Characterization of the I4399M Variant of Apolipoprotein(a): Implications for Altered Prothrombotic Properties of Lipoprotein(a)

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Characterization of the I4399M Variant of Apolipoprotein(a):
Implications for Altered Prothrombotic Properties of Lipoprotein(a)

By

Jackson Thomas McAiney

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

Windsor, Ontario, Canada

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Declaration of Co-Authorship

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

The experiments presented herein were the work of the author under the guidance and direction of Dr. Marlys Koschinsky and Dr. Michael Boffa. Figures 1.2, 1.3, and 1.4 were designed by and used with the permission of Dr. Marlys Koschinsky and Dr. Michael Boffa.

The recombinant-apo(a) stable lines were provided by Dr. Marlys Koschinsky and generated with the assistance of Corey Scipione, Ph. D. candidate at the University of Windsor, as outlined in Chapter 2.

Confocal experiments were performed with the assistance of the Zainab Bazzi, Ph. D. candidate at the University of Windsor. Fiber number measurements of patient sample confocal micrographs were conducted through ImageJ, using a macro designed by Dr. Fraser Macrae from the University of Leeds. The scanning electron microscopy experiments were performed with the assistance of Corey Scipione and Sharon Lackey, Facility Technician at ESEM Facility at the GLIER Institute at the University of Windsor.

Pulse-chase assay was optimized and performed with assistance of Matt Gemin, Masters candidate at the University of Windsor, as outlined in Chapter 2.

Molecular dynamic simulations were performed by Dr. James Gauld, Associate Professor at the University of Windsor, and his Master’s student, Dan Simard. Mass spectrometry spectra were generated by Corey Scipione with the assistance of Janeen Auld, Mass Spectroscopy Instrument Specialist at the University of Windsor.
I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.
Abstract

Elevated plasma levels of lipoprotein(a) (Lp(a)) are a causal risk factor for CHD. Lp(a) closely resembles LDL, but contains an additional glycoprotein apolipoprotein(a) (apo(a)) that is structurally homologous to the fibrinolytic proenzyme plasminogen. This has led to speculation that Lp(a) can oppose the fibrinolytic functions of plasminogen. A single nucleotide polymorphism (SNP) in the \textit{LPA} gene encoding apo(a) results in an Ile to Met substitution at position 4399 in the protease-like domain. In population studies, this variant has been correlated with elevated plasma Lp(a) levels and with higher CHD risk. We undertook a functional characterization of the effect of the I4399M substitution in apo(a). Molecular dynamics simulations of wild-type (wt) apo(a) and the Met variant revealed a shift from a buried (Ile) to slightly exposed (Met) environment. Subsequent MALDI-TOF mass spectrometry analysis demonstrated the presence of a methionine sulfoxide moiety at this position in the Met variant. When 17-kringle recombinant forms of apo(a) were included in a plasma clot lysis assay, both the wt apo(a) and Met variant inhibited lysis, but the Met variant had a 50% greater effect. However, the Met variant was equally as efficient as wt apo(a) in inhibiting plasminogen activation on a fibrin surface. The Met variant was also able to significantly shorten coagulation time and result in greater turbidity for clots made from either purified fibrin or lipoprotein-deficient plasma compared to wild-type. In agreement with these findings, SEM and confocal microscopy of clots made from fibrin and citrated plasma showed that compared to wt apo(a), the Met variant resulted in significant alteration of the fibrin network. Together, our data suggest that the Met4399 variant differs structurally from wt apo(a), which may underlie key differences related to its effects on fibrin clot architecture and fibrinolysis.
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First and foremost, I would like to most sincerely thank my supervisor Dr. Marlys Koschinsky for her patience, her support, her feedback, and mentorship during the challenges and achievements of my graduate studies. The last two years have been a very rewarding and incredible learning experience and I am very grateful for the opportunity to be able to work in your research laboratory.

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### Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>10-P</td>
<td>truncated recombinant apo(a) variant containing KIV&lt;sub&gt;10&lt;/sub&gt;, KV, and protease domain</td>
</tr>
<tr>
<td>10-P I4399M</td>
<td>10-P r-apo(a) variant possessing Ile to Met mutation</td>
</tr>
<tr>
<td>17K</td>
<td>wild type, 17 kringle-containing recombinant form of apo(a)</td>
</tr>
<tr>
<td>17K I4399M</td>
<td>17K recombinant apo(a) variant with Ile to Met mutation</td>
</tr>
<tr>
<td>ε-ACA</td>
<td>epsilon-aminoaprotic acid</td>
</tr>
<tr>
<td>apo(a)</td>
<td>apolipoprotein(a)</td>
</tr>
<tr>
<td>apo B-100</td>
<td>apolipoprotein B-100</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cells</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDP</td>
<td>fibrin degradation products</td>
</tr>
<tr>
<td>Fg</td>
<td>fibrinogen</td>
</tr>
<tr>
<td>FPA</td>
<td>fibrinogen fibrinopeptide A</td>
</tr>
<tr>
<td>FXR</td>
<td>farnesoid X receptor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>----------</td>
<td>-----------------------------------------------</td>
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<tr>
<td>FPB</td>
<td>fibrinogen fibrinopeptide B</td>
</tr>
<tr>
<td>HBS</td>
<td>HEPES buffered saline</td>
</tr>
<tr>
<td>HNF4α</td>
<td>hepatocyte nuclear factor 4α</td>
</tr>
<tr>
<td>HEK293</td>
<td>human embryonic kidney cells</td>
</tr>
<tr>
<td>HepG2</td>
<td>human hepatoma cells</td>
</tr>
<tr>
<td>K</td>
<td>kringle domain</td>
</tr>
<tr>
<td>Ks</td>
<td>Darcy constant (units: cm$^2$)</td>
</tr>
<tr>
<td>IIa</td>
<td>activated thrombin</td>
</tr>
<tr>
<td>LBS</td>
<td>lysine binding site</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>low-density lipoprotein receptor</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>lipoprotein(a)</td>
</tr>
<tr>
<td>LPA</td>
<td>gene encoding apo(a)</td>
</tr>
<tr>
<td>LPDP</td>
<td>lipoprotein-deficient plasma</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential media</td>
</tr>
<tr>
<td>Opti-MEM</td>
<td>serum free, conditioned media</td>
</tr>
<tr>
<td>OxPLs</td>
<td>oxidized phospholipids</td>
</tr>
<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>Pg</td>
<td>Plasminogen</td>
</tr>
<tr>
<td>r-apo(a)</td>
<td>recombinant apo(a)</td>
</tr>
<tr>
<td>RMSD</td>
<td>root mean squared deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cells</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>TFPI</td>
<td>tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue-type plasminogen activator</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular-cell adhesion molecule-1</td>
</tr>
<tr>
<td>VTE</td>
<td>venous thromboembolism</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood cells</td>
</tr>
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</table>
Chapter 1

Introduction

1.1 Relationship Between Atherosclerosis and Thrombosis in Atherosclerotic Plaque Formation

Atherosclerosis a progressive, inflammatory disease affecting the large arteries of the body and is the primary underlying cause of cardiovascular diseases (CVD), including coronary artery disease (CAD), ischemic stroke, and heart failure (1-4). According to the World Health Organization, over 17 million people die each year from CVDs and 80% of all CVD deaths are due to myocardial infarctions and strokes (5). Atherosclerosis is characterized as the thickening of the arterial intima due to the build up of lipids and fibrous materials, resulting in the formation of atherosclerotic lesions within vasculature (6).

Atherogenesis begins at the site of endothelial damage within an artery, caused by factors such as oxidative stress, environmental irritants like tobacco, bacterial or viral infection, and turbulent blood flow or shear stress on the arterial wall (7). Atherogenesis is stimulated by the invasion of lipid containing molecules, such as low-density lipoprotein (LDL), into the intimal layer (8). Movement of these lipid-containing molecules across the endothelial cell layer triggers cellular activation, resulting in the production of cell surface adhesion molecules on the ECs, including vascular-cell adhesion molecule-1 (VCAM-1) (6). These receptors promote the recruitment of lymphocytes and monocytes to endothelial cells at the inflammatory site. Recruitment of these WBCs produces chemokines and growth factors within the intima, allowing the
monocytes to transmigrate into the intimal space and differentiate into inflammatory macrophages (8). The accumulated LDL in the intima is exposed to oxidative and enzymatic modification; subsequent phagocytosis of these molecules by the macrophages results in the formation of “foam cells” (6, 9). Differentiation into foam cells results in ER stress and apoptosis of the macrophage, resulting in cytokine release and increased recruitment of monocytes to the lesion, increasing the size of the plaque (10). As the lesion grows in size, circulating platelets deposit at the site of injury and release platelet-derived growth factor (PDGF) and other mitogenic factors that penetrate the vascular wall (11). These factors cause smooth muscle cells (SMC) to proliferate and migrate into the intima, producing an extracellular matrix (ECM) that encloses the necrotic core of lesion, consisting of lipid components and necrotic debris (6). The smooth muscle cells and macrophages that constitute the fibrous connective tissue enclosing the necrotic core is referred to as the “fibrous cap” (12). The lesions will continue to increase in size and may rupture, releasing their contents into the lumen, causing arterial blockage than can lead to myocardial infarctions/stroke (13). Enzymes and cytokines, produced by immune cells within the core, weaken the fibrous cap surrounding the core and render the plaque vulnerable to rupture. Upon rupture, tissue factor and platelet-adhesion factor within the intima come into contact with the flowing blood in the arterial lumen, which results in immediate thrombosis formation (8).

1.2 Fibrin Clot Formation/Lysis and Plasminogen Structure and Function

Fibrinogen is a ~340kDa zymogen that plays a role in blood clotting, fibrinolysis, inflammatory response, and wound healing. Fibrinogen is composed of three homologous peptide chains (Aα, Bβ, and γ) that are encoded in a cluster in a region of approximately
50 kb on chromosome 4 and is synthesized in hepatocytes to form a hexamer composed of two sets of the three polypeptides (14). In response to injury or vascular disease, fibrinogen is cleaved by the active enzyme thrombin (Ila), exposing binding sites to form insoluble fibrin clots (15). When the fibrinogen is cleaved, fibrin fibers begin to assemble into half-staggered double-stranded structures called protofibrils and will continue to grow by associating laterally with other protofibrils (16, 17), forming a network of fibrin fibers. Multiple factors affect the strength and stability of fibrin clots and various arterial and venous diseases have been shown to alter the fibrin network, leading to altered fibrinolysis and clot stability (18, 19).

There is an important balance between clot formation in the body and subsequent lysis to prevent blockage of vasculature. The protein responsible for breakdown of fibrin clots is called plasminogen. Plasminogen (Pg) is a single-chained circulating zymogen that becomes cleaved to form the activated serine protease plasmin by the physiological activators: urokinase-type Pg activator (uPA) or tissue-type Pg activator (tPA) (20). While uPA is immobilized on the surface of endothelial cells, the circulating tPA is synthesized in endothelial cells of the vascular wall and is secreted as a single chain polypeptide, released in response to numerous stimuli (21). One such stimuli is thrombin, the serine protease responsible for the conversion of soluble fibrinogen to insoluble fibrin to form a clot (22). This conversion by thrombin causes a conformational change in fibrin’s structure, exposing tPA and plasminogen binding sites (23, 24). A tertiary complex is formed between tPA, plasminogen, and fibrin at these locations, resulting in efficient plasminogen activation (25). Similarly, immobilization of plasminogen on cell surfaces allows for more efficient activation by tPA or uPA (26). The primary in vivo
function of activated plasmin is to degrade the fibrin networks into individual components called fibrin degradation products (FDP).

Plasminogen is composed of 791 amino acids, arranged into a N-terminal pre-activation peptide, 5 tandem kringle domains (KI-KV), and an active protease domain containing the catalytic triad His$^{603}$, Asp$^{646}$, and Ser$^{741}$ (20). The kringle domains are composed of a triple looped structure, held by 3 disulfide bonds, and are found in many other proteins, particularly those associated with fibrinolysis and blood coagulation (27, 28). Kringles I, IV and V in plasminogen are characterized as possessing lysine-binding sites (LBS), which are clefts or pockets within the larger domain that allow for protein-protein interactions. The LBS possesses cationic and anionic residues at opposite ends deep within the cleft, with a region of highly conserved aromatic residues that creates a hydrophobic environment (29). These cationic and anionic residues within the cleft form ion pair interactions with the carboxyl and ammonium groups of the ω-amino acid, such as lysine (30). Plasminogen is cleaved to form the active plasmin by a cleavage of the Arg$^{561}$-Val$^{562}$ peptide bond in the C-terminal domain by plasminogen activators. Following cleavage, the heavy chain of plasminogen, containing the kringle domains, and the light chain, containing the catalytic domain, through two disulfide bonds (31). The activated plasminogen then subsequently degrades the fibrin clots into fibrin degradation products (32).

There are two main forms of plasminogen: the most abundant type is called Glu$^1$-Pg, which is resistant to activation due to its tight, spiraled structure that blocks tPA from reaching the activation cleavage site (33, 34). The second type is the result of plasmin cleavage of the Lys$^{77}$-Lys$^{78}$ peptide bond in native Glu$^1$-Pg, releasing the N-terminal activation peptide and forms Lys$^{78}$-Pg. Release of the N-terminal activation peptide
results in a conformational change that exposes the activation cleavage site, making the Lys²⁷⁸-Pg a better substrate for tPA and accelerating plasminogen activation. Active plasmin can also cleave the Arg²⁷⁵-Ile²⁷⁶ bond of the single chain tPA, resulting in a double chained version, which has increased plasminogen activation ability (35). Likewise, initial cleavage of fibrin by plasmin reveals C-terminal lysine residues that act like high affinity binding sites for plasminogen and tPA (36). The LBS in the kringle domains of plasminogen can interact with these exposed lysine residues, meaning this modified fibrin is a better cofactor for tPA-mediated plasminogen activation.

1.3 Fibrinogen Structure and Conversion to Fibrin

A fibrinogen molecule is an elongated 45nm structure consisting of two outer D domains connected to a central E domain connected by a coiled-coil segment (37). It is composed of two sets of three polypeptide chains, termed Aα, Bβ, and γ, which are joined together at the N-terminal E domain with 5 symmetrical disulfide bridges, while non-symmetrical disulfide bridges form a ring structure in this area (38-40). The Aα, Bβ, and γ chains are 610, 461, and 411 residues long, respectively, with the γ chain possessing a minor and major form arising from alternative splicing of the mRNA transcript (37). Each Aα subunit contains a N-terminal fibrinopeptide A (FPA) that is cleaved by the enzyme thrombin (Ila), exposing a polymerization site, termed Eₐ, that results in a structure called des-AA fibrin (Fig 1.1) (41-43). Each Eₐ site combines with a complementary-binding pocket on neighboring fibrinogen molecules in the D domain, termed Dₐ, located on the γ chain between the 337 and 379 residues (44, 45). The interaction of Eₐ and Dₐ results in the alignment of fibrin molecules in a staggered, overlapping arrangement, forming a double-stranded, twisted structure called a fibrin protofibril (16, 17, 46). To strengthen
this structure, these protofibrils undergo lateral associations, allowing the creation of multi-stranded fibers (47, 48). These protofibrils can form two different types of junctions with other fibrils; 1) laterally with another fibril to form a four-stranded fibril, called a “bilateral” junction, and 2) “equilateral” junctions, formed between the three fibrin molecules, giving rise to three double-stranded fibrils (49). Equilateral junctions occur more frequently when FPA cleavage is slower, resulting in more branches and a tighter fibrin matrix (50). Similarly, another fibrinopeptide, fibrinopeptide B (FPB), is slower than the release of FPA and results in des-BB fibrin (41-43). Cleavage by thrombin exposes an independent polymerization site, termed E_B, that interacts with a complementary D_b site in the β-chain segment of the D domain (51). Polymerization of des-BB fibrin results in the same type of protofibril structure as with des-AA fibrin (50), but the clot strength is much weaker than that of des-AA fibrin (51). The resulting clot architecture plays an important role in its stability and lysis potential; any abnormalities can lead to disrupted thrombolysis (52, 53). Two of the most common measures of fibrin clot structure are fibrin diameter and pore sizes within the clot network. Dense clots are characterized as possessing smaller diameter fibers with increased stiffness, as well as small pores, leading to increased clot lysis times (54). It has been shown that small diameter fibers are dissolved at faster rates than thicker fibers, suggesting that the rate of lysis is largely a function of pore size (53).
Figure 1.1. Schematic of fibrin clot formation from the fibrinogen zymogen. Fibrinogen molecules are cleaved by thrombin (IIa), resulting in the release of fibrinopeptides FPA and FPB, forming des-AA or des-BB fibrin monomers. Fibrin monomers align in a staggered, over-lapping pattern and cross-link to form clusters of monomers called protofibrils. The newly formed protofibrils form junctions to other fibrils and the collection of these cross-linked fibrils results in an insoluble fibrin clot. [Modified from Mega, JL (55)]
1.4 Lipoprotein(a): A Potential Link Between the Processes of Atherosclerosis and Thrombosis

Lipoprotein(a) [Lp(a)] was discovered by Kåre Berg in 1963 (56). It is a unique lipoprotein that circulates in human plasma. It is similar to LDL in terms of lipid content, containing about 45% cholesterol, and the presence of apolipoprotein B-100 (apoB-100), but differs by the presence of a unique glycoprotein moiety called apolipoprotein(a) [apo(a)] (57). Apo(a) is covalently attached to the apoB-100 component of LDL by a single disulfide bond (Fig 1.2). Apo(a) differs from other apolipoproteins in that it contains extensive carbohydrate modification, and is hydrophilic, lacking amphipathic alpha helices typically found in other apolipoproteins (58, 59). The heavy glycosylation is approximately 30-35% by weight and consists of N- and O-linked glycans attached to free serine, threonine or asparagine residues in the interkringle regions of apo(a). Cloning of apo(a) from human liver cDNA library revealed a striking homology between the amino acid sequence of apo(a) and the human serine protease zymogen plasminogen (60). This is an interesting result as plasminogen is responsible for the degradation of insoluble fibrin clots, indicating Lp(a)/apo(a) may exert its pathogenic effects by interfering with plasminogen’s normal functions.

Apo(a) is structurally homologous to plasminogen in that it possesses domains resembling the plasminogen kringle IV, kringle V, and protease domain, the last of which is inactive in apo(a) due to a series of mutations (Fig 1.3). Apo(a) possesses 10 subclasses of plasminogen-like kringle IV domains, denoted KIV₁ through KIV₁₀, that differ from each other by changes in amino acid sequence (60). These domains in apo(a) have been found to exhibit between 75-84% amino acid identity with the kringle IV domain in plasminogen. Similar to plasminogen, the kringle domains in apo(a) exhibit the
characteristic tri-looped structure, composed of 80 amino acids held together by three conserved disulphide bonds (61). The kringle domains are joined by a sequence of approximately 30 amino acids, referred to as the interkringle region. This region may provide flexibility to the quaternary structure of apo(a) (62). The plasminogen-like kringle KIV₁, and KIV₃-KIV₁₀ are each present in one copy in apo(a) (63). However, the KIV₂ domain is present in a varying number of identical copies, ranging from 3 to >40 repeats (64, 65). The variable number of KIV₂ repeats results in Lp(a) isoform size heterogeneity, a result of allelic variation in the number of repeated sequences encoding the KIV₂ domain in the LPA gene (66). Several of the other plasminogen-like kringle IV domains in apo(a) are also of interest in terms of their properties. KIV₅-KIV₈ domains each possess weak lysine binding sites (LBS); the LBS in KIV₇ and KIV₈ are thought to mediate the initial non-covalent interactions between apo(a) and the N-terminal lysine resides of apoB-100 (Lys⁶₈⁰ and Lys⁶₉₀) prior to covalent Lp(a) assembly (67-69) (Figure 1.3). The plasminogen-like KIV₉ domain in apo(a) contains a free cysteine which forms a covalent linkage with the apoB-100 of LDL. However, the identity of the extracellular oxidase enzyme that has been postulated to catalyze this reaction has yet to be determined (57, 70). The plasminogen-like KIV₁₀ domain possesses a stronger LBS comparable to the one in plasminogen KIV and is thought to mediate interactions with a key biological substrates including fibrin (71, 72), which has been shown to influence plasminogen activation on the fibrin surface (71). The plasminogen-like protease domain in apo(a) is catalytically inactive due to an arginine to serine substitution in apo(a) that removes the cleavage site that is recognized by plasminogen activators (73).
Figure 1.2. Structure of Lipoprotein(a). Lp(a) is composed of an LDL-like moiety attached to the heavily glycosylated, unique protein apolipoprotein(a) through a single disulfide bond with apoB-100. There are also lysine dependent, non-covalent interactions between the N-terminal lysine residues of apoB-100 of LDL and the lysine binding sites (LBS) of the KIV7-KIV8 domains of apolipoprotein(a) that play a role in Lp(a) particle assembly. Analysis of a liver cDNA library by McLean and colleagues (60) revealed a striking homology between apo(a) and the serine protease zymogen, plasminogen. [Adapted from Koschinsky ML and Marcovina SM (74)]
Figure 1.3. Schematic of the structural homology between the domains of plasminogen and apolipoprotein(a). Plasminogen consists of an amino-terminal tail domain (T), five kringle domains (numbered sequentially from I through V) and a serine protease domain (P). Apo(a) possesses 10 copies of sequences that resembles the plasminogen KIV domain, sharing between 75-84% amino acid identity, followed by a single copy of sequences resembling the plasminogen KV domain and protease domain. Each KIV domain is present in a single copy, except for KIV_{2}, which can be repeated between 3 and >40 times, accounting for the size heterogeneity of Lp(a). KIV_{5}-KIV_{8} containing weak lysine binding sites (LBS), and the LBS on KIV_{7} and KIV_{8} mediate the interaction with apoB-100 on LDL prior to Lp(a) assembly. KIV_{9} possesses a free cysteine residue that is involved in the covalent linkage between apo(a) and apoB-100 on LDL. KIV_{10} possesses a strong LBS and has been shown to interact with biological substrates, such as fibrin. Unlike in plasminogen, the protease domain in apo(a) contains critical amino acid substitutions that renders the catalytic domain inactive. [Adapted from Koschinsky ML (59)]
The inactive protease domain in apo(a) possesses a nine amino acid deletion within a highly conserved region in serine proteases, indicating that even in the absence of Arg→Ser substitution, the domain would still be inactive (73).

1.5 Evolution and Characteristics of LPA Gene

Lp(a) is present in Old World monkeys, apes, and humans. Previous comparison of the untranslated regions of human apo(a) and plasminogen cDNA sequences reveal that the two genes arose from a duplication event about 40 million years ago, roughly around the time of the divergence of the Old World and New World monkeys (60). Therefore, it was proposed that the apo(a) gene arose from a duplication of the plasminogen gene, followed by exon deletions, multiplication, and single base substitutions, such as the ones present in the plasminogen-like protease domain. Interestingly, however, an Lp(a)-like particle was found in European hedgehogs (75), which suggests either that the LPA gene arose before the major divergence of the Old World apes, or that the apo(a) in hedgehogs arose from an independent duplication of the plasminogen gene that is distinct from that which gave rise to apo(a) in Old World monkeys and humans; the latter is supported by the work by Lawn et al. (76).

The human gene encoding apo(a) is located on chromosome 6, within the cytogenetic band 6q26 (77). As stated above, alleles of LPA contain variable numbers of identical sequences encoding the apo(a) KIV<sub>2</sub> domain. This gives rise to apo(a) of different isoform sizes, which forms the basis of Lp(a) size heterogeneity in the population, and ranging from a minimum of 3 KIV<sub>2</sub> repeats to greater than 40 (64, 65). Null alleles have also been identified in which one allele with an excessively large amount number of KIV<sub>2</sub> repeats does not produce a secreted protein (78) or the allele
possesses a mutation that alters a splicing site, preventing normal mRNA splicing and resulting in a truncated, defective polypeptide that becomes degraded (79).

1.6 Plasma Lipoprotein(a) Levels - Relationship With Apo(a) Isoform Size

Plasma Lp(a) levels in the human population vary 1000-fold, ranging from <0.1 to >100 mg/dL (80). Work by Rader et al. showed through radiolabeling of Lp(a) that isoform size does not influence the rate of Lp(a) catabolism (80). Therefore, the inverse association of isoform size and Lp(a) concentrations is due to differences in Lp(a) production rate. Unlike LDL, plasma Lp(a) levels are primarily genetically determined (81). In Caucasians, the LPA gene accounts for ~90% of the observed variation in plasma Lp(a) levels (82), while only ~78% of the variation in the levels in African Americans can be explained by the gene itself (83). A general inverse correlation exists in the human population between apo(a) isoform size and Lp(a) concentrations, with smaller isoforms correlated with higher Lp(a) protein levels (84, 85). This has been suggested to arise due to less efficient secretion of larger apo(a) isoforms from hepatocytes, a result of longer retention times in the endoplasmic reticulum and increased intracellular degradation (86). Alternatively, mRNA transcript stability, translational efficiency and efficiency of Lp(a) assembly may also contribute to the inverse correlation, although each individual contribution to this process is still undetermined (59). Studies have shown that in Caucasians, elevated Lp(a) levels were found to be associated with small isoforms in over 80% of subjects; while African Americans, elevated Lp(a) levels are distributed over a broad range of isoform sizes (87, 88). As well, African Americans were shown to have higher Lp(a) levels than Caucasians who posses the same apo(a) isoform size (88). Smaller apo(a) isoforms (<22 KIV domains) have been linked by association to increased
instances of atherosclerosis and CVD due to elevated Lp(a) levels in these individuals (87, 89, 90).

1.7 Transcriptional Regulation of $LPA$

In addition to size of the $LPA$ gene, $cis$-acting elements may influence transcription of apo(a), thereby modulating Lp(a) levels in plasma (91). For example, work done by Chennamsetty et al. has shown that farnesoid X receptor (FXR), which is a bile acid-activated receptor that is expressed in the liver, strongly controls $LPA$ by binding to a negative control element located at the -826-bp region of the gene (92). It was also shown to interfere with hepatocyte nuclear factor 4α (HNF4α) mediated activation of $LPA$ transcription.

Owing to the strong regulation of Lp(a) plasma concentrations by the $LPA$ gene itself, levels of this lipoprotein are relatively resistant to conventional lipid-lowering approaches, such as diet, exercise, and pharmaceutical interventions, including statin therapy (93). Niacin, or vitamin B₃, was able to reduce Lp(a) levels up to 35% after the 26th week of treatment in a clinical trial (94), but recent studies have shown that niacin therapy is also associated with moderately increased risk for developing diabetes (95) and a clinical study showed it trended towards increased development of new-onset diabetes (96). Recent therapeutic strategies have focused on inhibitors of proprotein convertase subtilisin/kexin type 9 (PCSK9), which has been shown to decrease plasma Lp(a) by up to 30% (97) potentially through the up-regulation of the LDL receptor (LDLR) (98).
1.8 Identification of Two SNPs that Contribute Significantly to Plasma Lp(a) Levels and Risk

Three consecutive case-control studies were conducted of men and women who had undergone coronary angiography and were screened for 12,077 single nucleotide polymorphisms (SNPs) in over 7,000 genes for association with severe CAD (99). After screening all three study groups, an SNP was discovered in the \textit{LPA} gene encoding apolipoprotein(a) that was associated with severe CAD. This SNP, denoted rs3798220, causes a missense mutation in the inactive protease domain of apo(a), resulting in an Ile to Met substitution at the 4399 residue of apo(a). Carriers of this SNP accounted for 5.2% of the sample population and had increased adjusted odds ratio for serve CAD compared to non-carriers and carriers of the I4399M allele had significantly higher levels of Lp(a) compared to the non-carriers. Further analysis of this mutation revealed that carriers of the SNP had smaller apolipoprotein(a) isoform sizes and significantly higher levels of OxPLs/apoB levels compared to non-carriers (100). However, the mechanism or cause of its advanced pathogenic properties is still unknown. Another SNP in \textit{LPA} (rs10455872) has been identified in intron 25, resulting in an A\textrightarrow{}G substitution, which was also found to be associated with elevated Lp(a) levels and increased CAD risk (101). Recent meta-analysis of other SNPs outside the \textit{LPA} locus showed that no candidate genes have an effect on Lp(a) levels (102). Interestingly the effects of the rs3798220 SNP were shown to be present in carriers of European background, it has been shown that carriers of East and Southeast Asian background had no association with smaller isoforms or elevated Lp(a) concentrations and the variant was not found in African participates (103). While the association of these two SNPs with high Lp(a) levels has been well documented, it has
been postulated that the increased risk of CAD in carriers of these SNPs is abolished when adjusting for Lp(a) levels (104).

1.9 Pathogenicity of Lipoprotein(a)

Owing to key genetic studies performed within the last decade, Lp(a) is now conclusively classified as an independent and causal risk factor for cardiovascular disease (87, 105-108). High plasma Lp(a) concentrations have been determined to be a risk factor for numerous atherosclerotic diseases, which includes coronary artery disease (CAD) (93, 109), peripheral vascular disease (110, 111), as well as thrombotic disorders that are not secondary to atherosclerosis. In this regard, clinical studies have shown that high Lp(a) levels (>30mg/dL) can be directly correlated to instances of ischemic stroke in children (112, 113) and venous thromboembolism (114), although some studies have questioned this direct relationship to instances of VTE (115, 116). Using immunohistochemistry, Lp(a) has also been observed in the arterial intima at a site of an atherosclerotic plaque (117), and it has been shown to preferentially accumulate within the lesion compared to LDL alone (118). The extent of Lp(a) accumulation within the plaque was directly related to the plasma levels of this lipoprotein(a). Due to the structural similarities between Lp(a) and both LDL and plasminogen, it has been hypothesized that Lp(a) can contribute to both proatherosclerotic and prothrombotic processes, both of which contribute to cardiovascular events (Fig 1.4) (119). Mechanisms that are potentially proatherogenic include increases in macrophage foam cell formation, smooth muscle cell (SMC) proliferation and migration, monocyte chemo-attractant activity and endothelial cell adhesion molecule expression (93, 120). Likewise, mechanisms that could be potentially prothrombotic include increased platelet responsiveness (121), inhibition of fibrin clot
Figure 1.4. Potential pathogenic mechanisms of Lipoprotein(a). Various *in vitro* and animal studies have been conducted with demonstrates Lp(a) can contribute to both proatherogenic (left side) and prothrombotic mechanisms (right side) by which Lp(a) exerts its pathogenic effects. EC, endothelial cells; SMC, smooth muscle cells; PAI-1, plasminogen activator inhibitor-1; TFPI, tissue factor pathway inhibitor [Adapted from Koschinsky ML (59)]
lysis (122, 123) and tPA-mediated plasminogen activation (124), which have been observed in vitro and in vivo.

Although Lp(a) has been identified as an independent risk factor for CAD, the exact mechanism of action of Lp(a) is still not fully understood. It is postulated that in an atherosclerotic lesion, Lp(a) can interact with the plaque elements through binding of the apo(a) moiety to matrix components such as fibrin, fibronectin, and laminin (125). The strong LBS of KIV₁₀ of apo(a) has been shown to mediate increased endothelial cell contraction and permeability by a Rho/Rho kinase-dependent pathway (126). In addition, the inflammatory environment of arterial lesions containing oxidized lipid moieties and cellular debris may potentially interact with the Lp(a) molecule, enhancing its pathogenetic effects through its effect on fibrinogen and other blood proteins (109, 127). It is also hypothesized that the apo(a) component of Lp(a) upregulates plasminogen activator inhibitor-1 (PAI-1) expression in ECs, preventing the cleavage of the plasminogen pro-enzyme (128). As well, inhibition of plasmin formation prevents the plasmin-mediated activation of transforming growth factor-β (TGF-β), resulting in the inhibition of SMC migration and proliferation (129, 130). Since plasminogen activation and fibrinolysis are critical mechanisms in many cellular processes, knowledge of the exact mechanisms by which Lp(a)/apo(a) exert its downstream effects are crucial in disease prevention.

1.10 Inhibition of Plasminogen Activation and Fibrinolysis by Lp(a)/apo(a)

Inhibition of fibrinolysis has also been cited as a risk factor for atherosclerosis, leading to increased fibrin deposition in vasculature and the persistence of mural thrombi within atherosclerotic plaques (131, 132). Lp(a)/apo(a) has been shown to inhibit
fibrinolysis, contributing to its prothrombotic potential (133). Due to the structural similarities between apo(a) and plasminogen, Lp(a) is therefore able to inhibit fibrinolysis through binding to the free C-terminal lysine residues on fibrin, creating a quaternary complex with plasminogen on the fibrin surface, preventing tPA-mediated plasminogen activation (71). Hancock et al. investigated the effect of the domains of apo(a) on plasminogen activation on the fibrin surface and determined that the strong LBS in KIV10, the KV domain and the amino terminus of apo(a) were required for maximum inhibition (71). Similarly, it was also determined that apo(a) isoform size does not affect degree of inhibition of activation (71). Apo(a) is also able to inhibit pericellular plasminogen activation on endothelial cells and THP-1 monocytes in vitro through binding of cell surface plasminogen receptors by the strong LBS in KIV10 and KV domain, preventing binding by plasminogen (134). Finally, it has been shown that apo(a) is able to interfere with the positive feedback mechanism of Glu1-Pg to Lys78-Pg conversion, with the KIV5-9 domains being critical in this effect and effect was shown to be independent of isoform size (135).

1.11 Lipoprotein(a) and Oxidation

The mechanisms underlying the atherogenic action of Lp(a)/apo(a) has been attributed, in part, to the presence of oxidative modification on both the lipid and protein component (apo(a) and apoB-100) of Lp(a) (136-138). This modification on Lp(a) causes diverse biochemical (139) and functional changes that promote atherogenesis (140). For example, oxidation of Lp(a) has been reported to: increase PAI-1 production in vascular endothelial cells (141), impair endothelium-dependent vasodilation (142), allow Lp(a) uptake by macrophages though scavenger receptors (143), stimulate SMC proliferation
(144), and induce adhesion of monocytes onto endothelial cells (145). Lp(a) functions in a similar way to LDL to enter the intima at the site of an atherosclerotic plaque through leaky junctions in the endothelial layer (146), however Lp(a) has a longer half-life than LDL and therefore results in larger accumulation of Lp(a) within an atherosclerotic plaque compared to LDL (137). This prolonged accumulation of Lp(a) within the sub-intima relative to LDL is hypothesized to be due to Lp(a)’s affinity for the extracellular matrix (ECM) and fibrin (72, 147). Furthermore, there are lower antioxidants associated with Lp(a) than LDL, which together with the increased accumulation, promotes preferential Lp(a) oxidative modification compared with LDL (148).

More recently, Lp(a) has been shown to be a preferential carrier of oxidized phospholipids (OxPLs) (149). OxPLs can covalent modify proteins, such as apo(a), through a process by which the phospholipid oxidizes to form aldehydes, which can then react with ε-amino groups on lysine residues to form Schiff base adducts (150) or through Michael addition (151). It has been shown that apo(a) is modified by the addition of oxidized phosphatidylcholine (OxPtdC), however the exact location of the modification and the functional consequences have not been fully investigated (152). It has been shown that apo(a) elicits a dose-dependent increase in interleukin-8 (IL-8) mRNA and protein production in macrophages through signaling pathways with CD36 and TLR2 and the OxPL on ap(a); mutating apo(a) in a way that abolishes the OxPL blunts this inflammatory response (153).

1.12 LPA rs3798220 SNP and Thrombotic Risk

As stated above, carriers of the rs3798220 SNP are associated with elevated Lp(a) levels and increased adjusted odds ratio for severe CAD (99). A recent randomized trial
of low-dose aspirin therapy was conducted on a group of 25,131 healthy Caucasian women to deduce whether aspirin reduced cardiovascular risk in minor allele carriers (154). They determined that carriers of the allele possessed elevated Lp(a) levels and increased cardiovascular risk, but the carriers benefitted over two-fold compared to non-carriers following the trial. More recently, work done by Rowland et al. showed that the clot properties of carriers of the SNP depended on ethnicity, where Caucasians had decreased permeability and longer lysis time, and non-Caucasians had increased permeability and shorter lysis times (155).

1.13 Objectives

Clinical data has shown that carriers of the rs3798220 SNP have altered permeability and lysis times compared to non-carriers, and more effectively responds to aspirin therapy. However, the mechanism and biochemical rationale behind this observation has not been fully investigated. The purpose of this thesis will be to characterize the I4399M variant apo(a) using a variety of biochemical approaches. The Met variant will be introduced into a physiologically relevant apo(a) variant containing 17 KIV domains (17K) and compared to wild-type 17K without the mutation. Specific objectives of this research project were developed as follows:

1) Perform pulse-chase assays in HepG2 cells to determine whether an altered secretion rate of the Met variant contributes to the elevated Lp(a) plasma concentrations

2) Analyze the effect of the apo(a) Met variant on fibrin clot properties compared to wild-type apo(a), such as fibrinolysis, coagulation, and fibrin clot architecture
3) Determine if the Met variant causes a structural change in apo(a) or possible modification of apo(a) through sulfoxidation

1.14 Hypothesis

The 17K I4399M variant is structurally and therefore functionally different from wild-type 17K, resulting in the increased CAD risk with enhanced prothrombotic potential associated with the Met variant.
Chapter 2

Methods

2.1 Cell Culture

2.1.1 Construction and expression of recombinant apo(a) variants

Plasmids encoding a 17-kringle-containing form of recombinant apo(a) (17K r-apo(a)), as well as KIV\textsubscript{10}-P, a truncated version of 17K containing the plasminogen-like kringle IV\textsubscript{10}, kringle V, and the inactive protease domain; were constructed and expressed in human embryonic kidney (HEK 293) cells. 17K I4399M and KIV\textsubscript{10}-P I4399M variants, corresponding to the rs3798220 single nucleotide polymorphism, were generated by site directed mutagenesis using the QuikChange II-XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) with the mutagenic primer pair; sense 5’-CAT GTT CAG GAA ATg GAA GTG TCT AGG CTG-3’ and antisense 5’-CAG CCT AGA CAC TTC cAT TTC CTG AAC ATG-3’. The constructed vectors were co-transfected with MegaTran 1.0 transfection reagent (OriGene, Rockville, MD, USA) and pRSV-Neo vector (ATCC, Manassas, VA, USA), and dilution cloned to create stable cell lines.

2.1.2 Propagation of cell lines

Cell culture materials, including culture dishes, 96 well plates, and tubes (Sarstedt, Montreal, Quebec, Canada) were used in a sterile class-II type Biosafety Cabinet (NuAire, Plymouth, MN, USA). Human embryonic kidney (HEK 293) cells (ATCC) were grown with minimum essential media (MEM, Thermo Fischer Scientific, Waltham,
MA, USA), completed with 10% fetal bovine serum (FBS, Thermo Fischer) and 1% (v/v) antibiotic solution (100X Anti-Anti, Thermo Fischer). Human hepatoma (HepG2) cells (ATCC) were grown in MEM (Thermo Fischer), completed with 10% ATCC FBS (ATCC), and 1% (v/v) antibiotic solution (Thermo Fischer). All cells were grown at 37°C in 5% CO₂ in a CO₂ incubator with a HEPA filter (Forma Scientific, Marietta, Ohio, USA), with new media being supplemented every 2 days.

2.1.3 Purification of recombinant apo(a) variants

All recombinant apo(a) variants were purified from conditioned medium of stably expressing HEK293 cell lines using lysine-Sepharose affinity chromatography (GE Healthcare, Mississauga, ON, Canada), as previously described (Hancock 2003). Briefly, conditioned media (Opti-MEM, Thermo Fischer) was collected every three days from the stably transfected cells in 2L Corning roller bottles (Sigma-Aldrich, Oakville, ON, Canada). The media was loaded in a 50mL column containing lysine-Sepharose CL-4B (GE Healthcare), pre-equilibrated with phosphate-buffered saline (PBS, 137mM NaCl, 2.68mM KCl, 10.14mM Na₂HPO₄, 1.38mM KH₂PO₄, pH 7.4). The loaded column was washed with 10 column volumes of PBS and eluted with 200mM ε-aminocaproic acid (ε-ACA, Sigma-Aldrich) in PBS buffer. The fractions were collected and analyzed spectrophotometrically at 280nm and protein-containing fractions were pooled. The pooled fractions were dialyzed three times in Hepes-buffered saline (HBS, 150mM NaCl, 20mM HEPES, pH 7.4) at 4°C and dehydrated using 20,000 MW polyethylene glycol (PEG, Sigma-Aldrich). The concentration was determined spectrophotometrically for all apo(a) variants (corrected for Rayleigh scattering), using the following molecular weights and molar extinction coefficients: 17K and 17K I4399M (Mr ~ 278,219; ε₂₈₀nm =
2.07); KIV$_{10}$-P and KIV$_{10}$-P I4399M (M$_r \sim 52,040$; $\varepsilon_{280\text{nm}} = 2.16$). All proteins were assessed for purity by SDS-PAGE and silver staining, under reduced (containing 10mM dithiothreitil) and non-reduced conditions. Aliquots of the purified proteins were stored at -70°C.

### 2.2 Characterization of lipoprotein(a) in human plasma samples

Blood samples were collected in citrated tubes and DNA was extracted using the GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific). An additional blood sample was collected, with minimal stasis, into an 8.5 ml Vacutainer® Glass citrated tube (Product Number 364606; BD, Franklin Lakes, NJ). This tube was centrifuged at 3500 g for 15 min and plasma immediately frozen at −80 °C in aliquots of 0.5 ml. rs3798220 genotype was determined by sequencing a 427 base pair amplicon of the LPA gene performed by the London Regional Genomics Centre (Robarts Research Institute, London, Ontario). Sequences were analyzed using DNAman 3.0 software (Lynnon Biosoft, Quebec, Canada). Primers for PCR were 5-GAA GGG GCT GGA CCA TAT TT-3 and 5-CGG GTA ACA GGG TAT CTC TTT-3 (156). 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 were carried out using Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswitch, MA, USA) as per manufacturer’s instructions. Plasma samples were reduced and assessed for lp(a) isoform size using a SDS-Agarose gel electrophoresis, and were compared to a ladder of recombinant apo(a) isoform standards. Lp(a) plasma levels were determined using the Macra® Lp(a) Enzyme
Linked Immunosorbant Assay (Mercodia, Uppsala, Sweden) as per manufacturer’s protocol.

2.3 Lipoprotein-Deficient Plasma

Lipoprotein-deficient plasma (LPDP) was prepared from human plasma collected from healthy individuals that did not possess the rs3798330 genotype. Full blood was spun at 15,000rpm at 4°C for 15 minutes, the plasma was removed from the cells and the density was adjusted from 1.006 g/mL to 1.21 g/mL with NaBr according to the following equation:

\[
\text{NaBr (g)} = \frac{\text{[mL Plasma x (\rho_{\text{Final}} - \rho_{\text{Initial}})]}}{(1 - 0.245 x \rho_{\text{Final}})}
\]

where \(\rho_{\text{Final}} = 1.21 \text{ g/mL}\), \(\rho_{\text{Initial}} = 1.006 \text{ g/mL}\) and 0.245 mL/g is the partial specific volume of NaBr. Sealable centrifuge tubes (Beckman Coulter, Mississauga, ON, Canada) were filled with the plasma, sealed shut and spun at 45,000rpm for 20h at 40°C in a Beckman Ultracentrifuge with the type 70.1 Ti Rotor. The plasma separated into two distinct layers and the top layer; containing LDL, VLDL, Chylomicrons, and Lp(a), was carefully removed with a syringe. The lipoprotein-free layer was dialyzed against HBS buffer at 4°C and aliquots were stored at -20°C prior to use.

2.4 Plasminogen activation on fibrin or cell surfaces

THP-1 monocytes were seeded at a density of 200,000 cells per well. Cells were washed three times with HBS containing 0.4% (w/v) BSA prior to adding reaction mixture. Reaction mixtures contained: 600 nM plasma derived plasminogen, 15 nM tPA
(Alteplase), H-D-Val-Leu-Lys-7-amido-4-methylcoumarin (Bachem), and 400nM 17K or 17KI4399M protein. Plasmin formation was monitored over 1 hours at 37°C at an excitation wavelength of 370 nm and an emission wavelength of 470 nm and emission cutoff filter of 455 nm using a plate reading fluorescence spectrometer (SpectraMax M5®, Molecular Devices). The rate of plasminogen activation was taken as the initial slope of the plot of RFU against min⁻² from 10 to 40 mins as performed previously (157). Rates obtained for each individual experiment performed in triplicate were normalized to the rate of plasminogen activation in the absence of apo(a), or to 17K treatment.

Similarly, plasminogen activation on the surface of a fibrin clot was conducted by methods previously published (71). Recombinant plasminogen containing an serine to cysteine mutation in the active site (S741C) was expressed and purified from baby hamster kidney cells (BHK-21) and was labeled with 5-iodoacetamidofluorocein, as described previously (34). Cleavage of Flu-Plasminogen was analyzed in a 96-well Microfluor2 black plates (Costar) pre-coated with HBST (150mM NaCl, 20mM HEPES, pH 7.4, 1% (v/v) Tween 80). Flu-Plasminogen was titrated in triplicate at 6 different concentrations (0, 0.3, 0.6, 0.9, 1.2, and 1.5 μM) and added to HBST (0.02% (v/v) Tween 80) containing human fibrinogen (1mg/mL final) and wild-type or I4399M variant. This mix was added to wells containing small aliquots of Ila, CaCl₂, and tPa, and final concentrations of 1U/mL, 10mM, and 5nM, respectively. The quenching of fluorescence was measured under the following conditions: 37°C, excitation wavelength, 495nm; emission wavelength, 535nm; cutoff wavelength, 530nm; sensitivity normal; PMT setting, low; run-time, 1h; 36 second intervals. Raw fluorescence measurements were corrected for the buffer blank and corrected 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:

\[
d[\text{Pn}]/dt = (\text{dI/dt})*(1/(r*I_0))*([\text{P}]_0/[\text{A}]_0)
\]

where \(d[\text{Pn}]/dt\) represents the rate of plasmin formation per mole of tPa (s\(^{-1}\)), \(\text{dI/dt}\) is the initial rate of fluorescence decrease, \(r\) is the relative maximum change in fluorescence intensity (0.5 for fibrin surface (34)), \(I_0\) is the initial fluorescence intensity, \([\text{P}]_0\) is the initial Flu-plasminogen concentration, and \([\text{A}]_0\) is the initial tPA concentration. The rates of plasminogen activation are the average of triplicate measurements.

2.5 Assessment of plasma clot permeability

Fibrin clot permeability was determined using a pressure-driven system, briefly, 20mM CaCl\(_2\) and 1 U/mL human thrombin (Haemotological Technologies Inc.) were added to 120 μl citrated plasma and transferred to clear glass tubes to hold the clot. After 2 hours of incubation 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 within 60 minutes. A permeation coefficient, referred to as a Darcy constant, \((K_s)\), was calculated from the equation:

\[
K_s = (Q \times L \times \eta)/(t \times A \times \Delta p)
\]

where \(Q\) was the flow rate in time \(t\); \(L\), the length of a fibrin gel; \(\eta\), the viscosity of liquid (in poise); \(A\), the cross-sectional area (in cm\(^2\)), and \(\Delta p\), the pressure differential (in
dyne/cm). The resulting Darcy constants were expressed in units of cm², representing the intrinsic permeability of the clot.

2.6 In Vitro fibrinolysis and coagulation assay

Clot lysis assays were performed in similar manner to that previously published with some modification (158). Pooled lipoprotein-deficient plasma (LPDP) was diluted 1:1 with TBST solution (0.02M Tris, 0.15M NaCl, 0.01% Tween 20, pH 7.4) and spiked with 3 different amount of recombinant wild-type and I4399M apo(a). The reaction mixture was added to wells containing small, separated aliquots of CaCl₂, human thrombin (Sigma-Aldrich), and tPa (Alteplase) at a final concentration of 10mM, 1U/mL and 3nM, respectively. The kinetics of lysis was analyzed spectrophotometrically at 405nm at 37°C for 3 h to measure the change in turbidity in the well. The time required for 50% decrease in clot turbidity (t⁵₀%) was reported. Fibrinolysis assay was conducted again for human plasma samples that were homozygous carriers and non-carriers of the SNP by clotting with 20mM CaCl₂ and 1U/mL of IIa; the t₁/₂ values were also reported. Plasma coagulation assays were performed in similar manner to the clot lysis assay, but was clotted with CaCl₂ and human thrombin (Sigma) at a final concentration of 20mM and 1U/mL, respectively, in the absence of tPa. The time required to reach ½ max absorbance in clot turbidity (t₁/₂) and the extent of clot formation (ΔAbsorbance) were reported.

2.7 Analyzing the interaction of I4399M apo(a) with soluble fibrinogen

3mg/mL Fibrinogen (Calbiochem, Mississauga, ON, Canada) was mixed together with the wild type and I4399M variant to a final concentration of 3μM, vortexed, and
incubated for 10 mins at room temperature. The samples were then prepared with the
addition of 4X SDS reducing sample buffer and boiled at 90°C for 10 mins. Samples
were analyzed on a 4-15% SDS-PAGE gel and stained using Imperial Stain (Thermo
Scientific) for visualization of proteins.

2.8 Imaging of Fibrin Clots with Confocal and Scanning Electron Microscopy

For scanning electron microscopy (SEM), clots were generated by clotting
3mg/mL fibrinogen (Calbiochem, Mississauga, ON, Canada) with 1U/mL thrombin
(Haemotologic Technologies) and 20mM CaCl₂, in the absence and presence of wild-type
and mutant I4399M apo(a), at a final concentration of 1μM. A mixture of Fg and apo(a)
was added to a microdrop of 20mM CaCl₂ and 1U/mL IIa on a SEM peg with conductive
tape and the clot matured for 3h in a moist chamber box. The clots were fixed in 2.5%
Glutaraldehyde (Thermo Fisher) for 30min, followed by four 30 minute washes in dH₂O.
For human plasma samples, plasma was clotted with 20mM CaCl₂ and 1U/mL IIa in a
glass tube and matured for 2 hours. The clots were permeated for 1h, removed from glass
tube, fixed for 30min, and washed four times in dH₂O for 30min. The clots were imaged
using field-emission environmental scanning electron microscope (ESEM, FEI
QUANTA-200 FEG type, Holland FEI Company). Images were taken at a pressure of
70kPa, operating at an accelerating voltage of 10kV and approximately a 10mm working
distance. The images were observed at 15,000X and 30,000X magnification. ImageJ was
used to count fiber widths on the resulting images

In the case of confocal microscopy images, clots were formed with plasma from
patients without the rs3798220 SNP and homozygous carriers of the genotype with
20mM CaCl2 and 1U/mL of IIa. The mixtures were supplemented with 0.3mg/mL Alexa Fluor® 568 labelled fibrinogen (Calbiochem, Mississauga, Canada), fluorescent labelling was performed using Alexa Fluor® 568 Protein Labeling Kit (Invitrogen). Clots were made in glass tubes and matured for 2h. Following maturation, the clots were permeated for 1h and placed on microscope slides. The resulting fluorescent clots were imaged on an Olympus FV1000 confocal microscope at 60X magnification. ImageJ was used with a macro to count the number of fibers per 100μm.

2.9 Pulse Chase Secretion Assay

HepG2 cells were transiently transfected with an apo(a) expressing construct (pRK5ha17) encoding 17 KIV repeats without the mutation and containing the I4399M mutation. The cells were grown to 90% confluency and split into a 6 well flask (Sarstedt) the day before the experiment. On the day of the experiment, the cells were starved for 1h with methionine- and cysteine-free DMEM without serum, pulse-labeled for 1h in the same media containing 200 μCi/mL [S\textsuperscript{35}]methionine, and chased in complete media containing 10nM methionine. The media was collected off the cells at 0, 30, 60, 120, 240, and 480 min. Following removal of the media, the cells were washed twice with PBS and the cellular extract was collected after treating the cells with cold lysis buffer (100mM Tris, pH 8.0, 100mM NaCl, 10mM EDTA, 1% Triton X-100, 0.1% SDS, 100mM ε-aminoaproic acid) containing protease inhibitors. Cellular debris was removed from media and cellular extract through centrifugation, and both samples were pre-cleared by incubation with gelatin-agarose (Sigma) for 2h at 4\textdegree{}C. After centrifugation, the supernatants were incubated with an anti-apo(a) primary antibody overnight at 4\textdegree{}C. The following day, r-protein-G (Invitrogen) beads were added to the samples and incubated
for another 2h at 4°C. Following centrifugation, the resulting pellets were washed twice in PBS (137mM NaCl, 2.7mM KCl, 10.1mM Na₂HPO₄, 1.8mM KH₂PO₄, pH 7.4) and once in TE buffer (10mM Tris, 1mM EDTA, pH 7.0). The pellets were then boiled for 10mins in SDS 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 image plate was then imaged using a Molecular Imager® FX (Bio-RAD) and the appearance and intensity of the bands were analyzed.

2.10 Molecular dynamics simulations and protease domain modeling

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. All chemical models were derived from the crystal structure obtained by Gabel et al (73). More specifically, water molecules present in the crystallographic structure were deleted. Hydrogens and protons were then added to the model using the default method in MOE with water molecules then added using the TIP3P water model. 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(6) until the root-mean-square gradient of the total energy was below 0.05 kcal mol⁻¹ Å⁻¹. The same procedure was used to generate both initial solvated structures; the wild-type apo(a) and the I4399M 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 MDs ran for 4.90
ns with a time step of 2 fs.

2.11 Detection of methionine sulfoxide residue on KIV\textsubscript{10}-PI4399M

To characterize recombinant protein samples, 15\textmu g of either KIV\textsubscript{10}-P or KIV\textsubscript{10}-
PI4399M protein was reduced and labeled with 100mM iodoacetamide (Sigma) for 60
min, shaking, at room temperature in the dark. Samples were then subjected to a buffer
exchange with ice cold trichloroacetic acid (15% v/v), 20 min incubation at 4\textdegree C, followed
by 3X acetone washes of the pellet and re-suspended 4X reducing sample buffer. Samples
were then purified using SDS-PAGE followed by visualization of proteins by Imperial
stain, as per manufacturer’s protocol, excision of the protein band, and subjected to a 16
hour \textit{in-gel} tryptic digest with a ratio of \textasciitilde80:1 apo(a):Trypsin Gold (Promega) (159).
Tryptic peptides were then 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).

2.12 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.
Chapter 3

Results

3.1 Structural Differences in Apo(a) Variants Predicted by Molecular Dynamic Simulations

The rs3798220 SNP in the \textit{LPA} gene, first identified by Luke et al. (99) causes a missense mutation, resulting in an isoleucine to methionine substitution at the 4399 position of the protease domain of apo(a). However, resulting conformational changes of the protease domain caused this mutated residue have yet to be studied. Therefore, we began our investigation by using Molecular Operating Equipment (MOE) software to conduct molecular dynamic simulations, using apo(a) variants, with and without the mutation, derived from the crystal structure of the protease-like domain of apo(a) obtained from Gabel et al (73).

Average occupancy images of the wild-type and I399M variant were generated to determine if any structural differences exist between the Met and Ile variants (Fig 3.1). Comparison of the root-mean-square deviation (RMSD) of the coordination sphere of the two variants showed no gross conformational changes of the protease domain in either protein. However, it was observed that in the wild-type apo(a) variant, the 4399Ile residue is buried within the inactive protease domain, shielded from the solvent by an adjacent Arg residue at position 4420. With the introduction of the 4399Met residue, the adjacent 4420Arg residue no longer shields the residue and is in fact pushed into the solvent. This allows the mutant Met residue to be exposed to the environment, making it accessible to possible modifications.
Figure 3.1. Molecular dynamic simulations of wild-type and mutant apo(a) variants. 
A. To determine any structural differences between the wild-type and mutant variants, Molecular Operating Environment (MOE) software was used to produce average occupancy images using the NAMD program for the two apo(a) variants derived from the crystal structure reported by Gabel et al (73). B. Root mean squared deviation (RMSD) analysis was conducted on the gamma carbon of Ile and gamma sulfur of Met versus the proximal guanidino nitrogen of the adjacent Arg. The average distance between the two atoms over the course of the simulation was reported.
Looking further into this result, RMSD analysis was conducted on the average distance (Å) between the gamma carbon (4399Ile residue) or sulfur (4399Met residue) and the proximal guanidino nitrogen (4420Arg residue) (Fig 3.1). Over the course of the simulation, 4399Ile and 4420Arg have no significant interaction, seen by the fluctuation in the distance between the two atoms (Average distance; 4.37 ± 0.12Å), while the 4399Met and 4420Arg stay in relatively close proximity (Average distance; 3.39 ± 0.03Å), even with both residues exposed to the environment, and may coordinate to remain in this low energy state.

Despite the observation that the 4399Met residue does not cause any gross conformational changes in the protease-like domain, the residue becomes exposed to the external environment and could be potentially modified. Furthermore, the Met residue may coordinate with a proximal Arg residue, a structural conformation not seen in wild-type apo(a). Therefore, the effect of the structural difference of I4399M variant on the processes of thrombosis and fibrinolysis, compared to wild-type apo(a), were further studied.

3.2 Secretion of Wild-Type and Mutated r-Apo(a) Variants from HepG2 cells

Previous studies on the I4399M apo(a) variant have demonstrated that carriers of the corresponding rs3798220 SNP are associated with smaller apo(a) isoform sizes, as well as high plasma Lp(a) concentrations (100). It has also been reported that apo(a) variants with smaller isoform sizes secrete more efficiently than larger apo(a) isoform sizes (160). This suggests that increased secretion of apo(a) containing the mutation from hepatic cells could be contributing to the high Lp(a) levels in carriers of this SNP. The purpose of this assay was to determine if this observation was the result of the association
of this mutation with smaller isoform sizes or if the mutated residue is affecting its secretion.

To address this, we conducted an *in vitro* pulse-chase secretion assay in cultured human hepatoma (HepG2) cells transfected with plasmids containing the cDNA of recombinant apo(a) variants, containing 17 KIV domains, with and without the mutation (Figure 3.2). This experiment was conducted by incubating the transfected HepG2 cells with 200 μCi/mL [S\(^{35}\)]methionine and chased in complete media, followed by the collection of the media and lysates of the cells at 6 increasing time points. Samples were immunoprecipitated, run on an SDS-PAGE gel and exposed to a screen for 3 days. Before conducting the assay, it was first important to determine the time points to collect samples to ensure that the decrease in apo(a) in the lysates and subsequent increase in apo(a) in the media could be visualized. It was concluded that the time points 0, 30, 60, 120, 240, and 480 minutes effectively showed this trend in the secretion profiles. Following the exposure to the screen, the intensity of bands for the 17K and 17K I4399M variants in the media and lysates were normalized against the maximum band intensity, and plotted as a function of time (Figure 3.3). As anticipated, the intensity of apo(a) in the lysates decreased, while the intensity of apo(a) in the media increased as a function of time. However, compared to the 17K wild-type apo(a), there was no significant difference in the intensity of apo(a) bands at any time point in the media or lysates compared to the 17K I4399M variant. Both variants showed maximum apo(a) intensity in the media at 30 minutes, and at 240 minutes in the lysates following pulse.
Figure 3.2 Schematic representation of the recombinant apo(a) variants utilized in the proposed studies. The 17K recombinant apo(a) construct shown on the top line represents a physiologically relevant apo(a) isoform that contains 17 plasminogen-like kringle IV domains, denoted 1-10, and 8 KIV$_2$ repeats. KV represents the plasminogen-like kringle V domain and P is the inactive protease domain. The white circle within the second construct, labeled 17K I4399M, represents the substitution in the protease domain of apo(a) introduced using site directed mutagenesis.
Figure 3.3 Pulse-chase analysis of the secretion of 17K versus 17K I4399M from HepG2 cells. Transfected HepG2 cells were pulse labeled with \([S^{35}]\)methionine and chased in completed media. Media and lysates were collected at 0, 30, 60, 120, 240, and 480 minutes, run on SDS-PAGE gel and exposed on a screen for 3 days before imaging. A. Representative images of the apo(a) intensity in the media and lysates of the apo(a) variants at the six different time points. B. The intensity of apo(a) for the media and lysates were normalized to maximum intensity and plotted as a function of time. The results represent the mean ± SEM of four independent experiments.
Therefore, this result suggests that the presence of the mutation does not alter the rate of secretion in apo(a) variants of the same length. Using this knowledge, these two apo(a) variants of same length were used in subsequent assays to deduce the specific effect of this mutation on the coagulation and fibrinolytic pathways.

3.3 Effect of I4399M Variant on Prothrombotic Mechanisms Compared to Wild-Type Apo(a)

Previous studies have shown that Lp(a)/apo(a) has the capability to inhibit in vitro fibrinolysis through a mechanism involving binding to lysine residues of fibrin, preventing tPA-mediated plasminogen activation on the fibrin surface (134, 161) and therefore inhibiting lysis of the fibrin network (140, 162). Similarly, it has also been shown that apo(a) is able to inhibit pericellular plasminogen activation on multiple cell types through binding to cell surface receptors and preventing the binding of plasminogen (134). Therefore, we investigated the effect of the I4399M variant on these processes compared to the wild-type apo(a) variant.

We began by performing a tPA-mediated plasminogen activation assay on a fibrin surface with 0.9μM of catalytically-inactive, fluorescently-labeled plasminogen in the presence of wild-type and mutant apo(a). The rate of fluorescence decrease measured by spectrophotometry was determined and inserted into the derived equation to determine the rate of plasmin formation per mole of tPA. These values were then normalized against the rate with no apo(a) and presented as the relative % change at different concentrations of apo(a) (Figure 3.4). At an apo(a) concentration of 0.5μM, the wild-type variant inhibited the rate of plasmin formation by 25% compared to no apo(a) control, while the I4399M variant inhibited the rate by 27%. When the concentration was increased to 1μM,
the wild-type and I4399M variant inhibited plasmin formation rate by 44% and 46% respectively. As expected, increasing concentration of apo(a) results in greater inhibition of plasmin formation on the fibrin surface (71). However, there was no statistical difference between the percentage of inhibition caused by wild-type and mutant apo(a) variants. To examine the effect of the mutant on pericellular plasminogen activation, an assay was conducted where the purified apo(a) variants were incubated with THP-1 monocytes and a fluorescent substrate of plasmin. The rate of plasminogen activation was taken as the initial slope of RFU change per min$^2$ and normalized against the rate with no apo(a) present (Figure 3.5). As expected, the wild-type apo(a) variant inhibited plasminogen activation on the cell surface by ~30%. Surprisingly, the I4399M variant showed no inhibition of pericellular activation compared to the normal and resulted significantly less inhibition compared to the wild-type variant.

To expand on the preceding result, we investigated how the I4399M variant affects fibrinolysis compared to wild-type. A fibrinolysis assay was conducted, where lipoprotein-deficient plasma (LPDP) was incubated with increasing concentrations of the wild-type and I4399M apo(a) variants and the change of absorbance was measured by spectrometry at $37^\circ$C for 3h. The absorbance profiles were generated and the $\frac{1}{2}$ time required for clot lysis was reported ($t_{50\%}$) (Figure 3.6). At an apo(a) concentration of 0.5$\mu$M, the $t_{50\%}$ lysis of the wild-type apo(a) was determined to be 2182 seconds, ~3X greater than control (740 seconds), while the I4399M variant had a $t_{50\%}$ of 3020 seconds, ~4X greater than the control. At 1$\mu$M apo(a) concentration, the $t_{50\%}$ times for the 17K and I4399M variant were ~5.5X greater (3987 seconds) and ~7X greater (5023 seconds) than control, respectively.
Figure 3.4 tPA-mediated plasminogen activation assay on a fibrin surface in presence of r-apo(a) variants. Purified fibrinogen was clotted with 1U/mL IIa, 10mM CaCl$_2$, and 5nM tPa in the presence and absence of wild-type and mutant apo(a). Samples were spiked with a catalytically inactive variant of plasminogen labeled with 5-iodoacetamide. The quenching of fluorescence was measured by spectroscopy at 37$^\circ$C; excitation: 495nm, emission: 535nm, cutoff: 530nm. The rate of plasmin formation per mole of tPa was calculated and was represented as a relative percentage change of the rate without apo(a). *: p < 0.05 by two tailed ANOVA. The data represent the means ± SEM of four independent experiments each conducted in triplicate.
Figure 3.5 Plasminogen activation assay on the surface of THP-1 monocytes. THP-1 monocytes, seeded at a density of 200,000 cells per well, were incubated with 0.6μM plasminogen, 15nM tPa, and a fluorescence substrate of plasmin (H-D-Val-Leu-Lys-7-amido-4-methylcoumarin) in the presence and absence of wild-type and mutant apo(a). Plasmin formation was monitored for 1h at 37°C by spectrometry; excitation: 370 nm, emission: 470 nm, and cutoff: 455 nm. The rate of plasminogen activation was represented as the initial slope of the plot of RFU against min² and normalized against control. *: p < 0.05 by two-tailed ANOVA. The data represents the means ± SEM of three independent experiments each conducted in triplicate.
Finally, at an apo(a) concentration of 1.5μM, the $t_{50\%}$ for the wild-type and I4399M variant were ~8X greater (5867 seconds) and ~9.5X greater (6623 seconds) than control, respectively. As previously observed, increasing concentration of apo(a) results in a greater inhibition of fibrinolysis compared to no apo(a) control. Interestingly, at all three concentrations of apo(a), the I4399M variant significantly increases the inhibition of fibrinolysis compared to wild-type.

As the previous results suggest, the increase in inhibition of fibrinolysis seen with the I4399M mutant compared to wild-type can not be explained by the inhibition of plasminogen activation on the fibrin surface. Therefore, we investigated how the apo(a) variants affect clot formation through an absorbance based coagulation assay. The assay was conducted in a similar manner to the previous assay in the absence of tPA and the change of absorbance was measured by spectrometry at 37°C for 1h. The absorbance profiles were generated and the difference between the maximum and minimum absorbance, representing the turbidity of the sample, was calculated. The ½ time required for clot formation were reported (Figure 3.7). Looking at the sample turbidity, at all three concentrations of apo(a), the wild-type variant shows ~11% increase in absorbance compared to no apo(a) control, although the difference is not statistically significant. However, the I4399M variant was shown to significantly increase the sample turbidity at 0.5μM, 1μM, and 1.5μM of apo(a) by 20%, 25%, and 44%, respectively, compared to the wild-type apo(a) variant. Similarly, the observed ½ clotting time for the wild-type variant at the three apo(a) concentrations significantly decreased by 23%, 30%, and 34%, respectively, compared to the no apo(a) control.
Figure 3.6 Absorbance-based fibrinolysis assay with LPDP in presence and absence of apo(a). To study clot formation and subsequent lysis, lipoprotein-deficient plasma (LPDP) was clotted with 1 U/mL thrombin (IIa), 20 mM CaCl$_2$, and 3nM tPA in the absence and presence of wild-type and mutant apo(a) and analyzed by spectroscopy at 405nm. The changes in turbidity were measured at 37°C for 3 hr, normalized against maximum absorbance and plotted as a function of time for A. 0.5μM, B. 1μM, and C. 1.5μM of apo(a). D. The time to reach 50% lysis (t$_{50%}$) at the three concentrations were reported. *: p < 0.05 versus wild-type, **: p < 0.02 versus no apo(a) by two tailed ANOVA. The data represent the means ± SEM of three independent experiments each conducted in triplicate.
Figure 3.7 Absorbance-based coagulation assay with LPDP in presence and absence of apo(a). To study clot formation, lipoprotein-deficient plasma (LPDP) was clotted with 1 U/mL thrombin (Ila), 20 mM CaCl$_2$, in the absence and presence of wild-type and mutant apo(a) and analyzed by spectroscopy at 405nm. The changes in turbidity were measured at 37°C for 1 hr, normalized against minimum absorbance and plotted as a function of time for A. 0.5μM, B. 1μM, and C. 1.5μM of apo(a). D. The turbidity of the solution and E. The time to reach 50% coagulation at the three concentrations were reported. *: p < 0.05 versus wild-type by two tailed ANOVA, +: p < 0.05 versus no apo(a) by t-test, ++: p < 0.05 versus wild-type by t-test. The data represent the means ± SEM of three independent experiments each conducted in triplicate.
Interesting, it was seen that the I4399M variant significantly decreased the ½ clotting time compared to the wild-type at all concentrations of apo(a). The resulting ½ clotting times decreased by 27%, 35%, and 37% in the presence of the I4399M variant, respectively, compared to wild-type.

Overall, these results suggest that while the I4399M variant does not differ in the degree of inhibition of plasminogen activation on the fibrin surface, it does alter the clot properties compared to wild-type apo(a), seen by the increased t50% values and increased turbidity of the sample, as well as decreased clotting time. Therefore, the next step was to understand how this mutant affects the fibrin clot architecture in a way that results in the observed affects on fibrinolysis and coagulation.

3.4 The Relationship Between the I4399M Variant and Fibrinogen/Fibrin Clot Architecture

In order to understand the increased pro-thrombotic potential of the I4399M variant, the effect of the variant on the fibrin clot architecture was studied. This was achieved through scanning electron microscopy (SEM) of fibrin clots formed in the absence or presence of the I4399M and wild-type variant. Clots were formed on SEM pegs and subjected to low-vacuum. Images were captured at 15,000X and 30,000X magnification. The 30,000X images were analyzed using the imaging software ImageJ and the average width of a minimum of 25 fibers were reported (Figure 3.8). It was found that clots containing the wild-type apo(a) possessed a greater number of fibers compared to no apo(a) control, while the fiber width was not significantly different than the control. However, the I4399M clots had a much more disorganized structure and possessed multiple nodules throughout the clot, resulting from aggregated fibrin fibers.
Figure 3.8 SEM imaging of fibrin clots formed in the absence or presence of either the I4399M or wild-type apo(a). A. Purified fibrinogen in the absence and presence of wild-type and mutant apo(a) was clotted with 1U/mL IIa and 20mM CaCl₂ and visualized using environmental scanning electron microscopy (SEM). Images were taken at 15,000x magnification with a HFW of 6.67 microns. B. Fiber widths from 30,000X images of the clots from A were calculated using the imaging software ImageJ, with a minimum of 25 fibers measured per field of view. *: $p < 0.05$ by two tailed ANOVA. The data represent the means ± SEM of four separate images from the same clot.
Figure 3.9 Imperial stain of samples of I4399M and wild-type apo(a) variant in solution with fibrinogen. A. 3 mg/mL of purified human fibrinogen was incubated with 3μM of wild-type and I4399M apo(a) for 10 minutes at RT. Samples were vortexed and the I4399M mixture was centrifuged. The mixtures and resulting I4399M mixture pellet and supernatant were run on a 4-15% gradient SDS-PAGE gel and imaged with Imperial stain.
The resulting fibers were ~46% thicker than no apo(a) control and wild-type apo(a).

When optimizing the various plate based assays with purified fibrinogen, high concentrations of the 17K I4399M variant (~3μM) resulted in a turbid mixture, a result not seen with the wild-type 17K apo(a) at the same concentration. Therefore, in order to understand this observation, purified fibrinogen was incubated with 3μM of both apo(a) variants and the resulting I4399M mixture was centrifuged to separate any insoluble material. The supernatant and pellet were run on an SDS-PAGE gel and Imperial stained (Figure 3.9). The 17K and 17K I4399M variants in solution with fibrinogen were effectively visualized, showing the three bands of fibrinogen resulting from treatment with a reducing agent. Following centrifugation, the supernatant of the 17K I4399M and fibrinogen mixture contained a strong band for apo(a), but very weak bands for fibrinogen. Surprisingly, most of the fibrinogen in the mixture was found in the pellet, along with some apo(a). Overall, we have shown that the I4399M variant is able to alter the fibrin clot network in a way that differs from wild-type and is able to precipitate soluble fibrinogen.

3.5 Analysis of Clot Structure and Properties of Plasma from Homozygotes of the rs3798220 SNP

While the investigation up to this point was focused on the purified mutant apo(a) component, we then wanted to examine how a Lp(a) molecule containing the Met mutation would affect the fibrin clot structure and its subsequent properties. We obtained 4 patient samples that are homozygous carriers of the rs3798220 SNP and the samples were phenotyped to determine apo(a) isoform size. We then screened multiple people to identify wild-type controls; these samples were genotyped to determine if they possessed
the SNP and phenotyped to match the isoform sizes (data not shown). We were able to match one homozygote, which was a low Lp(a) expresser ([Lp(a)] \sim 5 \text{ mg/dL}), with a non-carrier of similar Lp(a) concentration and isoform size ([Lp(a)] \sim 4 \text{ mg/dL}, \text{ Size} = 23\text{K}). Similarly, we were able to match a highly expressing homozygote ([Lp(a)] \sim 40 \text{ mg/dL}) to a single non-carrier with similar Lp(a) concentration and isoform size ([Lp(a)] \sim 35 \text{ mg/dL}, \text{ Size} = 14\text{K}).

The first experiment using these samples was a clot permeability assay to determine the effect of the mutation on fibrin clot pore size. Previous studies have shown that Lp(a) is able to produce thinner fibers with tighter pores, making the clot less permeable and less susceptible to lysis (53). In our study, clots from homozygotes and non-carriers were formed in glass tubes, hooked to a buffer reservoir, and the time between drops and the weight of the drops were recorded. These values were placed into a derived equation and the values are reported as Darcy constants (k_s), with units of cm^2, representing the surface area of the pores in the clot (Figure 3.10). For the low [Lp(a)] samples, the non-carrier and homozygote samples had a Darcy constant of 7.10x10^{-9} \text{ cm}^2 and 7.52x10^{-9} \text{ cm}^2, respectively. For the high [Lp(a)] samples, the non-carrier had a Darcy constant of 4.51x10^{-9} \text{ cm}^2, while the homozygote had a Darcy constant of 5.89x10^{-9} \text{ cm}^2. This suggests that there is no significant difference in pore size between the homozygote and non-carrier clots at both the low and high Lp(a) concentrations.
Table: Sample [Lp(a)] (mg/dL) Phenotype

<table>
<thead>
<tr>
<th>Sample</th>
<th>[Lp(a)] (mg/dL)</th>
<th>Phenotype</th>
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<tbody>
<tr>
<td>Low [Lp(a)]</td>
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<td></td>
</tr>
<tr>
<td>Non-Carrier</td>
<td>4.55</td>
<td>23K</td>
</tr>
<tr>
<td>Homozygote</td>
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<td>24K</td>
</tr>
<tr>
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<td>Non-Carrier</td>
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<td>18K</td>
</tr>
<tr>
<td>Homozygote</td>
<td>46.68</td>
<td>17K</td>
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</table>

**Figure 3.10 Permeation and fibrinolysis values of patient plasma samples.** A. Plasma from paired patients that were non-carriers as well as patients homozygous for the polymorphism were clotted with 1U/mL IIa and 20mM CaCl$_2$ in a clear glass tube and allowed to mature in wet box for 2hrs. The tubes were then connected via tubing to a reservoir containing a buffer (0.01M Tris, 0.1M NaCl, pH 7.5) and the volumes flowing through the fibrin gels were used to calculate darcy constants ($k_s$). B. Lipoprotein-deficient plasma (LPDP) in the presence or absence of non-carrier or homozygote plasma samples were clotted with 1 U/mL thrombin (IIa) and 20 mM CaCl$_2$ and analyzed by spectroscopy at 405nm, measuring the changes in turbidity at 37°C for 3 hr. The time needed to reach $\frac{1}{2}$ lysis time was compared to LPDP control and presented as relative $\frac{1}{2}$ lysis time. *: p < 0.05 by two tailed ANOVA. The data represent the means ± SEM of three independent experiments each conducted in triplicate.
We were able to show that purified I4399M mutant can inhibit fibrinolysis to a greater degree than wild-type apo(a). Therefore, we conducted the same fibrinolysis assay using these homozygote and non-carrier samples to determine if the same result can be observed. Samples were spiked with tPA and clotted with CaCl$_2$ and Ila and the change of absorbance was measured for 3 hours. The t$_{50\%}$ times were reported, normalized against the LPDP control with no Lp(a) and reported as relative lysis times compared to control (Figure 3.10). As anticipated, the samples with low Lp(a) concentrations had similar lysis times; the non-carrier and homozygote samples had relative t$_{50\%}$ times of 1.215 and 1.219, respectively. As for individuals with high Lp(a) concentrations, the non-carrier had a relative t$_{50\%}$ time of 1.354, while the homozygotes had significantly inhibited fibrinolysis to a greater degree than the non-carrier, resulting in a relative t$_{50\%}$ time of 1.519. Overall, these patient samples have shown that low expressers of Lp(a), regardless of carrier status, have similar permeation and fibrinolysis values, while homoygotes that have high Lp(a) concentrations have similar Darcy constants than non-carriers, but are able to significantly increase inhibition of fibrinolysis compared to wild-type. Based on these observations, it was imperative to visualize these clots to see if the clot architecture is altered in a way similar to the results seen with the purified apo(a) variants.

Confocal microscopy and SEM was used to visualize fibrin clot architecture in the homozygote and non-carrier clots. For confocal microscopy, clots were made in glass tubes similar to the permeability assay and spiked with fluorescently labeled fibrinogen. Mature clots were washed, placed on slides and imaged at 60X. The resulting images were analyzed using ImageJ software using a designed macro, which overlays a grid to measure the number of intersecting fibers in a given field of view. The results are the averages of the counts from 6 different images of the same clot and the values are
reported as the number of fibers per 100μm (Figure 3.11). At low concentrations of Lp(a), both the non-carrier and carrier samples have nearly identical number of fibers per 100μm. As expected, at a high concentration of Lp(a), the non-carrier has a greater number of counted fibers, resulting in a denser clot with tighter pores. However, the homozygote samples do not follow this same type of trend and have significantly lower numbers of fibers compared to the non-carrier samples. This would suggest that the clots produced by the carrier plasma are not as dense and may possess larger pores. To expand on this result, SEM was utilized to measure the fiber width of the clots. This was conducted similarly to the confocal procedure, without the addition of labeled fibrinogen. The paired homozygote and non-carrier samples were washed and visualized under low vacuum at 15,000X and 30,000X magnification. Using the ImageJ software, the widths of at least 25 fibers of 6 different images of the same clot were calculated and the average fiber width was reported in μm (Figure 3.11). The samples with low Lp(a) concentrations showed that the carriers had significantly thicker fibers compared to the non-carriers, with average fiber widths of 0.066μm and 0.054μm, respectively. Surprisingly, the high [Lp(a)] non-carrier sample was shown to have ticker fibers than the non-carrier with a lower Lp(a) concentration. However, as anticipated, the high [Lp(a)] carrier had significantly thicker fibers compared to both the high [Lp(a)] non-carrier and the low [Lp(a)] carrier samples. Overall, these results would suggest that homozygous carriers of the apo(a) mutation have the same permeability as non-carrier clots but possesses less fibers in a given field of view, as suggested by the permeation and confocal data. However, the homozygote clots have thicker fibers and has the capability to inhibit fibrinolysis to a greater degree than non-carriers.
Figure 3.11 Confocal microscopy analysis of clot formation using homozygote and non-carrier plasma samples. Plasma samples from homozygous carriers and non-carriers of the rs3798220 SNP were clotted with 20mM CaCl2 and 1U/mL of IIa and were supplemented with 0.3mg/mL Alexa Fluor® 568 labelled fibrinogen. Clots were washed for one hour and transferred to a microscope slide. The resulting fluorescent clots were imaged on an Olympus FV1000 confocal microscope at 60X magnification. A. Representative images of the micrographs from confocal microscopy of the plasma samples at 60X magnification. B. ImageJ software was used to analyze the images and a collaborator-designed macro was used to determine the number of fibers per 100μm. *: p < 0.05 by two tailed ANOVA. The data represent the means ± SEM of 6 different images of the same clot.
Figure 3.12 Scanning electron microscopy and fiber width measurements in clots formed from homozygote and non-carrier patient samples. Plasma samples from homozygous carriers and non-carriers of the rs3798220 SNP were clotted with 20mM CaCl$_2$ and 1U/mL of IIa. Clots were fixed, washed for one hour and transferred to a SEM pegs. The resulting clots were imaged on a field-emission environmental SEM under low vacuum at 15,000X and 30,000X magnification. A. Representative images of the micrographs from SEM of the plasma samples at 15,000X magnification. B. Fiber widths from 30,000X images of the clots from A were calculated using ImageJ, with a minimum of 25 fibers measured per field of view *: $p < 0.05$ by two tailed ANOVA, ++: $p < 0.05$ vs non-carrier by two tailed ANOVA. The data represent the means ± SEM of 6 different images of the same clot.
3.6 Identification of a Modification on the Substituted Methionine Residue in the 4399M Variant

The molecular dynamics data suggested that the substituted methionine residue is exposed to the environment, possibly becoming susceptible to potential modification. Previous studies have shown that methionine residues can undergo oxidative modification to methionine sulfoxide, which plays a role in many cellular functions (163). Therefore, we investigated whether this exposed Met on the apo(a) residue can be modified to a methionine sulfoxide moiety. We conducted mass spectroscopy of the 4399Met mutation in a smaller variant of apo(a), containing KIV$_{10}$, KV and inactive protease domains (Figure 3.12). Two theoretical masses of the tryptic peptide containing the mutation were calculated, possessing either an unmodified Met residue and or an oxidized Met residue (2505.2595 and 2521.2544, respectively). The actual MS spectra contains no observable peak for the peptide of mass 2505.2595 and a major peak that corresponds to the peptide of mass 2521.2544, indicating that the presence of this 4399Met mutation in apolipoprotein(a) is susceptible to oxidative modification and can result in a methionine sulfoxide moiety on this residue.
Figure 3.13 Mass spectroscopy of a truncated apo(a) variant containing the I4399M mutation. A truncated recombinant apo(a) was constructed containing the KIV_{10}, KV, and inactive protease domains, either with (denoted KIV_{10-34399M}) with and without (denoted KIV_{10-P}) the polymorphism. The KIV_{10-P} I4399M was subjected to in-gel tryptic digestion and then analyzed by MALDI-TOF mass spectrometry using a Waters SYNAPT G2-Si instrument. The spectra were observed using MassLynx software and compared to two theoretical masses for the potential tryptic peptide.
Chapter 4

Discussion

4.1 The I4399M Variant Secretion Rate Does Not Differ from Wild-Type

In this study, we utilized the human heptoma cell line, HepG2, in order to analyze the secretion rate of recombinant 17-kringle containing variants of apo(a). This cell line was deemed appropriate for our in vitro assay as it has been shown that lipoproteins, and specifically Lp(a), is synthesized by hepatic cells in the liver (164, 165). In the hepatocytes, apo(a) is initially formed as a low molecular mass precursor, and then modified in the endoplasmic reticulum (ER) before entering the Golgi and becoming glycosylated (166). Therefore, using a cell line other than hepatocytes would not produce the mature apo(a) structure with the proper modifications, which could ultimately affect its secretion rate from that specific cell.

In our study, we found that when comparing two apo(a) variants of the same length, the mutation itself can not alter the secretion rate from hepatocytes. When looking at the images for the wild-type and I4399M variant, it is seen that the apo(a) in the lysates reaches maximum at 30 minutes and then the intensity decreases as the time of the assay increases. This decrease in intensity in the lysates is opposed by an increase in the media signal for both variants as the assay progressed. The maximum signal for both variants in the media was seen at 240min, and a dip in band intensity was seen at 480min, which most likely due to degradation of the mature apo(a). We use protease inhibitors in our lysis buffer to prevent degradation during immunoprecipitation, but it is not used on the media samples. During the assay, the samples are kept on ice to prevent enzymatic
degradation, so one would expect a minimal amount of degradation from the assay. However, the last time point is collected after 8 hours and the stress caused by the assay could cause the release of inflammatory cytokines from the hepatocytes (167) that could cause the recruitment of proteases which would lead to degradation of apo(a).

Since it was determined that the mutation alone cannot account for the elevated Lp(a) levels in carriers, there are two likely explanations that could account for this observation. First, carriers of this mutation have been correlated with smaller isoform sizes (99) and smaller isoforms are associated with higher Lp(a) protein levels (84, 85). Therefore, when comparing levels in a population, carriers possessing smaller apo(a) isoforms would have higher levels than non-carriers with larger isoform sizes. Secondly, the I4399M mutant may have a longer turnover time than the wild-type variant or may not be as efficiently catabolized, resulting in increased plasma Lp(a) concentration. Further studies must be conducted to analyze the internalization of this I4399M variant and its subsequent turnover rate compared to wild-type. Furthermore, the mutation can be introduced to a smaller variant and its secretion rate compared to wild-type variant of the same size could be determined to confirm the results of our study.

4.2 Analysis of the I4399M Variant Effect on Plasminogen Activation on Different Surfaces

Previous studies have shown that Lp(a)/apo(a) has the capability to inhibit plasminogen activation on a fibrin surface by binding to free lysines on fibrin (71) to prevent tPA-mediated plasminogen activation and on the cell surface by binding to cell surface plasminogen receptors (134). Similarly, it was also shown that the strong LBS of KIV_{10}, the KV domain, and the amino-terminal amino acids were critical for the
inhibition of plasminogen activation in the fibrin surface (71) and removal of the strong LBS in KIV_{10} or the KV domain blunts the inhibition of activation on the cell surface (134). Therefore, one would predict that a mutation in the protease domain would not result in a change in the inhibition of plasminogen activation on these surfaces.

As predicted, the I4399M variant inhibited activation to the same degree as wild-type on a fibrin surface at multiple apo(a) concentrations. The assay relied on a quenching of a fluorescent signal to calculate the rate of plasmin formation, which is much more efficient for this use than an absorbance based assay. An absorbance assay would rely on decreasing turbidity of the clot as it is lysed and would not give information on the rate of plasminogen cleavage. On the other hand, using fluorescence would allow one to calculate the amount of plasmin formed per mole of tPA (71). The derived equation takes into account the background fluorescence within the spectrometer to try to eliminate the error that is associated with fluorescence-based assays. While the predicted result was seen on the fibrin surface, an unexpected result was seen on the surface of THP-1 monocytes. Wild-type apo(a) showed a 30% decrease in activation by competing with plasminogen for binding sites, which agrees with literature (134, 168), while the I4399M variant showed no inhibition of activation compared to the no apo(a) control. This fluorescence-based assay relies on quenching of fluorescence as well, where a fluorescent substrate of plasminogen is cleaved and results in quenching. There are two potential reasons for this blunting of inhibition seen with the I4399M variant. First, the fluorescent substrate could be binding to the I4399M variant, and thus making it unavailable to plasminogen, or it can become post-transnationally modified, therefore preventing it from entering plasmin’s active site. In order for this to happen, the mutated residue in this variant would have to play a role in this interaction as the two apo(a) variants used are of
the same length. Secondly, the mutated residue could be interfering with apo(a) binding to the cell surface receptors, leaving more receptors open for the plasminogen conversion. Previous studies have shown that a point mutation in KIV\textsubscript{10} domain can result in blunting of inhibition as well (134, 168), and while the I4399M mutation is not occurring in one of the domains needed for binding, it could be acting through a different mechanism to prevent plasminogen binding to the cell surface. Overall, this data would suggest that the inhibition of plasminogen activation on the fibrin surface cannot account for the increased lysis time and altered coagulation seen with this I4399M variant.

4.3 I4399M Variant Alters Fibrinolysis Time and Coagulation Compared to Wild-Type

It has been shown that Lp(a)/apo(a) has the ability to inhibit the rate of fibrinolysis \textit{in vitro} in a dose-dependent manner and there is evidence that isoform size may also contribute to the degree of inhibition (161, 162, 169). It achieves this through preventing the conversion of Glu- to Lys-plasminogen, as well as blunting inhibition of the plasminogen activator inhibitor-1 (162). We were able to show that the I4399M variant was able to significantly increase fibrinolysis time compared to wild-type at all three concentrations of apo(a) in a dose dependent manner. Taking the plasminogen activation data into account, this suggests that the I4399M variant is achieving this response in a mechanism separate from inhibiting plasminogen activation. It has been shown that thinner fibrin fibers lyse quicker than thick fibers, but apo(a) interacting with fibrinogen results in thinner fibrin fibers in a denser fiber network, decreasing permeability and resulting in increased lysis time (52, 54). However, a recent study has shown that thicker fibers are more likely to elongate at first rather than being lysed, compared to thinner
fibers which are immediately lysed, resulting in increased lysis time (170). Therefore, it could be suggested that the potential explanation for the effect of the I4399M variant is that the mutant creates a tighter, denser clot that is less permeable and less susceptible to lysis. On the other hand, the mutation could cause thicker fibrin fibers, which are less susceptible to lysis and result in the increased lysis time. Further studies were needed on the structure of the fibrin clot to understand this result.

To further analyze this result, coagulation assays were conducted in the absence of tPA to understand if the I4399M variant alters the way the clot is formed. Literature has shown that many factors influence how a fibrin clot is formed, such as thrombin concentration, pH, ionic strength, and concentrations of calcium (171-173). Higher concentrations of IIa result in thicker fibers and a less dense network, while low IIa concentrations result in thinner fibers and a more dense network (170). The assay is characterized as possessing a “lag” phase with no change in turbidity as the protofibrils are initially formed (16). As the protofibrils laterally aggregate, the turbidity begins to increase. The magnitude of turbidity relates to the structure of the fibrin network, with thicker fibers causing a greater increase in final turbidity (90, 174). In our assay, controlling the amount of IIa added and other conditions would allow us to see changes caused by the apo(a) variants. We showed that the ½ coagulation time of the I4399M variant was significantly shorter compared to wild-type and the clots of the I4399M variant were significantly more turbid that the wild-type variant. The greater turbidity of the I4399M variant clot would suggest a different clot architecture than the wild-type and could insinuate that the clot is made of thicker fibers, causing the higher turbidity of the sample. Overall, the results from the fibrinolysis and coagulation assay indicated that the fibrin network may be different for the clots of the I4399M variant compared to wild-type
that could account for the differences seen. The next step was to visualize the fibrin clot to understand how the I4399M variant affects clot architecture.

4.4 The I4399M Alters Fibrin Clot Architecture and Causes Precipitation of Fibrinogen

As previously discussed, Lp(a)/apo(a) has been shown to result in an altered clot structure, possessing thinner fibers in a more dense network that results in increased lysis time (175, 176). However, our data would suggest that the increased lysis time and increased turbidity of the clot seen with the I4399M variant may be a result of different clot architecture compared to wild-type. SEM was used to image the clots due to its high-resolution capabilities and clots being made in the absence of cells, allowing the clots to be effectively washed and therefore visualized. The wild-type variant resulted in a denser clot with thinner fiber widths (Average = ~75μm). Unexpectedly, the I4399M variant resulted in a less dense clot with fibers that were significantly thicker than wild-type (Average = ~312μm). As well, the I4399M variant produced a clot that did not form the organized pore network seen with the wild-type variant. It was also characterized as possessing multiple fibrin nodules at the branching points of the fibrin network. The thicker fibers and nodules can account for the greater turbidity of the solution seen in the clotting absorbance assay. Although the I4399M clot looks less dense than the wild-type clot, the thicker fibers and nodules at the branching points could perhaps explain the increased lysis time in the mechanism described by Bucay et al. (170), where thicker fibers elongate and delay lysis times. Our study did not investigate the interaction of this mutant variant with other proteins or factors in the coagulation cascade, but the data
suggests that I4399M variant is able to alter the fibrin clot network to account for its increased prothombotic potential.

Through optimization of the previous assays, when high concentrations of 17K I4399M are incubated with fibrinogen, the mixture became very turbid. The mixture was centrifuged, run on a gel, and Imperial stained to visualize the protein. Imperial stain was used over a silver stain due to its specificity and purity, while an immunoblot of the samples would be ineffective as the precipitate was unknown. The staining showed that after centrifugation, almost all the fibrinogen in the mixture is in the pellet while the majority of 17K I4399M remained in the supernatant. This suggests that the apo(a) is interacting with the soluble fibrinogen and causing it to precipitate. The apo(a) band in the pellet could be the result of the apo(a) that is bound to the exposed lysines of the fibrinogen molecules or potentially co-precipitation of apo(a) with the fibrinogen molecule. Precipitation of soluble fibrinogen is usually only achieved through a rapid heat-precipitation technique (177) or in the presence of a high concentration of Ca$^{2+}$ on a fibrin gel (178). Since the I4399M variant only differs by the single Met residue compared to wild-type, this residue could potentially be interacting with the fibrinogen molecule, although this has to be further analyzed. Taken together, the data suggest that the I4399M variant interacts with fibrin that results in a unique clot network with thicker fibers and aggregated nodules at branching points, and is able to cause precipitation of fibrinogen at high concentrations.
4.5 Lp(a) from Homozygous Carriers of the SNP Affect Fibrin Clot and Its Properties Similar to I4399M Apo (a) Variant

The data generated in the preceding assays were in the presence of purified 17K I4399M apo(a). Although the effect of Lp(a) should be studied in parallel with the apo(a) data, samples from carriers of this SNP were difficult to obtain due to the low frequency of this mutation, and we currently do not possess a method to produce Lp(a) in vitro. We were able to obtain 4 individual samples from homozygous carriers of the SNP to analyze with our biochemical assays. The 2nd patient sample was very turbid upon arrival, signifying the plasma was coagulated and therefore was not used. The other three samples were phenotyped through immunoblotting to determine their isoform size. Previous studies on this mutation have used patient samples in various biochemical assays and accounted for race, gender, and ethnicity, but did not match the carriers with non-carriers of similar Lp(a) levels and isoform sizes. The results of these studies could therefore be a result of the association of the mutation with higher levels of Lp(a), accounting for the increased risk, rather than observing the direct effect of the mutation itself. Therefore, we screened multiple people for the SNP by genotyping to determine suitable non-carrier controls. These samples were phenotyped and the Lp(a) levels were determined via ELISA and matched to carrier samples with similar isoform sizes and Lp(a) levels. We were able to match a non-carrier to a carrier with very low Lp(a) levels and an isoform size of 23K. Similarly, we were able to match two carrier samples to a single non-carrier sample with very high Lp(a) levels and an isoform size of 17K. While more patient samples should be used to generate more complete conclusions, matching the non-carrier and carrier samples together can provide insight into effect of the I4399M Lp(a) on the various prothrombotic mechanisms.
We started with permeation of the clots in glass tubes to determine the Darcy constants, representing the area of the pores in the clot. As previously described, wild-type Lp(a)/apo(a) has been shown to result in a tighter, denser clot with a smaller Darcy constant, which relates to its ability to inhibit fibrinolysis (19, 52, 54). The matched homozygote and non-carrier pair with low Lp(a) levels showed no difference in Darcy constant, indicating the Lp(a) levels were too low to impact the fiber network. At the higher Lp(a) concentration, the homozygote and non-carrier had similar Darcy constants that were both significantly lower than the samples with low Lp(a) levels. This would suggest that the Met variant does not significantly alter the clot permeability compared to wild-type, regardless of Lp(a) levels. However, images from SEM shows the I4399M apo(a) variant causes significantly thicker fibers than clots with non-carrier plasma. We conducted the previously described fibrinolysis assay with the paired plasma samples. Following data generation, the samples were controlled against a no-apo(a) control and shown as the relative increase in clot lysis time compared to the control. The non-carrier and homozygote pair with low Lp(a) levels did not show any difference between each other in relative lysis time, agreeing with the permeation result. The samples with high Lp(a) levels both had increased relative lysis times compared to the low Lp(a) levels, however, the homozygote had a significantly longer relative lysis time compared to the non-carrier. Taking the permeation and fibrinolysis data together, the homozygote with high Lp(a) levels agrees with the data from the assays in the presence of purified I4399M apo(a), suggesting the homozygous clots have thicker fibers in an altered fibrin network, contributing to the increased lysis times.

To strengthen the data set, the clots of these homozygote and non-carrier pairs were visualized using confocal microscopy and SEM. Confocal microscopy was utilized
first to generate a larger field of view of the clot so that the fiber densities of the clots could be determined. Labeled-fibrinogen was added to the mixture prior to clotting so that it accounted for 1/100 of the total volume as to prevent over-saturating with fluorescent signal. Following the generation of the images, ImageJ was utilized with the collaborator-designed macro to determine clot density. The macro overlays a 10x10 grid over the image and counts the number of fibers that intersects this grid, giving values of the number of fibers per 100μm. In order to eliminate background noise from the actual fibers, the images were first converted to binary before analysis. As anticipated from the clot permeation results, the homozygote and non-carrier pair with low Lp(a) levels had similar fiber counts, indicating the clots had comparable densities. On the other hand, for the samples with high Lp(a) concentrations, the non-carrier had significantly more fibers in a given field than the homozygote carriers. While the resulting Darcy constants of the wild-type and mutant clots were comparable at both Lp(a) concentration intervals, the homozygote with high Lp(a) levels clots had significantly lower fiber counts compared to the non-carrier clot with high Lp(a) levels. While the presence of less fibers in the carrier clots would suggest much permeable fibrin clots, the comparable Darcy constants indicates that the fibers in the mutant clots are altered in such a way to account for this discrepancy. It must be stated that confocal imaging of fibrin clots using fluorescently labeled fibrinogen is somewhat flawed, as it does not completely constitute all the fibers within the clot (only 1/100 of the volume) and it is much more difficult to determine fiber edges using a light based method. Therefore, SEM was also used as it provides more accurate results in terms of quantitative measurements, not only due to the difference in how images are generated, but the increased magnification compared to confocal microscopy. In order to take SEM images of the plasma clots, the clots needed to be
intensely cleaned to remove any debris or excess ions that may interfere with the imaging (179). Therefore, the clots were made in glass tubes, connected to a reservoir to permeate the clot and then removed from the tube onto a SEM peg. In keeping with the results seen from the SEM images with purified apo(a), the homozygote with low Lp(a) levels had significantly thicker fibers than in the non-carrier clot. The same trend was also seen in the pair with the high Lp(a) levels, with the homozygote having significantly thicker fibers than the non-carrier. Finally, both the homozygotes and non-carriers had thicker fibers at higher Lp(a) levels compared to low Lp(a) levels (p < 0.05 and p < 0.01, respectively). These data can be considered to be more accurate than the confocal as the SEM is able to differentiate the edges of the fibers.

Overall, the data from the Lp(a) of homozygote carriers of the I4399M mutation agree with the data from the apo(a) I4399M variant. The clots made from this variant are characterized as having altered fibrin networks, with thicker fibers and nodules at the branching points of the clot. The clots from the homozygotes have less fibers than the non-carriers at high Lp(a) levels, but have comparable Darcy constants and result in increased clot lysis times compared to wild-type. This may be explained by the fibers elongating and delaying lysis, thereby increasing lysis time (170) or by some other unknown mechanism. In terms of the risk of severe CAD associated with this mutant, we suggest that the pathogenic effects seen are a result of the I4399M variant affecting the fibrin clot network, resulting in increased clot lysis time. As well, there may be a role for the isoform size of carriers of this SNP, which can contribute to this effect.
4.6 The I4399M Variant Possesses a Methionine Sulfoxide Moiety Which May Contribute to the Prothrombotic Potential of this Variant

The preceding data suggests that the mutated Met residue in the I4399M variant of apo(a) may be contributing to the prothrombotic potential of this variant. Although unmodified Met residues are not usually involved in signaling or other pathways, exposed Met residues have the capability to become oxidized to methionine sulfoxide moieties, which can have many physiological consequences on signaling or fibrin clot properties (180). It has also been determined that several important proteins in vascular biology have been shown to contain oxidative-sensitive methionine residues that have potential regulatory roles in pathogenesis of vascular or thrombotic diseases (180). Therefore, we investigated whether the exposed Met mutant in the I4399M variant can be modified by oxidation.

The molecular dynamic simulations were conducted on the wild-type and I4399M variant of apo(a); the protease domains were exposed to hydration and average occupancy images were generated. In the wild-type apo(a), the Ile residue remained buried throughout the simulation with a proximal Arg residue at 4420 acted as a cap. In the I4399M variant, the 4420Arg residue opens, exposing the 4399Met residue to the environment. The RMSD analyzes the relative change in distance between two residues over a given time. The analysis showed that in this open conformation, there was minimal movement between the Arg and Met residues, suggesting that they may be interacting and coordinating this open conformation. While this coordination the 4420Arg residue was not confirmed, it gave the basis that the Met residue is exposed for potential modification.

To determine if a methionine sulfoxide moiety is present in the I4399M variant, we conducted mass spectroscopy on a truncated version of apo(a). This variant only
possessed the KIV\textsubscript{10}, domain, the KV domain, and the protease domain containing the mutant Met residue. This truncated variant was required for mass spec in order to produce a clear signal with less tryptic peptides to analyze. The peptide containing the mutation was a good flyer for mass spectroscopy and two theoretical masses for this peptide were generated, one for the unmodified Met residue, and one possessing the methionine sulfoxide moiety. The spectra showed an intense peak for the theoretical mass with the Met sulfoxide moiety and did not produce a visible peak for the other theoretical mass, therefore confirming the ability of the I4399M mutant to possess a methionine sulfoxide moiety. This result could help explain the pathogenic affects of this I4399M mutant, as oxidative modification can be transferred to other proteins through redox reactions (180). Studies have shown that fibrinogen can be modified by oxidation through environmental factors like smoking, altering the fibrin clot properties of these individuals (181). This suggests that the fibrinogen itself may be directly interacting with the methionine sulfoxide residue in the I4399M variant to become oxidized, or it may affect fibrinogen in a mechanism that differs from the previously described ones. Therefore, the mechanism by which the I4399M alters the fibrin network is still to be deduced.

4.7 Conclusions

This present study has demonstrated that the rs3798220 SNP in \textit{LPA}, which results in an Ile to Met at the 4399 residue of the protease domain, alters fibrin clot architecture in the vasculature, which can account for the altered prothrombotic properties of this variant. Lp(a) from carriers of the SNP and purified apo(a) containing the mutation results in thicker fibrin fibers and aggregated nodules at the branching points of a fibrin clot. Clots from individuals homozygous for the mutation with high Lp(a) levels are less
dense than clots formed from comparable individuals lacking the mutation, but are comparable with respect to permeability. As well, clots corresponding to the Met variant are significantly more turbid and possess significantly longer fibrinolysis times compared to wild-type. This variant was also found to cause precipitation of soluble fibrinogen. We did not observe an effect of the Met variant on the rate of secretion of 17K from cultured hepatocytes, suggesting that this mechanism does not explain elevated levels of Lp(a) containing this polymorphism in the human population. Upon analysis of the mutated residue, it was found that Met in the I4399M variant has the capability to become modified to a methionine sulfoxide residue. Overall, our data suggests that this modification affects fibrinogen and the formation of the fibrin clot, which accounts for the affect of the I4399M variant on coagulation and fibrinolysis. However, the exact mechanism for these effects remains to be discovered. Further studies are needed to understand the full mechanism by which the Met residue becomes oxidized, as well as how this mutated residue interacts with fibrinogen to cause the altered fibrin structure that we observed in this study.
References


63. van der Hoek YY, Wittekoek ME, Beisiegel U, Kastelein JJ, Koschinsky ML. The apolipoprotein(a) kringle IV repeats which differ from the major repeat kringle are present in variably-sized isoforms. Human molecular genetics. 1993;2(4):361-6.


75. Laplaud PM, Beaubatie L, Rall SC, Jr., Luc G, Saboureau M. Lipoprotein[a] is the major apoB-containing lipoprotein in the plasma of a hibernator, the hedgehog (Erinaceus europaeus). Journal of lipid research. 1988;29(9):1157-70.


78. Gaw A, Boerwinkle E, Cohen JC, Hobbs HH. Comparative analysis of the apo(a) gene, apo(a) glycoprotein, and plasma concentrations of Lp(a) in three ethnic groups. Evidence for no common "null" allele at the apo(a) locus. The Journal of clinical investigation. 1994;93(6):2526-34.


80. Rader DJ, Cain W, Ikewaki K, Talley G, Zech LA, Usher D, et al. The inverse association of plasma lipoprotein(a) concentrations with apolipoprotein(a) isoform size is


84. Gavish D, Azrolan N, Breslow JL. Plasma Ip(a) concentration is inversely correlated with the ratio of Kringle IV/Kringle V encoding domains in the apo(a) gene. The Journal of clinical investigation. 1989;84(6):2021-7.


86. White AL, Guerra B, Lanford RE. Influence of allelic variation on apolipoprotein(a) folding in the endoplasmic reticulum. The Journal of biological chemistry. 1997;272(8):5048-55.

87. Longenecker JC, Klag MJ, Marcovina SM, Liu YM, Jaar BG, Powe NR, et al. High lipoprotein(a) levels and small apolipoprotein(a) size prospectively predict


90. Wild SH, Fortmann SP, Marcovina SM. A prospective case-control study of lipoprotein(a) levels and apo(a) size and risk of coronary heart disease in Stanford Five-City Project participants. Arteriosclerosis, thrombosis, and vascular biology. 1997;17(2):239-45.


150. Friedman P, Horkko S, Steinberg D, Witztum JL, Dennis EA. Correlation of antiphospholipid antibody recognition with the structure of synthetic oxidized


169. Knapp JP, Herrmann W. In vitro inhibition of fibrinolysis by apolipoprotein(a) and lipoprotein(a) is size- and concentration-dependent. Clinical chemistry and laboratory medicine. 2004;42(9):1013-9.


175. Undas A, Ciesla-Dul M, Drazkiewicz T, Sadowski J. Altered fibrin clot properties are associated with residual vein obstruction: effects of lipoprotein(a) and apolipoprotein(a) isoform. Thrombosis research. 2012;130(3):e184-7.


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