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Oxidative Modifications of Apolipoprotein(a): Implications for Proinflammatory and Prothrombotic Roles of Lipoprotein(a) in the Vasculature

By

Corey A. Scipione

A Dissertation
Submitted to the Faculty of Graduate Studies
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy
at the University of Windsor

Windsor, Ontario, Canada

2016

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Oxidative Modifications of Apolipoprotein(a): Implications for Proinflammatory and Prothrombotic Roles of Lipoprotein(a) in the Vasculature

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DECLARATION OF CO-AUTHORSHIP AND PREVIOUS PUBLICATION

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

This dissertation incorporates the outcome of joint research in collaboration with Dr. Santica Marcovina, Dr. Sotorios Tsimikas, Dr. Robert Hegele, and Dr. Amir Ravandi under the supervision of Dr. Marlys Koschinsky. In all cases, key ideas, primary contributions, experimental design, execution, data analysis, and interpretation was performed by the author. Additionally, manuscripts were written initially by the author and revised and edited by Dr. Michael Boffa and Dr. Marlys Koschinsky for Chapters 2, 3, and 4.

- In chapter 2 of this dissertation; collaboration with Dr. Amir Ravandi was through providing analysis of samples by mass spectrometry. Dr. Rocco Romagnuolo contributed through intellectual contribution.
- In chapter 3 of this dissertation; collaboration with Dr. Santica Marcovina was through providing purified Lp(a) and anti-apo(a) antibody. Dr. Sotorios Tsimikas contributed through intellectual contribution. Ms. Sera Sayegh conducted replicates of the luciferase reporter experiments.
- In chapter 4 of this dissertation; collaboration with Dr. Robert was through providing patient plasma homozygous for the I4399M SNP.
- Collaboration with JT. McAiney, Z. Bazzi, D. Simard is covered in Chapter 4 of this dissertation; the contribution of Mr. McAiney was performing the assays and data analysis on *in vitro* coagulation and fibrinolysis assays, as well as participated in the permeation of plasma clots. Ms. Bazzi imaged the fluorescent clots by confocal microscopy. Computational simulations were performed by Mr. Simard. Dr. Rocco Romagnuolo consulted on plasminogen activation assays and contributed fluorescently labeled recombinant plasminogen.

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This dissertation includes 4 original manuscripts that have been previously published, submitted, or is in preparation for publication in peer reviewed journals, as follows:

Dissertation Chapter	**Publication Title and Full Citation**	**Publication Status**
Chapter 2	Scipione CA, Romagnuolo R, Boffa MB, Ravandi A, Koschinsky ML. Characterization of the oxidized phospholipid modification of apolipoprotein(a) kringle KIV_{10}: Insights into the site of oxPL addition.	In preparation
Chapter 3	Scipione CA, Sayegh SE, Romagnuolo R, Tsimikas S, Marcovina SM, Boffa MB, Koschinsky ML. Mechanistic insights into Lp(a)-induced IL-8 expression: a role for oxidized phospholipid modification of apo(a). J Lipid Res. 2015 Dec; 56(12):2273-85.	Accepted
Chapter 4	Scipione CA, McAiney JT, Simard DJ, Bazzi ZA, Romagnuolo R, Gauld JW, Boffa MB, Hegele RA, and Koschinsky ML. Characterization of the I4399M variant of apolipoprotein(a): implications for altered structure and prothrombotic properties of lipoprotein(a).	Prepared for submission

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ABSTRACT

Elevated plasma concentrations of lipoprotein(a) (Lp(a)) have been identified as a causal risk factor for calcific aortic valve disease (CAVD) and coronary heart disease (CHD). Relationships have recently been identified for genetic factors, such as single nucleotide polymorphisms (SNPs) in the *LPA* gene, specifically r10455872 and rs3798220, that have been correlated with increased Lp(a) plasma levels and risk of cardiovascular disease (CVD). Apo(a) bears striking homology with the zymogen plasminogen and possesses several similar structural features. A key feature shared between these proteins is the presence of multiple repeats of a kringle domain, which possesses the ability to bind to exposed lysine residues with high affinity. Apo(a) contains several copies of a plasminogen like KIV domain, one of which, KIV_{10}, has been implicated in many proinflammatory processes *in vitro.* It has been hypothesized that the proinflammatory potential of Lp(a)/apo(a) is derived from the ability to be covalently modified by an oxidized phosphatidylcholine (oxPC) moiety. The work in this dissertation assesses the mechanism by which the oxPC moiety on apo(a) stimulates interleukin-8 (IL-8) production in macrophages. Targeted mutagenesis was used to determine a role for the KIV_{10} strong lysine binding site (sLBS) in the covalent addition of the oxPC moiety on apo(a) and identified histidine 33 of KIV_{10} as the site of covalent oxPC modification. Furthermore, characterization of the I4399M variant of apo(a), resulting from the rs3798220 SNP, from a perspective of its distinct structural and functional properties, revealed roles for the polymorphism on the structure and permeability of purified fibrin and plasma clots. The enhanced prothrombotic potential of this variant may be a result of an oxidized methionine residue, as identified by mass spectrometry. The identification of distinct functional

properties associated with the oxidative modification of Lp(a)/ apo(a) offers insights into its proatherosclerotic and prothrombotic potentials.

I dedicate this work to my family and friends, for the unwavering support, guidance, and encouragement that you continue to share

ACKNOWLEDGEMENTS

I would like to extend my sincerest gratitude to my supervisor, Dr. Marlys Koschinsky for accepting me into her research lab. The support, knowledge, and challenges that you have given to me have not only changed my path but also the attitude with which I will approach my future. You have provided me with many great opportunities that I am grateful for. I would also like to thank Dr. Michael Boffa for all of your constructive feedback over the years. Especially for your ability to turn my words into gold.

I would also like to thank my committee members, Dr. Vacratsis and Dr. Hubberstey, as well as all other members of the Chemistry and Biochemistry department for many helpful conversations over the years, the use of equipment, and the support that was shown to me in my endeavours.

I would like to thank all of the current and past members of the Koschinsky and Boffa labs that have helped turn everyday into an experience to be remembered. I would like to extend a special thank you to my best friend and colleague, Dr. Rocco Romagnuolo. Whether you were teaching me, challenging me, or just being a friend; you have forever changed the person I am and the path my life will take. Another thank you to Dr. Zainab Bazzi for always showing me kindness. You are always around when I needed help, support, and making sure that I never go hungry. Thanks to my buddy Justin for always finding a way to brighten every day. Thank you to all other members of the of the lab who contributed to my graduate student experience, which include Ana, Jackson, Christina, Sera, Nicole, Tanya and Matt, Scott, Victor, Mousa, Dragana, Danielle, Emad, Sudipa, and Zach.

Thank you to members of the department for all of the opportunities and experiences that I will remember the support and laughter in the years to come; Ashley, Anna, Cody, Terrance, Artur, Bei, LeeAnne, Jill, Chris, Steph and any friends that I may have forgotten to mention.

Finally, I would like to extend a thank you to my sisters, for their continued friendship and support; my niece and nephew, for bringing so much joy to my life; and to my parents, who never cease in their constant strength, encouragement, and love.

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LIST OF ABBREVIATIONS

Apo(a)	Apolipoprotein(a)
$ApoB_{100}$	Apolipoprotein B-100
ASO	Antisense oligonucleotide therapy
Asp	Aspartic acid
Arg	Arginine
CAMs	Cell adhesion molecules
CAVD	Calcific aortic valve disease
CAVS	Calcific aortic valve stenosis
CHD	Coronary Heart Disease
ε-ACA	ε-aminocaproic acid
EC	Endothelial cell
eNOS	endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ECM	Extracellular matrix
FXR	Farnesoid X receptor
His	Histidine
HUVEC	Human umbilical vein endothelial cell
IL-6	Interleukin-6
IL-8	Interleukin-8
ICAM-1	Intracellular cell adhesion molecule 1
Glu	Glutamic acid
KI	Kringle 1
KIV	Kringle 4
KIV_n	Kringle 4, subtype *n*
KV	Kringle 5
LDL	Low density lipoprotein

LDLR	Low density lipoprotein receptor
LOX	Lipoxygenases
LOX-1	Lectin-like oxidized LDL receptor
Lp(a)	Lipoprotein(a)
Lys	Lysine
Met(O)	Methionine sulfoxide
MCP-1	Monocyte chemoattractant protein-1
MDA	malendialdehye
mRNA	Messenger ribonucleic acid
NO	nitric oxide
oxLDL	oxidized low density Lipoprotein
oxPC	Oxidized phosphatidylcholine
oxPL	Oxidized phospholipids
P	Protease domain
PCSK9	Proprotein convertase subtilisin/kexin type 9
PL	Phospholipid
PLA_2	Phospholipase A_2
PAI	Plasminogen activator inhibitor
PAF	Platelet activating factor
PDGF	platelet derived growth factor
Phe	Phenylalanine
PUFA	Polyunsaturated fatty acid
r-apo(a)	recombinant apolipoprotein(a)
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphism
sLBS	strong lysine binding site
SMC	Smooth muscle cell

T	Tail domain
TFPI	Tissue factor pathway inhibitor
TGF-β	Transforming growth factor beta
TNF-α	tumour necrosis factor alpha
tPA	Tissue-type plasminogen activator
uPA	Urokinase plasminogen activator
Trp	Tryptophan
VCAM-1	Vascular endothelial adhesion molecule 1

General Introduction

1.1 LIPOPROTEIN(a)

Lipoprotein(a) (Lp(a)) was discovered in 1963 by Kåre Berg and constitutes a distinct class of lipoprotein particle found within the human plasma.[1, 2] Lp(a) contains a lipoprotein moiety that is essentially indistinguishable from low density lipoprotein (LDL); and contains a cholesteryl ester and triglyceride rich core, a cholesterol/phospholipid outer monolayer) as well as apolipoprotein B_{100} (apoB_{100}). However, Lp(a) is clearly distinguished from LDL by the presence of a unique glycoprotein, apolipoprotein(a) (apo(a)), that is covalently linked to apoB_{100} by a single disulfide bond (Figure 1.1).[3, 4] The apo(a) moiety is a hydrophilic component due to its immense level of glycosylation with carbohydrates accounting for approximately 28% of the total mass of the molecule, and also lacks the large hydrophobic sequences usually observed in lipoproteins.[5] In 1987, analysis of the human liver apo(a) cDNA sequence identified a striking similarity of apo(a) to the fibrinolytic plasma proenzyme plasminogen.[6] Although the physiological function(s) of Lp(a) have not yet been fully elucidated, studies within the past 8 years have identified Lp(a) as a causal risk factor for coronary heart disease (CHD) [reviewed in ref. 7]. There is now evidence to suggest that Lp(a) may be the strongest genetic risk factor for CHD [Reviewed in ref. 8]. Recently, a causal relationship was described between elevated plasma Lp(a) levels and calcific aortic valve disease (CAVD) [9, 10]

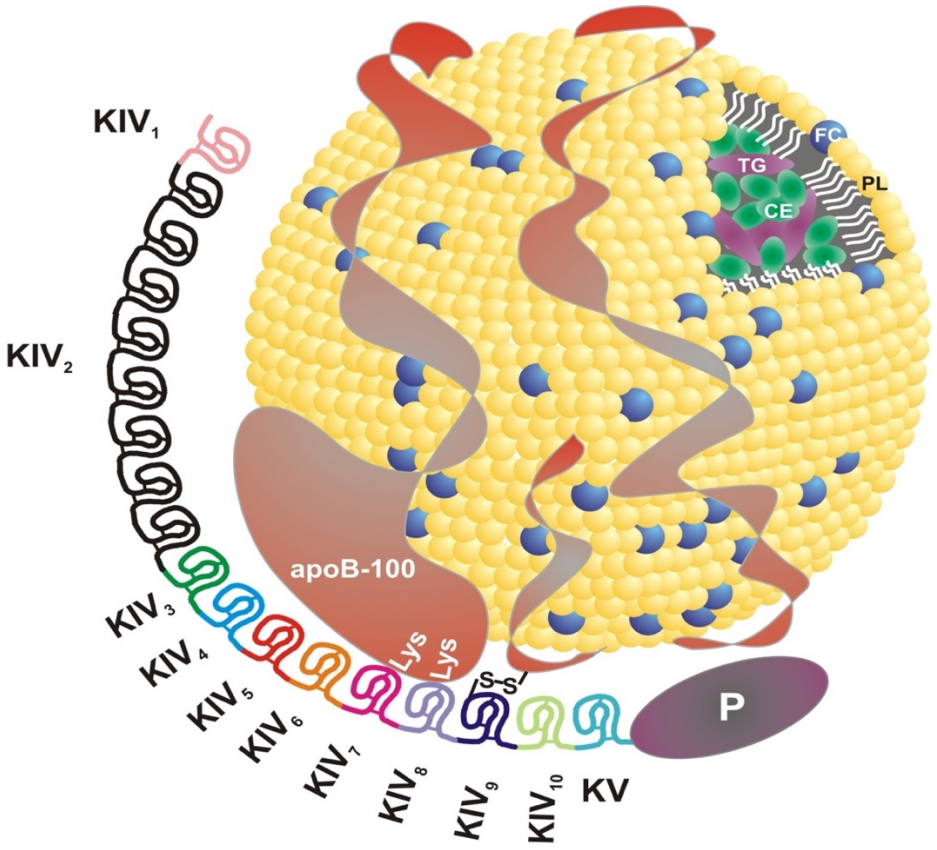

Figure 1.1 Representation of the structure of Lp(a).
Lipoprotein(a) (Lp(a)) is a distinct lipoprotein class that is similar to LDL in lipid composition and presence of apolipoproteinB-100 (apoB-100), but is distinguished from LDL by the presence of apolipoprotein(a) (apo(a)). The LDL-like moiety is composed of a triglycerides (TG) and cholesteryl esters (CE) core, surrounded by an outer monolayer of phospholipids (PL) and free cholesterol (FC). Apo(a) consists of multiple copies of a plasminogen-like kringle IV (KIV) domain, followed by a single copy of each of the plasminogen-like kringle V (KV) and a protease (P) domains. KIV domains in apo(a) can be further classified into 10 distinct subtypes (KIV_1 to KIV_{10}) based on amino acid sequence with the number of identically repeated KIV_2 domains varying in the population from 3 to >30. Lysine binding sites (LBSs) in KIV_7 and KIV_8 play a key role in non-covalent association with two lysine residues in the amino-terminal region of apoB-100. This initial non-covalent interaction between apo(a) and apoB-100 facilitates disulfide bond formation between a free cysteine in KIV_9 of apo(a) and apoB-100.[4] Adapted from reference 4.

Although the duality of the structure of this molecule, with similarities to both LDL and plasminogen, suggests that Lp(a) can elicit both proatherogenic and prothrombotic effects within the vasculature, there have been many potential pathophysiological roles proposed for Lp(a) that cannot be attributed to either the LDL or plasminogen-like components alone.

Circulating levels of plasma Lp(a) vary widely within the population, from nearly undetectable to greater than 100 mg/dL. The primary determinant of Lp(a) levels is the *LPA* gene itself, attributing for ~90% of Lp(a) plasma concentration.[11] The size of the *LPA* gene, as determined by the number of sequences encoding the KIV_2, accounts for approximately 69% of this variation, while 22% of the genetic variability in plasma Lp(a) levels is attributed to *cis*-acting sequences in the gene that are yet to be fully understood (reviewed further in Section 1.1.4).[11] Relationships have recently been identified for single nucleotide polymorphisms (SNPs), rs10455872 and rs3798220, in the *LPA* gene.[12] In general, an inverse correlation exists between apo(a) size and circulating Lp(a) levels, with small isoforms present in much higher concentrations than larger isoforms.[13, 14] This is explained at least in part by the inability of larger isoforms to be secreted from hepatocytes at the same rate as smaller isoforms.[14] It was determined that due to the complex folding required by multiple kringle domains, more time is required to properly fold an apo(a) molecule that contains a larger number of KIV domains. This results in an increase in endoplasmic reticulum (ER) retention time leading to ER assisted degradation, and thus lower amounts of secreted protein.[15] It is important to note that plasma levels of Lp(a) are dictated primarily by their rate of production and not by the efficiency by which they are

removed from circulation.[16] The specific pathways for Lp(a) catabolism are controversial, and the extent to which they contribute to Lp(a) plasma levels in both normal and disease states remains unclear.[17]

New drug therapies developed to specifically lower Lp(a) levels are currently under investigation. Antisense oligonucleotide (ASO) therapy targeting apo(a) is currently in Phase II clinical trials.[18] All currently available methods of lowering plasma Lp(a) levels also affect levels of other lipoproteins as well, as reviewed in Section 1.1.7. Thus, it has been difficult to assess whether lowering Lp(a) plasma concentrations reduces risk for CHD or other atherothrombotic disorders. A comprehensive understanding of the pathophysiological molecular mechanisms through which Lp(a) contributes to the progression of atherogenesis is currently lacking.[19] This has precluded the development of therapeutic strategies aimed at reducing the harmful effects of Lp(a) in the vasculature.

1.1.1 Structure of Lp(a)

Lipoprotein(a) presents as a unique lipoprotein particle identical to LDL with the exception of the unique glycoprotein apolipoprotein(a). Apo(a) bears striking homology to the human plasma zymogen plasminogen and possesses many of the same key features.[2] Plasminogen, in its proenzyme form, contains a short tail domain (T), followed by five sequential copies of a structural domain known as a kringle. These kringles vary in amino acid sequence from one another, and are annotated as kringle 1 (KI) to kringle 5 (KV). Following the kringle domains is a trypsin-like serine protease domain (P), which can be cleaved by urokinase plasminogen activator (uPA) or tissue-type plasminogen activator (tPA) resulting in the active fibrinolytic enzyme plasmin (Figure 1.2). Apo(a) contains ten

subtypes of a plasminogen-like kringle IV (KIV) domain, each present in a single copy, with the exception of apo(a) KIV_2 which can be present in a variable number of identically repeated copies (from less than 3 to greater than 30 identical copies; Figure 1.2).These kringle domains are annotated as kringle IV subtypes one through ten (KIV_1-KIV_{10}), and are connected in tandem by serine-, threonine-, and/or proline-rich inter-kringle polypeptide linkers.[20] Carboxyl-terminal to the KIV domains, apo(a) possesses domains that are highly similar to the plasminogen KV and protease domains (Figure 1.2). The protease-like domain in apo(a) has been rendered inactive by amino acid substitutions that convert the Arg-Val cleavage motif, recognized by plasminogen activators, to a Ser-Ile sequence, as well as a 9 amino acid deletion near the domain terminus.[21]

1.1.2 Lysine-binding properties of apo(a)

Kringle domains are present in many important proteins in the coagulation and fibrinolysis cascades, such as plasminogen, prothrombin, tPA, and Factors VII, IX, and X. In the case of plasminogen, the high affinity of kringle domains to exposed lysine residues allows plasminogen to interact with its ligands, such as fibrin. These binding interactions serve to act as a means to accelerate the activation of plasminogen to plasmin, and allow plasmin to act on its fibrin substrates.[22] The possession of lysine binding kringle domains is essential for the ability for plasmin to be generated and to dissolve fibrin clots. These structural kringle motifs are defined by a tri-looped structure consisting of approximately 80 amino acids, containing three invariant intramolecular disulfide bonds and are largely considered to function as protein binding domains.[23] The kringle domains within apo(a) vary in amino acid sequence and thus can possess distinct functional features. For example,

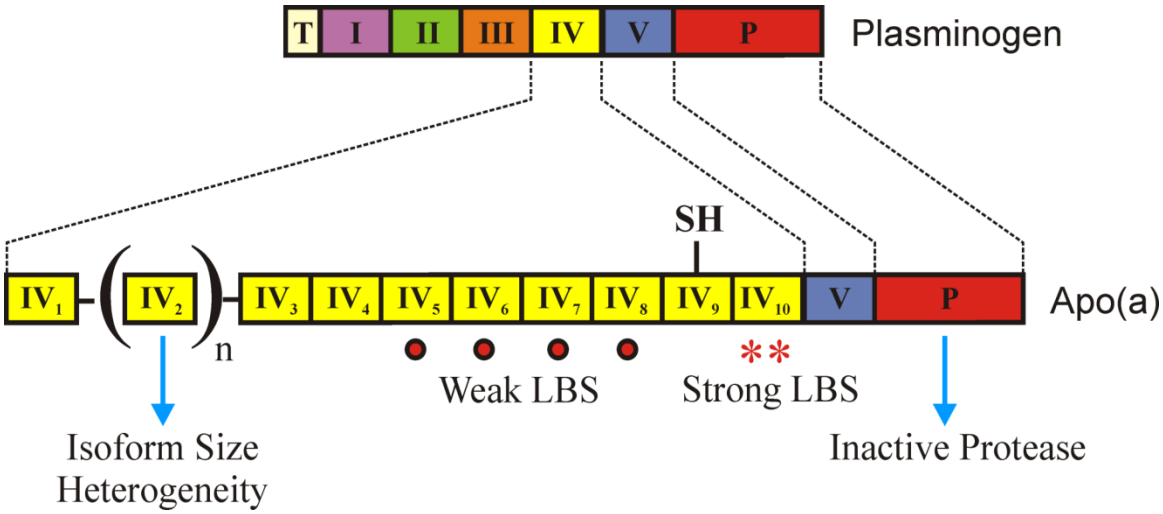

Figure 1.2 Structural similarities between plasminogen and apo(a).
Plasminogen consists of an amino terminal tail sequence (T) followed by five kringle domains (KI-KV) and a serine protease domain (P). Apo(a) contains a number of repeated domains which are highly similar to that of plasminogen kringle IV (KIV), followed by single copies of a plasminogen kringle V (KV) and protease-like (P) domains. Kringle IV domains of apo(a) can be grouped into 10 distinct types on the basis of amino acid sequence with each present in a single copy except for KIV type 2 (KIV_2). The number of KIV_2 domains ranges from 3 to greater than 30 identically repeated copies which gives rise to the Lp(a) isoform size heterogeneity observed in the population. Unlike plasminogen, the protease domain of apo(a) is catalytically inactive. The weak lysine binding sites (LBS) and strong LBS of apo(a) (indicated with circles and asterisks respectively) are important for many interactions between apo(a) and biological substrates. The cysteine (SH) present in KIV_9 of apo(a) is required for disulfide bond formation to the C-terminal region of apoB-100.[2] Adapted from reference 4.

apo(a) KIV_{5-8} each possess "weak" lysine binding properties based on their ability to bind to the lysine analog ε-aminocaproic acid (ε-ACA). The weak lysine binding sites (LBS) in KIV types 7 and 8 have been shown to mediate the non-covalent association of apo(a) with $apoB_{100}$ that precedes disulfide bond formation between the unpaired cysteine in apo(a) with apoB-100.[24, 25] The apo(a) KV domain also contains a weak lysine binding site and may play roles in endothelial cell tube formation and fibrin binding.[26-28] The KIV_{10} domain possesses the greatest similarity with plasminogen KIV and contains a relatively strong lysine binding site (sLBS).[29] This feature of KIV_{10} has been implicated in the ability of apo(a) to bind to exposed lysine residues on different substrates, such as fibrin and cell surface receptors and has been implicated in potential prothrombotic functions of apo(a).[30] Additionally, the sLBS in apo(a) has been implicated in vascular cell function.[31-36]

The structure of the lysine binding site has been well defined and generally consists of a hydrophobic trough that forms an indentation on the surface of the kringle; this trough is lined with hydrophobic amino acids and contains both a cationic and an anionic cluster at either end.[37] Overall, it has been determined that in apo(a) KIV_{10} the lysine binding pocket requires seven specific amino acids composing the hydrophobic trough (Trp^{60}, Phe^{62} and Trp^{70}), cationic centre (Asp^{54} and Asp^{56}) and anionic centre (Arg^{35} and Arg^{69}), in order to maximally bind to lysine or lysine analogs (Figure 1.3).[24, 29] Due to differences in the amino acid sequences of each apo(a) kringle domain, there are different theoretical and reported affinities for lysine and lysine analogues for each domain.[26, 29] For example, the KIV_5 LBS shares much homology to plasminogen KI but lacks a cationic centre and is predicted to have a reduced affinity for lysine.[38] X-ray crystallographic modelling of KIV_7

revealed a smaller LBS when compared to KIV_{10}, resulting from mutations of the critical residues Asp^{56} and Tyr^{62} in the LBS to Glu and Phe respectively.[39, 40] The Asp^{56} → Glu substitution is also present in KIV_{5-8}. A functional analysis of the KIV_7 binding domain reported a 10-fold decrease in the ability to bind lysine when compared to KIV_{10}[29]; these differences in affinity to lysine underlie important functional differences that have been reported in these apo(a) KIV domains.[41] KIV_9 does not contain the necessary requirements for an intact LBS and possesses very low affinity for lysine.[42] However this domain is unique in that it contains the only unpaired cysteine residue in the entire apo(a) molecule.[43] Using site directed mutagenesis, it was determined that this residue is responsible for the disulfide bond formation with the $apoB_{100}$ component of LDL.[3]

Each KIV sequence also includes an interkringle region that bridges each distinct domain. These polypeptide linkers are approximately 30 amino acids in length and are the location of the potential O-linked glycosylation sites on apo(a) due to the high Ser/Thr content of these sequences.[20] There is also at least one N-linked glycosylation site within each KIV domain.[44] Together, the O-and N-linked carbohydrates contribute approximately 28% of the mass of an apo(a) molecule, are rich in sialic acid, are heterogeneous in total carbohydrate content and are important for secretion of apo(a).[5, 44-46]

The complex nature of the structure of Lp(a) allows it to be a multi-ligand binding protein. In particular, the lysine binding potential of the apo(a) moiety has been implicated in many pathophysiological processes, both antifibrinolytic and proatherosclerotic.[47, 48]

1.1.3 Species Distribution of *LPA*

The gene encoding apo(a), *LPA*, resides on chromosome 6q26 and originated as a consequence of a gene duplication and remodeling event of *PLG*, the gene encoding plasminogen.[6, 49] The *PLG* gene is ubiquitous across all species, however *LPA* appeared late in primate evolution following the divergence of New and Old World monkeys approximately 80 million years ago.[49] As such, Lp(a) is only present in humans, apes, and Old World monkeys. The European hedgehog also contains an Lp(a)-like particle. However, hedgehog apo(a) is composed of multiple repeating copies of a plasminogen-like KIII domain.[49] This likely represented an independent *PLG* duplication event, distinct from the one that gave rise to human Lp(a).[49]

Analysis of the non-human primate *LPA* gene has yielded many insights into how sequence variations in the apo(a) KIV_{10} sequence can influence the lysine binding function. It is apparent that during the evolution of Lp(a) in apes and monkeys, the lysine binding properties of KIV_{10} were lost as a result of mutations distinct to each species; this is summarized in Table 1.1. A comparative analysis of rhesus monkey apo(a) cDNA with human liver cDNA revealed a similar structure with a series of plasminogen-like KIV domains immediately followed by a proteolytically inactive plasminogen-like protease domain.[50] As such, rhesus monkey Lp(a) lacks the KV domain. It was also identified that rhesus monkey apo(a) lacks a functional KIV_{10} LBS as a result of a $Trp^{70} \rightarrow$ Arg mutation in the lysine binding pocket.[31] Interestingly, this polymorphism has also been reported to be present in approximately 2% of the human population.[51] It was later determined that cynomologous monkeys share almost identical apo(a) domain organization with that of

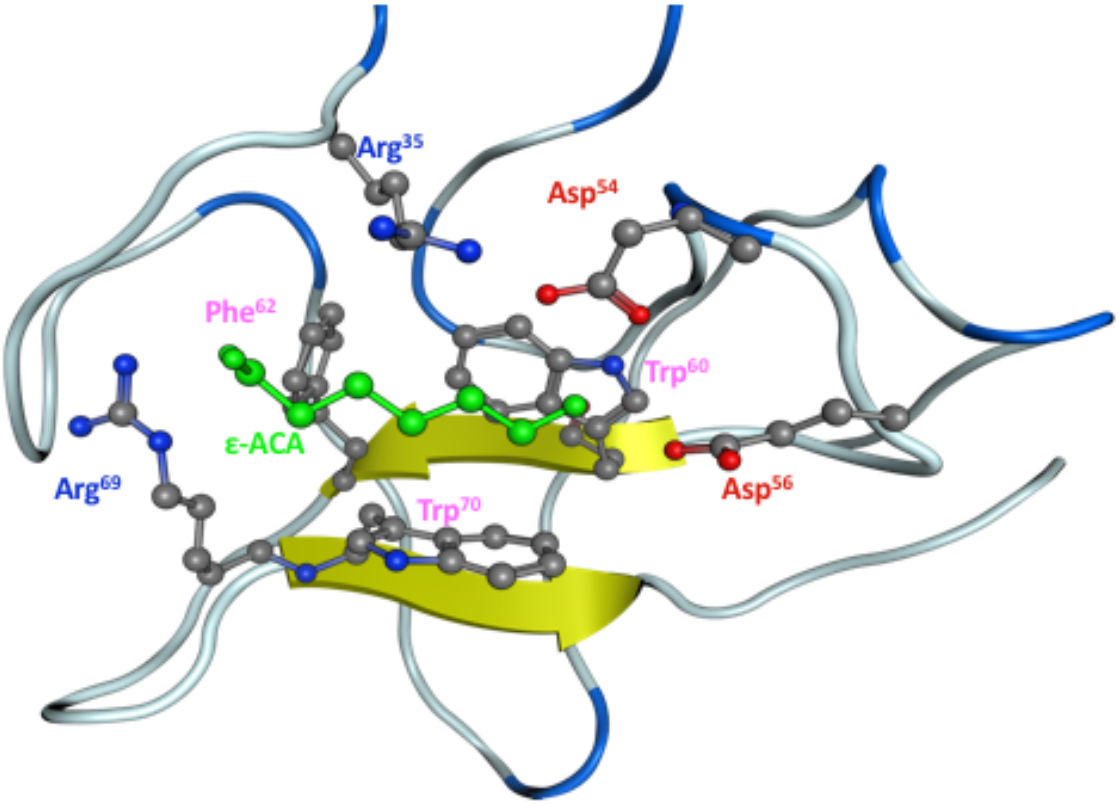

Figure 1.3 The Structure of the lysine binding site in apo(a) KIV_{10}
The lysine binding site in apo(a) KIV_{10} consists of seven critical amino acids collectively forming a hydrophobic depression in the surface of the domain with cationic and anionic centers. The following amino acids have been designated as critical for optimal lysine binding function; Arg^{35}, Asp^{54}, Asp^{56}, Trp^{60}, Phe^{62}, Arg^{69}, and Trp^{70}. Amino acids composing the; hydrophobic trough are indicated in fuschia; cationic centre are indicated in blue; and anionic centre in red.

rhesus monkeys, in that they both lack KV and possess the same LBS destabilizing mutation.[52] Analysis of baboon apo(a) also reported a significantly reduced ability to bind lysine-Sepharose and partially degraded fibrin; this was speculated to be due to conformational changes arising from the lack of a KV domain in these animals.[26] Unlike rhesus monkey apo(a), baboon apo(a) has all KIV_{10} amino acids required for lysine binding; interestingly, a Trp to Arg polymorphism has been reported at position 70, which corresponds to a minor allele in baboons.[26] The lack of ability of baboon KIV_{10} to bind to lysine despite the presence of an intact LBS highlights a potential role for KV in providing a conformation of the protein that facilitates the lysine binding function of KIV_{10}. In another study, chimpanzee apo(a) was assessed for its ability to bind to intact and plasmin-degraded fibrin. It was determined that an $Asp^{56} \rightarrow$ Asn mutation disrupts the sLBS in KIV_{10}.[53] This mutation is also present in gorilla.[52] The orangutan is the only reported species to maintain all of the amino acids required for the sLBS as well as a KV domain although Lp(a) from this species has not been isolated and studied.[52] It is intriguing that all species of Old World monkeys and apes, with the exception of the orangutan, have converged on a similar motif of inactivation of the KIV_{10} lysine binding ability, the advantages that may result from this remain to be determined.

1.1.4 Determinants of Plasma Lp(a) Concentration

With plasma Lp(a) levels ranging so widely within the population there has been much debate about whether synthesis, assembly, or clearance pathways ultimately dictate Lp(a) concentration. Apo(a) is synthesized and secreted primarily in hepatocytes.[54] The

Table1.1 Comparison of the structure and function of multiple species of apo(a)

A	Species	Kringles		Protease		Attachment to LDL
		IV	V	Domain	Activity	
Human		✔	✔	✔	-	✔
Bonobo		✔*	✔	✔	-	✔
Chimpanzee		✔*	✔	✔	-	✔
Gorilla		✔*	✔	✔	-	✔
Orangutan		✔	✔	✔	-	✔
Baboon		✔**	-	✔	-	✔
Cynomolgus		✔*	-	✔	-	✔
Rhesus monkey		✔*	-	✔	-	✔
Plasminogen		✔	✔	✔	✔	-
*Does not bind lysine due to a LBS mutation, **Does not bind lysine						

B	Lysine Binding Site	Key Amino Acid Differences
	Plasminogen KIV	Lys^{35} ⋮ Arg^{69}
		Asp^{54} ⋮ Asp^{56}
		Trp^{60} ⋮ Phe^{62} ⋮ Trp^{70}
	Human apo(a) KIV10	$Lys^{35} \rightarrow Arg^{35}$
	Cynomolgus & Rhesus monkey apo(a) KIV10 *Rare human polymorphism*	$Trp^{70} \rightarrow Arg^{70}$
	Chimpanzee & Gorilla apo(a) KIV10	$Asp^{56} \rightarrow Asn^{56}$

Table 1: Comparison of the structure and function of the KIV, V and protease-like domains of human apo(a) with apo(a) from Old World monkeys and apes **A**. Summary of the properties of KIV, KV and protease-like domains present in various species of apo(a). The potential of these variants to bind fibrin is a measure of their lysine binding ability. Species that have been directly assessed for their ability to bind to lysine or lysine analogs are annotated with an asterisk. **B.** Summary of the differences in amino acid sequence of the lysine binding site in humans, Old World monkeys, and apes

determinants of the level of Lp(a) in the plasma can be seen at the level of transcription, secretion, assembly, and catabolism.

With respect to the synthesis of apo(a), relatively little is known about factors that can modulate *LPA* expression. A number of studies have reported the ability of sex hormones to modulate Lp(a) levels. For example, in men with prostate cancer and receiving high dose estrogen therapy, Lp(a) levels were lowered by approximately 50%.[55] Similar effects on Lp(a) levels have been reported in postmenopausal women on estrogen replacement therapies, albeit to a lesser extent (~15-50% lowering).[56-58] A subsequent study showed that estrogen/tamoxifen treatment in transgenic apo(a) mice lowered hepatic *LPA* mRNA levels, as well as corresponding plasma apo(a) concentations.[59] Although the mechanism by which estrogens can lower apo(a) synthesis is unknown (no estrogen response elements have been identified in the *LPA* promoter) it has been hypothesized to be an indirect effect caused by the gene expression changes caused by estrogen.[60] Since the *LPA* promoter does contain interleukin 6 (IL-6) sensitive sites, it is possible that there may be some cytokine regulation of apo(a) encoding mRNA.[61] Wade *et al.* reported an approximately 5-fold enhancement in *LPA* reporter gene activity in HepG2 cells when exposed to IL-6.[62] A negative farnesoid X receptor (FXR) response element was also detected in the promoter region of the gene.[63] It is possible that bile acids can bind to this sequence and downregulate apo(a) mRNA levels, though this is yet to be investigated.

Several single nucleotide polymorphisms (SNPs) and other sequence variations, whether inside or adjacent to the gene, have been shown to effect *LPA* gene size and

potentially plasma Lp(a) concentration. Such sequence variants are a pentanucleotide repeat polymorphism ($TTTTA_n$) in the 5'-flanking region, though there is no influence for transcriptional regulation through this motif[64, 65], and several SNPs that exist within the gene sequence.[66, 67] Clarke and colleagues have recently identified an association with SNPs rs3798220 and rs10455872, with elevated Lp(a) levels and CHD.[12] Several null *LPA* alleles have also been reported, that result in sequence truncation, thus not giving rise to an apo(a) protein.[68, 69]

The involvement of *cis*-acting sequences in the transcription of *LPA* mRNA remains to be thoroughly investigated, with the exception of the effect of the quantity of KIV_2 repeats within the polypeptide sequence. Once translated, depending on the number of repeating KIV domains, apo(a) can be subjected to ER retention and possibly targeted for ER assisted protein degradation. Using steady state radiolabeling of primary baboon hepatocytes, White *et al.* determined that post-translational processing of apo(a) is a significant factor in Lp(a) production.[70, 71]The ultimate result of these studies was that smaller apo(a) molecules are synthesized and secreted at a faster rate than larger isoforms.[72] Interestingly, in individuals that are heterozygous for apo(a) isoform size, dominance is not always associated with the small apo(a) allele. Recent evidence suggests that size of the apo(a) allele not only effects plasma levels of that corresponding isoform, but also the levels of the other expressed apo(a) molecule.[73, 74]

The vast majority of apo(a) in plasma is present in Lp(a) particles, with very low levels of free apo(a) detectable in plasma.[75] This has been interpreted to suggest either rapid clearance of free apo(a), or highly efficient Lp(a) particle formation. Lp(a) assembly occurs

though a two-step mechanism, the first of which is the association of the weak LBS in apo(a) KIV types 7 and 8 with surface-exposed lysine residues in the amino terminus of $apoB_{100}$.[76, 77] There remains controversy, however, as to the location of covalent Lp(a) formation. Although there is a large body of evidence to suggest that the process of Lp(a) assembly occurs extracellularly, there are several studies that report the identification of covalent Lp(a) particles intracellularly.[78-80] In one report using baboon hepatocytes, it was suggested that Lp(a) can be assembled on the cell surface.[81] Support for extracellular assembly of Lp(a) has been provided by studies showing that an oxidase activity present in the medium of cultured hepatoma cells can significantly enhance covalent Lp(a) formation.[82] In *in vitro* studies, in the absence of this enzymatic activity, spontaneous covalent linkage of the apo(a) and apoB-100 components of Lp(a) progresses extremely slowly despite the efficiency of the initial non-covalent interaction.[82] It has also been reported that apo(a) supports a more open conformation when bound to lysine analogs, making it a better substrate for covalent bond formation.[82] As such, lysine-dependent binding of non-covalent apo(a)-apoB-100 complexes to cell surface receptors such as plasminogen receptors may represent a plausible mechanism by which apo(a) can adopt a conformation that accelerates covalent association with apoB-100. Taken together, it can be hypothesized that non-covalent interactions occur intracellularly, but covalent bond formation requires the cell surface and extracellular oxidase activity for optimal covalent Lp(a) formation.

The clearance of Lp(a) from the circulation has been under investigation for decades.[19] Several studies have reported a major role for the liver in the clearance of

Lp(a)[83]; a possible role for the kidney in Lp(a) catabolism has also been proposed. The latter stems from studies reporting that patients with renal disease have significantly elevated Lp(a) levels.[84, 85] This work was further addressed in an *in vivo* study reporting that the increases observed in Lp(a) plasma levels in hemodialysis patients were solely due to a decreased catabolic rate.[79]

In a powerful isotopic labelling study in humans, it was determined that Lp(a) levels are determined at the level of production rather than clearance, as there was no difference in the catabolic rates of large and small isoforms yet it is well established that they are synthesized at different rates.[16] It has been speculated over the years that Lp(a) catabolism can occur through different receptors, which is in keeping with the duality of its structure. The role of the low density lipoprotein receptor (LDLR) in Lp(a) catabolism has been a recurring question in the field of Lp(a) research, with evidence both for and against its involvement.[19] Evidence suggesting strongly against a role for the LDLR in Lp(a) clearance is the lack of ability of statins to significantly modulate Lp(a) levels.[86] Statins are HMG-CoA reductase inhibitors that cause an increase in the hepatic cell surface abundance of the LDLR, resulting in a significant decrease in plasma LDL levels.[87] Since statin therapy has little effect on Lp(a) levels, this has been interpreted to suggest that the LDL moiety of Lp(a) does not interact with the LDLR, perhaps due to steric hindrance by apo(a). It has been shown that exogenous apo(a) can effectively compete for the clearance of radiolabeled Lp(a) in mice. This suggests that an apo(a) receptor is primarily responsible for Lp(a) catabolism.[83] However, transgenic LDLR overexpressing mice were able to clear Lp(a) much more rapidly than wildtype, suggesting that the LDL moiety of Lp(a) may be

a ligand for this receptor.[88] The role of the LDLR has recently been under investigation once again in the context of the development of inhibitors of proprotein convertase subtilisin/kexin type 9 (PCSK9).[89] PCSK9 is a furin-like enzyme which is secreted in an inactive form, primarily from the liver.[90] It has been demonstrated that PCSK9 can bind to the LDLR and target it for intracellular degradation.[91] As such, increasing LDLR levels by inhibition of PCSK9 has become a viable therapeutic strategy to significantly lower plasma LDL *in vivo* (see section 1.1.5).[92] In addition to lowering of plasma LDL levels by up to 70%, PCSK9 inhibitors have been found to consistently lower Lp(a) levels by approximately 30%.[93] Evidence from our group[94] and others[92] suggest that under conditions of PCSK9 inhibition, the combination of low levels of competing LDL with a very high LDL receptor number result in the ability of the LDLR to internalize and degrade Lp(a). This has been a point of controversy in the field, particularly since some have failed to show a relationship in clinical studies between PCSK9-mediated LDL lowering and Lp(a) lowering, suggesting no role for the LDLR in Lp(a) catabolism.[95]

Under normal physiological conditions, the majority of Lp(a) is likely cleared through a receptor(s) that recognizes and binds to apo(a). In this regard, there have been reports of the existence of different receptors with the ability to bind Lp(a). In early studies, Tam *et al*. reported the presence of both low and high affinity apo(a)/Lp(a) receptors on the hepatic cell surface, with the low affinity component corresponding to plasminogen receptors that are capable of binding to the apo(a) component of Lp(a) in an ε-ACA-dependent manner [96] Recently it was reported that scavenger receptor-BI (SR-BI) deficient mice had significantly lower rates of Lp(a) clearance. However, >60% of Lp(a) was cleared

from circulation within 24 hours, thus only a minor role was determined for SR-BI.[97] Furthermore it was reported that the ability of Lp(a) to be a ligand for SR-BI is mediated by the presence of oxidized phospholipids (oxPLs) on Lp(a), rather than through its protein components.[98] With strong evidence for multiple receptors that can contribute to Lp(a) catabolism, the identification of the primary route for Lp(a) clearance *in vivo* remains to be determined. It is also worthwhile to note that roles for specific receptor pathways may vary in different physiological/pathophysiological settings.

1.1.5 The Role of Inflammation in Atherosclerosis and Atherothrombosis

Atherosclerosis is a progressive disease that is characterized by the accumulation of lipids and fibrous components within the walls of large arteries.[99] Atherosclerotic diseases are the leading cause of death in the industrialized world.[100] Atherogenesis is a complex pathophysiological process driven by responses to inflammation. The initiation of atherosclerotic plaque formation is caused by local inflammation in response to damage to the endothelial cell (EC) layer of the artery. Endothelial injury can be triggered by consuming a high saturated-fat diet, smoking, hypertension, mechanical trauma, hyperglycemia, obesity, or insulin resistance.[101] This ultimately leads to cellular stress and endothelial cell dysfunction.[102] The initial injury can also play a longer, sustained role in plaque development, which is primarily mediated by endothelial cells (ECs) as well as smooth muscle cells (SMCs), platelets, lymphocytes, monocytes, and macrophages. This is followed by the release of a broad array of cytokines, growth factors, proteases, and reactive oxygen species (ROS) from this group of cells within a developing lesion.[103]

Under normal conditions, ECs are anti-coagulant, anti-inflammatory, and function as a permeability barrier between the subendothelial space and the flowing blood in the lumen of the vessel.[104] The endothelium serves many homeostatic functions such as nitric oxide (NO) production and resistance to the adhesion of other cells.[105] However, EC damage can result in a proinflammatory state of the ECs and leaking of the components of blood into the intimal space. This damage usually occurs in areas that are prone to hemodynamic strain, such as the branch points of arterial vessels.[106] In a proinflammatory state, ECs will begin to upregulate chemokines, cytokines, and cell adhesion molecules (CAMs) such as vascular cell adhesion molecule 1 (VCAM-1), P-Selectin, E-Selectin, and intracellular cell adhesion molecule 1 (ICAM-1).[102] These CAMs, particularly VCAM-1, allow for the recruitment and attachment of leukocytes to the arterial wall. Oxidized LDL (oxLDL) can be internalized by the lectin-like oxidized LDL receptor (LOX-1) that is expressed throughout the vasculature of hypertensive, diabetic, and dyslipidemic individuals.[107] LOX-1 is also expressed on SMCs, monocytes and macrophages and has been proposed as a potential biomarker for cardiovascular diseases.[108] Oxidized LDL (oxLDL) can either directly or indirectly increase CAM expression; the latter occurs through decreasing endothelial nitric oxide synthase (eNOS) expression and nitric NO levels, which can also be an effector of CAM expression via NF-κB.[109]

The secretion of chemokines such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) by intimal cells (ECs and VSMCs) causes migration of the leukocytes through inter-endothelial junctions and into the subendothelial space.[106] Cytokines, namely macrophage-colony stimulating factor (M-CSF), induces

differentiation of resident leukocytes into macrophages.[110] During differentiation, epigenetic remodeling of macrophage DNA results in an upregulation of pattern-recognition receptors, such as scavenger receptors and toll-like receptors (TLRs).[111, 112] Scavenger receptors, such as scavenger receptor A (SRA) and CD36, are capable of internalizing a broad range of ligands including cell fragments, bacterial endotoxins, and oxLDL. The cholesterol taken up from oxLDL is either mobilized from the macrophages or deposited in lipid droplets within these cells.[112] In a case where lipid mobilization is impaired or if circulating cholesterol is high, extensive lipid deposition will transform macrophages into foam cells. Native LDL is not taken up rapidly enough for this to occur.[113] However, exposure to the oxidative waste of vascular cells (ie/ reactive oxygen species (ROS)) within the intima can promote oxidation, lipolysis, and aggregation of LDL allowing it to function as a ligand for more receptors, such as scavenger receptor 1A and LOX-1, and thus is more rapidly internalized.[114] The formation of foam cells is the major event that characterizes an early atherosclerotic lesion, as these cells become lipid enriched and accumulate a fatty streak will progress on the vessel lumen.[115] Over time, foam cells die and contribute to the formation of a necrotic core, a hallmark of late stage atherosclerotic lesions.[114]

Macrophages within lesions also contribute to the formation of an atherosclerotic plaque through the secretion of cytokines that promote the migration and proliferation of smooth muscle cells from the medial layer.[102] Decreased NO levels resulting from these cytokines also promotes platelet aggregation.[116] At the same time components of the blood, such as von Willebrand factor, promote platelet activation leading to release of platelet

derived growth factor (PDGF) that can further stimulate migration and proliferation of medial SMCs.[117] Activated platelets also stimulate transforming growth factor beta (TGF-β) secretion which stimulates interstitial collagen synthesis by SMCs. The combination of smooth muscle cell migration and extracellular matrix (ECM) protein deposition contributes to the formation of the fibrous cap.[110] The fibrous cap serves as a barrier between the lipid-rich thrombogenic material in the plaque and the coagulation proteins within the flowing blood.

Over time, the secretion of interstitial collagenases and matrix metalloproteinases (MMPs) is stimulated in macrophages, SMCs, and ECs by exposure of these cells to inflammatory ligands such as tumour necrosis factor alpha (TNF-α) and decreased NO levels.[118, 119] Due to the production of ECM proteins, secretion of macrophage proteases, and calcification of advanced lesions, the plaque will become unstable and will eventually rupture. Release of the thrombogenic material, namely the pro-coagulant tissue factor, in the necrotic core will stimulate the activation of the coagulation cascade resulting in the formation of an acute thrombus that can occlude the vessel.[110] The reduced blood flow can manifest as acute angina, myocardial infarction, an embolism, or a stroke.

1.1.5.1 The pro-atherogenic effects of LDL/Lp(a) on vascular cells

As described above, the initial steps in the progression of atherosclerosis are dependent on the interaction between the ECs, monocytes and macrophages within the arterial wall.[120, 121] The expression of cell adhesion molecules was found to be critical for the adherence of mononuclear cells to the vessel wall. It is well documented that LDL is able to increase VCAM-1 and E-Selectin expression, however oxidation of LDL

dramatically increases its ability to promote the expression of these molecules and ICAM-1 and P-Selectin expression as well.[122] Treatment of human umbilical vein endothelial cells (HUVECs) with Lp(a) has been reported to enhance the expression of all of the aforementioned CAMs, as well as PDGF.[123-126] Specifically, it was the apo(a) component of Lp(a) that was reported to trigger ICAM-1 expression in HUVECs.[123] Adhesion of monocytes to ECs was also reported to be stimulated through the binding of Lp(a) – in a lysine-dependent manner – to the β-integrin Mac-1. This Lp(a)-Mac-1 binding event was also shown to promote tissue factor secretion in THP-1 cells.[34] Lp(a)/apo(a) has also been shown to contribute to monocyte recruitment through enhancing secretion of chemokines I-309 and interleukin-8 (IL-8) from cultured macrophages.[127, 128]

Aside from CAM expression, a role for apo(a) has been defined in various other aspects of endothelial dysfunction. Treatment of HUVECs with recombinant apo(a) (r-apo(a)) leads to cytoskeletal rearrangement, which in turn results in a loss of cell-cell contact and increased HUVEC monolayer permeability.[36] The same system was later used to assess the role of apo(a) in inflammatory processes, as treatment of ECs with r-apo(a) was able to increase prostaglandin synthesis by means of stimulating the nuclear translocation of β-catenin.[35] Lp(a), in many ways, can participate in the atherosclerotic process in a manner similar to LDL. Like LDL, Lp(a) can be readily oxidized within the lesion and taken up by macrophages to form foam cells, allowing it to contribute to lesion development.[129]

Oxidized lipids have been shown to accelerate the onset of inflammatory processes in vascular cells.[130] Lp(a) has been shown to be the preferential carrier of oxidized

phospholipids (oxPL), when compared to LDL.[131] The apo(a) component of an Lp(a) particle also has an oxidized phospholipid moiety, which has been reported to induce apoptosis in endoplasmic reticulum (ER) stressed macrophages through a CD36 and TLR2 dependent manner, the same receptors used to initiate inflammatory responses by oxLDL.[132] These reports illustrate a clear role for Lp(a)/apo(a) in contributing to endothelial dysfunction and localized vascular cell inflammation, key steps in the initiation of atherosclerotic plaque formation.

Endothelial and smooth muscle cell migration are important steps in the formation and neovascularization of the fibrous cap in a mature plaque. Lp(a)/apo(a) is able to stimulate EC growth and migration by diminishing the pool of transforming growth factor-β (TGF-β) generated by HUVECs. The ability of apo(a) to mediate this effect was shown to be dependent on the presence of the LBS in KIV_{10} of apo(a), through binding to the integrin $\alpha_V\beta_3$ on the cell surface.[133] However, Lp(a) can inhibit *in vitro* EC tube formation by inhibiting the conversion of plasminogen to plasmin on the cell surface.[28] Similarly, apo(a) was able to stimulate SMC migration and proliferation by inhibiting the TGF- β, with a critical role defined for the inhibitory potential KIV_9 domain on pericellular plasmin formation.[134] Transgenic rabbits expressing a human r-apo(a) were found to have a distinct SMC phenotype prone to increased proliferation.[135] Furthermore, apo(a) has been shown to competitively inhibit pericellular plasminogen activation on the surface of ECs, monocytes and macrophages *in vitro*, in a KIV_{10} and KV dependent manner.[31] In the clinical setting, 90% and 31% of macrophages and SMCs, respectively, co-localized with Lp(a) in the coronary atheromas of patients.[136] The ability of Lp(a) to promote

inflammatory states in SMCs, ECs, monocytes and macrophages in both *in vitro* and *in vivo* by initiating distinct cell signaling processes proves that Lp(a) has strong proinflammatory potential. Taken together, these data strongly suggest a role for Lp(a)/apo(a) in the initiation and development of an atherosclerotic plaque.

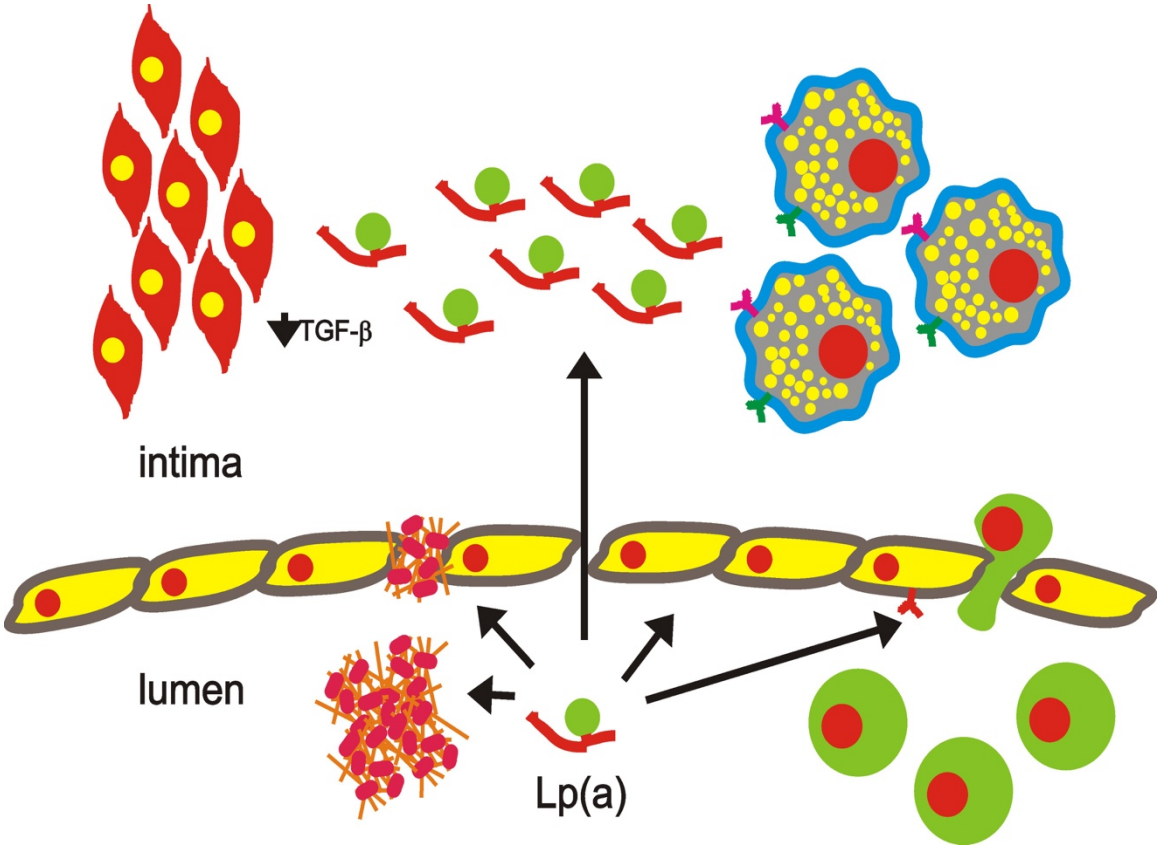

Figure 1.4 The effects of lipoprotein(a) on vascular cell phenotype.
Lp(a) has been reported to induce chemoattractant activity of circulating monocytes and to increase endothelial cell permeability. This may allow circulating monocytes to bind to endothelial cells, and to migrate into the vessel intima. Within the intima Lp(a) can become oxidized and contribute to the formation of macrophage foam cells, thus initiating the process of atherogenesis. Lp(a) has also been shown to increase smooth muscle cell migration and proliferation and, interfere with fibrinolysis in the vasculature, owing to its ability to inhibit plasminogen activation on the cell and fibrin surfaces.

1.1.5.2 The prothrombotic and antifibrinolytic effects of Lp(a)/apo(a)

Despite the remarkable homology between apo(a) and plasminogen, the direct role for Lp(a)/apo(a) in the thrombotic complications of atherosclerosis remains controversial. Though there is a growing body of evidence from *in vitro* studies to show that Lp(a) has procoagulant and antifibrinolytic effects, direct evidence for corresponding effects *in vivo* is currently lacking.

The initial reports of a possible prothrombotic role for Lp(a) were based on the ability of apo(a) to inhibit the binding of plasminogen to the cell surface receptors of ECs and monocytes.[137, 138] Recently it was reported that apo(a) and Lp(a) were not only able to inhibit the binding of plasminogen to the surface of ECs, monocytes and macrophages, but also that apo(a) could directly inhibit tPA-mediated plasminogen activation on the cell surface.[31] This effect was diminished in the presence of lysine analog ε-ACA and dependent on the sLBS in KIV_{10} as well as the KV domain of apo(a), underscoring a role for the lysine binding ability of apo(a) in this context. In addition, synthesis of plasminogen activator inhibitors (PAIs) is significantly increased in cultured ECs and male human monocytes when exposed to Lp(a), representing a mechanism by which apo(a) can limit plasmin generation.[139, 140] These findings implicate a role for Lp(a) in atherothrombosis, in the context of mural thrombus formation, cell migration, and angiogenesis.

More directly relevant to thrombotic disorders is the evidence that Lp(a)/apo(a) can inhibit tPA-mediated plasminogen activation in fibrin clots, thus attenuating fibrinolysis.[33] It has also been shown that Lp(a)/apo(a) can inhibit plasminogen activation by inhibiting the conversion of Glu-plasminogen to its more readily activatable Lys-plasminogen

form[141]; inhibition of Glu- to Lys-plasminogen conversion prevents a positive feedback mechanism that is required for maximal plasmin generation. Apo(a) can inhibit the binding of plasminogen to its substrate fibrin and can also form a quaternary complex with plasminogen, tPA, and fibrin that has a significantly reduced turnover rate when compared to the ternary complex lacking apo(a).[33, 142] *In vivo* models have supported these findings by showing attenuation of thrombolysis in rabbit jugular vein and transgenic apo(a) mouse models.[143, 144] It has also been shown that Lp(a) can enhance local thrombogenic potential by binding to and inactivating tissue factor pathway inhibitor (TFPI), a potent inhibitor of the tissue factor-initiated coagulation cascade.[145, 146] This could result in the unregulated activity of pro-thrombotic tissue factor upon plaque rupture. This evidence suggests that Lp(a) may possess roles in thrombus formation, though it is unclear if Lp(a) can directly promote clot formation or prevent lysis *in vivo*.

1.1.6 Lp(a) as a causal risk factor for vascular diseases

Lipoprotein(a) poses unique challenges to researchers who are striving to understand the contribution of this complex lipoprotein to the development of CVD. One of the greatest challenges is that the gene encoding apo(a), *LPA*, is only present in humans, apes and Old World monkeys[147] Without the ability to study human apo(a) in well characterized animal models for atherosclerosis, it is difficult to assess the pathophysiological roles of apo(a)/Lp(a) *in vivo*. Additionally, the measurement of plasma Lp(a) has been complicated by sensitivity to isoform size that has been shown in virtually all commercially available assays.[148] The method published by Marcovina and

colleagues[149] is considered to be the gold standard in the field and uses a 5-point calibrator system standardized traceable to an international reference material.

There have been many case-control studies which have consistently reported over more than 3 decades that elevated Lp(a) is a risk factor for CHD. Unfortunately, however, this type of study design cannot distinguish a causal role for Lp(a) in disease as opposed to a disease marker.[150, 151] Genetic studies less than a decade ago have provided evidence for a causal role for Lp(a) in CHD. In 2009, a Mendelian randomization study assessed genetic variation in the *LPA* gene by quantifying the number of sequences encoding KIV_2 domains in the *LPA* gene, and showed that the number of KIV_2-encoding sequences was inversely related to Lp(a) plasma levels and risk for myocardial infarction.[150] The design of this study eliminated selection bias and reverse causation, therefore making it possible to assess a causal role for Lp(a) in CHD. Genome-wide association studies have also determined that the size of the *LPA* gene is a major determining factor for CHD risk.[152, 153] Additional evidence for a causal role of Lp(a) in CHD came from a study by Clarke and colleagues in which two single nucleotide polymorphisms (SNPS) were identified in the *LPA* gene that are associated with increased Lp(a) levels and significantly increased risk for CHD.[12] SNPs were found to account for 36% of Lp(a) plasma levels in this study. The two SNPs are rs10455872, an intronic polymorphism, and rs3798220 which results in an Ile to Met substitution at the 4399 amino acid position in the protease-like domain of apo(a). For both SNPs, the association with risk was accounted for by their association with Lp(a) levels.[12]

Recently, the intronic SNP was found to be associated with aortic valve calcification and stenosis, although this has yet to be further investigated.[154, 155] Though,

the exact molecular mechanisms for a role for Lp(a) in CAVD have not been explored, with the exception of the recent discovery that Lp(a) can be a carrier of autotaxin in the plasma.[156] Prior to the study by Clarke and colleagues, the rs10455872 variant had been correlated with severe CAD and increased Lp(a) plasma levels.[157] The latter study also reported a strong correlation between this SNP and small apo(a) isoform sizes. The rs3798220 SNP was later investigated in a randomized trial of low-dose aspirin therapy. It was determined that women with the rs3798220 SNP had significantly higher Lp(a) levels and a 2-fold increased risk for cardiovascular disease.[158] This study further showed that in carriers of SNP, cardiovascular risk was lowered by over two-fold in response to the low-dose aspirin therapy compared to individuals without this polymorphism.[158] This variant has also been correlated with increased oxidized phospholipid content on the LDL component of Lp(a), possibly increasing the atherogenic potential of Lp(a) in individuals with this SNP.[159] In 2014, a study reported that elevated Lp(a) levels were correlated with reduced clot permeability and increased lysis times. Interestingly, polymorphism carriers, for the I4399M Lp(a) SNP, had ethnic-dependent variations in plasma clot permeability and lysis times, with Caucasians presenting with less permeable clots that were more resistant to lysis.[160] Although the basis for these observations were not explored in this study, it does highlight a potential functional role for this Lp(a) variant in thrombotic and/or fibrinolytic complications independent of its association with elevated Lp(a) levels.

It has been established that small isoform sizes of Lp(a) promote risk for CHD, but not independent of levels. A systematic review of 40 different studies (meta-analysis), showed that individuals with smaller apo(a) isoforms (less than 22 KIVs) have a 2-fold

higher risk of CHD and ischemic stroke than those with larger isoforms.[161] However, this remains to be a controversial topic in the field since it is not clear whether small isoform sizes of Lp(a) can confer risk independently of levels.

There has been controversy as to whether Lp(a) is a risk factor for venous thromboembolism (VTE). Several small studies have reported that high plasma concentrations of Lp(a) are a risk factor for VTE in both adults and children.[162-166] Recently it was reported that low KIV_2 repeat number and elevated Lp(a) levels are significantly and independently associated with VTE.[166] This study directly contradicts a large Danish genetic study of >41,000 individuals, that reported that Lp(a) is a causal risk factor for atherosclerosis but not venous thrombosis.[167] In fact, the vast majority of recent studies report that there is no association between genetically elevated Lp(a) levels and venous thrombosis.[168, 169] Though there is evidence for and against a role for Lp(a) in venous thrombotic disorders, the more recent, larger genetic studies almost exclusively discount a causal role for Lp(a) in venous thrombosis.

Taken together, the growing body of evidence from genetic studies shows that Lp(a) is a causal, independent risk factor for CHD, as well as other vascular diseases including CAVD (Figure 1.5). However, the mechanism by which Lp(a) contributes to vascular disease processes remains unclear. It is possible that the ability of Lp(a) to promote CVD may stem from its ability to be a carrier for proinflammatory oxidized phospholipids in plasma. This will be discussed in Section 1.2 in detail. Further investigation into the mechanisms of Lp(a) function may identify new strategies for the

development of therapeutics aimed at reduced the harmful effects of Lp(a) in the vasculature.

1.1.7 Therapeutic strategies to lower plasma Lp(a) levels

Currently, there are no available pharmaceutical treatments designed to specifically lower plasma Lp(a) levels. This has made it challenging to assess prospectively the effect of lowering of Lp(a) on cardiovascular outcomes. Statins, while very effective for LDL lowering, have little or no effect on Lp(a) levels.[86] Therapies that have been shown to lower Lp(a) by up to 30% also have effects on other components of the lipid profile including triglycerides, HDL and LDL. These include niacin[170, 171], and mipomersin[172].

PCSK9 inhibitors (monoclonal antibodies against proprotein convertase subtilisin/kexin type 9) have also been shown to lower Lp(a) levels by approximately 30%[93, 173, 174] and are highly effective LDL-lowering agents.[148,175] The mechanism by which PCSK9 inhibitors lower Lp(a) is controversial. Since PCSK9 functions to lower the number of low density lipoprotein receptors (LDLR) on the hepatocyte surface, removing PCSK9 from the system will increase LDLR numbers, this suggests that increased Lp(a) clearance -caused by PCSK9 inhibitors- is due to an increase in LDLR. This in turn, allows the LDLR to bind to its substrate LDL promoting rapid clearance of LDL cholesterol. Romagnuolo and colleagues, determined that Lp(a) (and not apo(a)) can be a ligand for the LDLR and that exogenous PCSK9 treatment of HepG2 cells can not only result in the expected decrease in LDLR but also a decrease in Lp(a) internalization.[94] However, this was only true in a situation where LDL levels were low and LDLR levels were elevated. In a study of 10 clinical trials, it was further shown that Lp(a) is a poor competitor for the

LDLR in the presence of LDL unless LDLR levels are significantly increased, in which case Lp(a) is a significant ligand for the receptor.[92] There is currently a growing body of early phase study evidence showing a decrease in CVD risk as a direct result of this therapeutic option.[176] However, since the decrease in plasma Lp(a) observed is at best ~30%, this study concluded that the LDLR is not the preferred route for Lp(a) clearance and another receptor(s) play a larger role in Lp(a) catabolism.[94]

Injectable antisense compounds that are specifically designed to target apo(a) are now in Phase II trials; results of the Phase I study showed that this compound can lower plasma Lp(a) levels by > 85% percent.[18, 177] This compound has the potential to be used in clinical trials designed specifically to look at cardiovascular outcomes in response to extreme Lp(a) lowering. A new ASO that is targeted to the KIV_2 subunit of apo(a) is currently under investigation. The ASO is conjugated to a liver specific glycan, for targeted RNA silencing in hepatocytes. Anti-apo(a) ASO, now known as ISIS-APO(a)Rx, therapy was able to reduce apo(a) by 86% in a 12K-apo(a) transgenic mouse model.[180] In a phase I study in normal volunteers, ISIS-APO(a)Rx was able to reduce plasma Lp(a) levels and associated oxPL by 89 and 93% respectively.[18] Another approach designed to specifically lower Lp(a) is Lp(a)-specific apheresis.[178] In a small study of 30 individuals with normal LDL levels that underwent Lp(a) apheresis treatments for 18 months a 73% reduction in plasma Lp(a) levels was reported, along with a significant regression in coronary atherosclerosis.[179] Though effective, Lp(a) apheresis is an invasive, costly procedure and on that basis is likely not feasible as a long term treatment option.

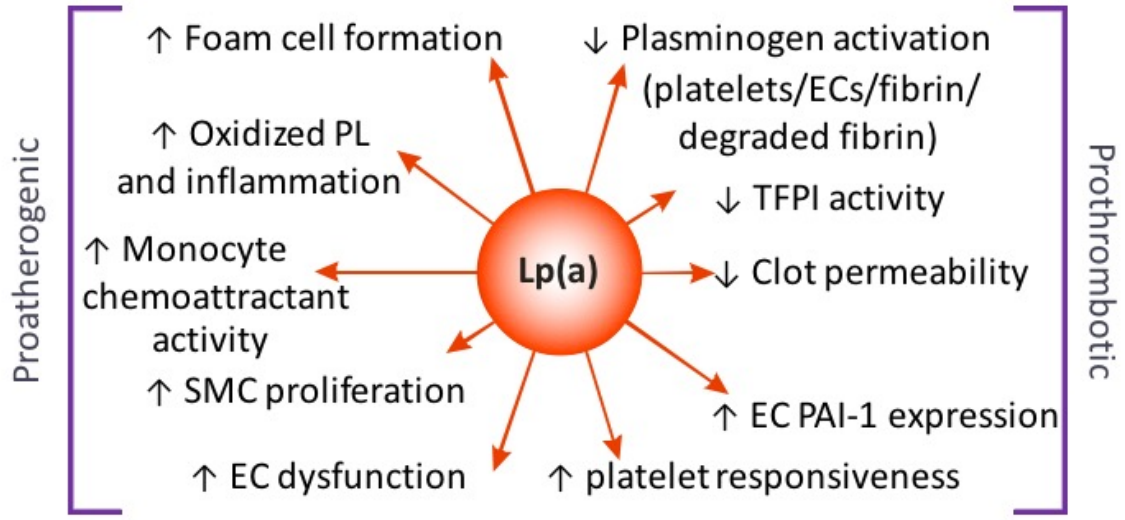

Figure 1.5 The multiple mechanisms of Lp(a) pathogenicity
Lp(a) has been shown to function through both proatherogenic and prothrombotic mechanisms as shown in in vitro and *in vivo* studies. Lp(a) may promote atherogenesis through its ability to modulate vascular cell phenotype or potentially through stimulating inflammation through its covalent oxPC moiety. Lp(a) may elicit prothrombotic effects through its ability to inhibit plasminogen activation, fibrinolysis, and due to its homology to plasminogen.

1.2 OXIDIZED PHOSPHOLIPID MODIFICATION OF LIPOPROTEIN(a) – NEW INSIGHTS INTO Lp(a) FUNCTION.

1.2.1 Formation and Structure of Oxidized Phospholipids

Glycerophospholipids are a class of lipid molecule found in many eukaryotic systems. They are composed of a glycerol backbone with the addition of two fatty acid residues and a phosphate-containing polar head group. A phospholipid (PL) that contains a fatty acid chain that has multiple sites of unsaturation is known as a polyunsaturated fatty acid (PUFA). PUFAs are prime candidates for oxidation due to the weaker carbon-hydrogen bonds found within the methylene groups in between double bonds. Oxidation of these molecules, through enzymatic or non-enzymatic means, can result in several distinct biologically active products, such as lysophospholipids. The mechanisms of non-enzymatic PUFA oxidation are well understood and usually require the presence of a free radical or ROS (Figure 1.6). Free radicals can be generated from exogenous sources such as ionizing radiation, UV-light, and smoking, or endogenous sources such as NADPH oxidase and the electron transport system in mitochondria. Radicals abstract a hydrogen atom from the bisallyic methylene found in unsaturated fatty acid carbon chains, forming a carbon-centered radical. This radical is prone to interact with molecular oxygen and will form a peroxyl radical. The formation of these peroxygen radicals is the first step in a

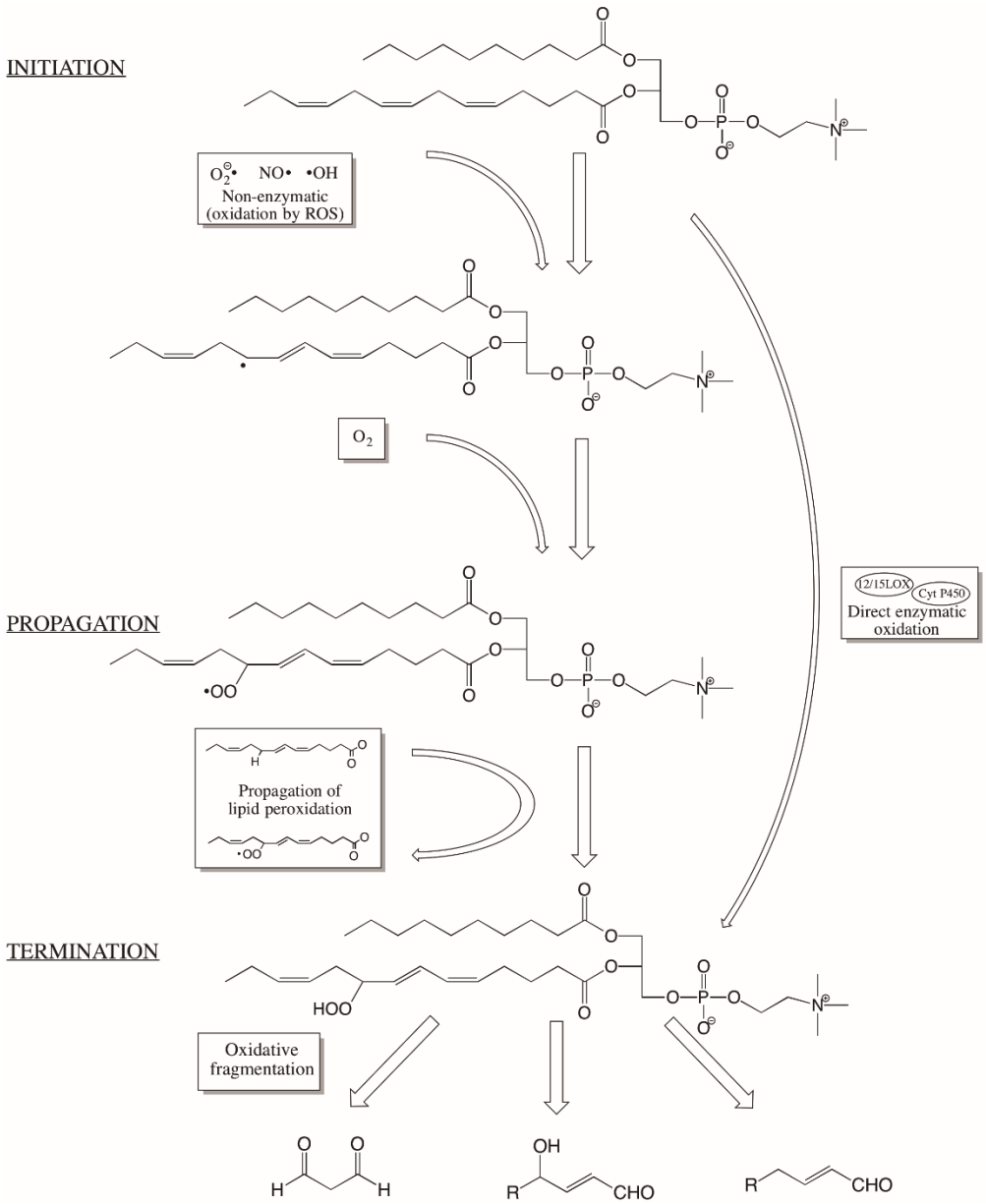

Figure 1.6 Oxidation of polyunsaturated fatty acid-containing phospholipids.
There are two main methods of PUFA oxidation; enzymatic and non-enzymatic. Non-enzymatic lipid oxidation requires the initiation of the lipid peroxidation reaction by a reactive oxygen species or radical. Either oxidation mechanism results in the formation of a hydroperoxide molecule that will rapidly decompose into reactive carbonyls, such as aldehydes or ketones. These reactive carbonyls can covalently modify DNA, proteins, or other lipid molecules.

canonical lipid peroxidation chain reaction, which terminates in the generation of oxPL. In contrast, enzymatic formation of oxPL is executed by a class of enzymes known as lipoxygenases (LOXs). The function of a LOX is to introduce molecular oxygen into 1,4-pentadiene motifs within a PUFA, creating a hydroperoxide molecule similar to the intermediates found in the lipid peroxidation reaction. Most of these enzymes recognize unesterified fatty acids. However, the 12/15-LOX group of enzymes is capable of oxidizing PL-esterified fatty acids. In fact, these enzymes have been shown to oxidize PL-PUFA substrates within the cell membrane and lipoprotein particles.[181, 182] Interestingly, 15-LOX produces (S)-isomers of PL-esterified hydroperoxides which have been found using chiral chromatography to be enriched in early atherosclerotic lesions.[183] Since non-enzymatic oxidation of lipids generates a racemic mixture of hydroperoxides, it is tempting to hypothesize an initial role for 15-LOX in *in vivo* atherogenesis.[183] It is important to note that advanced atherosclerotic lesions showed no enrichment in a specific enantiomer, suggesting a dominant role for non-enzymatic lipid oxidation as the lesion progresses.[183] It has also been reported that some members of the cytochrome P-450 family (classes 2C and 2J) can oxidized free PUFAs, which can then be esterified into oxPL.[184] Regardless of the mechanism of formation, peroxyl radicals and hydroperoxides can be further oxidized and fragmented in an arbitrary manner that leads to the formation of a wide spectrum of oxPLs with many unique functional groups. In particular, γ-hydroxy-α,β-unsaturated oxPLs containing terminal aldehydes have been shown to possess many different biological activities, including binding to macrophage CD36 and the induction of inflammatory effects in ECs.[185, 186] These oxPL species are also found to be enriched in plasma in cases of hyperlipidemia and atherosclerosis.[187] In addition, these oxPLs have been shown to form

protein adducts that have been detected as lipid-protein covalent complexes *in vivo*.[188] It has been reported that oxPL molecules that contain terminal aldehydes or α,β-unsaturated carbonyl groups can react with nucleophilic amino and thiol groups. This makes amino- and sulfhydryl-containing amino acids such as lysine, arginine, histidine, and cysteine potential targets for the formation of Michael adducts or Schiff bases.[189, 190]

1.2.2 Biological relevance of oxidized phospholipids in the vasculature

There is a significant body of data demonstrating a role for oxidized lipids as pathogenic molecules and indicators of disease. Specific to atherogenesis, oxidized-LDL is an example of the ability of lipid oxidation to influence changes in physiological processes, ultimately leading to pathogenic outcomes.[122, 191-193] Peroxidation of oxPLs can result in the generation of reactive molecules that form adducts with other lipids and protein molecules, thereby altering the normal physiology of signaling molecules and inducing immune responses.[194] Classic examples of such oxPLs are malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine (POVPC).[195, 196] It has been reported that several species of immunoglobulin, mainly IgG and IgM, can specifically detect these oxidized lipid products in oxLDL.[197, 198] These anti-oxLDL antibodies have been shown to be present in mouse models of atherosclerosis, as well as in humans with CVD.[199, 200] Recent studies report that individuals with higher levels of anti-oxLDL IgM antibodies had lower incidences of atherosclerosis, a result not observed for similar IgGs.[201] One of the cornerstones in the field of oxPLs and atherosclerosis was the discovery of an endogenous anti-oxPL IgM which was named E06. This antibody was derived from the spleen of $LDLR^{-/-}$ mice and is

able to bind to copper- oxidized LDL.[202] Intense characterization of this autoantibody led to the determination that it was specific to oxPLs that possessed a phosphocholine head group, and cannot recognize unoxidized PC residues.[203, 204] Sequencing of the heavy and light chain of E06 indicated that the antibody's variable region was 100% identical to the previously reported T15 human IgA that was crystalized with its ligand phosphocholine.[205] The E06 antibody was later determined to be able to histochemically stain atherosclerotic lesions, aside from is ability to bind to oxLDL and apoptotic cells. [206] E06 has become an important tool for characterizing the role of oxPLs in atherogenesis.[207]

Oxidized phospholipids (and oxPL-conjugated molecules) have also been shown to be ligands for various cellular receptors. The relationship of oxPLs with the scavenger receptor CD36 has been well characterized over the years, and has been shown to be important for the clearance of oxLDL by macrophages.[208] This has been reported as a crucial step in the progression of atherogenesis *in vivo*.[209, 210] Further study pointed to the requirement for a fragmented γ-hydroxy-α,β-unsaturated fatty acid in the *sn*-2 position of the PL for the covalent modification of proteins. This moiety was subsequently shown to covalently attach to Lys^{164} and Lys^{166} of $apoB_{100}$, by the formation of a Schiff base.[211] Binding of oxPL to CD36 has also been shown to activate platelets, by initiating JNK2 and MKK4 kinase signaling cascades.[212] Furthermore, these molecules have been shown to initiate signaling through vascular endothelial growth factor receptor-2 (VEGFR2), toll-like receptor-4 (TLR4), prostaglandin receptors, and peroxisome proliferator-activated receptors (PPARs).[212-215] As such, several signaling pathways can be initiated by exposure of cells to oxPLs; these pathways are involved in promoting monocyte chemotactic activity

and tissue factor expression in non-endothelial cells, as well as maintaining endothelial cell monolayer properties [reviewed in reference 215].

Overall, the accumulation of decades of evidence has resulted in the identification of oxidized phospholipids as proinflammatory molecules. Platelet activating factor (PAF)-like molecules, diacyl oxidized phospholipids, are able to significantly increase monocyte adhesion through the activation of β1-integrin and P-selectin on ECs.[216, 217] OxPLs have also been reported to stimulate expression and secretion of IL-8 and monocyte chemotactic protein-1 (MCP-1) in cultured ECs, linking the oxidation of lipids to atherogenesis.[213] PAF-like oxPLs and lysophosphatidic acid have also been shown to directly activate platelets and promote adhesion.[218] Pro-inflammatory ROSs have also been reported to be stimulated by oxPAPC, but not by native PAPC, in human and bovine aortic ECs, a process that could further promote oxidation and plaque formation.[219] Due to the various pathogenic mechanisms described, the potential for oxPLs to promote atherogenesis and enhance disease progression is clear and significant.

1.2.3 Oxidized phospholipids and lipoprotein(a)

Lipoprotein(a) has been proposed to initiate and contribute to atherothrombosis through a myriad of different potential mechanisms, as reviewed in Section 1.1.5 (Figure 1.5). Over the past decade evidence has accumulated suggesting that the pathogenicity of Lp(a) may be in part due to the presence of oxidized phospholipids on the Lp(a) particle.[220] In 2003, a study by Edelstein *et al.* determined that there are lysine-phosphatidylcholine adducts on the apo(a) component of Lp(a).[221] This work used the PC binding E06 IgM antibody to map the oxPC moiety to the carboxyl-terminal domains of apo(a), using

elastase digestion fragments, and thus determining that fragments including KIV_5-PD and KV-PD reacted with E06. It was determined that the oxPC moiety on apo(a) was located on the KV domain, as rhesus monkey apo(a) did not react with E06 and lacks a KV domain.[221] Furthermore, this study speculated that the oxPL moiety was the product of the formation of a Schiff base between the reactive aldehyde group of the oxidized glycerophosphocholine and lysine residues Lys^{12} and Lys^{42} of KV. Evidence for the involvement of lysine residues in coordinating the OxPL moiety was two-fold: two residues/mol of apo(a) were not able to be modified by 2,4,6-trinitrobenzene sulfonic acid. Specifically, Lys^{12} and Lys^{42} in the apo(a) KV domain were identified by computational modeling.[221] This was the first study to characterize a covalently-linked oxPL modification on the apo(a) component of Lp(a).

An E06 sandwich ELISA was developed that could be used to screen plasma samples for the quantification of oxPL on apoB containing lipoproteins.[222] Plasma from high-expressing apo(a)/$apoB_{100}$ double transgenic mice was found to have elevated levels of oxPL associated with their Lp(a) protein, where LDL from these mice (or from $apoB_{100}$ mice) did not.[223] Using the same sandwich E06 ELISA technique, a group of >500 patients was assessed for Lp(a) levels and oxPL:$apoB_{100}$ levels, and was subjected to coronary angiography. Elevated Lp(a) levels correlated with higher amounts of oxPL:$apoB_{100}$ and this was associated with incidence of obstructive coronary artery disease.[224] Similar results were reported from the Bruneck study where oxPL:$apoB_{100}$ levels and Lp(a) levels were strongly associated with each other and with the incidence and progression of carotid and femoral atherosclerosis.[225] In a follow up study it was determined that increasing

lipoprotein-associated phospholipase A_2 (Lp-PLA_2) activity enhanced CVD risk and that $apoB_{100}$-associated oxPL could predict cardiovascular events independent of Framingham Risk Score (FRS) information.[226]

The repeated correlation of high Lp(a) levels with elevated oxPL:$apoB_{100}$ suggests that the oxPL associated with the LDL component of Lp(a) may be a source of proatherogenic potential. Indeed, data from the Dallas Heart Study confirmed this finding and reported a correlation between small isoform sizes and increased oxPL:$apoB_{100,}$ which is consistent with the inverse correlation between Lp(a) levels and isoform size.[220] However, none of the studies to this point could differentiate which $apoB_{100}$-containing lipoprotein class actually carried the oxPL. Five years after the discovery of oxPL on Lp(a), Bergmark *et al.* characterized the distribution of oxidized phospholipids on $apoB_{100}$-containing lipoproteins using coimmunoprecipitation and ultracentrifugation experiments. First, it was determined that apo(a) pulldown decreased plasma E06 reactivity by 85%. Immunoblotting of ultracentrifugation fractions found that nearly all E06 positive fractions contained Lp(a), but not other lipoproteins.[131] Using organic extraction, approximately 50% of Lp(a)-associated lipids could be extracted and therefore were non-covalently associated. In addition, *in vitro* transfer studies showed that oxLDL donates oxPL to Lp(a), rather than to LDL. These data clearly identify Lp(a) as a preferential carrier of oxPL compared to other plasma lipoproteins.[131]

Further studies aimed to assess whether oxPL were associated with the apo(a) or LDL-like moieties of Lp(a). Using a gentle reduction technique, Lp(a) can be dissociated into apo(a) and the LDL-like lipoprotein (referred to as Lp(-a)). Using this technique, it

was determined that the apo(a) component of Lp(a) was able to react with the T15 anti-PC antibody, but Lp(-a) was not.[227] This study also showed no correlation between plasma Lp(a) levels or the number of KIV_2 repeats with the extent of oxPL modification of apo(a). Based on this evidence it was speculated that the oxPL on apo(a) might be derived from cellular origin, as a post-translational modification, and not picked up from the plasma pool of free oxPL, though the mechanism of oxPC modification of Lp(a) has yet to be confirmed.[227] Though there was no effect of the KIV_2 polymorphism on extent of oxPL modification of apo(a), it was found that carriers of the rs3798220 SNP (I4399M apo(a) variant) had increased oxPL:$apoB_{100}$.[159] This relationship was particularly strong for smaller isoforms of Lp(a) (<21 KIV_2 repeats), suggesting that the increase in CAD risk for I4399M carriers could be due not only to the increased Lp(a) levels that are associated with the presence of this SNP, but also to the elevated oxPL on Lp(a).

Interestingly, plasminogen was also found to contain oxPC moieties, and these remained present on plasmin after activation by uPA.[228] In lipoprotein-deficient fractions of human and monkey plasma, plasminogen was a significant carrier of oxPC indicating that plasminogen is a second major source of plasma oxPC in addition to Lp(a).[229] The relevance of the oxidized phospholipid on plasminogen is largely unknown. It was observed that oxPC levels on circulating plasminogen acutely increased following myocardial infarction.[229] A functional effect of oxPC on plasminogen function was shown in 2012, when removal of the oxPC on plasminogen (by treatment with PLA_2) was able to significantly prolong the lysis time of fibrin clots.[229] These data indicate that plasminogen may have oxPL-related pathophysiological roles in myocardial infarction and

atherothrombosis. Interestingly, the presence of oxPC seems to make plasminogen a more effective fibrinolytic agent, while the presence of oxPC on Lp(a) seems to promote vascular disease.

Only recently has the oxidized phospholipid on apo(a) been assessed directly from the perspective of structure and function. Leibundgut *et al.* screened Lp(a) from humans and five different primate species. Of note, the apo(a) from each of these species differs from human with respect to apo(a) KIV_{10} sequence and/or the presence of KV. Only human Lp(a) samples were positive for oxPC on apoB or apo(a) using E06 immunoblotting, where all primate species were negative for oxPC, consistent with the initial Edelstein study on rhesus monkey apo(a).[52, 221] However, the initial conclusion by Edelstein *et al.* of the oxPL moiety being present on the KV domain was contradicted by the fact that chimpanzee and gorilla Lp(a) do possess a KV domain but are E06 non-reactive. This lead to a comprehensive screening of more than 28 different recombinant human apo(a) variants, truncations, and isoforms in order to pinpoint a domain(s) of apo(a) that harbours the oxPC moiety. Figure 1.7 shows representative E06 (anti-PC) immunoblotting of all recombinant variants, Lp(a) and plasminogen. The data demonstrated that only apo(a) variants that contain components of the carboxyl-terminal portion are reactive with E06 (compare KIV_{1-4} to 6K, Fig. 1.7). In fact, systematic deletion of kringle domains in the carboxyl-terminal domain indicated that the presence of KIV_{10} is required for E06 reactivity.[52] Interestingly, the only variant that contained KIV_{10} but lacked immunoreactivity to E06 was 17KΔAsp[56], which contains all components of apo(a) but mimics the defective sLBS seen in gorilla and

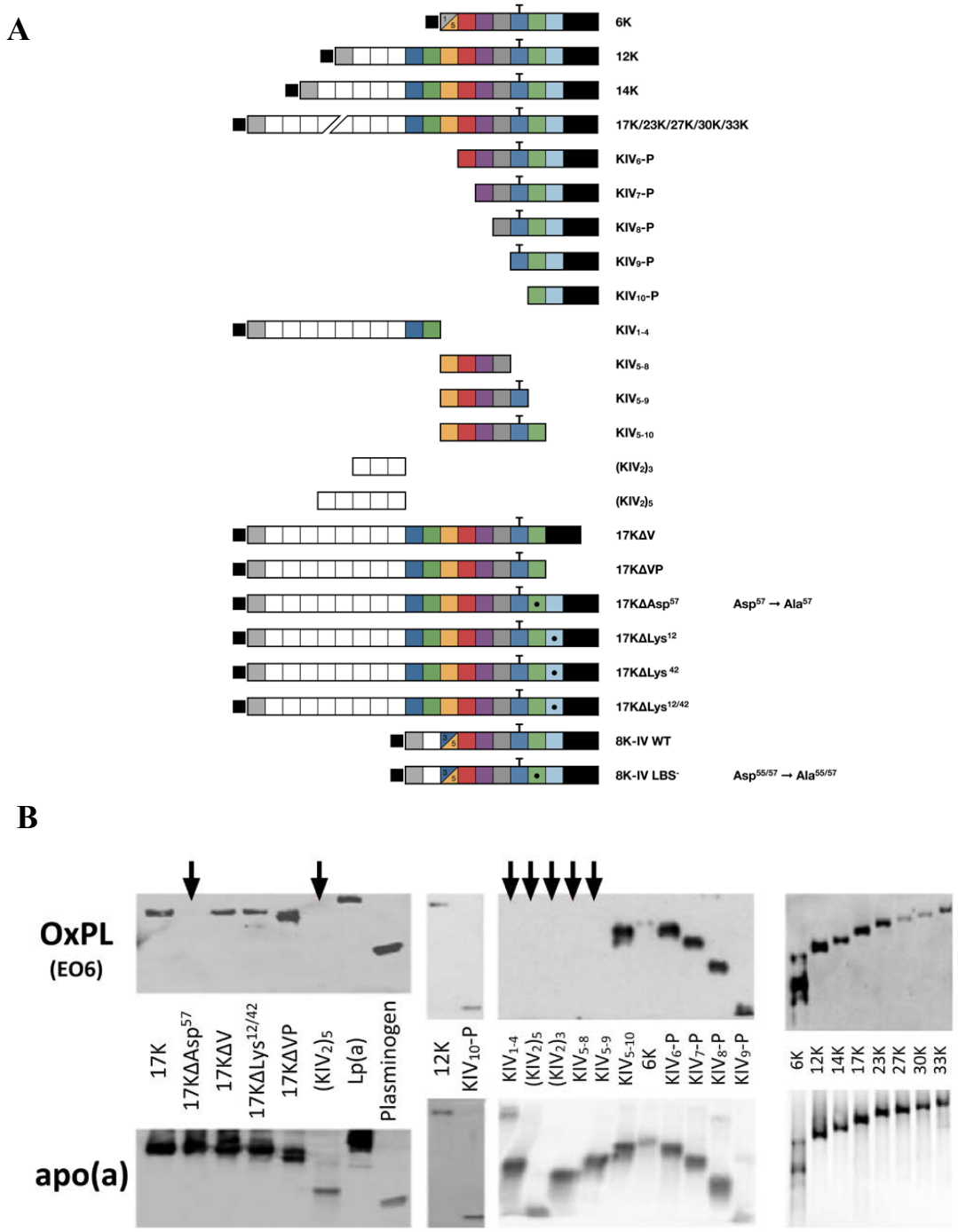

Figure 1.7 Mapping the domain of oxPL modification.
A. Schematic of recombinant apo(a) proteins. B. Immunoblotting of various r-apo(a) constructs and human plasminogen with E06 for PC-oxPL and an anti-apo(a) antibody. Individual constructs are explained in detail in figure 2. Arrows indicate where E06 activity is not present. In these immunoblots oxPL are denoted to reflect only E06-detectable oxPL and not all oxPL. Figure adapted with permission from reference 52.

chimpanzee species (see section 1.1.3). The presence of KV was clearly dispensable for E06 reactivity in the human apo(a) deletion variants (Fig. 1.7). Rhesus monkey apo(a),lacking a KV domain, also has a $Trp^{70} \rightarrow Arg$ mutation in KIV_{10}, which has a similar destabilizing effect on the sLBS. Transgenic mice expressing wild-type apo(a) contained oxPL, while mice expressing the ΔAsp^{56} apo(a) variant lacked oxPL.[52] In conclusion, this study determined that an intact, sLBS-containing KIV_{10} domain is required for E06 reactivity. Coupled with the lack of requirement for KV for E06 reactivity, the data strongly suggest that KIV_{10} harbours the oxPL moiety, not KV. The exact amino acid(s) modified by the oxPC moiety and the role of the sLBS in the mechanism of oxPC addition remains to be explained. The evidence in favour of a role for the oxidized phospholipids on Lp(a) in cardiovascular pathophysiology is strong, but the molecular mechanisms underlying the harmful effects of the covalent oxPC modification on apo(a) are still currently under investigation. A study by Seimon *et al.* recently reported the first direct functional role for the oxPC on apo(a) in an atherosclerotic process, in which Lp(a)/apo(a) was shown to induce apoptosis in ER-stressed macrophages.[132] Using a combination of trypsin and phospholipase A_2 treatments, they reported that removal of the oxPL from apo(a) abolished its ability to exert pro-apoptotic effects. The ability of the oxPL on apo(a), and other lipids in the study, to elicit apoptosis was determined to be dependent on TLR2/CD36 heterodimer mediated intracellular signaling.[132] These data suggest that the oxPL associated with apo(a) promote macrophage apoptosis within a developing atherosclerotic lesion, representing one mechanism linking oxPL on apo(a) to atherosclerosis. Given the strong correlation of the oxidized phospholipids on Lp(a) with incidence of CVD, it is clearly crucial to determine the various mechanisms by which the oxPC moiety on apo(a) directly influences the progression of disease.

1.3 RATIONALE AND OBJECTIVES

While elevated plasma Lp(a) levels have been documented to be a causal risk factor for cardiovascular diseases, the mechanisms of how Lp(a) contributes to atherothrombotic disorders are still not fully described. It is only in understanding these molecular processes that we can hope to develop appropriate therapeutic treatments aimed at preventing the pathogenic effects of Lp(a).

The identification of a correlation between elevated levels of oxidized phospholipids on Lp(a)/apo(a) and the incidence of coronary heart disease illuminates a new potential mechanism by which Lp(a) elicits its effects on the vasculature. It is important that the determinants of this oxidative modification be investigated; only by understanding the mechanism of oxPC addition to apo(a) can we develop the tools required for efficient study of its role in cardiovascular diseases. For instance, it has been shown that removal of the strong LBS in KIV_{10} decreases the proatherosclerotic effects of apo(a) in a transgenic mouse model[230]; since this LBS may also be required for oxPC addition, it is not clear if it is the LBS itself or the oxPC (or perhaps both) that is the entity causing harm.

It is well documented that oxidized lipid products play a progressive role in inflammation and atherosclerosis. The potential for the oxPC moiety on apo(a) to have effects on the atherogenicity of Lp(a) has not been intensively examined. Careful dissection of the known mechanisms of how apo(a) can influence vascular cells is required in order to determine if any of these roles are due, at least in part, to oxidative modification of apo(a). Understanding the link between *LPA* polymorphisms and their relation to

physiological processes, as well as the influence of the oxPL modification, is necessary for the development of appropriate pharmaceutical interventions.

We hypothesize that Lp(a)/apo(a) possesses an oxidized phosphocholine-derived modification on the KIV_{10} domain of apo(a), adducted to a specific amino acid residue, and that the strong lysine binding site within this domain is critical in this process. Furthermore, we hypothesize that there are numerous effects of oxidative modification of apo(a) reflecting the influence of pro-inflammatory processes related to disease progression.

In order to investigate these hypotheses, the objectives of this dissertation are:

1. To characterize, at the primary sequence level, the oxidized phosphocholine modification of apolipoprotein(a);
2. To elucidate the mechanism of lipoprotein(a) induced interleukin-8 expression in macrophages;
3. To determine the structural and functional effects of the rs3798220 (I4399M) single nucleotide polymorphism.

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Characterization of the oxidized phospholipid modification of apolipoprotein(a) kringle KIV_{10}: Insights into the site of oxPC addition

2.1 Summary

Elevated plasma levels of lipoprotein(a) (Lp(a)) have been identified as an independent and causal risk factor for coronary heart disease and calcific aortic valve stenosis. Lp(a) is an enigmatic lipoprotein that contains a moiety indistinguishable from low density lipoprotein (LDL) as well as the unique glycoprotein apolipoprotein(a). We have shown that apo(a) contains a covalent oxidized phosphocholine (oxPC) adduct on the KIV_{10} domain, and that perturbation of the strong lysine binding site in this kringle results in a lack of covalent oxPC addition. Since apo(a) from Old World monkeys and apes lacks covalent oxPC modification, sequence comparison with human apo(a) potentially offers insights into the identify of amino acids in human apo(a) that contain oxPC. In the current study, we constructed mutations in a $KIV_{10}KV$ di-kringle apo(a) species that contains covalent oxPC modification. All residues that are either substituted in primate apo(a) or that can act as acceptors for covalent oxPC addition were assessed using site-directed mutagenesis. The resulting variants were then subjected to immunoblotting analysis with E06, an IgM antibody that detects the presence of oxPC containing a phosphocholine head group. Each variant was also assessed for lysine-binding ability using lysine-Sepharose affinity chromatography. Our analysis has led to the identification of the His33 residue as the site of covalent OxPC addition in apo(a). Mutation of this residue to an alanine results in loss of OxPC modification of this variant, with concomitant retention of its lysine binding ability. In the current study we have also identified the presence of non-covalently associated oxPC present on the apo(a) component of Lp(a). Interestingly, the extent of non-covalently-bound OxPC species appears to be reduced in the apo(a) variant containing a mutation in the strong lysine binding site in KIV_{10}. Taken together, the ability to assess the

individual contributions of the lysine binding site and oxPC modification of apo(a) kringle IV type 10 will provide useful tools to advance our understanding of the mechanisms by which Lp(a) contributes to atherothrombotic diseases.

2.2 Background

Elevated plasma levels of lipoprotein(a) (Lp(a)) have been identified as an independent and causal risk factor for coronary heart disease and calcific aortic valve stenosis.[1] Lp(a) is an enigmatic lipoprotein that is indistinguishable from low density lipoprotein (LDL), both in the composition of lipids and the presence of apolipoprotein B_{100} (apoB_{100}). However, Lp(a) also contains the hydrophilic glycoprotein apolipoprotein(a) (apo(a)) that makes Lp(a) a unique class of lipoprotein.[2] The apo(a) component of Lp(a) is structurally similar to the plasma zymogen plasminogen. The *LPA* gene, encoding apo(a), originated from a duplication event of the plasminogen gene that occurred late in evolution and as such is only expressed in humans and Old World monkeys.[3] Apo(a) comprises ten domains with high sequence similarity to plasminogen kringle IV, denoted KIV_1-KIV_{10}. Each domain is present in a single copy with the exception of KIV_2 which can vary from <3 to >30 copies, giving rise to Lp(a) isoform size heterogeneity within the population.[4] Apo(a) also contains plasminogen-like KV and protease domains although the apo(a) protease domain is proteolytically inactive.[5]

Plasma Lp(a) levels vary widely within the human population, from less than 0.1 to greater than 100 mg/dL. Approximately 20% of individuals globally have elevated Lp(a) levels (defined clinically as >50 mg/dL).[6] However, determination of Lp(a) levels is not a routine procedure, due to challenges inherent in the measurement of this lipoprotein and lack of viable strategies for lowering of Lp(a) levels in the clinic. Additionally, the beneficial effect of Lp(a) lowering on clinical outcomes has not been establishment due to lack of agents designed to specifically lower Lp(a).

Lp(a). has been shown to stimulate atherthrombotic processes including endothelial cell (EC) cytoskeletal remodelling and proliferation[7], inhibition of plasminogen activation[8], and stimulation of monocyte chemotactic activity.[9]; these effects have been primarily attributed to the apo(a) component of Lp(a). Evidence generated within the past decade supports independent and potentially additive atherogenic effects of oxidized phosphocholine (oxPC) associated with Lp(a).[10] In this regard, a growing body of data has documented the presence of both covalent and non-covalent oxPC associated with plasma Lp(a). It has been determined that Lp(a) is a major class of oxPC-containing lipoprotein and that oxidized LDL (oxLDL) can donate oxidized lipids to Lp(a), thus has been named the preferential carrier of oxPC in the plasma.[11] Oxidized phospholipids have many well-documented pathophysiological effects and have been designated as damage-associated molecular patterns (DAMPs), eliciting many proinflammatory and proatherogenic effects in the vasculature. Several clinical studies have reported a strong correlation between the levels of Lp(a) and the oxPC content of apoB-containing lipoproteins; this was further associated with increased cardiovascular disease risk.[12-14] It is tempting to hypothesize that the effect of Lp(a) in atherogenesis may arise, at least in part, from the proinflammatory effects of oxPC present on this lipoprotein.

Understanding the association of oxPC with Lp(a) is critical for determining the exact role for this moiety on Lp(a) pathogenicity. The current understanding of the determinants of oxPC binding to Lp(a) was established through two main studies. The first of these studies, by Edelstein and colleagues, clearly demonstrated that the oxPC on Lp(a) is covalently associated with the apo(a) moiety of the molecule.[15] The study identified the lipid moiety on apo(a) as an oxidized phosphatidylcholine (oxPC), as it can be detected by

the E06 IgM autoantibody that is specific for oxPC-containing epitopes.[16] The E06-reactive epitope was determined to be present within the C-terminal region of apo(a). Rhesus monkey apo(a) lacks oxPC. Since this species lacks a KV domain, this was interpreted to suggest that oxPC modification of apo(a) occurs on KV.[15] Edelstein and colleagues speculated that the oxPC moiety was added to critical lysine residues in KV through Schiff base formation. Recently, a more comprehensive study screened several species of Old World monkeys and apes and determined that there was no E06 reactivity in any apo(a) from any of these species regardless of the presence or absence of the KV domain.[17] Furthermore, the investigators used human recombinant apo(a) variants containing systematic deletions analyzed by western blotting using E06 to deduce the location of the oxPC moiety. Using this approach, apo(a) KIV_{10} was identified as the site of oxPC addition. However, since all non-human primate apo(a) contains this domain yet lacks E06 reactivity, it was hypothesized that there may be a critical role for the lysine binding function of KIV_{10} for oxPC modification. Interestingly, only human apo(a) KIV_{10} contains a strong binding site (sLBS) in KIV_{10}. The lysine binding activity of apo(a) has been implicated in many of the effects of Lp(a) on vasculature, such as endothelial cell monolayer permeability and prostaglandin synthesis.[7, 18] As such, it can be hypothesized that the lysine-dependent pathogenic effects of Lp(a) may be due to oxPC present on apo(a) KIV_{10}.

In the present study we undertook a targeted mutagenesis approach to identify potential sites of oxPC addition on apo(a). Identification of these site(s) will allow us to assess the contribution that the oxidized phospholipid moiety on apo(a) makes to the pathogenic effects of Lp(a).

2.3 Experimental Methods

2.3.1 Construction, expression, purification and characterization of recombinant apo(a)

Expression plasmids encoding sequences spanning the entire KIV_{10} domain, and KIV_{10} plus KV domains were generated by amplification of the pRK5ha$KIV_{10\text{-}P}$ plasmid from the apo(a) signal sequence to the end of either KIV_{10} or KV, using primers sets A and B, or A and C respectively (Supplementary Table 2.1).[19] Bold face lower case letters in primers B and C indicate the mutations introduced to generate an *AgeI* restriction digest site. PCR reactions were carried out in a BioRad CFX96 thermocycler using Q5 High-Fidelity DNA Polymerase (New England Biolabs) under the following conditions: denaturation at 98°C (10 sec), annealing at 62°C (30 sec), and elongation at 72°C (20 sec), for a total of 40 cycles. The PCR product was digested with *EcoRI*-HF and *AgeI*-HF, (New England Biolabs); the pcDNA™4/*myc*-His was also digested with these enzymes, liberating the myc-tag. This reaction yielded a 438bp and 678bp fragment for KIV_{10} and KIV_{10}KV respectively. The fragments were agarose gel-purified and ligated with the vector at 16°C overnight using T4 DNA ligase (Invitrogen) to create the plasmids pcDNA4C.KIV_{10}.His and pcDNA4C.KIV_{10}KV.His.

Plasmids were transfected into HEK293 cells using MegaTran1.0 at 10 μg DNA/100mm plate and subjected to selection with 150 μg/mL Zeocin at 48 hours post-transfection, until a viable population was observed. Recombinant apo(a) was purified from the conditioned medium (CM) of stably-expressing cell lines. The CM was adjusted to 0.5 M NaCl containing, 0.2 mM β-mercaptoethanol, 5 mM imidazole, and 2% glycerol, pH 8.0,

and applied to a Ni^{2+}- Sepharose excel (GE Healthcare) column; the column was washed and eluted with 12 mM and 300 mM imidazole respectively. Eluted samples were concentrated with PEG-20,000 (Sigma) and dialyzed against HEPES-buffered saline (HBS: 20 mM HEPES and 150 mM NaCl, pH 7.4). Concentrations were determined using bicinchoninic acid assay (BCA assay; Pierce) with BSA as a standard. Purity of $KIV_{10}KV$ and KIV_{10} was assessed by SDS-PAGE followed by silver staining. Variants of KIV_{10} and $KIV_{10}KV$ (see Supplementary Table 2.1) were generated using the Quikchange II Site Directed Mutagenesis kit (Agilent) or the Q5 Site Directed Mutagenesis Kit (New England Biolabs), as per manufacturer's instructions, using primer pair sets shown in Supplementary Table 2.1.

2.3.2 Cell Culture

Human embryonic kidney (HEK293) cells were cultured in Minimal Essential Medium (MEM, Gibco) supplemented with 10% FBS (Gibco) and 1% antibiotic-antimycotic (Gibco). THP-1 acute monocytic leukemia cells (ATCC# TIB-202) were cultured in RPMI-1640 medium adjusted to contain 4.5 g/L glucose in 10 mM HEPES pH 7.4 containing a 1.0 mM sodium pyruvate, and supplemented with 10% (v/v) fetal bovine serum (ATCC), 1% (v/v) antibiotic-antimycotic, and 50 μM β-mercaptoethanol (complete medium). THP-1 monocyte cells were subcultured to a density of 4×10^5 cells/mL and their density did not exceed 1×10^6 cells/mL. All cells were grown in a humidified atmosphere (5% CO_2, 95% room air) at 37˚C. THP-1 monocytes were differentiated by the addition of complete growth medium enriched with 100 nM phorbol 12-myristate 13-acetate (PMA). Differentiated cells were plated in a 24-well plate at a density of 8×10^5

cells/mL (1 mL per well) and grown under the same conditions as for the THP-1 monocytes for 72 h. At this time, differentiated THP-1 cells were incubated with serum-free complete medium for 16 h after which time the medium was replaced with serum-free complete medium containing 500 nM of recombinant apo(a) variants $KIV_{10}KV$, KIV_{10}, or mutant variations of the former; the cells were incubated with the various protein additions for 12 hours.

2.3.3 Assessment of the presence of a covalent oxidized phospholipid moiety on apo(a)

SDS–PAGE was performed using 12% polyacrylamide gradient gels. Proteins were then transferred to a PVDF membrane (Millipore) in transfer buffer (25 mM Tris, 192 mM glycine, 10% (v/v) methanol). Blots were blocked in E06 blotting buffer (TBS buffer containing 1% BSA, 0.3 mM EDTA and 0.2% sodium azide) for 1 h, and then incubated with primary EO6 antibody (1:400) for 18 hours at 4°C. The membranes were washed with blotting buffer and incubated with an anti-IgM horseradish peroxidase conjugated secondary antibody (Sigma; 1:7500) for1 hour followed by a final wash in E06 blotting buffer. Immunoreactive bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) with a FluorChem Q Gel Imaging System (Alpha Innotech).[20] The same blots were then washed 5 times with 1X NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, and 0.05% Triton X-100) and blocked in 6% (w/v) non-fat milk in 1X NET for at least 1 h at room temperature. Blots were then incubated with a primary polyclonal sheep anti-Lp(a) antibody (1:15000) for at least 4 hours, washed three

times, and incubated with a rabbit anti-sheep HRP-conjugated secondary (1:7500) (GE Healthcare, USA). Blots were imaged as described above.

2.3.4 Lysine Binding Assay

Assessment of the lysine-binding ability of recombinant apo(a) proteins was performed using a modified lysine-Sepharose chromatography protocol.[21] Briefly, two mL of lysine-Sepharose resin (GE Healthcare) was equilibrated with wash buffer (0.5 M NaCl, 2.7mM KCl, 8 mM Na_2HPO_4, 1.46 mM KH_2PO_4; pH 7.4). Approximately 15 μg of protein was diluted approximately 20-fold with PBS and applied to the column. The column was then subjected to 6 X 1 mL washes. Fractions (1.5mL) were collected from gradient elution of the column with 0.1 μM to 200 mM of the lysine analog ε-aminocaproic acid (ε-ACA) in wash buffer. Fractions were assessed for the presence of recombinant apo(a) protein by immunoblotting analysis, using a polyclonal anti-Lp(a) antibody as previously described. Proteins were defined as being positive for lysine binding by their ability to remain bound to the lysine-Sepharose column in the presence of ε-ACA concentrations greater than 1mM.

2.3.5 Modeling of the Apo(a) KIV_{10} domain

The Molecular Operating Environment (MOE) program[22] was used for the modelling and input preparation for all molecular dynamics (MD) simulations, and analysis of the results obtained. All MD simulations were run using the NAMD program.[23] The X-ray crystallographic structure of the wild-type kringle IV 10/M66 variant of human apolipoprotein(a) with ε-aminocaproic acid bound (PDB ID: 3KIV) was used as a starting

template for all subsequent enzyme structures in the current study.[24] In particular, the His33Ala mutant was obtained from the wild-type enzyme via *in silico* mutagenesis using MOE.[22] Missing hydrogens were added using the default protocols in MOE[22] followed by spherical solvation around the boundaries of the protein to 6 Å. This solvated structure was then minimized using the Amber 12:EHT molecular mechanics (MM) forcefield.[25, 26]

The minimized structure was then subjected to MD simulation with no restraints applied to the atoms of the complex except the wall restraint that was applied to the spherical water droplet to ensure retention of the water sphere. The MD began with a simulated annealing in which, at a constant volume and pressure, the temperature was gradually raised from 150 to 300 K over 150 ps. This was followed by the production run for 4850 ps at a temperature of 300 K.

The root mean squared deviation (RMSD) of the lysine binding site in apo(a) KIV_{10} (the selected residues used were Arg35, Asp54, Asp56, Trp60, Phe62, Arg69, and Trp70) was monitored and examined by cluster analysis to generate 10 clusters in the context of H33A, and 8 clusters in the wild-type form. The average structure of the highest cluster was selected and minimized using the Amber 12:EHT MM forcefield.[25, 26] The calculated RMSDs also provided a statistical measurement of the deviation of the binding site of the mutant from that of the wild-type form.

2.3.6 Extraction and characterization of non-covalently bound phosphocholine containing lipids on apo(a)

The lipids were extracted from $KIV_{10}KV$ and $KIV_{10}KV\Delta Asp^{56}$ by the method previously described by Folch *et al.* with some modifications.[27, 28] The lipid extracts were stored in 2:1 chloroform:methanol, flushed with nitrogen and then capped and stored at -

80°C until analysis. The internal standard mixture spiked into each sample prior to extraction consisted of 1,2-dinonanoyl-*sn*-glycero-3-phosphocholine (09:0 P C), 1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (17:0 LPC) and 1,2-diarachidonyl-*sn*-glycero-3-phosphocholine (20:0 PC).[29] The oxidation products of PC were divided into two groups: fragmented (aldehydes and carboxylates), and non-fragmented (hydroxides, hydroperoxides).

The separation of oxidized PCs was carried out using reverse-phase (RP) chromatography as reported previously.[28, 30-33] Briefly, lipid extracts were reconstituted in RP solvent A (described below) immediately prior to injection. Thirty microliters of the sample was injected onto an Ascentis Express C18 HPLC column (15 cm × 2.1 mm, 2.7 µm; Supelco Analytical, Bellefonte, Pennsylvania, USA) with separation accomplished using a Prominence UFLC system from Shimadzu Corporation (Canby, Oregon, USA). Elution was performed by linear gradient of solvent A (acetonitrile/water, 60:40 v/v) and solvent B (isopropanol/acetonitrile, 90:10, v/v) with both solvents containing 10 mM ammonium formate and 0.1% formic acid. The time program used was as follows: initial solvent B at 32% until 4.00 min; switched to 45% B; 5.00 min 52% B; 8.00 min 58% B; 11.00 min 66% B; 14.00 min 70% B; 18.00 min 75% B; 21.00 min 97% B; 25.00 min 97% B; 25.10 min 32% B until the elution was stopped at 30.10 min. A flow rate of 260 µl/min was used for analysis, and the column and sample tray were held at 45°C and 4˚C, respectively.

The HPLC system was coupled to a 4000 QTRAP® triple quadrupole mass spectrometer system with a Turbo V electrospray ion source from AB Sciex (Framingham, Massachusetts, USA). For phosphatidylcholine, the detection was carried out in positive

ion mode by MRM of 84 transitions using a PC-specific product ion (184.3 *m/z*, Da) which corresponds to the cleaved phosphatidylcholine. The MRM settings were as follows: declustering potential=125, entrance potential=10, collision energy=53, collision cell exit potential=9 and dwell time= 50 msec. [34, 35]

The instrument was tuned by direct infusion of poly(propylene glycol) (PPG) in both positive and negative modes and external mass calibration was performed at regular intervals. The retention time window in MRM was set to detect peaks of significance within 60 seconds of confirmed retention time and data was collected utilizing Analyst® Software 1.6 (AB Sciex). Multi-quant® Software 2.1 (AB Sciex) was used to compare peak areas of internal standards and unknown analytes to quantitate the results.

2.4 Results

2.4.1 Identification of potential sites of covalent oxPC addition to the KIV_{10} and KV domains

In order to identify amino acids that can contain covalently-bound oxPC species, we undertook a site-directed mutagenesis approach. Oxidized phospholipids containing terminal aldehyde groups are the most reactive. As such, we exploited known mechanisms of covalent amino acid – aldehyde linkage. All amino acids which could form a Schiff base (arginine and lysine) or Michael adduct (histidine and cysteine) were considered candidates for oxPC addition. These residues were systematically mutated to an alanine, or glutamine in cases where a conservative mutation was thought to be appropriate. In this way all candidate lysine, arginine, and histidine residues were mutated. Cysteine residues were ruled out as candidate amino acids as all cysteine residues in apo(a) $KIV_{10}KV$ are involved in invariant disulfide bonds that are required to form the kringle structure. Additional mutations of Asp^{56} to Ala or Glu and Trp^{70} to Ala, respectively, were generated to further assess the requirement of an intact lysine binding site for covalent oxPC addition. These mutations were made based on the chimpanzee (Asp^{56} → Asn) and rhesus monkey (Trp^{70} → Arg) KIV_{10} domains, which lack lysine binding ability, or by the apo(a) KIV_7 weak lysine binding site (Asp^{56} → Glu). Figure 2.1 shows a representative structure of the $KIV_{10}KV$.his protein, with all candidate amino acids shaded in grey.

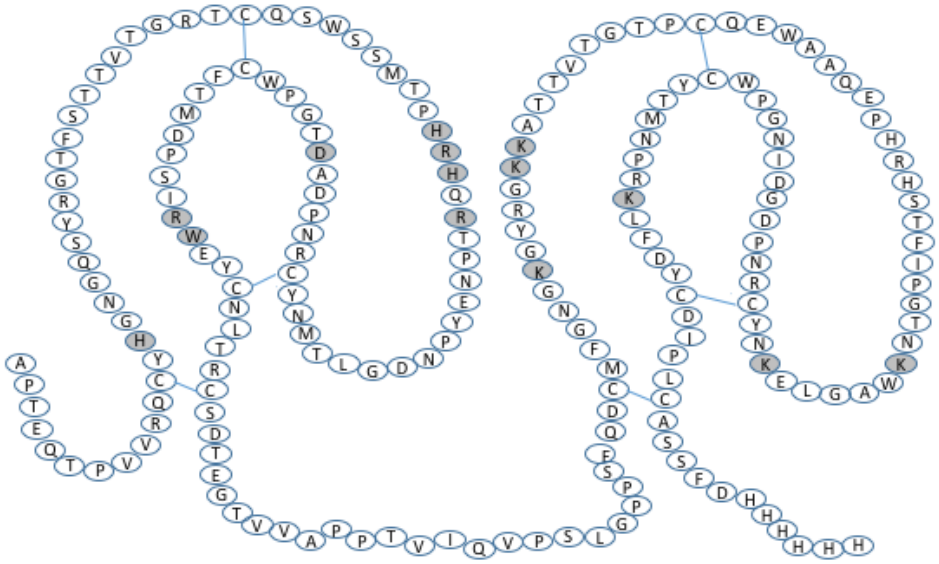


Figure 2.1 Candidate amino acids for covalent oxPC addition in $KIV_{10}KV$.
Truncated apo(a), $KIV_{10}KV$.his, was subjected to site directed mutagenesis to identify residue(s) required for oxPC addition. Critical amino acids were also mutated to assess the requirement for the strong lysine binding site for oxPC modification. The amino acids highlighted in grey are the amino acids that were systematically mutagenized.

2.4.2 Determination of covalent oxPC modification by E06 immunoblotting

Variants of the apo(a) $KIV_{10}KV$ construct were subjected to immunoblotting with the E06 antibody to identify the presence of covalently linked oxPC. Site directed mutagenesis of candidate residues in KIV_{10} revealed a clear role for the strong lysine binding site, as lysine binding deficient mutants Asp^{56} → Ala and Trp^{70} → Ala were all negative for the covalent oxPC modification (Figure 2.2A). The Asp^{56} → Glu variant retained E06 reactivity, albeit to a significantly reduced extent. All other apo(a) KIV_{10} domain variants were positive for E06 immunoreactivity, with the exception of the His^{33} → Ala variant. All variants generated in the KV domain of the $KIV_{10}KV$ variant (KV Lys → Ala substitutions) retained E06 immunoreactivity (Figure 2.2B). Screening of the apo(a) KIV_{10} domain alone revealed the presence of covalent oxPC modification, albeit to a lesser degree than that observed using $KIV_{10}KV$ (Figure 2.2C). No E06 immunoreactivity was observed using on the $KIV_{10}\Delta Asp^{56}$ variant; this was also observed using the $KIV_{10}KV\Delta Asp^{56}$ variant.

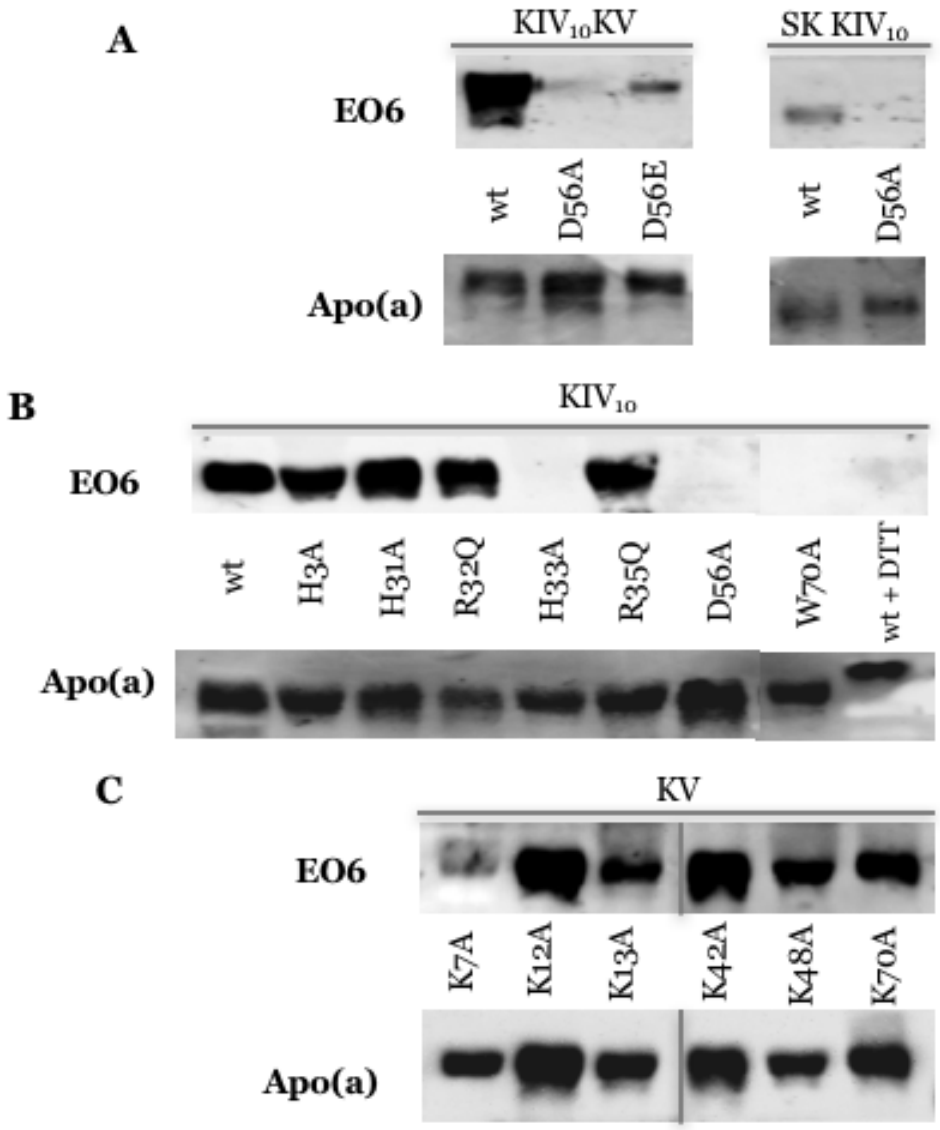


Figure 2.2 Covalent oxPC modification of apo(a) KIV_{10} and $KIV_{10}KV$ assessed by E06 immunoreactivity

Detection of covalent oxPC was detected by immunoblotting of apo(a) variants (see Fig. 2 1) with the E06 antibody. Total apo(a) protein was detected using a sheep polyclonal anti-apo(a) antibody. Lysine binding was assessed based on elution profiles of the apo(a) variants from a lysine-Sepharose column. **Panel A**. Apo(a) variants with candidate mutations in the lysine binding site of apo(a). Apo(a) kringle IV_{10} (SK) was also subjected to this analysis. **Panel B**. Analysis of candidate histidine and arginine residues oxPC in KIV_{10}. **Panel C**. Analysis of candidate lysine residues oxPC in KV.

2.4.3 Assessment of the lysine binding properties of E06-negative apo(a) variants

To further our analysis of the relationship between oxPC binding and the presence of a functional lysine binding site in KIV_{10}, we next examined the ability of apo(a) variants that are E06-negative by western blotting to bind to lysine-Sepharose columns. Subjecting wild-type $KIV_{10}KV$ and KIV_{10} to lysine-Sepharose chromatography followed by a gradient elution with the lysine analog ε-ACA revealed the ability of these proteins to bind to lysine (Figure 2.3). Both Asp^{56} → Ala variations of $KIV_{10}KV$ and KIV_{10} were eluted in the wash fractions; the Asp^{56} → Glu and Trp^{70} → Ala variants of $KIV_{10}KV$ also did not bind to the lysine column. Finally the His^{33} → Ala variant bound to the lysine-Sepharose column in a comparable manner to that observed for the $KIV_{10}KV$ and KIV_{10} wildtype proteins. As such, the His33 → Ala variant of $KIV_{10}KV$ does not contain covalent oxPC but retains lysine binding properties comparable to wild-type apo(a).

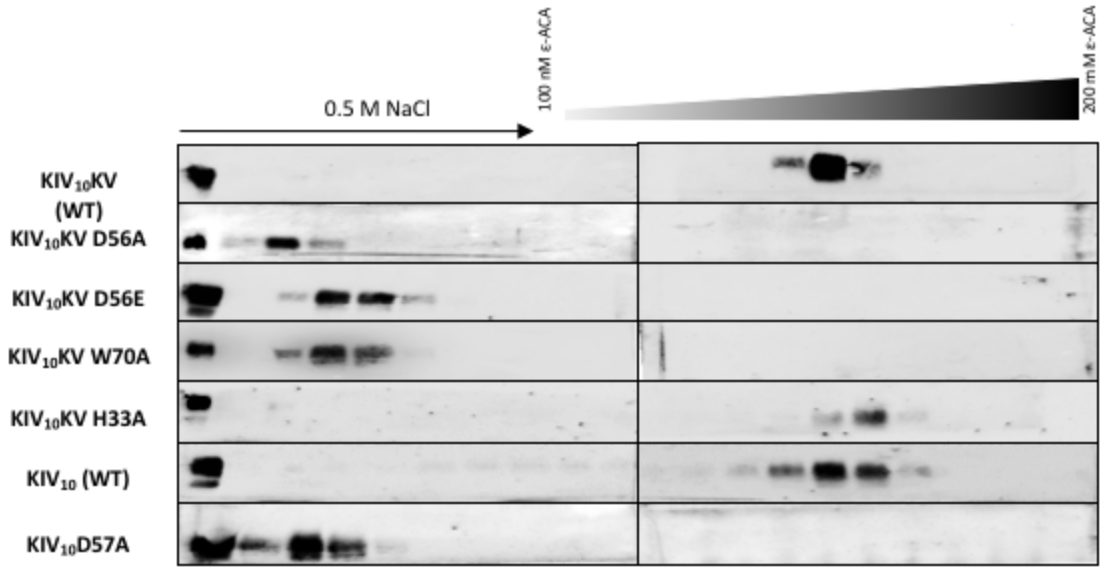


Figure 2.3 Assessment of lysine binding properties of E06 non-reactive apo(a) variants.
Lysine binding ability was assessed by applying 15 µg of each apo(a) variant to a lysine Sepharose column, followed by washing to remove non-specifically bound protein, and gradient elution using lysine analog ε-ACA to remove specifically-bound protein. Fractions were collected and analyzed by SDS-PAGE followed by anti-apo(a) blotting using a sheep polyclonal antibody.

Table 2.4 Summary of the investigation of covalent oxPC modification of apo(a) using site-directed mutagenesis of candidate residues in KIV_{10} and KV.

Domain	Amino Acid	Rationale	oxPL status	Binds Lysine
KIV_{10}	H3	Michael's Addition	✔	✔
	H31	Michael's Addition	✔	✔
	R32	Old World Monkeys	✔	✔
	H33	Michael's Addition	✘	✔
	R35	Michael's Addition	✔	✔
	D56A	Chimpanzee/Gorilla	✘	✘
	D56E	KIV_7 wLBS	✘*	✘
	W70	Rhesus/Cynomologous	✘	✘
KV	K7	Schiff Base	✔	✔
	K12	Schiff Base	✔	✔
	K13	Schiff Base	✔	✔
	K42	Schiff Base	✔	✔
	K48	Schiff Base	✔	✔
	K70	Schiff Base	✔	✔

** Significantly reduced E06 reactivity*

2.4.4 Computational assessment of KIV_{10} and KIV_{10} $H^{33}A$

Using a molecular dynamic simulation approach, we modelled the human KIV_{10} domain of apo(a) in complex with the lysine analog ε-ACA, both with and without the His^{33} mutation. This was performed to determine if there is a structural basis for the lack of oxPC addition to this residue. Analysis of the KIV_{10} domain allows for the observation of the His^{33} in its native environment (Figure 2.4). We observed that His^{33} does not appear to be within significant proximity of any amino acids that could participate in hydrogen bonding or salt bridge interactions. An overlay of the KIV_{10} and KIV_{10} $H^{33}A$ domains shows a lack of major changes in secondary structures or overall conformation as a result of the His^{33} →Ala substitution.

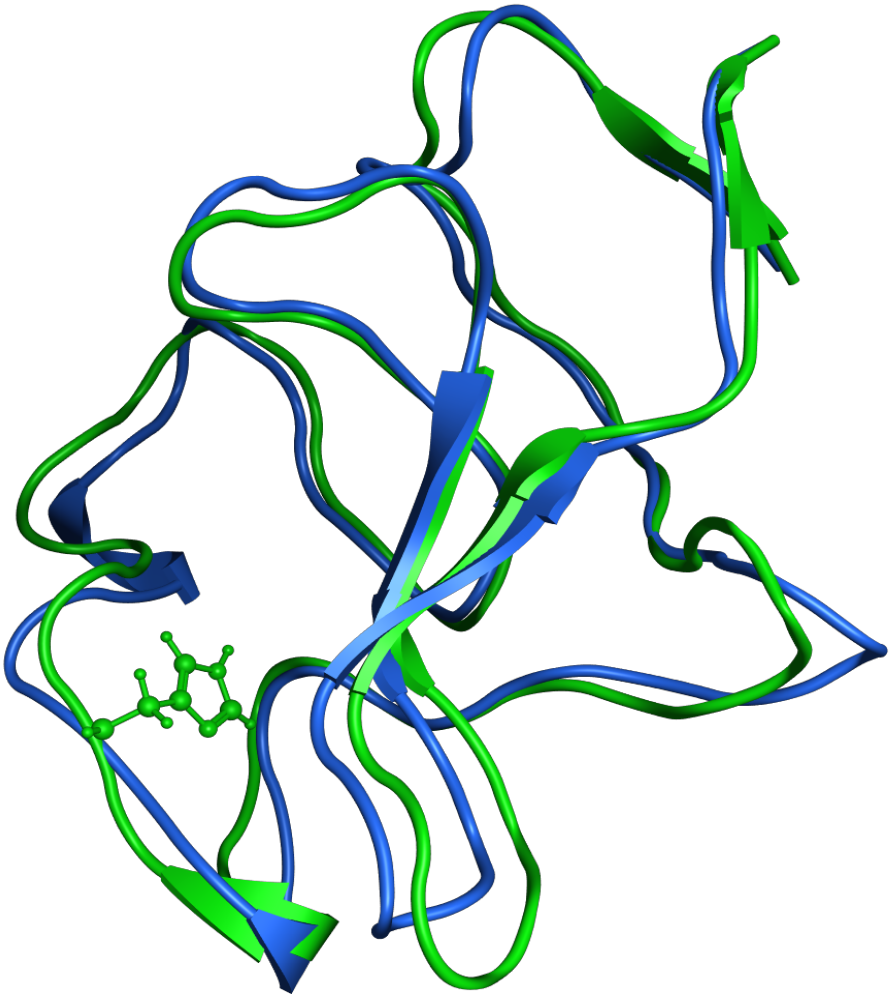
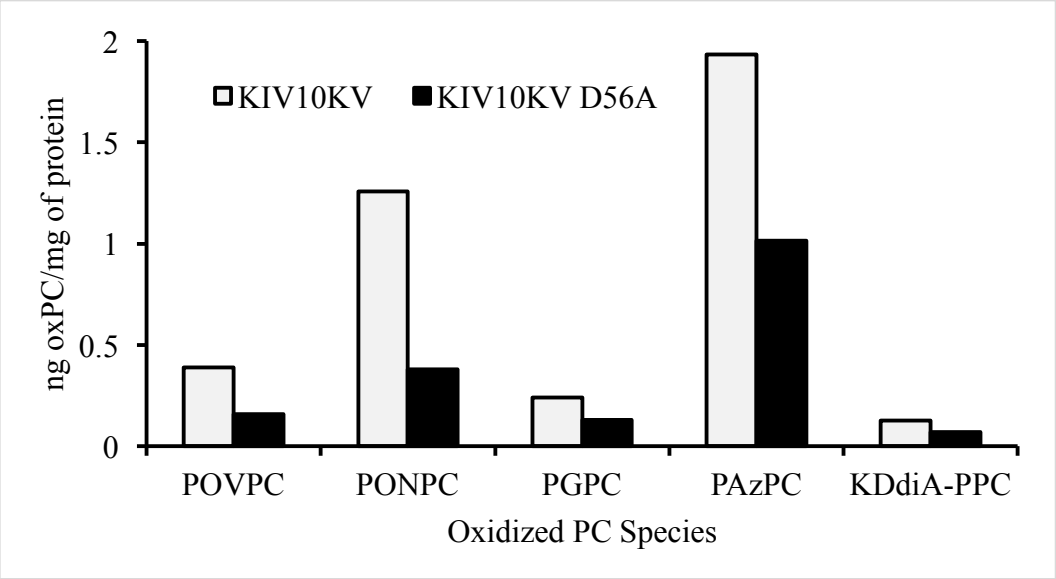


Figure 2.4 Computational assessment of KIV_{10} and KIV_{10} H33A.
A molecular dynamic simulation of KIV_{10} (green) complexed with ε-ACA (ligand not shown) was modeled and overlayed with a KIV_{10} variant containing a His^{33} → Ala mutation (blue). Models are representative of the average conformation of the respective domains over a 5 ns simulation. The position of His 33 is shown on the overlay of the wildtype KIV_{10} domain (green His 33 visualized).

2.4.5 Identification of non-covalent oxPC species on apo(a)

Using organic extraction, the non-covalently bound oxPC on $KIV_{10}KV$ or $KIV_{10}KV\Delta Asp^{56}$ were removed from their respective proteins and subjected to reverse phase chromatography in tandem with mass spectrometry using a multiple reaction monitoring approach in positive ion mode for 184.3m/z. This specific mass to charge ratio is indicative of the phosphocholine head group that is present on a subset of lipids that is of interest (Fig. 2.5A) The top 5 most abundant lipid species are shown in Figure 2.5B and include POVPC (1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine), PONPC (1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine), PGPC (1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine), PAzPC (1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine), and KDdia-PC (1-palmitoyl-2-(4-keto-dodec-3-ene-dioyl)-phosphatidylcholine). Amounts shown in Figure 2.5 are in ng oxPC per ng of protein.

A.



B.

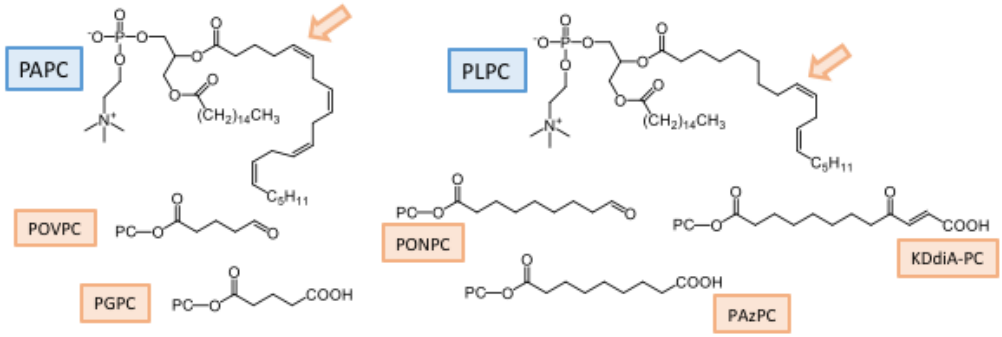


Figure 2.5 Apo(a) KIV10KV contains non-covalently associated oxidized phosphatidyl choline species.
A. Assessment of oxPC species that associate with the $KIV_{10}KV$ and $KIV_{10}KV$ D56A variants was conducted by extensive lipid extraction of the recombinant proteins followed by mass spectrometry. For phosphatidylcholine, detection was carried out in positive ion mode by MRM using a PC-specific product ion (184.3 m/z, Da) which corresponds to the cleaved phosphorylcholine head group. The top five most abundant species are represented in nanograms of oxPC per nanogram of protein, and are also represented as a schematic diagram in **B.**

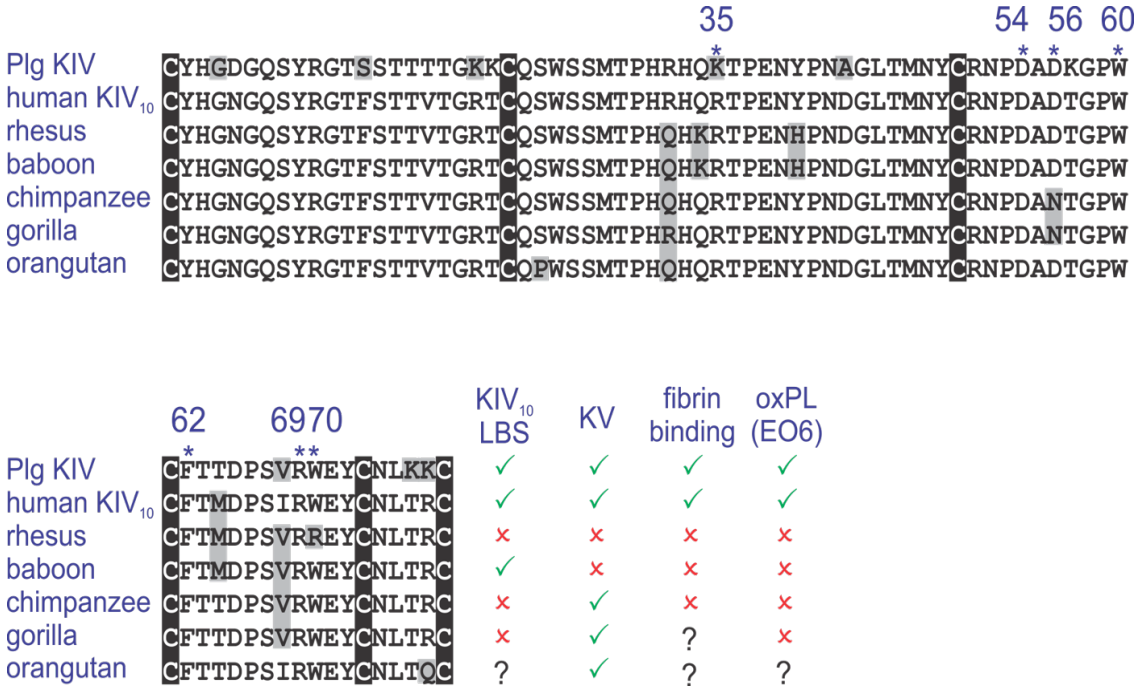


Figure 2.6 Sequence comparison of human and primate KIV_{10}, with insights to function.

Differences between human, ape, and Old World monkey apo(a). The sequences of KIV_{10} from the indicated species are aligned with human KIV_{10} as well as plasminogen (Plg) KIV. Substitutions relative to human KIV_{10} are shaded in grey, and the invariant cysteine residues involved in disulfide bond formation are shaded in black. Also shown are the residues (asterisked) of the functional lysine binding site. Summarized are the LBS activity based on amino acid sequence and the presence of KV, as well as the ability to bind fibrin and be recognized by E06.

2.5 Discussion

Lipoprotein(a) has been established as a causal risk factor for cardiovascular disease and calcific aortic valve stenosis.[36] The evidence for a pathophysiological role of Lp(a) is significant yet the mechanisms by which Lp(a) elicits its effects within the vasculature still remain unclear. Since its discovery in 2003 by Tsimikas and colleagues, the association of E06 reactive phospholipid species and Lp(a) has been proposed to be involved in the atherogenicity of Lp(a).[37, 38] The finding that the apo(a) moiety of Lp(a) possesses a covalently linked oxPC molecule, as indicated by E06 immunoblotting, highlighted a novel structural element to the apo(a) glycoprotein.[15] This finding provides additional rationale for the apo(a) component of the Lp(a) protein complex to be considered atherogenic, a title usually associated with the LDL-like moiety. The identification of a proinflammatory post-translational modification on apo(a) can potentially offer explanation to some of the proinflammatory effects associated with apo(a) on vascular cells, such as increased prostaglandin synthesis[18] and upregulation of cell adhesion molecules in endothelial cells[39], processes that are not yet fully understood. The exact molecular mechanisms by which this modification contributes to the proinflammatory and proatherogenic potentials of Lp(a) have only recently begun to be described.

The domain on apo(a) that harbours the oxidized phospholipid moiety has been previously investigated with the aid of non-human primate Lp(a), as well as several recombinant apo(a) variants. However, the location of the moiety on the primary sequence of apo(a) has yet to be determined. In this study, we subjected a recombinant human apo(a) variant to targeted alanine mutagenesis in order to deduce key structural features on the KIV_{10} and KV domains that are required of oxPC modification of apo(a). Initial studies by

Edelstein *et. al.*, suggested that the covalent oxPC moiety on apo(a) was present on either Lys^{12} or Lys^{42} of the KV domain.[15] This deduction was predicated by experiments performed on elastase fragments of human apo(a), with added insights from studies on rhesus monkey apo(a). Early studies hypothesized that the ability of apo(a) to induce IL-8 expression in macrophages was a product of the oxPC moiety.[40] Using a series of pancreatic and leukocyte elastase fragments it was determined that the oxPC moiety was present in the carboxyl-terminal portion of apo(a), as fragments 2 (KIV_5-P) and 7 (KV-P) reacted with E06. Analysis of rhesus monkey apo(a), which lacks KV, suggested that KV was the domain harbouring the oxPC moiety as this variant does not react with the E06 antibody.[15] However, optimal IL-8 stimulating ability was not possessed by fragment 7, suggesting the absence of an immunogenic oxidized phospholipid on fragment 7.[40] This was explored in detail in a later publication from our group and collaborators using human recombinant apo(a) variants, several of which entirely lacked the KV domain. In contrast to the report of Edelstein *et al.*, our study showed that proteins lacking KV (17KΔV, 17KΔVP, and KIV_{5-10}) could possess E06 immunoreactivity.[17] The battery of recombinant variants unveiled the importance of the KIV_{10} domain since all variants that lacked a KIV_{10} domain possessed no immunoreactivity to E06.

In order to further characterize the domains critical for oxPC addition in the present study, we first constructed a small apo(a) truncation including only the KIV_{10} exclusively, or the KIV_{10} and KV domains of apo(a) (Figure 2.1). Using these small apo(a) truncations we are able to confirm that these domains of apo(a) harbor the sequences giving E06 immunoreactivity, thus an oxidized phospholipid moiety (Figure 2.2A). These results conclusively define KIV_{10} as the carrier of covalent oxPC modification.

The finding of a relationship between KIV_{10} lysine binding ability and oxPC addition raises two key questions. First: is lysine binding by this kringle actually required for addition of oxPC? Second: when assessing the elimination of certain pathogenic effects of apo(a) when the KIV_{10} LBS is mutated (for example evoking EC dysfunction *in vitro*[7] or atherogenesis in transgenic mice *in vivo*[41]), are these effects due to loss of lysine binding or loss of oxPC addition or both? Our current study provides a definitive answer to the first, while also providing a way forward to address the second. Utilizing several mutants, in the context of $KIV_{10}KV$, we have demonstrated that mutation of human apo(a) at critical lysine binding residues Asp^{56} and Trp^{70} is able to block the ability of apo(a) to be modified by oxPC (Figure 2.2A). This result also is in agreement with the lack of E06 immunoreactivity observed previously in rhesus monkey apo(a), as this species possesses a Trp^{70} → Arg substitution.[15] Furthermore, using a more conservative Asp^{56} → Glu variant it can be observed that a significant reduction in the intensity of an E06 reactive band is present on this mutant, in contrast to the loss of E06 reactivity seen with the former lysine binding mutant. The conservative mutation mimics the key difference underlying the relatively weak lysine binding site present in human $KIV_{7.}$[21] Subjecting these variants to a lysine-Sepharose binding assay can confirm that these E06 unreactive proteins have no affinity for lysine, as Asp56 and Trp70 mutants elute from the resin without the addition of the lysine analog ε-ACA, whereas the non-mutated for remains about to the resin until the concentration of ε-ACA exceeds 100mM (Figure 2.3). However, even the weak lysine binding site variant, Asp^{56} → Glu, did not possess the ability to bind to lysine-Sepharose. This is potentially due to the use of 0.5 M NaCl in the wash buffer, which was used to prevent the non-specific interaction of the lysine-Sepharose and the glycans on apo(a). A

single KIV_{10} containing this mutation has been previously reported to possess lysine binding ability, albeit with 10-fold less affinity.[21] In the context of bacterially purified KIV_{10}, the D56E variant was able to withstand 0.15 M NaCl washes and up to 20 mM ε-ACA, whereas wildtype eluted at 200 mM ε-ACA.[21] The designation of this variant as a mutant with an intermediate ability to bind lysine provides support to the idea that lysine binding is important for the modification of this domain. The significant decrease in the ability of this variant to react with E06, without total abolishment of immunoreactivity, suggests an intermediate ability to be modified by phosphorylcholine containing oxPC. The extent of E06 reactivity positively correlates with the ability of apo(a) variants to bind lysine giving insights to the possible mechanism of oxPC addition. The realization of the importance of the lysine binding properties of apo(a) as a requirement for oxPC addition, allows for proper interpretation of the results seen in ape and Old World monkey apo(a) and may give insight into where the modification may be derived from. The interaction of apo(a) with various ligands in a manner dependent on the sLBS in KIV_{10}, is well documented with ligands such as fibrin[42], collagen[43], and cell surface receptors.[44, 45] The mechanism of oxPC addition to apo(a) is yet to be determined, however it is possible that the lysine binding properties of apo(a) may allow for apo(a) to interact with cell surface receptors and bring it into proximity of sources of oxidized phospholipids, such as the cell membrane.

The location of the oxPC moiety on the primary sequence of apo(a) has been a key research question. The early reports of the oxPC modification on apo(a) suggested Lys^{12} and Lys^{42} on KV as candidates of oxPC addition, based on the availability of these amino acids to participate in Schiff base formation.[15] Mutation of $Lys^{12/42}$ in combination was

found to have no effect on the ability of mutant apo(a) to react with the E06 antibody indicating that these two residues are not involved in oxPC addition.[17] Aldehyde containing oxidized phospholipids are known to form adducts with thiol and amido containing amino acid side chains such as lysine, cysteine, histidine, and arginine.[46] In this study we generated of 17 candidate proteins (derived from $KIV_{10}KV$) in order to unambiguously determine the specific amino acid location of the covalent oxidized phosphocholine modification on apo(a). Figure 2.2C indicates that no lysine mutation made in KV was able to prevent the oxPC modification of apo(a), as determined by E06 immunoreactivity (Summarized in Table 2.1). This is in agreement with direct proof that the KV domain does not harbor the E06 reactive lipid moiety.[17] In KIV_{10}, we examined residues His^{3}, His^{31}, Arg^{32}, His^{33}, and Arg^{35}. Figure 2.2B reveals that His^{3}, His^{31}, Arg^{32}, and Arg^{35} all retain reactivity to the E06 anti-phosphocholine antibody. However, the His^{33} → Ala variant did not possess any reactivity to E06, indicating that it lacks an oxPC moiety. Furthermore, subjecting this variant to lysine-Sepharose chromatography proved that this mutation has no effect on the ability of KIV_{10} to bind to lysine (Figure 2.3), as it binds to a similar apparent affinity as the non-mutated protein. In addition, we used molecular dynamic simulations of the KIV_{10} domain in the absence and presence of the His^{33} mutation and found no significant changes in the structure of the lysine binding domain, or the entire KIV_{10} in general (Figure 2.4). In fact, a close look at the coordination sphere surrounding His^{33} reveals that it is most likely not involved in any hydrogen bonding or salt bridge interactions, and is thus available for participation in a Michael addition reaction with phospholipid containing an oxidized fatty acyl chain terminating in a free aldehyde. We have, therefore, identified the long-sought location of the covalent oxPC modification of

apo(a). Moreover, we have managed to decouple the oxPC addition to KIV_{10} from its lysine binding function, a summary can be seen in Figure 2.6. Therefore, the identification of His^{33} as the site of oxPC modification will allow for the determination of which pathogenic processes that the oxPC modification of apo(a) stimulates.

Previous studies on the roles of oxPC in apo(a) elicited processes have highlighted the role specifically for the E06 reactive phospholipid in inflammatory processes. A recent study was able to block the apo(a) induced inflammatory response in human macrophages using an E06 blocking approach, by 50%.[47] Though E06 has a robust effect as a blocking agent, studies that used treatment of apo(a) with phospholipase A_2 were able to almost blunt the effect of oxPC on inflammatory processes entirely.[9, 48] This suggests that there are E06-unreactive pro-inflammatory oxPC on apo(a). Using a lipid extraction of $KIV_{10}KV$ and the associated Asp^{56} variant, we used a mass spectrometry approach and identified several species of oxPC that were non-covalently associated with apo(a) (Figure 2.6). The results from the organic extraction of the E06 negative Asp^{56} variant, revealed almost a 50% decrease in all of the detected oxPC species indicating that the lysine binding ability of apo(a) may also play a role in the ability to associate with non-covalent oxidized phospholipids. However, it can be noted that E06 remains able to identify a significant proportion of the oxPC on apo(a), as the wildtype apo(a) variant possessed greater than 50% more detectable lipid species. It should be noted, however, that the roles of these non-E06-detectable oxPC are unknown, since it is E06 reactivity that has been correlated to vascular risk in patients.

In conclusion, this study is the first report of a variant of apo(a) that lacks the ability to be modified by E06 reactive oxidized phosphocholine while retaining its ability to bind

to lysine through KIV_{10}. We have identified His^{33} within KIV_{10} as the long-sought site of oxPC addition. In addition, we have confirmed the association between the lysine binding ability of apo(a) and the ability for apo(a) to be modified by oxPC. The determination of the presence of non-covalent oxPC and the identification of the site of covalent oxPC addition will serve as valuable information in the elucidation of the roles for apo(a) in pro-inflammatory processes within the vasculature, and specifically which of those roles are elicited by atherogenic oxPC.

2.6 References

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Supplementary Table 0.1: Oligonucleotide primers used in this study

Primer	*Sequence*	*Rationale*
A	5'-TCCACTCCCAGGTCCAACTGCACCT-3'	Amplifies *EcoRI* restriction site before KIV_{10}
B	5'-CAAA**accggt**GTCTTGTTCAGAAGG-3'	*AgeI* restriction site after KIV_{10}
C	5'- GA**accggt**ACAGAGAGGATATCACAGTAGTCA -3'	*AgeI* restriction site after KV
D	5'-GTCCGGCAGTGCTAC**gcT**GGTAATGGCCAGAG-3'	KIV_{10} H3A
E	5'-CTCTGGCCATTACC**Agc**GTAGCACTGCCGGAC-3'	KIV_{10} H3A
F	5'-CATGACACCA**gcC**CGGCATCAG-3'	KIV_{10} H31A[c]
G	5'-GATGACCAAGATTGACATG-3'	KIV_{10} H31A[c]
H	5'-CATGACACCACAC**CaG**CATCAGAGGACCC-3'	KIV_{10} R32Q
I	5'-GGGTCCTCTGATG**CtG**GTGTGGTGTCATG-3'	KIV_{10} R32Q
J	5'-GGAATCCAGATGCC**GcT**ACAGGCCCTTGGTG-3'	KIV_{10} D56A
K	5'-CACCAAGGGCCTGT**AgC**GGCATCTGGATTCC-3'	KIV_{10} D56A
L	5'-CAGATGCC**GAa**ACAGGCCCTT-3'	KIV_{10} D56E[c]
M	5'-GATTCCTGCAGTAGTTCATTGTC-3'	KIV_{10} D56E[c]
N	5'-CAGCATCAGG**gcG**GAGTACTGC-3'	KIV_{10} W70A[c]
O	5'-GGGTCCATGGTAAAACAC-3'	KIV_{10} W70A[c]
P	5'-CTGTATGTTTGGGAATGG**gcA**GGATACCGGGGCAAGAAG-3'	KV K7A
Q	5'-CTTCTTGCCCCGGTATCC**Tgc**CCCATTCCCAAACATACAG-3'	KV K7A
R	5'-GGAAAGGATACCGGGGCgc**G**AAGGCAACCACTGTTA-3'	KV K12A
S	5'-TAACAGTGGTTGCCTT**Cgc**GCCCCGGTATCCTTTCC-3'	KV K12A
T	5'-AAGGATACCGGGGCAA**Ggc**GGCAACCACTGTTACTG-3'	KV K13A
U	5'-CAGTAACAGTGGTTGCC**gcC**TTGCCCCGGTATCCTT-3'	KV K13A
V	5'-AGCACGTTCATTCCAGGGACAAAT**gcA**TGGGCAGGTCTG-3'	KV K42A
W	5'-CAGACCTGCCCA**Tgc**ATTTGTCCCTGGAATGAACGTGCT-3'	KV K42A
Z	5'-ATAAATGGGCAGGTCTGGAA**gcA**AATTACTGCCGTAACCCTG-3'	KV K48A
AA	5'-CAGGGTTACGGCAGTAATT**Tgc**TTCCAGACCTGCCCATTTAT-3'	KV K48A
BB	5'-GAATCCAAGA**gcA**CTTTTTGACTACTGTGATATC-3'	KV K70[c]
CC	5'-ATTGTGTAGCACCAGGGAC-3'	KV K70[c]
DD	5'- ATGACTTCCAAGCTGGCCGTGGCT-3'	IL-8
EE	5'- TCTCAGCCCTCTTCAAAAACTTCTC-3'	IL-8
FF	5'- AGAGAGAGGCCCTCAGTTGCT-3'	GAPDH
GG	5'- TTGTGAGGGAGATGCTCAGTGT-3'	GAPDH

[a] *Lower case letters indicate mutated sequences.*
[b] *Plasmids containing KIV_{10}R36Q, KIV_{10}H33A, KIV_{10}R69Q mutations in the context of KIV_{10}KV were synthesized by Genscript®.*
[c] *Primers were designed for the generation of a linear blunt-ended product consistent with the Q5 site directed mutagenesis system (New England Biolabs).*

Mechanistic Insights into Lipoprotein(a)-Induced Interleukin-8 Expression: A Role for Oxidized Phospholipid Modification of Apolipoprotein(a)

3.1 Summary

Elevated lipoprotein(a) (Lp(a)) levels are a causal risk factor for coronary heart disease. Accumulating evidence suggests that Lp(a) can stimulate cellular inflammatory responses through the kringle-containing apolipoprotein(a) (apo(a)) component. Here, we report that recombinant apo(a) containing 17 kringle-IV domains (17K) elicits a dose-dependent increase in IL-8 mRNA and protein expression in THP-1 and U937 macrophages. This effect was blunted by mutation of the lysine binding site in apo(a) KIV_{10}, which results in the loss of oxidized phospholipid (oxPL) on apo(a). Trypsin-digested 17K had the same stimulatory effect on IL-8 expression as intact apo(a) while enzymatic removal of oxPL from apo(a) significantly blunted this effect. Using siRNA to assess candidate receptors, we found that CD36 and TLR2 may play roles in apo(a)-mediated IL-8 stimulation. Downstream of these receptors, inhibitors of MAP kinases JNK and ERK1/2 abolished the effect of apo(a) on IL-8 gene expression. To assess roles of downstream transcription factors, luciferase reporter gene experiments were conducted using an IL-8 promoter fragment. Apo(a) induced expression of this reporter construct was eliminated by mutation of IL-8 promoter binding sites for either NF-κB or AP-1. Our results provide a mechanistic link between oxPL modification of apo(a) and stimulation of proinflammatory intracellular signaling pathways.

3.2 Background

Atherosclerosis is the leading cause of death in the industrialized world.[1] The initiation of the development of an atherosclerotic lesion involves the activation of various vascular cell types, with macrophages playing a central role in this process as key mediators of the chronic inflammatory response.[2] Upon damage to the vessel wall, endothelial cells secrete chemoattractants that can interact with monocytes and promote their directional migration, thereby recruiting these cells to the site of the developing lesions where they become differentiated into macrophages. Activated macrophages secrete a number of pro-inflammatory cytokines including interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor α (TNFα) that contribute to lesion progression.[3, 4]

Elevated plasma concentrations of Lp(a) have been identified as a risk factor for vascular diseases such as peripheral vascular disease, stroke and coronary heart disease.[5-7] Lp(a) consists of a lipoprotein particle identical to low density lipoprotein (LDL) in both lipid composition as well as the presence of apolipoproteinB-100. However, Lp(a) is a unique lipoprotein class owing to the presence of the large glycoprotein apolipoprotein(a) (apo(a)) that is covalently linked to apoB-100 via a single disulfide bond.[8] Apo(a) bears striking homology to the human plasma zymogen plasminogen and contains 10 distinct domains each homologous to the plasminogen KIV sequence. Each of the 10 apo(a) KIV domains is present in a single copy with the exception of KIV type 2 (KIV_2) which can range from 3 to >30 identical copies in plasma Lp(a).[9] Apo(a) also contains single copies of carboxyl-terminal domains that closely resemble the kringle V (KV) and pro-protease domains of plasminogen. Apo(a) KIV types 5-8 (KIV_{5-8}) each possess a weak lysine binding site (LBS)[10], several of which are responsible for the initial non-covalent

interaction of apo(a) and apoB-100 prior to covalent bond formation.[11] KIV type 10 (KIV_{10}) is characterized by a strong LBS, which plays a role in apo(a)-mediated inhibition of plasminogen activation on fibrin and cell surfaces and binding of apo(a) to plasminogen receptors on the cell surface.[12-14]

Within the vascular milieu, the apo(a) component of Lp(a) has been reported to mediate a number of potential pro-inflammatory effects. In cultured vascular endothelial cells, Lp(a)/apo(a) has been shown to induce expression of the cell adhesion molecules ICAM, E-selectin and VCAM.[15, 16] Lp(a)/apo(a) also promote the assembly of actin stress fibers resulting in cell contraction and increased endothelial cell permeability.[17] Lp(a)/apo(a) has been implicated in pro-inflammatory processes through its ability to bind to the integrin Mac-1 on endothelial cells, resulting in the Mac-1-dependent promotion of monocyte adhesion and transendothelial migration.[18] The apo(a) component of Lp(a) has been shown to induce gene expression in THP-1 macrophages, including the upregulation of IL-8 expression; this effect was mapped to the carboxyl-terminal half of the apo(a) protein, with a role for the KV domain suggested.[19] However, definitive identification of the apo(a) sequences required for this response, as well as elucidation of the mechanisms underlying it, have not been accomplished to date.

In recent years much attention has been paid to the oxidized phospholipid (oxPL) modification of Lp(a) and the extent to which it underlies the proatherogenic properties of this lipoprotein.[20-22] It has been shown that, compared to LDL, Lp(a) is a preferential carrier of oxPL in plasma[23], and that the modification consists of a lysine-phosphatidyl choline adduct that may reside within the apo(a) KV sequence.[24] More recently, it has been demonstrated that oxPL addition to apo(a) requires the strong LBS in KIV_{10}, while apo(a)

species lacking the KV domain were reported to contain oxPL to an extent comparable to full-length apo(a).[25] OxPL modification of apo(a) was demonstrated to induce apoptosis in macrophages undergoing endoplasmic reticulum (ER) stress[26]; this response could be maintained upon trypsin digestion of the oxPL-containing apo(a) suggesting that the intact protein sequence was not required. In the same study, it was shown that the pro-apoptotic effects of apo(a) and Lp(a) were mediated by a TLR2/CD36–dependent signaling pathway.[26] Taken together, the data suggest that oxPL modification of apo(a) facilitates interaction of apo(a)/Lp(a) with CD36 and macrophage toll-like receptors that, in turn, activate cellular pathways responsible for apoptosis in the context of sustained ER stress. This pathway may underlie, in part, the ability of Lp(a) to promote the development of atherosclerotic plaque vulnerable to rupture and subsequent atherothrombotic events.

In the current study we sought to identify the cellular mechanisms by which apo(a) can induce a different pro-atherosclerotic mechanism: namely, induction of interleukin-8 expression in macrophages.

3.3 Experimental Methods

3.3.1 Expression, purification and characterization of recombinant apo(a) and plasma-derived Lp(a)

Plasmids encoding a 17-kringle-containing form of recombinant apo(a) (17K r-apo(a)), as well as 17KΔLBS10 (containing an Asp → Ala substitution at amino acid position 57 that abolishes the strong LBS in the KIV_{10} domain) and 17KΔV (containing a deletion of the KV domain) were constructed and expressed in human embryonic kidney (HEK 293) cells as previously described.[12, 27, 28] All r-apo(a) species were purified from the conditioned medium (CM) of stably-expressing cell lines by lysine–Sepharose (GE Healthcare) affinity chromatography as previously reported.[12, 28] Purified proteins were assessed for purity by SDS-PAGE followed by silver staining, and the absence of endotoxin was verified using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript) as per the manufacturer's protocol.

Lp(a) was purified from the plasma of a healthy volunteer with an apo(a) isoform containing 16 KIV domains using a previously described protocol.[14] Purified Lp(a) concentrations were determined by bicinchoninic acid assay (Pierce) using BSA as a standard. The integrity of the purified Lp(a) was assessed by SDS-PAGE (under either reducing or non-reducing conditions) followed by silver staining.

Assessment of the presence of an oxidized phospholipid moiety on purified recombinant apo(a) variants and purified Lp(a) was performed by western blotting as previously described using E06 IgM (Avanti Polar Lipids) which is specific for the phosphorylcholine head group of oxidized phospholipids. Total apo(a) protein was detected using an anti-apo(a) monoclonal antibody.

3.3.2 Cell Culture

THP-1 acute monocytic leukemia cells (ATCC# TIB-202) were cultured in RPMI-1640 medium adjusted to contain 4.5 g/L glucose, 10 mM HEPES pH 7.4 and 1.0 mM sodium pyruvate, and supplemented with 10% (v/v) fetal bovine serum (ATCC), 1% (v/v) antibiotic-antimycotic (Gibco), and 50 μM β-mercaptoethanol (complete medium). U937 histiocytic lymphoma cells (ATCC# CRL-1593.2) were cultured in RPMI-1640 medium adjusted to contain 1.0 mM sodium pyruvate, and supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic-antimycotic. All cells were grown in a humidified atmosphere (5% CO_2, 95% room air) at 37˚C. THP-1 monocytes were differentiated by the addition of complete growth medium enriched with 100 nM phorbol 12-myristate 13-acetate (PMA). U937 cells were differentiated following the same protocol, except that 250 nM PMA was used.[29] Differentiated cells were plated in a 24-well plate at a density of 8×10^5 cells/mL (1 mL per well) and grown under the same conditions as for the THP-1 monocytes for 72 h. At this time, differentiated THP-1 or U937 cells were incubated with serum-free complete medium for 16 h after which the medium was replaced with fresh serum-free complete medium containing treatments such as apo(a), Lp(a), and/or ε-ACA at the concentrations indicated in the Figure Legends; the cells were incubated with the treatments for 12 hours. In some experiments THP-1 cells were treated with specific inhibitors (Cayman Chemical) to assess downstream effectors according to the manufacturer's specifications as outlined in Supplemental Table 1. 17K r-apo(a) was added to the cells at a final concentration of 500 nM either concurrently or following treatment with the respective inhibitors; cells were then incubated for 12 hours.

3.3.3 Trypsin and Phospholipase A_2 (PLA_2) Treatment of Apo(a)

In some experiments, 17K r-apo(a) (42 μg) was subjected to treatment with 0.21 μg Trypsin Gold (Promega) for one hour at 37°C followed by quenching of the reaction by the addition of 0.4 μg soybean trypsin inhibitor (STI). Treatment of 17K r-apo(a) with PLA_2 was based on a protocol modified from Seimon *et al.*[26] Briefly, 17K r-apo(a) (42 μg) was dialyzed in Tris buffer (0.05 M Tris pH 7.4, 0.15 M NaCl), after which 10 U of PLA_2 (Sigma) supplemented with 5 mM $CaCl_2$ was added and the reaction incubated for one hour at 37°C. Following PLA_2 incubation some samples were further subjected to trypsin digestion as described above. After application of the reaction mixtures to THP-1 macrophages, the final apo(a) concentration in the medium was 500 nM in all cases.

3.3.4 Transfection of siRNA

Following 48 hours of PMA stimulation, THP-1 macrophages (seeded as described above) were transfected with CD36 siRNA, TLR2 siRNA, or scrambled control siRNA (Santa Cruz Biotechnology) each at a final concentration of 80 nM as per the manufacturer's protocol. The transfection mixture was incubated with the cells for 10 h followed by the addition of an equal volume of complete medium for 12 hours, and then incubated in serum-free complete medium for an additional 12 hours prior to 12 hours of apo(a) treatment (performed as described above). Cells were harvested and assayed 48 hours post transfection. Percent knockdown was determined using quantitative real-time PCR (qRT-PCR) analysis (see below), using primers pairs K/L and M/N (Supplemental Table 2) for CD36 and TLR2, respectively.

3.3.5 Quantitative Real Time PCR

THP-1 macrophage lysates were collected after apo(a) treatment using RLT Plus buffer (Qiagen) supplemented with 1% (v/v) 2-mercaptoethanol. Total RNA was isolated from the lysates using the RNeasy Plus kit (Qiagen) as per the manufacturer's protocol. IL-8 gene expression was assessed by qRT-PCR utilizing primer pair A/B (Supplemental Table 2) and normalized to GAPDH using primer pair C/D (Supplemental Table 2). All DNA primers utilized in this study were synthesized by Integrated DNA Technologies. Reactions were assembled in triplicate using the iTaq™ Universal SYBR® Green One-Step Kit (BioRad) according to the manufacturer's protocol and performed on a BioRad CFX 96 Thermal Cycler. Relative quantification of IL-8 mRNA was determined by normalization of the threshold cycle for each sample with that of GAPDH from the same reaction well, using BioRad CFX Manager Software Version 3.1 (Bio-Rad). All mRNA expression data are reported as a fold-increase compared to the control sample from cells not treated with apo(a).

3.3.6 IL-8 Protein Quantification in Conditioned Medium

CM was harvested from the THP-1 macrophages at the time of lysate preparation. The amount of IL-8 protein in the CM was measured in triplicate using a human IL-8 ELISA Kit (Invitrogen) according to the manufacturer's protocol after a 1/1000 dilution of CM.

3.3.7 Generation of IL-8 Reporter Constructs

Genomic DNA was extracted from THP-1 monocytes using the QIAamp DNA Blood Midi Kit (Qiagen) according to the manufacturer's protocol. A functional promoter

fragment of the human *CXCL8* gene was generated by amplification of the (-162) to (+54) sequence of *CXCL8* from the genomic DNA using primer pair E/F (Table 3.1); Primer E introduces an *Xho*I restriction site and Primer F overlaps with a native *Hin*dIII site. PCR reactions were carried out in a BioRad CFX96 thermocycler using Q5 High-Fidelity DNA Polymerase (New England Biolabs) under the following conditions: denaturation at 98°C (10 sec), annealing at 62°C (30 sec), and elongation at 72°C (20 sec) for a total of 40 cycles. The PCR product was digested with *Hin*dIII-HF and *Xho*1, (New England Biolabs); the pGL3 Basic firefly luciferase reporter vector (Promega) was also digested with these enzymes. The promoter fragment was agarose gel-purified and ligated with the vector at 16°C overnight using T4 DNA ligase (Invitrogen) to create the plasmid -162 IL-8. ΔNFκB and ΔAP-1 variants were generated by site-directed mutagenesis of -162 IL-8 using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) with the mutagenic primer pairs G/H and I/J (Table 3.1), respectively.

3.3.8 Dual-Luciferase Assays

THP-1 monocytes were transfected with either empty pGL3 Basic plasmid, the -162 IL-8 reporter construct or mutant constructs, together with pRL-TK renilla luciferase control plasmid (Promega) using Lipofectamine LTX (Invitrogen). After 48 hours, THP-1 monocytes were seeded into 24-well plates ($8x10^5$ cells/well) and differentiated into macrophages as described above. Quantification of luciferase activity of transfected THP-1 macrophages was performed using the Dual-Glo® Luciferase Assay System (Promega), as per the manufacturer's protocol. Firefly luciferase activity was divided by the renilla luciferase activity to correct for differences in cell number and transfection efficiency;

corrected luciferase activities are expressed relative to that from cells transfected with the -162 IL-8 construct and not treated with apo(a).

3.3.9 Statistical Methods

Statistical analyses were performed with the use of SPSS software, version 22.0 (SPSS Inc., Chicago, Illinois). Comparisons between samples were performed by one-way ANOVA using a Tukey post-hoc analysis. Statistical significance was assumed at $p < 0.05$.

3.4 Results

3.4.1 The apo(a) component of Lp(a) stimulates IL-8 gene expression and protein secretion from THP-1 macrophages in a dose-dependent manner

We incubated THP-1 cells – differentiated into macrophages by PMA treatment – with 350 nM of LDL, 17K r-apo(a), or Lp(a) and assessed IL-8 gene expression using qRT-PCR (Fig. **3.**1A). Treatment with apo(a) was shown to stimulate IL-8 gene expression by 8.4-fold compared to untreated control. The increase in IL-8 gene expression by Lp(a) was comparable to that observed for apo(a), while LDL treatment of the cells resulted in no significant increase in IL-8 gene expression (Fig. 3.1A). Treatment with apo(a) or Lp(a) resulted in a corresponding increase in IL-8 protein secretion into the conditioned medium while no appreciable increase was observed following LDL treatment (Fig. 3.1B). The effect of 17K on IL-8 mRNA expression was dose-responsive between 100 and 1000 nM 17K (corresponding to ~2.5 - 25 mg/dL) (Fig. 3.1C). Likewise, a dose-dependent effect of 17K on IL-8 protein secretion from THP-1 macrophages was observed (Fig. 3.1D).

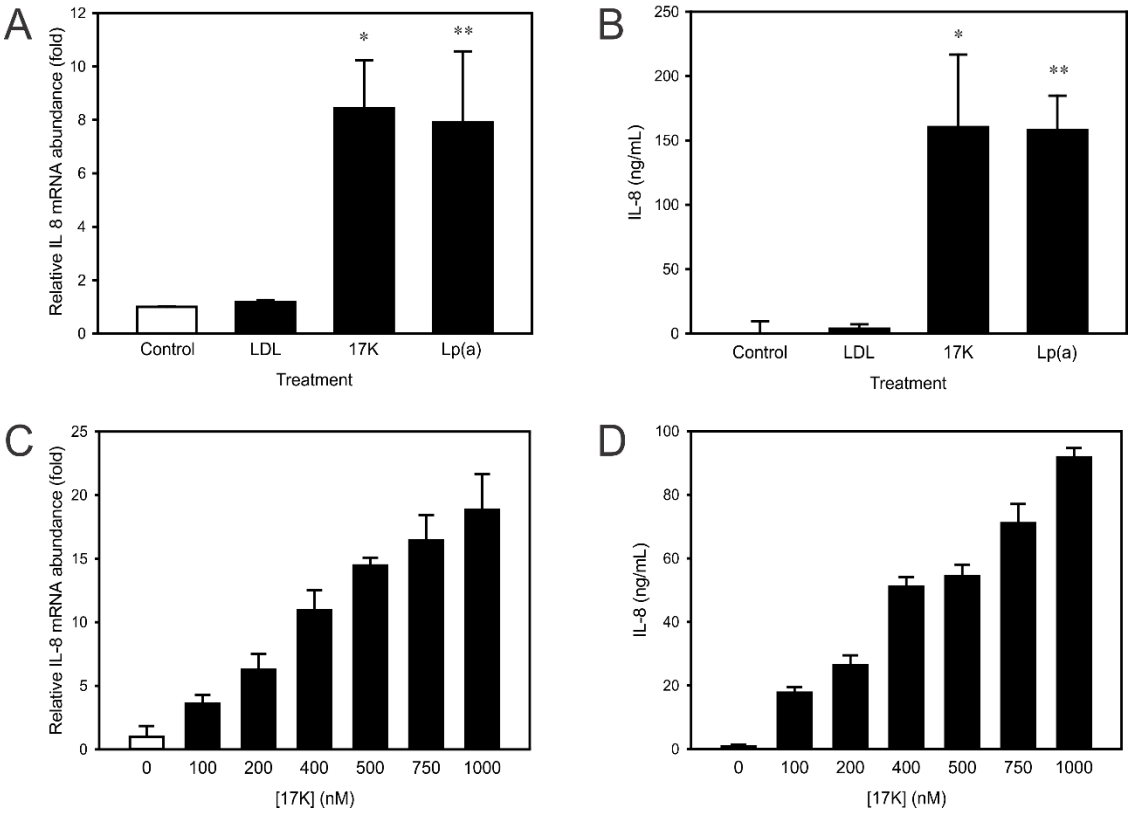


Figure 3.1 The apo(a) component of Lp(a) stimulates IL-8 expression in THP-1 macrophages

A. THP-1 macrophages were treated with 350 nM of each of LDL, 17K recombinant apo(a), or Lp(a) for 12 hours; control cells lacked apo(a). Total cellular RNA was then extracted for each condition and analyzed by real-time qRT-PCR using primers specific for IL-8 and GAPDH. IL-8 mRNA levels were normalized to GAPDH. The abundance of IL-8 mRNA is expressed relative to that in the untreated cells (Control). **B.** Conditioned medium harvested from treated cells in **A** was assayed for IL-8 protein expression using a human IL-8 ELISA kit. **C.** THP-1 macrophages were treated with a range of concentrations of 17K r-apo(a) (100 nM – 1000 nM) for 12 hours and IL-8 mRNA abundance was measured as above. The abundance of IL-8 mRNA is expressed relative to that in the cells not exposed to apo(a). **D.** Conditioned medium harvested from treated cells in **C** was assayed for IL-8 protein expression using a human IL-8 ELISA kit. In all cases, the data represent the means ± s.e.m. of at least three independent experiments each conducted in triplicate. *: $p < 0.05$ and **: $p < 0.01$ versus Control.

3.4.2 Apo(a) stimulation of IL-8 production in THP-1 macrophages requires the lysine-binding site in apo(a) KIV_{10} domain

To assess the mechanism by which apo(a) stimulates IL-8 expression, the role of lysine-dependent interactions and the contribution of specific domains in apo(a) to the effect was assessed. The addition of the lysine analogue ε-aminocaproic acid (ε-ACA) did not affect the ability of 17K to increase IL-8 expression (Fig. 3.2A). On the other hand, using a recombinant apo(a) variant lacking the intact strong lysine binding site (LBS) in KIV_{10} (17KΔLBS10; Fig. 3.2A), we found that treatment with this apo(a) variant significantly blunted (by 70%) the ability of apo(a) to induce IL-8 gene expression. To explore a role for the KV domain of apo(a) we utilized a recombinant variant of 17K apo(a) lacking this domain (17KΔV; Fig. 3.2A). Treatment of THP-1 macrophages with 17KΔV did not result in a significant change in IL-8 mRNA induction compared to treatment with 17K (Fig. 3.2A). As in our previous work, immunoblotting of these r-apo(a) species using the anti-oxPL antibody E06 revealed that both 17K and 17KΔV as well as Lp(a) contain oxPL while the 17KΔLBS10 lacks detectable oxPL (Fig. 3.2B).

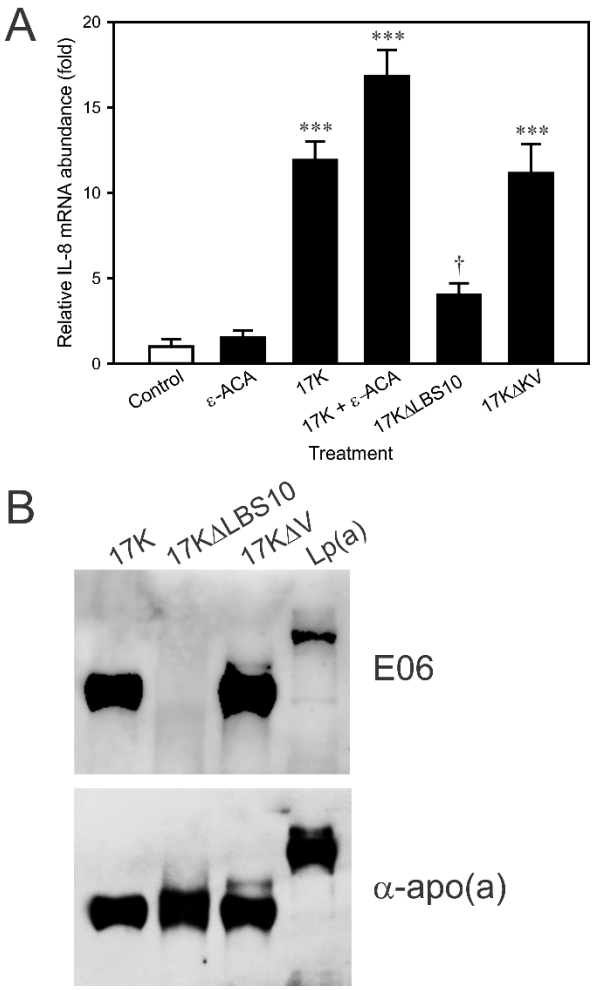


Figure 3.2 Role of lysine-dependent interactions on the ability of apo(a) to stimulate IL-8 gene expression in THP-1 macrophages.

A. THP-1 macrophages were treated with 500 nM each of 17K, 17KΔLBS10, or 17KΔV recombinant apo(a), and/or 200 mM ε-ACA in serum free medium for 12 hours. IL-8 mRNA levels were quantified by real-time qRT-PCR using primers specific for IL-8 and GAPDH. IL-8 mRNA levels were normalized to GAPDH and the relative abundance of IL-8 mRNA was expressed relative to the untreated control. The data represent the means ± s.e.m. of at least three independent experiments each conducted in triplicate. ***: $p < 0.001$ versus Control; †: $p < 0.05$ versus 17K. **B.** Immunoblotting of plasma purified Lp(a) and 17K r-apo(a) variants was performed using an E06 antibody specific for oxidized phosphocholine. A monoclonal anti-apo(a) antibody (α-apo(a)) was used to detect total apo(a) protein.

3.4.3 Apo(a) stimulation of IL-8 mRNA and protein expression in U937 macrophages is dependent on the KIV_{10} lysine-binding site

In order to confirm that the effect of Lp(a)/apo(a) on IL-8 expression we observed in THP-1 cells could also be shown using other macrophage-like cell lines, U937 histiocytic lymphoma cells differentiated with PMA were assayed in the same manner as THP-1 macrophages. We found that 17K apo(a) and Lp(a) treatment of the U937 cells resulted in a 6.4-and 3.4-fold increase in IL-8 mRNA, respectively (Fig. 3.3A). Treatment of these cells with 17KΔLBS10 resulted in significantly less of an increase in IL-8 expression compared to 17K-treated cells. In this cell line, 17KΔV treatment resulted in a significant increase in IL-8 mRNA compared to 17K (Fig. 3.3A). Similar trends were observed when secreted IL-8 protein was quantified after treatment with 17K, 17KΔLBS10, 17KΔV and Lp(a) treatment, (Fig. 3.3B).

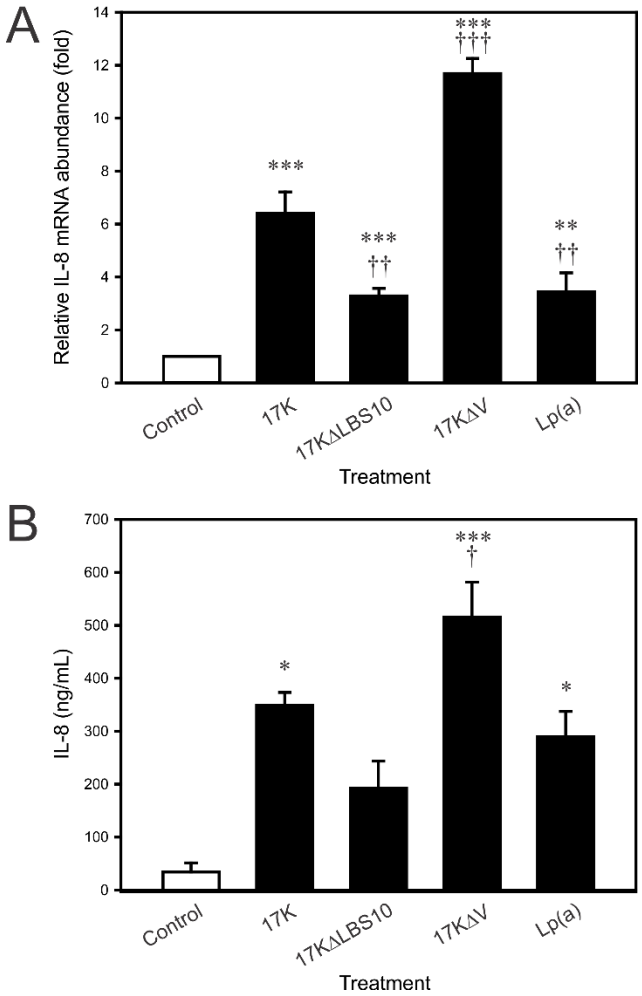


Figure 3.3 Apo(a)-induced IL-8 mRNA and protein expression in U937 macrophages is dependent on the KIV_{10} lysine-binding site.

A. Differentiated U937 macrophages were treated with 500 nM each of Lp(a), 17K, 17KΔLBS10, or 17KΔV recombinant apo(a) in serum free medium for 12 hours. IL-8 mRNA levels were quantified by real-time qRT-PCR using primers specific for IL-8 and GAPDH. IL-8 mRNA levels were normalized to GAPDH and the relative abundance of IL-8 mRNA was expressed relative to the untreated control. **B.** Conditioned medium harvested from treated cells in **A** was assayed for IL-8 protein expression using a human IL-8 ELISA kit. In all cases, the data represent the means ± s.e.m. of at least three independent experiments each conducted in triplicate. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$ versus Control; †: $p < 0.05$, ††: $p < 0.01$ and †††: $p < 0.001$ versus 17K.

3.4.4 Apo(a)-mediated stimulation of IL-8 expression is reduced by phospholipase A_2, but not trypsin treatment

We sought to demonstrate a direct role for apo(a)-associated oxPL in mediating the stimulatory effect of apo(a) on IL-8 expression in THP-1 cells. Treatment of cells with apo(a) that had undergone trypsin treatment resulted in no significant difference in IL-8 gene expression or protein production compared to undigested 17K (Fig. 3.4A, B). However, enzymatic removal of oxPL with PLA_2 resulted in a significant decrease in IL-8 mRNA expression of 53% compared to untreated 17K. (Fig. 3.4A). A similar result was seen when PLA_2 treatment was followed by tryptic digestion of 17K; in this case a 67% decrease in IL-8 gene expression was observed compared to treatment with 17K that had been subjected to tryptic digestion alone (Fig. 3.4A). Comparable trends were seen with respect to IL-8 protein expression, wherein PLA_2 treatment of apo(a) in the absence or presence of tryptic digestion resulted in 73% and 72% decreases in IL-8 protein levels, respectively, compared to the corresponding species not treated with PLA_2 (Fig. 3.4B).

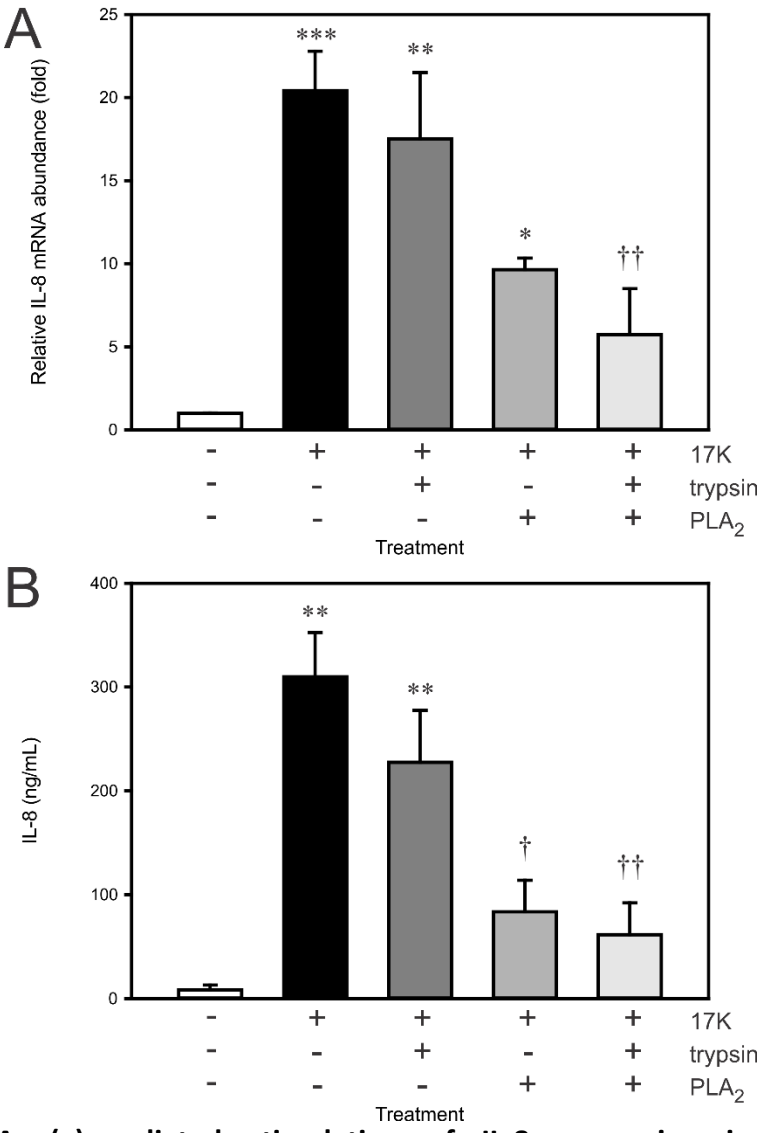


Figure 3.4 Apo(a)-mediated stimulation of IL-8 expression is reduced by phospholipase A_2, but not trypsin treatment.

A. THP-1 macrophages were treated with 500 nM 17K which was pretreated with either trypsin or PLA_2 or by PLA_2 followed by trypsin for 12 hours. IL-8 mRNA levels were quantified by real-time qRT-PCR using primers specific for IL-8 and GAPDH. IL-8 mRNA levels were normalized to GAPDH and the relative abundance of IL-8 mRNA was expressed relative to the untreated control. **B.** Conditioned medium harvested from treated cells in **A** was assayed for IL-8 protein expression using a human IL-8 ELISA kit. The data represent the means ± s.e.m. of three independent experiments each conducted in triplicate. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$ versus no treatment (open bars); †: $p < 0.05$ and ††: $p < 0.01$ versus untreated17K.

3.4.5 Identification of THP-1 cell surface receptors involved in mediating apo(a) induced IL-8 expression

To identify potential cell surface receptor(s) involved in binding to apo(a) and facilitating IL-8 expression, a candidate receptor approach was taken. The expression of candidate receptors CD36 and TLR2 – either alone or in combination – was reduced using siRNA methodology, following which the cells were treated with 17K for 12 hours. Knockdown of both CD36 and TLR2 receptors individually as well as together resulted in an apparent blunting of the apo(a)-mediated increase in IL-8 mRNA levels compared to the apo(a)-treated control siRNA sample (Fig. 3.5A). When corresponding IL-8 protein levels were measured the apparent blunting of the apo(a)-stimulated increase was only seen under the conditions of the double knockdown (Fig. 3.5B). qRT-PCR analysis of siRNA treated samples revealed a relatively modest mRNA knockdown of 27% and 35% for CD36 and TLR2 respectively (Fig. 3.5C).

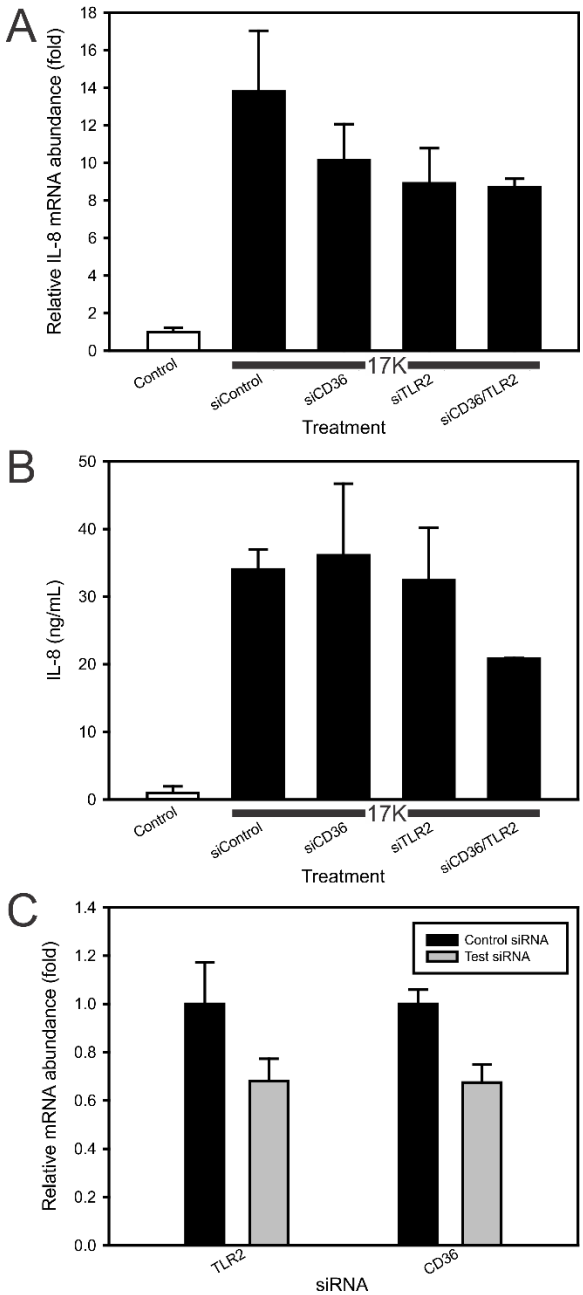

A
Relative IL-8 mRNA abundance (fold)
18
16
14
12
10
8
6
4
2
0
Control
siControl
siCD36
siTLR2
siCD36/TLR2
17K
Treatment
B
IL-8 (ng/mL)
50
40
30
20
10
0
Control
siControl
siCD36
siTLR2
siCD36/TLR2
17K
Treatment
C
Relative mRNA abundance (fold)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control siRNA
Test siRNA
TLR2
CD36
siRNA

Figure 3.5 The role of lipid-specific THP-1 macrophage cell surface receptors on apo(a)-stimulated IL-8 production.

THP-1 macrophages were transfected with control siRNA (siControl) or siRNA corresponding to CD36, TLR2 or CD36 and TLR2 in combination. After 12 hours, cells were treated with 500 nM 17K for an additional 12 hours. **A.** Total cellular RNA was extracted and analyzed by real-time qRT-PCR, using primers specific for IL-8 and GAPDH. IL-8 mRNA levels were normalized to GAPDH and the abundance of IL-8 mRNA was expressed relative to that in the cells not transfected with siRNA and not treated with 17K (Control). **B.** The amount of IL-8 protein secreted was measured in medium harvested from the cells treated with apo(a) in **A** using a human IL-8 ELISA. **C.** To determine receptor knockdown efficiency, real-time qRT-PCR was performed on RNA harvested from the cells in **A** using specific primer pairs for TLR2, CD36, and GAPDH. mRNA levels were normalized to GAPDH and expressed relative to those in cells transfected with the control siRNA. In all cases, data shown are the means ± s.e.m. of 2 – 4 independent experiments.

3.4.6 Characterization of the intracellular signaling pathways mediating apo(a)-induced IL-8 expression

To examine the molecular mechanisms underlying the apo(a)-stimulated IL-8 gene expression in THP-1 macrophages, a series of inhibitors for signaling pathways and transcription factors that are known to act downstream of TLR2 and CD36 was used. Compared to control cells treated with 17K but no inhibitor, induction of IL-8 mRNA levels was found to be decreased between 40% and 85% using the ERK-specific MEK inhibitors PD98059 and PD0325901 and the c-Jun N-terminal kinase (JNK) inhibitor SP600125 (Fig. 3.6A). Inhibition of p38 kinase using the inhibitor SB203580 resulted in no change in the ability of apo(a) to induce IL-8 gene expression. Inhibition of transcription factor NF-κB by the inhibitor BAY-11-7082 resulted in a dramatic decrease (approximately 70%) in the ability of apo(a) to stimulate IL-8 expression. No effect on IL-8 mRNA levels was observed upon inhibition of AP-1 with SR11302 (Fig. 3.6A). Measurement of IL-8 protein secretion in the corresponding samples showed similar trends to those observed for the mRNA abundance (Fig. 3.6B). Treatment with the inhibitors in the absence of 17K apo(a) did not result in an appreciable change in IL-8 expression (data not shown).

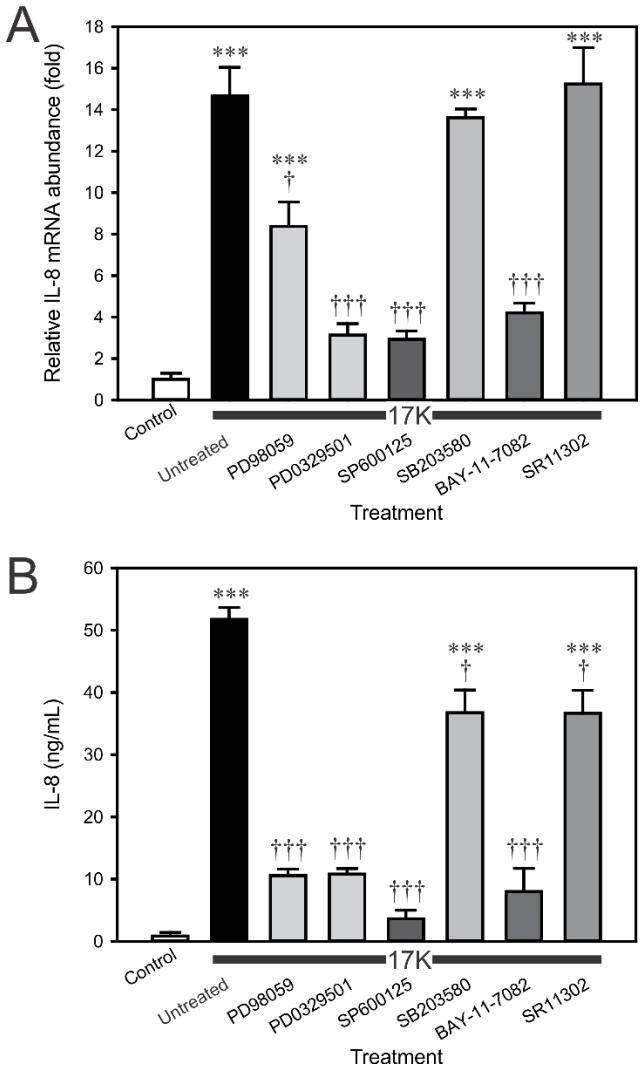
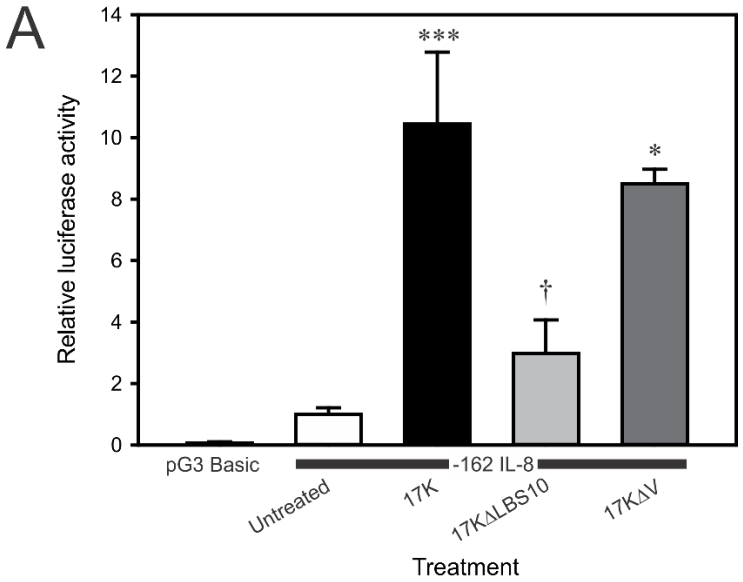


Figure 3.6 ERK1/2 and JNK signaling and activation of transcription factor NF-κB are required to stimulate IL-8 gene expression.

THP-1 macrophages were treated with 17K r-apo(a) alone or in combination with a variety of MAP Kinase pathway inhibitors (specific for ERK1/2 (PD98059, PD0325901), JNK (SP600125) or P38 (SB203580)), or inhibitors of downstream transcription factors NF-κB and AP1 (BAY-11-7082 or SR11302 inhibitors respectively). Conditions for inhibitor treatments were as outlined in Supplemental Table 1. **A.** IL-8 mRNA levels were quantified by real-time qRT-PCR, using primers specific for IL-8 and GAPDH. IL-8 mRNA levels were normalized to GAPDH and the abundance of IL-8 mRNA was expressed relative to the cells not treated with 17K or inhibitor (Control). **B.** The amount of IL-8 protein secreted was measured in medium harvested from the cells treated with apo(a) in **A** using a human IL-8 ELISA. The data represent the means ± s.e.m. of at least three independent experiments each conducted in triplicate. ***: $p < 0.001$ versus Control; ††: $p < 0.01$ and †††: $p < 0.001$ versus 17K alone.

3.4.7 Apo(a) stimulates transcription of the IL-8 promoter in THP-1 macrophages

In order to assess the ability of apo(a) to stimulate transcription of the human IL-8 promoter, a firefly luciferase reporter plasmid was assembled containing a functional 216-bp promoter fragment of the *CXCL8* gene (between -162 and +54). After transfection of THP-1 monocytes with the reporter plasmid followed by differentiation into macrophages, treatment with 17K resulted in a more than 10-fold increase in relative luciferase activity, compared to that observed in control transfected cells not treated with 17K (Fig. 3.7A). In accordance with our findings with endogenous IL-8 mRNA, treatment of the transfected cells with 17KΔV resulted in a similar increase in luciferase activity to that observed with 17K, while treatment with 17KΔLBS10 resulted in a significantly blunted increase (Fig. 3.7A). Luciferase assays were also conducted using cells transfected with -162bp IL-8 promoter constructs containing mutations in the NFκB and AP-1 transcription factor binding sites (Fig. 3.7B). The basal activities of both mutant promoters were reduced compared to the wild-type promoter, although they remained substantially higher than the activity observed from the promoterless control plasmid (pGL3 Basic) (Fig. 3.7B). The ability of 17K to enhance expression of the IL-8 promoter was almost abolished with either mutant (Fig. 3.7B).


A
Relative luciferase activity
14
12
10
8
6
4
2
0
***
*
†
pG3 Basic
-162 IL-8
Untreated
17K
17KΔLBS10
17KΔV
Treatment

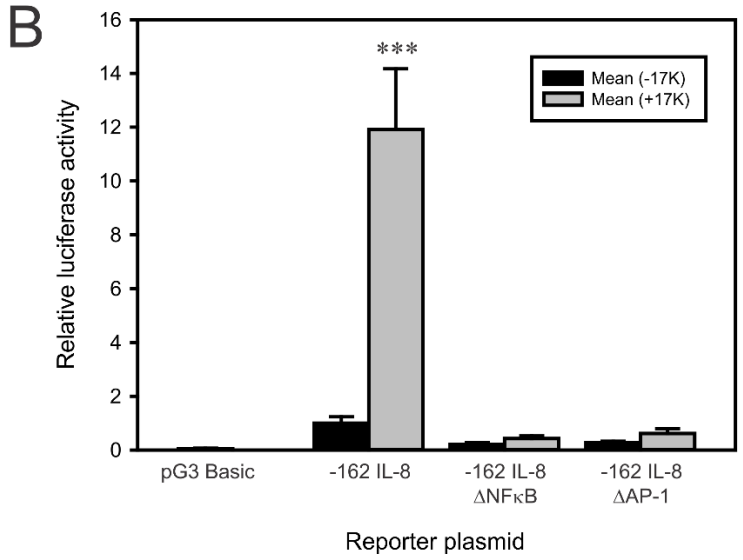

B
Relative luciferase activity
16
14
12
10
8
6
4
2
0
***
Mean (-17K)
Mean (+17K)
pG3 Basic
-162 IL-8
-162 IL-8
ΔNFκB
-162 IL-8
ΔAP-1
Reporter plasmid

Figure 3.7 Apo(a)-mediated up-regulation of the IL-8 promoter requires the apo(a) KIV_{10} domain and intact NF-κB and AP-1 binding sites.

THP-1 monocytes were co-transfected with either the empty pGL3 Basic firefly luciferase reporter plasmid or the -162 IL-8 firefly luciferase reporter plasmid, together with the pRL-TK renilla luciferase internal control plasmid. Following transfection, cells were differentiated into THP-1 macrophages using PMA. **A.** Cells transfected with the IL-8 promoter construct were treated for 12 hours with 500 nM each of 17K, 17KΔLBS10, or 17KΔV recombinant apo(a) proteins. Cellular lysates were then harvested and luciferase activity was quantified. Firefly luciferase activities were normalized to renilla luciferase activities, and expressed relative to that in cells transfected with -162 IL-8 but not treated with apo(a). The data represent the means ± s.e.m. of 3 – 7 independent experiments each conducted in triplicate. *: $p < 0.05$ and ***: $p < 0.001$ versus -162 IL-8 Untreated; †: $p < 0.05$, ††: $p < 0.01$ and †††: $p < 0.001$ versus 17K. **B.** THP-1 monocytes were transfected with the either the wild-type -162 IL-8 reporter plasmid, or corresponding promoter constructs containing mutated NFκB or AP-1 sites (ΔNF-κB or ΔAP-1, respectively); transfected cells were treated with or without 500 nM 17K apo(a). Lysates were prepared and relative luciferase activities determined as described in **A.** The data represent the means ± s.e.m. of 3 – 7 independent experiments each conducted in triplicate. ***: $p < 0.001$ versus no 17K treatment.

3.5 Discussion

Despite its recognition as a causal risk factor for vascular disease, the nature of the contribution of lipoprotein(a) to atherogenesis remains poorly understood. It has been known for over 25 years that Lp(a) can accumulate at the sites of developing lesions.[30] Evidence has been generated to suggest that the pathogenicity of Lp(a) thus stems from its proatherogenic potential, owing to its LDL-like moiety, and from the ability of the unique apo(a) component of Lp(a) to attenuate the plasminogen activation system.[31] In recent years, Lp(a) has been shown to preferentially associate with pro-inflammatory oxidized phospholipids which may underlie the risk associated with elevated plasma Lp(a) levels. It has been hypothesized that Lp(a) delivers oxidized phospholipids to the sites of atherosclerotic lesions, thereby contributing to chronic inflammation in this milieu.[23]

Atherogenesis is characterized by inflammation, mediated through the activity of a complex network of chemokines that play key roles throughout the process of lesion development. IL-8 (CXCL8) is a chemokine in the CXC subfamily that is expressed in macrophages present in atherosclerotic lesions.[32] In addition to its role as a monocyte and neutrophil chemoattractant and activator, IL-8 promotes monocyte adhesion to arterial endothelial cells in the early stages of lesion formation[33, 34], while contributing primarily to angiogenesis in the late stages of plaque formation.[35]

It was previously reported that Lp(a), through its apo(a) moiety, induces IL-8 gene expression in THP-1 macrophages.[19] In this study, the apo(a) kringle V domain was proposed to be critical for the enhancment of IL-8 expression, perhaps through the presence of pro-inflammatory oxPL postulated to be present in this sequence.[24] However, more recent findings from our group suggest that the strong LBS in KIV_{10} is required for

oxidized phospholipid modification of apo(a), and that removal of the KV domain does not affect oxPL addition.[25] In the present study we addressed the mechanism by which apo(a) enhances IL-8 expression in THP-1 macrophages and the role of oxPL modification of apo(a) in this process. In initial experiments, we demonstrated that the apo(a) component of Lp(a), represented by a 17-kringle recombinant form of the protein, was able to induce IL-8 expression while LDL was not (Fig 1A,B). Dose-reponse curves indicate that IL-8 expression is sensitive to apo(a) concentrations in the pathogenic range (> 75 nM) (Fig. 1C,D).

Once we established that use of a 17-kringle containing recombinant apo(a) was a suitable model to study apo(a)-mediated stimulation of IL-8 expression in THP-1 macrophages, we began to probe the mechanism underlying this observation. A number of processes have been reported in which the lysine binding properties of apo(a) KIV_{10} is implicated, including the ability of apo(a) to inhibit pericellular plasminogen activation and to increase vascular endothelial cell permeability.[13, 17] In these studies, the role of the lysine binding properties had been demonstrated using both the lysine analogue ε-ACA as well as an amino acid substitution in the apo(a) KIV_{10} domain that abolishes the lysine binding ability of this kringle. Interestingly, we have shown that the strong LBS in KIV_{10} is also required for oxPL modification of apo(a).[25] The exact site of oxPL addition is unclear, although evidence from our laboratory demonstrates that the modification resides in apo(a) KIV_{10}[25], rather than in the KV domain as previously suggested.[24]

In the current study, we first compared the ability of Lp(a), apo(a) and LDL, in equimolar concentrations, to stimulate IL-8 expression in THP-1 macrophages. Recombinant 17K apo(a) and plasma purified Lp(a) exhibited comparable effects of a

similar magnitude on both IL-8 mRNA and protein expression (Fig. 3.1A,B); no effect of plasma-derived LDL was observed relative to control conditions. This pointed to a role for the apo(a)-component of Lp(a), and suggested that apo(a) and Lp(a) are equally bioreactive in this system.

We next addressed whether the lysine binding function of apo(a) KIV_{10} *per se* is critical for IL-8 upregulation. While addition of the lysine analogue ε-ACA had no effect on the ability of 17K r-apo(a) to induce IL-8 mRNA expression, mutation of the strong lysine binding site in KIV_{10} (17KΔLBS10 variant) resulted in a markedly decreased induction (Fig. 3.2A). At a concentration of 200 mM of the lysine analogue ε-ACA used in this study, the lysine binding sites on 17K apo(a) are blocked, and thus cannot participate in lysine-mediated binding interactions with cell surface receptors. However, mutation of the lysine binding site also eliminated oxPL addition to apo(a) , as assessed by western blot analysis with the E06 antibody (Fig. 3.2B) Our findings thus point to a role for oxPL in mediating interactions with the cells that result in the stimulation of IL-8 expression. In support of a role for oxPL on apo(a), 17KΔV, which contains a comparable level of oxPL modification relative to 17K (Fig. 3.2B) stimulates IL-8 expression to a similar extent as the wild-type 17K (Fig. 3.2A). These key findings were recapitulated in the U937 human lymphocyte cell line following differentiation into a macrophage-like phenotype. We observed that Lp(a)/apo(a) retains the ability to induce both IL-8 gene and protein expression in this cell line. (Fig 3.3A,B) A comparable pattern of IL-8 stimulation was observed in U937 cells treated with the 17KΔLBS10, while the 17KΔV variant displayed an enhanced ability to stimulate IL-8 expression compared to that observed in THP-1 cells. (Fig. 3.3A, B).

Trypsin-degraded apo(a) stimulates mRNA production (and protein secretion) to a comparable extent as intact apo(a) protein (Fig. 3.4A,B). This provides indirect evidence for a role for the oxPL moiety of apo(a) since trypsin digestion would eliminate all functional protein-interacting domains in apo(a) while preserving the existence of the oxPL adduct capable of stimulating IL-8 expression. Pretreatment of 17K apo(a) with phospholipase A_2 either in the absence or presence of trypsin digestion results in a substantial decrease in the ability of apo(a) to stimulate IL-8 mRNA and protein expression (Fig. 3.4A,B). PLA_2 digestion results in release of a lysoPC moiety from apo(a)[24], thereby preventing delivery of the protein-bound oxPL to cell surface receptors. As such, the ability of PLA_2 treatment of apo(a) to inhibit its stimulatory effect on IL-8 expression provides direct evidence for a role for oxPL modification of apo(a) in mediating this effect.

Our identification of a critical role for the apo(a) KIV_{10} rather than the KV domain in stimulating IL-8 expression contradicts the earlier reports of Scanu and colleagues.[19, 24] In the first study a series of proteolytic fragments containing or lacking different kringles was used. While the F2 fragment (KIV_5 – P) had similar IL-8-inducing activity as full-length apo(a), none of the subfragments of F2 had comparable activity to F2, including the F7 fragment (KV-P) on which the conclusion for the role of KV rested. In the latter study, pointing to a role for the apo(a) kringle V domain in both binding to oxPL and up-regulating IL-8 expression, the rIII r-apo(a) variant did, in fact, contain both KIV_{10} and KV. Moreover, the observed lack of E06 reactivity (and hence oxPL content) of rhesus money apo(a), which lacks KV, can be explained by the fact that this species contains a Trp^{72}→Arg mutation in KIV_{10} which renders its strong LBS inactive.[36]

To identify THP-1 cell surface receptors that mediate the apo(a) up-regulation of IL-8 expression, we hypothesized a role for CD36 and TLR2. This is based on the study by Seimon and colleagues that demonstrated a role for activation of these receptors on macrophages via the oxPL component of apo(a)[26], possibly through the *sn*1 saturated fatty acyl moiety in conjunction with the phosphocholine headgroup. In their study, activation of these receptors in turn led to stimulation of apoptosis in ER-stressd macrophages.[26] However, the downstream signalling pathway associated with activation of these receptors by oxPL was not determined. In the present study, a quite modest effect of CD36 and TLR2 knockdown on apo(a)-mediated stimulation of IL-8 mRNA or protein in THP-1 macrophages was observed (Fig. 3.5A,B). These experiments were hampered by the small extent of siRNA-mediated knockdown of these receptors, in keeping with the observations of others.[37, 38] Thus, while our findings are not inconsistent with the notion that the oxPL modification of apo(a) allows it to induce IL-8 expression, further experiments will be required to conclusively demonstrate a role for these receptors, or others, in this effect.

Regulation of IL-8 gene expression has been extensively studied in a variety of cell types.[39] The most important factors mediating induction of IL-8 are NFκB and AP-1.[39] A plausible link between TLR2/CD36 activation and regulation of IL-8 through these factors is supplied by the observations that activation of these receptors induces MAPK pathways that result in activation of the respective transcription factors.[40] Interestingly, it has also been shown that NFκB activation accompanies Lp(a) stimulation of CCL1 expression in macrophages.[41] On this basis, we hypothesized a role for NFκB and AP-1 in the enhancement of IL-8 expression in THP-1 cells downstream of MAPK signalling pathway activation in response to apo(a) treatment. Using specific inhibitors we demonstrated that

apo(a) mediated activation of MAP kinases JNK and ERK1/2 – but not p38 – signficantly lowered both apo(a)-induced IL-8 mRNA and protein expression (Fig. 3.6). JNK and ERK1/2 act through AP-1 and NFκB, respectively, to activate IL-8 transciption and have been shown to play a role in IL-8 induction by several factors including TNFα, whereas p38 regulates IL-8 mRNA abundance through modulation of the stability of its mRNA.[42] Accordingly, we found that both the 17K apo(a) and the variant lacking the kringle V domain – but not the 17KΔLBS10 variant – resulted in activation of the IL-8 promoter (Fig. 3.7A). These findings not only provide further evidence for the critical role of the oxPL moiety on apo(a) in its ability to up-regulate IL-8 expression, but also demonstrate that this effect is mediated at the level of transcription of the IL-8 promoter. A role for both NFκB and AP-1 sites on the IL-8 promoter were identified as essential for apo(a)-stimulated IL-8 expression (Fig. 3.7B). These findings are consistent with previous data showing that both of these factors, which bind to adjacent sites on the IL-8 promoter (NFκB: -81 to -70; AP-1: -166 to -120)[43], are required for induction of IL-8 by factors such as TNFα and interferon γ.[39, 44, 45] On the other hand, our own inhibitor data indicate that the AP-1 inhibitor SR11302 had no effect on IL-8 induction by apo(a) (Fig. 3.6). The compound was incubated with the cells at its EC50 (1 μM), as recommended by the manufacturer, but this concentration has not always proved to be sufficient to abrogate the effects of AP-1.[46, 47]

Very recently, quite a distinct effect of Lp(a)-associated oxPL on gene expression was reported.[46] Specifically, it was found that Lp(a) upregulated ABCA1 expression in HepG2 (human hepatoma) cells in a manner associated with upregulation of LXRα and PPARγ. This effect was dependent on oxPL on Lp(a), but perhaps not oxPL covalently

associated with apo(a), as selective uptake of oxPL from Lp(a) by SR-B1 was implicated. Antibody blocking of the SR-B1 in our system did not, however, abrogate the stimulatory effect of apo(a) on IL-8 expression suggesting lack of a role for SR-B1 in this process (data not shown).

In conclusion, we have shown that apo(a) induces IL-8 expression in THP-1 macrophages through the oxPL modification of apo(a) that requires the presence of the strong lysine binding site in the kringle IV type 10 domain. The ability of the oxPL moiety on apo(a) to stimulate IL-8 expression occurs through a signalling pathway that may be initiated by oxPL-mediated toll-like receptor activation; this, in turn, up-regulates IL-8 transcription through activation of NFκB and AP-1 via upsteam signalling through ERK1/2 and JNK, respectively. This is a pivotal finding in the field in that it suggests an important dual role for the strong LBS in KIV_{10}. One of these roles involves mediating key lysine-dependent interactions involving the apo(a) component of Lp(a) that are critical to the inhibitory role of apo(a) in the plasminogen activation system.[12, 13] On the other hand, the strong LBS in KIV_{10} is also critical for the oxPL modification of apo(a). This modification has been proposed to play a key role in the pathogencity of Lp(a) that underlines its risk[21, 22], with a direct role reported in the initiation of apopotosis in macrophages undergoing ER stress.[26] The key role of the oxPL component of apo(a) in the activation of NFκB (and likely AP-1) may provide a common mechanistic basis for many of the observed effects of apo(a)/Lp(a) on induction of chemokines including CCL1[41], as well as adhesion molecules such as ICAM[15], and VCAM and E-selectin.[16] Caution must therefore be exercised in interpreting the results of experiments designed to elucidate the role of the apo(a) KIV_{10} strong lysine binding site in the pro-atherogenic effects of Lp(a).[47] Based on the present

study, the ability of KIV_{10} to mediate *bona fide* lysine-dependent interactions versus the importance of this kringle for the oxPL modification of apo(a) must be differentiated on a mechanistic basis going forward.

3.6 References

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Characterization of the I4399M variant of apolipoprotein(a):

Implications for altered prothrombic properties of lipoprotein(a)

4.1 Summary

Lipoprotein(a) (Lp(a)) is a causal risk factor for a variety of cardiovascular diseases. Lp(a) is distinguishable from LDL by the presence of the glycoprotein apolipoprotein(a) (apo(a)). Apo(a) is structurally homologous to the fibrinolytic zymogen plasminogen which has led investigators to hypothesize that Lp(a) can interfere with the normal fibrinolytic functions of plasminogen. This, in turn, may have implications for the persistence of fibrin clots in the vasculature, thereby contributing to both thrombotic and atherothrombotic diseases. A single nucleotide polymorphism (rs3798220) in the *LPA* gene has been reported that results in a missense mutation of Ile^{4399} → Met in the protease-like domain of apo(a) (I3499M variant). In population studies, the I4399M variant has been correlated with elevated plasma Lp(a) levels and higher CHD risk, and carriers of the SNP enjoyed increased cardiovascular benefit from aspirin therapy. Additional evidence from *in vitro* studies suggested an antifibinolytic role for Lp(a) containing this variant. In the present study, we performed a series of experiments to assess the effect of the Ile-Met substitution on fibrin clot formation and lysis as well as the architecture of the clots. We found that the Met variant decreased coagulation time and increased fibrin clot lysis time compared to wild-type apo(a). Further, we observed that the presence of the Met variant significantly increased fibrin fiber width, but not density, in plasma clots formed *ex vivo*. Mass spectrometry analysis of a recombinant apo(a) species containing the Met variant revealed sulfoxide modification of the methionine residue. Taken together, our data suggest that the I4399M variant differs structurally from wild-type apo(a), which may underlie key differences related to its effects on fibrin clot architecture and fibrinolysis.

4.2 Background

Elevated lipoprotein(a) has been identified as a causal risk factor for cardiovascular diseases such as coronary heart disease (CHD).[1] This complex lipoprotein consists of a low density lipoprotein (LDL) moiety covalently linked to the unique glycoprotein apolipoprotein(a) (apo(a)) by a single disulfide linkage.[2] Apo(a) contains ten distinct plasminogen-like KIV domains, with all but KIV type 2 (KIV_2) present as a single copy.[3] The KIV domains are immediately followed by sequences resembling plasminogen KV and the protease domain; the protease-like domain in apo(a) is catalytically inactive .[3] The KIV_2 domain of apo(a) can be present from <3 to >30 copies, giving rise to extreme isoform size heterogeneity within the population.[4]

It has long been hypothesized that, owing to the homology with plasminogen and lack of protease activity, the pathogenicity of Lp(a) may arise from its ability to interfere with the normal functions of plasminogen in the vasculature. In this regard, binding of Lp(a)/apo(a) to fibrin is well documented, and it has been reported that apo(a) can compete with plasminogen for binding to lysine residues present on fibrin and partially degraded fibrin thereby decreasing plasmin formation and fibrin clot breakdown.[5] An alternative mechanism has been proposed, whereby the apo(a) component of Lp(a) can form a quaternary complex with plasminogen, tPA and fibrin; this complex is catalytically inefficient with respect to plasmin formation compared to that observed in the absence of apo(a). Apo(a)/Lp(a) has also been shown to inhibit the conversion of Glu- to Lys-plasminogen on the fibrin surface, hence attenuating this key positive feedback mechanism in fibrinolysis.[6] In all of these studies, a role for the strong lysine binding site present in apo(a) KIV type 10 has been identified in mediating these effects.

Interestingly, it has been reported that apo(a) can promote thrombosis in a *Plg*$^{-/-}$ mouse model, suggesting a plasminogen-independent role for Lp(a).[7] In keeping with a such a mechanism, apo(a) also has been reported to bind to fibrinogen in a lysine-independent manner, with binding sites proposed on the αC region of fibrinogen through sites outside of the apo(a) KIV_{10} domain.[8]. The mechanism(s) by which Lp(a) can alter thrombotic processes in a plasminogen-independent context remains unclear. However, owing to its ability to directly bind fibrin in both lysine-dependent and independent mechanisms, it is tempting to hypothesize that Lp(a) may directly contribute to the structure, formation and lysis of fibrin clots.

Several genetic studies have identified an association with two single nucleotide polymorphism (SNPs) within *LPA*, the gene encoding apo(a). These two SNPs (rs3798220 and rs10455872) have been associated with increased plasma Lp(a) levels and increased risk for CHD.[1] The rs3798220 SNP results in a $Ile^{4399} \rightarrow$ Met substitution in the inactive protease domain of apo(a).[9] This variant of Lp(a) has been associated with a smaller number of KIV_2 repeats and elevated plasma Lp(a) levels and increased CAD severity.[9] The low allele frequency of this variant (<4%; ref.) has posed challenges for human studies. Interestingly, however, in a randomized trial of low-dose aspirin therapy in women, it was observed that while carriers of the Met variant had significantly elevated Lp(a) levels and a two-fold increased risk for CVD, they also exhibited a greater than two-fold decrease in cardiovascular risk in response to low dose aspirin, an effect not observed in non-carriers. This may suggest that the I4399M variant of Lp(a) contribute to risk for CVD over and above that arising from Lp(a) levels. In a recent study, elevated Lp(a) levels were correlated with reduced clot permeability and increased clot lysis times *in vitro*.[10] In a separate

investigation using similar *in vitro* methods, plasma from I4399M carriers showed ethnic-dependent variations in plasma clot permeability and lysis times, with Caucasians exhibiting less permeable clots with a decreased rate of lysis than non-Caucasians[11]. In the current study, we have utilized both recombinant apo(a) and human Lp(a) to assess the ability of the I4399M polymorphism to affect fibrin clot formation and clot breakdown, as well as the structure of fibrin clots. Additionally, we have identified a possible mechanism through which the I4399M apo(a) variant can alter the prothrombotic/antifibrinolyic potential of Lp(a).

4.3 Experimental Methods

4.3.1 Preparation of recombinant apo(a) and plasma-derived Lp(a)

Plasmids encoding a 17-kringle-containing form of recombinant apo(a) (17K r-apo(a)), as well as KIV_{10}-P (containing the KIV_{10}, KV and protease-like domains of apo(a)) were constructed and expressed in human embryonic kidney (HEK 293) cells as previously described.[12-14] Plasmids encoding the 17K/I4399M and KIV_{10}-P/I4399M variants corresponding to the rs3798220 SNP were generated by site directed mutagenesis using the QuikChange II-XL Site-Directed Mutagenesis Kit (Agilent Technologies) with the following mutagenic primer pair (mutagenic bases are boldface lowercase): sense 5'-CAT GTT CAG GAA AT**g** GAA GTG TCT AGG CTG-3'; antisense 5'-CAG CCT AGA CAC TTC **c**AT TTC CTG AAC ATG-3'. All r-apo(a) species were purified from the conditioned medium (CM) of stably-expressing cell lines by lysine–Sepharose (GE Healthcare) affinity chromatography as previously reported.[12]

Lp(a) was purified as previously described from the plasma of a healthy volunteer with an apo(a) isoform containing 16 KIV domains.[15] Purified Lp(a) concentrations were determined by bicinchoninic acid assay (Pierce) using BSA as a standard. The integrity of the purified Lp(a) was assessed by SDS-PAGE (under either reducing or non-reducing conditions) followed by silver staining.

4.3.2 *In Vitro* Fibrinolysis and Coagulation Assays

Clot lysis assays were performed in similar manner to that previously reported with some modifications.[16] Pooled, lipoprotein-deficient plasma (LPDP) was diluted 1:1 with TBST solution (0.02 M Tris pH 7.4, 0.15 M NaCl, 0.01% (v/v) Tween 20) and

supplemented with 3 different concentrations of 17K or 17K/I4399M. The reaction mixture was added to wells containing small, separated aliquots of $CaCl_2$ and human thrombin (Haemotological Technologies Inc.) (final concentrations of 10 mM and 1 U/mL, respectively). Clot formation was monitored by measurement of absorbance at 405 nm at 37°C in a plate-reading spectrophotometer (SpectraMax Plus 384; Molecular Devices). The time required to reach half-maximal absorbance (50% clot time) and the maximal change in absorbance (ΔAbs) were determined from the resultant curves. For measurement of clot lysis, a similar protocol was followed except that the wells also contained a small, separated aliquot of tPA (Alteplase CathFlo®, Roche) (final concentration 3 nM). The time required to achieve the absorbance at 405 nm half-way between the maximum and minimum excursions (50% lysis time) was assessed turbidometrically as described above.

4.3.3 Plasminogen activation assays

Plasminogen activation in the presence of fibrin was measured as we have previously described.[12] Briefly, recombinant plasminogen containing a serine to cysteine mutation in the active site (S741C) was expressed and purified from baby hamster kidney cells (BHK-21) and labeled with 5-iodoacetamidoflurocein.[17] Reaction were assembled in a 96-well Microfluor[2] black plates (Costar Inc.) pre-coated with HBS (150 mM NaCl, 20 mM HEPES, pH 7.4) containing 1% (v/v) Tween-80. Fluorescently-labeled plasminogen (0.9 μM, final) was combined with human fibrinogen (1 mg/mL, final) in the presence or absence of 17K or 17K/I4399M (0.5, 1, or 1.5 μM, final) in HBS containing 0.02% (v/v) Tween 80. This mixture was then added to wells containing small aliquots of $CaCl_2$, thrombin, and tPA, at final concentrations of 10 mM, 1U/mL, and 5 nM, respectively. The

quenching of fluorescence was monitored at 37°C in a SpectraMax M5e plate-reading fluorimeter (Molecular Devices) using the following settings: excitation wavelength: 495nm; emission wavelength: 535nm; cutoff wavelength: 530nm; sensitivity: normal; PMT setting: low. Raw fluorescence measurements were corrected for the buffer blank and for internal filter effects. Linear regression was used to determine the initial rates of fluorescence decrease and the rate of plasmin formation was given by the equation:

$$\frac{d[Pn]}{dt} = \left(\frac{dI}{dt}\right) \times \left(\frac{1}{\mathrm{r} \cdot \mathrm{I}_0}\right) \times \left(\frac{[P]_0}{[A]_0}\right)$$

where *d[Pn]/dt* represents the rate of plasmin formation per mole of tPA (s^{-1}), *dI*/*dt* is the initial rate of fluorescence decrease, r is the relative maximum change in fluorescence intensity (0.5 for fibrin surface)[18], I_0 is the initial fluorescence intensity, $[P]_0$ is the initial fluorescently-labeled plasminogen concentration, and $[A]_0$ is the initial tPA concentration. The rates of plasminogen activation are the average of triplicate measurements.

4.3.4 Human subjects

Subjects were either healthy volunteers or were ascertained through a tertiary referral lipid clinic. There were no specific inclusion or exclusion criteria. Informed consent was obtained from subjects prior to DNA collection and analysis using a protocol approved by the University of Western Ontario Ethics Review Board (#07920E). Blood was collected into EDTA or citrate anticoagulant and plasma was prepared by centrifugation. Plasma was aliquoted and stored in cryovial tubes at -80 C. Genomic DNA was isolated from buffy coat and red cells of the remaining sample of each patient using the Puregene DNA isolation kit (Gentra Systems, QIAGEN Inc) or the GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific). The rs3798220 variant in

LPA was genotyped using a validated TaqMan assay on a ViiA7 Real-Time PCR System and automated software (Applied Biosystems, Foster City, CA), or by direct sequencing of PCR products, at the London Regional Genomics Centre (www.lrgc.ca). For the latter, a 427-base pair amplicon was produced using the following primers: 5'-GAA GGG GCT GGA CCA TAT TT-3' and 5'-CGG GTA ACA GGG TAT CTC TTT-3'.[10] The PCR conditions used were: at 98 °C followed by 36 cycles of 98 °C for 30 sec; 53 °C for 30 sec; 72 °C for 40 sec, and a final extension for 10 min at 72 °C. The final reaction volume was 25 μL, and contained 10 ng genomic DNA, and was carried out using Q5® High-Fidelity DNA Polymerase (New England Biolabs) as per the manufacturer's instructions.

Lp(a) isoform size was determined in plasma samples by SDS-agarose gel electrophoresis[19], using an anti-apo(a) antibody raised against recombinant apo(a). Apo(a) isoform sizes were determined using a ladder of recombinant apo(a) isoform standards. Lp(a) plasma levels were determined using the Macra® Lp(a) Enzyme Linked Immunosorbent Assay (Mercodia, Uppsala, Sweden) as per manufacturer's protocol.

4.3.5 Assessment of plasma clot permeability

Fibrin clot permeability was determined using a pressure-driven system as previously described.[20] Briefly, 20 mM (final) $CaCl_2$ and 1 U/mL (final) human thrombin were added to 120 μL citrated plasma and the mixtures transferred to clear glass tubes to hold the clots. Following incubation for 2 h in a wet chamber, tubes containing the clots were connected via plastic tubing to a reservoir of a buffer (0.01 M Tris, 0.1 M NaCl, pH 7.5) and the volumes flowing through the fibrin gels were measured over the course of 60 mins. A permeation coefficient (Ks) was calculated using the following equation:

$$K_s = \frac{Q \times L \times \eta}{t \times A \times \Delta p}$$

where Q is the flow rate in time t; L, the length of a fibrin gel; η, the viscosity of liquid (in poise); A, the cross-sectional area (in cm^2); and Δp, the pressure differential (in dyne/cm).

4.3.6 Confocal and scanning electron microscopy of clots

Clots for scanning electron microscopy were prepared and subjected to permeation as described in Section 5.3.5, and then were fixed for 30 min in 1.4% (v/v) glutaraldehyde. Clots were then washed 3 times with phosphate-buffered saline (PBS) for 30 mins, followed by 3 times with distilled, deionized H_2O for a total of 30 mins. Images were acquired on a field-emission environmental scanning electron microscope (ESEM, FEI QUANTA-200 FEG type, Holland FEI Company). Acquisition conditions were as follows: pressure of 70 kPa; accelerating voltage of 10 kV; and a working distance of approximately 10 mm. Fiber widths were measured using ImageJ software; a minimum of 30 fibers were measured per image.

For confocal microscopy, clots were formed in a similar manner to that reported in Section 5.3.5, with the exception that the plasma was supplemented with 0.3 mg/mL Alexa Fluor® 568 labelled fibrinogen. The fibrinogen (Calbiochem, Mississauga, Canada) was labeled using an Alexa-568 Protein Labeling Kit (Invitrogen) according to the manufacturer's protocol. The clots were allowed to mature for 3 h in a moist chamber at room temperature at which time they were washed with TBS to removed excess label. The resulting fluorescent clots were imaged on an Olympus FV1000 confocal microscope. Images were analyzed for fiber density using ImageJ software with a custom-designed plugin.[20] In this method, a 10 × 10 grid is placed on individual images and the number of

fibers intersecting the grid lines is computed. Fiber densities of four different micrographs per sample were averaged.

4.3.7 Molecular dynamics simulations and protease domain modelling

The Molecular Operating Environment (MOE) software was used for model preparation, energy minimizations, and assessment of the generated trajectories. The molecular dynamics (MD) simulations were performed using the NAMD program[21] All chemical models were derived from a previously-published model of the apo(a) protease domain.[22] water molecules present in the modelled structure were deleted. Hydrogen and proton atoms were then added to the model using the default method in MOE; water molecules were then added using the TIP3P water model.[23] The latter added water molecules throughout the protein, where appropriate, and extending outwards from the edge of the protein to form a 6 Å layer of water molecules. The resulting solvated model was then minimized with the AMBER:12EHT force field until the root-mean-square gradient of the total energy was below 0.05 kcal mol^{-1} $Å^{-1}$.[24] The same procedure was used to generate both initial solvated structures of the wild-type apo(a) and the I4399M apo(a) variant. For all MD simulations the AMBER12:EHT force field was used as was the default velocity Verlet integration method to obtain the trajectories. The solvated systems were first equilibrated for 0.15 ns at 150 K, while the subsequent production MD simulations ran for 4.90 ns with a time step of 2 fs.

4.3.8 Mass spectrometry

15 μg of either KIV_{10}-P or KIV_{10}-P/I4399M protein was reduced with 16 mM DTT and labeled with 100 mM iodoacetamide (Sigma) for 60 min in the dark at room temperature with shaking. Samples were then subjected to a buffer exchange with ice cold trichloroacetic acid (15% v/v) followed by 20 min incubation at 4°C. After centrifugation, 3 acetone washes of the pellet were performed. The pellet was resuspended in 4× reducing sample buffer. Samples were then subjected to SDS-PAGE followed by visualization of proteins by Imperial stain, as per manufacturer's protocol (Thermo Scientific). Protein bands were then excised and subjected to a 16 h *in-gel* tryptic digest with a ratio of ~80:1 apo(a):Trypsin Gold (Promega).[25] Tryptic peptides were extracted, spotted in a 1:1 ratio with alpha-cyano-4-hydroxycinnamic acid and subjected to MALDI-TOF mass spectrometry using a Waters SYNAPT G2-Si instrument. The resulting spectra were analyzed using MassLynx Software (Waters).

4.3.9 Statistical methods

Comparisons between samples were performed by one-way ANOVA using a Tukey post-hoc analysis using SPSS software, Version 22.0 (SPSS Inc.) or by a two tailed Student's t-test using Microsoft Excel. Statistical significance was assumed at $p < 0.05$.

4.4 Results

4.4.1 The effect of the I4399M apo(a) variant on clot formation

In preliminary experiments, we noticed that addition of 17K/14399M to a solution containing physiological concentrations of fibrinogen resulted in a cloudy solution, whereas addition of 17K had no such effect. We determined that this phenomenon was caused by the 17K/I4399M-mediated precipitation of fibrinogen, which did not occur with 17K (Chapter 4 Supplementary Figure 1). This finding prompted us to investigate the effects of 17K/I4399M on plasma clot formation and lysis in lipoprotein-deficient plasma (LPDP).

We titrated 17K and 17K I4399M (0.5, 1.0, and 1.5 μM) into LPDP and initiated coagulation by the addition of $CaCl_2$ and thrombin. Addition of wild-type 17K had no effect on the maximum turbidity (change in absorbance) observed upon clot formation (Figure 4.1A). However, addition of 17K/I4399M resulted in a significant dose-dependent increase (17%, 21%, and 33%) in the maximum turbidity of the clots (Figure 4.1A). Supplementation of plasma with increasing concentrations of 17K resulted in a dose-dependent decrease in the time to 50% clotting of 23%, 30%, and 34% compared to clots formed in the absence of apo(a) (Figure 4.1B). The 17K/I4399M variant had a significantly greater effect, with additional decreases in time to 50% clotting of 27%, 34%, and 36% compared to that observed with 17K (Figure 4.1B).

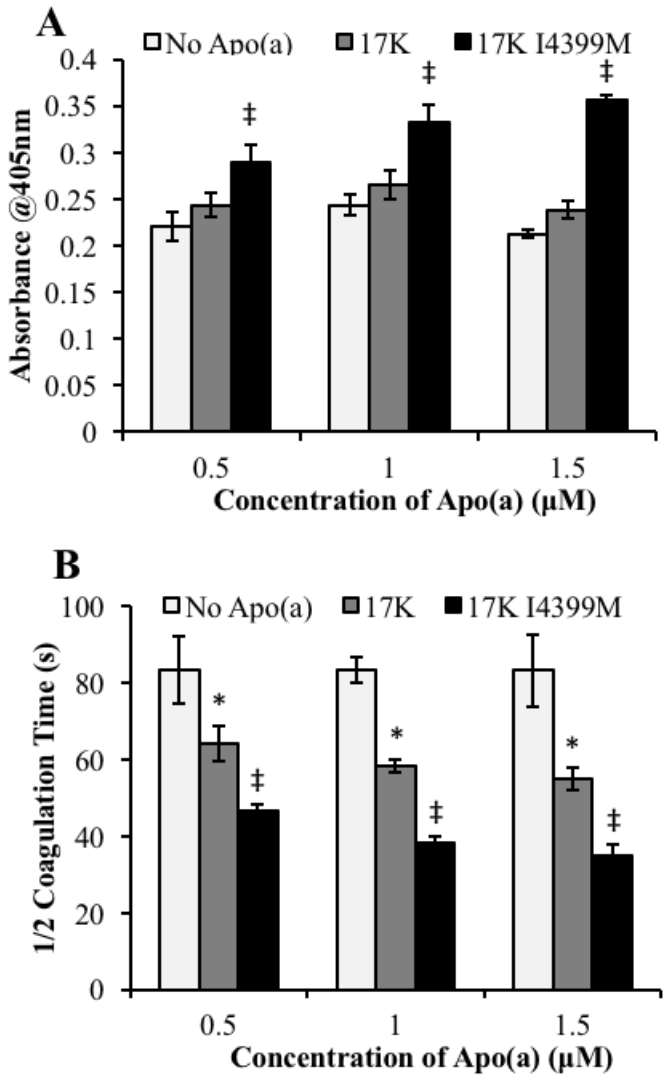


Figure 4.1 The effect of 17K and 17K/I4399M apo(a) on coagulation.
LPDP was clotted with 1 U/mL thrombin and 20 mM $CaCl_2$ in the absence and presence of the indicated concentrations of either wild-type 17K or 17K I4399M. Clot formation was monitored by the measurement of turbidity. Panel A. The maximal change in absorbance (ΔAbs) occurring upon clot formation. Panel B. Time to 50% clotting. Data are results of at least three independent experiments, and were analyzed using Student's t-test. Daggers: $p < 0.05$ versus 17K; asterisks: $p < 0.05$ versus no apo(a).

4.4.2 The I4399M apo(a) variant more potently inhibits fibrinolysis

We next compared the ability of 17K and 17K/I4399M to inhibit fibrinolysis, using a similar *in vitro* assay containing LPDP but with the addition of tPA. Compared to control samples lacking apo(a), clot lysis times were significantly decreased in the presence of 17K apo(a) (0.5, 1.0, and 1.5 μM), which extended lysis times by 2.9-, 5.6-, and 9.5-fold respectively (Figure 4.2A). The 17K I4399M variant (0.5, 1.0, and 1.5 μM) resulted in a significant additional 28%, 21%, and 11% increase in clot lysis times, respectively, compared to those observed with 17K (Figure 4.2A).

We then assessed if differences between 17K and the I4399M variant in inhibition of clot lysis might be related to differences in their ability to inhibit plasminogen activation. To do this, we measured the effect of these variants on the activation of a fluorescently-labelled plasminogen variant that is catalytically inactive upon cleavage by tPA (Figure 4.2B). Both apo(a) variants yielded a dose-dependent decrease in plasminogen activation (between ~25% and ~45%), but there was no significant difference between 17K and 17K/I4399M (Figure. 4.2B).

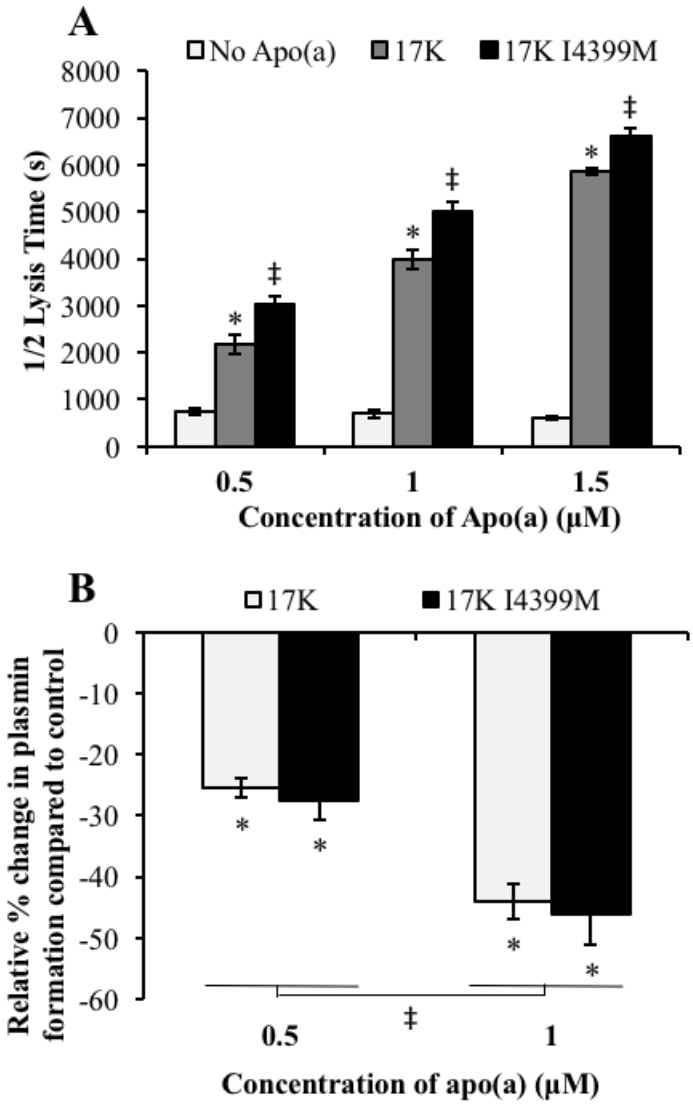


Figure 4.2 The effect of 17K and 17K/I4399M apo(a) on fibrinolysis.
Panel A. Clots containing tPA were formed in the absence or presence of the indicated concentrations of either 17K or 17K/I4399M and clot lysis was monitored turbidometrically. The time to reach 50% clot lysis. A minimum of three experiments were conducted per treatment condition. Asterisks: $p < 0.02$ versus no apo(a); daggers: $p < 0.05$ versus 17K. Panel B. Plasminogen activation assays utilizing tPA and fluorescently-labeled recombinant plasminogen were conducted in the presence of fibrin and in the absence or presence of 17K or 17K/I4399M. The rate of plasminogen activation per mole of tPA is presented as a rate relative to that observed in the absence of apo(a). The data represent the means ± S.E.M. of three independent experiments each conducted in triplicate. Asterisks: $p < 0.05$ versus no apo(a); daggers: $p < 0.02$ versus 0.5μM treatment.

4.4.3 The I4399M variant of apo(a) alters fibrin clot structure.

Fibrin clots were generated with purified components supplemented with 1.0 μM 17K or 17K/I4399M. After glutaraldehyde fixation and extensive washes, fibrin clots were subjected to low vacuum scanning electron microscopy (SEM) to assess clot structure. Figure 4.3A shows representative secondary electron images taken at 30,000× magnification. While there was no difference in the width of fibrin fibers between clots lacking apo(a) and containing 17K, clot containing 17K/I4399M containing clots had fiber widths that were significantly (50% increase) greater (Figure 4.3).

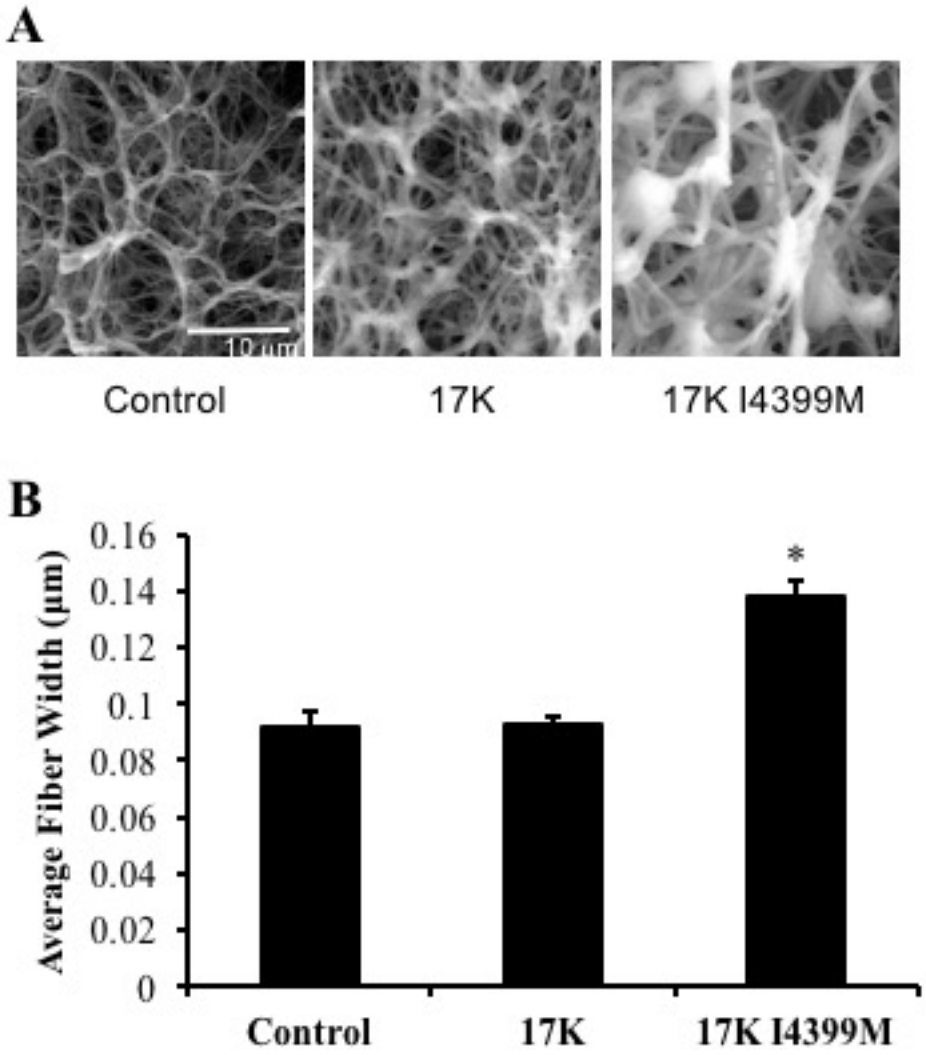
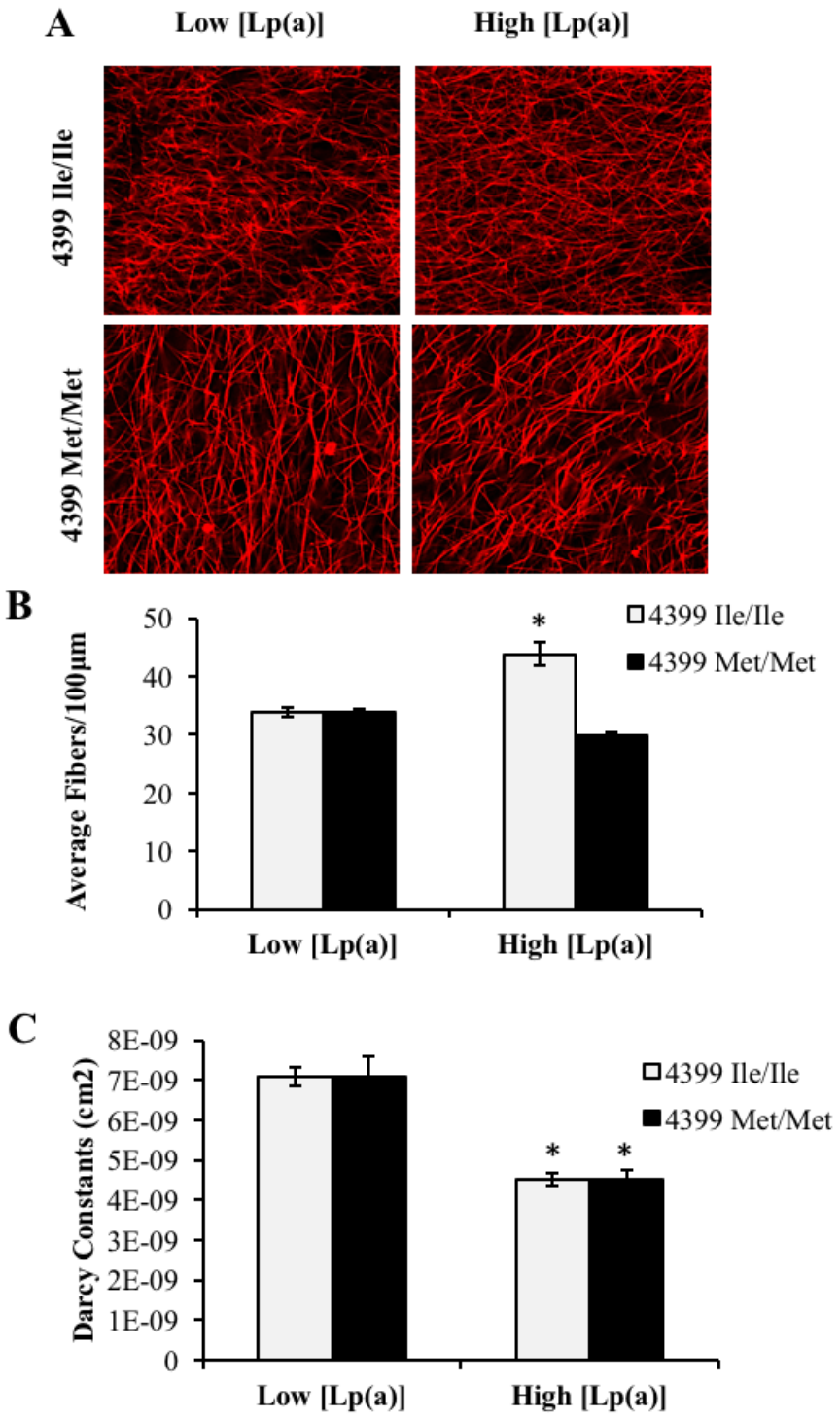


Figure 4.3 The effect of 17K and 17K/I4399M on fibrin clot structure.
Panel A. Purified fibrinogen in the absence and presence of the respective apo(a) variants (1 µM) was clotted with thrombin and visualized using low vacuum scanning electron microscopy. Shown are representative images were taken at 30,000× magnification with a HFW of 6.67 µm. Panel B. Fiber widths were calculated using ImageJ, with a minimum of 30 fibers measured on at least 4 images. *: $p < 0.0001$ versus control lacking apo(a).

4.4.4 Clots formed from the plasma of individuals homozygous for the rs3798220 SNP are structurally distinct

Clots formed in plasma from a total of four individuals with either high (>30mg/dL), or low (<10 mg/dL) plasma Lp(a), and homozygous for either the Met or Ile apo(a) variant were assessed for fibrin fiber density, plasma clot permeability, and average fibrin fiber width. Subjects of either genotype were matched for similar plasma Lp(a) concentration and apo(a) isoform size. Figure 4.4A shows representative plasma clots from each individual; clots were supplemented with 0.3mg/mL Alexa Fluor® 568 to label fibrin fibers and subjected to confocal microscopy to assess fiber density. Density was similar clot formed from the plasma of individuals of either genotype but with low Lp(a) levels (Figure 4.4B). Fiber density was significantly greater for the Ile/Ile homozygote with higher Lp(a) levels but not for the Met/Met homozygote with higher Lp(a) levels (Figure 4.4B).

Permeability of clots made from the plasma of each individual was assessed by a pressure-driven permeation method and measurements were reported by calculation of a permeability (Darcy) constant for the respective clots (Figure 4.4C). There was a significant decrease of approximately 30% in the Darcy constant of clots formed from the plasma of individuals with elevated plasma Lp(a) levels compared to those with low plasma Lp(a), irrespective of the genotype of the subject (Figure 4.4C). Plasma clots from each individual were also subjected to fixation and SEM imaging (Figure 4.4D) followed by determination of fibrin fiber width (Figure 4.4E). Fiber width was significantly greater for subjects with high Lp(a) levels; moreover, the Met/Met homozygotes had greater fiber width that Ile/Ile homozygotes for both ow and high Lp(a) levels.


A
Low [Lp(a)]
High [Lp(a)]
4399 Ile/Ile
4399 Met/Met
B
Average Fibers/100µm
50
40
30
20
10
0
*
4399 Ile/Ile
4399 Met/Met
Low [Lp(a)]
High [Lp(a)]
C
Darcy Constants (cm2)
8E-09
7E-09
6E-09
5E-09
4E-09
3E-09
2E-09
1E-09
0
*
*
4399 Ile/Ile
4399 Met/Met
Low [Lp(a)]
High [Lp(a)]

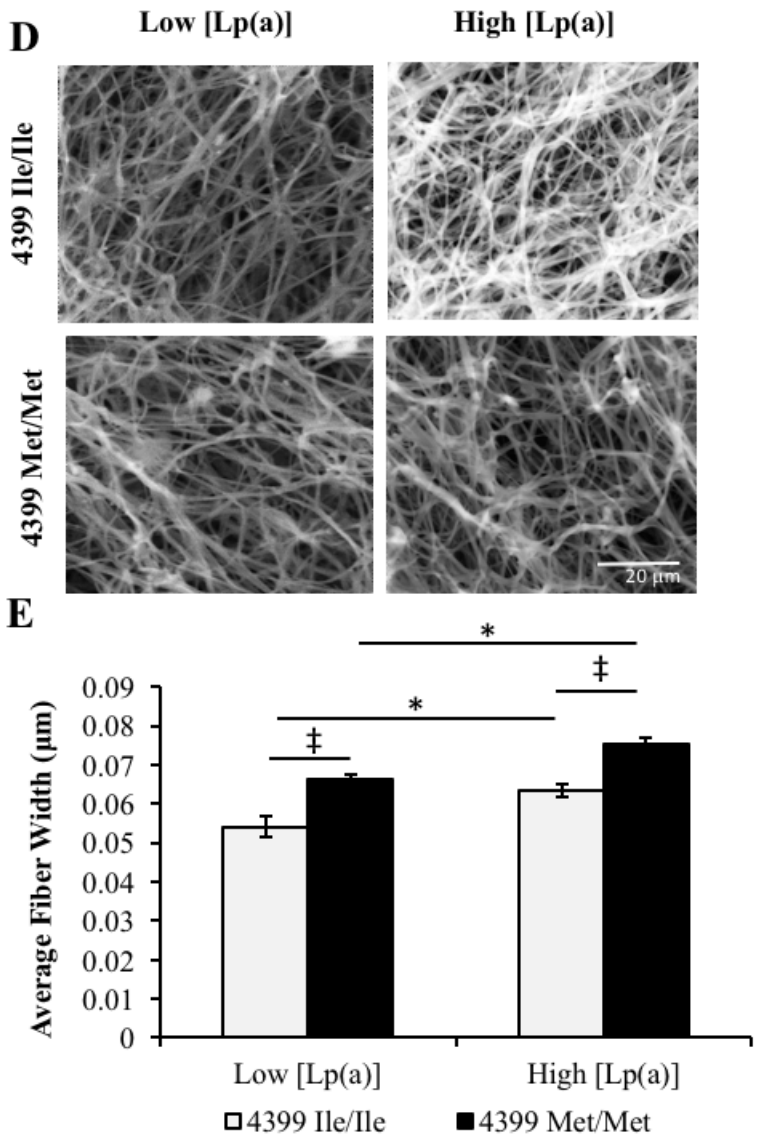

D
Low [Lp(a)]
High [Lp(a)]
4399 Ile/Ile
4399 Met/Met
20 µm
E
Average Fiber Width (µm)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
*
‡
*
‡
Low [Lp(a)]
High [Lp(a)]
4399 Ile/Ile
4399 Met/Met

Figure 4.4 Plasma clots from individuals homozygous for the Met/Met apo(a) variant are structurally distinct.

Individuals were genotyped for the rs3798220 *LPA* SNP; plasma Lp(a) concentrations were determined by ELISA. Panel A. Clots were formed in the presence of Alexa 568-labeled fibrinogen using the plasma of individuals with either low or high Lp(a) levels who were either Ile/Ile or Met/Met homozygotes. The clots were subjected to confocal microscopy. Representative clot images are shown. Panel B. The number of fibrin fibers per 100 μm area was determined using ImageJ software. The data represent the means ± S.E.M. of analysis of at least 4 images from each individual. Asterisk: $p < 0.05$ compared to individual of same genotype with low Lp(a) levels. Panel C. Clots prepared from individuals described in Panel A were subjected to a pressure-driven clot permeation assay. The data represent the means ± S.E.M. of analysis of at least 3 clots from each individual. Asterisk: $p < 0.05$ compared to individual of same genotype with low Lp(a) levels. Panel D. Plasma clots prepared as in Panel C were fixed and subjected to low vacuum SEM. Representative images, taken at 30,000× magnification, are shown. Panel E. Fibers from the clots shown in Panel D were assessed for average fiber width. Fibers were quantified by examining 30 different fibers from 4 - 5 different images for each individual. Asterisk: $p < 0.05$ versus individual with different Lp(a) levels but same genotype; dagger: $p < 0.05$ versus individual of same genotype but different Lp(a) level.

4.4.5 Modelling of the apo(a) protease domain containing the I4399M variant indicates structural differences compared to wild-type apo(a)

Using a model for the protease domain of apo(a)[22], average occupancy images were taken for a native apo(a) protease domain and a simulation incorporating an induced I4399M substitution. Figure 4.5A shows a comparison between the simulated domains. The first coordination sphere (with respect to the Ile/Met residue) suggests that the substitution of the hydrophobic isoleucine residue to the more polar methionine residue induces a conformational change in the local environment of this amino acid. In the native structure, Ile4399 is shielded from the bulk solvent by the adjacent Arg4420. When Met is in the 4399 position, however, the Arg4420 and Met4399 conform to a status where the Met is exposed to the solvent, and is no longer guarded by the neighbouring Arg. In Figure 4.5 B, a root mean squared deviation analysis of the distance between the δ-carbon on Ile, or the sulfur on the alternate Met, with the guanidine nitrogen on the adjacent Arg was explored to examine the relationship between these moieties. The 5 ns simulation suggests that the Met has a shorter average distance to the Arg of 3.39 ± 0.03 Å, implying that the newly exposed Met4399 may be in a more coordinated state. Analysis of the Ile residue shows a greater and much more erratic average distance of 4.37 ± 0.12 Å. This suggests that the I4399M substitution results in a distinct alteration in the conformation of the apo(a) protease domain in this area.

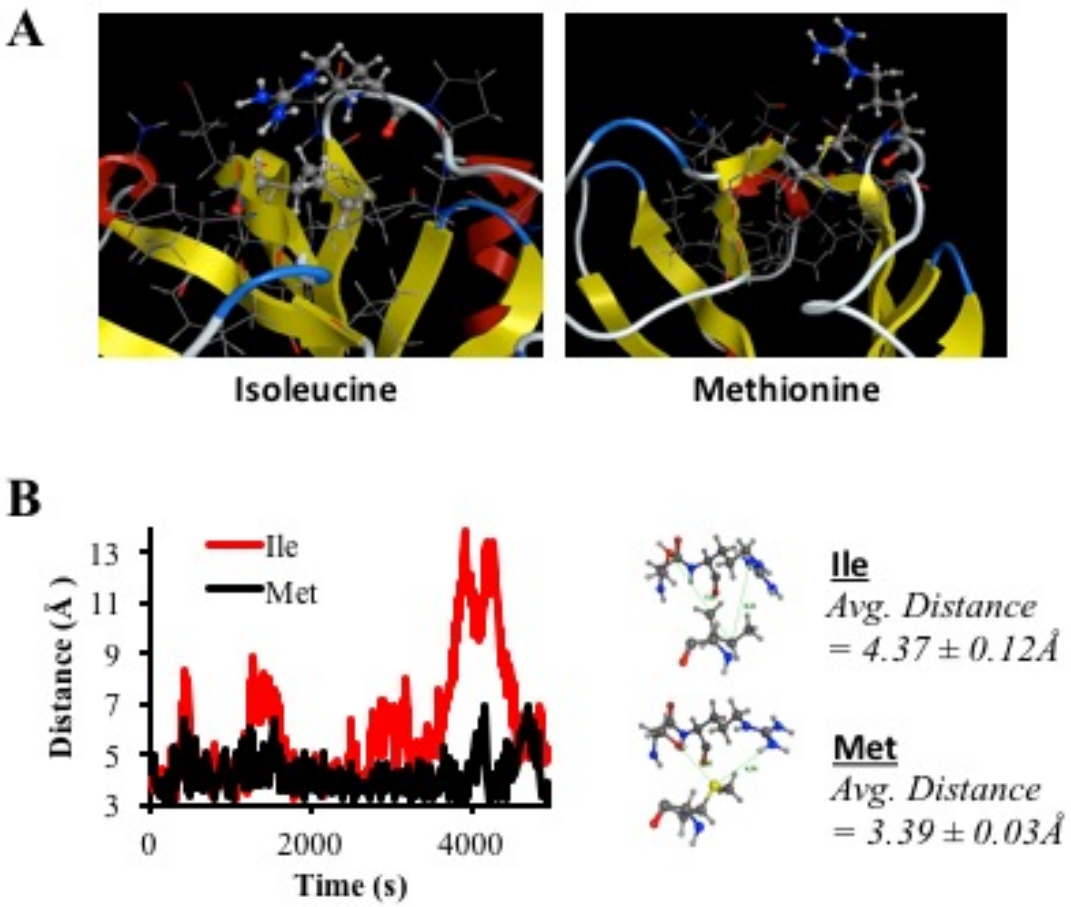


Figure 4.5 The I4399M substitution in the apo(a) protease-like domain perturbs the local environment.

Panel A. Average occupancy images of simulations of the Ile4399 (native structure) and Met4399 variants. The image on the left shows the Ile4399 residue buried underneath the adjacent Arg4420; this interaction is not observed in the simulation of the Met4399 variant. Introduction of the methionine at position 4399 alters the conformation of the domain allowing the Arg residue to adopt a new position such that the Met residue can interact with the solvent around the molecule. Panel B. RMSD analysis of the distances between the Ile/Met and the adjacent Arg reports a greater average distance between the Ile and Arg residues, suggesting that the polymorphic Met variant is possibly in a more coordinated state and likely has a distinct conformation.

4.4.6 Identification of an oxidized methionine residue on the I4399M apo(a) variant.

KIV_{10}-P and KIV_{10}-P/I4399M were generated in order to assess the presence of oxidative modifications in the protease-like domain of apo(a) using mass spectrometry. The smaller size of these variants was expected to yield more easily interpreted spectra. MALDI-TOF mass spectrometry of KIV_{10}-P tryptic peptides identified a peptide VILGAHQEVNLESHVQEIEVSR, which includes the Ile4399 residue, at the theoretical mass/charge ratio of 2487.3 m/z (data not shown). This indicates that this peptide is unmodified and present in its native form. Analysis of the corresponding peptide from the Met4399 variant was expected to be present at a mass of 2505.2 m/z (Figure 4.6A), but a peptide of this mass was not detected (Figure 4.6C). However, a peptide of an increased mass/charge ratio of 2521.2 m/z (Figure 4.6B), corresponding to the presence of an oxidized methionine residue in the peptide, was detected (Figure 4.6C).

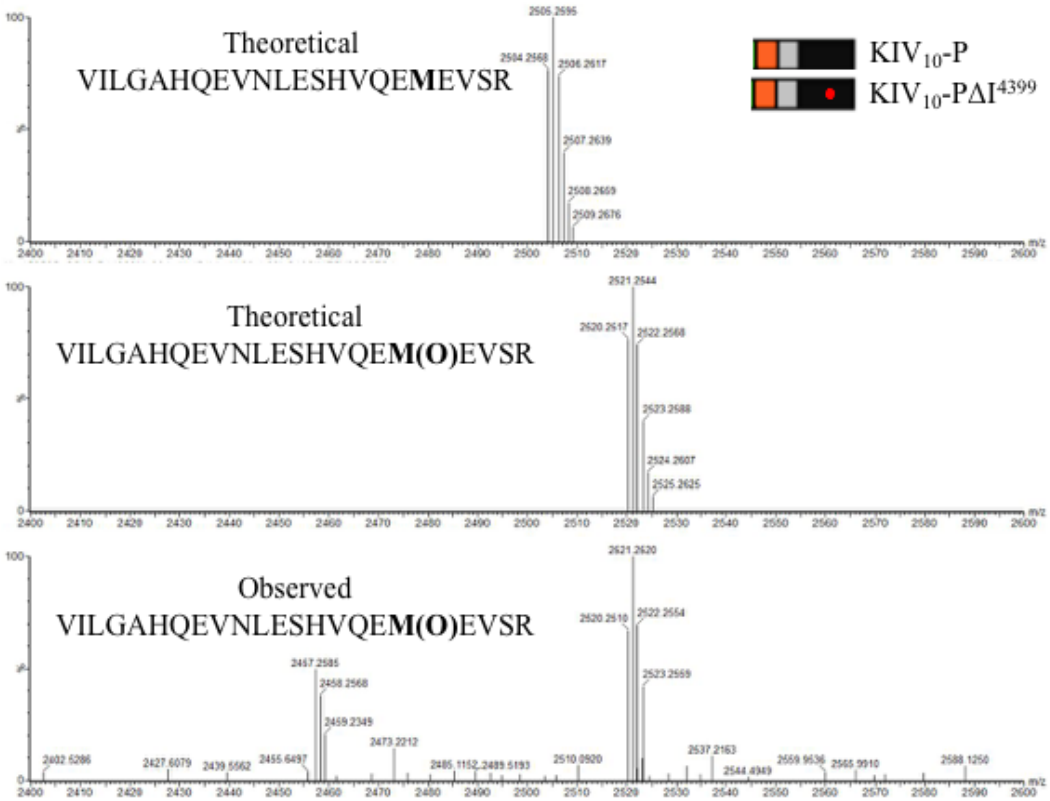


Figure 4.6 Identification of an oxidized methionine residue on the I4399M variant of apo(a).

Panels A and B. Theoretical MALDI-TOF mass fingerprints of the VILGAHQEVNLESHVQE**M**EVSR peptide and of the peptide containing the oxidized methionine residue. Panel C. The observed mass spectrum of KIV_{10}-P/I4399M tryptic peptides is shown.

4.5 Discussion

The rs3798220 in *LPA* which encodes an Ile to Met substitution in the protease domain of apo(a) has been associated with higher Lp(a) levels, smaller apo(a) isoforms, and greater risk of cardiovascular diseases.[1, 9, 26] It remains unclear whether Lp(a) containing Met at position 4399 is inherently more pathogenic or whether this polymorphism is associated with some other genetic factor predisposing to disease. For example, some studies have shown that the effect of rs3798220 on disease disappears after adjustment for Lp(a) levels.[1] On the other hand, it has been reported that that carriers of this SNP derive greater benefit from aspirin[11]; although it was found that there was no difference in event rate between carriers with high and low Lp(a) levels, possibly indicating a functional effect of the polymorphism, such a conclusion has to be tempered by the small number of events observed in the carrier group owing to the rarity of this SNP.[11] We have decided to directly examine the effects of the Met substitution on the pathogenic potential of Lp(a) by expressing and functionally characterizing a recombinant form of the variant, in the context of a physiologically-relevant 17-kringle isoform. The focus of our work has been on the effect of this mutation on coagulation, fibrinolysis, and fibrin clot structure.

It has been previously shown that Lp(a)/ apo(a) can inhibit fibrinolysis[5, 14], inhibit tPA-mediated plasminogen activation on the surface of fibrin[12], and inhibit the positive feedback step of plasmin-mediated conversion of Glu-plasminogen to Lys-plasminogen.[6] Inhibition of thrombolysis by apo(a) was also observed in a rabbit jugular vein model.[27] More recently, a report that apo(a) transgenic mice on a $Plg^{-/-}$ background had increased thrombotic potential when exposed to vascular injury lead to an alternative model for the effects of apo(a) on thrombolysis, possibly involving changes in clot structure.[7] Indeed,

elevated Lp(a) levels have been previously associated with altered fibrin clot structure, reduced permeability of fibrin clots and prolonged lysis times.[28, 29] Furthermore, human plasma from Caucasian patients carrying the rs3798220 SNP exhibited longer clot lysis times and increased clot permeability, compared to Caucasian patients who were not carriers.[10] Curiously, however, these trends were not observed in non-Caucasian patients.

In this study, we have shown that (i) apo(a) decreases coagulation time of plasma clots *in vitro* and the Met4399 variant has a more potent effect; (ii) the Met4399 variant uniquely increases the maximum turbidity of plasma clots formed *in vitro*, suggesting an effect on fibrin structure; (iii) the Met4399 variant is a more potent inhibitor of fibrinolysis of plasma clots *in vitro* without being more effective at inhibiting plasminogen activation; (iv) the Met4399 variant causes the formation of more disorganized pure fibrin clots with thicker fibrils; and (v) plasma clots formed from patients homozygous for the Met4399-encoding allele have an altered fibrin structure. Altogether, these data strongly suggest that the Met4399 variant has a greater prothrombotic/antifibrinolytic potential, at least in part through deleterious effects on fibrin structure. We present evidence from molecular modeling that the presence of Met at position 4399 alters the local structure of the protein, resulting in a more solvent-exposed and less mobile side chain. Indeed, we showed the Met residue to be oxidized by MALDI-TOF mass spectrometry, which provides a functional link to effects on fibrin structure (see below).

Given the association of rs3798220 with elevated plasma Lp(a) concentrations, we also took advantage of our recombinant expression system to directly address the proposition that the Ile to Met substitution increases the rate of apo(a) biosynthesis. Using a pulse chase secretion assay in HepG2 cells, we found no difference in the rate of secretion

of the Met variant compared to the Ile variant (Supplementary Figure 2). This result suggests that the association of the rs3798220 polymorphism and elevated Lp(a) levels may not be due to the SNP directly but likely due to the correlation of this variant with smaller apo(a) isoforms, which are more readily secreted.[30] It cannot be excluded that the I4399M substitution affects other processes that impact plasma Lp(a) concentrations such as Lp(a) assembly or catabolism. It has not yet been directly investigated if the elevated Lp(a) levels in carriers of this polymorphism reflect linkage disequilibrium with smaller apo(a) isoforms or other genetic variants that directly impact Lp(a) levels.

The known causal relationship of elevated Lp(a) levels to cardiovascular diseases such as coronary heart disease and ischemic stroke could reflect the contribution of Lp(a) to atherosclerotic processes, thrombosis, or both. Indeed, evidence for both roles has emerged from *in vitro* and animal model studies.[31] One approach to assessing if Lp(a) contributes to thrombosis has been to examine the relationship of Lp(a) to "pure" thrombotic disorders such as venous thromboembolism (VTE). The results of such studies have been controversial, however. Elevated plasma levels of Lp(a) have been correlated with idiopathic and recurrent VTE, and this was inversely correlated with the number of KIV_2 repeats.[32, 33] On the other hand, Mendelian randomization studies have suggested that Lp(a) is not a causal risk factor for venous thrombosis.[34] A recent study reported an independent link between low KIV_2 copy number and incidence of VTE.[32] Four *LPA* SNPs, including rs3798220, were found to have no association with VTE when assessed individually, although certain *LPA* haplotypes influenced disease susceptibility. No haplotypes containing the minor allele of rs3798220 were shown to be associated with increased VTE risk, however.

We found that apo(a) decreased the clotting time of plasma clots *in vitro* with 17KI4399M having the more potent effect (Figure 4.1A). In addition, this variant specifically increased the maximum turbidity of the clots (Figure 4.1B). Indeed, experiments in which purified apo(a) was combined with purified fibrin showed a marked effect of the Met4399 variant, but not the wild-type apo(a), on clot structure (Figure 4.3). Generally, increase in the turbidity of fibrin clots is strongly associated with changes in the structure of clots. More specifically, increased turbidity of a clot is indicative of thicker fibrin fibers and is generally associated with attenuation of prothrombin activation of thrombin activity[35], although such an effect would not be occurring in our purified system. It is accepted that increased clot turbidity is associated with increased lysis times.[36] Detailed microscopic studies have revealed that thinner fibers may be more susceptible to lysis than thick fibers [reviewed in ref. 37].

We also studied clot structure and permeability using, for the first time, plasma from individuals homozygous for the Met allele. Unlike previous studies[10], we found no difference in clot permeability between Ile/Ile and Met/Met subjects with similar Lp(a) levels, although the subjects with higher Lp(a) did, as expected, have lower clot permeability (Figure 4.4C). rs3798220 genotype did affect plasma clot fiber density (Figure 4.4B), and amplified the ability of elevated Lp(a) to promote thicker fibrils (Figure 4.4D,E). The latter result is in agreement with what we observed in a purified system using recombinant apo(a) of either genotype (Figure 4.3). It should be noted, however, that we were only able to obtain plasma from two subjects homozygous for Met at 4399, owing to the low frequency of this polymorphism. Nonetheless, our studies using purified components and naturally-occurring variants do support the idea that both Lp(a) levels and

rs3798220 influence fibrin clot structure, and hence the coagulability of plasma and its susceptibility to lysis. Accordingly, it is possible that the properties of the variant Lp(a) contribute to the increased risk for vascular disease in rs3798220 carriers through an effect on clot structure.

To investigate a possible structural and biochemical rationale for the effects of the Ile/Met substitution, we used a molecular dynamic simulation approach. We modelled the inactive protease domain of apo(a) with either an isoleucine or methionine residue in the 4399 position (Figure 4.5). In the native Ile containing domain, the Ile residue is kept in a buried orientation blocked from exposure to the protein-solution interface by adjacent Arg4420. Introducing the Met residue into the 4399 position results in a change in the orientation of the Arg residue such that the methionine can shift outward into a more exposed conformation, effectively altering the surface of the protease domain in this variant. Root mean squared deviation analysis between the Ile/Met and Arg residue show large fluctuations in the distance spanning the Ile-Arg residues, but a significantly more consistent distance measured between the Met-Arg suggesting that the Arg may stabilize the Met residue in its more solvent exposed conformation.

A possible ramification of this putative conformational change is that it may expose the polymorphic methionine residue to oxidative molecules during apo(a) biosynthesis in hepatocytes or within the plasma. Indeed, using MALDI-TOF mass spectrometry we determined that the I4399M variant possesses an oxidized methionine residue in its native form. Met(O) moieties have been implicated in the dysfunction of several other plasma proteins, such as apoA-I and fibronectin.[37, 38] The oxidation of Met on fibronectin was found to profoundly alter its ability to bind collagen, despite only a small conformational

change of the protein. Given that lysine-independent fibrinogen binding sites have been identified amongst the KV and protease domains of apo(a)[8, 39], it is tempting to speculate that the presence of the Met residue resulting from the rs3798220 SNP may influence the ability of the variant apo(a) to bind to fibrin in a distinct way, altering clot structure and potentially augmenting the pathogenic effects of Lp(a) thrombosis and fibrinolysis.

4.6 References

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4.7 Supplemental Data

4.7.1 Experimental Methods

4.7.1.1 SDS-PAGE analysis of apo(a)-fibrinogen interaction

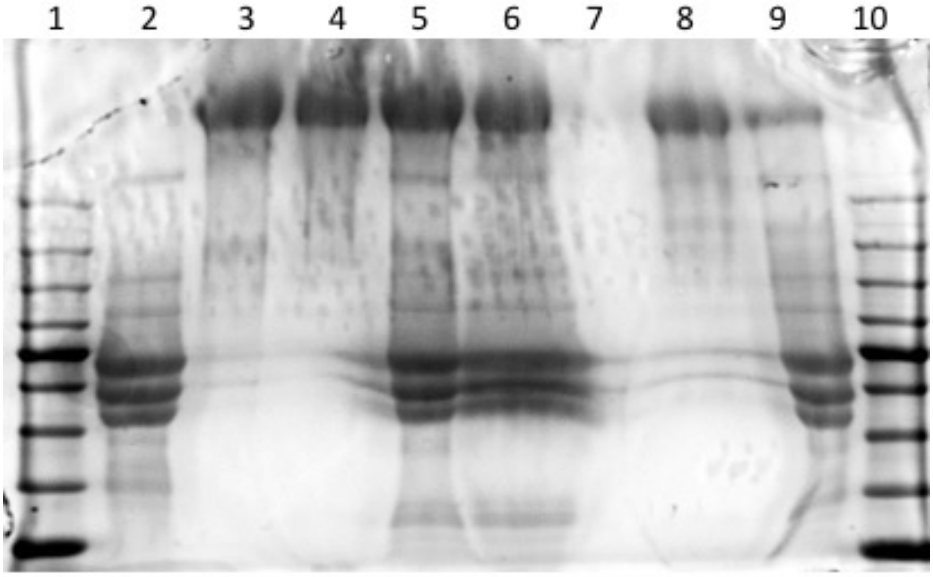
Fibrinogen (Calbiochem, Mississauga, ON, Canada) at a concentration of 3mg/mL was mixed together with the wild type and variant apo(a) to a final apo(a) concentration of 500nM, vortexed, and incubated for 10 mins at room temperature. Where indicated, samples were further subjected to centrifugation at 1000 x g for 5 min followed by separation of the supernatant and pellet. The samples were then prepared with the addition of 4X SDS reducing sample buffer and boiled for 10 min at 90°C. Samples were analyzed on a 4-15% SDS-PAGE gel and stained using Imperial Stain (Thermo Scientific, Waltham, MA, USA) for visualization of proteins

4.7.1.2 Pulse Chase Secretion Assay

HepG2 cells were transiently transfected with an apo(a) expression construct (pRK5ha.17) encoding 17 KIV repeats with and without the I4399M SNP.[13] The cells were grown to 90% confluency and split into a 6-well plate (Sarstedt) 16 hours prior to the experiment. Cells were then starved for 1h with methionine- and cysteine-free DMEM without serum, and pulse-labelled for 1h in the same media containing 200 μCi/mL [S^{35}]methionine, and chased in complete DMEM media. Media and lysates were harvested at 0, 30, 60, 120, 240, and 480 min intervals, after PBS washes of the cells, using cold lysis buffer (100mM Tris, pH 8.0, 100mM NaCl, 10mM EDTA, 1% Triton X-100, 0.1% SDS, 100mM ε-aminocaproic acid) containing protease inhibitors. Cellular debris was removed from media and lysates through centrifugation, and samples were pre-cleared by incubation

with gelatin-agarose (Sigma) for 2h at 4°C, followed by incubation with a monoclonal anti-apo(a) antibody overnight at 4°C. The following day, r-protein G beads (Invitrogen) were added to the samples and incubated for another 2h at 4°C. Samples were pelleted and washed twice with RIPA buffer (50mM Tris, pH 7.4, 0.15M NaCl, 1% Nonidet P- 40, 1% Sodium Deoxycholate, 0.1% SDS, pH 7.5) and once in TE buffer (10mM Tris, 1mM EDTA, pH 7.0). The pellets were then boiled for 10mins in 2X Laemmli sample buffer (4% SDS, 20% glycerol, 0.001% bromophenol blue, 125mM Tris, 100mM dithiothreitol, pH 6.8). Immunoprecipitated samples were run on 7% SDS-PAGE gels, dried, and exposed using autoradiography (Fujifilm). After 4 days, the screen was then imaged using a Molecular Imager® FX (Bio-RAD). Protein secretion rates were determined by densitometric analysis using Alpha View software (AlphaInnotech), and presented as a percentage of the maximum intensity of the pRK5ha.17 transfected sample.

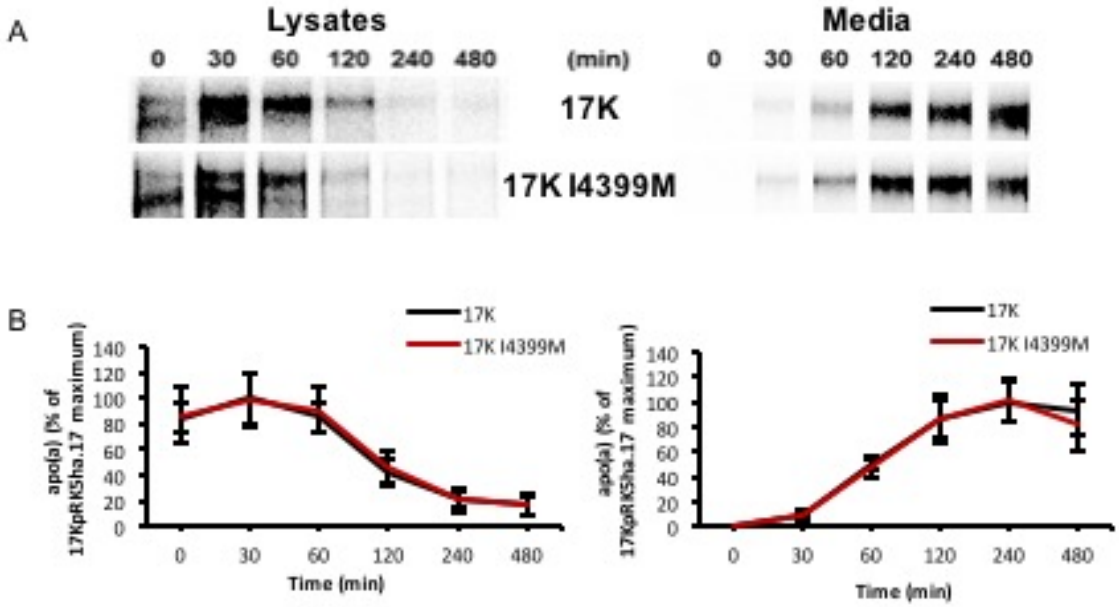
4.7.2 Supplemental Figures



Lane	Contents
1	MW marker
2	Purified Fibrinogen
3	17K r-apo(a)
4	17K I4399M r-apo(a)
5	17K + Fibrinogen
6	17K I4399M + Fibrinogen
7	Blank
8	17K I4399M + Fibrinogen supernatant*
9	17K I4399M + Fibrinogen pellet*
10	MW marker

** Sample prepared by centrifugation of the sample present in lane 6*

Supplemental Figure 1.1 17K I4399M stimulates aggregation of fibrinogen.
Fibrinogen at 3.0 mg/mL, or apo(a) at 500nM was incubated for 10 minutes at room temperature alone or in combination. In the case of turbid samples, debris was isolated using centrifugation. Samples were subjected to SDS-PAGE analysis followed by Imperial staining to visualize protein bands.



Supplemental Figure 1.2 Apo(a) I4399M variant does not have a different rate of secretion in HepG2 cells.

Pulse-Chase secretion assay was performed to determine the comparative secretion rate of 17K I4399M. **A.** Representative gel images for the anti-apo(a) immunoprecipitated samples for time points ranging from 0-480 min for both 17K and 17K I4399M transfected cells. **B.** Densitometric analysis of the immunoprecipitated bands represented as a fraction of the bands present for the equivalent time points in the 17K transfected samples, the data represent the means ± S.E.M of three independent experiments. No significant differences between protein secretion rates was determined.

General Discussion

Elevated levels of Lp(a) have been identified as a causal risk factor for calcific aortic valve stenosis (CAVS) and coronary heart disease (CHD).[1] Plasma Lp(a) levels very widely in the population and are primarily genetically determined.[2] The major genetic determinant of apo(a) production is the size of the *LPA* gene. However, there is evidence that single nucleotide polymorphisms may influence *LPA* levels as well, and additionally the extent of proinflammatory oxidative modification, and pathophysiology.[3] Although there is compelling evidence for the proatherogenic roles for Lp(a), and several studies indicating roles in thrombotic processes; the mechanisms by which it elicits its effects on the vasculature remain to be fully understood. The aim of this study was to assess the impact of oxidative modifications on the apo(a) component of Lp(a). In this regard, we evaluated the roles of the covalent oxPC modification of apo(a) KIV_{10} and the potential methionine oxidation in the protease domain arising from an I4399M polymorphism.

The location of the of the covalent oxidized phospholipid addition to the KIV_{10} domain of apo(a) was studied using a site directed mutagenesis approach of candidate apo(a) variants. The proinflammatory effect of the oxPL moiety was assessed with respect to its effect on inflammatory responses in macrophages. Investigation into the relationship between the lysine binding potential of apo(a) molecules and the extent of oxPC modification was determined using several different strong lysine binding site (sLBS) destabilizing mutations. In addition, the I4399M polymorphism in apo(a) was studied with respect to its effect on fibrin clot structure and the time required for clot lysis.

5.1 Lp(a)/apo(a) possesses a proinflammatory oxidized phospholipid moiety

Previous studies have reported the association of oxidized phospholipids with the $apoB_{100}$ -containing lipoproteins LDL and Lp(a).[4] The early studies reported a strong correlation between oxPL:$apoB_{100}$ with carotid and femoral atherosclerosis and with obstructive coronary artery disease.[5, 6] Since Lp(a) contains an LDL-like moiety, it is intuitive to hypothesize that the oxPL detected in these studies is derived from Lp(a), though it was not until 2008 that specific $apoB_{100}$ protein classes were differentiated with respect to oxPL:$apoB_{100}$ levels.[7] Lp(a) was found to contain approximately 85% of lipoprotein associated oxPL, thus represents almost the entire pool of oxPL:$apoB_{100}$ measured.[7] Due to the proinflammatory properties of most oxPL species, this finding suggests that this may represent a mechanism for the proatherogenic effects of Lp(a).[8, 9] It is important to note that all of the studies to date reporting the quantification of oxPL:apoB100 have used the E06 antiphosphocholine antibody to detect oxPC. The E06 mouse monoclonal IgM has been thoroughly investigated and has been proven to detect the phosphocholine (PC) head group of oxidized phospholipids, with high specificity.[10, 11] Thus the correlation between oxPL:$apoB_{100}$ and vascular diseases can be interpreted as a correlation between E06-reactive oxidized phospholipid molecules and inflammatory processes. In fact, E06 has been used to stain atherosclerotic plaques based on its ability to bind to atherogenic lipoproteins and apoptotic cells within an atherosclerotic lesion, suggesting that the antibody could be used as a biomarker for proatherosclerotic epitopes *in vivo*.[12] However, due to the ability of E06 to bind oxPC in a free and covalently conjugated form[10], it is important to take into consideration the possibility of detecting both non-covalent and covalently bound oxPC molecules. In the early covalent oxPL epitope

mapping of Lp(a)/apo(a) by Edelstein and coworkers, it was determined that E06 could act as an indicator of the ability of apo(a) to elicit interleukin-8 (IL-8) mRNA and protein expression, in an oxPL-dependent manner.[8] It was determined that only E06-reactive proteolytically cleaved fragments of apo(a) were able to stimulate IL-8 expression. This was the first indication that directly associated E06-reactive apo(a) fragments with the ability to enhance the pro-inflammatory potential and was later investigated in a follow up study, however it could only be inferred that this reflected a direct effect of the oxPL moiety.[13] The data represented in Chapter 3 is the first to report a direct effect of the covalent oxPL moiety on IL-8 expression in macrophages, describing clear mechanistic links to inflammatory signaling pathways.

In Chapter 2, we describe the use of E06 immunoblotting to detect specific sequences in apo(a) that are required for the addition of covalently-linked oxPC. Evidence from previous studies were able to identify the covalent attachment of an oxidized phospholipid modification on the apo(a) component of Lp(a), as seen by E06 immunoblotting.[8] Location of the apo(a) domain containing the covalent oxPL moiety was addressed in the studies of Leibundgut *et al.* where it was determined that the KIV_{10} domain houses the E06-reactive site in apo(a).[14] The use of human recombinant 17-kringle-containing apo(a) (17K) together with variants containing systematic deletions of the C-terminal domains of apo(a) allowed for the description of important information in the development of the current study. Interestingly, primate apo(a) was not able to be detected by E06 and was able to give insight into the sequence requirements for covalent oxPL binding to apo(a). Sequence analysis of several species of primate apo(a) identifies the major differences between the human and primate KIV_{10} and KV domains (see Figure 2.6).

Using a recombinant, minimally small version of apo(a) – represented in a $KIV_{10}KV$ (dikringle) construct – the exact amino acid linkage to the oxPL moiety was able to be determined (Chapter 2). By generating mutants of the KIV_{10} amino acid differences between primate and human apo(a), we were able to verify the requirement for an intact strong lysine binding site (sLBS) in KIV_{10} in order to facilitate covalent oxPC addition. Using the Asp^{56} → Ala and Trp^{70} → Ala variants in KIV_{10}, which are known to destabilize the sLBS[15, 16], we have shown that inactivation of the LBS through two different mutations results in a loss of E06 immunoreactivity. This result is in agreement with the data previously published by our group using a $17K\Delta LBS_{10}$ variant (contains the Asp^{56} → Ala substitution), as well as from the assessment of primate species containing Asp^{56} (gorilla/cynomologous) and Trp^{70} (chimpanzee/rhesus monkey) polymorphisms, which were also unable to be detected by E06.[14] Assessment of $KIV_{10}KV$ containing a conservative Asp^{56} → Glu mutation, similar to that in the weak lysine binding KIV_7, showed the retention of E06 reactivity, suggesting a decrease in the ability of this mutant to be covalently modified by oxPC. Prior characterization of this variant in the context of bacterially purified KIV10, showed that this mutation remains to permit lysine binding of this domain, but with a 10-fold decreased affinity.[17] Thus, the decrease in E06 reactivity is likely caused by the decrease in LBS activity. This mutant represents a useful tool that could aid in the description of effects of the sLBS in KIV_{10} without the complexity for a possible role for the covalent oxPC moiety on apo(a).

There is a population of individuals who possess a natural Trp^{70} → Arg polymorphism, giving rise to a pool of Lp(a) which is unable to bind lysine.[15] It would be interesting to assess if these individuals have "E06-negative" Lp(a), and to see if Lp(a)

retains a causal relationship with CHD and CAVS in these individuals. It has been noted that transgenic mice overexpressing a Asp^{56} → Ala mutation in the KIV10 domain of an 8-kringle construct were resistant to the formation of atherosclerotic lesions.[18] The lack of disease progression in these animals was hypothesized to be due to the lack of ability of apo(a) to inhibit plasminogen activation due to the loss of the sLBS in KIV_{10}, thus inhibiting TGF-β activation and subsequent smooth muscle cell (SMC) proliferation and migration.[18] However, oxPLs have been documented to play a role in stimulating SMC proliferation by initiating phenotypic switching *in vitro* and *in vivo*.[19] As such, it is also possible that the lack of oxPL on apo(a) transgenic animals lacking the sLBS in KIV_{10} resulted in an inability to initiate phenotypic switching in SMCs and thus, mice did not have the usual accelerated effects of Lp(a) on the development of atherosclerosis. The assessment of key components of the atherosclerotic process, that can be modulated by oxPL, should also be assessed in similar *in vivo* models. A role for the oxPC modification of apo(a) should be assessed in endothelial dysfunction, permeability, and lesional macrophage deposition in relevant atherosclerotic mouse model, as these are all critical initial steps of atherogenesis. However, the role for the KIV_{10} LBS cannot be decoupled from a role for the covalent oxPC adduct without the use of a novel apo(a) transgenic mouse lacking a covalent oxPC modification (defined in Section 5.2).

5.2 Differentiating roles for the KIV_{10} sLBS and covalent oxPL moiety on apo(a)

There have been many studies that suggest roles for the KIV_{10} sLBS on the proatherosclerotic properties of apo(a)/Lp(a), such as endothelial cell (EC) migration and cytoskeletal remodelling.[20, 21] However, it has not been clear whether this reflects a role for

the sLBS *per se*, or on the requirement of the sLBS for oxPL addition. In Chapter 3, we identified a critical role for the activation of NF-κB in the signalling pathways initiated by the oxidized phospholipid moiety on apo(a). The activation of NF-κB is implicated in chronic inflammation seen within a developing atherosclerotic plaque [reviewed in Ref. 22]. Previously described effects of apo(a) on vascular cell phenotype *in vitro* have linked a role for the intact sLBS in KIV_{10} to pathways that have been known to involve NF-κB. For example, ECs stimulated with 17K r-apo(a) have been shown to upregulate cyclooxygenase-2 (COX-2) and increase prostaglandin synthesis.[21] This effect was abrogated when the apo(a) KIV_{10} sLBS was eliminated by site-directed mutagenesis. The expression of endothelial cell adhesion molecules ICAM1, VCAM1, and E-selectin can also be up-regulated by a similar signalling pathway stimulated by oxPL.[23] Lp(a) and apo(a), but not LDL, are able to initiate an increase in mRNA of these inflammatory markers, although roles for the KIV_{10} sLBS or oxPL have not yet been explored.[24, 25] With new knowledge about the requirement of the sLBS in apo(a) for covalent oxPL addition, caution must now be exercised when determining roles for the lysine binding potential of apo(a), as elucidated with sLBS deficient mutants. Since oxPLs have a wide range of proinflammatory functions they may be responsible for many of the proinflammatory effects attributed to Lp(a), and its lysine binding potential. In order to assess a role for the oxPL on apo(a), while at the same time accounting for a potential role for the sLBS in KIV_{10}, a variant of apo(a) that lacks oxPL but maintains lysine binding ability is required. The use of a KIV_{10} Asp^{56} → Glu mutant can be used in this respect as well, as we have determined that it maintains the ability to be covalently modified by oxPC, but has impaired

lysine binding potential. We have gathered these essential tools using a targeted mutagenesis approach as described in Chapter 2.

The determination of the specific amino acid residue that participates in the formation of the oxPL adduct has been of interest since the discovery of the covalent oxPL modification of apo(a). In the 2003 study by Edelstein *et al.* the possibility of Schiff base adducts on KV was explored using 2,4,6-trinitrobenzene sulfonic acid (TNBSA), which modifies exposed lysine residues.[8] It was determined that two of the six KV lysine residues, Lys^{12} and Lys^{42}, were not able to react with TNBSA, and therefore hypothesized to be blocked by oxPL adducts. However, the study by Leibundgut *et al.* utilized a 17KΔLys12/42 double mutant, in addition to several recombinant apo(a) variants lacking the KV domain (17KΔV, 17KΔVP, and KIV5-10) and found no requirement for the KV domain amino acids in the addition of oxPC to apo(a).[14] We reported that all of these variants maintained E06 immunoreactivity, thus suggesting that the site of covalent OxPL addition to apo(a) does not occur within the KV sequence. [14] We exploited known mechanisms of reactive aldehyde covalent bonding by amido- and thiol-containing amino acid side chains; Schiff base and Michael addition reactions to generate recombinant apo(a) molecules for this study. We constructed several candidate mutations corresponding to substitutions in His, Arg and Lys residues in the context of a minimally small $KIV_{10}KV$.his apo(a) truncation, to aid in the identification of the site of oxPL addition. Similar to the previous results from our group on the 17KΔLys12/42 double mutant and KV lacking apo(a) truncations (17KΔV, 17KΔVP, and KIV5-10), we created KV lysine mutants in the context of $KIV_{10}KV$, thus confirming the results seen using the corresponding 17K variants. As seen in Table 2.2, all mutations in lysine residues studied within $KIV_{10}KV$ were able to be

detected by E06. Candidates in the KIV_{10} domain consisted of His → Ala mutations and conservative Arg → Gln mutations that were considered appropriate due to the fact that Arg35 is a component of the cationic cluster in the sLBS. E06 screening of these KIV_{10} variants identified KIV10KVΔH33A as the only variant that did not react with E06. Since this variant has not been previously studied, there was no prior knowledge as to the structural or functional effects of the His-Ala substitution. Molecular dynamic simulations of this mutant showed no significant changes in the overall structure of the KIV_{10} domain. In fact, assessment of the His33 residue in the modelling of KIV_{10} shows no likely intramolecular interactions of this amino acid at all, with the nearest potential interaction being over 3.0 Å away. The function of this variant was determined by characterizing its ability to bind to lysine-Sepharose, compared to the wild-type KIV_{10}KV. As seen in Chapter 2, there was no apparent difference in the ability of the His^{33} variant to bind to lysine-Sepharose compared to wildtype, making the H33A variant of KIV10KV the only E06 non-reactive KIV_{10} variant that possesses a fully functional strong lysine binding site. The ability to separate the lysine binding function of apo(a) KIV_{10} from its ability to covalently bind to oxPL will allow for mechanistic studies on the proatherogenic effects of Lp(a) in the vasculature. Using a full-length apo(a) variant containing the H33A mutation in KIV_{10}, we can now assess the role of the oxPL moiety on vascular cell phenotype compared to wild-type apo(a). In a recent study, E06 was used to block the effects of the oxPL on 17K r-apo(a).[26] Although E06 was able to block apo(a)-mediated IL-6 and TNFα secretion, it required the use of molar amounts of the antibody which may not be tolerated by all cell types [reviewed in Ref. 27]. ECs, SMCs, and monocytes express receptors capable of binding to immunoglobulins, thus stimulating inflammatory activation.[27, 28]

Solid-phase IgM incubation of U937 monocyte cells was recently shown to activate NF-κB and stimulate H_2O_2 production in a scavenger receptor-A dependent manner, thus provoking proinflammatory macrophage phenotypes.[28] There is also the concern that using a large IgM molecule (approximately 900 kDa) may sterically block apo(a)/Lp(a) from binding to cellular receptors via the sLBS, which is in close proximity to the E06 binding site on apo(a). While using E06 IgM as a tool to study the role of the oxPL modification of apo(a) may not be optimal, using H33A-containing apo(a) variants compared to wild-type apo(a) would provide the most direct assessment of the function of the covalent oxPC modification of apo(a).

It would also be interesting to generate a humanized $apoB_{100}$ transgenic mouse model that expresses wildtype apo(a), or variants containing KIV_{10}D56E or KIV_{10}H33A mutations. These animals could be used to determine the relative contributions of the oxPC moiety on apo(a) versus the sLBS. The use of the described set of transgenic animals would result in a *bona fide* Lp(a) particle lacking the sLBS but containing a covalent oxPC moiety, as well as the reverse situation where the LBS is intact but the proinflammatory oxPC moiety is not present. It has already been reported that mice expressing human apo(a) have significantly greater risk of developing atherosclerotic lesions compared to mice expressing an apo(a) with a defective sLBS.[18] The specific role of the oxPC moiety on apo(a) cannot be discerned here, as apo(a) from these mice were also unable to bind lysine to the same degree as wildtype apo(a) mice. The generation of recombinant apo(a) variants or apo(a) transgenic animals with the H33A mutation or the D56E mutation will serve as our most powerful tool yet in the determination of the role for the oxidized phospholipid moiety in the atherogenic potential of Lp(a). Introducing these human apoB/apo(a)

transgenic mice, on an LDLR$^{-/-}$ or apoE$^{-/-}$, background, will allow us to study the impact of the oxPC and sLBS on lesion development, lipid deposition, and recruitment of various vascular cells. We have reported evidence for a role for the oxPC moiety in monocyte recruitment (Chapter 3) and a role has been determined for the sLBS in EC[29], thus it would be interesting to profile the lesion immunohistochemically for the proportion of each cell type (including VSMCs, macrophages and foam cells) in the different proposed mouse genotypes.

The atherogenic potential of oxPLs has been shown to results from the stimulation of chronic inflammation.[19, 30, 31] Chapter 3 reports the specific ability of the oxPL on apo(a) to induce interleukin-8 mRNA and protein expression in cultured macrophage cells. There have been few reports of functions confirmed to be elicited by the oxPL moiety on apo(a). Seimon and colleagues determined that the oxPL on apo(a) had the ability to induce apoptosis in ER-stressed macrophages, using the same phospholipase A_2 digestion approach that we utilized in Chapter 3.[32] A role for the oxPL was also described in the priming of human monocytes to elicit inflammatory reponses.[26] The true scope of the effect of the covalent oxPC modification, as well as the non-covalent oxPLs on apo(a) on the functions of Lp(a) have not yet been defined. Though it seems from the studies by Seimon, van der Valk, and our group (Chapter 3), that the covalent oxPL moiety clearly plays a role in the chemotaxis, apoptosis and inflammatory signalling in monocyte/macrophage cells[26, 32]; roles for the oxPL component of Lp(a) has not been studied in SMCs and endothelial cells. Work generated from our lab has shown clear effects for the KIV10 sLBS in EC proliferation and migration[29], permeability and contractility[20], and prostaglandin synthesis[21]. These processes all potentially could arise from responses of ECs to the oxPL

moiety on apo(a). The KIV10 sLBS has also been shown to be involved in the migration of SMCs in a RhoA/ROCK dependent manner.[33] It should be noted that all cell signalling pathways reported from our lab in both ECs and SMCs are potential upstream affectors of NF-κB. It is possible that the cellular receptors triggered by the oxPL moiety on the apo(a) component of Lp(a) elicit a universal signalling cascade culminating in the activation of NF-κB (and likely AP-1), thus stimulating inflammatory phenotypes in vascular cells. As previously mentioned, the respective roles for the sLBS and oxPL on apo(a) have not been explored with respect to the reported increase in EC cell adhesion molecule expression observed upon apo(a)/Lp(a) treatment. [24, 25]

5.2 Future work on the characterization of oxidized phospholipids on Lp(a)/apo(a)

The association of elevated Lp(a) levels and oxPL with the increased incidence of CAVD and CHD as determined by E06 ELISA, suggests great functional importance of the covalent oxPC on Lp(a) in initiating inflammatory processes *in vivo* [reviewed in ref. 33]. The work presented in Chapters 2 and 3 clearly define the structural determinants required for covalent oxPL addition and a functional role for the oxPL moiety on apo(a). This work also illuminates key questions that have yet to be answered about the association of atherogenic oxPLs with the atherogenic potential of Lp(a). In addition to the potential pathophysiological processes that the covalent oxPL moiety on apo(a) may mediate (see Section 5.1), there remain a number of basic questions regarding i) role of a functional lysine binding site in oxPL addition (covalent or non-covalent); ii) the source of oxPL; a role for cellular receptors in the covalent oxPC modification of apo(a); iii) the role and significance of non-covalent oxPCs that are detected on the $KIV_{10}KV$ r-apo(a); and iv) the

role of the covalent oxPC moiety in other processes mediated by Lp(a)/apo(a), such as vascular cell migration or macrophage Lp(a) internalization.

Chapter 2 illuminates the importance of an intact lysine binding site in the KIV_{10} domain of apo(a) in covalent OxPC modification. This may provide insights into the regulation and mechanism of covalent oxPL addition to apo(a). The requirement of the lysine binding site in KIV_{10} raises the possibility that the addition of oxPL (both covalent and non-covalent) to apo(a) may require binding to exposed lysine residues on a cellular ligand. It has not been determined whether intracellular apo(a) has a covalent oxPC moiety. In this regard, it can be hypothesized that apo(a) requires molecular docking in close proximity to a cellular source of oxPL, such as a cell or microsomal membrane. Our group previously reported that recombinant apo(a) can bind to several distinct high and low affinity receptors on the surface of hepatocytes with the identity of the low affinity binding component being plasminogen receptors.[34] Owing to the structure of free apo(a) and the presence of a covalent oxPC moiety on plasminogen[35], it is tempting to speculate that the binding of apo(a)/plasminogen to a plasminogen receptor may be required for covalent oxPC addition to apo(a)/plasminogen, through lysine binding domains. It is also unclear if all secreted Lp(a) molecules have a covalent oxPC on apo(a) KIV_{10}. Understanding the process by which apo(a) acquires the proinflammatory lipid moiety may give insight to the potential for therapeutic options aimed at decreasing the atherogenic potential of Lp(a) through blocking the covalent OxPL modification.

Lp(a)/apo(a) has been documented to interact with a plethora of cellular receptors that may contribute to the pathophysiological functions of Lp(a). However, the nature of the interaction of apo(a) with several of these receptors is unclear. In Chapter 3 of this

thesis and in the work of Seimon *et al.*, the effects of apo(a) on macrophage inflammation and apoptosis were proposed to be initiated by the binding of the covalent oxPC moiety to the lipid specific receptor CD36 and to toll-like receptors 2, 4, and 6.[32] Each of these receptors are expressed at varying levels, and has the potential for different effects on different vascular cell types.[36] An investigation into the major apo(a) oxPC receptor and the downstream effects on inflammatory processes is a vital step in determining the contribution of the covalent oxPC moiety on apo(a) to the role of Lp(a) in atherosclerosis. In the same way a role should be defined for the non-covalent oxPL species associated with $KIV_{10}KV$ that were observed by lipid extraction of apo(a) as reported in Chapter 2. To study potential roles for non-covalent oxPC in the inflammatory properties of apo(a), first the effect of the covalent oxPC must be defined using a H33A apo(a) variant. E06 pretreatment of the H33A variant of apo(a) may give an additional decrease in the pathogenic potential of apo(a), this would infer proinflammatory roles for the non-covalent oxPC on apo(a). The initial study identifying Lp(a) as an E06-reactive lipoproteins (as quantified by sandwich ELISA) reported temporal changes in the amounts of oxPL detected as a function of the amount of time elapsed post-MI[4]; this trend was also reported with respect to plasminogen-associated oxPL.[35] It has been proposed that Lp(a) might act as a scavenger of oxPL in these cases, picking up excess oxidized material generated as a product of the acute phase reactants PLA_2 and platelet activating factor-acetylhydrolase.[37] It would be interesting to quantify and profile the different non-covalent oxPL species associated with Lp(a) from humans with different disease states (ie/ CAVS, myocardial infarction (MI), CHD, etc.). In this way we can further understand the roles for the non-covalent oxPL associated with Lp(a) and potentially use these profiles to predict disease

risk. It was also recently reported that Lp(a) can transport autotaxin (ATX) and oxPL to mineralized aortic valves, thus contributing to the development of calcific aortic valve disease.[38] It is possible that the affinity of ATX for the oxPL on Lp(a) facilitates the ATX-Lp(a) interaction, this remains to be further investigated.

Due to the proximity of the covalent oxPC moiety on Lp(a)/apo(a) to the sLBS, it would be interesting to see if loss of the oxPC on KIV_{10} could alter the function of Lp(a)/apo(a) in non-inflammatory processes. Roles for Lp(a) in the inhibition of fibrinolysis have been largely determined to be dependent on the lysine binding activity of apo(a).[40-42] Since we currently do not know if 100% of plasma Lp(a) particles contain covalent oxPC, it is possible that there is a pool of Lp(a) that lacks covalent oxPC; this population may have an altered affinity for fibrin or plasmin modified fibrin. In a study on the covalent oxPC on plasminogen, it was determined that the oxPC on plasminogen was required for optimal clot lysis, as enzymatic removal of the oxPC resulted in a 30% decrease in clot lysis.[35] It has yet to be determined, however, if the oxPC on plasminogen has a role in fibrin binding in the context of plasminogen activation or in the proteolytic activity of plasmin.[43] It can be hypothesized that if there is a pool of Lp(a) lacking a covalent oxPC modification, there may be a difference in the affinity of this Lp(a) for fibrin, thus potentially altering the effects of Lp(a) on the coagulation and/or fibrinolytic systems. Variants of apo(a) that possess lysine binding potential but lack an oxPC moiety might be interesting to assess in the context of plasminogen activation, binding, as well as the formation of the apo(a)/plasminogen/tPA/fibrin complex, *in vitro*.

5.4 Roles for Lp(a)/apo(a) in thrombotic and fibrinolytic processes

Due to the high degree of sequence similarity between apo(a) and plasminogen, and the lack of a functional serine protease domain (PD) in apo(a), it has been hypothesized that the pathogenicity of Lp(a) may stem from its ability to interfere with plasminogen *in vivo*. It has been previously reported that Lp(a)/apo(a) can bind to plasminogen[41], fibrin and partially degraded fibrin.[40] It has been shown that apo(a) can compete with plasminogen for the exposed lysine residues on fibrin, thus decreasing the ability of plasmin to be generated thereby reducing clot dissolution. Chapter 4 describes a role for the fibrin binding ability of a polymorphic form of apo(a) that results in alterations in the structure of clots, as well as a decrease in the ability of clots to be lysed.

The role for Lp(a)/apo(a) in thrombotic disorders has been thought to be largely dependent on the ability of Lp(a) to inhibit plasminogen activation and fibrinolysis. This work has been extensively investigated by our group in *in vitro* models, yielding the conclusion that apo(a) can inhibit plasminogen activation on the surface of fibrin and partially degraded fibrin.[40, 44] It was further concluded that the robust inhibitory potential of apo(a) on plasminogen activation is due to the ability of apo(a) to participate with plasminogen, tPA, and fibrin in the formation of a quaternary complex that has a decreased turnover rate compared to complexes lacking apo(a).[44] A later study concluded that apo(a) also inhibits this process by decreasing the extent of conversion of Glu-plasminogen to the more readily activatable Lys-plasminogen on the fibrin surface.[45] However, there have been reports *in vivo* of apo(a) promoting coaguability in a plasminogen-independent manner. An increase in thrombotic potential was reported in a transgenic mouse model

overexpressing apo(a) on a $Plg^{-/-}$ background.[46] This strongly suggests that apo(a) can have a direct effect on thrombotic processes.

The role for Lp(a) in "purely thrombotic" disorders, such as venous thromboembolism (VTE), has been a controversial topic for many years [reviewed in ref. 48]. There has been a significant body of *in vitro* data suggesting thrombotic roles for Lp(a). However, there has been limited progress on identifying a direct role for Lp(a) in thrombotic disorders within human population studies. A study from our group suggests roles for Lp(a)/apo(a) in platelet aggregation and granule release in the presence of the thrombin receptor activating peptide SFLLRN.[49] Conversely, Lp(a) was shown to decrease the aggregation of platelets in the presence of platelet-activating factor[50] and collagen; this may be due the ability of Lp(a) to directly bind to the latter two substrates.[51, 52] The involvement of Lp(a) in platelet stimulation would occur in the context of the arterial system, where the prominent atherosclerotic potential of Lp(a) could be considered a major contributing factor to thrombotic disease progression, and thus may increase the atherogenic property of Lp(a). The roles for Lp(a) in thrombotic processes in the milieu of fibrin-rich and platelet poor venous blood are contradictory and inconclusive. Initial studies on the topic reported that Lp(a) is an independent risk factor for VTE.[53-56] However, recent large population studies appear to show no causal relationship between Lp(a) and VTE.[57, 58]

Chapter 4 contains data showing that purified fibrin clots can form at an increased rate, and that the maximum turbidity of these clots containing 17K I4399M apo(a) is greater than that of clots formed in the absence of apo(a) or with 17K. It is generally accepted that more turbid clots (as measured by increased maximal absorbance) are associated with

thicker fibrin fibrils that result in an altered clot architecture [reviewed in ref. 46]. We report that clots with thicker fibrin fibers have prolonged lysis times. This contradicts the existing paradigm that thicker fibrin fibers result in a "loose" clot that is susceptible to lysis. However, assessment of clot structure and real-time lysis by microscopy has revealed that thinner fibers tend to lyse more rapidly, and this process may be obscured when assessing lysis by decreased turbidity at A_{405} nm; this may be attributable to the increased turbidity in the presence of thicker fibrin fibrils.[47] It can also be noted that alterations in fiber thickness are associated with variations in the ability to generate thrombin, thus limiting the rate of clot formation, a factor not relevant in the context of our assays using purified components. The presence of thicker fibrils seen in the polymorphic clots in Chapter 4 using SEM assessment of plasma and especially purified fibrin clots, suggests that the increased fiber thickness is due to the I4399M polymorphism exclusively in the purified fibrin system and due to the presence of Lp(a) in the context of plasma, with additional increase in fiber thickness seen in plasma clots from a rs3798220 homozygote. The results suggest distinct and additive effects of this polymorphism on fibrin clots, above the effects of Lp(a) alone.

It has been reported that the rs3798220 single nucleotide polymorphism is associated with increased plasma Lp(a) concentrations and increased levels of CHD.[59] This variant results in a missense mutation in the inactive protease-like domain of apo(a), resulting in an Ile → Met substitution at the 4399 amino acid position. This variant has also been correlated with smaller apo(a) isoforms and increased CAD severity.[60] The I4399M polymorphism has also been shown to be correlated with reduced clot permeability and increased clot lysis times *in vitro*.[61] However, there was a significant influence of patient

ethnicity in the effect of the I4399M polymorphism on clot permeability and lysis which complicated the study.[61]

Chapter 3 clearly identifies distinct properties of the I4399M polymorphism in assays using clots formed from purified components. In this context, 17K r-apo(a) containing the Met polymorphism (17K I4399M) had an increased ability to inhibit fibrinolysis compared to clots formed in the presence of wild-type 17K apo(a). Assessment of the ability of 17K and 17K I4399M to inhibit plasmin formation revealed no difference between their ability to inhibit this process. It is important to note that our plasmin generation assay looks directly at the cleavage of Glu-plasminogen to Glu-plasmin by tPA, and is not complicated by the positive feedback mechanism in which plasmin converts Glu-plasminogen to Lys-plasminogen. Apo(a) has been shown to have a direct inhibitory effect on Glu- to Lys-plasminogen conversion on the fibrin surface, thus enhancing the antifibrinolytic role of apo(a) in systems that generate functional plasmin.[45] Since we observed no distinct ability of the 17K I4399M variant to inhibit plasmin generation, but an increased ability to prolong fibrinolysis; it is possible that the conformational change in the protease domain to this SNP may have an effect on Glu- to Lys-plasminogen conversion. It has been proposed that the protease domain of apo(a) may influence the positive feedback mechanisms of plasminogen activation in the context of pericellular plasminogen activation[62], but a specific role for the PD in this process has not yet been investigated.

Apo(a) has also been shown to directly compete with plasminogen for the binding of fibrin and partially degraded fibrin.[41] The effect of the I4399M polymorphism was not assessed on these processes. It is possible, however, that investigation of the affinity of the

apo(a) I4399M variant for fibrin surfaces compared to wild-type apo(a) or plasminogen might offer mechanistic information about the effect of the I4399M polymorphism on clot formation and lysis. Indeed, molecular dynamic simulation analysis indicates that introduction of Met4399 disrupts the native conformation of the inactive protease domain of apo(a). The shift in structure from a buried Ile residue to a solvent exposed Met residue illuminates a novel surface epitope on apo(a) that requires characterization in the context of the pathophysiological processes of Lp(a). A lysine- independent binding interface between apo(a) and fibrinogen has been previously described to be outside of the KIV10 domain, likely in the region spanning KV-PD.[63, 64] It should be investigated if apo(a) variants lacking the protease domain or containing the I4399M variant of apo(a) can interact with fibrinogen in this way. The identification of a methionine sulfoxide (Met(O)) residue at the Met4399 offers added complexity to this idea. Oxidation of methionine residues is currently under intense investigation as a mechanistic switch in many physiological processes involved in thrombosis and vascular function [reviewed in ref. 64]. Since there was no peptide detected in our mass spectrometric study with a native methionine residue it can be inferred that the Met4399 residue is irreversibly oxidized. However, this remains to be verified experimentally. Methionine sulfoxide reductases have been shown to reduce Met(O) residues on plasma Apo A-I, thrombomodulin and von Willebrand factor thereby modulating their functions in different disease states.[65] Fibronectin has been shown to exhibit a minute conformational change upon oxidation, although the oxidation of a specific methionine in this context can completely abolish the ability of fibronectin to bind to its ligand collagen.[66]

The assessment of human plasma samples homozygous for the I4399M polymorphism revealed that this variant of apo(a) can cause increased fibrin fibril thickness, in agreement with results observed using clots formed from purified components in the absence or presence of apo(a) – additive to the effects observed by elevated plasma Lp(a) alone. This suggests independently that there may be a CVD risk for carriers of this polymorphism, regardless of their Lp(a) plasma levels.

The implications of the I4399M variant on thrombotic disorders are difficult to assess in the human population owing to the low frequency of this variant. In order to understand the degree of CVD risk by the I4399M variant of apo(a) in disease, a much larger data set containing a greater subpopulation of rs3798220 carriers needs to be assessed, accounting for Lp(a) plasma concentrations and controlled for isoform size. *In vitro* studies looking at the role of the I4399M variant of apo(a) in clot formation and lysis can be expanded to explore the possibility of altered Glu- to Lys-plasminogen conversion or fibrin binding affinity associated with this variant. Due to the ability of aspirin to have an effect on fibrin clot structure and lysis[67], studies should include a determination to the potential benefits of aspirin therapy in a more diverse group of individuals with the I4399M polymorphism, that are not seen in those with elevated Lp(a) who lack this polymorphism.

5.5 Closing remarks

This study outlines defined roles for oxidative modifications on apo(a) in the context of the prothrombotic and proatherogenic potential of Lp(a)/apo(a). Identification of Lp(a) as a causal risk factor for vascular diseases has sparked interest in the development for potential therapeutics that are directed at lowering plasma Lp(a) levels. The

development of therapeutic options that counteract the effects of the oxidative modifications on Lp(a) can potentially benefit those with elevated Lp(a). New therapeutic strategies aimed at lowering plasma Lp(a) levels through the use of PCSK9 monoclonal antibodies can lower Lp(a) levels by approximately 30%.[68] There may be a benefit to additional aspirin therapy in individuals carrying the rs3798220 SNP, as some individuals will have clinically elevated Lp(a), despite anti-PCSK9 intervention. With the potential for oxidative modification of Lp(a) causing distinct effects on atherothrombotic processes, antioxidant therapy could be used prophylactically, to help abrogate the effects caused by other risk factors that can generate ROS. There have been reports of eicosapentaenoic acid having preventative effects on the oxidation of apoB-containing lipoprotein particles, when used in combination with triglyceride lowering therapies.[69] Treatment options preventing the proinflammatory effects of Lp(a) on the vasculature should be explored in individuals with elevated plasma levels, especially when considering individuals who are prone to elevated levels of reactive oxygen species in the plasma, such as individuals who smoke or suffer from insulin resistance.

The development of potent Lp(a) lowering agents, such as antisense oligonucleotides (ASOs) targeting apo(a), will be key to improving the outcome of individuals with elevated Lp(a). Apo(a)-ASO therapy has been shown to lower Lp(a) levels by >80%, in a Phase 1 clinical trial.[70] Targeted anti-apo(a) ASO therapy will allow for the assessment of causal roles for Lp(a) in processes where is it difficult to determine if there is a causal role, as seen in the controversial studies on plasma Lp(a) levels and VTE. Ultimately, only by the robust lowering of plasma Lp(a), that has only been seen in the

early clinical trials of ASO therapy, can we begin to determine the true benefit of specific Lp(a) lowering on cardiovascular disease outcome.

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SAM

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> On Jul 15, 2016, at 7:45 PM, Corey Scipione <scipionc@uwindsor.ca> wrote:
>
> Hello Dr. Tsimikas,
>
> I hope all is going well.
> I am contacting you today as I am currently writing up my dissertation in the Koschinsky lab and I would really like to use some of the figures that were generated for the 2013 Leibundgut paper, "Determinants of Binding of Oxidized Phospholipids on Apolipoprotein (a) and Lipoprotein (a)."
>
> Most specifically, the mapping of the oxPL using recombinant human apo(a) constructs that I performed for Fig. 8 of the paper. The identification of the KIV10, rather than the KV, domain as the site of addition and the requirement of an intact KIV10 LBS for addition are critical components of the apo(a) oxPL story.
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HONOURS and AWARDS

CSATVB 2016 Travel Award for Young Investigators	2016
Ontario Student Opportunity Trust Fund Graduate Student Bursary	2016
Canadian Lipoprotein Conference Trainee Poster Presentation Award	2015
A.R. and E.G. Ferris Award	2015

ATVB 2014 Travel Awards for Young Investigators	2014
Ontario Graduate Scholarship (3 years)	2013-2015
Verdecchia Family - Health Science Scholarship	2013
University of Windsor Doctoral Tuition Scholarship	2011 – present

PUBLICATIONS

Journal Articles

1. **Scipione CA**, McAiney JT, Simard DJ, Gemin M, Gauld JW, Boffa MB, Hegele R, and Koschinsky ML. Characterization of the I4399M variant of apolipoprotein(a): Implications for altered prothrombic properties of lipoprotein(a). *Submitted to ATVB.*
2. Romagnuolo R, **Scipione CA**, Marcovina SM, Gemin M, Seidah NG, Boffa MB, Koschinsky ML. Roles of the low density lipoprotein receptor and related receptors in inhibition of lipoprotein(a) internalization by proprotein convertase subtilisin/kexin type 9. *Submitted to PLoS One.*
3. Oliveira C, Fournier C, Descamps V, Morel V, **Scipione CA**, Romagnuolo R, Koschinsky ML, Boullier A, Marcelo P, Domon J, Brochot E, Duverlie G, Francois C, Castelain S, Helle F. Apolipoprotein(a) inhibits Hepatitis C Virus entry. *Submitted to Hepatology. (HEP-16-1756)*
4. Van der Valk FM, Bekkering S, Kroon J, van den Bossche J, van Buul JD, Nederveen AJ, Verberne HJ, **Scipione CA**, Nieuwdorp M, Joosten LAB, Netea MG, Koschinsky ML, Witztum JL, Tsimikas S, Riksen N, Stroes ESG. (2016). Oxidized phospholipids on lipoprotein(a) elicit arterial wall inflammation and an inflammatory monocyte response in humans. *Circulation.* 2016 Aug 5. pii: CIRCULATIONAHA.116.020838.
5. Bi X, Song J, Gao J, Zhao J, Wang M, **Scipione CA**, Koschinsky ML, Wang ZV, Xu S, Fu G. (2016). Activation of liver X receptor attenuates lysophosphatidylcholine-induced IL-8 expression in endothelial cells via the NF-κB pathway and SUMOylation. *J Cell Mol Med*. 2016 Aug 4. doi: 10.1111/jcmm.12903.
6. **Scipione CA**, Sayegh SE, Romagnuolo R, Tsimikas S, Marcovina SM, Boffa MB, Koschinsky ML. Mechanistic Insights into Lipoprotein(a)-Induced Interleukin 8 Expression: A Role for Oxidized Phospholipid Modification of Apo(a). *J Lipid Res.* 2015 Dec;56(12):2273-85. doi: 10.1194/jlr.M060210.
7. Bouchareb R, Mahmut A, Nsaibia MJ, Boulanger MC, Dahou A, Lépine JL, Laflamme MH, Hadji F, Couture C, Trahan S, Pagé S, Bossé Y, Pibarot P, **Scipione CA**, Romagnuolo R, Koschinsky ML, Arseneault BJ, Marette A, Mathieu P. Autotaxin Derived from Lipoprotein(a) and Valve Interstitial Cells Promotes Inflammation and Mineralization of the Aortic Valve. *Circulation*. 2015 Aug 25;132(8):677-90. doi: 10.1161/CIRCULATIONAHA.115.016757.
8. Romagnuolo R, **Scipione CA**, Boffa MB, Marcovina SM, Seidah NG, Koschinsky ML. Lipoprotein(a) Catabolism is Regulated by Proprotein

Convertase Subtilisin/Kexin Type 9 Through the Low Density Lipoprotein Receptor. *J Biol Chem.* 2015 May 1;290(18):11649-62.

9. Komnenov D, **Scipione CA**, Bazzi ZE, Garabon JJW, Koschinsky ML, Boffa MB. Pro-inflammatory cytokines reduce human TAFI expression via tristetraprolin-mediated mRNA destabilisation and decreased binding of HuR. *Thromb Haemost*. 2015 Jul 28;114(2):337-49.
10. Leibundgut G, **Scipione C**, Yin H, Schneider M, Boffa MB, Green S, Dennis E, Witztum JL, Koschinsky ML, Tsimikas S. Determinants of binding of oxidized phospholipids on apolipoprotein (a) and lipoprotein (a). *J Lipid Res.* 2013 Oct;54(10):2815-30.
11. Cho T, Romagnuolo R, **Scipione C**, Boffa M B, Koschinsky M L. Apolipoprotein(a) Stimulates Nuclear Translocation of β-Catenin: A Novel Pathogenic Mechanism for Lp(a). *Mol. Biol. Cell.* 2013 Feb.
12. Leibundgut G, Arai K, Orsoni A, Yin H, **Scipione C**, Miller ER, Koschinsky ML, Chapman MJ, Witztum JL, Tsimikas S. Oxidized phospholipids are present on plasminogen, affect fibrinolysis, and increase following acute myocardial infarction. *J Am Coll Cardiol.* 2012 Apr 17;59(16):1426-37.
13. Lin JH, Garand M, Zagorac B, Schadinger SL, **Scipione C**, Koschinsky ML, Boffa MB. Identification of human thrombin-activatable fibrinolysis inhibitor in vascular and inflammatory cells. *Thromb Haemost*. 2011 Jun 6;105(6):999-1009.

Manuscripts in Preparation

1. **Scipione CA**, Romagnuolo R, Boffa MB, Ravandi A, Koschinsky ML. Characterization of the oxidized phospholipid modification of apolipoprotein(a) kringle KIV10: Insights into the site of oxPC addition.
2. Romagnuolo R, **Scipione CA**, Gemin M, Seidah NG, Koschinsky ML. Lipoprotein(a) catabolism is regulated by sortilin in hepatocytes.
3. Gemin M, Romagnuolo R, **Scipione CA**, Seidah NG, Koschinsky ML. Apolipoprotein(a) secretion is dependent on sortilin in hepatic cells

Conference Publications

1. Oliveira C, Fournier C, Descamps V, Morel V, **Scipione CA**, Koschinsky ML, Boullier A, Marcelo P, Domon JM, Brochot E, Duverlie G, Francois C, Castelain S, Helle F. Apolipoprotein(a) inhibits hepatitis C virus entry. *J. Clin. Virology*. 2016.(Vol. 82 S82-S83)
2. Koschinsky ML, Gemin M, **Scipione C**, Boffa MB, Seidah NG, Romagnuolo R. Evaluating the roles of PCSK9 and specific receptors in lipoprotein(a) catabolism. *J Clin Lipidol.* 2016.
3. McAiney JT, **Scipione CA**, Simard DJ, Gauld JW, Boffa MB, Koschinsky ML. Characterization of the I4399M Variant of Apolipoprotein(a): Implications for Altered Prothrombotic Properties of Lipoprotein(a). *Arterioscler Thromb Vasc Biol.* 2016.
4. **Scipione CA**, Igbokwe VC, Romagnuolo R, Koschinsky ML. Lipoprotein(a) Stimulates Pro-Inflammatory Phenotypes in Vascular Cells: Demonstration of a Role for the Lysine Binding Properties of Apolipoprotein(a). *Arterioscler Thromb Vasc Biol.* 2016

5. **Scipione CA**, Sayegh SE, Boffa MB, and Marlys L Koschinsky. Elucidation of the Mechanism of Apolipoprotein(a)/Lipoprotein(a)-Induced Interleukin 8 Gene Expression: Insight into Potential Receptors and Signaling Pathways and the Role of Oxidized Phospholipids. *Arterioscler Thromb Vasc Biol.* 2014;34:A2
6. Leibundgut G, **Scipione C**, Yin H, Schneider M, Boffa MB, Green S, Dennis EA, Witztum JL, Koschinsky ML, Tsimikas S. Oxidized Phospholipids on apoliproprotein(a) are Only Present on Human Lp(a): Implications for Understanding Lp(a) Atherogenics. *J Am Coll of Cardiol.* 2013 Mar 12 (Vol. 61, Issue 10 Page E2037)

CONFERENCES

1. Gemin M, Romagnuolo R, McIntyre AD, **Scipione CA**, Seidah NG, Boffa MB, Hegele RA, Koschinsky ML. Sortilin Promotes Secretion of Apolipoprotein(a) by Hepatic Cells. American Heart Association Scientific Session 2016, New Orleans, United States. (2016) Poster Presentation.
2. Scipione CA, Smith SA, Romagnuolo R, Boffa MB, Ravandi A, Koschinsky ML. Identification of the site of oxidized phospholipid modification on apolipoprotein(a) kringle KIV10. 41st Annual Canadian Lipoprotein Conference 2016, St. John's, Canada. (2016) Oral Presentation.
3. Bekkering S, Van Der Valk F, Kroon J, Yeang C, Van Den Bossche J, Van Buul J, **Scipione C**, Nieuwdorp M, Joosten L, Netea M, Koschinsky M, Witztum J, Tsimikas S, Riksen N, Stroes E. Oxidized phospholipids on lipoprotein(a) induce epigenetic reprogramming and an increased pro-atherogenic response in human monocytes. International Lp(a) Satellite Meeting, Innsbruck, Austria. (2016) Oral Presentation.
4. Van Der Valk F, Bekkering S, Verweij S, Kroon J, Van Den Bossche J, Van Buul J, Nederveen A, Verberne H, **Scipione C**, Joosten L, Netea M, Koschinsky M, Witztum J, Tsimikas S, Riksen N, Stroes E. Oxidized phospholipids on lipoprotein(a) elicit arterial wall inflammation and an inflammatory monocyte response in humans. International Lp(a) Satellite Meeting, Innsbruck, Austria. (2016) Oral Presentation.
5. Koschinsky ML, Gemin M, **Scipione C**, Boffa MB, Seidah NG, Romagnuolo R. Evaluating the roles of PCSK9 and specific receptors in lipoprotein(a) catabolism. National Lipid Association Scientific Sessions 2016, New Orleans, United States. (2016) Poster and Oral Presentation **Top ranked abstract.*
6. McAiney JT, **Scipione CA**, Simard DJ, Gauld JW, Boffa MB, Koschinsky ML. Characterization of the I4399M Variant of Apolipoprotein(a): Implications for Altered Prothrombotic Properties of Lipoprotein(a). Arteriosclerosis, Thrombosis, and Vascular Biology | Peripheral Vascular Disease 2016 Scientific Sessions, Nashville, United States. (2016) Poster Presentation.
7. **Scipione CA**. Igbokwe VC, Romagnuolo R, Koschinsky ML. Lipoprotein(a) Stimulates Pro-Inflammatory Phenotypes in Vascular Cells: Demonstration of a Role for the Lysine Binding Properties of Apolipoprotein(a). Arteriosclerosis, Thrombosis, and Vascular Biology | Peripheral Vascular Disease 2016 Scientific Sessions, Nashville, United States. (2016) Poster Presentation.

8. **Scipione CA**, Koschinsky ML. Lp(a)/apo(a) promotes pro-inflammatory responses in vascular cells: A role for the oxPL moiety on apo(a). 40th Annual Canadian Lipoprotein Conference 2015, Toronto, Canada. (2015) Poster Presentation.
9. Romagnuolo R, Gemin M, **Scipione CA**, Seidah NG, Koschinsky ML. Evaluating the role of potential receptors in Lp(a) Catabolism. 40th Annual Canadian Lipoprotein Conference, Toronto, Canada. (2015) Poster Presentation.
10. Bouchareb R, Mahmut A, Nsaibia M, Boulanger M, Dahou A, Lépine J, Laflamme M, Fayez H, Couture C, Trahan S, Pagé S, Bossé Y, Pibarot P, **Scipione CA**, Koschinsky ML, Arsenault B, Marette A, Mathieu P. Autotaxin enhances aortic valve mineralization through a NF-κB/IL-6/BMP-2 dependent inflammatory pathway. 2015 Canadian Cardiovascular Congress, Toronto, Canada. (2015) Oral Presentation.
11. **Scipione CA**, Sayegh SE, Boffa MB, Koschinsky ML. Elucidation of the Mechanism of Apolipoprotein(a)/Lipoprotein(a) Induced Interleukin 8 Gene Expression: Insight into Potential Receptors and Signaling Pathways and the Role of Oxidized Phospholipids. American Heart Association Scientific Sessions 2014, Chicago, United States (2014) **Invited poster presentation.*
12. **Scipione CA**, Sayegh SE, Boffa MB, Koschinsky ML. Elucidation of the Mechanism of Apolipoprotein(a)/Lipoprotein(a) Induced Interleukin 8 Gene Expression: Insight into Potential Receptors and Signaling Pathways and the Role of Oxidized Phospholipids. Arteriosclerosis, Thrombosis, and Vascular Biology 2014 Scientific Sessions, Toronto, Canada. (2014) Oral Presentation. **Top ranked abstract*
13. **Scipione CA**, McAiney JT, Simard DJ, Boffa MB, and Koschinsky ML. Characterization of the I4399M variant of apolipoprotein(a): implications in structure and pro-thrombotic potential. 39th Annual Canadian Lipoprotein Conference 2014, Saskatoon, Canada. (2014) Oral Presentation.
14. Leibundgut G, **Scipione CA**, Yin H, Schneider M, Boffa MB, Green S, Dennis EA, Witztum JL, Koschinsky ML, Tsimikas ML. Oxidized Phospholipids on apoliproprotein(a) are Only Present on Human Lp(a): Implications for Understanding Lp(a) Atherogenics. American College of Cardiology Annual Session and Expo. 2013, San Francisco, United States. (2013) Poster Presentation.
15. **Scipione CA**, Boffa MB, Koschinsky ML. Elucidation of the mechanism of apo(a)/Lp(a)-induced IL-8 gene expression: Insight into potential receptors and signaling pathways. 38th Annual Canadian Lipoprotein Conference 2013, Mont Tremblant, Canada. (2013) Oral Presentation.
16. **Scipione CA**, Boffa MB, Koschinsky ML. The Effect of Apo(a) and Plasminogen on Vascular Cell Phenotype: A Role for Oxidized Phospholipid Modification?. Windsor Celebrates the International Year of Chemistry, Windsor, Canada (2011) Public Poster Presentation.
17. **Scipione CA**, Fochesato L, Kim DY, Tanha J, Jarrell H, Pandey S. Identification and functional characterization of pharmacophores capable of inhibiting neuronal cell death following stroke by blocking the pro-apoptotic protein Bax. Southwestern Ontario Undergraduate Chemistry Conference, London, Canada (2010) Oral Presentation.