotted for using an anti-apo(a) antibody (a5) and gel images of each cell type: HEK293 (A) and HepG2 (B) were obtained. Variants were compared to a purified 17K apo(a) standard (17K std) to assess correct size. In both cell types, the lysates demonstrated a lower precursor form of apo(a) (p-apo(a)) representing the hypoglycosylated, immature form of apo(a) and
a higher band representing a mature form of apo(a) (m-apo(a)). In both cell types, one apo(a) band was detected in the conditioned medium (CM), corresponding to the mature, secretable form of apo(a). The same trend was observed for the wild-type (WT) variant of apo(a) as in the mutant forms: P52L, S37F, R18W, T23P, and G17R.

A.2 Assessing stably expressing HEK293 cell lines with mutant apo(a) variants

After confirming the expression and secretion of the mutant variants, it was important to generate stably expressing HEK293 cell lines as described in Chapter 2.5. Conditioned media from these stably expressing cells were harvested and mutants were purified using Lysine-Sepharose affinity chromatography, as previously described [51]. Purified mutants were immunoblotted for and compared to a 17K apo(a) standard.

Figure A.2: Detection of mutant variants from stably expressing HEK293 cells. Conditioned medium was harvested from HEK293 cell lines stably expressing mutants: P52L, S37F, R18W, and T23P. Mutants were purified and subjected to immunoblotting using an anti-apo(a) antibody. One band was detected from each of the mutants and compared to a purified 17K apo(a) standard.
Purity was confirmed with the lack of nonspecific bands and the comparably equal size when comparing relative mobility with 17K std.

A.3 Transfection efficiency & pulse-chase data

Pulse-chase analysis of the apo(a) variants was achieved as described in Chapter 2.6. HepG2 cells were co-transfected with pRL-TK Renilla Luciferase reporter vector and luciferase activity was detected for each apo(a) variant and trial in order to account for differences in transfection between different samples and trials. Densitometric values of pulse-chase experiments were normalized to the corresponding luciferase activity. An example of the calculation is demonstrated in Table 5.1. CM BC Avg and Lys BC Avg represent the raw data obtained from densitometric analysis. This raw data was normalized to its specific transfection efficiency illustrated by the luciferase activity (Table 5.1; Trans. Eff.) by dividing the raw data by this value. This provides us with data that takes into consideration the transfection efficiency (Table 5.1; Norm. TE CM; for normalized conditioned media, Norm. TE LYS; for normalized lysates). After taking into account transfection efficiency, the resulting values were then normalized to the greatest WT value in either the conditioned media or lysates to give rise to the data used for statistical analysis (Table 5.1; Norm. CM; normalized conditioned media, Norm. LYS; normalized data). Mutants of the same trial were normalized to the greatest WT value in either the conditioned media or lysate. Statistical analysis was achieved using values corresponding to the Norm. CM and Norm. LYS for each apo(a) variant (WT and mutants) and calculating the average of all trials. After which, a two-tailed student’s T-test assuming unequal variances was performed in order to determine statistical significance.
Table A.1: Example calculation of transfection efficiency and pulse-chase normalization

<table>
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<tr>
<th>Time (mins)</th>
<th>Trans. Eff.</th>
<th>Norm. TE CM</th>
<th>Norm. TE LYS</th>
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<td>Lys BC Avg.</td>
<td>Norm. CM</td>
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<tr>
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A.4 Detection of LDL in purified sample

LDL was purified from the blood of a patient, with written consent, who possesses no detectable form of Lp(a). This was achieved as described in Chapter 2.10. The detection of LDL was assessed using SDS PAGE, followed by silver staining analysis and imaged. Successfully purified LDL was subjected to r-Lp(a) assembly assays.
**Figure A.3: Silver stain analysis of LDL purification from patient.** Purified low density lipoprotein (LDL) samples were subjected to SDS PAGE, followed by silver staining analysis. The first lane contains a protein ladder standard (std.) and the second lane contains a single band corresponding to purified LDL. kDa; kilodaltons.

**A.5 Pulse-chase data without normalizing to luciferase activity**

The data generated in Figure 3.2 represents results normalized to luciferase activity to account for differences in transfection efficiency and cell numbers on the experimental basis that cells will express both plasmids in a 1:1 ratio. However, large variability was observed in the Renilla luciferase values, which we would have expected to be similar between different wells. It is possible, therefore, that accounting for the Renilla luciferase activity may have added variability to the data, rather than controlling for it. As such, data for pulse-chase experiments were generated without normalizing to luciferase activity (Appendix A.5; Figure A.4). Table A.2 summarizes the data obtained from both data analyses. Effects that reached statistical significance are denoted with asterisks on the Table.

**Table A.2 Summary of data generated from apo(a) secretion and r-Lp(a) assembly assays**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>In vivo Effects</th>
<th>Effects on intracellular processing</th>
<th>Effects on apo(a) secretion</th>
<th>Normalized to Luciferase Activity</th>
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<td></td>
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<td>Effects on intracellular processing</td>
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<tr>
<td>S37F in KIV(_6)</td>
<td>↓[Lp(a)] in plasma</td>
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<td>↓ efficiency*</td>
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<tr>
<td>G17R in KIV(_8)</td>
<td>↓[Lp(a)] in plasma</td>
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<td>↓ efficiency*</td>
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<td>T23P in KIV(_8)</td>
<td>↓[Lp(a)] in plasma in homozygotes ↑[Lp(a)] in plasma in heterozygotes</td>
<td>↓ efficiency*</td>
<td>No apparent change</td>
<td>↓ efficiency*</td>
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<tr>
<td>P52L in KIV(_8)</td>
<td>↓[Lp(a)] in plasma</td>
<td>No apparent change</td>
<td>↑ efficiency</td>
<td>No apparent change</td>
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<tr>
<td>R18W in KIV8</td>
<td>↑[Lp(a)] in plasma</td>
<td>No apparent change</td>
<td>No apparent change</td>
<td>No apparent change</td>
</tr>
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</table>

In contrast with the results obtained in Figure 3.2B-C that consider transfection efficiency, the data obtained in Figure A.4A-B appear to represent a more precise analysis as indicated by the error bars. The two data sets demonstrate a similar trend when comparing intracellular processing of the apo(a) variants and differs by a few observations regarding the efficiency of apo(a) variant secretion. The R18W mutant demonstrated no apparent change in apo(a) secretion that was not statistically significant in the non-normalized data. P52L demonstrated the same trend between both sets, but was statistically significant in the normalized data. The T23P and G17R mutant demonstrated statistical significance in both sets of intracellular processing data. The non-normalized data suggests no apparent change, whereas the normalized data suggests that T23P and G17R only slightly increased apo(a) secretion efficiency, reaching statistical significance in only T23P. The S37F mutant demonstrated statistical significance in the non-normalized data and the trend differed between the two sets with regard to apo(a) secretion. The non-normalized data demonstrates a decrease in apo(a) secretion efficiency, while the normalized data demonstrates an increased. Presently, it is difficult to say which data set is more representative of the secretion assays since considerable variability is presented in both cases. As such, more independent trials need to be performed.
Figure A.4: The effect of 17K apo(a) mutant variants on apo(a) secretion from HepG2 Cells. Pulse-chase analysis of HepG2 cells transiently expressing either WT, P52L, S37F, T23P, G17R or R18W. Cells were starved in Cys-/Met- medium for 60 minutes, pulse-labelled for 60 minutes with $^{35}$S-Met/Cys, and subsequently chased in unlabelled media for 0, 30, 60, 120, 240, and 480 minutes. (A) Graphical representation of the resulting densities of the intracellular apo(a) variants (combination of both mature and immature apo(a) forms) at the corresponding chase
times. (B) Graphical representation of the resulting densities of secreted apo(a) variants at the corresponding chase times. Densitometric analysis was performed using AlphaView where the mutant variants were normalized to the maximum density observed for the wild type apo(a) variant and plotted as a function of time. Significance compared to wildtype apo(a) are represented by asterisks, where *p<0.05 and n=5 (5 independent experiments). The colours correspond to the different mutant as indicated in the legend.
A.6 R-Lp(a) Assay

The quantitative data generated for the r-Lp(a) assay discussed in Chapter 3.1 was the average experimental values obtained from two independent trials. The second immunoblot involved in generating the data in Chapter 3.1 is shown below.

![Western blot image of apo(a) variants WT, P52L, S37F, T23P, and R18W purified from stably expressing HEK293 cells coupled with purified LDL to form r-Lp(a). Recombinant Lp(a) was separated from free apo(a) by SDS PAGE on a 5% gel and visualized by immunoblotting and imaging on a Fluorochrom Q Imager.]

**Figure A.5: Immunoblot of r-Lp(a) assembly.** Western blot image of apo(a) variants WT, P52L, S37F, T23P, and R18W purified from stably expressing HEK293 cells coupled with purified LDL to form r-Lp(a). Recombinant Lp(a) was separated from free apo(a) by SDS PAGE on a 5% gel and visualized by immunoblotting and imaging on a Fluorochrom Q Imager.
VITA AUCTORIS

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PUBLICATIONS:


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