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Resolving the Longitudinal Triglyceride Phenotype of Heterozygous LPL and Apo A-V Deficiency

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Abstract

Hypertriglyceridemia (HTG) is a risk factor for cardiovascular disease. However, only the triglyceride (TG) phenotype produced by biallelic loss-of-function (LOF) variants in the canonical TG metabolism genes is well understood. The TG phenotype produced by monoallelic LOF variants is poorly understood. We aimed to evaluate the TG phenotype associated with monoallelic LOF variants in the canonical TG metabolism genes.

Next-generation sequencing panel was employed to identify patients heterozygous for LOF variants in two of the canonical TG metabolism genes *LPL* and *APOA5*, followed by chart review to determine baseline and longitudinal TG phenotype in these patients.

My findings suggest that heterozygosity for LOF variants in these genes is associated with highly variable TG phenotype, both at baseline and longitudinally. Thus, my findings describe a unique, previously underappreciated TG phenotype and identification of these variants may serve as an early warning to physicians regarding difficulty in treating HTG in these patients.

Keywords

Complex traits and disease, dyslipidemia, genetics, triglycerides, hypertriglyceridemia, next-generation sequencing, rare variants, pathogenic variants, lipoprotein lipase, apolipoprotein A-V, cardiovascular disease risk.

Summary for Lay Audience

Cardiovascular diseases (CVD) are the leading cause of death and reduced quality of life globally. While effective public policy and improved emergency care and screening have reduced CVD deaths in many places, the rapidly aging and growing global population has caused these reductions to stagnate and even reverse in some areas. This has highlighted a need for better understanding of the underlying risk factors for CVD and their influences so that we can better combat CVD. One of the major risk factors for the development of CVD is elevated triglyceride (TG) levels in an individual's blood – called hypertriglyceridemia (HTG) – which have been observed to be associated with increased CVD risk. Genetics (i.e., DNA) play a prominent role in the development of HTG. DNA changes (called genetic variants) can render an encoded protein non-functional. These are called loss-of-function (LOF) variants. Given that humans have two copies of every gene, complete loss of a protein's functionality usually requires LOF variants in both copies. This is well understood to cause severe HTG resistant to treatment when it affects the proteins controlling TG levels. The impact of a LOF variant in only a single copy of these genes (called heterozygosity) on TG levels is not well understood. To better understand this, I studied the DNA and TG levels of 22 patients over varying time frames in a clinical setting who had a LOF variant in one of their TG level controlling genes and determined that LOF variants in these genes is associated with highly variable TG levels both at baseline and over time, with their TG levels often ranging between normal and extreme HTG levels, with no discernable pattern, which seems to be unique to patients with this genetic profile. Therefore, my research efforts have demonstrated clearly that heterozygosity for LOF variants in these genes produces a unique TG profile in these patients. Detection of heterozygosity for these variants may serve as an early warning for physicians regarding the potential difficulty in treating the HTG of these patients and indicate the need for careful monitoring of their TG levels.

Co-Authorship Statement

For each manuscript incorporated into my thesis, I am the first-listed author and I have indicated which manuscripts were adapted in each Chapter of this thesis. In each of the listed research articles, I contributed towards the study's design, data collection, identification of rare variants of interest to our studies, data visualization, performed any and all data analyses, and wrote each manuscript. In the review article listed, I compiled the data on each of the variants listed in that review from the literature and various genetic databases, performed all data visualization done, and I wrote more than 85-90% of the manuscript.

My supervisor, Dr. Robert A. Hegele provided excellent supervision and guidance, funding support, and patient samples and availability to clinic charts for all research analyses conducted. He also contributed to study design; advised on data collection, visualization, and analysis; and assisted greatly with manuscript preparation and revisions. These contributions were consistent across all manuscripts.

Technical assistance for each research manuscript was provided by core members of the Hegele Lab, including: 1) Adam D. McIntyre, for (i) extracting and isolating DNA from patient samples being studied, (ii) assisting data curation by assisting in pathogenicity classifications of all variants under study, and (iii) providing revisions and comments during initial manuscript preparation; 2) Dr. Jian Wang, for (i) providing access to clinical databases that were utilized during data collection, (ii) assisting data curation by assisting in pathogenicity classifications of all variants understudy, and (iii) providing revisions and comments during initial manuscript preparation, and 3) Brooke Kennedey, for maintaining all ethical protocols and study approvals. Additionally, while the results of the investigation have yet to be published, Ericka Simon assisted greatly in the investigation discussed in the sections of Chapter 3 discussing study 2 of that chapter, where she assisted greatly by providing biochemical and clinical demographic data for 366 patients from the Lipid Genetics Clinic. Dr. Jian Wang also assisted with study 2 of Chapter 3 by providing the data for the normal controls used in that investigation.

External assistance was provided by Dr. Jacqueline S. Dron (Center for Genomic Medicine, Massachusetts General Hospital, Boston, Massachusetts, USA) who (i) assisted and advised on study design for the work described in Chapter 2, (ii) assisted and advised on data analysis and visualization for the work described in Chapter 2, and (iii) provided excellent mentorship. Many of the lessons I learned from her were carried over to the investigation described in the sections pertaining to study 1 of Chapter 3.

Dedication

For Ammi, Thaththi, and Kishen.

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List of Abbreviations

ABCA1	ATP-binding cassette transporter A1
ABCG5	ATP-binding cassette transporter G5
ABCG8	ATP-binding cassette transporter G8
ACAT	Acetyl-CoA cholesterol acyl transferase
Acetyl-CoA	Acetyl Coenzyme A
ACMG	American College of Medical Genetics and Genomics
ANGPTL3	Angiopoietin-like protein 3
ANGPTL4	Angiopoietin-like protein 4
ANGPTL8	Angiopoietin-like protein 8
Apo	Apolipoprotein
APOBEC-1	Apo B mRNA editing enzyme catalytic subunit 1
ASCVD	Atherosclerotic cardiovascular disease
BMI	Body mass index
CAD	Coronary artery disease
CETP	Cholesteryl ester transfer protein
C.I.	Confidence interval
CIHR	Canadian Institutes for Health Research
CM	Chylomicron

CMR	Chylomicron remnant
CNV	Copy-number variant
CVD	Cardiovascular disease
dNTP	Deoxyribonucleotide
ddNTP	dideoxyribonucleotide
DGAT	Diacylglycerol transferase
DNA	Deoxyribonucleic acid
DNL	*De novo* lipogenesis
EL	Endothelial lipase
ER	Endoplasmic reticulum
FA	Fatty acid
FATP4	Fatty acid transport protein 4
FCS	Familial chylomicronemia syndrome
FFA	Free fatty acid
FH	Familial hypercholesterolemia
gnomAD	Genome Aggregation Database
GPIHBP1	Glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1
HDL	High-density lipoprotein
HL	Hepatic lipase

HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HSPG	Heparan sulfate proteoglycan
HTG	Hypertriglyceridemia
IDL	Intermediate density lipoprotein
Indel	Insertion or deletion
K	Kringle
LCAT	Lecithin-cholesterol acyltransferase
LCT	Long chain triglyceride
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LMF1	Lipase maturation factor 1
LOF	Loss-of-function
LOVD3	Leiden Open Variation Database 3.0
Lp(a)	Lipoprotein(a)
LPL	Lipoprotein lipase
LRP1	Low-density lipoprotein-related protein 1
MAF	Minor allele frequency
MCS	Multifactorial chylomicronemia syndrome
MCT	Medium chain triglycerides

MGAT	Monoacylglycerol transferase
MI	Myocardial infarction
MTTP	Microsomal triglyceride transfer protein
NGS	Next-generation sequencing
NPC1L1	Niemann-Pick C1 like protein 1
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin/kexin type 9
PLTP	Phospholipid transfer protein
PTV	Protein-truncating variant
RCT	Reverse cholesterol transport
SDC1	Syndecan-1
SEL1L	Sel-1 suppressor of Lin-12-Like 1
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
SorLA-1	Sortilin-related receptor
SR-BI	Scavenger receptor class B type 1
SREBP	Sterol regulatory element binding protein
SV	Structural variant
TG	Triglyceride

TGRL	Triglyceride rich-lipoprotein
UTR	Untranslated region
VLDL	Very-low-density lipoprotein
VUS	Variant of uncertain significance

Chapter 1 - Introduction

1.1 Atherosclerotic cardiovascular disease

Clinical lipidology, the study of lipids and their related biochemical processes with regards to human health, is an exponentially growing field [1] with ever-increasing importance. Several circulating lipid species, namely cholesterol, triglycerides, and their carriers in plasma, represent the most important biochemical risk factors and contributors to the development of cardiovascular diseases (CVDs), and more specifically, atherosclerotic cardiovascular diseases (ASCVDs), the leading cause of death and reduced quality of life globally [2–4].

1.1.1 Definition of atherosclerotic cardiovascular disease

CVD is the generic term for the group of diseases impacting the proper functioning of ether specific components of the human cardiovascular system (i.e., blood vessels, valves, cardiac tissue, etc.) or the whole of the cardiovascular system. ASCVD is a sub-category of CVDs that are caused by the development of atheromatous plaques on arterial blood vessel walls [5]. Atherosclerosis is the process by which these plaques form and it represents the primary risk factor for ASCVDs. Plaques, at the most basic level, are composed of a lipid-rich core separated from the blood vessel lumen by a cap of fibrillar collagen [6]. Additionally, the plaque may undergo calcification as it matures and grows but this is not necessarily correlated with plaque vulnerability [5]. These plaques can cause partial or total occlusion of the blood vessel as they grow, which may lead to catastrophic and potentially lethal loss of blood supply [5]. Additionally, as the plaque develops it may rupture and the lipid-rich thrombotic core may be released from the arterial wall leading to the production of an embolus that will circulate throughout the cardiovascular system, eventually lodging in distal arteries where it obstructs blood flow [5]. This is a blood clot [5]. Blood clots within the coronary arteries of the heart cause myocardial infarction (MI), colloquially referred to as a heart attack, and blood clots within arteries supplying the brain produce ischemic stroke which leads to potentially lethal and often debilitating loss of brain tissue [5].

1.1.2 Current statistics on atherosclerotic cardiovascular disease

While CVD mortality rates have plummeted by 40-80% in high-income countries since the 1970s [7–11] due to effective public policy and advancements in screening and emergency care [11–18], all measures of global CVD burden have significantly increased from 1990 to 2019 [19], with the primary reasons being population growth and an aging population [19]. Additionally, the burden of disease has shifted greatly to low- and middle-income countries which have not seen nearly the same degree of CVD mortality reduction as high-income countries in the same time frame. Currently, >80% of CVD cases and deaths occur in low- and middle-income countries [20]. The relatively low progress made in these countries is primarily due to the high economic burden and lack of healthcare resources available to effectively combat CVD in these countries [21]. For example, between 2011 and 2015, the economic burden due to CVDs in low- and middle-income countries was measured to be $3.7 trillion [22]. Furthermore, and rather alarmingly, it appears the long-term decline in CVD mortality in high-income countries is stagnating and in some cases reversing [11,19]. Thus, there is a renewed need for better characterization and understanding of CVD risk factors so that we may develop more effective clinical risk prediction tools and prognosis indicators, as well as designing more effective and potentially more economical CVD interventions and risk management strategies.

1.1.3 Risk factors for atherosclerotic cardiovascular disease

The primary risk factors for ASCVD include, but are not limited to, age [23,24], sex [24,25], smoking [26,27], hypertension [28–30], diabetes mellitus (primarily type 2) [31–33], obesity [34,35], and plasma lipid disturbances (dyslipidemias) [2]. Additionally, low educational, occupational, and socioeconomic status have been shown to increase prevalence for several of these CVD risk factors [36]. Dyslipidemias, namely disturbances in plasma levels of cholesterol and triglycerides (TG), and their lipoprotein carriers, represent the primary biochemical risk factor and thus, represent a particularly important target for management of ASCVD risk as they are a risk factor that is typically both highly heritable and highly modifiable. An overwhelming body of literature has established that there are 4 major classes of lipoproteins (and the lipids contained within

them) that are implicated in determining ASCVD risk associated with dyslipidemias: 1) low-density lipoproteins (LDL) [37–41], 2) high-density lipoproteins (HDL) [42,43], 3) lipoprotein (a) [Lp(a)] [44], and 4) triglyceride (TG)-rich lipoproteins (TGRLs) [45–48].

There is a wealth of clinical, genetic, mechanistic, and epidemiological evidence and a consensus agreement that the primary causal factor for the development of ASCVD is elevated LDL [39,41]. The mechanistic role of LDL in the disease initiation and progression of atherosclerosis and ASCVD has been well established, though some of the specific molecular and biochemical interactions are still being investigated [41]. Briefly, a combination of stressors such as, but not limited to, low wall shear stress (i.e., the frictional force blood flow exerts on the blood vessel walls) [49–54], local (i.e., vascular endothelium) and systemic inflammation [55], hypertension [56], diabetes mellitus [57], and genetic susceptibility of the arterial wall to pro-atherogenic processes [58,59] can damage and/or prime sites along arterial walls such that in the event that LDL levels are elevated, as is the case in hypercholesterolemia, the most common form of dyslipidemia [37], the cholesterol-rich LDL particles are actively transported to the arterial intima where they accumulate [41]. This is the initiating event for atherosclerotic plaque formation. Given its importance as the primary driving force of atherosclerosis as well as the prevalence of hypercholesterolemia as the most common form of dyslipidemia, a large array of pharmaceutical interventions have been discovered and invented over the last few decades for the treatment of elevated LDL and consequently, elevated LDL-cholesterol (LDL-C) levels. Most notable are the statins [60], Niemann-Pick C1-Like 1 (NPC1L1) inhibitors [61], and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors [62], which have allowed for extremely effective treatment and reduction of ASCVD risk and events. In summary, the role of LDL and LDL-C in ASCVD disease risk and progression has been firmly established and a wide array of treatment options are available for the management of LDL and LDL-C associated ASCVD risk.

The negative correlation between plasma HDL-C levels and ASCVD risk has been well-established for decades now [42,43,63]. Consequently, increasing plasma HDL-C concentrations was considered a target of great interest for lowering ASCVD risk for many years. However, clinical trials of HDL-C raising therapies, namely extended-

release niacin and cholesteryl ester transfer protein (CETP) inhibitors, have failed to demonstrate any reduction in ASCVD events due to raising HDL-C concentrations [64–68]. Additionally, there is a mounting body of evidence indicating that the correlation between HDL-C levels and ASCVD morbidity and mortality follows a U-shaped association, in which both extremely low and high HDL-C levels are considered indicative of ASCVD risk [69–72]. Additionally, as reviewed by Kjeldsen and colleagues, several mendelian randomization studies have been conducted to assess the role of HDL-cholesterol concentration in causing ASCVD and the majority consensus from these studies is that plasma HDL-cholesterol concentration itself may not be a causal factor for ASCVD [63]. Instead, it is now thought that HDL functionality may be a more relevant target for HDL-based therapies rather than HDL-C specifically. For example, HDL particles facilitate reverse cholesterol transport (see section 1.2.2.3 for more details) which is a process that can remove excess cholesterol from various cells and redirect that cholesterol to be excreted from the liver through the bile and ultimately excreted from the body altogether through the feces [73]. This is particularly promising as cholesterol-laden foam cells, a characteristic component of atherosclerotic plaques, can have their cholesterol content reduced via this process, as reviewed by Ouimet and colleagues [74]. However, there is a lack of definitive research demonstrating that raising the level of functional HDL (in reference to its reverse cholesterol transport functionality) is a viable therapy option for managing ASCVD risk, though there have been some promising clinical study results [75–78]. Taken together, these data have revealed a need to identify and better understand how HDL composition, structure, plasma concentration, and functionality are mechanistically linked to ASCVD progression. Thus, the presence and extent of a causal relationship between extreme HDL-C concentrations in plasma and ASCVD remains unclear [42,63].

A wealth of epidemiological, genome-wide association, and Mendelian randomization studies have established Lp(a) as an independent, causal, and almost exclusively genetically-determined risk factor for the development and progression of ASCVD [44,79–83]. Given its structural similarities to LDL (i.e., apoB100-containing lipoprotein with a primarily cholesterol ester core), elevated Lp(a) levels contributes to ASCVD in

similar ways, but the apolipoprotein(a) [apo(a)] component of Lp(a) that differentiates it from LDL also confers unique pro-atherogenic properties to Lp(a) [44]. Specifically, Lp(a) has been found to have unique pro-inflammatory and pro-calcific functionalities in ASCVD progression. Firstly, despite being found in lower numbers compared to LDL even in high Lp(a) conditions, Lp(a) has been found to be preferentially retained in the arterial wall over LDL during plaque formation due to the apo(a) component found in them binding to extracellular matrix proteins [84,85]. Secondly, Lp(a) can be considered an aggregator of oxidized phospholipids which have pro-inflammatory and pro-calcific properties in the ASCVD process. Apo(a) covalently binds to oxidized phospholipids and Lp(a) particles also contain oxidized phospholipids in their lipid cores. Consequently, Lp(a) exerts significant pro-atherogenic effect through the accumulation of these oxidized phospholipids in the arterial wall during plaque formation [86]. Additionally, Lp(a) has been found to attract monocytes to them and as a consequence, promote inflammation at the arterial wall when the Lp(a) is lodged there [87]. Lp(a) may also contribute to atherothrombotic disease (i.e. plaque rupture and clot/embolus formation) but this is still the subject of investigation [88]. In conclusion, Lp(a) is now a well-established ASCVD risk factor with more potential roles in atherosclerotic and atherothrombotic disease under investigation.

Finally, the existence of a causal relationship between elevated plasma TG levels and ASCVD is not clear and has been the subject of extensive debate. Recently, analysis of randomized control trial results established that elevated TGRL levels in plasma are associated with residual ASCVD risk in patients after risk reduction due to LDL-C lowering therapies [89–92], an association that is supported by Mendelian randomization studies as well [93–96]. However, plasma TG level alone is inconsistently associated with ASCVD. Specifically, while univariate analyses consistently demonstrate association between elevated plasma TG and ASCVD risk, these associations become non-significant after adjustment for total cholesterol and/or LDL-C, as reviewed by Singh and Singh [97]. Additionally, an assessment of over 68 prospective studies accounting for >300 000 participants also found that association of elevated TG with coronary artery disease (CAD) risk was rendered non-significant after adjustment for HDL-C and non-

HDL-C [98]. Recently, it has been hypothesized that rather than the TG component of TGRLs, the remnant cholesterol present in these lipoproteins is more important for the atherogenic potential of TGRLs [99]. There is a mounting body of evidence to support this hypothesis [47,89–91,100,101], explaining why triglycerides alone are not consistently associated with ASCVD. Thus, rather than being atherogenic agents themselves, which has long been suspected, TGs serve as a biomarker for the concentration of TGRL and TGRL remnants in plasma. It should be noted that while elevated TG itself may not be directly atherogenic, severe elevations in plasma TG levels, specifically greater than or equal to 10 mmol/L, are a significant risk factor for development of acute pancreatitis [102]. Given that elevated TGRL concentrations likely explain the extensive residual risk for ASCVD observed in many patients with well-controlled LDL-C levels, it is now exceedingly necessary to establish a strong understanding of the genetic and biochemical mechanisms at play controlling TGRL levels in plasma and how these may impact ASCVD progression. This will enable us to identify early markers of altered TG metabolism as well as new potential pharmaceutical targets. Expanding our understanding of the underlying causative and contributing factors to altered TG metabolism will enable early disease identification and intervention, better and more efficient screening and monitoring strategies, and should enable us to tackle the current stagnation in global ASCVD risk and mortality reduction.

1.2 Triglyceride Metabolism

Triacylglycerols aka triglycerides (TG) are a class of non-polar lipid molecules comprising the most abundant form of fat in human adipose tissue and invariably make up the vast majority of dietary fat intake in humans. Structurally, TGs are composed of a glycerol backbone esterified to 3 fatty acid (FA) molecules (**Figure 1.1**). The properties of these FA components can be used to further classify specific TG species. Most commonly, triglycerides can be classified as saturated, if there are only carbon-carbon single bonds in the FA components or unsaturated if there are carbon-carbon double bonds in the FA components of TGs. Unsaturated TGs and FAs can be further classified as monounsaturated (only a single carbon-carbon double bond) or polyunsaturated (more than 1 carbon-carbon double bond). Additionally, TG can be further classified based on

the length of the aliphatic chains of the FA. Long chain triglycerides (LCTs) are defined when the aliphatic chains of the FA components are >12 carbons long whereas medium chain triglycerides (MCTs) are defined as have FAs with aliphatic chains of length 6-12 carbons.

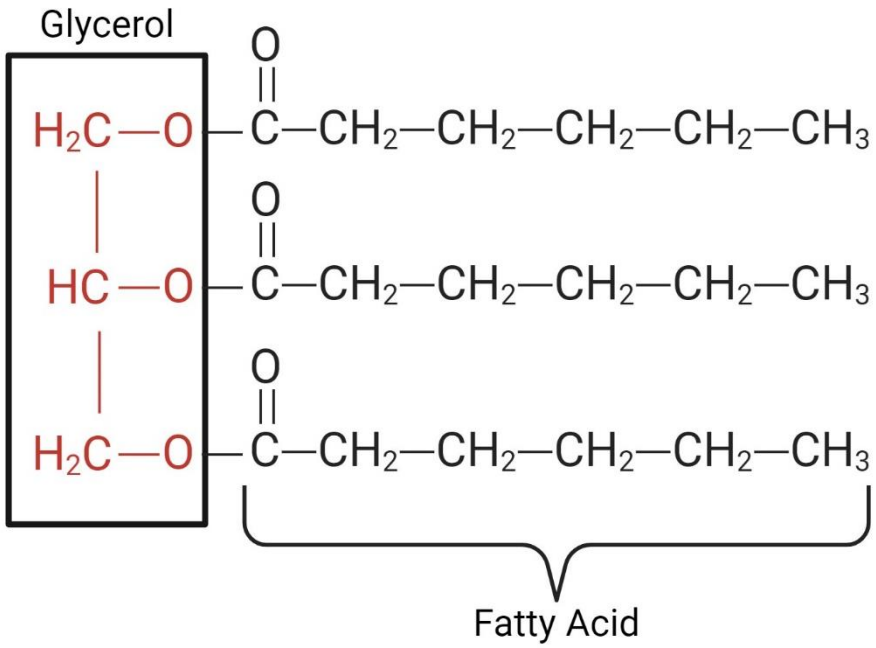

Figure 1.1. Chemical structure of triglyceride molecule.

Triglycerides (TG) are a class of organic ester molecules composed of a glycerol backbone linked to 3 fatty acids (FA). The 3 fatty acid substituents of a TG molecule do not necessarily have to be identical and are usually different from one another. The length of the FA chains is variable. Long-chain TGs are defined as TG with FA chains with >12 carbons. Medium chain TGs contain FA chains with 6-12 carbons. Depicted in the figure is an example of a medium chain TG. Created with Biorender (https://www.biorender.com/).

TGs play several vital roles in human physiology and biochemistry. They are used primarily as a form of energy storage but also as a form of transport for FAs both intracellularly and in plasma. TGs used for energy storage are stored primarily in adipose tissue which release the TG into circulation upon hormone signaling (such as glucagon and epinephrine) [103] when needed by skeletal muscle, cardiac muscle, and kidneys when glycogen stores become scarce and gluconeogenic pathway precursors are not available. The FA components of circulating TG are separated from the glycerol backbone via hydrolysis. These free fatty acids (FFAs) are the taken up by oxidative tissues (skeletal muscle and cardiac muscle) where the FFAs undergo fatty acid oxidation, primarily beta-oxidation, to produce acetyl coenzyme A (acetyl-CoA) which is a vital molecule utilized in the Krebs cycle and mevalonate pathway to produce energy [104]. The secondary function of TGs to transport FAs is due to the diverse and varied roles FAs play in a multitude of cellular, biochemical, and physiological processes and the need to store and transport these FAs as needed by the body. FAs have structural roles as components of phospholipids that form cellular membranes, metabolic roles as an important source of energy (see above), and a number of FA species are known to regulate the activity and/or expression of some transcription factors, meaning FAs also impact gene expression [105]. FAs and TGs can be synthesized by the body, but the primary source is dietary intake of, mainly, animal and vegetable fats.

1.2.1 Sources of plasma triglycerides

1.2.1.1 Exogenous sources

TGs are the major form of dietary lipid intake and are found primarily in 2 sources in the diet, animal fats and vegetable oils. Following ingestion of these foods, gastric lipases in the stomach break down TGs to form diacylglycerols and FFAs [106]. These partial glycerides and FFAs help facilitate and begin the emulsification of dietary lipid content. Upon gastric emptying into the duodenum of the small intestine, hepatically synthesized bile acids further emulsify the dietary lipid content, composed primarily of TGs and TG-derived molecules (partial glycerides and FFAs), dietary cholesterol, plant sterols, fat-soluble vitamins and phospholipids [107]. This emulsification enhances the lipolytic activity of pancreatic lipases which are secreted into the duodenum of the small intestine.

Pancreatic lipases hydrolyze TGs to monoacylglycerol and FFAs. Dietary cholesterol, usually found in the form of cholesterol esters, are hydrolyzed as well to free cholesterol and FAs by cholesterol esterase. The free cholesterol is incorporated into forming mixed micelles which also contain bile acids, phospholipids, FFAs and monoacylglycerols [106,107]. These micelles and their contents are transported across the intestinal unstirred water layer to the brush border of the enterocytes for uptake.

Cholesterol absorption by enterocytes is well understood. The majority of enterocyte cholesterol absorption is mediated by NPC1L1, accounting for ~70% of cholesterol absorption in mice [108,109]. Conversely, FFA uptake is not as well understood. FFAs are absorbed from the intestinal lumen into enterocytes via passive diffusion through the apical membrane when luminal FFA concentrations are greater than enterocyte FFA concentrations. But they are also absorbed via active transport mechanisms. These are not well understood but it is hypothesized by some that these mechanisms exist to ensure that essential FAs (FAs our body needs for various functions but cannot synthesize on its own) are still taken up even if there is low luminal FFA concentrations, though this hypothesis still requires further study as noted by Ko and colleagues [106]. There are two main proteins implicated in the FFA enterocyte uptake process. First is CD36, which is highly expressed in the small intestine, especially the proximal third, and highly localized to the villi of the small intestine [106,107]. However, the exact mechanisms at play are not well understood. Second is fatty acid transport protein 4 (FATP4). Interestingly, while mouse knockdown models of *FATP4* demonstrate reduced FA uptake by enterocytes [110], this effect is not due to the transport function of the protein but instead due to the enzymatic activity of FATP4 which is involved in intestinal phospholipid and TG synthesis via catalyzing the esterification of FAs with coenzyme A [111]. However, the essentiality of FATP4 is questionable as studies that have investigated complete knockout in small intestinal tissue have observed no change in lipid absorption compared with wild-type mice [112].

After uptake into enterocytes, TGs and cholesterol esters are re-synthesized for lipoprotein, specifically chylomicron, assembly and subsequent transport to the

circulation. Chylomicron assembly and secretion into circulation are discussed in section 1.2.2.1. Chylomicron structure and function are further summarized in section 1.2.3.1.

1.2.1.2 Endogenous sources

For both TG and cholesterol, endogenous synthesis occurs in the liver. With regards to TG, in summary, when carbohydrates are abundant, the hepatocytes initiate *de novo* lipogenesis (DNL), which converts glucose to FA [113] and glycerol generated from the normal glycolysis pathway can be utilized along with the generated FAs to synthesize TGs [114]. With regards to cholesterol, *de novo* cholesterol synthesis occurs in conditions of low intracellular free cholesterol concentrations, specifically, low endoplasmic reticulum (ER) free cholesterol levels [115]. In such conditions, in summary, inactive sterol regulatory element-binding protein 2 (SREBP2) located on the ER membrane is triggered to undergo a maturation process that ultimately results in upregulation of several cholesterol metabolism regulators, including 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase which catalyzes the rate limiting step of the *de novo* cholesterol synthesis pathway by catalyzing a reaction that forms mevalonate from a precursor molecule whose synthesis was initiated using acetyl-CoA as a starting molecule [115]. The mevalonate is then converted to 2 activated isoprenes which then undergo a series of condensation reactions that produces squalene, a 30-carbon linear precursor to all steroid molecules, including cholesterol [115]. The squalene then undergoes several more reactions before finally being transformed into cholesterol [115]. These hepatically synthesized TGs and cholesterol molecules are then assembled into hepatically-derived lipoproteins and secreted into circulation. The 2 major lipoproteins synthesized and assembled in the liver are LDLs and very-low-density lipoproteins (VLDL). Given that LDLs and Lp(a) are not major players in the transport and metabolism of TGs, they are beyond the scope of this thesis and will not be discussed further in detail. VLDL, however, like chylomicrons, are TG-rich lipoproteins and their assembly and metabolism are discussed in section 1.2.2.2 and their structure and functions are further summarized in section 1.2.3.2.

1.2.2 Lipoprotein metabolism in general

TGs and cholesterol are highly non-polar molecules and are thus insoluble in blood plasma which is highly polar. To transport non-polar lipids throughout the circulation, the human body packages them into amphipathic macromolecule complexes of lipid and protein called lipoproteins. The generic structure of all lipoproteins is that they have a hydrophilic surface membrane surrounding a hydrophobic lipid core, enabling the transport of non-polar lipids through the circulation. The hydrophilic surface membrane is composed of a phospholipid monolayer incorporated with cholesterol and a special class of proteins known as apolipoproteins (apo). Apolipoproteins play structural and metabolic roles and different lipoprotein classes possess distinct apolipoprotein profiles. The hydrophobic core contains TG and cholesterol esters.

There are seven major classes of lipoproteins, distinguished by their size, specific lipid composition, apolipoprotein profile, and in some cases, electrical charge: 1) chylomicrons (CMs), 2) chylomicron remnants (CMRs), 3) very-low-density lipoproteins (VLDL), 4) intermediate-density lipoproteins (IDLs), 5) low-density lipoproteins (LDLs), 6) high-density lipoproteins (HDLs), and 7) lipoprotein(a) [Lp(a)] (**Figure 1.2**) [107,116]. CMs and VLDL particles are the primary transporters of TG in plasma and are classified as TG-rich lipoproteins (TGRLs). HDL particles may sometimes carry TG as well depending on the action of specific enzymes, such cholesterol ester transfer protein (CETP) which can transfer TG from other lipoproteins to HDL in exchange for cholesterol esters carried by HDL particles (see section 1.2.2.3 for more details). CMRs, IDLs, LDL, and Lp(a) are not majorly involved in TG metabolism and contain very little TG themselves and as such, will not be discussed further as this thesis revolves around aspects of TG metabolism. CMRs and IDLs will be mentioned in sections 1.2.2.1 and 1.2.2.2 as they are the products of CM metabolism and VLDL metabolism, respectively.

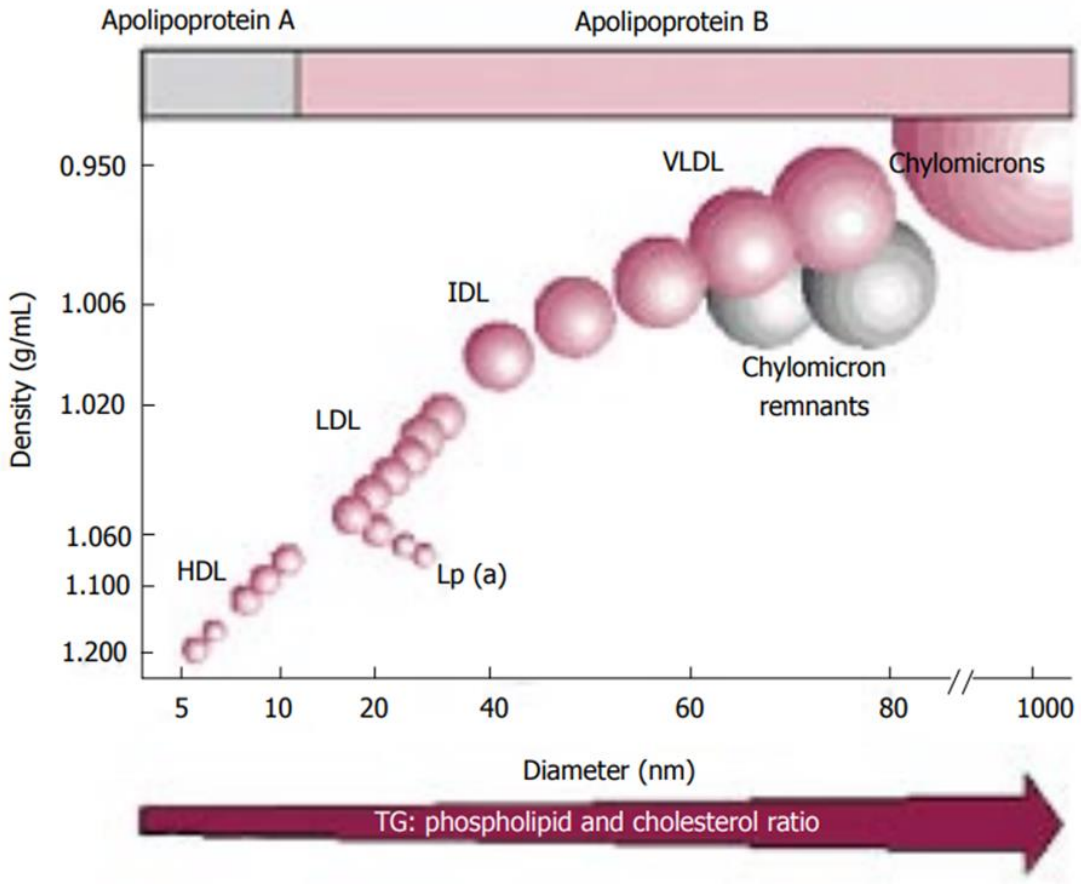

Figure 1.2 Size and density distribution of various lipoprotein species.

Lipoproteins are classified primarily according to 4 factors: size, density, lipid composition, and apolipoprotein profile. Rarely, electrical charge differences may also be utilized to classify lipoproteins. Abbreviations: VLDL = Very low-density lipoprotein, IDL = Intermediate density lipoprotein, LDL = Low-density lipoprotein, Lp(a) = Lipoprotein(a), and HDL = High-density lipoprotein. Figure is adapted from: Sabnis N, Bowman W, Lacko A. Lipoprotein based drug delivery: Potential for pediatric cancer applications. World J. Pharmacol. 4, 172 (2015) [117].

As discussed in detail in sections 1.2.1.1 and 1.2.1.2, TGs have two sources, dietary lipid intake (exogenous) and *de novo* hepatic TG synthesis (endogenous). CMs (see section 1.2.2.1 for more details) are responsible for exogenous TG transport from the small intestine to the rest of the body while VLDL particles (see section 1.2.2.2 for more details) transport endogenous TG from the liver to the circulation. HDL plays a relatively minor role in plasma TG metabolism in comparison to CM and VLDL which are responsible for the vast majority of plasma TG transport.

1.2.2.1 Chylomicron metabolism

Following absorption of dietary lipid intake by enterocytes of the small intestine (see section 1.2.1.1 for more details), TGs and cholesterol esters are re-synthesized from the absorbed chemically digested lipids. Briefly, monoacylglycerol acyltransferases (MGAT) and diacylglycerol transferases (DGAT), endoplasmic reticulum transmembrane proteins with active site facing the cytosol [118,119], catalyze the synthesis of TG from absorbed FFAs and 2-monoacylglycerol [120]. Specifically, MGAT catalyzes the addition of one FFA molecule to a molecule of 2-monoacylglycerol molecule to form a diacylglycerol molecule [120]. Then DGAT catalyzes the addition of one FFA molecule to diacylglycerol to form triacylglycerol aka TG [119,120]. Cholesterol absorbed by the enterocyte has one of two fates. Around 50% will be re-secreted back into the intestinal lumen [120] while most of the remaining absorbed cholesterol is utilized to synthesize cholesterol esters. ATP binding cassette subfamily G (ABCG) member 5 (ABCG5) and member 8 (ABCG8), which are enterocyte apical surface transmembrane proteins, facilitate cholesterol efflux from enterocytes back into the intestinal lumen by utilizing energy from ATP hydrolysis to pump cytosolic cholesterol across the apical membrane of enterocytes (and in hepatocytes as well but this functionality is not relevant here) [121]. Cholesterol ester synthesis in enterocytes is facilitated by the action of acyl-CoA cholesterol acyl transferases (ACATs), transmembrane endoplasmic reticulum proteins with their active sites facing the cytosol. ACATs catalyze an esterification reaction between FFA and absorbed free cholesterol [119,120,122].

In the fed state (i.e. proceeding absorption of dietary lipids), TG and cholesterol esters synthesized in the enterocyte are then used to assemble chylomicrons in the ER of these cells [123,124]. This occurs via two major steps [125]. Broadly, the first step is the formation of a primordial CM around apolipoprotein B-48 (apoB48) as it is being translated and transported into the ER lumen via the translocons [125,126]. ApoB48 is an isoform of the full apolipoprotein B protein, dubbed apoB100, that acts as the major structural component of CMs. Briefly, in the enterocytes of the small intestine in humans, the apoB100 mRNA is transcribed but then edited by apoB mRNA editing enzyme catalytic polypeptide 1 (APOBEC-1) such that a cytosine residue at position 6666 of the apoB100 mRNA is deaminated and converted to uracil, producing a UAA stop codon that results in a apoB protein 48% the length of the full apoB100 protein [127–129]. The primordial CM consists of the mature apoB48, phospholipids, cholesterol (utilized in the membrane of the primordial CM), and minor amounts of TG and cholesterol ester. Then, in the smooth ER, TG and cholesterol esters synthesized in the ER membrane are transported into the ER lumen and via the action of microsomal triglyceride transfer protein (MTTP) the lipid is moved to assemble around the apoB48 backbone of the primordial CM. MTTP is vital to this process and loss of MTTP leads to inability to form CMs [107]. This structure before exiting the ER is called the pre-chylomicron particle. After formation of the pre-chylomicron particle, it is transported to the Golgi apparatus and then to the enterocyte cytoplasm where several lipoproteins other than apoB48 are added to it [130]. Specifically, apolipoproteins A-I (apo A-I), A-IV (apo A-IV), and A-V (apo A-V) are added to the pre-chylomicron particle to form the mature CM particle [130]. The size and composition of the CM via this process is determined by the amount of dietary lipid ingested and absorbed by the enterocyte as well as the type of lipid absorbed [107,124]. It should be noted that in the fasted state, a separate process generates primarily VLDLs in the enterocytes [131], though not all of the mechanistic details of this process appear to be known yet to the best of our knowledge, though there is good evidence that this occurs via a separate pathway than CM assembly [124].

After formation of the mature CM, it is secreted from the basolateral membrane of the enterocytes of the small intestine into the lymphatic system which eventually allows the

CMs to enter the circulatory system as the lymph eventually drains into circulatory system via the thoracic duct which empties into the junction of the left subclavian and internal jugular veins [107,132]. During this process, the CMs are further modified via the addition of more apolipoproteins, namely apolipoproteins C-II (apo C-II), C-III (apo C-III), and E (apo E) [107].

Upon entering the circulation, mature CMs will eventually encounter lipoprotein lipase (LPL), the primary enzyme responsible for catalyzing the hydrolysis of TG in TG-rich lipoproteins to FFAs and glycerol [133]. This is the rate limiting step in the clearance of TG from circulation [133]. LPL is found anchored to the luminal surface of endothelial cells found in capillaries of skeletal muscle, cardiac muscle, and adipose tissue [133]. Adipose tissue primarily utilizes the liberated FFAs and glycerols for energy storage. In the muscle tissues, it is typically utilized immediately for energy or for various other metabolic processes.

The catabolic action of LPL is regulated by the activity and/or association of several major apolipoprotein species found on the circulating CMs and the actions/functions of several other proteins to ensure that LPL is expressed on the endothelial cell surface and anchored appropriately. Firstly, regarding the apolipoproteins involved, apo C-II and apo A-V, both found on CMs, are required for both initiation and efficient catabolic activity of LPL [134]. Apo C-II has long been recognized as necessary for the interaction of circulation TG-rich lipoproteins with LPL [134]. The exact mechanism by which apo C-II does this is not fully understood or known. Apo A-V (discussed at length in chapter 3) is also involved in the activation and enhancement, both direct and indirect, of LPL. Of its 4 known major functions in regulating plasma TG levels, 2 are related to LPL [135]: 1) apo A-V is thought to directly enhance LPL activity by strengthening the association of apo A-V containing lipoproteins with endothelial cell surface components associated with LPL [140–142] and 2) indirect enhancement of LPL activity by competing with LPL for binding to a unique LPL-inhibitory epitope present in the angiopoietin-like protein 3/8 (ANGPTL3/8) complex [139,140]. The direct enhancement function of APOA5 is specifically thought to be due to the interaction of a positively charged apo A-V region

interacting with an acidic region of glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 (GPIHBP1) [140–142].

Apart from the apolipoproteins discussed above, 2 other proteins are essential for the proper expression and activity of LPL. Firstly, lipase maturation factor 1 (LMF1) is a membrane-bound chaperone protein expressed in the ER that ensures that ensures that LPL folds correctly upon translation [141]. Secondly, GPHBP1 serves two primary functions that facilitate the catabolic activity of LPL: 1) following LPL synthesis and secretion into the extra cellular space around endothelial cells, GPIHBP1 binds LPL and transports it across endothelial cell membrane to the luminal endothelial cell surface [142], and 2) as discussed above, GPIHBP1 is responsible for anchoring LPL to the luminal surface of endothelial cells after it transports LPL from the extracellular space to the luminal endothelial cell surface [142].

After the clearance of TGs from a CM, the remaining particle/complex is considered a CMR (one of the seven major lipoprotein classes outlined in section 1.2.2). With regards to major structural differences between CMs and CM remnants, CM remnants lack apo C-II, which is removed from CMs by HDL particles (discussed in section 1.2.2.3) [143] and are enriched in apo E by interaction with HDL particles [107,144]. Regarding their lipid composition, CMRs have little TG content, and their non-polar cores are primarily composed of cholesterol esters. CM remnants are cleared from circulation by hepatocytes via receptor-mediated endocytosis initiated by interaction of apo E with, primarily, the LDL receptor (LDLR) expressed on the hepatocyte cell surface [107,145]. The endocytosed CMRs deliver dietary cholesterol to the liver. CMRs are hydrolyzed within hepatocytes and the liberated cholesterol esters are utilized for one of 3 major functions: 1) VLDL synthesis, 2) bile acid synthesis, or 3) direct secretion back into the small intestine via the bile [107].

1.2.2.2 Very-low-density lipoprotein metabolism

While VLDL is synthesized by enterocytes in fasting conditions [131], the predominant source of VLDL found in circulation is the liver. VLDL is the primary agent by which endogenously synthesized lipids are exported from the liver (see section 1.2.1.2 for more

details on endogenous sources of lipids) into the circulation. VLDL, like CMs, is classified as a TG-rich lipoprotein. Like CMs, apoB also constitutes the primary structural apolipoprotein feature of VLDLs [146]. The key difference though is that VLDLs contain apoB100 and not apoB48. This is because APOBEC-1 is not expressed in hepatocytes in humans [146].

Just like in enterocytes, within the ER of hepatocytes, hepatic MTTP mediates the assembly and aggregation of TG, cholesterol esters and phospholipids to the apoB100 backbone to form the pre-VLDL particle [107,146]. The TG incorporated into VLDLs is of primarily endogenous origin, but the cholesterol esters may be of either endogenous origin or taken from the endocytosed CM remnants. Like the process pre-chylomicron particles undergo in enterocytes, as the pre-VLDL particle is transported through the ER lumen and subsequently the hepatocyte to its secretory pathway, it incorporates several apolipoproteins, namely apo C-II, C-III, and E. However, unlike CMs, once VLDLs enter circulation they may import additional cholesterol esters from circulating HDL particles (discussed in more detail in section 1.2.2.3) through the action of cholesterol ester transfer protein (CETP) in exchange for TGs [147]. This produces TG-enriched HDL particles and cholesterol-enriched VLDL and LDL particles.

Upon secretion into circulation from the liver, VLDL particles undergo essentially the same metabolic pathway that CMs undergo, which is discussed in detail in section 1.2.2.1. Similar to CMs, after hydrolysis and clearance of their TG content, VLDLs shrink and lose some of their apolipoproteins and surface lipids features [107]. These VLDL remnants, called IDLs, also undergo the same apo E enrichment that CMRs do through interaction with HDL particles [107,144]. This allows IDLs to be taken up by hepatocytes like CMRs via the same pathway. However, IDLs are not as readily sequestered from circulation via this pathway as CM remnants are. Rather, most IDLs undergo further hydrolysis of their remaining TG content by hepatic lipase (HL) and thus undergo further modification of their surface features by transference of most of the remaining exchangeable apolipoproteins to other circulating lipoprotein particles, leaving apoB100 as the only remaining apolipoprotein [91,107,148]. This results in the formation of LDL particles. LDL particles are predominantly composed of cholesterol esters

assembled around an apoB100 backbone. As discussed in section 1.1.3, LDL particles and specifically LDL-C (LDL cholesterol) are one of the best established atherogenic lipid agents/species.

1.2.2.3 High-density lipoprotein metabolism

HDLs are a highly important yet poorly understood lipoprotein species as mentioned in section 1.1.3. This class of lipoproteins is extremely heterogenous, with extensive intra-particle variation observed in protein composition, lipid composition, shape, size, and electrical charge [147]. Subsequently, there are two major sub-categories of HDL particles based on their density. HDL_2 refers to larger less-dense (i.e. higher lipid-to-protein ratio) particles whereas HDL_3 refers to smaller, more dense particles (i.e. lower lipid-to-protein ratio) [147]. Additionally, unlike the previously discussed CMs, CMRs, VLDLs, IDLs, and LDLs, HDL particles do not contain any apoB isoform. Instead, the primary structural apolipoprotein in HDLs is apo A-I [147].

HDL particle assembly begins with apo A-I synthesis. Apo A-I is primarily expressed and synthesized in enterocytes of the small intestine (as previously mentioned in section 1.2.2.1) and hepatocytes. Intestinal apo A-I is a component of mature CMs but is released from CMs into the plasma after they undergo LPL-mediated hydrolysis of their TG content [149–151]. Ultimately, both intestinal and hepatic apo A-I are secreted into plasma, not bound to any lipid. The lipid poor apo A-I secreted into plasma interacts with cell membranes throughout the body where ATP binding cassette transporter (ABCA1) effluxes free cholesterol and phospholipids from cell membranes. The lipid poor apo A-I interacts with these effluxed lipids to form a discoidal proto-HDL particle with two apo A-I molecules [147]. The discoidal shape is due to the particle currently lacking a cholesterol ester core as all of the effluxed cholesterol from ABCA1 is free cholesterol that is incorporated into the surface membrane of the discoidal HDL particle [147]. Hepatically synthesized and secreted into circulation lecithin cholesterol acyltransferase (LCAT) is activated by the apo A-I component of the discoidal HDL particle which leads to esterification of the free cholesterol content of the discoidal HDL particle with FAs derived from phospholipids, ultimately leading to the formation of a cholesterol ester

core [107,147]. This leads to transformation of the discoidal HDL particle to proper, spherical HDL particles. Initially, these particles are of the HDL_3 subclass but as the HDL particle acquires more free cholesterol, which LCAT converts to more cholesterol esters, and phospholipid, from the action of phospholipid transfer protein (PLTP) which catalyzes the transfer of multiple lipid species including phospholipids between lipoprotein particles, the HDL acquires enough lipid that it can be classified as HDL_2 [147]. HDL_2 particles may be modified by cholesterol ester transfer protein (CETP). Specifically, CETP, synthesized primarily in the liver and secreted into circulation bound to HDL particles, catalyzes the transfer of cholesterol esters and TG between lipoproteins [152]. CETP catalyzes the transfer of cholesterol esters from the bound HDL particle to other lipoproteins such as CM, CMR, VLDL, IDL, and/or LDL in exchange for TG [152]. The HDL_2 particles enriched with TG from the action of CETP may undergo hydrolysis of its new TG content by HL or hydrolysis of its phospholipid content by endothelial lipase (EL) [107]. The catabolic activity of these enzymes degrades the HDL_2 particle back to the smaller, denser HDL_3 subclass. Finally, HDL particles may interact with scavenger-receptor class B type I (SR-BI), which is a cell membrane protein found on a large array of tissues including but not limited to liver, macrophages, and monocytes. SR-BI enables selective uptake of HDL cholesterol (HDL-C), specifically cholesterol esters by the liver.

The sum of the actions of HL, EL, and SR-BI interacting with HDL particles is the production of smaller HDL particles from formerly large ones. These smaller particles continue to circulate and repeat the above-described processes.

Reverse cholesterol transport (RCT) can be said to be the primary function of the HDL particle life cycle described above. Briefly, as the HDL particle goes through its life cycle, it uptakes cholesterol (and phospholipid and sometimes TG as well) from various peripheral tissues and ultimately, through the interaction with SR-BI, deposits the cholesterol into the liver for excretion or production of either bile acids or bile [147]. While this area has been of particular interest in ASCVD research, as highlighted in section 1.1.3, our understanding of HDL is poor and requires more investigation.

However, this functionality of the HDL life cycle does show promise for both treating and understanding the atherosclerotic process better.

1.2.3 Structure of lipoproteins

1.2.3.1 Chylomicrons

CM is the lipoprotein that transports absorbed dietary lipids from small intestine enterocytes to the rest of the body [153]. CM are the largest lipoprotein class by size, with average diameters ranging vastly from 75 to 1200 nm [107,154] with the amount and type of dietary lipid, especially TG, intake [155–157] and the rate of lipid absorption [153] determining the size. Mature CM contain apo A-I, A-II, A-IV, A-V, B48, C-II, C-III, and E [107,153]. CM contain exactly one apoB48 molecule which acts as the main non-exchangeable and core structural protein of CM [158]. Chylomicrons, morphologically, are spherical and macroscopically amphipathic complexes [119]. CM have a highly non-polar, TG and 2-monoacylglyceride rich core with some cholesterol ester content surrounded by a phospholipid monolayer with the hydrophilic face of the phospholipids facing outwards towards the plasma while the hydrophobic tails of the phospholipids face inwards towards the lipid core [107,119,153]. The phospholipid monolayer also contains free cholesterol, which functions similarly to its role in regulating cell membrane fluidity and stability. **Figure 1.3** shows a diagram of the structure of CM.

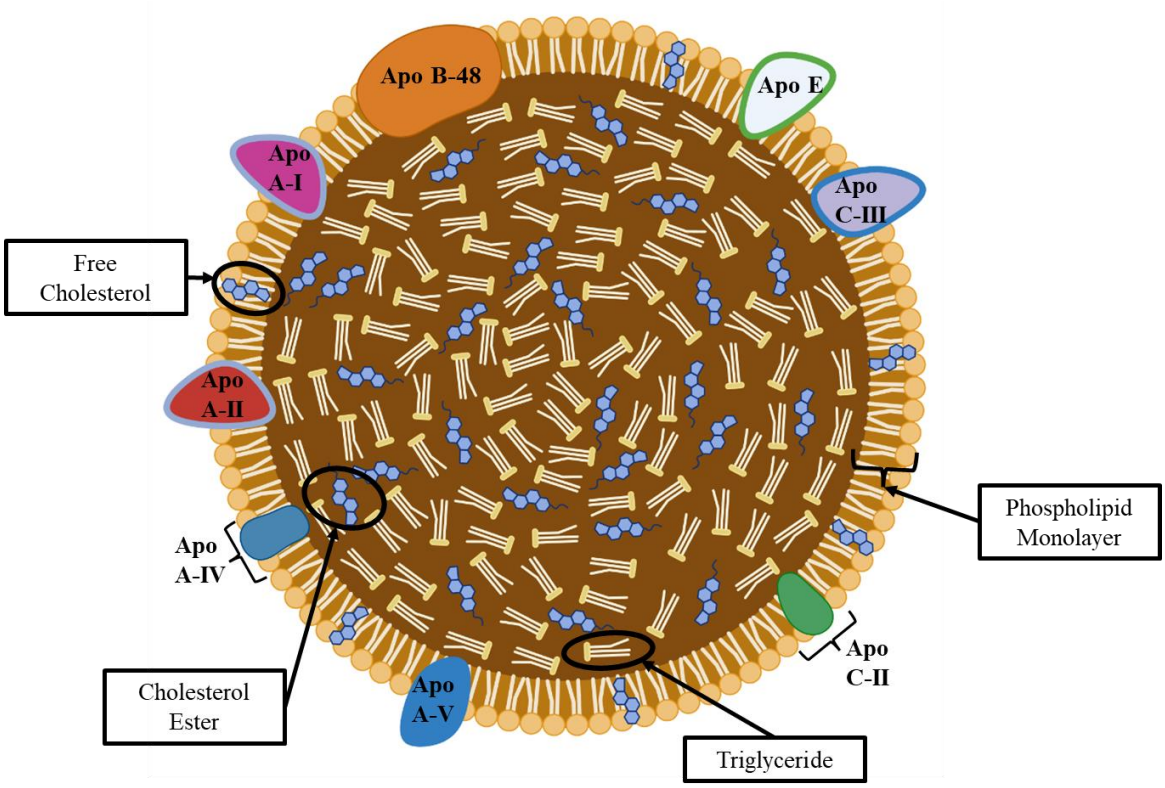

Figure 1.3. Structure of chylomicrons.

Diagram depicting a cross-sectional view of a chylomicron. Chylomicrons are composed primarily of triglycerides and 2-monoacylglyceride (not shown) with some cholesterol ester content surrounded by a phospholipid monolayer, which has some free cholesterol incorporated within it. The primary apolipoproteins of chylomicrons are depicted within the diagram: apolipoprotein (apo) A-I, A-II, A-IV, A-V, B48, C-II, C-III, and E. Apo B-48 is the primary structural, non-exchangeable apolipoprotein of this lipoprotein. Created in Biorender (https://www.biorender.com/).

1.2.3.2 Very-low-density lipoproteins

VLDL is the TGRL that transports the majority of endogenously synthesized TG from the liver to the rest of the body [107,146]. VLDL particles are quite large at diameters between 30 to 80 nm, with only CM being larger [107,159]. VLDL size is primarily determined by the quantity and type (in reference to the FA components of the TG) of TG carried like CM [107] but may also vary with genetic and environmental factors [159]. Increased hepatic TG synthesis corresponds with increased VLDL size. With regards to apolipoprotein profile, VLDL contains apo A-V, apoB100, apo C-I, apo C-II, apo C-III, and apo E [107,160]. Like apoB48 in CM, apoB100 in VLDL serves as the core structural protein around which the lipoprotein complex is assembled [107]. Each VLDL particle contains only one apoB100 molecule. Morphologically, VLDL particles are extremely similar to CM, with the only major differences being their size and apolipoprotein profile. **Figure 1.4** shows a diagram of the structure of VLDL particles.

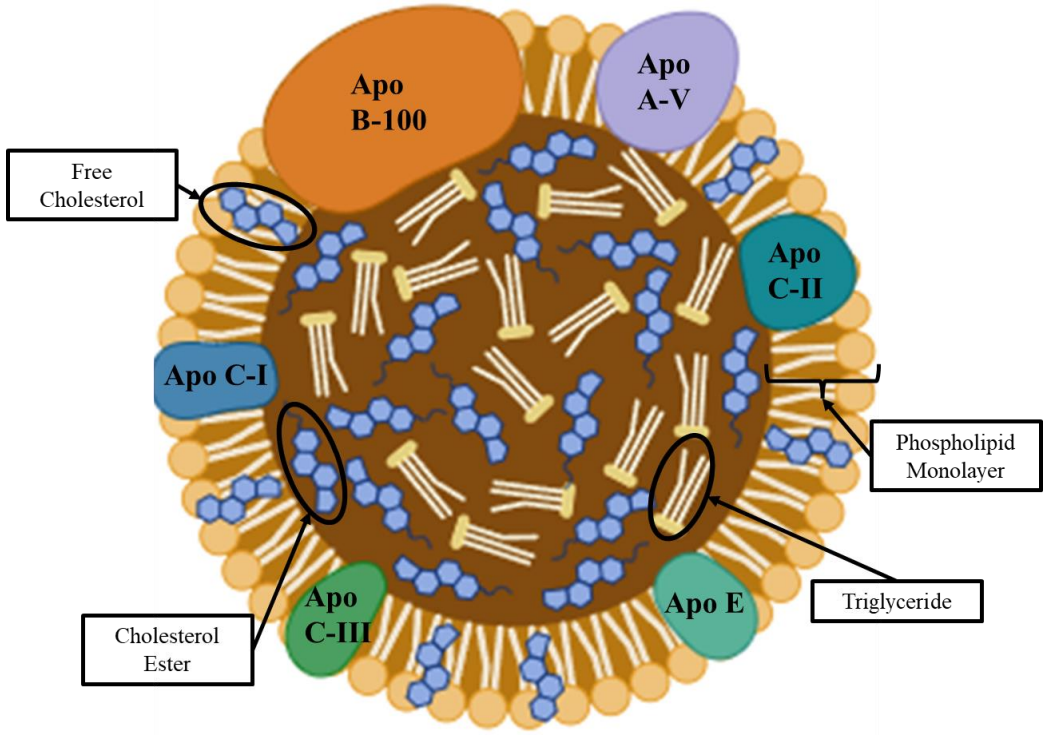

Figure 1.4. Structure of very-low-density lipoproteins.

Diagram depicting a cross-sectional view of a very low-density lipoprotein (VLDL). VLDL are composed primarily of triglycerides with some cholesterol ester content surrounded by a phospholipid monolayer, which has some free cholesterol incorporated within it. The primary apolipoproteins of VLDL are depicted within the diagram: apolipoprotein (apo) A-V, B100, apo C-I, apo C-II, apo C-III, and apo E. Apo B-100 is the primary structural, non-exchangeable apolipoprotein of this lipoprotein. Created in Biorender (https://www.biorender.com/).

1.2.3.3 High-density lipoproteins

HDL is a unique, non-apoB containing class of lipoproteins whose primary functionality is in facilitating RCT through its biochemical behavior and life cycle (see section 1.2.2.3 for more details) from various peripheral tissues to the liver [147]. However, they do play a minor role in plasma TG metabolism via the action of CETP which exchanges cholesterol esters contained within the HDL particle with TG contained in other circulating lipoproteins, leading to various downstream interactions of HDL with HL and EL (see section 1.2.2.3 for more details). As discussed in section 1.2.2.3, HDL particle size varies throughout its life cycle but they are generally quite small lipoproteins, ranging from 1.063 to 1.210 nm in diameter [107]. HDLs may be sub-classified based on their density and size (see section 1.2.2.3). HDL contains apo A-I, A-II, A-IV, C-I, C-II, C-III, and E, though the specific apolipoprotein profile of this class of particles may vary drastically depending on what stage of the life cycle it is in [107,150]. Apo A-I is the primary structural protein component of HDL particles. However, unlike CM and VLDL which contain only one molecule of their respective primary structural non-exchangeable apolipoprotein component (apoB48 and apoB100, respectively), HDL particles may contain multiple apo A-I molecules [147]. Morphologically, mature HDL particles are generally spherical, but their size and density may vary depending on the stage of its life cycle. Additionally, nascent HDL takes on a discoidal shape as opposed to a spherical shape prior to the formation of its lipid rich core. HDL surface hydrophilic layers are functionally and structurally similar to CM and VLDL and other lipoproteins. The lipid core of HDL is primarily cholesterol esters but may also contain some TG after the action of CETP. **Figure 1.5** shows a diagram of a mature HDL particle.

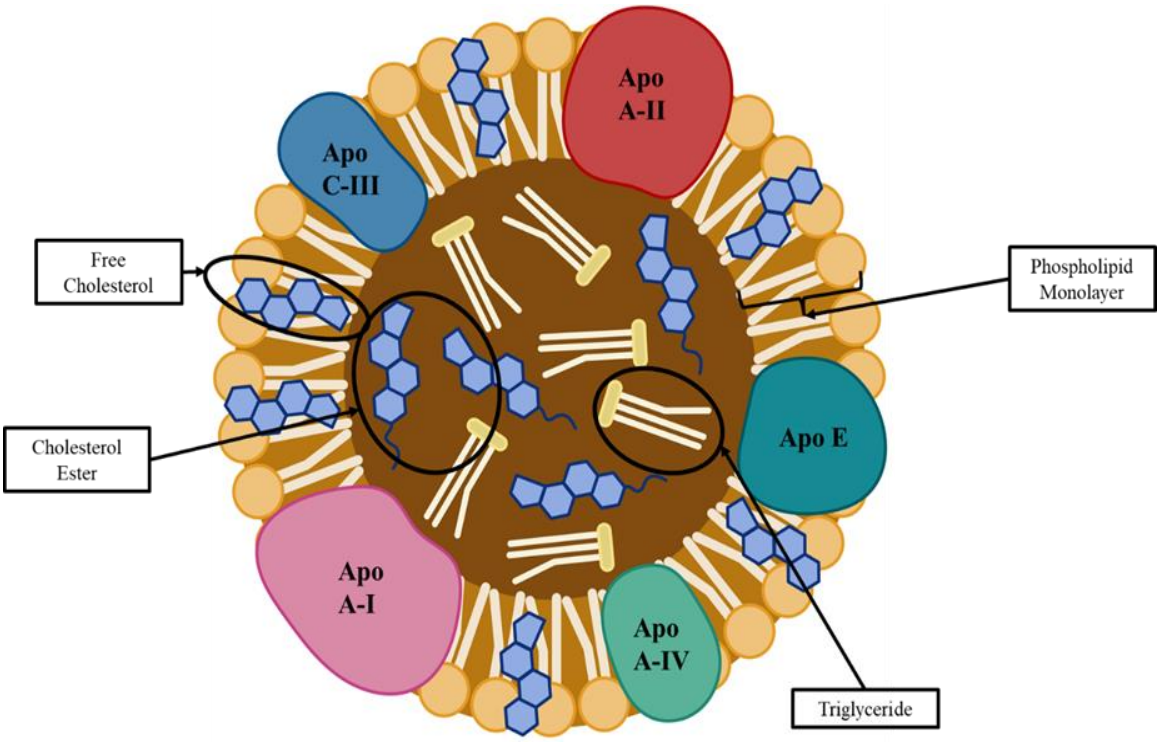

Figure 1.5. Structure of high-density lipoproteins.

Diagram depicting a cross-sectional view of a high-density lipoprotein (HDL). HDL are composed primarily of cholesterol esters with some minimal triglyceride content surrounded by a phospholipid monolayer, which has some free cholesterol incorporated within it. The primary apolipoproteins of VLDL are depicted within the diagram: apolipoprotein (apo) A-I, A-II, A-IV, C-I (not shown), C-II (not shown), C-III, and E is the primary structural, non-exchangeable apolipoprotein of this lipoprotein. Created in Biorender (https://www.biorender.com/).

1.3 Genetic variation and disease in humans

Genetic variation in humans refers broadly to differences, both large and small, in DNA sequences between the genomes of different individuals in a given population. Genetic variation is responsible for much of the interindividual variability we see between human individuals. Most genetic variants are benign and considered inconsequential to human health. However, rarely, genetic variants can impact human health, through directly causing disease themselves, modulating disease susceptibility, modifying individual responsiveness to various clinical interventions and environmental factors, and determining interindividual differences in clinical outcomes [161].

Genetic variants are typically classified according to two major criteria 1) frequency in a population (covered in section 1.3.1) and 2) DNA sequence change mechanism/type (covered in sections 1.3.2 and 1.3.3). Additionally, clinicians may utilize additional classifications for determining the pathogenicity of a given genetic variant (see section 1.3.4) [162].

1.3.1 Variant frequency

Variant frequency refers to how often a given variant is observed in a population under study. Thanks to extensive collaborative efforts over the last few decades, there now exists multiple publicly available population-level genetic databases [163–168]. These have enabled scientists to determine the frequencies at which all forms of genetic variation in humans occurs in given populations. The frequency of a given genetic variant in a population of interest is typically referred to as the minor allele frequency (MAF). The terminology of "minor" is an artifact of classical genetics terminology in which the terms "major" and "minor" refer to the more or less common allele, respectively. MAF are relative measures as they are calculated based on specific populations of study. Therefore, the MAF of a variant in one population may not be the same when determining its MAF in a 2nd separate population. A related consequence of this is that a "minor" allele in one population may be the "major" allele in another and vice versa.

There are several large-scale population genetic mechanisms that may impact MAF of a given variant in a population of interest. Firstly, natural selection, the process by which individuals differentially survive and reproduce based on survival and reproductive advantages they possess. This includes sexual selection. The genetic variations underpinning or contributing to the differential survival and successful reproduction rates will propagate throughout a population over time, altering MAF of variants present in the population. There is a wealth of evidence that indicates this still occurs in humans [169–174]. Secondly, genetic drift is the process by which allele frequencies (including MAF) within a population are altered due to the fact that the alleles present in the offspring are always a random sample of the alleles present in the parents [175]. Unlike natural selection in which reproductive success rates preferentially determines the variant frequency of a given variant, in genetic drift, changes to variant frequencies are totally random. This can even cause genetic variants to disappear completely from a population reducing its overall genetic variability. Thirdly, gene flow is the process by which two distinct populations "trade" genetic material (i.e., one member of a population A reproduces with a member of population B), increasing the overall genetic variability in the two populations [176,177]. In the absence of natural selection and genetic drift, gene flow will eventually result in equalization of the variant frequencies of the two populations exchanging genes and essentially, make them functionally a single population. Finally, and perhaps most relevant to this thesis, is the founder effect. This term refers to loss of genetic variation that occurs when a non-representative (from a genetic variation standpoint) small sample of individuals from a larger population are used to establish a new population [178]. This mechanism is particularly relevant to this thesis as it is largely agreed that a founder effect is responsible for the ~100-fold greater prevalence of familial chylomicronemia syndrome (see section 1.4.3.1.1 for more details regarding this condition) in the French-Canadian population, especially in the Charlevoix-Saguenay-Lac-Saint-Jean region in Eastern Quebec [179–183].

1.3.1.1 Rare variants

Rare variants are typically defined as variants with a MAF less than 0.01 in the population of interest [184]. Variants impacting human health with large effect size are

usually rare variants. According to evolutionary theory and natural selection (briefly discussed in section 1.3.1), this is because deleterious variants negatively impacting human health are likely to impact ability to survive and/or impact reproductive fitness. As a result, it is difficult for these variants to propagate in a population. It should be noted that most rare variants are not particularly deleterious and in fact it is likely that most rare variants have no phenotypic impact. We know this because the extensive development of population genetics databases [163–168] has revealed thousands of new rare variants in healthy populations.

1.3.1.2 Common variants

Common variants are typically defined as variants with MAF greater than 0.05 in the population of interest [184]. Variants with MAF between 0.01 and 0.05 are defined as less common. Most common variants are largely benign with no major impact on human health. However, some have been found to impact human health, though not with particularly large effect sizes. Single-nucleotide polymorphisms (SNPs) (see section 1.3.2 for extensive discussion on this) are the most common form of genetic variation among humans and are rarely deleterious to fitness. SNPs have been extensively utilized to investigate and probe the etiology of complex traits and diseases in humans and other organisms. With regards to dyslipidemias, they have been used to construct risk scores for determining genetic predisposition to the development of various dyslipidemias [185,186].

1.3.2 Single nucleotide variants

Single nucleotide variants (SNVs) are variants that involve alterations to a single nucleotide within a DNA sequence [187,188]. SNPs are a subcategory of SNVs that are particularly common. Specifically, SNVs are considered SNPs if they have MAF greater than or equal to 0.01, although a critical value of 0.05 is sometimes suggested.

SNVs are primarily classified based on their impact on DNA sequence ontology and their location within the DNA sequence. Beginning with SNVs located within the protein coding region of a gene, they can be broadly classified as synonymous or non-synonymous. Synonymous SNVs are variants that produce no amino acid changes in the

protein encoded by the affected gene due to redundancy in the codon triplet code for encoding amino acids [189]. Briefly, this is because there are 64 possible codons (3 letter DNA segments encoding a specific amino acid or a protein translation stop site), 61 encoding amino acids and 3 encoding stop codons, while there are only 20 amino acids that are coded for [189]. Thus, these SNVs are "synonymous" in that they do not change amino acid sequence encoded by the gene. Non-synonymous SNVs are the opposite: they alter the encoded amino acid sequence. Non-synonymous SNVs can be further sub-classified based on exactly how they impact the amino acid sequence: 1) missense variants, which are defined as coding region SNVs that alter a codon such that the protein produced from the gene has an altered amino acid sequence which may significantly alter protein functionality and/or viability and 2) nonsense variants, defined as coding region SNVs that alter an amino-acid coding codon to a stop codon, resulting in production of a truncated protein. Protein truncation is a well-known mechanism for disease via loss of or significant alteration to normal gene/protein function [190]. Variants that lead to premature truncation are called protein-truncating variants (PTVs). Apart from nonsense variants, the only other SNVs that may lead to protein truncation are variants impacting post-transcriptional pre-mRNA splicing activity. Specifically, SNVs found in the splice acceptor and splice donor sites located at the beginning and end of intronic regions, respectively, may lead to loss of ability of the splicing machinery to recognize the site or aberrant splicing activity. This may introduce a stop codon prematurely that is found within intronic sequences that were meant to be excised from the pre-mRNA molecule [190].

Non-coding region SNVs are primarily classified according to their location relative to the coding region. The major categories are SNVs in the promoter, introns, 5' and/or 3' untranslated regions (UTR), enhancer, and silencer regions [191]. Non-coding SNVs can also have significant impact on sequence ontology and complex diseases and traits [191].

As discussed briefly in section 1.3.1.2, SNPs are a rough and loosely defined sub-category of SNVs. SNVs that are relatively prevalent within a population may be dubbed as SNPs. SNPs have been the subject of extensive study and investigation for determining

risk and polygenic contributions to a wide range of diseases, including dyslipidemias [185,186].

1.3.3 Structural variants

Structural variants (SVs) are typically defined as large genomic alterations encompassing at least 50 base pairs [192]. However, this is a rather arbitrary definition. The definition of SVs may be extended to encompass all non-SNV genetic variations, including those ranging in size from 1 to 50 affected base pairs as well. The latter definition is what will be used here. SVs can be classified according to the specific impact they have on the affected sequence: insertions, deletions, duplications, inversions, and translocations. Given the size and physical impact of SVs on the impacted sequences, it is not surprising that SVs are often implicated in disease etiology. However, it should be noted that not all SVs are necessarily pathogenic. It is thought that SVs account for a significant degree of normal interindividual variability in humans. In fact, average interindividual genomic variation in humans is only 0.1% when looking only at SNVs but expands to ~1.5% when accounting for SVs [193].

1.3.3.1 Insertions and deletions

Insertions and deletions (collectively referred to as indels) are structural variants that involve the addition or loss, respectively, of nucleic acids from a DNA sequence. These can range in size, with anywhere from 1 to 50 bases pairs being impacted.

Since mRNA is read by ribosomes in blocks of 3 nucleic acids at a time (i.e. the codons), insertion or deletion of nucleotides into the protein coding sequence of a gene in numbers not divisible by 3 can disrupt the grouping of these nucleotides, altering the protein produced once the produced mRNA is read by ribosomes [194]. These are referred to as frameshift variants, as the insertion or deletion of nucleotides in numbers not divisible by 3 shifts the grouping of nucleotides read as codons by ribosomes (which is typically referred to as the open reading frame of a gene). Frameshift variants can have devastating consequences for sequence ontology. Firstly, frameshift variants can produce premature stop codons leading to production of truncated protein like nonsense variants. Thus, some frameshift variants are PTVs. Secondly, frameshift variants can result in loss of the

standard stop codon leading to aberrantly extended translation of the mRNA molecule, producing an abnormally elongated protein product which is likely to have significant functional differences to the wild-type protein. Finally, in-frame indels refers to indels that occur in numbers divisible by 3, thus preserving the open reading frame of the gene. In-frame indels may also induce catastrophic consequences for sequence ontology if the indels impact and interrupt regions of the coding sequence that code for important structural or functional domains of the mature protein or if they impact regions important for ensuring proper folding of the mature protein.

1.3.3.2 Copy number variants

The human genome contains multiple copies of various sequences at various levels. For example, because humans are diploid organisms, we possess two copies of every somatic chromosome allele, one from each parent. At an even finer level, certain genes contain repeated segments of DNA in their protein coding sequences that correspond to production of a repeated domain or motif in the protein product of that gene. The number of copies of a given genetic locus can have important functional implications for a large array of biological features. For example, with regards to ASCVD risk and lipid metabolism, Lp(a) levels in plasma are largely genetically determined by a copy number variation (CNV) mechanism [44]. Specifically, apolipoprotein(a), the hallmark apolipoprotein of Lp(a), contains several Kringle (K) domain structures, most notably K-IV type 2 which is repeated multiple times within apolipoprotein(a) structure [44]. It is the number of K-IV type 2 copies within the apolipoprotein(a) that ultimately determines the plasma levels of Lp(a) and the number of K-IV type 2 units within apolipoprotein(a) is genetically determined, with K-IV type 2 repeat number inversely related to Lp(a) plasma level [44]. The reason this CNV in the *LPA* gene encoding apolipoprotein(a) determines plasma levels of Lp(a) is because smaller CNVs have lower retention time in the endoplasmic reticulum as well as relatively reduced pre-secretion degradation rates than larger CNVs, which gives smaller apolipoprotein(a) isoforms more efficient secretion than larger ones, hence the inverse correlation between apolipoprotein(a) isoform size and plasma Lp(a) levels [195–197]. Thus, CNVs can be broadly defined as molecular phenomena that impact the number of copies of a given genetic locus and the

number of repeats is variable between individuals of the population of study [198]. The primary SVs at play in CNV are deletions and duplications, which decrease and increase, respectively, the copy numbers of impacted genetic loci. CNVs represent the most common form of SVs in the human genome [199,200]. The impact of CNVs on sequence ontology and human health depends on the impacted locus but can range from deleterious to beneficial. Using the earlier example, decreased copy number of K-IV type 2 domains in apolipoprotein(a) corresponds to increased plasma Lp(a) levels which is detrimental to human health as elevated Lp(a) is a strong independent risk factor for ASCVD [44].

1.3.3.3 Chromosomal alterations

Chromosomal alteration is the umbrella term for extremely large-scale alterations to standard wild-type chromosomal structure. Translocations refer to transfer of a genetic segment from one chromosome to a different chromosome or to the opposing allele of the same chromosome. These can be incredibly disruptive to both the donor and recipient chromosomes, with extent depending on the exact loci that are impacted on each. Translocations are well known to play major roles in several human diseases, including, but not limited to, several forms of cancer such as myelomas, leukemias, and musculoskeletal neoplasms [201–205]. Inversions are the alteration of the orientation of a DNA segment within a chromosome from its original orientation to the reverse orientation [206]. Partial deletion of chromosomal segments has also been observed to produce several diseases [207–209]. Partial duplication of chromosomal segments is also associated with several diseases [210–213]. Finally, the most extreme chromosomal alterations abnormalities in total chromosome count, generically termed aneuploidy. Perhaps most well-known of these is the aneuploidy that produces Down syndrome due to presence of a full extra copy of chromosome 21 in humans, typically termed as trisomy 21 [214]. In a sense, aneuploidies are chromosomal level CNVs.

1.3.4 Sequence variant interpretation guidelines

1.3.4.1 American College of Medical Genetics and Genomics guidelines for pathogenicity of sequence variants

The American College of Medical Genetics and Genomics (ACMG) developed guidelines for the interpretation and classification of sequence variants based on strict evidence-based criteria for both clinical and research use [162,215]. These guidelines outline a strict process, criteria, and evidentiary standards for determining and classifying the pathogenicity of genetic variants. Typically, utilizing these guidelines, genetic variants may be categorized as benign, likely benign, uncertain significance, likely pathogenic, and pathogenic [162]. The ACMG criteria facilitate variant pathogenicity interpretation by evaluating the variants in several categories such as population MAF, predictions from *in silico* algorithms regarding the functional impact of the variant, actual functional experimental data, zygosity data, disease-variant segregation studies, and several other forms of evidence.

1.3.5 Methods for studying genetic variation

1.3.5.1 Sanger sequencing

Developed in 1977, Sanger sequencing [216] was a breakthrough DNA sequencing technique that led in part to the development and production of the first commercial DNA sequencing machines [217]. Sanger sequencing incorporated radio-labelled dideoxyribonucleotides (ddNTPs), chemical analogues of deoxyribonucleotides (dNTPs), the monomers that form DNA, to produce DNA strands of every possible length via a series of terminating DNA extension reactions. The ddNTPs terminate DNA extension reaction because they lack the 3’ hydroxyl group on the sugar backbone which is required for DNA extension as the 3’ hydroxyl group is the site where the 5’ phosphate of subsequent dNTPs is supposed to bind. DNA extension reaction mixtures incorporated with radio-labelled ddNTPs at a fraction of the concentration of dNTPs produce DNA molecules of varying lengths as the ddNTPs are randomly incorporated into the forming strands. The original technique employed four parallel reactions, one for each ddNTP base, and then running the results of the reactions on 4 lanes of a polyacrylamide gel

[216]. Autoradiography, due to the radio-labelled ddNTPs, would highlight the positions of the ddNTPs in order and would allow one to infer the nucleotide sequence of the original template molecule being sequenced based on the order and where the ddNTP radioactive signals are seen on the gel [216]. The ease of use of the ddNTPs as well as the increased accuracy and robustness of the Sanger sequencing technique compared to previous techniques propelled the technique to become the single most common sequencing methods for years to come [218]. Sanger sequencing was eventually mostly phased out of use due to high costs and the laborious nature of the technique in favor of more effective alternatives like next-generation sequencing.

1.3.5.2 Next-generation sequencing

Next-generation sequencing (NGS) refers to a collection of techniques facilitated by new technology that has superseded Sanger sequencing as the most common and widely used sequencing technique currently. In summary, NGS sequences millions of DNA fragments in parallel which are then aligned and pieced together through bioinformatics analyses which map the individual reads of these fragments to the human reference genome [219]. NGS is a high read depth technique, meaning that each of the nucleotide bases fed into the process is sequenced multiple times which produces more accurate data and provides insights into genetic variation present in the sample [219]. NGS can be used to perform sequencing of whole genomes, whole exomes, or of a panel of specific genes of interest (e.g., LipidSeq, which sequences genes associated with dyslipidemias and various metabolic disorders [220,221]; see section 1.3.5.2.1 for more details regarding LipidSeq).

NGS possess several important advantages compared to Sanger sequencing. Primarily, unlike Sanger sequencing which can only sequence one sample at a time, NGS can be utilized to sequence multiple samples simultaneously and it can be used to sequence extremely large samples such as whole genomes or exomes quite quickly compared to Sanger sequencing. This facilitates large scale genetics studies on larger groups of people that would not have been possible during the Sanger sequencing era due to how cost-prohibitive and inefficient it would have been [218,222].

The basic methodology of NGS follows several major steps. For DNA, these are DNA fragmentation, followed by library preparation, sequencing, bioinformatic analysis and variant annotation and interpretation. Firstly, DNA fragmentation refers to the collection techniques that may be utilized to break a DNA sample into small fragments roughly 100 to 300 base pairs long. Mechanical methods, enzymatic digestion of the sample, sonication, or nebulization may be employed to do this [223]. For cases in which only specific DNA sequences are of interest, either a hybridization capture assay, which utilizes complementary probes to isolate target sequences [224,225], or amplicon assay, utilizing polymerase chain reaction (PCR) to amplify the targeted sequences [226,227], are employed to isolate the target sequences. The isolated DNA fragments are then used for library preparation which is a process that modifies each DNA fragment so that they can have a sample-specific index (essentially a sample identification tag) which identifies the source of the sample (for example, a specific patient in a cohort that is being sequenced). During this process, sequencing adapters can be added to the DNA fragments. These are small oligonucleotides that attach to and flank fragments which contain the sequence(s) of interest, and perform 3 primary functions: 1) they enable binding of the sequence(s) of interest to the sequencing platform, 2) they contain the primer binding site for the sequence(s) of interest, which enables binding of sequencing primers for enabling proper, targeted polymerase action, and 3) they may contain tags (often called index or barcode regions) which enable the pooling of multiple samples to be sequenced within a single run without getting the different samples mixed up [228]. Multiple parallel sequencing is then performed to sequence the prepared library using an NGS sequencer. Different NGS sequencers may have differing processes primarily at this step due to different NGS strategies/approaches that can be employed [229]. Regardless of the specific technology utilized by different sequencers, they all enable massively parallel simultaneous sequencing of all the DNA fragments. The generated sequencing information is fed through a bioinformatics pipeline which calls the bases, aligns the reads to the reference genome, and identifies variants and annotates them based on their possible clinical significance by comparing the reads to the reference genome [230]. Read alignment facilitates the construction of the whole sequence of the original sample.

These outputs are all sent back to the scientist. Refer to section 1.3.4.1 for more information on variant annotation.

1.3.5.2.1 LipidSeq: a next-generation targeted resequencing panel for the study of dyslipidemias

LipidSeq, initially developed in 2013 by our lab, is an NGS sequencing panel targeting 69 genes and 185 SNPs that have been found to be causally-linked to or associated with various dyslipidemias and metabolic disorders [220,221]. LipidSeq has been utilized by past and present members of our lab to describe novel variants impacting human lipid and metabolic health, uncover the polygenic determinants of complex dyslipidemias like HTG [185,186], and unearth novel disease mechanisms [221]. Clinically, LipidSeq has facilitated DNA-based diagnoses for a great number of patients at the London Lipid Genetics Clinic which has provided relief to many patients in various ways. For example, LipidSeq has provided answers to some patients who struggled for a long time without diagnosis and provided them with a clear direction forward while for other patients it enabled them to secure private coverage for therapies to treat their conditions [221].

1.3.5.3 Microarrays

Microarrays at their most basic level are a collection of nucleic acid probes bound to a solid surface that can be utilized to analyze gene expression and/or transcription factor binding and perform genotyping of SNPs [231]. They are popular method for genome wide association studies, but the SNPs genotyped using a microarray are rarely causative for the disease or trait under study as the vast majority of SNPs genotyped via these methods are located within intergenic regions and thus it is more likely that these SNPs are acting as tags associated with the variant that is mechanistically responsible for the disease or trait under investigation [231].

1.4 Hypertriglyceridemia

Hypertriglyceridemia (HTG) is defined as fasting plasma TG concentrations that are abnormally elevated, typically defined as concentrations above 2.0 mmol/L. It can be further classified as either mild-to-moderate HTG for fasting plasma TG concentrations

between 2.0 and 10.0 mmol/L or severe HTG for fasting plasma TG concentrations exceeding 10 mmol/L. Sometimes a further designation is made for very severe HTG, usually defined as fasting plasma TG exceeding 20.0 mmol/L. As discussed briefly in section 1.1.3, while TG themselves may not be directly atherogenic, the other contents of their lipoprotein carriers are almost definitely atherogenic and likely accounts for the residual ASCVD risk present in patients with well-controlled LDL-C. Additionally, severe HTG is a significant predictor and cause of acute pancreatitis, which is potentially lethal. Elevated plasma TG levels are determined by a multitude of factors. HTG is usually due to aberrant elevations in plasma concentrations of chylomicrons (see sections 1.2.2.1 and 1.2.3.1 for more details about chylomicrons) and/or very-low-density lipoprotein (see sections 1.2.2.2 and 1.2.3.2 for more details about very-low-density lipoproteins) which are the TG-rich lipoproteins responsible for the vast majority of plasma TG transport.

1.4.1 Causes of and risk factors for hypertriglyceridemia

HTG is multifactorial in its nature. The primary processes/factors that contribute to the development of HTG are 1) increased hepatic production of TGs (see section 1.2.1.2) and 2) impaired clearance of TGRLs (chylomicrons and VLDLs) from circulation. Genetics play a large role in both processes. The role of genetics in hypertriglyceridemia are discussed in section 1.4.3.

There is a large array of secondary factors that contribute to and may cause HTG. Environmental factors include, but are not limited to, excessive caloric intake (excessive lipid and/or carbohydrate intake) and excessive alcohol intake [232]. Some medical conditions may also cause HTG. Rarely, pregnancy may induce HTG, which can be life threatening [233]. This is because, during the late-second and third trimesters, increased estrogen levels in the mother upregulate hepatic VLDL production while reducing LPL-mediated lipolysis in the mother, which occurs concurrently with increased human placental lactogen levels in the mother, which induces adipose tissue lipolysis in the mother to create more substrate for hepatic VLDL production [233,234]. While these elevations in plasma TG levels are usually well tolerated, rarely, if the mother has

compromises in her metabolic pathways, sometimes due to genetic factors, this can lead to pregnancy-induced HTG [233]. More commonly, endocrine disorders are known to be secondary causes of HTG. Uncontrolled diabetes mellitus (both type 1 and 2), central obesity, metabolic syndrome, hypothyroidism and hypercortisolism (can be caused by Cushing's syndrome) are all well-known secondary causes of HTG [232,235]. Renal disease, specifically nephrotic syndrome and renal failure, may also cause HTG [236]. Acute hepatitis may also produce HTG [237]. Autoimmune conditions such as systemic lupus erythematosus [238], rheumatoid arthritis [239], and Sjögren's syndrome [240] have also been shown to elevate plasma TG and produce HTG. Both human immunodeficiency virus infection and the highly active antiretroviral therapy used to treat it are known to induce metabolic disturbances including inducing HTG [241]. Finally, there is a relatively extensive list of medications for which there is some evidence indicating they may produce HTG. Both selective and non-selective beta-blockers [242], thiazides [242], corticosteroids [243], Tamoxifen [244], Raloxifene [245], oral estrogens [246], protease inhibitors [247], oral vitamin A and synthetic retinoids [248], Isotretinoin [249], Sirolimus and other immunosuppressants [250,251], L-asparaginase [252], bile acid binding resins [253], phenothiazine and some second generation anti-psychotics [254], and rosiglitazone [255] have been identified as having varying extents of plasma TG elevating effect.

1.4.2 Clinical features of hypertriglyceridemia

Physical manifestations of elevated TG in plasma (HTG) are rare but do occur, primarily in severe HTG cases. Severe HTG is defined as fasting plasma TG exceeding 10 mmol/L. Severely elevated TG may physically manifest in one or more of the following ways depending on the specific dyslipidemia responsible and the extent of plasma TG elevation: 1) eruptive xanthomatosis (**Figure 1.6A**), skin lesions that form when excessive lipoprotein in plasma invade the subcutaneous space by passing through blood vessel walls and are taken up by macrophages in those areas [256]; 2) tuberous xanthomas (**Figure 1.6D**), non-tender skin lesions that form via a similar process to eruptive xanthomas. In dysbetalipoproteinemia, a form of HTG caused primarily by homozygosity for the apolipoprotein E2 isoform, these lesions are commonly observed

on the skin around extensor joints [256,257]; 3) palmer crease xanthomas (**Figure 1.6E**), lipid deposits created via a similar process to eruptive and tuberous xanthomas but in the creases of the palms. This specific physical presentation is indicative for familial dysbetalipoproteinemia [256]; and 4) lipemia retinalis (**Figure 1.6C**), milky white appearance of retinal blood vessels and pink colored retina which occurs at extremely elevated TG levels only, usually starting around 35 mmol/L [256]. Additional possible manifestations include hepatosplenomegaly, focal neurological symptoms (e.g., irritability), and recurrent epigastric pain [256]. Additionally, acute pancreatitis may occur as a result of severely elevated plasma TG levels, which is particularly concerning as it is a potentially lethal condition [258]. Perhaps the most striking manifestation of severely elevated plasma TG is lipemic plasma (**Figure 1.6B**), in which the significant accumulation of TG-rich lipoproteins, primarily CM, in plasma leads to it developing a milky white appearance as opposed to the standard clear color it usually has when centrifuged [256]. If the manifestation of severe HTG is due exclusively to chylomicron accumulation, as is the case in familial chylomicronemia syndrome (see section 1.4.3.1.1 for more details), these patients do not have elevated ASCVD risk as CM are too large to embed in arterial walls and form plaques and their accumulation in plasma leads to almost complete loss of smaller downstream lipoprotein production and synthesis [256,259].

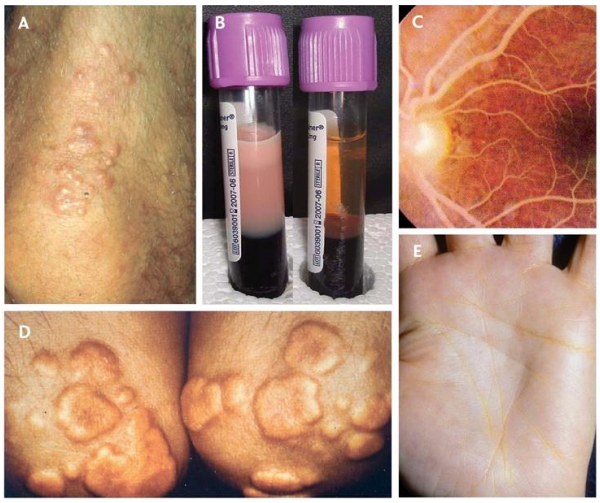

Figure 1.6. Physical manifestations of severe hypertriglyceridemia.

A: Eruptive cutaneous xanthomas (here on a patient's knee). Most often associated with markedly elevated plasma chylomicrons in cases of familial chylomicronemia (hyperlipoproteinemia type 1) or primary mixed dyslipidemia (hyperlipoproteinemia type 5), they usually occur in clusters on the skin of the trunk, buttocks or extremities. **B:** Lipemic plasma. Whole blood has been allowed to stand at 4°C overnight. The sample on the left comes from a patient whose fasting total cholesterol result was 14.2 mmol/L and triglyceride concentration was 41.8 mmol/L. The sample on the right comes from a normolipidemic subject. **C:** Lipemia retinalis. A milky appearance of the retinal vessels and pink retina can be seen when plasma triglyceride concentration exceeds 35 mmol/L. **D:** Tuberous xanthomas, filled with foam cells, appear as reddish or orange, often shiny nodules, up to 3 cm in diameter. They are usually moveable and nontender. In patients with familial dysbetalipoproteinemia (hyperlipoproteinemia type 3), they usually appear on extensor surfaces; these are on a patient's elbows. **E:** Palmar crease xanthomas are filled with foam cells and appear as yellowish deposits within palmar creases. These skin lesions are pathognomonic for familial dysbetalipoproteinemia (hyperlipoproteinemia type 3). Figure was adapted from Yuan G, Al-Shali KZ, Hegele RA. Hypertriglyceridemia: its etiology, effects and treatment. CMAJ Can. Med. Assoc. J. 176(8), 1113–1120 (2007) [256].

Mild-to-moderate HTG, usually defined as fasting plasma TG between 2.0 and 9.9 mmol/L, does not usually result in physical manifestations like severe HTG. The major clinical concern with mild-to-moderate HTG is ASCVD risk [256].

1.4.3 Role of genetics in hypertriglyceridemia

HTG is a complex disease that has both genetic and environmental factor basis. Genetics do play a prominent role in the development of hypertriglyceridemia. There are several major enzymes, proteins, and apolipoproteins whose proper function is essential for facilitating plasma TG metabolism and clearance and genetic variation in many of the genes encoding these proteins have been identified to be causal for HTG.

1.4.3.1 Monogenic hypertriglyceridemia

Monogenic HTG refers to HTG caused by rare pathogenic genetic variations in singular genes. There is only one true monogenic form of HTG: familial chylomicronemia syndrome (FCS) [260].

1.4.3.1.1 Familial Chylomicronemia Syndrome

FCS represents the only true monogenic HTG. It is an ultrarare condition, with estimated prevalence of 1 in 100 000 to 1 in 1 million [261,262], with a notably ~100-fold increased prevalence in the French-Canadian population, especially the population located near Charlevoix-Saguenay-Lac-Saint-Jean region in Eastern Quebec [179–183]. It is caused by biallelic (i.e., homozygous or compound heterozygous) pathogenic variants in the *LPL* gene, which encodes lipoprotein lipase (LPL), or in one of four other genes which encode essential LPL-interacting proteins, which are *APOC2*, *APOA5*, *GPIHBP1*, and *LMF1*, which encode apo C-II, apo A-V, GPIHBP1, and LMF1, respectively [260,263]. The vast majority of FCS cases (~80%) are caused by the presence biallelic pathogenic variants in LPL, with the remaining 20% caused by biallelic pathogenic variants in the genes encoding the 4 major LPL-interacting proteins [263]. FCS follows a classical autosomal recessive inheritance pattern [262].

FCS is characterized by sustained, refractory, and severe HTG (fasting plasma TG concentration ≥ 10.0 mmol/L) brought about by persistence of CMs in circulation due to

loss of LPL-mediated lipolytic pathway due to pathogenic variants in the major genes encoding the LPL lipolytic pathway. FCS patients, due to the severe, refractory HTG, may also present clinically with systemic manifestations such as failure to thrive, lipemia retinalis, eruptive xanthomatosis focused on the trunk, buttocks and extremities, hepatosplenomegaly, focal neurological symptoms, usually irritability, recurrent epigastric pain, nausea, and vomiting [256]. Clinical diagnosis usually occurs in infancy when CM accumulation in plasma begins but can occur as late as early adulthood [261,262].

Interestingly, FCS patients do not experience elevated ASCVD risk related to the severe, refractory HTG [259]. This is because CMs are too large to implant in arterial walls and initiate the atherosclerotic process and in FCS patients the loss of LPL-mediated lipolysis prevents plasma TG metabolism so remnant lipoprotein particles which are small enough to initiate atherosclerosis are not produced [259].

1.4.3.1.2 Monoallelic FCS gene pathogenic variant induced hypertriglyceridemia – an under-investigated and poorly understood form of hypertriglyceridemia

The etiology, progression, treatment, and phenotypic presentation of FCS has been well studied over the last 5 decades and continues to be investigated. Thus, the phenotype produced by biallelic pathogenic variants in major plasma TG metabolism genes, *LPL, APOC2, APOA5, GPIHBP1,* and *LMF1,* is thoroughly understood. However, rather interestingly, the much more common condition of heterozygosity (monoallelic) for pathogenic variant in the FCS genes is far less familiar and has been less systematically evaluated [260].

Heterozygosity for pathogenic variants in *LPL* is relatively common in the general population, up to 3% in some studies [185,186,260], as opposed to biallelic condition which is 1 in 100 000 to 1 in a million [263]. Despite being much more common there is less familiarity with the TG phenotype of heterozygous carriers. This is perhaps due to a lack of modern work characterizing the TG phenotype in individuals heterozygous for pathogenic variants in *LPL*. While there have been some studies [264–268] done to

characterize the phenotype of this group, the vast majority of them took place prior to the advent of NGS in the 2000s. Additionally, heterozygosity for pathogenic variants in *APOC2*, *APOA5*, and *LMF1* have been observed to be associated with expression of HTG [263,269–271]. However, similar to the case with heterozygosity for pathogenic variants in *LPL*, the TG phenotype produced by heterozygosity for pathogenic variants in *APOC2*, *APOA5*, and *LMF1* is not familiar and has yet to be systematically evaluated. The case of heterozygosity for pathogenic variants in *GPIHBP1* is an exception since heterozygotes are largely normolipidemic [272].

Additionally, some physicians have extrapolated the clinical expression of heterozygous LPL deficiency from the pattern observed in autosomal dominant familial hypercholesterolemia (FH), in which untreated heterozygotes display a fully expressed, stable phenotype that is about half as severe as in homozygotes [273]. However, the biology of the mutated cell surface receptor in FH does not automatically provide an appropriate model for the mutated plasma enzyme in LPL deficiency. Thus, there is a need to address this assumption with empirical evidence.

Investigating the TG phenotype in patients possessing monoallelic pathogenic variants in the FCS genes is particularly important. While FCS patients are not susceptible to ASCVD [259] due to their total loss of the LPL-mediated lipolytic pathway, the same cannot be said for monoallelic carriers who still possess healthy alleles of the FCS genes apart from the specific allele they are carrying in heterozygous state. Consequently, these patients still produce remnant lipoprotein particles such as LDL which can initiate and contribute to progression of ASCVD. Given that HTG has been shown to account for some of the continued ASCVD risk in patients with well-controlled LDL-C, clarifying the TG phenotype of heterozygous carriers of pathogenic variants in the FCS genes may potentially give us better understanding of this residual risk as well as enable us to better monitor and understand the full range of TG phenotypes possible in the population.

1.4.3.2 Polygenic Hypertriglyceridemia

Polygenic HTG refers to HTG caused, at least in part, by the accumulation or presence of multiple phenotype affecting variants across the genome, both common small-effect

variants and rare large effect variants, with no biallelic loss-of-function variants involved [185,186,260,274]. Extreme accumulation of these variants leads to patients becoming highly susceptible to developing HTG, usually secondary to or exacerbated by non-genetic factors such as diet and lifestyle [260,274]. These variants may be SNPs and/or SVs, with phenotypic impact depending usually on location of the variant within the genome. Variants impacting the coding region of a gene are more likely to produce larger, direct impacts on TG phenotype (e.g., interfering with or disrupting protein active site) while non-coding region variants (which are statistically more common) are more likely to have smaller, indirect impacts on TG phenotype (e.g., slightly altering expression levels) [275]. Polygenic HTG accounts for the vast majority of all HTG cases with genetic underpinnings, both in the mild-to-moderate HTG and severe HTG range [185,186].

Multifactorial chylomicronemia syndrome (MCS) is the polygenic equivalent of FCS, but there are several key differences between the two conditions [274,276–278]. While the exact prevalence of MCS is difficult to pin down, the prevalence of severe HTG in North America is estimated to be between roughly 1 in 600 to 1 in 250 people [279–281]. Given how rare FCS is, MCS is the most common cause of severe HTG as the development of severe HTG almost always requires some genetic involvement [260,274,278]. Despite both being causes of severe HTG, the biochemical phenotypes and clinical presentation of patients with FCS and MCS are quite different. The polygenic nature of MCS means that LPL-mediated lipolysis is not totally abolished unlike in FCS. As result, MCS patients present with a more diverse lipoprotein population than FCS patients. Specifically, MCS patients have elevated levels of CMs, VLDL, IDL, and other remnant lipoprotein species. As a result, MCS patients have a much worse lipoprotein profile for atherosclerosis than FCS patients [260]. This is why MCS patients have elevated ASCVD risk while FCS patients do not. Another consequence of retaining some level of lipolytic activity is differences in the clinical presentation of these two conditions. Firstly, MCS has a significantly later age of onset than FCS, likely owing to the polygenic nature of MCS requiring secondary non-genetic factors to induce severe HTG [260,276]. Secondly, the HTG phenotype is much more severe in FCS than it is in MCS, even when FCS

patients are placed on intense treatment regiments while MCS patients are not [276]. This is primarily because MCS patients still retain some LPL-mediated plasma lipolytic activity whereas FCS patients have no LPL-mediated lipolytic activity. The two main consequences of this are that 1) MCS patients tend to respond better to standard HTG treatments than FCS patients [276] and 2) certain physical manifestations of extreme chylomicronemia are not typically observed in MCS patients while they are commonly found in FCS patients, such as lipemia retinalis and eruptive xanthomatosis [262,277]. Absolute risk of acute pancreatitis is high for both groups compared to the general population, but the prevalence of acute pancreatitis is significantly lower in MCS patients versus FCS patients, with one study finding acute pancreatitis rates of 37% in their MCS cohort and 75% in their FCS cohort which were similarly sized [276].

1.5 General thesis outline

1.5.1 Rationale and Hypothesis

To date, a large body of research has accumulated describing in detail the impact of biallelic pathogenic variants in the FCS genes (*LPL*, *APOC2*, *APOA5*, *GPIHBP1*, and *LMF1*) on human health. However, the impact of monoallelic pathogenic variants in these same genes on human health, specifically on the TG phenotype of these patients, is less familiar. The vast majority of research on such patients took place prior to the genomic era brought on by the advent of NGS.

Thus, the primary aim of my MSc research was to characterize the baseline and longitudinal behaviour of the TG phenotype in heterozygous carriers of pathogenic variants in FCS genes in a clinical setting. My secondary aim was to determine if prior assumptions about phenotypic expression of pathogenic *LPL* variants follows an FH model or not. In other words, my secondary aim was to answer the following question: do *LPL* pathogenic variant heterozygotes have an abnormal phenotype that is stable and intermediate in severity between a homozygous pathogenic variant condition and healthy condition?

We hypothesized that heterozygosity for pathogenic variants in the FCS genes confers a unique TG phenotype not seen in other groups. Additionally, the biochemical phenotype of individuals heterozygous for pathogenic variants in FCS genes does not fit an FH model as the biology of mutated cell surface receptors (as is the case in FH) is quite different from the biology of mutated plasma enzyme (as is the case with LPL and its supporting proteins).

1.5.2 Research Objectives

My first objective was to assess the baseline TG phenotype of heterozygous carriers of pathogenic variants in *LPL*, the gene encoding the central enzyme of plasma TG metabolism, lipoprotein lipase, and to observe how the TG phenotypes of these patients evolved over the course of their treatment at the Lipid Genetics Clinic located in London, Ontario, Canada. These efforts are detailed in Chapter 2.

My second objective was to assess the baseline TG phenotype of heterozygous carriers of pathogenic variants in *APOA5*, the gene encoding one of the major LPL-interacting proteins, apolipoprotein A-V, and observe how the TG phenotypes of these patients evolved over the course of their treatment at the Lipid Genetics Clinic located in London, Ontario, Canada. These efforts are detailed in Chapter 3. Additionally, following this investigation, I conducted a review of all *APOA5* variants reported as disease-causing or disease-associated in various public databases and synthesized my results with the literature. Some of the work done for this review is reflected in the introduction and discussion sections of Chapter 3. I also performed a preliminary investigation into the association between a common *APOA5* SNP, *APOA5* p.Ser19Trp, and HTG. These results are also presented in Chapter 3.

I also had an objective to evaluate the baseline and longitudinal TG phenotypes of heterozygous carriers of pathogenic variants in *APOC2*, *GPIHBP1*, and *LMF1* as well but there were not enough patients with heterozygosity for pathogenic variants in these genes in the Lipid Genetics Clinic population for me to study.

1.6 Summary

This thesis details my research characterizing the baseline and longitudinal TG phenotype and behaviour of this phenotype in a clinical setting. The LipidSeq NGS panel was used to determine the genetic profile of all subjects studied. Review of clinical charts was employed to obtain retrospective biochemical data on the lipid and metabolic profiles of patients studied. These data were utilized to further our understanding of the complexities of plasma lipid metabolism, specifically plasma TG metabolism as my work has now highlighted a unique highly variable TG phenotype found in patients heterozygous for pathogenic variants in *LPL* and *APOA5*.

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Chapter 2 - The longitudinal triglyceride phenotype in heterozygotes with *LPL* pathogenic variants

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Original Research

The longitudinal triglyceride phenotype in heterozygotes with *LPL* pathogenic variants

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KEYWORDS
Complex trait;
Familial chylomicronemia syndrome;
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Hyperlipoproteinemia type 5;
Multifactorial chylomicronemia syndrome;
Hypertriglyceridemia;
Lipoprotein lipase deficiency;
Next-generation DNA sequencing;
Pathogenic variant

Background: Biallelic pathogenic variants in lipoprotein lipase (LPL) cause familial chylomicronemia syndrome with severe hypertriglyceridemia (HTG), defined as plasma triglycerides (TG) > 10 mmol/L (> 885 mg/dL). TG levels in individuals with one copy of a pathogenic *LPL* gene variant is less familiar; some assume that the phenotype is intermediate between homozygotes and controls.
Objective: We undertook an evaluation of the longitudinal TG phenotype of individuals heterozygous for pathogenic *LPL* variants.
Methods: Medically stable outpatients were evaluated based on having: (1) a single copy of a rare pathogenic *LPL* variant; and (2) serial fasting TG measurements obtained over > 1.5 years of follow-up.
Results: Fifteen patients with a single pathogenic *LPL* variant were followed for a mean of 10.3 years (range 1.5 to 30.3 years). TG levels varied widely both within and between patients. One patient had normal TG levels < 2.0 mmol/L (< 175 mg/dL) continuously, while four patients had at least one normal TG level. Most patients fluctuated between mild-to-moderate and severe HTG: five patients had only mild-to-moderate HTG, with TG levels ranging from 2.0 to 9.9 mmol/L (175 to 885 mg/dL), while 6 patients had at least one instance of severe HTG. Of the 203 total TG measurements from these patients, 14.8%, 67.0% and 18.2% were in the normal, mild-to-moderate and severe HTG ranges, respectively.
Conclusion: The heterozygous LPL deficient phenotype is highly variable both within and between patients. Heterozygosity confers susceptibility to a wide range of TG phenotypes, with severity likely depending on secondary factors.

Perera SD, Wang J, McIntyre AD, Dron JS, Hegele RA. The longitudinal triglyceride phenotype in heterozygotes with LPL pathogenic variants. J. Clin. Lipidol. 17(1), 87–93 (2023).

2.1 Introduction

2.1.1 Lipoprotein Lipase

The lipolytic action of what we now know to be lipoprotein lipase (LPL) was first observed in 1943 by Dr. Paul F. Hahn [1] in dogs with visibly lipemic plasma (i.e. that it had creamy white appearance), which we now know to be indicative of chylomicronemia (though in his experiments this was due to blood plasma being taken postprandially rather than due to any genetic factor we know of in the animals). Hahn observed that upon injection of heparinized whole blood or plasma from donor dogs into the lipemic dogs, lipemia was abolished [1]. However, the specific heparin-induced clearing agent was not specifically identified and characterized as "lipoprotein lipase" until 1955 by Dr. Edward Korn [2,3]. In the proceeding decades, a rich body of research has accumulated regarding the specific functionality, biochemistry, regulation, and underlying genetics of LPL, establishing LPL as a key player in plasma lipid metabolism by catalyzing the rate-limiting step in clearing plasma TG from circulation, specifically by catalyzing the hydrolysis of TG in TGRL to FAs and monoacylglycerides [4].

2.1.1.1 Synthesis and expression of lipoprotein lipase in plasma lipoprotein metabolism

LPL is primarily expressed in adipose tissue, skeletal muscle, and cardiac muscle tissue [5]. LPL is synthesized by the parenchymal cells of these tissues and ultimately expressed in the vascular lumen bound to the cell membranes of the endothelial cells of the capillaries supplying these tissues [6]. It is also highly expressed in lactating mammary glands but the LPL in this case comes from adipocytes in the gland, not from the mammary epithelium itself [7].

LPL expression is highly regulated and complex, involving multiple mechanisms and pathways with various physiological states, such as fasting and fed states, triggering differential LPL expression patterns in various tissues [8]. The basic sequence of events leading to mature LPL expression in the capillary lumen of adipose and oxidative (skeletal and cardiac muscle) tissues is as follows. First, upon translation of the signal peptide sequence, the nascent LPL peptide is targeted to and transported into the

endoplasmic reticulum (ER) by the signal peptide. Then, in the ER, the nascent LPL peptide is completely translated and then undergoes N-linked glycosylation, after which the action of lipase maturation factor 1 (LMF1), one of the 5 proteins in which biallelic loss-of-function variants in the gene encoding it have been implicated in causing familial chylomicronemia syndrome (FCS), and Sel-1 suppressor of Lin-12-Like 1 (SEL1L) found on the ER membrane ensure proper folding of the peptide and its secretion from the ER to the Golgi apparatus for packaging and transport to the secretory pathways [9–11]. Each LPL molecule is further modified with the addition of two complex oligosaccharide chains in the Golgi apparatus [12]. Then, in the trans-Golgi network, LPL molecules are directed to one of two primary fates: 1) if they bind to Syndecan-1 (SDC1), a heparan sulfate proteoglycan (HSPG), they are incorporated into secretory vesicles of the sphingomyelin secretion pathway [13] or 2) if they bind to sortilin-related receptor (SorLA-1), which occurs in neutral and acidic conditions, the LPL is targeted to a lysosomal degradation pathway [14]. LPL stored in secretory vesicles may form catalytically inactive helical polymers which are hypothesized to act as a form of LPL storage prior to their secretion into the interstitial space via the sphingomyelin secretory pathway [13,15]. LPL secreted to the interstitial space via this pathway binds to negatively-charged HSPGs on the parenchymal cell surface, via a positively-charged region in LPL, where, eventually, the LPL interacts with and is captured by glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 (GPIHBP1) expressed on the surface of adjacent endothelial cells [16–18]. The newly formed LPL-GPIHBP1 complex undergoes transcytosis from the interstitial space to the capillary lumen cell surface of the endothelial cell [19], where LPL catalyzes the hydrolysis of circulating TGs in various lipoproteins, primarily chylomicrons (CMs) and very-low-density lipoproteins (VLDLs) [4].

Post-translationally, there are two main groups of proteins that regulate LPL expression and activity: 1) the angiopoietin-like (ANGPTL) protein family, specifically ANGPTL3, ANGPTL4, and ANGPTL8 [5,8,20,21], and 2) several apolipoproteins, namely apolipoprotein A-V (apo A-V), apolipoprotein C-I (apo C-I), apolipoprotein C-II (apo C-II), apolipoprotein C-III (apo C-III), and apolipoprotein E (apo E) [5,8,20,22–31].

Briefly, the ANGPTLs listed above all inhibit LPL activity and are involved in differential tissue expression of LPL in fed versus fasted states [8,32–34]. With regards to the apolipoproteins, apo A-V and apo C-II are essential for proper LPL activity, primarily through facilitating interaction of LPL with circulating TG-rich lipoproteins [8,24,35–39], while apo C-III has been found to inhibit LPL activity by inhibiting LPL bound to GPIHBP1 and/or causing displacement of LPL from TG-rich lipoproteins [28,40]. Apo C-I may also inhibit LPL activity by causing displacement of TG-rich lipoproteins from LPL [28]. The role of apo-E in regulating LPL activity is dependent on the isoform expressed. Of the 3 common isoforms expressed in humans, apo E2, E3, and E4, E3 and E4 have been found to limit LPL-mediated TG hydrolysis [41]. The specifics of how all these proteins interact with LPL is beyond the scope of this thesis and will thus not be discussed further. The role of apo A-V and the ANGPTLs will be further discussed in Chapter 3 which discusses the role of apo A-V in plasma TG metabolism in more detail.

2.1.1.2 Protein structure of lipoprotein lipase

Because GPIHBP1 binding to LPL is required to stabilize LPL and prevent spontaneous unfolding of the catalytic hydrolase domain, there has been a great degree of difficulty in characterizing the molecular structure of LPL [42,43]. In 2019 though, two teams characterized the crystal structure of the LPL-GPIHBP1 complex instead of LPL alone, allowing for new insights into the functional unit of plasma TG metabolism [43,44]. Prior to this, we could only make inferences regarding the structure of LPL based on a homology model constructed from the structure of pancreatic lipase, a distantly related member of the same lipase protein family [45].

After cleavage of the 27 amino acid signal peptide, mature LPL is composed of 448 amino acids with molecular mass of ~55 kDa [46]. Note that the numbering used for residues discussed in this section includes the signal peptide as residues 1-27. The previous homology model indicated that like pancreatic lipase, LPL should have two primary functional domains, 1) a N-terminal α/β-hydrolase domain containing a catalytic triad, and 2) a C-terminal lipid-binding domain formed by a β-barrel [45]. Both 2019 studies on the LPL-GPIHBP1 complex crystal structure confirmed that this prediction

was true [43,44]. The larger N-terminal α/β-hydrolase domain is specifically composed of 6 α-helices and 10 β-strands with 3 notable regions: 1) serine protease-like catalytic triad (Ser159, Asp183, His268); a lid region (residues 245-265), which likely regulates substrate availability to the active site residues by adopting open or closed conformations; and 5 residues (Ala194, Arg197, Ser199, Asp201, and Asp202) that are involved in coordination of a calcium ion into the LPL molecule [43,44]. There is also an oxyanion hole formed by residues Trp82 and Leu160 in the N-terminal domain [44]. The smaller C-terminal region is composed of a flat β-barrel, containing a tryptophan-rich lipid binding region (residues 412-422) which is likely responsible for substrate recognition [43,44,47]. It should be noted that some hydrophobic residues of the lid region in the N-terminal domain have also been implicated in lipid binding [43,44]. Additionally, the C-terminal region is responsible for LPL binding to GPIHBP1 and HSPGs. GPIHBP1 interacts with LPL C-terminal domain via a Ly6/uPAR domain in GPIHBP1 which adopts a 3-fingered fold conformation stabilized by 5 disulfide bonds [43,48–50]. Specifically, residues 443-447 and 465-466 interact with finger 1, residues 447-448 and 463-467 interact with finger 2, and residues 367, 369, 374, 403-406, 447, and 464 interact with finger 3 [43].

Historically, it was thought that LPL monomers were non-functional and head-to-tail homodimerization, thought to be facilitated by LMF1 in the ER, was required to produce a functional LPL unit [10,46,51,52]. In fact, the crystallographic studies of the LPL-GPIHBP1 complex in 2019 also observed the complexes in head-to-tail homodimer configuration [43,44]. However, there is a major caveat to this assumption: logically, the head-to-tail homodimer configuration produced by the intercalation of the tryptophan-rich lipid-binding region and active site of opposite monomers would prevent lipid/lipoprotein binding and catalytic activity, a fact discussed in both crystallographic studies [43,44]. However, the later of the two studies determined that the homodimer structure they observed was likely of no physiologically relevance as they found that the structure they observed was an artifact of the crystal packing interaction utilized in the study [44]. Recently, the historically held assumption of head-to-tail homodimers as the functional unit has been called into question as it has been reported that LPL, both alone

and complexed to GPIHBP1, are indeed catalytically active [46]. Additionally, the later of the two crystallographic studies also confirmed this and showed that the head-to-tail homodimer configuration observed in crystallographic studies was most likely due to crystal packing interactions rather than a physiologically relevant protein monomer interaction [44]. Thus, it was concluded that the LPL-GPIHBP1 complex monomer was the functional unit. Recently, though, it was shown that LPL may form helical polymers in secretory vesicles prior to their secretion, indicating a new physiologically relevant form of LPL, albeit one that is not catalytically active [15]. However, a new catalytically active form of LPL has been described, complicating the picture even further [53]. Specifically, a novel homodimer scheme for LPL has been discovered, in which the C-terminals of adjacent LPL monomers interface (i.e., a tail-to-tail homodimer) to produce a catalytically active homodimer [53]. It should be noted that this homodimer would not be bound to the endothelial cell surface and would instead likely circulate through the plasma [53]. This finding, while *in vitro,* is consistent with findings of dimeric LPL bound to circulating TG-rich lipoproteins and hydrolyzing them [54,55]. In fact, recent work has found that upon association of the LPL-GPIHBP1 complex with chylomicrons and in the presence of excess FFA, likely from hydrolysis of chylomicron contents, LPL dissociates from the complex which may help explain how LPL homodimer would form *in vivo* [53,56]. Additionally, resolution of the LPL homodimer structure revealed a hydrophobic pore adjacent to the active site, which was shown to be able to accommodate TG fatty acid chains and was hypothesized to provide substrate specificity and/or allow unidirectional release of FFA from TG hydrolysis in contrast to the bidirectional release observed with LPL-GPIHBP1 complex mediated hydrolysis of TG [53].

2.1.1.3 Genomic structure of *LPL*

The gene encoding LPL in humans, namely *LPL*, is located on the short arm of chromosome 8 (8p21.3) and contains 10 exons and 9 introns spanning roughly 30 kb [57,58]. Exons 1-9 are of similar size but exon 10 is notably larger, containing the entire 3’ untranslated region [57,58]. LPL is a member of the lipase superfamily which includes hepatic lipase and pancreatic lipase and given the structural similarities of these proteins,

the gene encoding these proteins likely derive from a common ancestral gene [58]. *LPL* as previously stated, is expressed in adipose tissue, skeletal muscle and cardiac muscle [5].

2.1.1.4 Biallelic *LPL* variants and familial chylomicronemia syndrome

As previously discussed in Chapter 1 section 1.4.3.1.1, familial chylomicronemia syndrome (FCS) is an ultrarare phenotype, with prevalence estimated at one in 100,000 to 1,000,000 [59–61]. FCS is caused by biallelic pathogenic variants in *LPL* or in one of four other genes which encode LPL-interacting proteins apo C-II, apo A-V, GPIHBP1 and LMF1 [62]. FCS is characterized by sustained refractory severe HTG and systemic manifestations including lipemia retinalis, eruptive xanthomatosis and hepatosplenomegaly [63]. About 70-80% of FCS cases are caused by biallelic pathogenic variants in *LPL* (synonymous with LPL deficiency or former Frederickson hyperlipoproteinemia type 1), with the remaining cases resulting from biallelic variants in the other four genes [62].

2.1.2 Monoallelic lipoprotein lipase deficiency is an underappreciated risk factor for hypertriglyceridemia

The phenotype of FCS - and specifically LPL deficiency - has been well studied over the past half century. However, the phenotype resulting from the more common situation of a single copy of pathogenic variant in *LPL* (i.e. heterozygous LPL deficiency) is less familiar and has been less systematically evaluated [60]. Some physicians have extrapolated the clinical expression of heterozygous LPL deficiency from the pattern observed in autosomal dominant familial hypercholesterolemia (FH), in which untreated heterozygotes display a fully expressed, stable phenotype that is about half as severe as in homozygotes [64]. However, the biology of the mutated cell surface receptor in FH does not automatically provide an appropriate model for the mutated plasma enzyme in LPL deficiency. Therefore, there is a need to clarify if phenotypic expression of pathogenic *LPL* variants follows a model akin to the FH model. In other words, there is a need to answer the following questions: 1) do heterozygotes for pathogenic *LPL* variants have an abnormal phenotype that is only intermediate in severity between a homozygous

individual and a healthy individual with two normal alleles?; and 2) is the phenotype of these heterozygotes stable over time? We previously showed that heterozygotes for rare, pathogenic variants in *LPL* are significantly overrepresented – by 3- to 4-fold – in cohorts with both severe and mild-to-moderate HTG but are also present in a small proportion (~3%) of adults with normal TG levels [65,66]. The wide range of TG phenotypes suggests that the analogy with heterozygous FH might be inappropriate.

2.1.3 Study description and objective

Thus, we evaluated TG levels in individuals heterozygous for pathogenic *LPL* variants who were followed for multiple years (see section 2.3.5 for more details on specific range of years). We found that the TG phenotypes were highly variable both within and between patients, ranging from normal to severe HTG, at baseline and longitudinally.

2.2 Materials and methods

2.2.1 Study subjects

Individuals were patients at the Lipid Genetics Clinic, London, Ontario, Canada who were referred for management of their lipid levels. Inclusion criteria were: (1) heterozygosity for a pathogenic *LPL* variant; (2) at least three TG measurements taken over a minimum of 1.5 years; and (3) patient was medically stable over the duration of follow-up. The project was approved by the Research Ethics Board of Western University (protocol number 0379; **Appendix B**) and all participants provided informed consent.

2.2.2 Biochemical, clinical, and demographic information

As part of routine lipid profiling after an 8 to 12 hour fasting period, plasma TG measurements were collected for all patients at baseline and at routine follow-up visits to the Lipid Genetics Clinic. Additional historical fasting plasma TG measurements were collected from referral notes provided to us at each patient's initial visit to the Lipid Genetics Clinic. Lipid profiles were measured using the Roche Cobas C502 Analyzer (Hoffmann La Roche, Mississauga, ON, Canada), as reported previously [65,66]. Clinical

and demographic data were collected at the time of the initial visit to the Lipid Genetics Clinic.

2.2.3 DNA preparation and sequencing

Genomic DNA isolation and gene sequencing protocols have been described previously [65,67,68]. Briefly, genomic DNA from whole blood was enriched for our LipidSeq panel, targeting 69 genes and 185 single nucleotide polymorphisms (SNPs) associated with dyslipidemia and other metabolic disorders [69]. Sequencing was performed using standard protocols at the London Regional Genomics Centre on a MiSeq personal sequencer (Illumina, San Diego CA).

2.2.4 Genetic Analysis

Our standard bioinformatic processing and annotation pipeline was used to call variants [65]. Briefly, CLC Bio Genomics Workbench (version 12.0; CLC Bio, Aarhus, Denmark) was first used to align sequencing reads for each patient sample against the human reference genome (build hg19) and was then used to call variants.

2.2.5 Variant pathogenicity classification

Pathogenicity of *LPL* variants was first determined using the VarSome ACMG tool (https://varsome.com/about/resources/acmg-implementation/). In 2015, the American College of Medical Genetics and Genomics (ACMG) established a system for interpreting the pathogenicity of sequence variants [70]. The VarSome ACMG tool was developed to computerize this system originally designed for manual use by experienced clinicians. VarSome served as the first filter to identify pathogenic or likely pathogenic variants. We manually confirmed the pathogenicity of each variant using ACMG guidelines, resulting in a cohort of patients whom we were confident were heterozygous carriers of rare pathogenic *LPL* variants. For patients included in our study, pathogenic rare variants were only found in *LPL* and none of the other canonical FCS genes (i.e. *APOC2*, *APOA5*, *GPIHBP1* and *LMF1*).

2.2.6 Polygenic risk score for elevated triglyceride levels

We utilized a weighted 16-SNP polygenic risk score (PRS) previously developed by our lab to assess the cumulation of common TG-raising alleles in each patient [65]. More information on calculation and interpretation of polygenic risk scores are discussed by Dron et al [71].

2.2.7 Statistical analysis

Calculation of means and standard deviations was performed using GraphPad Prism 9.3.1 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). Means are reported as mean ± standard deviation. Figures were generated in R version 4.1.3 (R Foundation for Statistical Computing, Vienna, Austria, https://www.r-project.org/) using the ggplot2 package [72].

2.3 Results

2.3.1 Study subjects

The filtering algorithm for patient inclusion is shown in **Figure 2.1**. Forty patients were heterozygous for a rare *LPL* variant. Of these, 19 were identified by the VarSome ACMG tool as having a pathogenic or likely pathogenic variant in *LPL*. Upon manual curation of variants in these 19 patients following the ACMG guidelines, two were excluded from further analysis as manual review concluded that variants possessed by those patients were variants of uncertain significance, not pathogenic or likely pathogenic. Two more patients were excluded due to an insufficient number of TG measurements available for review. Our final cohort consisted of 15 unrelated patients with pathogenic *LPL* variants (**Table 2.1**).

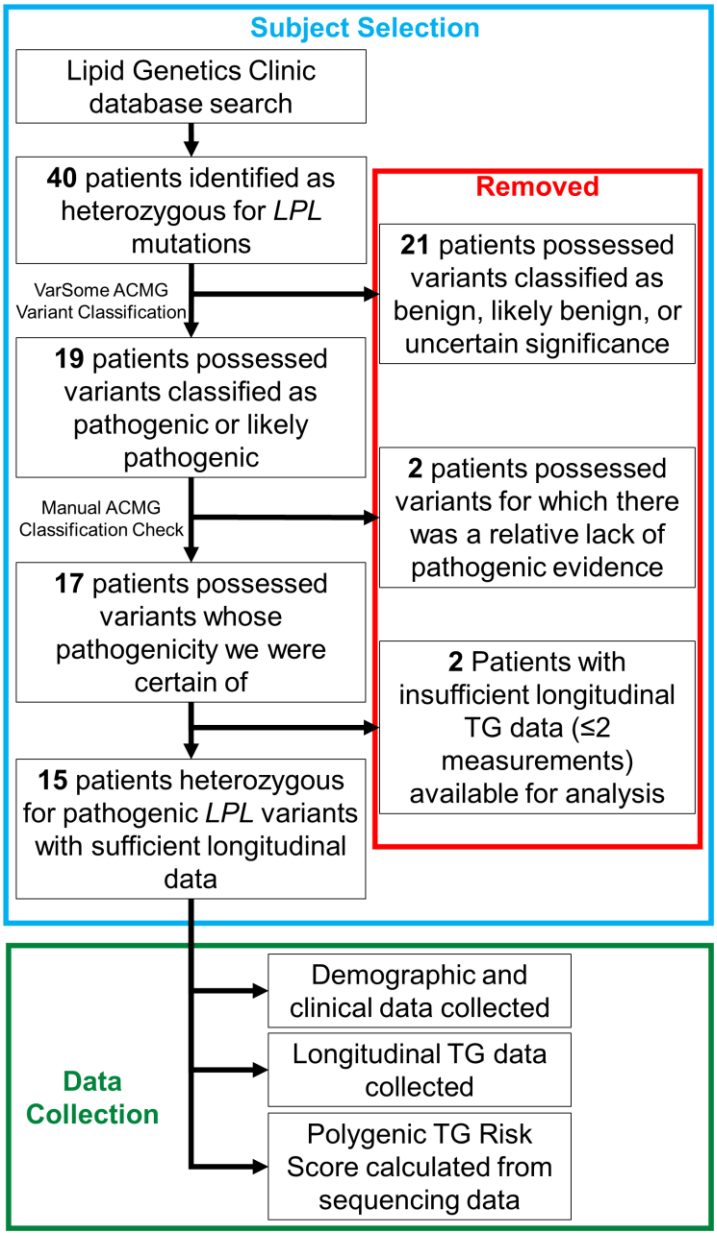

Figure 2.1. Subject filtering and selection.

Flowchart depicting subject selection and filtering process. Subjects were included in the study if they were heterozygous for pathogenic *LPL* variants and if 3 or more TG measurements were available for analysis. Of 40 patients identified as heterozygous for any *LPL* variants, 15 were found to possesses pathogenic variants and have 3 or more TG measurements available for analysis.

Table 2.1. Unique *LPL* variants found.

Variant (Exon:DNA change:Protein Change)	VarSome ACMG Classification	Number of Patients in Cohort (N = 15)	Previously Reported?
exon1:c.46_47del:p.Q16fs*24[a]	Likely Pathogenic	2	Yes [73]
exon3:c.G286C:p.V96L	Pathogenic	2	Yes [74]
exon3:c.G292A:p.A98T	Pathogenic	1	Yes [75]
exon5:c.T626G:p.L209Term[b]	Pathogenic	1	No
exon5:c.G644A:p.G215E	Pathogenic	5	Yes [76,77]
exon6:c.C808T:p.R270C	Pathogenic	2	Yes [78]
exon6:c.G809A:p.R270H	Pathogenic	1	Yes [78,79]
exon6:c.G1018A:p.V340I	Pathogenic	1	Yes [80]

[a] The notation ‘fs*(number)’ indicates that the frameshift variant results in a stop codon at the position (number) residues downstream of the variant site.

[b] Term = Termination; indicates a variant that results in a prematurely truncated protein due to premature stop codon

2.3.2 Baseline demographic information

Demographic information and lipid-related clinical characteristics of our cohort are summarized in **Table 2.2**. Mean age and body mass index (BMI) were 48.1 ± 7.1 years and 30.2 ± 3.8 kg/m^2 respectively. Two subjects were of non-Northern European ancestry (patient 5 was of South Asian ancestry and patient 6 was of Afghan ancestry; see section 4.3.2 for more details regarding the relevance of this factor), two each had a history of pancreatitis (patients 7 and 11) and ASCVD (patients 7 and 12), and four had well-controlled type 2 diabetes mellitus (patients 2, 7, 13, and 15). Over the duration of follow-up, no patient developed new-onset diabetes, or thyroid, renal or liver disease. In addition, medication use, including the use of lipid-lowering medications, was stable over the duration of follow-up. Only three patients were female (patients 1, 9, and 11). Only one patient was < 40 years of age (patient 4) and his baseline TG was 8.19 mmol/L. Only three patients had BMI < 27 kg/m^2 (patients 5, 10, and 14) and their baseline TG levels were 1.50, 2.03 and 3.70 mmol/L, respectively. Over the study duration, BMI remained stable for most patients. Over the duration of the study, 14 patients were continuously on a statin (all patients except patient 8), 12 also took a fibrate (all patients except patient 2, 5, and 9), one took niacin (patient 8), 7 took ezetimibe (patients 1, 5, 6, 8, 11, 12, and 15) and 4 took over the counter omega-3 fatty acids (patients 6, 11, 13, and 15). Prescriptions and dosages were consistent over > 90% of study visits and compliance was judged as satisfactory for all study participants.

Table 2.2. Baseline characteristics in patients heterozygous for pathogenic *LPL* variants.

Number of patients/females	15/3
Age (years)	48.1 ± 7.1
Body mass index (kg/m^2)	30.2 ± 3.8
Non-Northern European ancestry[a]	2
Type 2 diabetes mellitus	4
History of acute pancreatitis	2
History of atherosclerotic cardiovascular disease	2
Current or former smoking history	5
No alcohol consumption	6
Total cholesterol (mmol/L)	7.36 ± 3.30
Triglycerides (mmol/L)	13.2 ± 14.1
High-density lipoprotein cholesterol (mmol/L)	0.66 ± 0.29

Values shown are mean ± standard deviation or number of patients unless otherwise indicated.

[a] See section 4.3.2 for more details on the relevance of non-northern European ancestries.

2.3.3 Pathogenic *LPL* variants

Six unique missense variants of *LPL* were found in our cohort: p.Val96Leu in two patients, p.Ala98Thr in one patient, p.Gly215Glu in five patients, p.Arg270Cys in two patients, p.Arg270His in one patient, and p.Val340Ile in one patient. One frameshift variant resulting in a premature stop codon, p.Gln16fs*24, was found in two patients. One nonsense variant, p.Leu209Term, was found in a single patient. All variants were previously reported, except p.Leu209Term (**Table 2.1**). The positions of each variant along the *LPL* gene are shown in **Figure 2.2**.

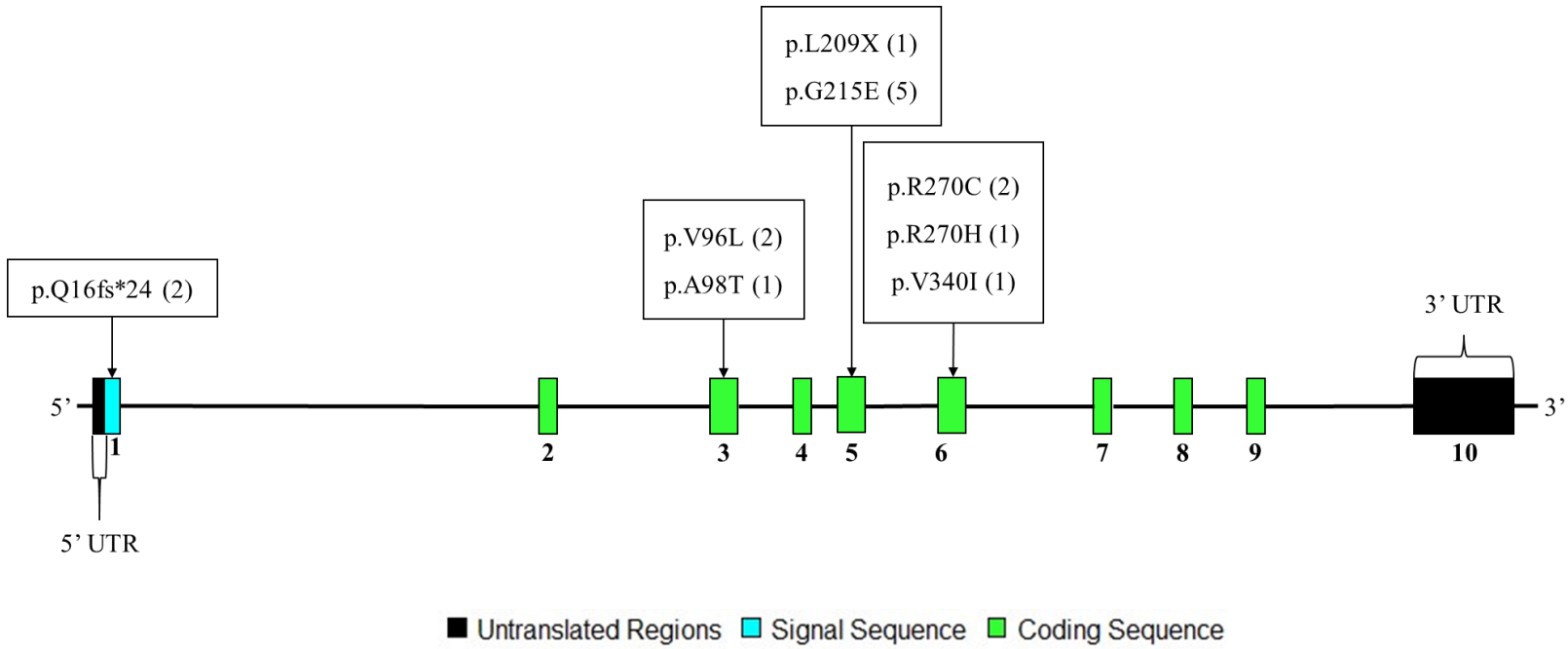
p.L209X (1)
p.G215E (5)
p.R270C (2)
p.R270H (1)
p.V340I (1)
p.V96L (2)
p.A98T (1)
p.Q16fs*24 (2)
3' UTR
5'
3'
1
2
3
4
5
6
7
8
9
10
5' UTR
Untranslated Regions
Signal Sequence
Coding Sequence

Figure 2.2. Genetic map of pathogenic *LPL* variants identified in study.

Genetic map displaying the locations of *LPL* variants analyzed in this study. Major structural features are color-coded. Black boxes indicate untranslated sequences, blue boxes indicate sequences coding for the LPL signal peptide, and green boxes indicate sequences coding for the mature protein. Regarding variant nomenclature, p.Q16fs*24 (p.Gln16fs*24); p.V96L (p.Val96Leu); p.A98T (p.Ala98Thr); p.L209X (p.Leu209Term); p.G215E (p.Gly215Glu); p.R270C (p.Arg270Cys); p.R270H (p.Arg270His); and p.V340I (p.Val340Ile). Number in brackets shows the number of patients carrying the indicated variants in our cohort.

2.3.4 Variation in baseline triglycerides

At baseline, two (13.3%), seven (46.7%), and six (40%) patients had normal TG levels, mild-to-moderate HTG, and severe HTG, respectively. The average baseline lipid profile at the initial visit to Lipid Genetics Clinic is shown in **Table 2.2**.

2.3.5 Distribution of longitudinal fasting plasma triglycerides in *LPL* heterozygotes

The average duration of follow-up was 10.3 ± 8.5 years (range was 1.5 to 30.3 years). During follow-up, patients had on average 13 ± 8 TG measurements. Marked variability was observed in TG levels between patients at baseline (**Figure 2.3**). Wide within-patient variability of TG levels over time was common: only one patient (6.7%) continuously had normal TG levels. Five patients (33.3%) had TG levels only within the mild-to-moderate HTG range continuously, while no patients had TG levels only within the severe HTG range. Six patients (40.0%) had TG levels that fluctuated between mild-to-moderate and severe HTG ranges. TG levels in one patient (6.7%) fluctuated between normal and the mild-to-moderate HTG range, without entering the severe HTG range. Two patients (13.3%) had at least one TG reading in the normal, mild-to-moderate, and severe HTG ranges at different time points. Among the 203 total TG measurements taken, 136 (67.0%) fell in the mild-to-moderate range, with the remainder divided between the normal and severe HTG ranges at 30 (14.8%) and 37 (18.2%), respectively.

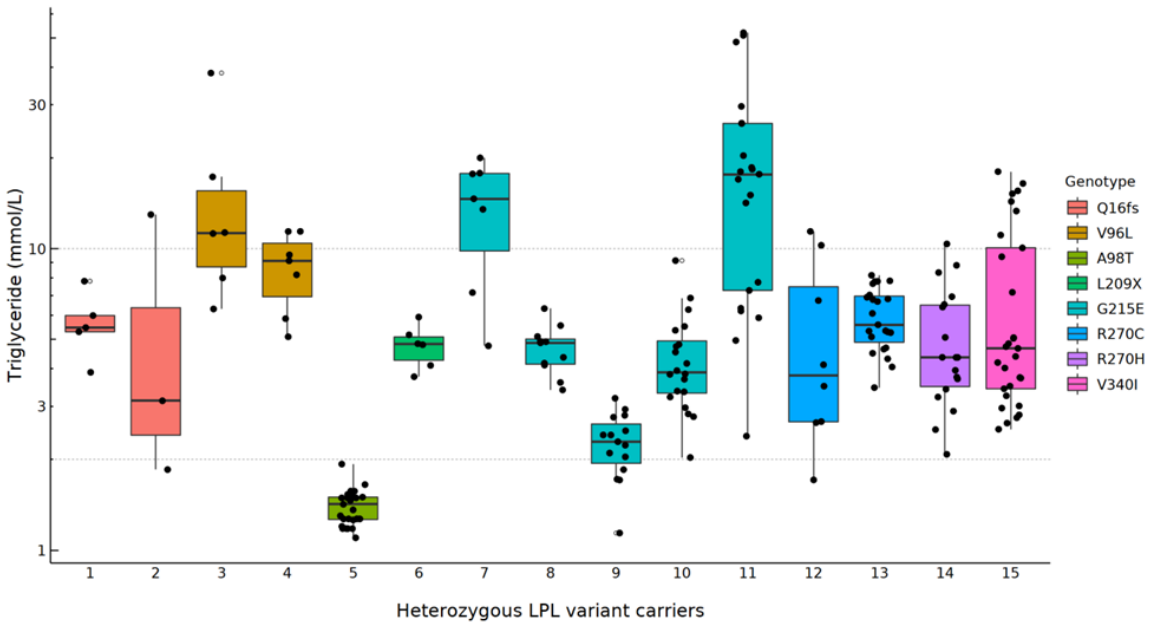

Figure 2.3. Variable triglyceride phenotypes in patients heterozygous for pathogenic *LPL* variants.

Distribution of longitudinal TG measurement data from heterozygous carriers of pathogenic *LPL* variants (N = 15) was graphed as individual box-and-whisker plots. Boxes are color-coded according to genotype. Each patient's median TG level is represented as the middle line of each box, while the whiskers display the interquartile range of triglycerides achieved by each patient. Individual TG measurements are represented as black dots. Fasting plasma TG (mmol/L) are displayed using a $\log_{10}$ scale. Horizontal lines at 2 and 10 mmol/L delineate the cut-offs for TG severity classifications (mild-to-moderate HTG is defined as TG between 2 and 9.9 mmol/L while severe HTG is TG > 10 mmol/L).

2.3.6 Association between pathogenic *LPL* variants and longitudinal TG phenotype

No consistent pattern in longitudinal TG measurements was observed to be associated with a particular variant, and instead variability appeared to be a common feature of the heterozygotes for pathogenic *LPL* variants (**Figure 2.4**). The five patients with the *LPL* p.Gly215Glu variant showed marked between-patient variability (**Figure 2.4E**). There was phenotypic variability observed in carriers of other variants, such as p.Arg270Cys and p.Val96Leu (**Figure 2.4F** and **2.4B**, respectively).

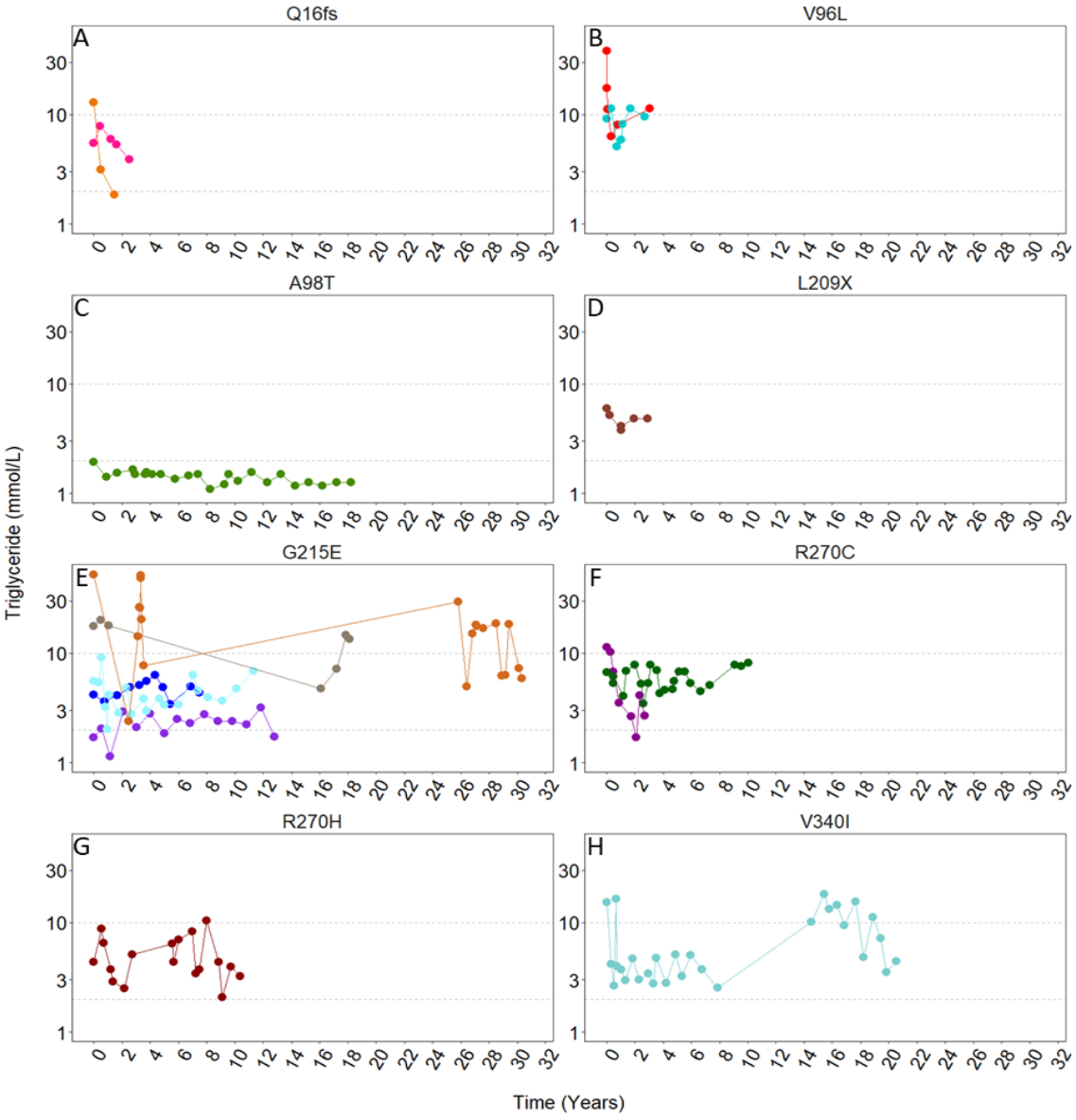

Figure 2.4. Triglyceride trajectories of individuals heterozygous for pathogenic *LPL* variants.

Longitudinal TG data for all patients (N = 15) plotted as years elapsed from first measurement (X-axis). Y-axis displays fasting plasma triglyceride level (mmol/L) using a $\log_{10}$ scale. Trajectories for patients heterozygous for **A)** p.Q16fs (p.Gln16fs*24), **B)** p.V96L (p.Val96Leu), **C)** p.A98T (p.Ala98Thr), **D)** p.L209X (p.Leu209Term), **E)** p.G215E (P.Gly215Glu), **F)** p.R270C (P.Arg270Cys), **G)** p.R270H (p.Arg270His), and **H)** p.V340I (p.Val340Ile).

2.3.7 Polygenic influences of triglyceride phenotype

PRS data were available for all patients. Patients 1 to 15 had PRSs in the 50th, 5th, 75th, 23rd, 23rd, 3rd, 83rd, 75th, 97th, 75th, 83rd, 75th, 23rd, 75th, and 92nd percentiles for elevated TG, respectively. In our clinic, a PRS exceeding the 90th percentile is considered to be indicative of clinically relevant polygenic risk accumulation for HTG. Therefore, only 2 of the 15 patients, specifically patients 9 and 15, are considered to have clinically relevant PRSs, meaning that the majority of our cohort likely does not have background polygenic susceptibility contributing to their TG phenotype.

2.4 Discussion

We report marked within and between patient variability of the TG phenotype in heterozygotes for pathogenic *LPL* variants, some of whom were evaluated for decades. Baseline untreated TG levels ranged from normal to severely elevated, and over time they fluctuated between the normal and mild-to-moderate HTG ranges, and mild-to-moderate and severe HTG ranges. About two-thirds of all TG measurements taken from all 15 patients were in the mild-to-moderate HTG range, and the remaining one-third were about equally divided between the normal and severe HTG ranges. Heterozygous LPL deficiency is not analogous to heterozygous FH, in which low-density lipoprotein (LDL) cholesterol levels do not fluctuate so widely within and between patients.

Heterozygosity for pathogenic variants in *LPL* is relatively common in the general population compared to homozygosity: up to 3% in our studies [60,65,66]. In contrast, biallelic rare *LPL* variants in FCS are associated with fully expressed severe HTG, but fortunately this is an ultrarare condition [60]. Heterozygotes are thus many times more prevalent in the population, but there is less familiarity with their phenotype. Others have reported variability of the TG phenotype previously [76,81–84]. For instance, in the pre-genomic era, John Brunzell and colleagues imputed heterozygote status in the parents of children with complete biochemical LPL deficiency [81]. They observed that one-quarter of parents had normal fasting TG levels, one half had mild-to-moderate HTG, and the remainder had severe HTG [81]. Although the DNA basis for the pathogenic *LPL*

variants was not determined, the authors nonetheless commented on the wide variability of TG phenotypes in obligate heterozygotes for LPL deficiency [81].

In the early genomic era, Wilson and colleagues studied large multigenerational Utah kindreds extended from the first index case of FCS with two copies of the *LPL* variant p.Gly215Glu, (also known as p.Gly188Glu, depending on the numbering of the propeptide sequence) [76,82]. *LPL* p.Gly215Glu is recognized as the archetypal non-functional *LPL* missense variant, with a complete lack of lipolytic activity *in vitro* [76]. *LPL* p.Gly215Glu is also the most common pathogenic variant seen in cohorts of FCS patients [62]. Among genotyped heterozygotes, Wilson and colleagues noted a wide range of possible fasting TG levels [82]. Data from our five heterozygotes for *LPL* p.Gly215Glu, similarly showed marked between-patient variability in TG levels (**Figure 2.3** and **Figure 2.4E**).

What factors might explain the variability in TG phenotype in heterozygotes? We observed no clear relationship with degree of TG perturbation depending on the variant subtype (e.g., missense versus nonsense). It is possible that residual LPL activity in heterozygotes could contribute to a variable TG phenotype, although earlier studies showed that post heparin LPL activity in heterozygotes was highly variable – even above the normal range in some cases - and not clearly correlated with the TG level [81]. Furthermore, background polygenic predisposition to HTG [65] is well known to be a major contributor to the prevalence of severe HTG in the population. However, only 2 patients within our cohort were considered to have clinically relevant PRSs, indicating that polygenic risk does not appear to be playing a major role in influencing the majority of the TG phenotypes we observed in our cohort. But the earlier studies of Wilson and colleagues may provide some clues. Among Utah heterozygotes for *LPL* p.Gly215Glu, variability in TG levels was related to age: specifically, 94% of heterozygotes < 40 years old had normal TG levels while two-thirds of those > 40 years old had HTG, of whom 15% had severe HTG [82]. The average age at referral in our cohort was 48.1 years, which is consistent with age-related predisposition to at least mild-to-moderate HTG and sometimes severe HTG. We did not observe clear temporal or age-related trends for

increased TG levels. Also, our three female patients were not obviously different from males with respect to baseline TG and variability.

Other studies of kindreds with heterozygous *LPL* pathogenic variants reported that obesity, insulin resistance and type 2 diabetes mellitus worsened the severity of the HTG phenotype [83–85]. Unfortunately, our sample is too small – e.g., only four patients had diabetes - to evaluate the relationship of obesity and/or diabetes on HTG severity. However, our patients were all following medical advice, including stable adherence to medications.

It is interesting to consider that some of the variability between patients with different variants may be due to the specific molecular defect produced by their variants. We observed 8 unique variants in our cohort (**Table 2.1**). Regarding the earliest occurring variant in the *LPL* sequence we observed in our cohort, to the best of our knowledge, there have been no dedicated functional analyses for the *LPL* p.Gln16fs*24 variant, though this is not surprising considering the rather obvious molecular defect produced by this variant [73,86]. This frameshift variant produces a premature stop codon at residue 40 of the amino acid sequence, resulting in over 90% of the LPL peptide not being synthesized. Consequently, heterozygotes for this variant possess only a single functional *LPL* allele. To the best of our knowledge, haploinsufficiency is not a mechanism of disease of *LPL* pathogenic variants in humans, so the elimination of a single allele is likely not sufficient on its own to produce HTG. In fact, as discussed in more detail later in this discussion, normolipidemic carriers of monoallelic pathogenic *LPL* variants are common [62,65,66]. However, some preliminary evidence from a study of the *LPL* gene in Mediterranean river buffalo indicates that haploinsufficiency may be a regulatory mechanism present in that species [87]. In-depth studies in humans will be needed to determine if this mechanism may be at play with regards to human *LPL* variants. Next, *LPL* p.Val96Leu variant has been observed previously in patient with LPL deficiency [74]. The *LPL* p.Val96Leu variant was previously observed in compound heterozygosity with *LPL* p.Gly215Glu, together causing LPL deficiency in a patient [74]. *In vitro* studies conducted by this team found that the *LPL* p.Val96Leu variant protein demonstrated significantly lower catalytic activity at 26 ± 4% of wild-type LPL activity but stability

comparable to wild-type protein [74]. This is rather interesting considering that the valine and leucine side chains have similar molecular structure. Bruin et al. propose that the reduction in catalytic activity is likely due to the fact that the Val96 residue is located in the center of an alpha-helix structure in the wild-type protein and the small difference between the molecular structures of valine and leucine may be sufficient to induce a physiologically relevant structural change in the alpha-helix [74]. We know that the N-terminal region alpha-helices comprise a major component of the α/β-hydrolase catalytic domain of LPL, which explains the catalytic defect associated with this variant [43,44]. In fact, this also explains why there is not stability defect. Since valine and leucine are structurally similar, it is unlikely that the structural changes produced by this variant are sufficient to disrupt protein folding significantly enough to alter the structure of the C-terminal domain which is responsible for stabilizing the mature LPL protein by binding to GPIHBP1 [42,43]. Thus, it is likely that the *LPL* p.Val96Leu variant is pathogenic, but the specific molecular defect is less obvious and less damaging as some residual LPL activity is retained by the variant LPL protein unlike with p.Gln16fs*24 variant peptide. Next, the *LPL* p.Ala98Thr variant has been well established to produce significantly reduced catalytic activity and secretion of LPL protein [75]. While this variant does not seem to directly impact the active site of the protein, it does impact an alpha helix in the α/β-hydrolase catalytic domain of LPL similar to the previously discussed *LPL* p.Val96Leu [43,44]. The secretion defect likely arises from the significantly greater structural differences between alanine and threonine leading to more significant structural and/or stability defects in the variant protein. While the *LPL* p.Leu209Term variant has not been previously reported, premature protein truncation is a well-known mechanism of disease in LPL [88]. The premature stop codon in this case leads to complete loss of the C-terminal domain which contains the lipid binding loop as well as the features that enable LPL to interact with GPIHBP1 and itself [43,44,53], thus rendering the variant peptide non-functional. The clinical phenotypes associated with *LPL* p.Gly215Glu variant heterozygosity have already been extensively discussed earlier in this section, but the molecular defect has not been. Briefly, it has been long established that this is one of the classical examples of a loss-of-function variant in *LPL* as the glycine residue at this position is the only amino acid which can meet the structural constraints present in this

location for proper functioning and folding of the LPL protein [89]. Additionally, it has been established that the presence of this variant reduces protein synthesis of the variant peptide by ~50% and ~80% reduced secretion rate [90]. This variant did not impact protein degradation rate though [90]. Interestingly, this indicates that there may be a possible pre-translational mechanism acting on variant mRNA to prevent protein synthesis of this variant peptide. To the best of our knowledge, no such mechanism has been investigated nor described yet, but this may be an additional mechanism of disease in *LPL* pathogenic variants. The *LPL* p.Arg270Cys and p.Arg270His variants occur in extremely close proximity to the His268 residue of LPL which is the final catalytic triad residue of the protein [43,44]. Both variants at residue 270 have been observed to produce total LPL deficiency in the homozygous state [78]. Both cysteine and histidine have significantly different molecular structures compared to the arginine they are replacing. It is likely, that given these structural differences, the substitution of either of these amino acids for arginine at residue 270 is likely to significantly alter the local environment near the His268 residue of the catalytic triad which may interfere with its proper functioning and/or result in structural defect that prevents it from properly forming the active site of the protein. However, to the best of our knowledge, there have been no modelling or structural analyses that have been performed regarding this. Finally, to the best of our knowledge, there have not been any structural or functional analyses performed with regards to the *LPL* p.Val340Ile variant which has been previously reported in an HTG genetic screening study [80]. Thus, we can only pose conjecture on the molecular defect associated with this variant. This variant is significantly upstream of the lipid binding loop located at residues 412-422 so it is unlikely that it interferes with the functional domain specifically. However, it is in close proximity to some of the residues associated with GPIHBP1 binding, specifically binding to finger 3 of the GPIHBP1 Ly6/uPAR domain [43]. However, valine and isoleucine are structurally similar so the degree to which such a change would impact GPIHBP1 binding is questionable. More investigations are needed to determine what the exact defect produced by this variant is.

With regards to the two premature truncation variants we observed, *LPL* p.Gln16fs*24 and p.Leu209Ter, it should be noted that to the best of our knowledge that nonsense mediated decay is not an established mechanism of disease with regards to *LPL* loss of function variants. It has only been previously presumed to be the mechanism of disease with no direct evidence [91]. Additionally, to the best of our knowledge, dominant negative variants are not known to occur in *LPL*. However, it is possible they might exist. While no solid evidence is available to the best our knowledge, it was proposed by Bruin et al. in 1994 that a heterodimer of two variant LPL peptides would be highly unstable, primarily due to the instability caused by one of the variants (*LPL* p.Gly215Glu) as the other variant was highly stable (*LPL* p.Val96Leu) [74]. However, this would not impact LPL complexed with GPIHBP1 which is monomeric [46] and instead impact circulating LPL species such as the proposed homodimer recently described [53]. More investigation is needed regarding this matter.

With growing availability of genetic testing, heterozygosity for pathogenic *LPL* variants is increasingly being reported in genetic test results for patients with severe HTG. Our understanding of the clinical consequences of heterozygous LPL deficiency indicates that this genotype cannot be the direct cause of HTG. This is because up to 3% of people with normal lipids also have a heterozygous pathogenic *LPL* variant [62,65,66]. Furthermore, only 10% of patients with mild-to-moderate HTG [66] and only 15–20% of patients with severe HTG [65] are heterozygotes for pathogenic variants. Heterozygosity is a risk factor that raises the odds of expressing HTG but is not causal. Furthermore, most heterozygotes with pathogenic *LPL* variants have normal lipids: 3% of the general population, who are never referred to clinic and will never undergo sequencing for HTG genes. The absolute number of normolipidemic heterozygotes dwarfs the 15-20% of heterozygotes seen among the 1 in 400 individuals referred with severe HTG. Because genetic testing for *LPL* is not pursued clinically for healthy people, the predominant relationship of this genotype with a normal lipid profile will not be appreciated by clinicians.

Genetic testing in adult patients is pursued in the context of severe HTG, which is operationally equivalent to multifactorial chylomicronemia syndrome (MCS) [92]. In our

experience some clinicians who order genetic testing in patients with severe and refractory HTG are expecting to find recessive biallelic pathogenic variants in *LPL*, *APOC2*, *APOA5*, *GPIHBP1* or *LMF1*, consistent with FCS. However, this genotype is present in only 1-5% of adults with severe HTG [60,62,65,73,93,94]. Instead, 15–20% of severe HTG patients are heterozygous for a single copy of such a variant in one of these five genes [60,65,94]. Thus, heterozygosity is consistent with predisposition to MCS or severe HTG, but it cannot be considered causal [60,65,66]. A single copy of a pathogenic variant in an FCS gene like *LPL* merely raises the risk for developing MCS/severe HTG.

In our experience, some clinicians when seeing the report of a heterozygous pathogenic *LPL* variant in a patient with severe HTG assume that this indicates "autosomal dominant HTG". But such a condition does not exist. Evidence against existence of autosomal dominant HTG is: (1) absence of vertical transmission, i.e. no co-segregation of heterozygous *LPL* pathogenic variants and HTG in multigenerational families [76,82–85]; (2) the large absolute number of normolipidemic individuals with heterozygous pathogenic variants [62,65,66]; (3) wide variability of the TG phenotype in heterozygotes over time; and (4) the dependence of expression of the phenotype on non-genetic factors such as increased age, obesity and diabetes [82–85].

Finally, compared to other variables in the standard lipid profile, TG routinely shows much wider variability over time [95]. The intrinsic dynamic variability of TG could also have contributed to the observed fluctuations in our heterozygotes. In addition, colleagues from Denmark used a different design but similarly found that genetic determinants of baseline mild-to-moderate HTG predicted future pancreatitis, an endpoint that is associated typically with severe HTG [96]; this again suggests potential wide variability of TG levels over time.

Thus, genetic susceptibility to severe HTG or MCS in adults follows probabilistic and not deterministic principles [62]. 15–20% of those with severe HTG are susceptible because of heterozygosity for a pathogenic variant in an FCS gene. Another 35–50% have a high polygenic risk score for elevated TG, compared to only 10% of the general population [60,65,97]. Genetically susceptible adults can also have secondary non-genetic

exacerbating factors such as older age, obesity, diabetes, insulin resistance, alcohol use, poor diet, etc. Finally, the degree of TG elevation in MCS/severe HTG can sometimes be just as severe as in FCS [92]. Operationally and clinically, these both warrant similar acute management and assertive novel treatments for long term control [97]. But in absolute terms, complex MCS/severe HTG is at least 100-times more prevalent than true recessive FCS, of which 80% is LPL deficiency [60].

Thus, the TG phenotype of individuals heterozygous for *LPL* pathogenic variants is highly variable both within and between patients. *LPL* heterozygotes have TG phenotypes ranging from normal to severe HTG, even over relatively short periods of time. The analogy with heterozygous FH is inappropriate given the marked variability in the phenotype. HTG is a less stable phenotype than hypercholesterolemia, with more types of genetic determinants, including polygenic determinants, and secondary non-genetic factors playing a large role in phenotypic expression. Additionally, TG measurements at a single time point in a heterozygote may not sufficiently describe the TG phenotype. Finally, it would be of interest to determine whether heterozygotes for pathogenic variants in the other four FCS genes show similar phenotypic variability.

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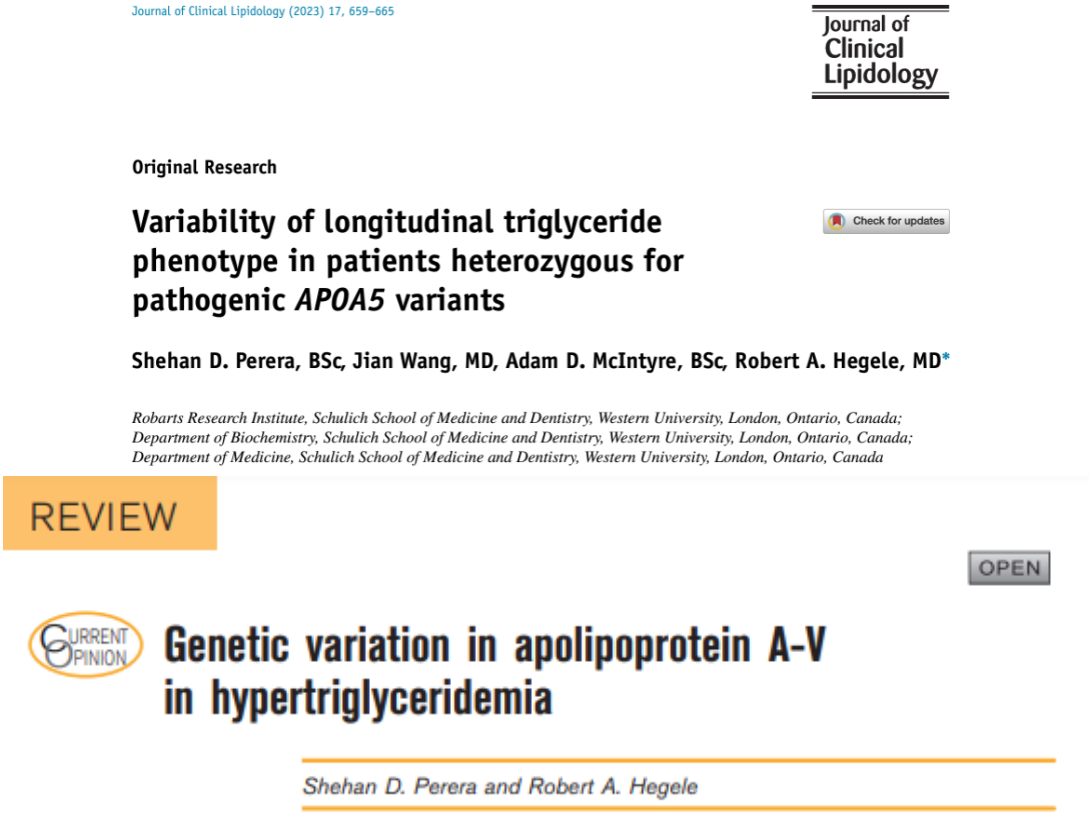
Chapter 3 - Investigating the associations between rare and common variants in *APOA5* and hypertriglyceridemia

A substantial proportion of the work contained in this chapter has been adapted from two previous publications, one in the *Journal of Clinical Lipidology* and the other in *Current Opinion in Lipidology*, and their content has been edited from their original publications for clarity, completeness, and to ensure consistency throughout this thesis.



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Original Research

Variability of longitudinal triglyceride phenotype in patients heterozygous for pathogenic *APOA5* variants

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REVIEW

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CURRENT OPINION

Genetic variation in apolipoprotein A-V in hypertriglyceridemia

Shehan D. Perera and Robert A. Hegele

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3.1 Introduction

3.1.1 Apolipoprotein A-V

Apolipoprotein (apo) A-V is a key regulator of plasma triglyceride (TG) levels [1]. Biallelic (homozygous or compound heterozygous) loss-of-function (LOF) variants in the *APOA5* gene, which encodes apo A-V, are a well-documented cause of familial chylomicronemia syndrome (FCS) [2]. FCS is characterized by severely compromised plasma lipolysis resulting in pathogenic elevation predominantly of intestinally-derived chylomicrons, refractory hypertriglyceridemia (HTG), characteristic physical findings, abdominal pain with failure to thrive, and high lifetime risk of pancreatitis. The consequences of heterozygosity – that is, a monoallelic pathogenic variant of *APOA5* – is less well appreciated and the consequences of common SNPs is often misunderstood and exaggerated. Thus, there is a need to investigate and examine the association of such variants with HTG.

3.1.1.1 Expression and physiological roles of apolipoprotein A-V in lipoprotein metabolism

Apo A-V was one of the first human proteins that were identified utilizing a primordial artificial intelligence approach leveraging comparative DNA sequence analysis of human and mouse genomic DNA, specifically utilizing the VISTA tool, to identify evolutionarily conserved sequences with similar structure to the already well-characterized apolipoprotein genes *APOA1*, *APOC3*, and *APOA4* [3,4]. Initial studies indicated that it is expressed exclusively in the liver and secreted into circulation with TG-rich lipoproteins, primarily very-low density lipoproteins (VLDLs) [3]. It was later identified to also be expressed in the small intestine, though to a much lesser extent [5]. Intestinally secreted apo A-V is presumed to circulate with intestinally produced lipoproteins, namely chylomicrons (CMs). Interestingly, apo A-V is also found in the bile produced by the liver, making hepatic apo A-V an exocrine secretion which may reach the intestinal lumen and exert some effects there [6]. Compared to other apolipoproteins, apo A-V has very low absolute concentration in plasma (<1 μg/mL) [7]. Despite this, it is an extremely potent regulator of plasma TG concentrations [8].

There are currently 4 primary mechanisms by which Apo A-V is known to regulate circulating plasma TG levels. First, increased apo A-V expression causes intracellular apo A-V to interfere with hepatic synthesis of VLDL particles in a dose-dependent manner, preventing their secretion into the circulation [9–11]. Specifically, it is thought that intracellular apo A-V may interfere with the lipidation of the forming VLDL particle within the endoplasmic reticulum where it normally acquires the majority of its lipid content prior to secretion from hepatocytes [11].

Second, apo A-V bound to circulating TG-rich lipoproteins (CMs and VLDLs) [7] in plasma directly enhances the catalytic activity of lipoprotein lipase (LPL) to clear TG from circulation in concert with the functionality of another essential apolipoprotein for efficient LPL activity, namely apo C-II [7,11,12], and the endothelial cell membrane protein that anchors LPL to the endothelial cell surface, namely glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 (GPIHBP1) [13–15]. This function of TG-rich lipoprotein-bound apo A-V is likely mediated by its ability to bind to and/or interact with heparan sulfate proteoglycans (HSPGs) [13] and GPIHBP1 [14,15] on the endothelial cell surface. GPIHBP1 is the major capillary lumen binding site/anchor for LPL [16] and is the primary platform upon which LPL-mediated hydrolysis of circulating TGs occurs [17,18]. The apo A-V interaction with GPIHBP1 likely enhances association of apo A-V containing lipoproteins with LPL and its associated endothelial cell surface features thereby enabling more efficient LPL-mediated lipolysis.

Third, there is some evidence that apo A-V mediates endocytosis-mediated hepatic uptake of TG-rich lipoproteins and their remnants (i.e., CM and VLDL remnants) through interaction with members of the low-density lipoprotein (LDL) receptor (LDLR) family [19].

Finally, a novel role for apo A-V was recently found by which circulating apo A-V indirectly enhances LPL catalytic activity by competing with LPL for binding to a unique inhibitory epitope that was found to be present in the ANGPTL3/8 complex, thereby suppressing the inhibitory effects of the complex on LPL [20,21]. As briefly mentioned

in chapter 2 section 2.1.1.1, the angiopoietin-like proteins (ANPTLs), specifically the ANGPTL3/4/8 family, have been implicated in the regulation of LPL activity. ANGPTL3 (expressed almost exclusively by the liver) and ANGPTL4 (expressed by the liver, adipose tissue, brain, intestine, thyroid, kidney, and heart) are well characterized inhibitors of LPL [22–37] which were recently shown to have their inhibitory effects enhanced and decreased, respectively, upon complexing with ANGPTL8, whose expression is induced by insulin signaling [38].

The ANGPTL3/8 complex possesses a unique inhibitory epitope that recognizes and interacts with LPL to inhibit it, but apo A-V has been recently shown to compete with this epitope, thereby indirectly enhancing LPL catalytic activity [20,21]. Additionally, it was found that this interaction between apo A-V and the ANGPTL3/8 complex plays a role in regulating selective tissue uptake of circulating TG in the fed versus fasted state [20,21,38–43]. Specifically, in the fed/post-prandial state, increased insulin signaling induces the expression of ANGPTL8 in the liver and adipose tissue while downregulating hepatic apo A-V production and adipose ANGPTL4 expression. This leads to an increase in circulating ANGPTL3/8 complex uninhibited by apo A-V competition, enabling it to inhibit LPL activity in skeletal and cardiac muscle tissues. Concurrently, there is a decrease in circulating ANGPTL4 and an increase in ANGPTL4/8 complex, the latter of which has reduced ability to inhibit LPL, leading to a relative increase in adipose tissue LPL-mediated lipolysis.

Additionally, ANGPTL4/8 interacting with adipose tissue LPL prevents ANGPTL3/8 and ANGPTL4 from associating with and inhibiting adipose tissue LPL. Taken together, this leads to the majority of circulating TG in the post-prandial state being hydrolyzed in adipose tissue where the products of LPL-mediated lipolysis are stored for energy. In the fasted state, ANGPTL4 expression in adipose tissue is upregulated while ANGPTL8 expression in both hepatic and adipose tissues is downregulated (due to lack of insulin signaling). Thus, adipose ANGPTL4/8 levels and circulating ANGPTL3/8 concentrations are low. Concurrently, hepatic apo A-V expression is increased, leading to suppression of any ANGPTL3/8 complex activity while ANGPTL4 potently inhibits adipose LPL. The next effect is that skeletal and cardiac muscle tissues hydrolyze the majority of

circulating TG in the fasted state leading to energy that can be used by those tissues. On a related note, recent experimental evidence has suggested that ANGPTL4/8 may have effects that connect the coagulation system with LPL-mediated lipolytic activity, though this promising and interesting interaction requires further investigation [44].

It should be noted that given the role of apo A-V in promoting LPL-mediated lipolysis, its plasma levels should be inversely correlated with plasma TG levels. However, rather paradoxically, it has long been known that there is instead a positive correlation between circulating plasma TG levels and circulating apo A-V concentrations [45]. This is similar to the correlation observed between circulating plasma TG concentrations and apo C-III, an inhibitor of LPL activity that opposes the functionality of apo A-V [46]. One explanation posited for this seemingly paradoxical correlation is that apo A-V circulates primarily as a component of TG-rich lipoproteins, and thus, in a macro-biochemical context, it seems natural that apo A-V plasma concentrations are positively correlated with circulating TG concentrations. However, at the micro-biochemical regional level in close proximity to LPL, such as the endothelial cell or adipocyte, apo A-V enhances lipolysis, eventually resulting in TG reduction, but not at an immediate or sufficiently large scale as to be reflected in total plasma TG concentration, whose decline would be delayed and reactive, adhering to a slower time course.

3.1.1.2 Protein structure of apolipoprotein A-V

After cleavage of its 23 amino acid signal peptide, the mature apo A-V protein consists of a ~39 kDa protein composed of 343 amino acids [3,47]. Apo A-V is secreted from the liver as a component of high density lipoproteins (HDL) and VLDL and from the small intestine as a component of chylomicrons [3,9,47]. Apo A-V has two coiled-coil domains and a large α-helical content with the most recent predictions of apo A-V structure indicating ~35% α-helical content in the lipid-free state (i.e., not associated with lipoproteins) which increased to ~45% α-helical content upon association with lipid, corresponding to the elongation and stabilization of α-helix segments [48].

Apo A-V has 3 primary functional domains. First, the N-terminal region spanning residues 1 to 146 of the mature protein (note this numbering does not include the signal

peptide which is 23 residues in length). This region is hydrophilic with the α-helices formed by the residues in this region adopting a water-soluble helix bundle configuration [48]. Secondly, the C-terminal region spanning residues 295-343 of the mature apo A-V protein are highly hydrophobic [10,47,48] and have been identified as having lipid binding properties [47–50]. Finally, the central intervening region between the terminal regions spanning residues 147–294 contains a string of residues associated with enhancement of LPL activity by interacting with GPIHBP1. Specifically, a positively charged region spanning residues 186–227 is involved in binding to HSPG [13], LDLR receptor family members [19], and GPIHBP1 [18].

There is strong evidence that this positively charged region of apo A-V and the acidic domain of GPIHBP1 are both required for the proper interaction between apo A-V and the LPL-GPIHBP1 complex that facilitates the LPL catalytic activity enhancing functions of apo A-V [14]. In summary, enhancement of efficient LPL-mediated lipolysis of TGRLs requires coordination between GPIHBP1, apo A-V and LPL [47]. Another region of note within the central intervening region of apo A-V is the hydrophobic region formed by residues 161-181 preceding the positively charged region. This hydrophobic region has been implicated in facilitating the binding of apo A-V to the surface of intracellular lipid droplets [51]. This may explain the function of apo A-V to reduce hepatic VLDL secretion, although this may also result in concurrent hepatic lipid accumulation [47].

Currently, the exact region of apo A-V associated with its ability to bind the ANGPTL3/8 complex is unknown [20,21] although we may be able draw some inferences based on properties of the apo A-V-interacting epitope of the ANGPTL3/8 complex. The epitope seems to be composed of a hydrophilic leucine zipper-like motif [21]. From this, we can infer that apo A-V may interact with this epitope either through its hydrophilic N-terminal domain [48] and/or through residues in the central intervening region such as the above described positively charged region [49]. It is unlikely that apo A-V interacts directly with the LPL-inhibitory epitope of the ANGPTL3/8 complex via its C-terminal region as this region is hydrophobic and responsible for apo A-V binding to lipoproteins [10,48]. More research is required regarding this.

3.1.1.3 Gene structure of *APOA5*

The gene encoding apo A-V, namely *APOA5*, is located on the long arm of chromosome 11 (11q23.3) within the apolipoprotein gene cluster that also includes *APOA1*, *APOC3*, and *APOA4* [2,3]. The *APOA5* gene is composed of four exons and three introns spanning roughly 3.05 kb on the reverse strand. As previously mentioned, this gene is expressed almost exclusively in the liver with some expression observed in the small intestine.

3.1.2 Role of *APOA5* variants in disease

3.1.2.1 Biallelic *APOA5* variants and familial chylomicronemia syndrome

APOA5 is one of the five genes in which biallelic pathogenic variants have been implicated in causing familial chylomicronemia syndrome (FCS) [52] (see section 1.4.3.1.1 for more details), which as previously discussed, is characterized by sustained, refractory, and severe HTG. In the case of apo A-V deficiency, this is specifically due to loss of LPL-mediated lipolysis caused by loss of the LPL activity enhancing functions of apo A-V leading to extreme chylomicron accumulation in plasma [53]. While the intracellular function of apo A-V to inhibit hepatic synthesis and secretion of VLDL is likely also lost in apo A-V deficiency conditions, this has minimal clinical impact as in FCS conditions, production of lipoprotein species other than chylomicrons is extremely limited if not completely abolished [52]. FCS patients may also present with a variety of physical manifestations (see sections 1.4.2 and 1.4.3.1.1 for more details), the most concerning being significantly elevated risk for the development of acute pancreatitis due to severe HTG [1,52].

3.1.2.2 Monoallelic variants in *APOA5*

The phenotype produced by biallelic pathogenic variants in *APOA5* is well characterized and understood [2,52]. However, the phenotype associated with monoallelic pathogenic variants in *APOA5* is not. There are conflicting and variable reports regarding the TG phenotype severity seen in patients with such variants. Some studies have found that heterozygosity for pathogenic variants in *APOA5* is associated with either normal TG

levels or mild-to-moderate HTG [12] while other studies have identified individuals with similar genotype possessing severe HTG, citing secondary TG elevating factors such as obesity and diabetes as aggravating factors leading to the development of severe HTG in patients with this genotype [54] (see section 3.4.1 for more details on these studies). Unfortunately, there is very little work dedicated to studying the monoallelic carriers of these variants. Most descriptions of the TG phenotype associated with monoallelic pathogenic variants in *APOA5* comes from cross-sectional investigations of families ascertained via a homozygous proband [12,54,55]. Thus, there is a lack of dedicated research on the TG phenotype associated with monoallelic pathogenic variants in *APOA5* as well as a paucity of longitudinal phenotyping in patients with this genotype.

3.1.2.3 Common single nucleotide polymorphisms in *APOA5*

Many studies on *APOA5* genetics have highlighted the TG-elevating effects of several common polymorphisms [56]. With respect to HTG, the *APOA5* promoter polymorphisms c.-1131T>C [57–59] and c.-3A>G [60]; protein coding sequence polymorphisms c.56C>G (corresponding to p.Ser19Trp) [2,45,58,61], c.553G>T (corresponding to p.Gly185Cys) [2,62,63], and c.725C>G (corresponding to p.Leu242Val) [56,64]; intronic polymorphism c.162-43G>A (also known as IVS3 + 476G>A) [56,65,66]; and 3' untranslated region (UTR) polymorphism c.*158T>C [67] have all been associated with elevated TG in numerous small case-control association studies, and more recently as small effect signals for slight deviations in TG levels in genome-wide association studies.

Many of these common *APOA5* polymorphisms have been previously cited as major contributors to the large variability in HTG severity individuals heterozygous for true pathogenic variants in *APOA5* [12,54,55,68–70]. Of particular note is the c.56C>G (p.Ser19Trp) polymorphism. This is perhaps the best known and most studied common *APOA5* polymorphism. *In vitro* experiments have found that the c.56C>G (p.Ser19Trp) SNP alters the signal peptide of nascent apo A-V, resulting in a ~49% reduced secretion of apo A-V from the cell due to less efficient translocation of the signal peptide across the endoplasmic reticulum [61]. However, it should be noted that the extent of the clinical

significance of these common SNPs in *APOA5* is debatable. Common *APOA5* polymorphisms, especially the c.56C>G (corresponding to p.Ser19Trp), are often interpreted as being major contributors to the large variability in HTG severity observed in heterozygotes for true rare *APOA5* LOF variants, such as truncating variants, as previously noted. They are even sometimes erroneously reported as being causal for FCS on clinical genetic reports of next-generation sequencing analysis. In reality, these are merely risk alleles that raise the probability or odds of developing HTG if the metabolic context allows. Their high frequency in normolipidemic populations, ranging from 5-15% [71], indicates clearly that these SNPs, at least in isolation, are not as clinically significant as rare pathogenic variants are, despite what many studies attempt to imply. While isolated occurrences of these SNPs are likely not clinically significant, it has been shown that extreme accumulation of many common SNPs associated with elevated plasma TG, including some *APOA5* SNPs, is sufficient to induce mild-to-moderate [72] and even severe HTG [73]. This is typically referred to as polygenic HTG. To this end, these *APOA5* SNPs are utilized as components of polygenic risk scores for HTG [74]. Given that the phenotypic impact of these SNPs is equated by some researchers and clinicians with true pathogenic variants in *APOA5*, there is a need to clarify the association between these SNPs in isolation and hypertriglyceridemia, particularly regarding the c.56C>G (p.Ser19Trp) polymorphism which is among the most cited SNPs for elevating the severity of TG phenotypes observed in heterozygotes for pathogenic *APOA5* variants.

3.1.3 Description of studies and objectives

3.1.3.1 Study 1: Variability in longitudinal triglyceride phenotype in patients heterozygous for pathogenic *APOA5* variants

Thus, to properly characterize the longitudinal TG phenotype in heterozygotes for pathogenic *APOA5* variants, we evaluated TG levels in individuals heterozygous for pathogenic *APOA5* variants who were followed for multiple years (see section 3.3.1.5 for more details on specific range of years). We found that the TG phenotypes of these patients were highly variable both within and between patients, ranging from normal to severe HTG, at baseline and longitudinally.

3.1.3.2 Study 2: Investigating the association of common *APOA5* p.Ser19Trp SNP and its association with hypertriglyceridemia in a clinic population

Thus, to clarify and investigate the association of the common *APOA5* c.56C>G (p.Ser19Trp) SNP with HTG, we evaluated the historically greatest TG levels of homozygous and heterozygous carriers for the c.56C>G (p.Ser19Trp) found in a clinic population and compared these groups to each other and to a control group. We found that average historically highest TG levels in heterozygotes and homozygotes for the p.Ser19Trp SNP were not significantly different from each other, but both were significantly different from healthy controls.

3.2 Materials and methods

3.2.1 Study 1: Variability of longitudinal triglyceride phenotype in patients heterozygous for pathogenic *APOA5* variants

3.2.1.1 Study subjects

Following our established procedures [72,73,75], data were collected from patient charts of patients at the Lipid Genetics Clinic, London, Ontario, Canada who were referred for management of their lipid levels. Inclusion criteria were: 1) heterozygosity for a pathogenic *APOA5* variant; 2) at least three TG measurements available for analysis; and 3) patient was medically stable and on stable treatment over the duration of follow-up. Patients with additional rare pathogenic HTG-associated variant(s) were excluded. The project was approved by the Research Ethics Board of Western University (protocol number 0379; **Appendix B**) and all participants provided informed consent.

3.2.1.2 Biochemical, clinical, and demographic information

After 8 to 12 hours of fasting, plasma lipid profiles were determined at baseline and at routine follow-up visits to the Lipid Genetics Clinic. Clinical and demographic data were collected at the time of initial visit to the Lipid Genetics Clinic and historical fasting plasma TG measurements were collected from referral notes. Lipid profiles conducted at the Lipid Genetics Clinics were measured using the Roche Cobas C502 analyzer (Hoffman La Roche, Mississauga, ON, Canada), as reported previously [72,73].

3.2.1.3 DNA preparation and sequencing

Genomic DNA isolation from whole blood and gene sequencing protocols used have been described previously [73,76,77]. We used our LipidSeq panel, targeting 69 genes and 185 single nucleotide polymorphisms (SNPs) associated with dyslipidemia and other metabolic disorders [78]. Sequencing was performed at the London Regional Genomics Centre on a MiSeq personal sequencer (Illumina, San Diego, CA).

3.2.1.4 Genetic analysis

Our standard bioinformatic processing and annotation pipeline was used to call variants [73]. Briefly, CLC Bio Genomics Workbench (version 12.0; CLC Bio, Aarhus, Denmark) was first used to align sequencing reads for each patient sample against the human reference genome (build hg19) and was then used to call variants.

3.2.1.5 Variant pathogenicity classification

Pathogenicity of *APOA5* variants was first determined using the Franklin by Genoox tool (https://franklin.genoox.com) to determine the American College of Medical Genetics and Genomics (ACMG) pathogenicity classification of each *APOA5* variant, as previously described [78]. The ACMG established a system for interpreting the pathogenicity of sequence variants in 2015 [79]. Franklin is a computerized implementation of these guidelines which were originally developed for use by clinicians to assess variants. To maximize confidence in classification, we manually confirmed the pathogenicity of each variant using ACMG guidelines. We also excluded patients with additional pathogenic rare variants in any of the other canonical FCS genes (i.e., *APOC2*, *GPIHBP1*, *LMF1*, or *LPL*).

3.2.1.6 Polygenic risk score for elevated triglyceride levels

We utilized a weighted 16-SNP polygenic risk score (PRS) previously developed by our lab to assess the cumulation of common TG-raising alleles in each patient [73]. Details on calculating and interpreting polygenic risk scores are discussed by Dron et al [74].

3.2.1.7 Statistical analysis

Calculation of means and standard deviations was performed using R version 4.1.3 (R Foundation for Statistical Computing, Vienna, Austria, https://www.r-project.org). Means are reported as mean ± standard deviation. Figures were generated in R version 4.1.3 (R Foundation for Statistical Computing, Vienna, Austria, https://www.r-project.org) using the ggplot2 package [80].

3.2.2 Study 2: Investigating the association of the common *APOA5* p.Ser19Trp SNP and its association with hypertriglyceridemia

3.2.2.1 Study subjects

Following our established procedures [72,73,75], data were collected from patient charts of patients at the Lipid Genetics Clinic, London, Ontario, Canada who were referred for management of their lipid levels. Subjects were identified via a clinic database search and separated into 3 groups based on the number of p.Ser19Thr alleles they possessed: 1) patients heterozygous (monoallelic) for the p.Ser19Trp SNP in *APOA5*, 2) patients homozygous (biallelic) for the p.Ser19Trp SNP in *APOA5*, and 3) normal healthy controls with no copies of the p.Ser19Trp SNP in *APOA5*. Healthy control samples were obtained from volunteers such as current and former laboratory staff, previous students, and sometimes normolipidemic family members of patients. The project was approved by the Research Ethics Board of Western University (protocol number 0379; **Appendix B**) and all participants provided informed consent.

3.2.2.2 Biochemical information

Historically highest fasting (8-12 hour fast) plasma TG measurements for each subject were extracted from charts, drawing from measurements made both by the Lipid Genetics Clinic and from before they were referred to the Lipid Genetics Clinic. Historically highest fasting plasma TG was used as an indicator of TG phenotype severity. Lipid profiles conducted at the Lipid Genetics Clinics were measured using the Roche Cobas C502 analyzer (Hoffman La Roche, Mississauga, ON, Canada), as reported previously [72,73].

3.2.2.3 DNA preparation and sequencing

See section 3.2.1.3.

3.2.2.4 Genetic analysis

See section 3.2.1.4.

3.2.2.5 Polygenic risk score for elevated triglyceride levels

See section 3.2.1.6.

3.2.2.6 Statistical analysis

Statistical analyses were performed using R version 4.1.3 (R Foundation for Statistical Computing, Vienna, Austria, https://www.r-project.org). Welch's analysis of variance (ANOVA) with Games-Howell post-hoc test was performed to assess differences between groups. Means are reported as mean ± standard deviation. One-way Welch's ANOVA and Games-Howell post-hoc test were utilized as both make no assumptions regarding equal samples sizes and equal population variances. Specifically, as described in section 3.3.2.1, the sample sizes for all 3 groups are unequal. Additionally, we are not prepared to assume that population variance of historically highest TG levels is similar in all 3 groups, nor do we have any good reason to believe it would be similar. Therefore, we utilized the one-way Welch's ANOVA with Games-Howell post-hoc test for our analysis. Figure was generated in R version 4.1.3 (R Foundation for Statistical Computing, Vienna, Austria, https://www.r-project.org/) using the ggplot2 package [80].

3.3 Results

3.3.1 Study 1: Variability of longitudinal triglyceride phenotype in patients heterozygous for pathogenic *APOA5* variants

3.3.1.1 Study subjects

We identified 165 patients within the Lipid Genetics Clinic patient population who were heterozygous for a rare coding region variant in *APOA5*. Of these, 21 were identified by the Franklin bioinformatics process as having a single pathogenic or likely pathogenic

variant in *APOA5*. Upon manual curation, all 21 were found to be definite pathogenic variants. However, 14 patients were excluded due to an insufficient number of TG measurements available for review. Our final cohort consisted of 7 unrelated patients with a single copy of a pathogenic *APOA5* variant and sufficient long-term follow-up. The filtering algorithm is shown in **Figure 3.1**.

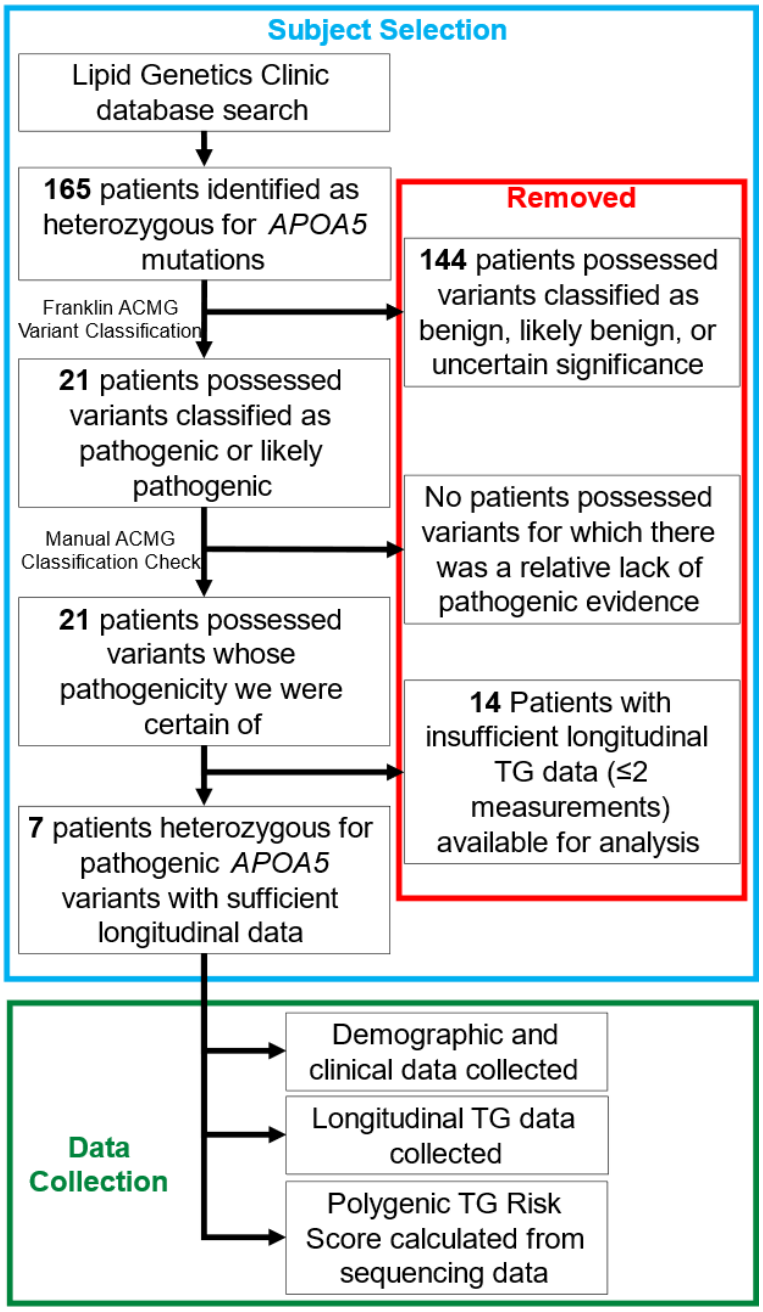

Figure 3.1. Subject filtering and selection.

Flowchart depicting subject selection and filtering process. Subjects were included in the study if they were heterozygous for pathogenic *APOA5* variants and if 3 or more TG measurements were available for analysis. Additionally, patients with pathogenic variants in other TG-affecting genes were excluded from analysis. Seven patients were identified in the Lipid Genetics Clinic database to meet these criteria.

3.3.1.2 Baseline demographic information

Demographic information and lipid-related clinical characteristics of study subjects at baseline are summarized in **Table 3.1**. All subjects were of Northern European ancestry (see section 4.3.2 for more details on the relevance of ancestry). Mean age and body mass index were 47.7 ± 15.5 years and 31.2 ± 5.1 kg/m^2, respectively. None had diabetes mellitus. Two subjects (patients 1 and 7) had a history of HTG-related pancreatitis, with patient 7 having developed chronic pancreatitis by the time they were referred to our clinic. Patient 1 had a history of ASCVD. Over the duration of follow-up, no patient developed new-onset diabetes, but patient 1 experienced an episode of acute pancreatitis with new-onset glucose intolerance. No patient showed thyroid, renal, or liver disease at baseline or over the course of follow-up. Additionally, medication use, including the use of lipid-lowering therapies, was stable over the duration of follow-up for all patients.

Table 3.1. Baseline characteristics in patients heterozygous for pathogenic *APOA5* variants.

Number of patients (male|female)	7 (3|4)
Age (years)	47.7 ± 15.5
Body mass index (kg/m^2)	31.2 ± 5.1
Non-Northern European ancestry[a]	0
Type 2 diabetes mellitus	0
History of acute pancreatitis	2
History of atherosclerotic cardiovascular disease	1
Current or former smoking history	3
No alcohol consumption	1
Total cholesterol (mmol/L)	5.6 ± 2.1
Triglycerides (mmol/L)	7.5 ± 5.2
High-density lipoprotein cholesterol (mmol/L)	0.89 ± 0.15

Values shown are mean ± standard deviation or number of patients unless otherwise indicated.

[a] See section 4.3.2 for more details on the relevance of ancestry

3.3.1.3 Pathogenic *APOA5* variants

Four unique nonsense variants of *APOA5* are represented in our cohort: p.Gln97Term (p.Q97X) in four patients, p.Glu98Term (p.E98X) in one patient, p.Gln275Term (p.Q275X) in one patient, and p.Gln305Term (p.Q305X) in one patient. The position of each variant within the *APOA5* gene is shown in **Figure 3.2**.

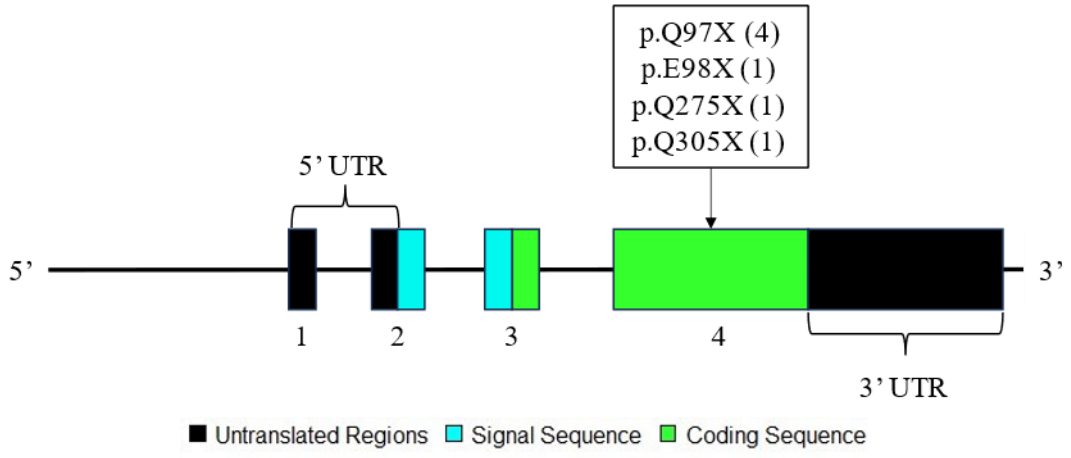

Figure 3.2. Genetic map of pathogenic *APOA5* variants observed in study.

Genetic map displaying locations of the *APOA5* variants possessed by patients analyzed in this study. Major structural features are color-coded. Black boxes indicate untranslated sequences, blue boxes indicate sequences coding for the apo A-V signal peptide, and green boxes indicate sequences coding for the mature protein. Regarding variant nomenclature, p.Q97X (p.Gln97Term), p.E98X (p.Glu98Term), p.Q275X (p.Gln275Term), and p.Q305X (p.Gln305Term). Number in brackets shows the number of patients carrying the indicated variant in our cohort.

3.3.1.4 Variation in baseline triglycerides

At baseline visit to Lipid Genetics Clinic, one (14.3%), four (57.1%), and two (28.6%) patients had normal TG levels, mild-to-moderate HTG, and severe HTG, respectively. The average baseline lipid profile for our study group at initial visit to the Lipid Genetics Clinic is shown in **Table 3.1**.

3.3.1.5 Longitudinal fasting plasma triglycerides in *APOA5* heterozygotes are highly variable

On average, duration of follow-up with patients in our cohort was 5.3 ± 3.7 years (range was 0.5 to 11.8 years), with a mean of 7.1 ± 2.6 TG measurements over that period. The longitudinal TG phenotype of patients in our cohort showed marked variability both within and between patients (**Figure 3.3**). Two (28.6%) of patients had TG levels that fluctuated between normal TG levels and mild-to-moderate HTG, four (57.1%) had TG levels that fluctuated between mild-to-moderate and severe HTG, and one patient (14.3%) had TG levels in all three ranges (normal, mild-moderate HTG, and severe HTG). Of the 50 total lipid measurements taken over time, 18 (36%) were in the severe HTG range, 27 (54%) were in the mild-to-moderate HTG range, and 5 (10%) were in the normal range.

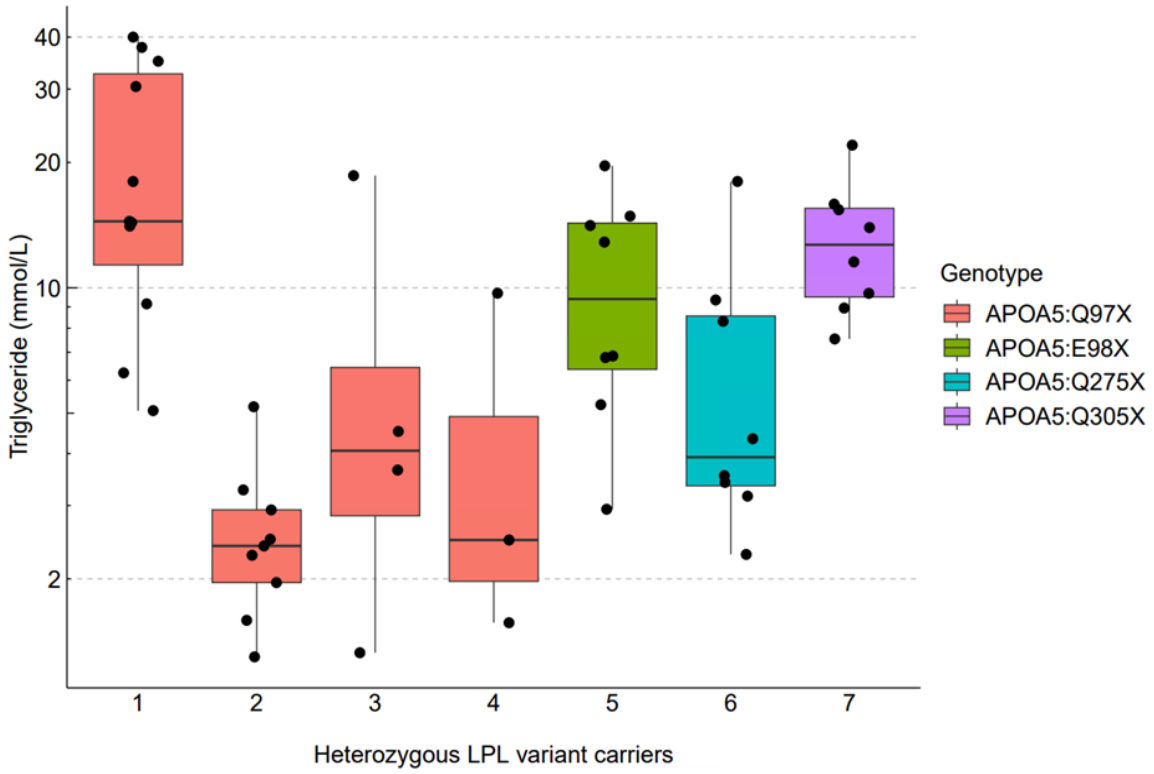

Figure 3.3. Variable fasting plasma triglyceride phenotypes in patients with pathogenic monoallelic *APOA5* variants.

Distribution of longitudinal triglyceride (TG) measurement data from pathogenic monoallelic *APOA5* variant carriers (N = 7) is graphed as individual box-and-whisker plots. Boxes are color-coded according to genotype. The midline of each box represents median fasting plasma TG level and whiskers represent interquartile range of TG levels for each patient. Individual measurements are represented as black dots. Y-axis displays fasting plasma TG (mmol/L) on $\log_{10}$ scale. Dotted, horizontal lines at 2 and 10 mmol/L delineate HTG severity cut-offs (normal fasting plasma TG is defined to be fasting plasma TG <2 mmol/L; mild-to-moderate HTG is defined to be fasting plasma TG between 2 and 9.9 mmol/L; severe HTG is defined as fasting plasma TG >10 mmol/L).

3.3.1.6 Association between pathogenic *APOA5* variants and longitudinal triglyceride phenotype

No consistent pattern in longitudinal TG measurement trajectories was observed to be associated with the *APOA5* p.Q97X variant (**Figure 3.4A**). No comments regarding variant specific patterning or effects can be made definitively based on data for the other three *APOA5* variants observed in our cohort due to small sample size. Common to all variants is the non-refractory but highly variable TG phenotype. The four *APOA5* p.Q97X heterozygotes showed marked between-patient variability in TG levels over time.

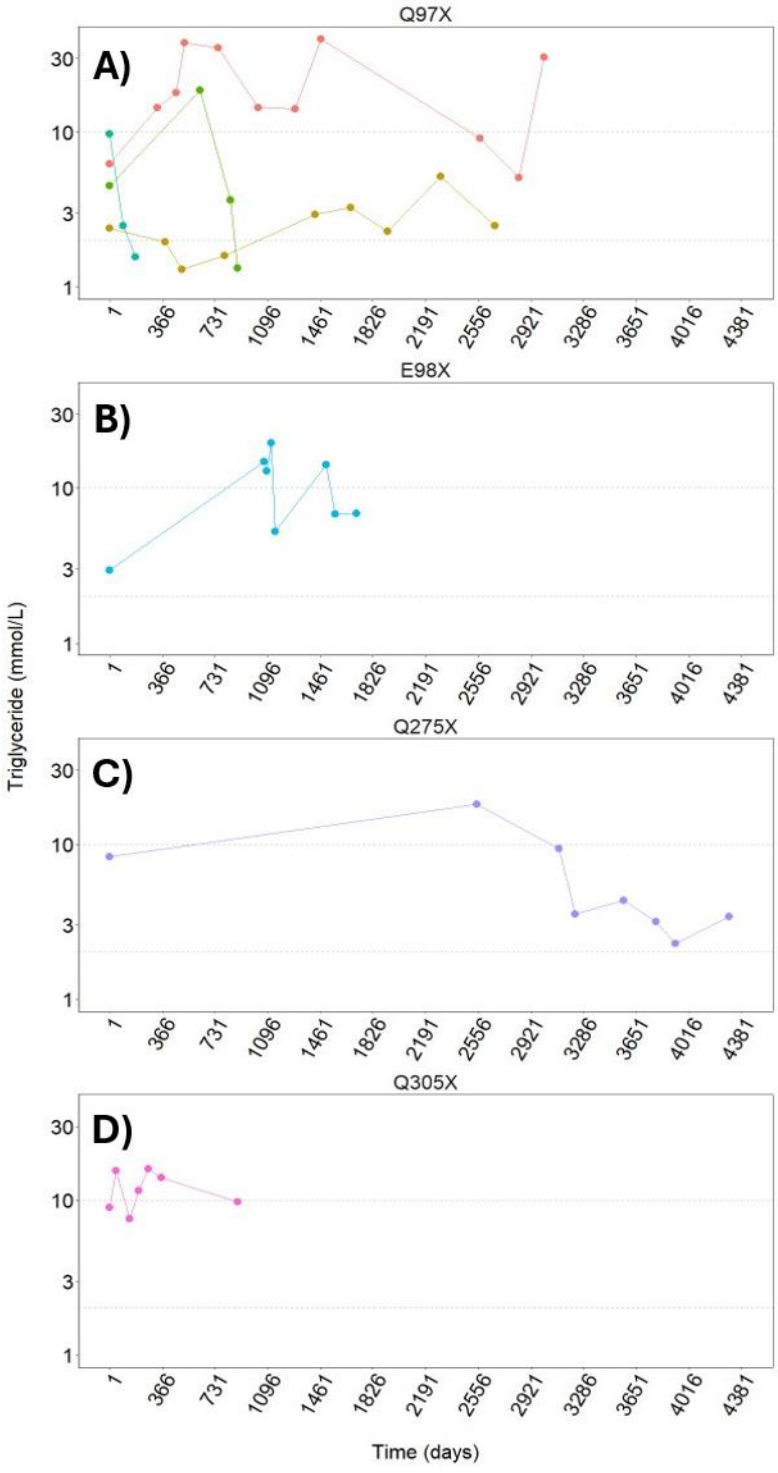

Figure 3.4. Longitudinal triglyceride trajectories of patients heterozygous for pathogenic *APOA5* variants.

Longitudinal TG data for all patients (N = 7) are plotted as days elapsed from first measurement (X-axis). Y-axis displays fasting plasma TG level (mmol/L) using a $\log_{10}$ scale. Trajectories for patients heterozygous for *APOA5* A) p.Q97X, B) p.E98X, C) Q275X, and D) p.Q305X.

3.3.1.7 Polygenic influence on triglyceride phenotype

PRS data were available for patients 1-6, who had scores in the 79th, 42nd, 96th, 31st, 97th and 79th percentiles for raised TG, respectively. Sequencing data that were needed to assess polygenic risk and construct a PRS for patient 7 were not available. In our clinic, a PRS exceeding the 90th percentile is considered to indicate clinically relevant polygenic risk for hypertriglyceridemia. Thus, only two patients, patients 3 and 5, had a high polygenic score, indicating that in most cases background polygenic susceptibility was not incrementally contributing to hypertriglyceridemia. With regards to the *APOA5* p.Ser19Trp SNP that is the variant of interest in Study 2, patients 3 and 4 were found to be heterozygous for this SNP.

3.3.2 Study 2: Investigating the association of the common *APOA5* p.Ser19Trp SNP and its association with HTG in clinical cohort

3.3.2.1 Study subjects

We identified within the Lipid Genetics Clinic patient population 345 patients who were heterozygous for the common *APOA5* p.Ser19Trp SNP, 21 patients who were homozygous for the common *APOA5* p.Ser19Trp SNP, and 36 normal healthy controls who did not have any copies of the common *APOA5* p.Ser19Trp SNP.

3.3.2.2 Association of *APOA5* p.Ser19Trp SNP with TG phenotype in a lipid genetics clinic population

The homozygous group had the greatest mean historically highest TG of the three groups at 10.79 ± 11.53 mmol/L, followed by the heterozygous group at 8.37 ± 10.49 mmol/L, with the normal controls having the lowest mean historically highest TG of the three groups at 0.98 ± 0.46 mmol/L. Both the homozygous and heterozygous groups demonstrated marked variability in the severity of their TG phenotype (**Figure 3.5**). Within the homozygous group, 3 (14.3%) patients demonstrated historically highest TG in the normal range, 11 (52.4%) in the mild-to-moderate HTG range, and 7 (33.3%) in the severe HTG range. Within the heterozygous group, 77 (22.3%) patients demonstrated historically highest TG in the normal range, 174 (50.4%) in the mild-to-moderate HTG

range, and 94 (27.2%) in the severe HTG range. For comparison, the normal control group had 21 (97.2%) patients whose historically highest TG was in the normal TG range, 1 (2.8%) in the mild-to-moderate HTG range, and none in the severe HTG range.

A one-way Welch's ANOVA comparing the mean historically highest TG (mmol/L) of the three study groups indicated that there was a statistically significant difference in the means between at least two groups ($F(2, 50.577) = 90.225$, $p < 2.2 \times 10^{-16}$). A post-hoc Games-Howell test showed that the mean historically highest TG (mmol/L) was significantly different between p.Ser19Trp homozygotes and normal controls ($p = 0.002$, 95% C.I. = [-16.168,-3.432]) and significantly different between p.Ser19Trp heterozygotes and normal controls ($p < 0.001$, 95% C.I. = [-8.731,-6.048]). There was no statistically significant difference in mean historically highest TG (mmol/L) between p.Ser19Trp homozygotes and heterozygotes ($p = 0.625$). These results are summarized in **Figure 3.5**.

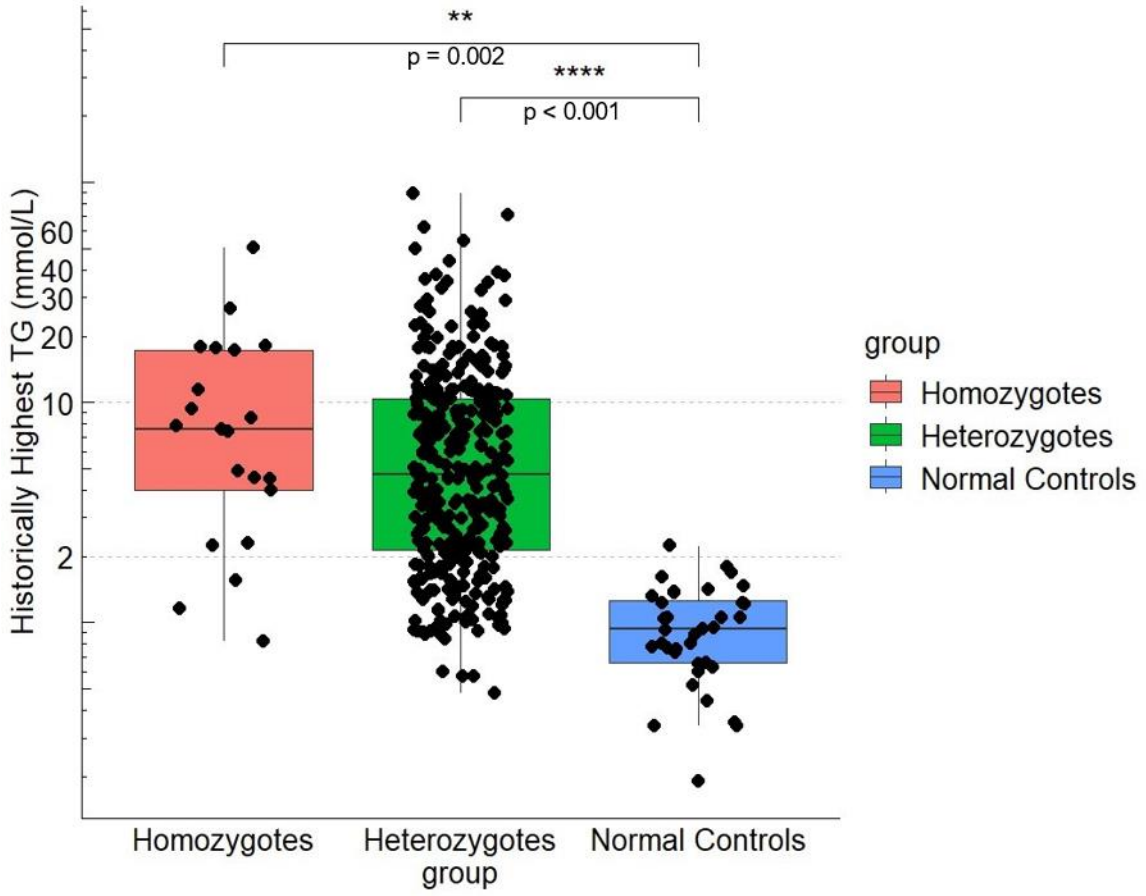

Figure 3.5. Variable fasting plasma triglyceride phenotype severities within carriers of varying numbers of *APOA5* p.Ser19Trp alleles.

Distribution of historically highest fasting plasma triglyceride (TG) measurement data among 3 groups: homozygotes (*N* = 21) for *APOA5* p.Ser19Trp ("Homozygotes" in figure), heterozygotes (*N* = 345) for *APOA5* p.Ser19Trp ("Heterozygotes" in figure), and normolipidemic controls (*N* = 36) lacking the *APOA5* p.Ser19Trp in either allele ("Normal Controls" in figure). Data are graphed as box-and-whisker plots. Boxes are color-coded according to genotype group. The midline of each box represents median fasting plasma TG level and whiskers represent interquartile range of each group. Black dots represent the historically highest fasting plasma TG measurement of each subject. Mean historically highest TG values of all three groups were compared using a one-way Welch's ANOVA with post-hoc Games-Howell test. There was significant statistical intergroup difference detected ($F(2, 50.577) = 90.225$, $p < 2.2 \times 10^{-16}$). Games-Howell

post-hoc test revealed that both the homozygous and heterozygous groups were significantly different from the normolipidemic control group ($p = 0.002$ and $p < 0.001$, respectively; represented in figure as ** and ****, respectively). Y-axis displays fasting plasma TG (mmol/L) on $\log_{10}$ scale. Dotted, horizontal lines at 2 and 10 mmol/L delineate HTG severity cut-offs (normal fasting plasma TG is defined to be fasting plasma TG <2 mmol/L; mild-to-moderate HTG is defined to be fasting plasma TG between 2 and 9.9 mmol/L; severe HTG is defined as fasting plasma TG >10 mmol/L).

3.4 Discussion

3.4.1 Study 1: Variability of longitudinal triglyceride phenotype in patients heterozygous for pathogenic *APOA5* variants

We found that patients heterozygous for pathogenic *APOA5* variants showed marked within and between patient variability of TG phenotypes, similar to what we previously observed in patients heterozygous for pathogenic *LPL* variants [75] (see Chapter 2 for this study). Specifically, we found that the untreated, baseline TG levels of these patients ranged from normal to mild-to-moderate to severe HTG, and that TG levels within patients fluctuated across these three strata over time. 54.0% of all TG measurements taken were in the mild-to-moderate HTG range, consistent with the findings of our previous study of heterozygous *LPL* variants, where two-thirds of all measurements were in this range [75]. But unlike *LPL* pathogenic variant heterozygotes, in whom the remaining measurements were roughly equally distributed between the normal and severe HTG ranges, most of the remaining measurements (36%) in the *APOA5* variant heterozygotes we studied were in the severe HTG range, with only a minority (10%) in the normal TG range. Thus, similar to heterozygous pathogenic *LPL* variants [75], heterozygous pathogenic *APOA5* variants have extremely variable associations with TG levels, rather than a stable consistent autosomal dominant pattern, as observed in familial hypercholesterolemia.

APOA5 is one of five causal genes for FCS, an extremely rare condition with prevalence of 1-10 in a million that is characterized by severe, sustained, refractory HTG and systemic manifestations such as lipemia retinalis, eruptive xanthomatosis, hepatosplenomegaly and acute pancreatitis [52]. FCS is caused by biallelic (homozygous or compound heterozygous) LOF variants in one of *LPL*, *APOA5*, *APOC2*, *GPIHBP1*, or *LMF1* genes, which encode the proteins LPL, apo A-V, apo C-II, glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 (GPIHBP1), and lipase maturation factor 1 (LMF1), respectively [52]. However, the phenotype produced by monoallelic LOF variants in these genes is less well appreciated. We showed that heterozygosity for rare variants in one of the five FCS genes in the

general population is relatively common at ~3-4%, with 3- and 5-fold enrichment in patients with mild-to-moderate and severe hypertriglyceridemia, respectively [2,72,73].

Previous descriptions of the phenotype seen in heterozygosity for pathogenic variants in *APOA5* come mainly from cross-sectional studies of families ascertained via a homozygous proband [12,54,55]. Thus, in addition to a lack of dedicated research on the heterozygous phenotype, there is also a paucity of longitudinal phenotyping in these patients. Oliva and colleagues reported 10 individuals in a single family who were heterozygous for *APOA5* p.Q148X (also called p.Q145X due to a different numbering convention) [12]. Half of these individuals had mild-to-moderate HTG while the rest had a normal lipid profile [12]. Marçais et al. found that 8 heterozygotes for the *APOA5* p.Q139X nonsense variant had severe HTG [54], although most had additional TG-raising factors, such as diabetes or obesity [54]. Our data similarly show variation at baseline and longitudinally between and within heterozygotes for *APOA5* nonsense variants.

We observed four unique variants, of which *APOA5* p.E98X was novel (**Table 3.2**). Of the three previously reported variants, *APOA5* p.Q97X was recurrently associated with HTG [69,70,81–86]. While longitudinal TG data were not reported, there was considerable variability in the baseline TG levels of *APOA5* p.Q97X heterozygotes, ranging from normal TG levels [82], to mild-to-moderate [81], and severe HTG [69,70,81,85]. Such variability, plus the observation of heterozygosity for *APOA5* variants among young and healthy normolipidemic individual without obvious TG-raising factors is consistent with the idea that secondary factors can worsen the TG phenotype in such heterozygotes. In our cohort, all subjects had one or more risk factors associated with HTG, including advanced age and increased BMI. Dysglycemia and diabetes were not seen in our cohort.

Direct comparison of the phenotypic effects of the *APOA5* p.Q275X and p.Q305X variants are challenging due to small numbers of affected individuals. For instance, the *APOA5* p.Q275X variant has only been reported together in an oligogenic interaction state with another pathogenic variant that could affect triglycerides [86,87]. In our cohort,

carriers of these variants had advanced age and elevated BMI, which could have worsened the phenotype. Furthermore, the *APOA5* p.Q305X variant has not been reported alongside individual phenotypic data [88]. However, since even more severe prematurely truncated variants have been found to be secreted into plasma, e.g., *APOA5* p.Q139X variant [54], it is possible that these downstream truncations may not affect secretion of the variant protein, although function could still be compromised, e.g., through elimination of the C-terminal lipid binding domain at residues 301-343 of mature apo A-V [49].

Our lab previously showed that most cases of severe HTG in the population have a high polygenic score for TG [73]. However, polygenic risk does not appear to be playing a role in the phenotype of most of the *APOA5* variant heterozygotes evaluated here.

As we previously noted [75], some clinicians assume that heterozygosity for pathogenic variants in severe HTG patient implies autosomal dominant expression of HTG. However, the aggregate of data suggests that, unlike autosomal dominant familial hypercholesterolemia, autosomal dominant HTG does not exist because there is: 1) a large number of normolipidemic heterozygous carriers of pathogenic variants in FCS gene including *APOA5* in the general population [72,73]; 2) a lack of consistent co-segregation of heterozygous pathogenic *APOA5* variants and HTG in multigenerational families [12,54,81,82]; 3) wide variability of TG phenotype in *APOA5* heterozygotes at baseline and over time; and 4) strong modulating effects of secondary non-genetic factors such as age, BMI, and diabetes [12]. Thus, heterozygous pathogenic variants in *APOA5*, like those in *LPL*, contribute to HTG susceptibility via a probabilistic, not deterministic model [75].

Among the 1 in 400-500 individuals with severe HTG in the general population [89], only 1-5% possess biallelic pathogenic variants in FCS genes [2,52,73,90–92], while 15-20% are heterozygous for pathogenic variants in FCS genes [2,73,92]. Another 35-50% have a high polygenic SNP score for TG underlying their HTG, compared to only 10% in the general population [2,53,73]. Genetically susceptible adults mainly display HTG in

the presence of non-genetic exacerbating factors such as increased age, BMI, diabetes, alcohol use, poor diet, physical activity level, etc. [12,93–103].

In summary, the TG phenotype of individuals heterozygous for *APOA5* pathogenic variants is highly variable both within and between patients. TG phenotypes in these patients range from normal to severe HTG both at baseline and over time. This complexity rules out a simple highly penetrant autosomal dominant influence or model to explain the genetics and phenotype. TG levels are a genetically more complex and less stable phenotype than other lipid traits, e.g., LDL cholesterol, with much greater influence from polygenic factors and also secondary non-genetic exacerbating factors. Additionally, single time point TG measurements in individuals heterozygous for pathogenic variants in *APOA5* may not satisfactorily describe the TG phenotype. Heterozygosity for a pathogenic *APOA5* variant might warn the clinician of the potential risk of severe HTG and that controlling the high triglyceride level might represent more of a challenge compared to patients without such variants. Future research could examine if screening for such variants at birth or another time point would be an effective strategy for ASCVD risk reduction and management by informing patients about predispositions they have to developing HTG.

3.4.2 Study 2: Investigating the association of the common *APOA5* p.Ser19Trp SNP with HTG in a clinical cohort

We found that both heterozygous and homozygous carriers of the common p.Ser19Trp SNP in *APOA5* demonstrated marked variability in the severity of their plasma TG phenotypes. Specifically, when observing the historically highest recorded TG measurements for our cohort, patients in both the homozygous and heterozygous groups demonstrated TG levels ranging from normal to mild-to-moderate HTG to severe HTG. The proportion of patients within each TG severity range were comparable between the homozygous and heterozygous groups, indicating that these groups are likely not phenotypically different. This was confirmed by statistical analysis. Specifically, one-way Welch's ANOVA with post-hoc Games-Howell test was performed to compare mean historically highest TG between all 3 study groups and revealed that while both the homozygous and heterozygous groups were significantly different from control on

average ($p = 0.002$ and $p < 0.001$, respectively), they were not significantly different from each other on average ($p = 0.625$). The variability of TG phenotype severity we observed, especially in the homozygous group, indicates that the *APOA5* p.Ser19Trp SNP is not causative for HTG and in fact, individuals with this SNP are not guaranteed to display HTG, definitively showing that this SNP is not as deleterious as a true pathogenic variant.

Our results are consistent with population data, which find that common SNPs such as *APOA5* p.Ser19Trp have high frequency in the normolipidemic population, in the range of 5 to 15% [71]. This suggests greatly that at least in isolation, these common SNPs that have been observed to have or are associated with TG-elevating effects are not causal for HTG. However, in combination with multiple other SNPs, they can predispose to both mild-to-moderate [72] and severe HTG [73]. While common SNPs like *APOA5* p.Ser19Trp have been previously thought to be the major contributor to the variability of HTG severity seen in heterozygotes for pathogenic APOA5 variants [12,54,55,68–70], the results we observed in Study 1 (see section 3.3.1.7) clearly show that this variability is not dependent on the presence of TG-elevating SNPs, nor on the *APOA5* p.Ser19Trp SNP specifically. Therefore, while these SNPs may contribute to the variability and/or severity of HTG observed in patients heterozygous for pathogenic variants in *APOA5*, they are not likely to be the major secondary contributor, at least not when in isolation. My work seems to indicate that even in patients heterozygous for pathogenic variants in *APOA5*, significant accumulation of common TG-elevating SNPs is required for them to exert a clinically significant effect.

However, my current analysis is only a preliminary analysis. Additional data collection and a more robust filtering process could reveal further insights regarding the true clinical impact of this common SNP. Currently, a minority of patients within this dataset possess rare pathogenic variants within the FCS genes. Additionally, filtering out patients with clinically significant PRS for elevated TG is also needed. Additional data collection for various other variables such as age, BMI, and other known TG-elevating demographic factors (see section 1.1.3 for more details) would allow for a more thorough analysis of the clinical significance of the common *APOA5* p.Ser19Trp SNP in isolation and its

interaction with other secondary factors. The present results, though, do highlight that, at the very least, *APOA5* p.Ser19Trp is not directly causative for HTG as previously discussed.

3.4.3 A review of genetic variation in *APOA5*

There are currently at least 118 unique rare variants of *APOA5* reported in the literature and/or various genetic databases as being associated with or have evidence suggesting possible associations with phenotypes such as FCS and, more broadly, with HTG, but also with atherosclerotic cardiovascular disease (ASCVD). We have summarized the coding sequence variants resulting in amino acid changes alongside the relative positions of the major functional domains of apo A-V in **Figure 3.6**. Additionally, the nucleic acid changes for noncoding region variants are summarized in **Figure 3.7**. **Table 3.2** contains a list of these variants with notes on the suspected and/or confirmed molecular defects caused by these variants.

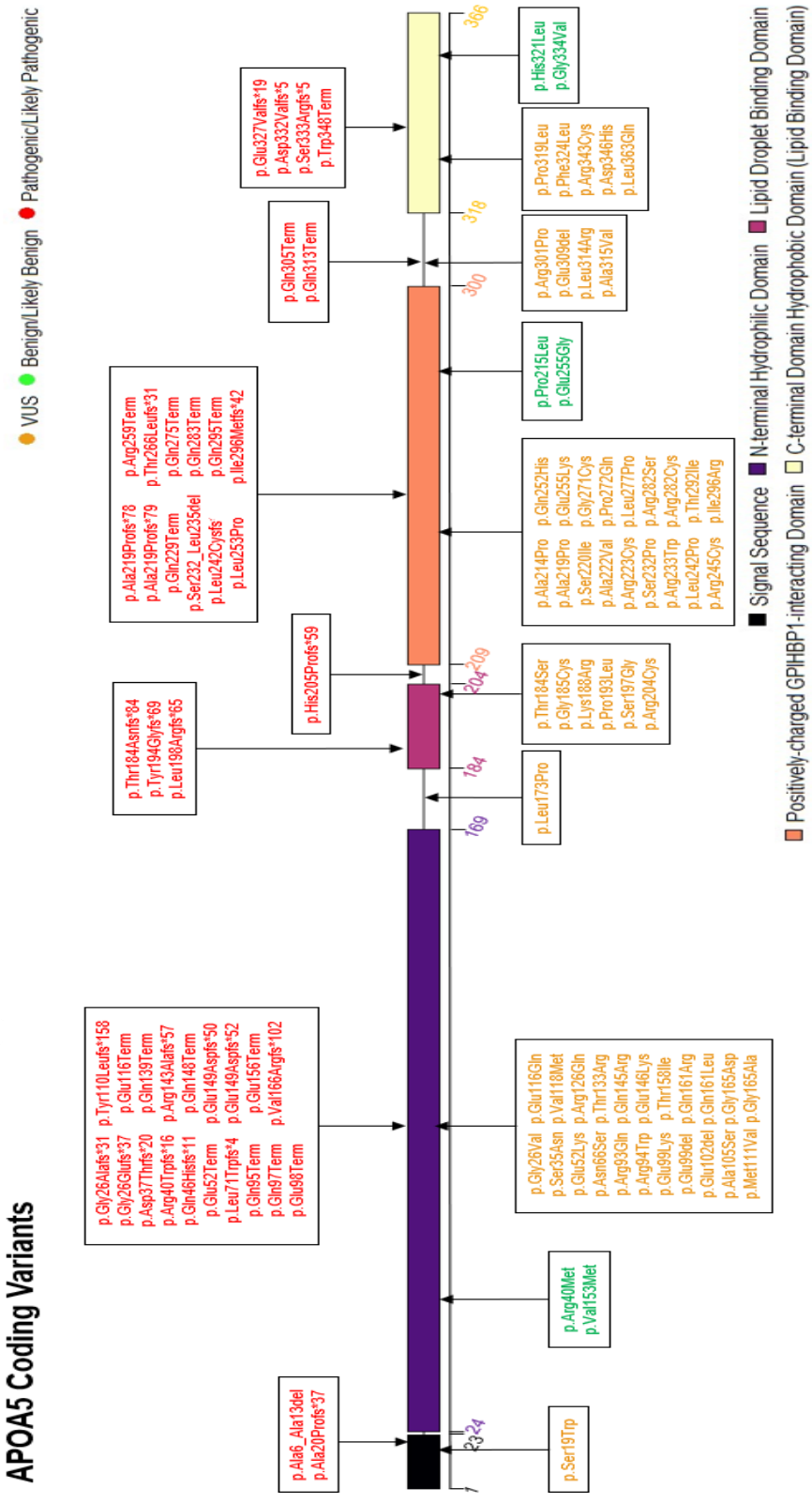

Figure 3.6. Map of reported *APOA5* coding sequence variants.

Boxes represent functional domains of the apo A-V peptide: Black represents the signal peptide, purple represents the N-terminal hydrophilic domain, magenta represents the lipid droplet binding domain, orange represents the positively charged GPIHBP1-interacting domain, and the yellow represents the C-terminal hydrophobic domain (lipid binding domain). Axis numbering represents the amino acid residue number in the primary structure of the newly synthesized apo A-V peptide and the specific residues indicated represent the first and last residues of the domains they share color with. All variants are color-coded according to pathogenicity classification according to ACMG guidelines: red indicates pathogenic or likely pathogenic, orange indicates a variant of uncertain significance (VUS), and green indicates benign or likely benign. Due to size of the figure and technical constraints of this thesis, the figure quality is reduced to fit the document size. A higher quality figure is available online within the review article from which this figure has been adapted as noted at the beginning of this chapter: Perera SD, Hegele RA. Genetic variation in apolipoprotein A-V in hypertriglyceridemia. Curr. Opin. Lipidol. 35(2), 66–77 (2024) [104].

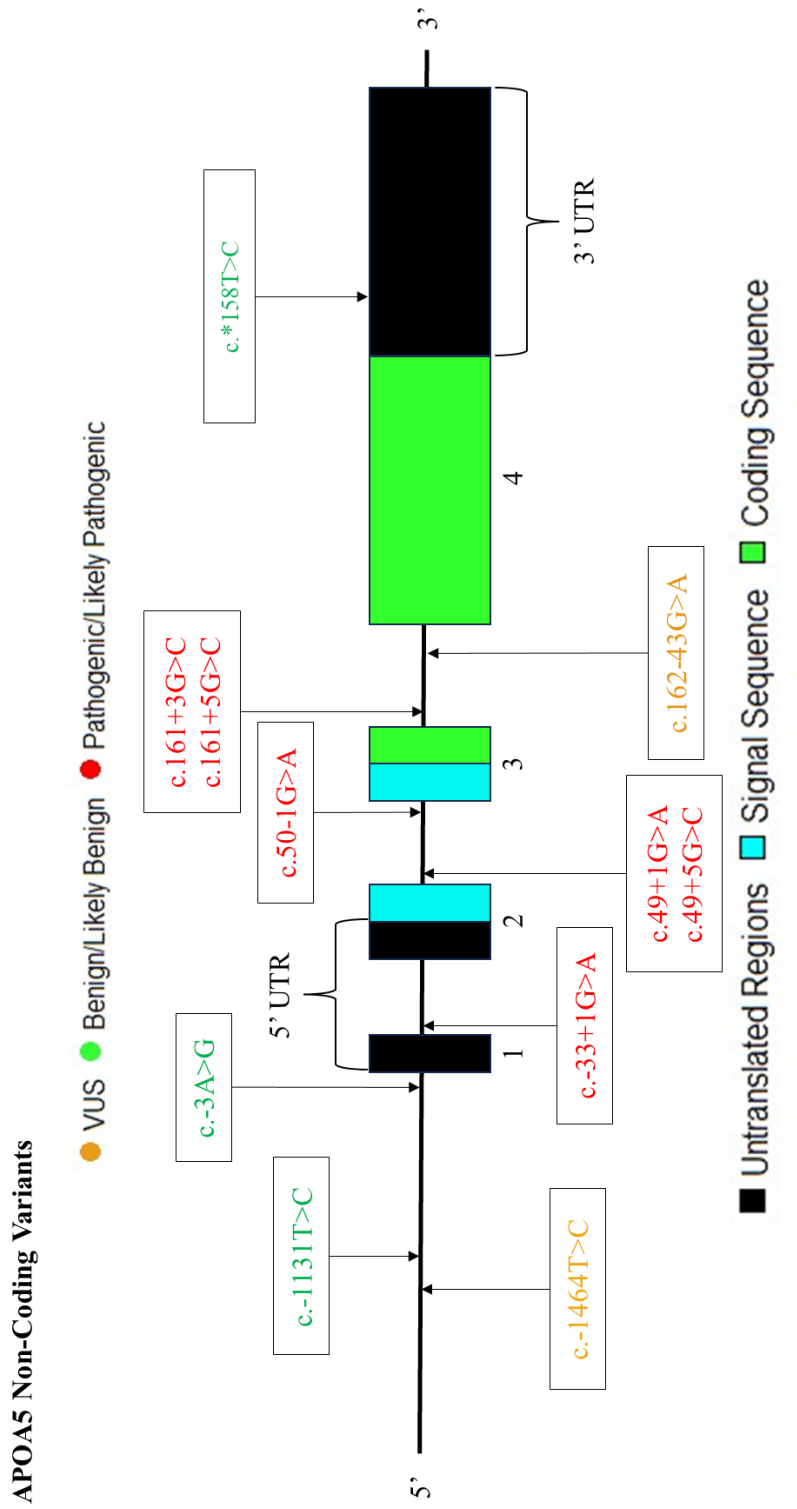

Figure 3.7. Map of reported *APOA5* noncoding variants.

Gene map of *APOA5* annotated with variants discovered in the regulatory regions (5’ and 3’ untranslated regions (UTRs), promoter region, etc.), splice donor and acceptor sites, and introns. Numbering underneath boxes indicates exons. Major structural features are color-coded. Black boxes indicate untranslated sequences, blue boxes indicate sequences coding for the apo A-V signal peptide, and green boxes indicate sequences coding for the mature protein. Variants are presented using nucleic acid changes. All variants are color-coded according to pathogenicity classification according to ACMG guidelines: Red indicates pathogenic or likely pathogenic, orange indicates a variant of uncertain significance (VUS), and green indicates benign or likely benign.

Table 3.2. *APOA5* variants reported in literature and/or clinical testing.

Variant Type	Nucleotide Change	Amino Acid Change[a,b, c]	ACMG classification[d]	Molecular defect (if applicable) and related notes	Previously reported in literature?
Regulatory	c.-1464T>C	N/A	VUS	Creates a putative vitamin D receptor binding site which increases *APOA5* promoter activity. Also associated with decreased HDL-C in vitamin D deficient patients.	Yes [105]
Regulatory	c.-1131T>C	N/A	Benign	In 100% linkage disequilibrium with c.-3A>G and c.*158T>C. Together, these 3 variants have been	Yes [57,58,106–109]

				shown to reduce *APOA5* expression, though the exact role of c.-1131T>C in this is unclear, unlike the other two variants. Component of *APOA5**2 Haplotype.	
Splicing	c.-33+1G>A	N/A	Likely Pathogenic	Splicing donor site null variant that results in skipping of exon 1 during transcription, which contains a portion of the signal peptide of apo A-V.	Yes [78]
Regulatory	c.-3A>G	N/A	Benign	In 100% linkage disequilibrium with c.-1131T>C and c.*158T>C. Together,	Yes [109–111]

				these 3 variants have been shown to reduce *APOA5* expression. Specifically, this variant has two purported functional impacts. Firstly, the transcription factor GATA4 only binds the wild-type allele of this variant. Secondly, this variant impacts the Kozak sequence of *APOA5*, which is thought to impact translation initiation. Component of *APOA5**2 Haplotype.	
Gross Deletion	c.16_39del	p.Ala6_Ala13del	Likely pathogenic	Partial deletion of signal sequence leads	Yes [112]

				to hepatic missorting of protein to lipid droplets which subsequently results in impaired secretion of protein.	
Splicing	c.49+1G>A	N/A	Likely Pathogenic	Abolishes donor splice site of intron 2 leading to altered mRNA that encodes a truncated protein.	Yes [113]
Splicing	c.49+5G>C	N/A	Likely Pathogenic	Predicted to abolish donor splice site of intron 2 leading to altered mRNA that encodes a truncated protein.	Yes [114]
Splicing	c.50-1G>A	N/A	Likely	Predicted to abolish	Yes [115]

			Pathogenic	functionality of acceptor splice site of intron 2 leading to altered mRNA that encodes a truncated protein.	
Missense	c.56C>G	p.Ser19Trp	VUS	Variant produces a less efficient signal peptide that is predicted to reduce apo A-V secretion by up to 49%. Variant is considered VUS due to preserved protein function, inconsistent segregation with disease, high population frequency, etc. Best regarded as a risk factor and not a	Yes [45,58,61,116–118]

				disease-causing variant in isolation. Only component of *APOA5**3 haplotype.	
Small Deletion	c.58delG	p.Ala20Profs*37	Likely Pathogenic	Deletion alters reading frame, resulting in altered mRNA that encodes a truncated protein. Truncation at amino acid 37 eliminates ~90% of the protein, abolishing all functional domains of apo A-V.	No (found in our own clinical testing)
Small duplication	c.73_76dup	p.Gly26Glufs*37	Likely Pathogenic	Duplication alters reading frame resulting in mRNA encoding truncated protein. ~90% of the	No (reported in LOVD3 only)

				protein is lost, abolishing all functional domains.	
Missense	c.77G>T	p.Gly26Val	VUS	Unknown	Yes [119]
Small Deletion	c.77delG	p.Gly26Alafs*31	Likely Pathogenic	Deletion alters reading frame resulting in mRNA encoding truncated protein. ~90% of protein is lost, abolishing all functional domains of apo A-V.	Yes [78]
Missense	c.104G>A	p.Ser35Asn	VUS	Unknown	Yes [119]
Small Deletion	c.109delG	p.Asp37Thrfs*20	Likely Pathogenic	Deletion alters reading frame resulting in mRNA encoding truncated protein. >90% of protein is	No (found in our own clinical testing)

				lost, abolishing all functional domains of apo A-V.	
Small Deletion	c.117_120del	p.Arg40Trpfs*16	Likely Pathogenic	Deletion alters reading frame resulting in mRNA encoding truncated protein. >90% of protein is lost, abolishing all functional domains of apo A-V.	No (reported in ClinVar only)
Missense	c.119G>T	p.Arg40Met	Likely Benign	Unknown. Considered benign due to gnomAD maximal non-founder subpopulation allele frequency of 0.269% which is greater than threshold for disease	Yes [84]

				(0.1%).	
Small Deletion	c.138del	p.Gln46Hisfs*11	Likely Pathogenic	Deletion alters reading frame resulting in mRNA encoding truncated protein. >90% of protein is lost, abolishing all functional domains of apo A-V.	No (reported in LOVD3 only)
Missense	c.154G>A	p.Glu52Lys	VUS	Unknown	Yes [120]
Nonsense	c.154G>T	p.Glu52Term	Likely Pathogenic	Nonsense SNP produces mRNA that encodes truncated protein due to premature stop codon. ~85% of the protein is eliminated resulting in loss of all functional	No (reported in ClinVar only)

				domains.	
Splicing	c.161+3G>C	N/A	Likely Pathogenic	Abolishes donor splice site of intron 3 leading to altered mRNA that skips exon 3 entirely and results in a truncated peptide that is a predicted 18 amino acids long.	Yes [55]
Splicing	c.161+5G>C	N/A	Likely Pathogenic	Predicted to severely decrease the binding capacity of the donor splice site of intron 3 suggesting splicing defect. Patient homozygous for this variant and seemingly no other rare pathogenic *APOA5*	Yes [78,121,122]

				variants was apo A-V deficient.	
Splicing	c.162-43G>A	N/A	VUS	Unknown	Yes [3]
Missense	c.197A>G	p.Asn66Ser	VUS	Unknown	Yes [88]
Small Deletion	c.211delC	p.Leu71Trpfs*4	Likely Pathogenic	Deletion alters reading frame resulting in mRNA encoding truncated protein. ~80% of protein is lost, abolishing all functional domains of apo A-V.	Yes [78]
Missense	c.278G>A	p.Arg93Gln	VUS	Unknown	Yes [84]
Missense	c.280C>T	p.Arg94Trp	VUS	Unknown	Yes [84]
Nonsense	c.283C>T	p.Gln95Term	Likely Pathogenic	Nonsense SNP produces mRNA that encodes truncated	Yes [84]

				protein due to premature stop codon. ~75% of the protein is eliminated resulting in loss of all functional domains.	
Nonsense	c.289C>T	p.Gln97Term	Likely Pathogenic	Nonsense SNP produces mRNA that encodes truncated protein due to premature stop codon. ~75% of the protein is eliminated resulting in loss of all functional domains.	Yes [69,70,78,81–86,123]
Nonsense	c.292G>T	p.Glu98Term	Likely Pathogenic	Nonsense SNP produces mRNA that encodes truncated protein due to	Yes [78,123,124]

				premature stop codon. ~75% of the protein is eliminated resulting in loss of all functional domains.	
Missense	c.295G>A	p.Glu99Lys	VUS	Unknown	Yes [119]
Small Deletion	c.295_297delGAG	p.Glu99del	VUS	Predicted to disrupt amphipathic N-terminal domain α-helix configuration due to elimination of negatively-charged amino acid on the hydrophilic site of the helix.	Yes [68,78]
Small Deletion	c.305_307del	p.Glu102del	VUS	Unknown. Does not appear to be an α-helix forming	No (reported in LOVD3 only)

				residue[68,125,126] so effect is difficult to predict.	
Missense	c.313G>T	p.Ala105Ser	VUS	Unknown	Yes [84]
Small Insertion	c.326_327insC	p.Tyr110Leufs*158	Likely Pathogenic	Insertion alters reading frame resulting in mRNA encoding truncated protein. >50% of protein is lost. N-terminal hydrophilic domain is in-tact. All other functional domains are interrupted or eliminated.	Yes [115,127]
Missense	c.331A>G	p.Met111Val	VUS	Unknown	Yes [78]
Missense	c.346G>C	p.Glu116Gln	VUS	Unknown but has been studied amongst	Yes [128–130]

				numerous other variants as part of several investigations into the role *de novo* variants in Autism Spectrum Disorder.	
Nonsense	c.346G>T	p.Glu116Term	Likely pathogenic	Nonsense SNP produces mRNA that encodes truncated protein due to premature stop codon. ~70% of the protein is eliminated resulting in loss of or serious interruption of all functional domains.	Yes [84]
Missense	c.352G>A	p.Val118Met	VUS	Unknown	Yes [78]
Missense	c.377G>A	p.Arg126Gln	VUS	Unknown	No (reported in LOVD3

					only)
Missense	c.398C>G	p.Thr133Arg	VUS	Found in heterozygosity in patient with normal post-heparin LPL mass but no post-heparin LPL activity.	Yes [131]
Nonsense	c.415C>T	p.Gln139Term	Likely Pathogenic	Nonsense SNP produces mRNA that encodes truncated protein due to premature stop codon. >60% of the protein is eliminated resulting in loss of and serious interruption of all functional domains.	Yes [54,127]
Small	c.427delC	p.Arg143Alafs*57	Likely	Deletion alters reading	Yes [78,84,120,132–136]

Deletion			Pathogenic	frame resulting in mRNA encoding truncated protein. ~45% of protein is lost, leading to loss of GPIHBP1 and Heparin binding domain and C-terminal Lipid Binding Domain.	
Missense	c.434A>G	p.Gln145Arg	VUS	Unknown	Yes [84,120]
Missense	c.436G>A	p.Glu146Lys	VUS	Unknown	Yes [78]
Nonsense	c.442C>T	p.Gln148Term	Likely pathogenic	Nonsense SNP produces mRNA that encodes truncated protein due to premature stop codon. ~60% of the protein is eliminated resulting in	Yes [12]

				loss of and serious interruption of all functional domains.	
Small Deletion	c.447_450delGCAG	p.Glu149Aspfs*50	Likely Pathogenic	Deletion alters reading frame resulting in mRNA encoding truncated protein. ~45% of protein is lost, leading to loss of GPIHBP1 and Heparin binding domain and C-terminal Lipid Binding domain.	Yes [78]
Small Insertion-Deletion	c.447delGinsCTC	p.Glu149Aspfs*52	Likely Pathogenic	Insertion-deletion alters reading frame resulting in mRNA encoding truncated protein. ~45% of protein is lost, leading	

				to loss of GPIHBP1 and Heparin binding domain and C-terminal Lipid Binding domain.	
Missense	c.457G>A	p.Val153Met	Benign	Unknown. Benign because of very high gnomAD maximal non-founder and founder subpopulation allele frequencies of 11.917% and 4.936%, respectively. It has also been observed in the homozygous state in population databases more than expected for disease.	Yes [119,132,138,139]
Nonsense	c.466G>T	p.Glu156Term	Likely	Nonsense SNP	No (reported in ClinVar

			Pathogenic	produces mRNA that encodes truncated protein due to premature stop codon. ~60% of the protein is eliminated resulting in loss of and serious interruption of all functional domains.	only)
Missense	c.473C>T	p.Thr158Ile	VUS	Unknown	Yes [78]
Missense	c.482A>G	p.Gln161Arg	VUS	Unknown	No (reported in LOVD3 only)
Missense	c.482A>T	p.Gln161Leu	VUS	Unknown	No (reported in LOVD3 only)
Missense	c.494G>A	p.Gly165Asp	VUS	Unknown	Yes [120]
Missense	c.494G>C	p.Gly165Ala	VUS	Unknown	Yes [132]
Small	c.494dup	p.Val166Argfs*102	Likely	Duplication alters	No (reported in ClinVar

duplication			Pathogenic	reading frame resulting in mRNA encoding truncated protein. Loss of 201 amino acids, leading to loss of the lipid droplet binding domain, GPIHBP1 and Heparin binding domain and C-terminal Lipid Binding domain. Additionally, ClinVar comments indicate that this variant has been observed in a patient with chylomicronemia.	only)
Missense	c.518T>C	p.Leu173Pro	VUS	Unknown	Yes [85]

Small Duplication	c.550dup	p.Thr184Asnfs*84	Likely Pathogenic	Duplication alters reading frame resulting in mRNA encoding truncated protein. Loss of ~50% of protein leading to elimination of the lipid droplet binding domain, GPIHBP1 interacting binding domain, and C-terminal lipid binding domain.	No (reported in LOVD3 only)
Missense	c.551C>G	p.Thr184Ser	VUS	Unknown	Yes [78,140,141]
Missense	c.553G>T	p.Gly185Cys	VUS	Unknown. Likely a risk factor much like S19W or -1131T>C are in some	Yes [84,142–144]

				populations. Interestingly, this variant is negatively associated with obesity risk in the Chinese population while still raising TG.	
Missense	c.563A>G	p.Lys188Arg	VUS	Unknown	Yes [124]
Missense	c.578C>T	p.Pro193Leu	VUS	Unknown	Yes [78]
Small Deletion	c.579_592del14	p.Tyr194Glyfs*69	Likely Pathogenic	Deletion alters reading frame resulting in mRNA encoding truncated protein. ~28% of protein is lost, leading to loss of GPIHBP1 and Heparin binding domain and C-terminal Lipid Binding	Yes [145]

				domain.	
Missense	c.589A>G	p.Ser197Gly	VUS	Unknown	Yes [84]
Small Deletion	c.593_606del14	p.Leu198Argfs*65	Likely Pathogenic	Deletion alters reading frame resulting in mRNA encoding truncated protein. ~28% of protein is lost, leading to loss of GPIHBP1 and Heparin binding domain and C-terminal Lipid Binding domain.	Yes [135]
Missense	c.610C>T	p.Arg204Cys	VUS	Unknown	Yes [78]
Small Deletion	c.614_624del11	p.His205Profs*59	Likely Pathogenic	Deletion alters reading frame resulting in mRNA encoding truncated protein. ~28% of protein is	Yes [146]

				lost, leading to loss of GPIHBP1 and Heparin binding domain and C-terminal Lipid Binding domain.	
Missense	c.640G>C	p.Ala214Pro	VUS	Unknown	Yes [78]
Missense	c.644C>T	p.Pro215Leu	Likely Benign	Unknown. Considered benign because allele frequency in gnomAD maximal non founder subpopulations is higher than expected for disease at 0.216%. It has also been observed in the homozygous state in population databases.	Yes [84,127]
Small	c.653_654dup	p.Ala219Profs*79	Likely	Duplication alters	No (reported in LOVD3

Duplication			Pathogenic	reading frame resulting in mRNA encoding truncated protein. ~18% of protein is lost, leading to loss of C-terminal portion of the GPIHBP1 and Heparin binding domain and C-terminal Lipid Binding domain.	only)
Small Deletion	c.654delC	p.Ala219Profs*78	Likely Pathogenic	Deletion alters reading frame resulting in mRNA encoding truncated protein. ~18% of protein is lost, leading to loss of C-terminal portion of the GPIHBP1 and Heparin binding	Yes [146]

				domain and C-terminal Lipid Binding domain.	
Missense	c.655G>C	p.Ala219Pro	VUS	Unknown	Yes [147]
Missense	c.659G>T	p.Ser220Ile	VUS	Unknown	Yes [119]
Missense	c.665C>T	p.Ala222Val	VUS	Unknown	Yes [84]
Missense	c.667C>T	p.Arg223Cys	VUS	Unknown	Yes [119]
Nonsense	c.685C>T	p.Gln229Term	Likely Pathogenic	Nonsense SNP produces mRNA that encodes truncated protein due to premature stop codon. ~37% of the protein is eliminated which eliminates a portion of the GPIHBP1/Heparin binding domain and	No (reported in LOVD3 only)

				also eliminates the C-terminal lipid binding domain.	
Missense	c.694C>T	p.Ser232Pro	VUS	Unknown	Yes [148]
Small Deletion	c.694_705del12	p.Ser232_Leu235del	Likely pathogenic	Impaired binding to immobilized heparin due to slower association, somewhat defective sortilin interaction and complete SorLA/LR11 binding deficiency.	Yes [69]
Missense	c.697C>T	p.Arg233Trp	VUS	Unknown	Yes [84]
Small Deletion	c.724delC	p.Leu242Cysfs*55	Likely Pathogenic	Deletion alters reading frame resulting in mRNA encoding truncated protein. ~18% of protein is	Yes [120]

				lost, leading to loss of C-terminal portion of the GPIHBP1/Heparin binding domain and C-terminal Lipid Binding domain.	
Missense	c.725T>C	p.Leu242Pro	VUS	Unknown. Was found in hyperchylomicronemia proband but its involvement is unclear.	Yes [70]
Missense	c.733C>T	p.Arg245Cys	VUS	Unknown	Yes [84]
Missense	c.756G>C	p.Gln252His	VUS	Unknown	Yes [120]
Missense	c.758T>C	p.Leu253Pro	Likely pathogenic	Decreased liposome binding, almost completely deficient in sortilin and	Yes [68,69,78,115]

				SorLA/LR11 binding, and finally variant potently inhibits LPL activity.	
Missense	c.763G>A	p.Glu255Lys	VUS	Unknown	Yes [84,120]
Missense	c.764A>G	p.Glu255Gly	Benign	Unknown. There is some evidence that it has some reduced ability to enhance LPL but allele frequency is too high for what is expected of disorder according to gnomAD (0693%) and has been observed in homozygous state in population databases more than is expected for disease.	Yes [149]

Nonsense	c.775A>T	p.Arg259Term	Likely Pathogenic	Nonsense SNP produces mRNA that encodes truncated protein due to premature stop codon. ~30% of the protein is eliminated which eliminates a portion of the GPIHBP1/Heparin binding domain and eliminates the C-terminal lipid binding domain.	No (reported in ClinVar only)
Small Deletion	c.795del	p.Thr266Leufs*31	Likely Pathogenic	Deletion alters reading frame resulting in mRNA encoding truncated protein leading to loss of C-terminal portion of the	No (reported in ClinVar only)

				GPIHBP1/Heparin binding domain and C-terminal Lipid Binding domain.	
Missense	c.811G>T	p.Gly271Cys	VUS	Forms dimers and multimers due to formation of disulfide bonds at this position being available. This variant does not bind LDL-family receptors, LR8 or LRP1. Does not seem to impact LPL activity directly.	Yes [149]
Missense	c.815C>A	p.Pro272Gln	VUS	Unknown	Yes [84]
Nonsense	c.823C>T	p.Gln275Term	Likely Pathogenic	Nonsense SNP produces mRNA that encodes truncated	Yes [78,86,87,150–153]

				protein due to premature stop codon. ~25% of the protein is eliminated which eliminates a portion of the GPIHBP1/Heparin binding domain and eliminates the C-terminal lipid binding domain.	
Missense	c.830T>C	p.Leu277Pro	VUS	Unknown	Yes [78,84,124]
Missense	c.844C>A	p.Arg282Ser	VUS	Associated with a reduction in TG levels and serum APOAV levels.	Yes [78,154]
Missense	c.844C>T	p.Arg282Cys	VUS	Unknown	Yes [84]
Nonsense	c.847C>T	p.Gln283Term	Likely Pathogenic	Nonsense SNP produces mRNA that	No (reported in ClinVar only)

				encodes truncated protein due to premature stop codon. ~20% of the protein is eliminated which eliminates a portion of the GPIHBP1/Heparin binding domain and eliminates the C-terminal lipid binding domain.	
Missense	c.875C>T	p.Thr292Ile	VUS	Unknown	Yes [78,84]
Nonsense	c.883C>T	p.Gln295Term	Likely Pathogenic	Nonsense SNP produces mRNA that encodes truncated protein due to premature stop codon. ~20% of the protein is eliminated which	Yes [78,84,120,132]

				eliminates a portion of the GPIHBP1/Heparin binding domain and eliminates the C-terminal lipid binding domain.	
Missense	c.887T>G	p.Ile296Arg	VUS	Unknown	Yes [78,146]
Small Deletion	c.888delA	p.Ile296Metfs*42	Likely Pathogenic	Deletion alters reading frame resulting in mRNA encoding truncated protein. Disrupts the C-terminal lipid binding domain.	Yes [78]
Missense	c.902G>C	p.Arg301Pro	VUS	Unknown	Yes [120]
Nonsense	c.913C>T	p.Gln305Term	Likely Pathogenic	Nonsense SNP produces mRNA that encodes truncated	Yes [88]

				protein due to premature stop codon. C-terminal lipid binding domain is disrupted.	
Small Deletion	c.926_928delAGG	p.Glu309del	VUS	Unknown	Yes [120]
Nonsense	c.937C>T	p.Gln313Term	Likely Pathogenic	Nonsense SNP produces mRNA that encodes truncated protein due to premature stop codon. C-terminal lipid binding domain is disrupted.	Yes [84,155]
Missense	c.941T>G	p.Leu314Arg	VUS	Unknown	Yes [120]
Missense	c.944C>T	p.Ala315Val	VUS	Unknown but evidence currently	Yes [78,84,124,156]

				suggests that in isolation this variant is not pathogenic but since it has increased frequency in HTG patient population, it may be interact with other variants to cause HTG.	
Missense	c.956C>T	p.Pro319Leu	VUS	Unknown	Yes [84]
Missense	c.962A>T	p.His321Leu	Benign	Unknown but is considered benign since it has been reported in the homozygous state in population databases more than is expected for disease-causing variant (gnomAD	Yes [84,149]

				homozygous count is 4 individuals)	
Missense	c.972C>G	p.Phe324Leu	VUS	Unknown	Yes [124]
Small Deletion	c.980_981delAG	p.Glu327Valfs*19	Likely Pathogenic	Deletion alters reading frame resulting in mRNA encoding truncated protein. Disrupts the C-terminal lipid binding domain.	Yes [120]
Small Deletion	c.990_993delAACA	p.Asp332Valfs*5	Likely Pathogenic	Deletion alters reading frame resulting in mRNA encoding truncated protein. Disrupts the C-terminal lipid binding domain.	Yes [69,78,86,118,119,136]
Small	c.995_998delACAG	p.Asp332Valfs*5	Likely	Deletion alters reading	Yes [88]

Deletion			Pathogenic	frame resulting in mRNA encoding truncated protein. Disrupts the C-terminal lipid binding domain.	
Gross Insertion	c.999insGGCAAGG TTGTGAGCAAGCT GCAGGCCC	p.Ser333Argfs*5	Likely pathogenic	Large insertion alters reading frame resulting in mRNA encoding truncated protein. Disrupts the C-terminal lipid binding domain.	Yes [120]
Missense	c.1001G>T	p.Gly334Val	Likely Benign	Unknown. Benign as this variant has been observed in homozygous state in gnomAD population database.	Yes [84]

Missense	c.1027C>T	p.Arg343Cys	VUS	Unknown. Interestingly, even in the homozygous state, this variant does not seem to impact LPL activity.	Yes [84,127,132]
Missense	c.1036G>C	p.Asp346His	VUS	Unknown	Yes [78]
Nonsense	c.1044G>A	p.Trp348Term	Likely Pathogenic	Nonsense SNP produces mRNA that encodes truncated protein due to premature stop codon. C-terminal lipid binding domain is disrupted.	No (reported in ClinVar only)
Missense	c.1088T>A	p.Leu363Gln	VUS	Unknown	Yes [84]
Regulatory	c.*158T>C	N/A	Likely Benign	Creates a functional	Yes [3,67,157,158]

				miRNA (miR-485-5p) binding site in the 3' UTR of the *APOA5* gene, which enables miRNA-mediated degradation of the mRNA, thereby reducing allele expression. Does not seem to be pathogenic in isolation. Component of *APOA5**2 haplotype.	

[a] For frameshift variants resulting in premature stop codon, the notation "fs*(number)" indicates that the frameshift variant results in stop codon at the position (number) residues downstream of the variant site.

[b] N/A = Not Applicable

[c] Term = Termination; indicates a variant that results in a prematurely truncated protein

[d] VUS = Variant of Uncertain Significance

This curated list was produced by compiling *APOA5* variants listed in three databases (the Human Gene Mutation Database (HGMD) [159], ClinVar [160], Leiden Open Variation Database 3.0 (LOVD3) [161]) as disease-causing or associated variants and then double-checking the reporting of these variants in the literature where available. Pathogenicity for all variants listed was then independently assessed using our laboratory pipeline as we have previously reported [78]. Specifically, we utilized the Franklin by Genoox tool (https://franklin.genoox.com) paired with manual curation to determine the pathogenicity classifications of all the variants according to the American College of Medical Genetics and Genomics (ACMG) guidelines [79]. We also included variants found in our own clinical testing at the Lipid Genetics Clinic, London, Ontario, Canada, if they were considered pathogenic or likely pathogenic under the ACMG guidelines. We note that eight variants reported in ClinVar, namely p.Arg40Trpfs*16, p.Glu52Term, p.Glu156Term, p.Val166Argfs*102, p.Arg259Term, p.Thr266Leufs*31, p.Gln283Term, and p.Trp348Term, nine reported in LOVD3, namely p.Gly26Glufs*37, p.Gln46Hisfs*11, p.Glu102del, p.Arg126Gln, p.Gln161Arg, p.Gln161Leu, p.Thr184Asnfs*84, p.Ala219Profs*79, and p.Gln229Term, and the two novel variants found in our clinic, p.Ala20Profs*37 and p.Asp37Thrfs*20, have been included without any prior literature citations associated with them. While they lack previous reporting in literature, their inclusion is warranted as they most of them are predicted to produce premature stop codons in a gene in which premature protein truncation is an accepted mechanism of disease. [2].

Interestingly, our curation indicates that of the 118 variants with evidence suggesting association with disease, only 50 are pathogenic or likely pathogenic according to the ACMG guidelines. This is because most rare variants in *APOA5* are missense variants and almost all of these lack functional research demonstrating their pathogenicity. Compared to the obvious molecular lesions caused by premature stop codons resulting in truncated protein product, the consequences of most missense variants in *APOA5* are less obvious. Dedicated functional, structural, and/or family studies of the protein produced by missense variants are needed to determine the pathogenicity of these variants and the specific molecular defects they may induce.

Currently, 47 of 50 variants classified as pathogenic predict premature protein truncations of varying severities (**Figures 3.6** and **3.7**). Even small truncations eliminating small portions of the protein are deleterious as the lipid binding properties of apo A-V are enabled by the C-terminal lipid binding domain [47–50]. The remaining three pathogenic variants have all been determined to cause significant functional defect. Firstly, the p.Ala6_Ala13del variant codes for a gross deletion of eight amino acids from the signal sequence and was found to result in missorting and impaired secretion of the otherwise fully functional apo A-V protein [112]. Secondly, the p.Ser232_Leu235del codes for a deletion of 4 amino acids from the GPIHBP1-interacting domain, which rather logically resulted in impaired ability of the abnormal apo A-V protein to interact with heparin and GPIHBP1 [14,18,69]. Interestingly, this variant also results in impaired apo A-V binding to sortilin and SorLA/LR11, which are thought to mediate the ability of apo A-V to reduce hepatic VLDL synthesis and secretion [69,162]. Thirdly, p.Leu253Pro is a missense variant that results in multiple functional defects [68,69]. Currently, p.Leu253Pro is the only known pathogenic missense variant in *APOA5*. This variant was observed in the homozygous state in a 5-year old female patient with severe HTG, with no other TG-elevating variants detected [69]. Functional analysis [69] revealed three consequences of this variant: 1) the secreted abnormal apo A-V protein had impaired liposome binding ability; 2) the abnormal apo A-V protein had complete loss of sortilin and SorLA/LR11 binding ability, which as noted above is thought to mediate the ability of apo A-V to reduce hepatic VLDL synthesis and secretion [162]; and finally, 3) the secreted abnormal apo A-V protein rather interestingly was found to potently inhibit LPL activity instead of enhancing it. *In silico* modeling suggested that the deleterious impact of this missense variant is because the leucine at residue 253 in wild-type apo A-V interfaces between two α-helices via hydrogen bonding interactions [69]. Therefore, given the high α-helical content of mature apo A-V and the functional importance of these structures [47], it is likely that a missense variant that disrupts the formation and/or interaction of these α-helices would be pathogenic. Therefore, while most missense variants in *APOA5* are considered variants of uncertain significance (VUS), it is likely that at least some are in fact pathogenic variants and one of the potential mechanisms may be via disruption of key α-helical structures. Thus, there is a need for improved

understanding of the role of missense variants in *APOA5*-associated disease as there is a paucity of both *in vitro* and *in vivo* functional evidence evaluating the molecular defects associated with these variants.

Premature protein truncation leading to disruption and/or elimination of key functional domains and/or misfolding of the truncated peptide are the obvious molecular defect produced by premature stop codons (both from nonsense and frameshift variants). To the best of our knowledge, nonsense-mediated mRNA decay [163] is not a known mechanism by which LOF occurs in this gene. In fact, given that it has been previously shown that even severely prematurely truncated *APOA5* variants are synthesized and secreted [54], it is unlikely that even in the future this will be found to be a mechanism of disease associated with this gene.

Homozygosity and compound heterozygosity for pathogenic variants leads to severe loss of apo A-V mass and activity in plasma [164] leading in turn to the development of FCS as discussed previously in sections 1.4.3.1.1 and 3.1.2.1. However, the phenotype produced by and/or associated with the presence of monoallelic pathogenic *APOA5* variant condition is not clear. As discussed throughout this chapter, previous descriptions of heterozygous carriers of LOF variants in *APOA5* found considerable variability in baseline TG levels, ranging from normal TG [12,82] to mild-to-moderate HTG [12,81] to severe HTG [54,69,70,81,85]. This is consistent with the findings described in Study 1 (see sections 3.3.1 and 3.4.1). Taken together, alongside findings of young, healthy heterozygous carriers of *APOA5* LOF variants [82], this suggests that secondary factors, such as increased age and body mass index (BMI), are likely needed to force expression of clinical HTG in *APOA5* LOF variant carrying heterozygotes.

However, the exact molecular mechanism by which the heterozygous state for a pathogenic variant predisposes to HTG is unclear. In the monoallelic state, the wild-type allele should theoretically provide sufficient compensation capacity to offset the loss of activity from the non-functional protein produced by the variant allele. This is consistent with the findings of normolipidemic heterozygous carriers of *APOA5* LOF variants [82]. This suggests that haploinsufficiency, where two healthy alleles are required to express a

completely normal phenotype, is not a likely mechanism for producing HTG in patients with monoallelic pathogenic *APOA5* variants. Indirect evidence suggests that *APOA5* variants may exert a dominant-negative effect, where the variant protein interferes with the normal functionality of the wild-type protein.

Specifically, preliminary evidence suggests that certain truncated apo A-V proteins that are expressed can interfere with the ability of wild-type apo A-V protein to associate with lipoprotein particles during their formation in cells [54]. Given that normolipidemic carriers of *APOA5* LOF variants are prevalent in the population [72,73,82], this dominant-negative effect of truncated apo A-V variants and the nonfunctional variant protein alone are insufficient to induce phenotypic HTG. Instead, it is likely that the heterozygous state compromises the homeostatic capacity of the lipolytic cascade such that it is more easily saturated in the presence of secondary TG-elevating risk factors, such as elevated age, increased BMI, polygenic risk accumulation, excessive calorie intake, etc. [165]. A more easily saturated lipolytic capacity would more readily precipitate HTG, and even severe HTG if the secondary stresses were large enough.

However, this explanation is just speculative at this time and the actual mechanistic picture underlying this dominant-negative interaction requires additional study. If this dominant-negative effect is the primary molecular mechanism by which heterozygosity for *APOA5* variants producing prematurely truncated apo A-V protein impacts TG metabolism, this suggests that carriers of such variants are at constant risk of developing HTG and even severe HTG. This may be a good argument for early genetic testing of the lipid metabolism genes like *APOA5* so that patients can have more awareness and options with regards to determining their health and various lifestyle practices; this can be formally tested with a future research trial.

3.5 References

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Chapter 4 – Discussion

Some of the work contained in this chapter has been adapted from three previous publications, two in the *Journal of Clinical Lipidology* and one in *Current Opinion in Lipidology*, and their content has been edited from their original publications for clarity, completeness, and to ensure consistency throughout this thesis.

Perera SD, Wang J, McIntyre AD, Dron JS, Hegele RA. The longitudinal triglyceride phenotype in heterozygotes with LPL pathogenic variants. J. Clin. Lipidol. 17(1), 87–93 (2023).

Perera SD, Wang J, McIntyre AD, Hegele RA. Variability of longitudinal triglyceride phenotype in patients heterozygous for pathogenic APOA5 variants. J. Clin. Lipidol. 17(5), 659–665 (2023).

Perera SD, Hegele RA. Genetic variation in apolipoprotein A-V in hypertriglyceridemia. Curr. Opin. Lipidol. 35(2), 66–77 (2024).

4.1 Overview

In this thesis, I have described my efforts to characterize definitively the range of longitudinal fasting plasma triglyceride (TG) phenotypes of lipid clinic patients heterozygous for rare, pathogenic/loss-of-function (LOF) variants in the *LPL* and *APOA5* genes, which encode the key plasma TG metabolism proteins lipoprotein lipase (LPL) and apolipoprotein A-V (apo A-V), respectively. I additionally conducted preliminary analyses investigating the extent of the association of a common SNP in *APOA5*, that is sometimes erroneously reported as causative for hypertriglyceridemia (HTG), with HTG. The analyses were conducted by analyzing the fasting plasma TG data from the charts of patients treated at the Lipid Genetics Clinic, London, Ontario, Canada. Genetic analyses of these patients was made possible by utilizing a next-generation sequencing panel, LipidSeq, which targets genes identified in research as being causal for and/or associated with dyslipidemias, including HTG, and other metabolic disorders [1].

4.2 Summary of research findings

Prior to my work, the TG phenotype associated with and produced, at least in part, by heterozygosity for rare pathogenic variants in *LPL* and *APOA5* (i.e., heterozygous LPL and apo A-V deficiency) was not well defined, especially regarding the longitudinal behaviour over many years. For *LPL*, this was primarily because most studies investigating the TG phenotype associated with heterozygosity for pathogenic variants in the gene were conducted prior to the turn of the century, resulting in them being less familiar to researchers and clinicians in the modern age (see section 2.4 for a discussion of the findings of these past studies) [2–6]. And for *APOA5*, most descriptions of the TG phenotype produced by heterozygosity for pathogenic variants in the gene came from cross-sectional studies of families ascertained via a proband who was homozygous for a pathogenic variant in the gene, leading to a lack of research dedicated to studying the heterozygous phenotype specifically [7–9]. Additionally, there is a paucity of longitudinal TG data available for patients with these genotypes. Consequently, there is less familiarity with the TG phenotype associated with heterozygosity for pathogenic variants in both genes than there is with the familial chylomicronemia syndrome (FCS)

phenotype produced by biallelic pathogenic variants in these genes. This has led to assumptions regarding the TG phenotype associated with heterozygosity for pathogenic variants in these genes.

With regards to heterozygosity for pathogenic variants in *LPL*, there are two primary assumptions made by some physicians. First, some physicians assume that heterozygous LPL deficiency can only produce stable HTG with severity intermediate between normal (fasting plasma TG <2 mmol/L) and severe HTG (fasting plasma TG >10 mmol/L). This likely arises from some analogizing heterozygous LPL deficiency to autosomal dominant familial hypercholesterolemia (FH), in which untreated heterozygotes display a fully expressed, stable phenotype that is half as severe as found in homozygotes [10]. Second, some physicians when observing heterozygosity for pathogenic variants in *LPL* in severe HTG patients assume that this indicates the existence of "autosomal dominant HTG". My work investigating and addressing these assumptions along with characterizing the longitudinal TG phenotype of patients heterozygous for pathogenic variants in *LPL* is found fully detailed in Chapter 2 and summarized in section 4.2.1 below.

With respect to heterozygosity for pathogenic variants in *APOA5*, while severe HTG has been observed previously in heterozygotes, it has been often assumed that the development of severe HTG in this group is largely dependent on the co-occurrence of common TG-elevating *APOA5* polymorphisms. However, given the overrepresentation of these common SNPs in normolipidemic populations [11], this explanation seems unlikely. My work addressing these assumptions along with characterizing the longitudinal TG phenotype of patients heterozygous for pathogenic variants in *APOA5* is found fully detailed in Chapter 3 and summarized in sections 4.2.2 below. A preliminary investigation into the association of a common *APOA5* SNP associated with elevated plasma TG, p.Ser19Trp (which is one of the polymorphisms often implicated in causing severe HTG in *APOA5* pathogenic variant heterozygotes), with HTG was also conducted to further investigate the true role and impact of this common SNP with respect to HTG severity. This investigation is also detailed in Chapter 3 and the results are summarized in section 4.2.3 below.

The main findings of my work in the context of knowledge prior to my work are summarized in **Figure 4.1**.

Before my work

1. It is sometimes assumed that heterozygosity for rare pathogenic variants in *LPL* produces only stable, mild-to-moderate HTG.
2. Severe HTG in patients heterozygous for rare pathogenic variants in *APOA5* occurs only in the presence of other common *APOA5* SNPs associated with elevated plasma TG.
3. The common *APOA5* SNP, p.Ser19Trp, has been implicated in some clinical genetic testing reports and some research to be as deleterious as true rare pathogenic variants.

After my work

1. Heterozygosity for rare pathogenic variants in *LPL* is associated with highly variable TG phenotype ranging from normal TG to severe HTG both between patients and overtime within the same patient.
2. Heterozygosity for rare pathogenic variants in *APOA5* is also associated with a similar phenotype to that described in 1. Additionally, severe HTG was observed in this group even in patients with minimal polygenic influences/lack of common *APOA5* SNPs associated with elevated plasma TG.
3. Heterozygotes and homozygotes of the *APOA5* p.Ser19Trp SNP in aggregate demonstrate extremely similar TG phenotypes, with no significant differences observed between these two groups ($p = 0.625$).

Figure 4.1. Summary of research findings.

State of knowledge in the field prior to my research work is summarized in the "Before my work" box. The updated knowledge of these areas after my research work are summarized in the "After my work" box.

4.2.1 Longitudinal triglyceride phenotype in patients heterozygous for pathogenic variants in *LPL*

My research described in Chapter 2 details the longitudinal behavior of plasma TGs in a previously underappreciated population, heterozygous carriers of pathogenic variants in *LPL*, and clarifies and corrects several assumptions regarding the TG phenotype of these individuals, ultimately providing a clearer understanding of the underlying disease risk in this population [12].

Demographic and longitudinal fasting plasma TG data were collected via chart review of 15 medically stable patients from the Lipid Genetics Clinic (London, Ontario, Canada) identified as heterozygous carriers of rare pathogenic variants in *LPL*, with no pathogenic variants located in other major TG metabolism genes. Genetic screening was performed using LipidSeq next-generation sequencing panel [1]. Demographic information are detailed in **Table 2.2** (located in Chapter 2, section 2.3.2). Within our cohort, 8 unique pathogenic variants in *LPL* were observed, one of which was novel (see Chapter 2, **Table 2.1** and **Figure 2.2** for more details on variants).

Patients were followed for an average of 10.3 ± 8.5 years (ranging from 1.5 to 30.3 years) over which patients had an average of 13 ± 8 TG measurements performed. Marked variability in TG levels was observed between patients at baseline, with 13.3% (2/15), 46.7% (7/15), and 40.0% (6/15) patients demonstrating normal fasting plasma TG levels, mild-to-moderate HTG, and severe HTG, respectively. Highly variable within-patient and between-patient variability of TG levels was observed over time. Of the total of 203 total TG measurements taken amongst our cohort over the follow-up duration, 14.8% (30/203), 67.0% (136/203), and 18.2% (37/203) were in the normal TG, mild-to-moderate HTG, and severe HTG ranges, respectively. No patients demonstrated only severe HTG over the course of follow-up, but most patients demonstrated variable TG with no clear pattern to longitudinal variation (i.e., no consistent increase or decrease over time nor any cyclic behaviour nor any variant specific behaviour): 6.7% (1/15) displayed only normal TG levels, 33.3% (5/15) only demonstrated mild-to-moderate HTG, 6.7% (1/15) varied between normal and mild-to-moderate HTG, 40.0% (6/15)

varied between mild-to-moderate and severe HTG, and 13.3% (2/15) varied between normal TG levels, mild-to-moderate HTG, and severe HTG.

Because polygenic risk allele accumulation has been previously shown by our lab to be a significant cause of both mild-to-moderate and severe HTG in the population [13,14], we also evaluated the potential role of polygenic influences as contributors to the highly variable TG phenotypes observed using polygenic risk score for elevated TG previously developed by our lab [13]. Of the 15 patients studied, only 2 patients demonstrated clinically significant (>90th percentile) polygenic risk factor accumulation, indicating that polygenic influences were not a major contributor to the TG phenotypes we observed in our cohort.

These findings clearly demonstrate that previous assumptions regarding the TG phenotype of *LPL* pathogenic variant heterozygotes are incorrect. Firstly, as clearly demonstrated by 53.3% (8/15) patients in our study, severe HTG can indeed occur with this genotype thus demonstrating that analogy of heterozygous LPL deficiency with FH is not appropriate. Secondly, the assumption of "autosomal dominant HTG" being produced by this genotype is clearly not true, as demonstrated by the existence of a patient in our study who possessed completely normal TG over the entire course of their follow-up. This is supported by the observation that there is no co-segregation of heterozygosity for rare pathogenic *LPL* variants and HTG in multi-generational families [3–6,15], the large number of normolipidemic carriers demonstrating heterozygosity for rare pathogenic *LPL* variants in population studies [13,14,16], and the dependence of the expression of HTG phenotype on non-genetic secondary factors, such as age, obesity, and diabetes [4–6,15].

In summary, the longitudinal TG phenotype of heterozygous carriers of pathogenic variants in *LPL* is highly variable, both between patients and within a patient at both baseline and over time. Additionally, previously held assumptions regarding the nature of the TG phenotype produced by heterozygosity for rare pathogenic variants in *LPL* are incorrect. TG are a naturally more temporally variable phenotype than cholesterol [17], with more types of genetic determinants, including polygenic risk factors, and secondary, non-genetic risk factors. Thus, the susceptibility of patients with heterozygous rare

pathogenic variants in *LPL* to HTG follows a probabilistic model, and not a genetically deterministic model as was previously assumed [16].

4.2.2 Longitudinal triglyceride phenotype in patients heterozygous for pathogenic variants in *APOA5*

My research described in Chapter 3 (specifically Study 1 of this chapter) details the longitudinal behavior of plasma TGs in heterozygous carriers of pathogenic variants in *APOA5* and clarifies and corrects several assumptions regarding the TG phenotype of these individuals, ultimately providing a clearer understanding of the underlying disease risk in this population [18]. Additionally, to the best of my knowledge, this may be one of the first, if not the first, dedicated investigations of the longitudinal TG phenotype in this group to date.

Demographic and longitudinal fasting plasma TG data were collected via chart review of 7 medically stable patients from the Lipid Genetics Clinic (London, Ontario, Canada) identified as heterozygous carriers of rare pathogenic variants in *APOA5*, with no pathogenic variants located in other major TG metabolism genes. Genetic screening was performed using LipidSeq next-generation sequencing panel [1]. Demographic information are detailed in **Table 3.1** (located in Chapter 3, section 3.3.1.2). Within our cohort, 4 unique pathogenic variants in *APOA5* were observed, one of which was novel (see Chapter 3, **Table 3.2** and **Figure 3.2** for more details on variants).

Patients were followed for an average of 5.3 ± 3.7 years (range of 0.5 to 11.8 years) over which patients had an average of 7.1 ± 2.6 TG measurements performed. Marked variability in TG levels was observed between patients at baseline, with 14.3% (1/7), 57.1% (4/7), and 28.6% (2/7) patients demonstrating normal fasting plasma TG levels, mild-to-moderate HTG, and severe HTG, respectively. Highly variable within-patient and between-patient variability of TG levels was observed over time. Of the 50 total TG measurements taken amongst our cohort over the follow-up duration, 10.0% (5/50), 54.0% (27/50), and 36.0% (18/50) were in the normal TG, mild-to-moderate HTG, and severe HTG ranges, respectively. No patients demonstrated only severe HTG over the course of follow-up, but most patients demonstrated variable TG with no clear pattern to

longitudinal variation (i.e., no consistent increase or decrease over time nor any cyclic behaviour nor any variant specific behaviour): 28.6% (2/7) varied between normal and mild-to-moderate HTG, 57.1% (4/7) varied between mild-to-moderate and severe HTG, and 14.3% (1/7) varied between normal TG levels, mild-to-moderate HTG, and severe HTG.

As polygenic risk allele accumulation has been previously shown by our lab to be significant cause of both mild-to-moderate and severe HTG in the population [13,14], we also evaluated the potential role of polygenic influences as contributors to the highly variable TG phenotypes observed using polygenic risk score for elevated TG previously developed by our lab [13]. Of the 7 patients studied, only 2 patients demonstrated clinically significant (>90th percentile) polygenic risk factor accumulation, indicating that polygenic influences were not a major contributor to the TG phenotypes we observed in our cohort. However, sequencing data necessary to construct the risk score for patient 7 was not available.

These findings clearly demonstrate that previous assumptions regarding the TG phenotype of *APOA5* pathogenic variant heterozygotes are incorrect. Only 2 of the 7 patients in our cohort demonstrated clinically significant polygenic risk accumulation, while 5 of the 7 patients displayed severe HTG at least once, indicating the polygenic risk accumulation is not necessary for the development of severe HTG in this group.

In summary, the longitudinal TG phenotype of heterozygous carriers of pathogenic variants in *APOA5* is highly variable, both between patients and within a patient at both baseline and over time. Additionally, previously held assumptions regarding the nature of the TG phenotype produced by heterozygosity for rare pathogenic variants in *APOA5* are incorrect. Additionally, these data also demonstrate that heterozygous apo A-V deficiency does not produce “autosomal dominant HTG” for similar reasons as were observed in the previously described study in Chapter 2 and section 4.2.1. Additionally, like the heterozygous carriers of rare pathogenic *LPL* variants, secondary factors appear to be the major contributors to the development of extreme TG profiles in *APOA5* pathogenic variant heterozygotes. Thus, the susceptibility of patients with heterozygous

rare pathogenic variants in *APOA5* to HTG follows a probabilistic model, and not a genetically deterministic model as was previously assumed [16].

4.2.3 The common *APOA5* p.Ser19Trp SNP is not consistently associated with hypertriglyceridemia

Also detailed in Chapter 3 (specifically in reference to Study 2 in this chapter) is a preliminary investigation of the association of the common single nucleotide polymorphism (SNP) of *APOA5*, p.Ser19Trp, with HTG. This SNP is among several *APOA5* SNPs commonly cited as major contributors to the development of severe HTG in *APOA5* pathogenic variant heterozygotes, like those described in Study 1 of Chapter 3 (findings from this study are summarized in Section 4.2.2). Additionally, this SNP is sometimes reported as being causal for FCS on some clinical genetic reports from next-generation sequencing analyses. However, the extent of deleterious impact of this SNP is questionable considering its extremely high prevalence in normolipidemic populations of 5-15% [11]. The clinic population of the Lipid Genetics Clinic (London, Ontario, Canada) provides a unique opportunity to examine the association of both homozygosity and heterozygosity of this common SNP with HTG in a large cohort.

Historically highest fasting plasma TG (mmol/L) measurement data for 402 patients from the Lipid Genetics Clinic (London, Ontario, Canada) was collected. 85.8% (345/402) patients were identified as heterozygous for the common *APOA5* p.Ser19Trp SNP, 5.2% (21/402) were identified as homozygous, and 8.9% (36/402) were normolipidemic controls lacking the SNP. Marked variability in the TG phenotype severity was observed in both the homozygous and heterozygous groups. Within the homozygous group, 14.3% (3/21) patients demonstrated historically highest TG in the normal range, 52.4% (11/21) in the mild-to-moderate HTG range, and 33.3% (7/21) in the severe HTG range. Within the heterozygous group, 22.3% (77/345) patients demonstrated historically highest TG in the normal range, 50.4% (174/345) in the mild-to-moderate HTG range, and 27.2% (94/345) in the severe HTG range. For comparison, the normal control group had 97.2% (35/36) patients whose historically highest TG was in the normal TG range, 2.8% (1/36) in the mild-to-moderate HTG range, and none in the severe HTG range. A one-way Welch's ANOVA comparing the mean historically highest TG (mmol/L) of the three

study groups indicated that there was a statistically significant difference in the means between at least two groups ($F(2, 50.577) = 90.225$, $p < 2.2 \times 10\text{-}16$). A post-hoc Games-Howell test showed that the mean historically highest TG (mmol/L) was significantly different between p.Ser19Trp homozygotes and normal controls ($p = 0.002$, 95% C.I = [-16.168,-3.432]) and significantly different between p.Ser19Trp heterozygotes and normal controls ($p < 0.001$, 95% C.I = [-8.731,-6.048]). There was no statistically significant difference in mean historically highest TG (mmol/L) between p.Ser19Trp homozygotes and heterozygotes ($p = 0.625$).

These preliminary results indicate that *APOA5* p.Ser19Trp zygosity is not a significant determinant of fasting plasma TG severity, at least in isolation. This confirms also that treating this SNP as a causal variant for HTG and/or FCS is not appropriate as homozygosity for this variant is not consistently associated with production of HTG, yet alone severe HTG.

4.2.4 Genetic variation in *APOA5*

Chapter 3, section 3.4.3, details the work done to produce the most up-to-date review of genetic variation in *APOA5* to date [19]. Interestingly, in compiling, analyzing, and comparing all variants of *APOA5* reported in literature and various genetic variation databases as being as being associated with various pathologies, we found that premature truncation is the only well characterized mode by which apo A-V dysfunction may occur. However, of the 118 variants in *APOA5* reported in the literature and various genetic variation databases as associated with disease, only 39.8% (47/118) are premature truncation variants. Missense variants are the most common form of rare variant reported in *APOA5*, making up 51.7% (61/118) of all reported *APOA5* variants. However, only a single *APOA5* missense variant, p.Leu253Pro, has been established as pathogenic and causal for severe HTG [20]. Thus, we have identified that the molecular defect produced by some missense variants in *APOA5* are poorly understood and likely form a previously underappreciated mode by which apo A-V dysfunction may occur.

4.3 Research strengths, limitations, and caveats

4.3.1 Strengths

A major strength of my investigations into the longitudinal TG phenotypes of *LPL* and *APOA5* pathogenic variant heterozygotes (findings summarized in sections 4.2.1 and 4.2.2, respectively) was my access to over 25 years of patient data from the Lipid Genetics Clinic (London, Ontario, Canada) patient population. Due to nature of the cases seen at the Lipid Genetics Clinic, I had access to a unique patient population that is enriched for individuals with extreme dyslipidemia phenotypes compared to the general population. For reference, the prevalence of severe HTG in the general population is ~1 in 400 people [21] but due to the unique nature of the cases attending the Lipid Genetics Clinic, I was able to study multiple individuals with severe HTG in all 3 of my investigations. Specifically, I observed 8 individuals in my study of *LPL* heterozygotes (findings summarized in section 4.2.1) [12], 5 in my investigation of *APOA5* heterozygotes (findings summarized in section 4.2.2) [18], and a total of 101 individuals across the homozygous and heterozygous groups in my *APOA5* p.Ser19Trp study who displayed severe HTG (findings summarized in section 4.2.3). Due to the clinic population being enriched with individuals with extreme lipid profiles, it increased the likelihood of observing patients with the genotypes I wanted to study. In combination with the long-standing nature of the Lipid Genetics Clinic, this allowed me to conduct unique longitudinal investigations into the progression of the TG phenotype of patients heterozygous for pathogenic variants in *LPL* and *APOA5* in a clinical setting.

Another major strength of my research was the use of the LipidSeq next-generation sequencing panel for the genetic analysis of all patients examined at the Lipid Genetics Clinic (London, Ontario, Canada). LipidSeq was designed by our lab specifically for investigating the genetic basis of the dyslipidemias observed in patients referred to the Lipid Genetics Clinic (London, Ontario, Canada) [1,22]. Specifically relevant to my research was that LipidSeq allows for simultaneous assessment of rare variants and common SNPs across multiple genomic loci [1,22].

4.3.2 Limitations

The primary limitation of my longitudinal TG phenotype investigations (findings summarized in sections 4.2.1 and 4.2.2) is the relatively small sample size of both investigations. My investigation of patients heterozygous for pathogenic *LPL* variants had a final cohort of 15 patients, consisting of 12 males and 3 females [12]. My investigation of patients heterozygous for pathogenic *APOA5* variants had a final cohort of 7 patients, of which 3 were male and 4 were female [18]. Additionally, it can be argued that both longitudinal investigations have sampling bias. The Lipid Genetics Clinic (London, Ontario, Canada) tends to mainly see and treat patients referred for management of extreme lipid profiles. It was previously established by our lab that ~3-4% of the normolipidemic population are heterozygous for rare pathogenic variants in the FCS genes [13]. Thus, while the Lipid Genetics Clinic (London, Ontario, Canada) patient population provides a unique opportunity by increasing likelihood for observing dyslipidemia cases with genetic basis due to it being enriched for individuals with extreme lipid profile, it can be argued that, from a generalizability standpoint to the general population, both of my longitudinal investigations suffer from sampling bias.

Another limitation of my research was the lack of consistent longitudinal data for several clinical and demographic variables such as BMI, activity level, diet, stress, lifestyle, etc. Secondary environmental factors have been previously indicated by multiple studies to be important determinants of HTG development and severity in heterozygotes for pathogenic variants in *LPL* and *APOA5*, which I summarized in the discussion sections of Chapters 2 and 3 and in the published versions of these investigations [12,18,19]. If these data were available, I may have been able to conduct more robust statistical analyses regarding the longitudinal TG phenotypes observed in my study cohorts, which may have revealed new information regarding gene-environment interactions at play in patients under study.

An additional limitation of my work, which may be argued to have sampling bias and limited generalizability to the general population, is that the majority of patients examined in both of my longitudinal investigations were of European ancestry [12,18],

with only 2 patients in the study of *LPL* pathogenic variant heterozygotes being of non-European ancestry [12]. This is arguably the most significant limitation of my research since different population groups can have very different background genetic profiles and therefore, considerably different risk alleles or SNPs for various conditions, including HTG, as well as considerably different frequencies of various SNPs that may be common in one population but rare in another. This is demonstrated well by *APOA5* as there is considerable population differences in the common SNPs observed among different population groups. For example, the *APOA5* p.Ser19Trp SNP that I studied is primarily a SNP found in Caucasian populations while another common *APOA5* SNP, p.Gly185Cys, is found primarily in Asian populations [19,23–27].

Finally, I was unable to examine the longitudinal TG phenotypes of patients heterozygous for pathogenic variants in the other FCS genes, namely in *APOC2*, *GPIHBP1*, and *LMF1*, due to a lack of data and eligible patients within the Lipid Genetics Clinic patient population. Given the complexity of plasma TG metabolism and its regulation, it is not possible to determine with any confidence what the longitudinal TG phenotype associated with heterozygosity for pathogenic variants in these genes would be from the data I was able to collect in my longitudinal investigations. It is possible that the longitudinal TG phenotypes associated with heterozygosity for pathogenic variants in these genes would be unique.

4.3.3 Caveats

There is one major caveat that should be considered when interpreting the findings I have presented. This primarily has to do with the fact that missense variants in *APOA5*, apart from the p.Leu253Pro variant which has been demonstrated to be pathogenic [20], are not considered pathogenic under any circumstances currently. This is because there is a severe lack of any functional investigations into these variants of *APOA5* despite many of them being reported as associated with various disease states, which I summarized in my review of genetic variation in *APOA5* [19], which is also discussed in Chapter 3, section 3.4.3. Many of the patients that were excluded from analysis in my longitudinal investigation of patients heterozygous for pathogenic *APOA5* variants were carriers of

these missense variants. Consequently, it is possible that the TG phenotype produced by heterozygosity for a pathogenic missense variant is unique from that produced by heterozygosity for a premature truncating variant in *APOA5*. This is because it has been noted previously that prematurely truncated apo A-V may exert a dominant-negative effect [8]. The exact molecular mechanism by which truncated apo A-V exerts this dominant-negative effect is unknown and requires further investigation. Thus, it is possible that while missense and truncating variants in *APOA5* can both produce non-functional protein, the mechanisms by which these mutant proteins are dysfunctional may be distinct and may result in different TG phenotype behaviours.

Another major caveat is regarding the preliminary study I conducted investigating the association of *APOA5* p.Ser19Trp zygosity with HTG. This was a preliminary analysis, and as I noted in discussing it in Chapter 3, section 3.4.2, further filtering of the patients included is possible to remove those with rare pathogenic variants in FCS genes (which are a minority of patients in that cohort) and remove those with extreme polygenic risk factor accumulation. Doing so would allow for a more isolated investigation of the *APOA5* p.Ser19Trp allele and its association with HTG. However, given the size of the cohort, it is unlikely that this would majorly impact the findings of that preliminary investigation.

4.4 Applications and future directions

My research has identified a novel genotype-phenotype relationship regarding regulation of plasma TG levels. I have characterized and identified unique features of this genotype-phenotype relationship which may be utilized to develop and/or inform better clinical monitoring and treatment practices for this patient group.

4.4.1 Investigating the longitudinal TG phenotype associated with heterozygosity for pathogenic variants in *APOC2*, *GPIHBP1*, and *LMF1*

Future studies should examine the longitudinal TG phenotype associated with heterozygosity for pathogenic variants in the remaining FCS genes that I could not study due to lack of eligible patients. Interestingly, heterozygosity for pathogenic variants in

APOC2 leading to heterozygous apo C-II deficiency are inconsistently associated with the occurrence of HTG [28–30]. This is broadly similar to what I observed in my investigations into the longitudinal TG phenotypes associated with heterozygosity for pathogenic variants in *LPL* and *APOA5* [12,18]. Heterozygosity for pathogenic variants in *LMF1* have been observed to produce severe HTG [31,32] but interestingly, these are the first two reports of heterozygosity for a nonsense variant in *LMF1* producing severe HTG. According to Chen et al., all reports of heterozygosity for nonsense variants in *LMF1* prior to these reported normal TG or at most borderline mild-to-moderate HTG [31]. According to Chen et al., it seems secondary factors are required to precipitate severe HTG in heterozygotes for nonsense variants in *LMF1* [31]. Specifically, Chen et al. observed severe HTG in a heterozygote for *LMF1* nonsense variant who also had severe obesity and heavy tobacco use, both factors being known to be associated with elevated plasma TG [31], while the severe HTG observed in a heterozygote for *LMF1* nonsense variant by Dancer et al. was in an 8-month old infant who also presented with acute gastroenteritis, a condition which is known to produce mild-to-moderate HTG on its own [32]. Finally, rather interestingly, heterozygosity for *GPIHBP1* knockout in mice is not associated with HTG and heterozygous *GPIHBP1* knockout mice were normolipidemic [33,34]. However, to the best of my knowledge, this has not been investigated in humans yet. It is possible that it is different in humans, but it is equally likely that instead, in the heterozygous knockout genotype in both humans and mice, the healthy allele is sufficient to maintain the normal phenotype, indicating that haploinsufficiency, at least in mice, is not a mechanism of diseases with regards to *GPIHBP1* variants.

Therefore, future studies should examine the longitudinal TG phenotypes associated with heterozygosity for pathogenic variants in the remaining FCS genes, especially in *APOC2* and *LMF1*, for which the occurrence of HTG has been previously observed. I would also be interested in a thorough functional investigation of heterozygous *GPIHBP1* knockout/loss-of-function in humans. The existence of circulating catalytically active dimeric form of LPL [35] as well as the expression of LPL without association with GPIHBP1 [34,36,37] may at least partially explain why heterozygous knockout of

GPIHBP1 is not sufficient to induce HTG as the healthy allele likely produces enough GPIHBP1 to maintain the normal phenotype.

4.4.2 Investigating the specific biochemistry and molecular mechanism underlying the dominant-negative effect of truncated apo A-V protein on healthy protein

As discussed in Chapter 3, section 3.4.3, it has been previously reported that a prematurely truncated apo A-V protein, encoded by the nonsense variant of *APOA5*, p.Gln139Term, exerts a dominant-negative effect on healthy apo A-V protein, interfering with its ability to interact with forming lipoprotein particles in the liver [8]. Unfortunately, since the initial report of this phenomenon in 2005, to the best of our knowledge, this mechanism has not been further investigated. Consequently, the molecular mechanisms and biochemistry underlying this effect remain unknown. This begs the question of whether all premature truncating variants in *APOA5* produce truncated peptides exerting this effect. Additionally, is the dominant negative effect a constant, or does its impact vary under varying physiological conditions? All of the variants I observed in my longitudinal TG phenotype investigation of heterozygosity for pathogenic *APOA5* variants were nonsense variants resulting in premature truncation of the encoded peptide. I still observed variable TG phenotypes in these patients, but in aggregate, the proportion of severe HTG measurements observed in the *APOA5* pathogenic variant heterozygotes I studied was greater than the proportion of severe HTG measurements I observed in the aggregate of all longitudinal TG measurements I collected from *LPL* pathogenic variant heterozygotes I also studied. It is possible that the dominant-negative effect of truncated apo A-V is responsible for this difference. Either way though, given that apo A-V deficiency due to biallelic pathogenic variants in *APOA5* produces FCS [16,23], then it is possible, that at least while the dominant-negative effect is active and preventing the association of healthy apo A-V with lipoprotein particles, that heterozygotes for truncating variants in *APOA5* have a plasma lipid profile and phenotype mimicking the FCS phenotype. Thus, further investigations are needed to understand the extent of this dominant-negative effect and the exact molecular mechanisms and biochemical factors needed for this effect to take place.

4.4.3 Investigating the functional defects produced by missense variants in *APOA5*

As discussed in Chapter 3, section 3.4.3, the majority of variants in *APOA5* reported to be associated with disease in both the literature and in various genetic variation databases are missense variants. However, only one missense variant in *APOA5*, p.Leu253Pro, has been established as a pathogenic *APOA5* variant [20]. The Leu253 residue in healthy apo A-V interfaces between two α-helices via hydrogen bonding interactions [20] and it is suspected that the missense variant altering this residue to a proline interferes with this functionality. Given the high α-helical content of mature apo A-V and the functional importance of these structures [38], we postulated that it is likely that missense variants resulting in similar disruptions to α-helix formation and/or α-helix interactions are pathogenic. Given that most variants associated with disease in *APOA5* are missense variants but only one has been established as pathogenic, it is likely that missense variant are an underappreciated causes of apo A-V dysfunction related disease. Therefore, future work focusing on characterization of the structural and functional defects in apo A-V due to missense variants in *APOA5* are of great interest.

4.5 Conclusions

Plasma TG metabolism and its regulation are complex and often this complexity is not fully appreciated by both physicians and researchers alike, as demonstrated by previous assumptions about the TG phenotype of heterozygotes for pathogenic *LPL* variants analogizing it to autosomal dominant familial hypercholesterolemia. My research work detailed in this thesis has clarified some mysteries and corrected some assumptions regarding plasma TG metabolism and its regulation, but it has raised just as many if not more questions. I have assessed impact of heterozygosity for pathogenic *LPL* and *APOA5* variants on longitudinal fasting plasma TG behaviour, demonstrating that these variants are associated with extreme variability in plasma TG levels both at baseline and longitudinally, both within and between patients, ranging from normal to mild-to-moderate HTG to severe HTG (Chapter 2 and Chapter 3, Study 1) [12,18]. I have additionally conducted a preliminary investigation demonstrating that the common *APOA5* p.Ser19Trp SNP, which has been previously implicated and treated as if it were

as deleterious as a rare, truly pathogenic variant is in fact not consistently associated with HTG and homozygosity for the SNP does not produce statistically different TG phenotype compared to heterozygosity for the SNP (Chapter 3, Study 2). Finally, I also conducted and compiled the most up-to-date review of *APOA5* genetic variation to date, highlighting gaps in our understanding of the molecular mechanisms underlying disease produced by variation in the *APOA5* gene (Chapter 3, Section 3.4.3). By establishing a better understanding of the genetic architecture and molecular mechanisms underlying healthy and diseased plasma TG metabolism, we may be able to discover novel targets and/or strategies for tackling HTG and its related co-morbidities, including but not limited to ASCVD and acute pancreatitis.

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Appendices

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Perera SD, Wang J, McIntyre AD, Dron JS, Hegele RA. The longitudinal triglyceride phenotype in heterozygotes with LPL pathogenic variants. *J. Clin. Lipidol.* 17(1), 87–93 (2023).

The longitudinal triglyceride phenotype in heterozygotes with LPL pathogenic variants

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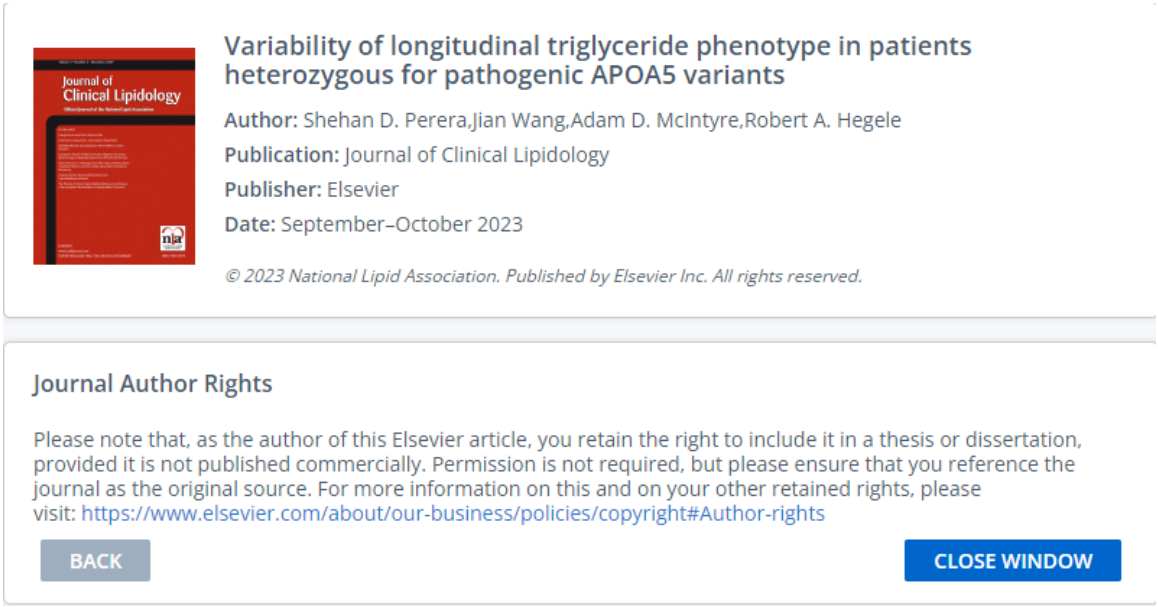
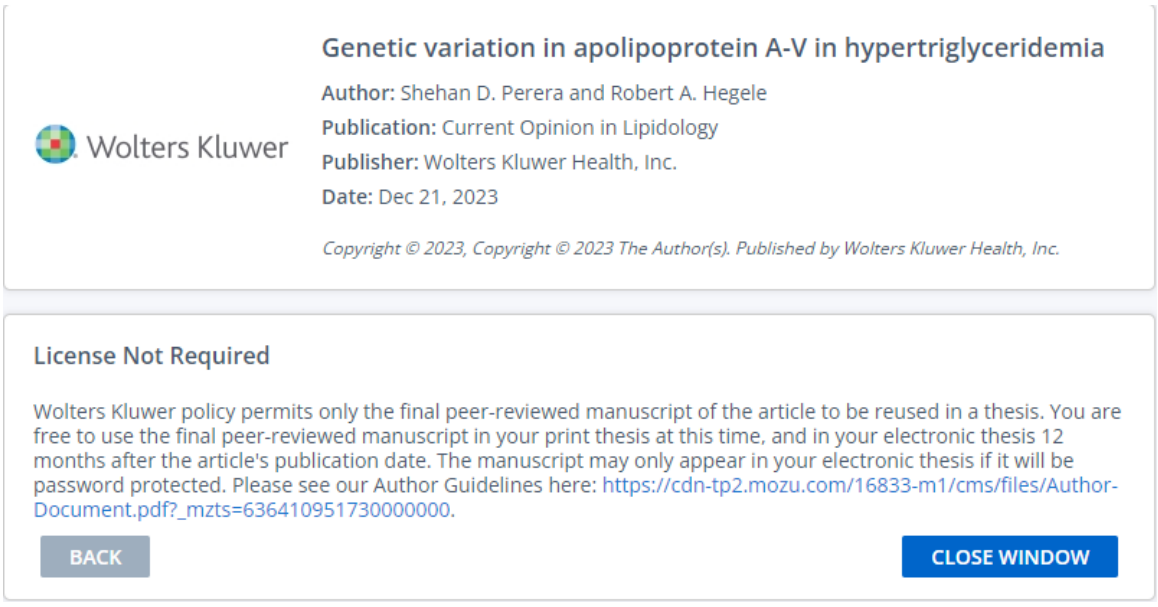
Perera SD, Wang J, McIntyre AD, Hegele RA. Variability of longitudinal triglyceride phenotype in patients heterozygous for pathogenic APOA5 variants. *J. Clin. Lipidol.* 17(5), 659–665 (2023).



Perera SD, Hegele RA. Genetic variation in apolipoprotein A-V in hypertriglyceridemia. *Curr. Opin. Lipidol.* 35(2), 66–77 (2024).



Appendix B. University of Western Ontario - Ethics Approval.

Western Research

Date: 26 September 2023

To: Dr. Robert Hegele

Project ID: 0379

Review Reference: 2023-0379-84294

Study Title: Candidate gene sequencing, genetic and genomic analysis for identification of new genetic determinants of intermediate traits of atherosclerosis, dyslipidemia, diabetes, obesity, hypertension, lipodystrophy and other rare metabolic or cardiovascular disorders in the human population. 07920E

Application Type: HSREB Amendment Form

Review Type: Delegated

Full Board Reporting Date: 10/Oct/2023

Date Approval Issued: 26/Sep/2023 09:57

REB Approval Expiry Date: 03/Nov/2023

Dear Dr. Robert Hegele ,

The Western University Health Sciences Research Ethics Board (HSREB) has reviewed and approved the WREM application form for the amendment, as of the date noted above.

Documents Approved:

Document Name	Document Type	Document Date	Document Version
26 Sep 2023 REB #379 Delegated HSREB Protocol Form Addition of Study Team Member for submission	Protocol	26/Sep/2023	

Documents Acknowledged:

Document Name	Document Type	Document Date	Document Version
21 Sep 2023 Summary Letter of Amendment to REB 379 Revised Study Team Members	Summary of Changes	21/Sep/2023	
26 Sep 2023 REB #379 Delegated HSREB Protocol Form Addition of Study Team Member Tracked Changes	Summary of Changes	26/Sep/2023	

REB members involved in the research project do not participate in the review, discussion or decision.

The Western University HSREB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Electronically signed by:

Nicola Geoghegan-Morphet , Ethics Officer on behalf of Dr. Naveen Poonai, HSREB Chair, 26/Sep/2023 09:57

Reason: I am approving this document.

Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).

Curriculum Vitae

WANNIVIDULEGE SHEHAN DINUKA PERERA

Post-Secondary Education and Degrees

Western University, London, Ontario, Canada

Master of Science (MSc) candidate in Biochemistry (September 2021 – Present)

- Thesis title: Resolving the longitudinal triglyceride phenotype of heterozygous *LPL* and *APOA5* deficiency
- Supervisor: Robert A. Hegele, MD, FRCPC, Cert Endo, FACP

Bachelor of Medical Sciences (BMSc) (September 2017 – April 2021)

- Honours, Western Scholars
- Major in Physiology, Major in Medical Cell Biology

Academic Honours, Awards, and Achievements

- Graduated as "Western Scholar" in BMSc with Honours
- Dean's Honour Roll every year throughout undergraduate degree (2017-2021)

Presentations

- Robarts Research Retreat Poster and Pitch Presentations (2023)
    - Title: Monoallelic loss-of-function variants in *LPL* and *APOA5* predisposes patients to hypervariability of their plasma triglycerides
- London Health Research Day (LHRD) Poster Presentation (2023)
    - Title: Monoallelic loss-of-function variants in *LPL* and *APOA5* predisposes patients to hypervariability of their plasma triglycerides
- Department of Biochemistry Spring Symposium Seminar Presentation (2023)

    - Title: Heterozygosity for Rare Pathogenic Variants in *LPL* and *APOA5* Produces Hypervariability of Plasma Triglycerides
- Robarts Research Retreat Poster Presentation (2022)
    - Title: Variable expressivity of hypertriglyceridemia in heterozygotes for loss-of-function variants in lipoprotein lipase
- London Health Research Day (LHRD) Seminar Presentation (2022)
    - Title: Variable Expressivity of Hypertriglyceridemia in Heterozygotes for LOF variants in *LPL*
- Harold Stewart Lecture and Research Showcase Poster Presentation (2022)
    - Title: Large Triglyceride Differences Among *LPL* Heterozygotes

Related Work Experience

- Teaching Assistant for Laboratory Skills and Research Experiences in Interdisciplinary Medical Sciences (MEDSCIEN 4990E) at Western University, London, Ontario, Canada (2021-2022, 2022-2023)

Publications:

Perera SD, Muleta AD, Vlasschaert C, Hegele RA. Preprint servers in lipidology: current status and future role. *Curr. Opin. Lipidol.* 33(2), 120 (2022).

Perera SD, Wang J, McIntyre AD, Dron JS, Hegele RA. The longitudinal triglyceride phenotype in heterozygotes with LPL pathogenic variants. *J. Clin. Lipidol.* 17(1), 87–93 (2023).

Perera SD, Wang J, McIntyre AD, Hegele RA. Variability of longitudinal triglyceride phenotype in patients heterozygous for pathogenic APOA5 variants. *J. Clin. Lipidol.* 17(5), 659–665 (2023).

Perera SD, Hegele RA. Genetic variation in apolipoprotein A-V in hypertriglyceridemia. *Curr. Opin. Lipidol.* 35(2), 66–77 (2024).