Markers of Non-Fasting Lipids, Remnant Metabolism, and CVD Risk in Youth and Adults

by

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Abstract

Cardiovascular disease (CVD) is a leading cause of morbidity and mortality worldwide. Abnormal levels of circulating lipids (dyslipidemia) is a well-known risk factor for CVD. Traditionally, fasting lipids, inclusive of low-density lipoprotein cholesterol (LDL-C) and total cholesterol (TC), have been used as lipid risk markers for CVD. However, many individuals, especially those with type 2 diabetes and the metabolic syndrome, still suffer from CVD despite normal LDL-C levels. More recently, non-fasting remnant cholesterol has emerged as a novel CVD lipid risk marker. Non-fasting remnant cholesterol has been found to be causally associated with CVD risk and in some cases has a greater relationship to CVD than traditional fasting parameters. Intestinal remnant cholesterol (measured as plasma apolipoprotein (apo) B48) is significantly elevated in adults with CVD, obesity, and other adverse metabolic conditions. Following a high-fat meal, adults with obesity have an exaggerated and prolonged lipid response in TG and apoB48, called postprandial dyslipidemia.

The aim of this thesis research was to explore the role of non-fasting lipids in CVD-risk across the lifespan. To achieve this, we conducted 4 separate studies at various age points and demographics. The aim of the first study was to elucidate the concentration of non-fasting remnant cholesterol in a large Canadian cohort (Alberta's Tomorrow Project (ATP)) and its association with CVD. Secondly, we measured apoB48 remnant lipoproteins in adults with genetic variants that are protected from CVD. Lastly, we determined the role of apoB48 remnant lipoproteins in early metabolic risk in two separate studies.

Firstly, non-fasting remnant cholesterol was calculated in a Canadian cohort (ATP; n=16,251, average age: 62.32 ± 9.69 years) and the relationship between non-fasting remnant

cholesterol and adverse CVD events was determined. It was found that non-fasting remnant cholesterol was significantly elevated in females with prevalence of CVD compared to those without ((0.73 ± 0.36 mmol/L versus 0.83 ± 0.38 mmol/L (12%)) and this was independent of changes in LDL-C. Additionally, we found that increasing quartiles of non-fasting remnant cholesterol positively associated with prevalent and incident CVD case numbers (n=2,936, n=1,169, respectively) whereas LDL-C had no relationship.

Secondly, the postprandial lipid response was compared in adults with proprotein convertase subtilisin kexin 9 (*PCSK9*) -LOF mutations (n=22; 59.0 ± 12.9 years) to those without variants (n=23; 63.2 ± 9.1 years). PCSK9 is a novel CVD risk marker and individuals with *PCSK9*-LOF variants have been shown to be protected against CVD. Individuals with *PCSK9*-LOF had significantly lower fasting TG (-21%, p=0.03) and apoB48 (-26%; p=0.03) compared to non-carrier controls (n=23). LOF variants also had significantly reduced postprandial total-apoB (-17%, p=0.04), apoB48 (-23%, p=0.04), and TG (-18%, p=0.01). The lifelong reductions in postprandial lipids in *PCSK9*-LOF may confer protection from CVD and further validate the use of PCSK9 inhibitors to lower CVD risk.

The third objective in this thesis was to determine to role of apoB48 remnant lipoproteins in early CVD risk. The first part of this objective examined the relationship between fasting apoB48 and cardiometabolic risk factors in 17-year old adolescents from the Western Australian Pregnancy Cohort (Raine) Study (n=1045). Fasting plasma apoB48 was significantly higher in male (15.28 ± 2.95 ug/mL) compared to female (12.45 ± 2.43 ug/mL) adolescents. Additionally, fasting apoB48 was increased by 21% (3.60 ug/mL) in adolescents in a high-risk metabolic cluster and this increase was more pronounced in males (31%, 6.15 ug/mL). Fasting plasma apoB48 was positively associated with fasting plasma TG, total-cholesterol, insulin, leptin, HOMA-IR, and the

anthropometric parameters waist-circumference and skinfold-thickness. Fasting plasma apoB48 was also inversely associated with fasting plasma HDL-C and adiponectin. This study determined that fasting apoB48 remnant lipoprotein concentrations are associated with cardiometabolic risk factors in youth. The second part of objective three aimed to determine the fasting and postprandial lipid response to a high-fat meal in youth with and without obesity. Fasting plasma TG (0.86 ± 0.42 mmol/L versus 1.23 ± 0.54 mmol/L; 30%, $p=0.03$) and apoB48 (8.09 ± 2.70 versus 18.04 ± 7.07; 55%, $p<0.0001$) were significantly elevated obese youth compared to their normal weight peers. Additionally, the postprandial apoB48 (65%, $p<0.0001$), TG (31%, $p=0.005$), and TC (20%, $p=0.002$) response following a high-fat meal was also significantly elevated in youth with obesity. Overall, the studies in this thesis support the hypothesis that intestinal-derived remnant cholesterol plays an important role in CVD-risk at different stages in life. The above evidence provides a rationale to further implement non-fasting lipid screening measures in Canada and to push towards assessing non-fasting lipids in youth with obesity and other adverse metabolic conditions.

Preface

The present thesis dissertation is original work by Jacqueline Krysa. The research projects, of which this thesis is a part, received research ethics approval from the University of Alberta Health Research Ethics Board. The contributions made by the candidate, Jacqueline Krysa, and the co-authors of these studies are described below.

Chapter 3. This chapter presents initial analysis from a larger project (Pro00073641: 'Non-fasting lipids to better predict heart disease using ATP cohort'), which I applied for ethics approval and for data access to Alberta's Tomorrow Project. The work from this project is in collaboration with the Alliance for Canadian Health Outcomes Research in Diabetes (ACHORD) under the supervision of Dr. Dean Eurich and assistance of Ming Ye.

Chapter 4 forms part of a collaboration led by Dr. Teik Ooi at the University of Ottawa, and Dr. Spencer Proctor at the University of Alberta. The study was approved by the Research Ethics Board of the Ottawa Hospital Research Institute approved the study protocol. In Chapter 4, I analysed plasma samples collected from the Ottawa PCSK9 cohort following a high-fat meal challenge for postprandial total apoB, apoB48, triglycerides and total cholesterol. I also performed subsequent statistical analysis on all other biochemical and anthropometric measures taken from this study. I compiled the data from this study and wrote the manuscript. This chapter has been published as a manuscript: Ooi TC, Krysa JA, Abujrad H, Mayne J, Henry K, Cousins M, Raymond A, Favreau C, Taljaard M, Chrétien M, Mbikay M, Proctor S, Vine D. The Effect of PCSK9 LOF Variants on the Postprandial Lipid and ApoB Lipoprotein Response. *J Clin Endocrinol Metab*. 2017; 102(9);3452-3460. doi: 10.1210/jc.2017-00684 (Please see Appendix A). Additionally, I published a review exploring the relationship between nutrition and PCSK9: Krysa JA, Ooi TC, Proctor SD, Vine DF. Metabolic-Dietary modulation of PCSK9: Effects of

Plasma Lipids and CVD risk. *Journal of Nutrition*. 2017; 174(4):473-481. doi: 10.3945/jn.116.235069 (Please see Appendix B).

Chapter 5 is part of an international research collaboration with Dr. Trevor Mori and Dr. Lawrence Beilin at the University of Western Australia and Dr. Spencer Proctor and Dr. Donna Vine at the University of Alberta. The data used to generate Chapter 5 was analyzed as part of a larger, cross-sectional population from the Raine cohort in Western Australia. The study was approved by the Human Ethics Committee at King Edward Memorial Hospital and Princess Margaret Hospital for Children in Perth, Western Australia. Complementary approval was also obtained from the University of Alberta (Pro00001941). Plasma apoB48 was previously analyzed in plasma samples by ELISA. In Chapter 5 I analyzed and interpreted apoB48 data in the Raine cohort at age 17 years old and wrote the corresponding manuscript, which is submitted for publication in the International Journal of Obesity: Krysa JA, Vine DF, Beilin LJ, Burrows S, Huang RC, Mori TA, Proctor SD. The Relationship of Fasting Plasma ApoB48 to Cardiometabolic Risk Factors in Adolescents (Please see Appendix C).

Chapter 6. This chapter is written in manuscript format and is in preparation for publication as: Krysa JA, Vine DF, Jetha M, Ball GD, Proctor SD. "ApoB-Lipoprotein Remnant Dyslipidemia and High-Fat Meal Intolerance is Associated with Increased Cardiometabolic Risk in Youth" and was approved as Pro00001941: 'Childhood lipid risk study'. Recruitment of youth with obesity was facilitated through the Pediatric Centre for Health and Weight in the Edmonton General Hospital through Dr. Geoff Ball and Mr. Marcus O'Neill. I was responsible for writing ethics approval, recruiting, coordinating study participants, and independently organizing and operating study clinic days. I also analyzed plasma for biochemical data, interpreted the data and wrote the manuscript, which is in preparation for publication.

"The Road goes ever on and on

Down from the door where it began.

Now far ahead the Road has gone,

And I must follow, if I can,

Pursuing it with eager feet,

Until it joins some larger way

Where many paths and errands meet.

And whither then? I cannot say"

- J.R.R. Tolkien (The Lord of the Rings: The Fellowship of the Ring)

Dedication

In memory of my grandfather, Dr. James Waring. Thank you for inspiring my scientific curiosity, passion, and pursuit of knowledge.

Acknowledgements

I would like to express my deepest gratitude to Dr. Spencer for accepting me as a graduate trainee and for always providing me with support and mentorship throughout my graduate program. Thank you for all the wonderful research and training opportunities you provided me throughout my PhD.

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My heartfelt gratitude also goes to my loving grandmother, brother, and my best friend Morgan Peacock. Most importantly, I would like to thank my mother for her love and encouragement. I would not be where I am today without your unwavering support throughout my academic and life journey.

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List of Abbreviations

ABCA1	ATP Binding Cassette A1
ACE	Adverse cardiovascular event
AIS	Acute ischemic stroke
ANGPTL	Angiopoietin-like Proteins
ANOVA	Analysis of variance
ATGL	Adipose Triglyceride Lipase
APO	Apolipoprotein
APOBEC1	ApoB-Editing Catalytic Component 1
ASCVD	Atherosclerotic Cardiovascular Disease
ATP	Alberta's Tomorrow Project
CABG	Coronary artery bypass graft
CALIPER	Canadian Laboratory Initiative on Pediatric Reference Intervals
CE	Cholesterol Ester
CETP	Cholesterol Ester Transport Protein
CM	Chylomicron
CCHS	Copenhagen City Heart Study
CHMS	Canadian Health Measures Survey
CGPS	Copenhagen General Population Study
CIHDS	Copenhagen Ischemic Heart Disease Study
CRP	C-Reactive Protein
CT	Computed Tomography
CVD	Cardiovascular Disease
DHA	Docosahexaenoic Acid
DGAT	Diacylglycerol Acyl Transferase
EGF-A	Epidermal Growth Factor-Like Repeat A
EPA	Eicosapentaenoic Acid
ER	Endoplasmic Reticulum
FATP	Fatty Acid Transport Proteins
FFA	Free Fatty Acids
FH	Familial Hypercholesterolemia
FRS	Framingham Risk Score
FTT	Fat Tolerance Test
GLP-1	Glucagon-Like Peptide 1
GOF	Gain of Function
HDL-C	High-Density Lipoprotein Cholesterol
HF	Heart Failure
HMGCR	3-Hydroxy-3-Methyl-Glutaryl-Coeznyme A Reductase
HSL	Hormone Sensitive Lipase
IHD	Ischemic heart disease

IL	Interleukin
IR	Insulin Resistance
LCAT	Lecithin: Cholesterol Acyltransferase
LDL-C	Low-Density Lipoprotein Cholesterol
LDL-R	Low-Density Lipoprotein Receptor
LOF	Loss of Function
LPL	Lipoprotein Lipase
LRP	Lipoprotein Receptor Related Protein
MAG	Monoacylglycerol
MetS	Metabolic Syndrome
MGAT	Monoacylglycerol Acyl Transferase
MI	Myocardial Infarction
MTP	Microsomal Triacylglycerol Transfer Protein
NAFLD	Non-alcoholic fatty liver disease
NPC1L1	Niemann-Pick C1 like 1
Non-HDL-C	Non-High-Density Lipoprotein Cholesterol
OCP	Oral Contraceptive Pills
oxLDL	Oxidized LDL
PA	Physical Activity
PCI	Percutaneous coronary intervention
PCOS	Polycystic ovarian syndrome
PCSK9	Proportein Convertase Subtilisin Kexin 9
PCTV	Prechylomicron Transport Vesicles
PPAR	Peroxisomal Proliferator Activated Receptor
PPL	Postprandial Lipemia
PUFA	Polyunsaturated Fatty Acid
RC	Remnant Cholesterol
RCT	Reverse Cholesterol Transport
SGLT2	Sodium-Glucose Transport Protein 2
SPPARMα	Selective Peroxisome Proliferator Activated Receptor alpha Modulators
SR-B1	Scavenger Receptor Class B Type 1
SREBP	Sterol-Response Element Binding Protein
T2D	Type 2 Diabetes
TAG	Triacylglycerol
TC	Total Cholesterol
TG	Triglyceride
TIA	Transient ischemic attack
TNF	Tumor Necrosis Factor
TRL	Triglyceride-Rich Lipoprotein
VLDL	Very-Low Density Lipoprotein

Chapter 1: Literature Review

1.1 Cardiovascular Disease (CVD) and Pathophysiology

1.1.1 Introduction

Cardiovascular disease (CVD) is one of the leading causes of morbidity and mortality worldwide. In Canada, over 2.4 million individuals are living with CVD, with expected health costs exceeding 7.6 billion dollars[1]. A central pathology of CVD is the formation of atherosclerotic plaques in the major coronary vessels. Atherosclerotic CVD (ASCVD) is defined as pathological conditions that originate from atherosclerosis and include: coronary heart disease, cerebrovascular disease and peripheral artery disease[2]. Important research considerations for CVD include risk prediction and reduction strategies that would decrease the eventual burden of disease. In the following introduction, several topics in the scope of CVD will be covered including: the etiology of atherosclerosis, modifiable and non-modifiable CVD risk factors, diagnostic lipid targets of CVD, and potential CVD therapies. The overall goal of this chapter is to develop a broader understanding of the complexities of CVD, particularly the early pathogenesis and development of novel lipid therapeutic targets that can improve health outcomes.

1.1.2 Etiology of Atherosclerosis

Atherosclerosis is defined by the progressive thickening of the arterial intima. The atherosclerotic plaque is comprised of immune cells, lipids, as well as vascular endothelial and smooth muscle cells. The formation of the atherosclerotic plaque begins with the appearance of a fatty streak. Fatty streaks can progress to form an atheroma, which contains foam cells, and lipid droplets and is capped by smooth muscle cells and collagen. Growth of the atheroma overtime may restrict or prevent blood flow through major arteries of the body promoting adverse cardiovascular events (ACE) such as a myocardial infarction (MI) or heart attack[3].

1.1.2.1 The Traditional Hypothesis of Atherosclerosis

An early determinant of atherosclerosis is endothelial dysfunction, which is characterized by an increased expression of vascular adhesion molecules that promote the adhesion of inflammatory cells to the arterial wall. Part of the resolution process includes the stimulation of vascular macrophages to engulf excess cholesterol in attempt to stabilize the pro-inflammatory

processes. Unregulated uptake of cholesterol by macrophages creates a lipotoxic cascade resulting in a microscopic "foaming" appearance of the macrophage[4]. According to the traditional hypothesis of atherosclerosis: cholesterol derived from circulating low-density lipoprotein cholesterol (LDL-C) particles undergoes oxidation (oxLDL) and is subsequently engulfed by macrophages that activate endothelial and smooth muscle cells, which release additional inflammatory mediators and adhesion molecules[5]. Lipid loaded macrophages or 'foam cells' can then exacerbate local vascular damage by secreting proinflammatory and prothrombogenic factors enhancing the atherosclerotic process[5]. Ultimately, this results in the blockage of central coronary vessels leading to ischemic events or a rupture of the plaque resulting in a thrombus (Figure 1-1).

1.1.2.2 Sources of Plaque: Cholesterol and Remnant Lipoproteins

Remnant lipoproteins of hepatic and intestinal origin (apolipoprotein B48 (apoB-48) chylomicron remnants (CM-r) and very-low density lipoprotein remnants (VLDL-r)) are small, cholesterol dense particles that have been shown to penetrate the arterial wall through similar mechanisms as LDL-C[6,7]. Herein, remnant cholesterol particles can also be engulfed by macrophages and result in foam cell formation and inflammation, thereby promoting atherosclerosis. Unlike LDL-C, remnant particles do not require oxidative modification to be taken up by macrophages[8]. Additionally, remnants contain significantly more cholesterol per particle than LDL-C and therefore may contribute more cholesterol per particle to the vessel wall than LDL-C and therefore, may be a more important contributor to atherosclerosis[9]. In the following introduction, remnant lipoproteins and their role in CVD progression, and risk prediction will be discussed in greater detail.

a.)

b.)

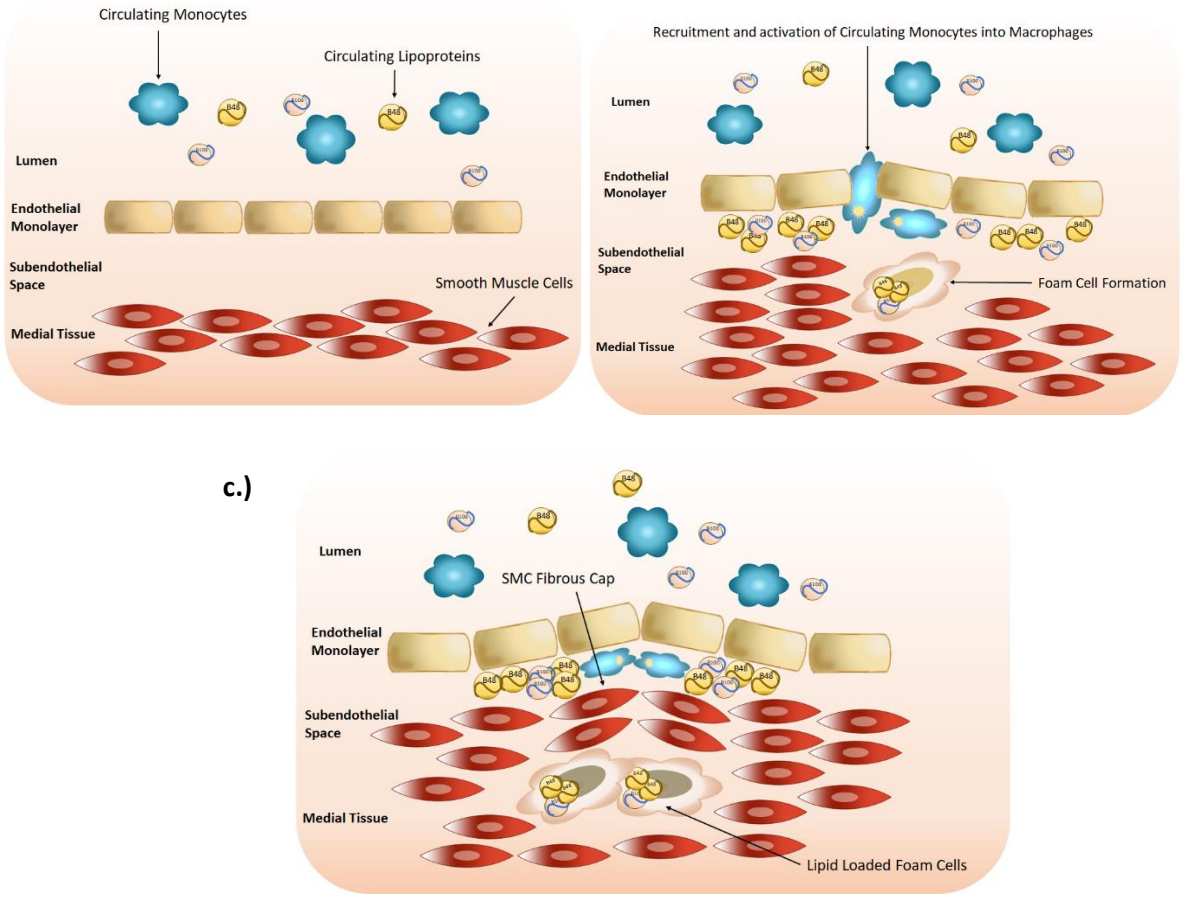

Figure 1-1: *The pathophysiology of atherosclerosis.* (a) Normal Artery, (b) Fatty Streak Formation, (c) Mature Lesion. Figure by Jacqueline Krysa©

1.1.3 CVD Risk Factors

Lifestyle, genetic, and disease risk factors enhance CVD progression. The major non-modifiable risk factors for CVD include: age, genetic makeup, and type 1 diabetes. The primary modifiable-risk factors that enhance CVD risk include: tobacco and alcohol use, physical inactivity, obesity, malnutrition, high blood pressure, use of oral contraception, stress, as well as high plasma lipids and glucose[10].

1.1.3.1 Genetic, Lifestyle Factors, Obesity, and CVD

1.1.3.1.1 Genetic Makeup

The genetic makeup of an individual plays a vital role in determining their risk of developing chronic disease. Importantly, many CVD risk factors are influenced by genetics and therefore, variants in these genes result in elevated risk. Common genetic mutations that influence CVD risk include those that significantly increase plasma lipid levels. Notably, familial hypercholesterolemia (FH) is a genetic disorder that enhances blood cholesterol due to mutations in important genes involved in lipid metabolism. Individuals with FH may have up to a 13-fold increase risk of developing CVD compared to those without FH[11]. Other examples include mutations in the genes coding for apolipoproteins (such as apolipoproteins B and E), lipid metabolism enzymes, receptor proteins, as well as novel emerging genes such as proprotein-convertase subtilisin kexin type 9 (*PCSK9*), which will be discussed later in further detail[12].

1.1.3.1.2 Physical Inactivity

Several studies have found a significant inverse relationship between physical activity (PA) and CVD risk. Importantly, low PA is often associated with overweight and obesity and in youth, seems to underpin the progression of poor nutrition choices and early obesity[13,14]. There are several potential mechanisms by which PA may alter CVD risk: PA has been shown to be important in regulating blood coagulation and fibrinolysis, which are vital for blood clot formation and breakdown within blood vessels, respectively[15]. It has also been demonstrated that PA influences vascular remodelling. Vascular remodelling including angiogenesis, vasculogenesis, and arteriogenesis. These promote the growth of new capillaries from existing ones and enhances blood flood through cardiovascular tissues, which may reduce the incidence of ischemic events[16]. Importantly, PA also influences blood pressure and plasma lipids. Several studies have demonstrated that regular exercise significantly lowers blood pressure and plasma lipids thereby reducing hypertension, dyslipidemia, and overall CVD risk[10]. In addition to the information above, regular PA is often associated with healthier dietary choices.

1.1.3.1.3 Diet

Adequate nutrition is paramount to survival and proper metabolic functioning. The maintenance of nutrient balance is necessary to maintain physiological homeostasis and prevent communicable and non-communicable disease. There is a plethora of evidence to demonstrate a link between diet and CVD risk. Diets rich in fruit, vegetables, whole grains, fibre, fish and those low in sodium and saturated fats have been shown to significantly reduce CVD risk[10]. Conversely, diets high in saturated fat, sodium, sugar, and cholesterol or 'Western-type' diets significantly enhance CVD risk. The mechanisms by which specific macro and micro nutrients enhance or prevent CVD are complex and not well understood[10].

The continuing rise in obesity, particularly in youth, may be related adverse changes in overall eating patterns as opposed to individual nutrients[17]. Adverse changes in eating patterns can include irregular eating patterns, increased portion size, and meal frequency. These collectively effect both the quantity and quality of the diet, which may underpin the relationship between nutrient intake and chronic disease[17]. Diet quality can be difficult to assess as single nutrients can act synergistically or antagonistically with other dietary components and influence the overall quality[18]. Diet indices such as the Healthy Eating Index[19], the Diet Quality Index[20] , and the Healthy Diet Indicator[21] are metrics to assess overall diet quality and have been highly correlated with the intake of several macro-and micronutrients[18]. However, diet quality indices have not been shown to be accurate predictors for CVD risk and other chronic diseases in larger populations[22,23]. Therefore, additional markers, such as blood markers of diet status, are needed to predict chronic disease risk.

1.1.3.1.4 Oral Contraception

Oral contraceptive pills (OCP) are a widely used method of birth control worldwide. OCPs have been shown to significantly increase CVD risk in women[24] and enhance CVD risk factors in adolescents[25]. OCPs have prothrombotic effects that can enhance the incidence of clots that reduce blood flow through major coronary vessels[26]. OCPs also enhance CVD risk factors including raising blood lipids, blood pressure and blood glucose[24]. Therefore, OCP-use is of interest when studying factors of CVD risk in larger populations and forms a part of my research question in chapter 5.

1.1.3.2 Lipid Risk Factors

Described in further detail (section 1.3). Abnormal changes in circulating blood lipids increase CVD risk by enhancing atherosclerosis.

1.1.3.2.1 Classic Versus Remnant and Non-Fasting Lipid Determinants

To assess for CVD risk in the general population, physicians will often screen adults for fasting lipids, LDL-C and total cholesterol (TC). Both LDL-C and TC are 'classic lipid risk markers' that have been shown to accurately predict CVD risk in adults[27]. In Canada, optimal LDL-C cholesterol levels are defined as less than 3.5 mmol/L, TC as less than 5.2 mmol/L, and non-HDL-C as less than 4.3 mmol/L[1]. Despite this, there are several at-risk populations, including those with diabetes and the metabolic syndrome, that still have CVD despite normal LDL-C and TC levels[28]. While the traditional fasting lipid panel is still recommended to assess CVD risk, many countries are shifting towards measuring CVD-risk in a non-fasting state[29]. In the non-fasting state, there are novel risk factors that have recently been introduced and appear to better capture at risk subpopulations for CVD. The premise for measuring lipids in the non-fasting state can be rationalized by several points: LDL-C, TC, and HDL-C do not significantly fluctuate in the fed state, while TG and remnant cholesterol can peak around 4 hours postprandially Impaired TG metabolism (hypertriglyceridemia) is emphasized in the non-fasting state and is an independent risk marker of major CV events. Furthermore, there is increased convenience of sampling in the non-fasting state[6].

Non-fasting remnant cholesterol is derived from the hydrolysis of triglyceride-rich lipoproteins (TRL) (VLDL and CM) into smaller, cholesterol dense remnant particles (VLDL-r and CM-r). Remnant lipoproteins are small enough to enter the intima of the arterial wall and contribute to atherogenesis in a similar mechanism as classic lipids lipoprotein fractions[7]. Non-fasting remnant cholesterol has recently been shown to be causally associated with CVD in Europe and has a greater association with CVD than LDL-C[30]. Interestingly, non-fasting remnant cholesterol is also significantly elevated in individuals with diabetes or the metabolic syndrome and may be able to better predict CVD risk in these populations[31–33]. The above findings suggest that non-fasting remnant cholesterol is an important contributor to CVD risk and may be a better risk predictor for at-risk populations and is explored further in Chapter 3 of this thesis.

1.1.3.2.2 Residual risk

In North America, current clinical guidelines use fasting LDL-C as a focal CVD risk marker and treatment target for lipid lowering therapies[27,34]. Statins are the most commonly prescribed lipid lowering therapy[35]. However, almost 30% of individuals are statin-intolerant and do not achieve target reductions in LDL-C. Additionally, substantial proportions of the population, including those with insulin resistance and diabetes, still suffer residual CVD risk with normal fasting LDL-C values[28]. The above suggests that lowering LDL-C alone is not an optimal treatment target for patients with diabetes and the metabolic syndrome to prevent CVD and suggests that alternative lipid targets must also be lowered to prevent residual-risk. Novel observations from Europe published during my PhD program have confirmed that non-fasting lipids are causally associated with CVD and are as equally important when considering CVD risk[30].

1.1.4 At-risk Populations

1.1.4.1 Elderly

Age is one the most common non-modifiable risk factors associated with CVD and other chronic diseases. As the body ages, tissue integrity declines alongside increased exposure to metabolic risk factors. Indeed, it is well established the insulin sensitivity declines during aging, along with adverse changes to blood lipids and blood pressure. Additionally, aging is associated with changes in the properties of the arterial wall including wall thickening and reduced elasticity[36]. Overall, these changes will increase the susceptibility of developing CVD in the elderly[10]. Although most end-stage CVD events occur in the adult and elderly populations, youth are also at risk of developing CVD. However, the mechanisms that drive early CVD-risk is not well understood. Chapter 5, and 6 in my thesis aim to elucidate metabolic pathways involved in early CVD risk.

1.1.4.2 Diabetes

Diabetes is a chronic disease that is a consequence of a lack of or uncontrolled insulin secretion from pancreatic β-cells. In healthy conditions, insulin regulates fuel homeostasis by facilitating glucose uptake into peripheral tissues while concomitantly suppressing the release of lipids from the adipose tissue. There are two types of diabetes: type 1 diabetes and type 2 diabetes. Type 1 diabetes is an autoimmune condition that results in the destruction of pancreatic beta cells

leading to an absence of insulin secretion. Type 2 diabetes (T2D) is often associated with obesity and usually manifests when chronic obesity is coupled with genetic susceptibility leading to impaired insulin signalling or insulin resistance. Impaired insulin signalling during T2D results in hyperglycemia as a consequence of impaired insulin stimulated glucose uptake and uncontrolled hepatic glucose production[37]. Diabetes also impairs the suppression of fatty acid release from the adipose resulting in high circulating blood lipids (dyslipidemia). Other mechanisms that promote diabetic dyslipidemia are described below.

Patients with diabetes are at a significantly elevated risk of developing CVD compared to those without diabetes. The mortality of patients with diabetes typically associates with CVD complications[38]. With the growing state of obesity and metabolic dysfunction, T2D has become more prevalent and is occurring in younger age groups. Diabetes is associated with macrovascular (ischemic heart disease) and microvascular (retinopathy, neuropathy, and nephropathy) complications. Importantly, the macrovascular vascular complications present in diabetes are associated with structural and functional changes in major arteries of the body that enhance the incidence of an adverse cardiovascular events[39]. Additionally, the majority of patients with diabetes have dyslipidemia, which will enhance atherosclerosis and promote CVD in these patients[40]. Our laboratory and others have demonstrated that patients with both type 1 and type 2 diabetes have elevated non-fasting remnant lipoproteins[32,41]. Additionally, in pre-clinical models, we have shown rodents with insulin resistance have distinct changes in proteoglycan expression on the arterial surface, which contributes to enhance remnant cholesterol retention within the arterial wall and may further promote atherosclerosis during the diabetic condition[42].

1.1.4.3 Obesity

Chronic over-nutrition enhances the storage of lipids in the adipose tissue resulting in the expansion of adipocytes. During obesity, there is excessive storage of lipids in the adipose and dysfunctional release of fatty acids or 'fatty-acid spillover' into the plasma. Excessive plasma fatty acids results in lipotoxicity, which is an accumulation of toxic triglyceride metabolites in ectopic tissues including the liver, muscle and pancreas[43]. Fatty acid spillover and lipotoxicity further promote oxidative stress and cellular dysfunction of peripheral organs. Excessive lipid storage in adipocytes also enhances the production of pro-inflammatory mediators including tumor necrosis factor (TNF)-α as well as interleukin (IL)-1 and 6. Collectively, the increase in circulating free

fatty acids and adipocyte-derived pro-inflammatory mediators enhance atherogenesis by promoting early endothelial dysfunction, enhancing fatty streak formation as well as late-plaque formation, rupture and thrombosis[44].

1.1.4.4. Childhood and Youth CVD Risk

Despite most adverse CVD outcomes manifesting in adulthood, the pathophysiology of atherosclerosis has its origins in childhood[45]. Autopsy studies have confirmed that presence of atherosclerosis in children, predominately as a fatty streak. The Bogalusa Heart Study investigated the relationship between metabolic risk factors and presence of early CVD from autopsies of children that died from accidental causes[45]. The Bogalusa Heart Study and the Pathological Determinants of Atherosclerosis in Youth (PDAY) study demonstrated that the extent of atherosclerosis on the surface of the arterial intima increases with age, and approximately 70% of young adults test positive for fatty streaks and fibrous plaque[45,46]. It was also observed that high concentrations of circulating lipids (TC, LDL-C, TG) in youth were significantly associated with increased atherosclerotic lesion area as well as anthropometric factors including obesity and higher waist circumference[46]. Despite these findings, there remains little knowledge on lipid metabolism during childhood obesity and how it relates to CVD risk. Furthermore, classic fasting lipid risk markers do not also accurately track with obesity and other anthropometric CVD risk factors at this age[47,48], suggesting that more research is needed to elucidate early CVD-risk markers in youth.

1.1.5 Diagnostic and Screening Approaches to CVD

There are numerous screening and diagnostic tools that physicians employ to detect and estimate CVD risk in adult patients. In Canada, physicians use the Modified Framingham Risk Score (FRS) to estimate CVD risk in adults, which considers patient age, high density lipoprotein cholesterol (HDL-C), total cholesterol (TC), systolic blood pressure and whether the patient is a smoker or has diabetes. Screening with the FRS is recommend to begin at 40 years of age and repeated every 5 years[49]. Most CVD risk assessment tools, including the FRS, have not been validated for use in younger populations[50] and therefore, more research is needed to elucidate novel risk markers and tools to assess CVD risk in younger populations. Markers of inflammation are also used to detect CVD as they are known to play an important role in atherosclerosis. Common inflammatory markers used to quantify CVD risk include plasma C-reactive protein (CRP) and homocysteine. Imaging tools such as computed tomography (CT) and carotid artery ultrasounds

can also be used as markers of atherosclerosis. Screening for lipids in adults is widely supported in most countries as dyslipidemia is a modifiable risk factor that can be effectively lowered by lifestyle changes and pharmacological therapies. Therefore, physicians can use the absolute benefit of treating dyslipidemia as a function of baseline risk[51]. More recently, non-fasting lipids have been recommended to assess CVD risk as they provide a greater appreciation of the overall lipid panel[52]. Non-fasting lipid assessments have been introduced in Europe and more recently in Canada[52,53]. However, there are no current reference ranges of non-fasting lipids in the updated Canadian Cardiovascular Guidelines[1]. In Chapter 3 we will investigate concentrations of non-fasting lipids in a Canadian cohort and their relationship to CVD.

1.2 Lipoprotein Metabolism

1.2.1 Classes of Lipoproteins

Lipoproteins are micelle macromolecules comprised of lipids and proteins that function to transport fat soluble components through the circulation and are responsible for regulating lipid synthesis and catabolism[54]. Lipoproteins are comprised of a hydrophobic core of non-polar lipids (primarily cholesterol esters and triglycerides) surrounded by a hydrophilic membrane comprised of phospholipids, free cholesterol and apolipoproteins. Apolipoproteins (apo) are proteins present on lipoproteins that play a critical role in lipoprotein structure, acting as ligands for lipoprotein receptors, dictating movement of lipoproteins and acting as inhibitors or activators of enzymes involved in lipoprotein metabolism[55]. Lipoproteins originate from both the liver and the intestine and are further described in the sections below.

Classes	Density Range (g/mL)	Particle Size (diameter, nm)	Apolipoproteins
CM	<0.95	80-500	B48, A-I, A-II, A-IV, C, E
CM Remnant	<1.006	>50	B48, E
VLDL	<1.006	30-80	B100, C, E
IDL	1.006-1.019	25-35	B100, C, E
LDL	1.019-1.063	18-25	B100
HDL	1.063-1.210	5-12	A-I, A-II

Table 1-1: *Classes of Lipoproteins*[55,56]

1.2.1.1 Very low-density lipoprotein (VLDL)

Endogenously produced lipids are synthesized and secreted by the liver as very low-density lipoprotein (VLDL), a large, triglyceride rich lipoprotein (TRL). VLDL is one of the major apoB-containing lipoproteins found in the plasma[57]. Every VLDL particle contains one apoB100 protein, which is an integral part of the lipoprotein structure. Other apolipoproteins present within the VLDL particle include apoCI, apoCII, apoCIII and apoE. In the circulation, VLDL particles are subject to lipolysis mainly through the action of lipoprotein lipase (LPL) and to a lesser extent, hepatic lipases. LPL is located on the endothelium of capillaries, adipose, myocardium, and skeletal muscle and promotes the hydrolysis of TG on the VLDL particle to liberate free fatty acids (FFA), which can be taken up by peripheral tissues and used for energy metabolism and storage. LPL is activated by the apolipoprotein, apoCII, and inhibited by apoCIII. Upon lipolysis of FFA, VLDL particles become smaller, cholesterol dense indeterminate density lipoprotein (IDL) particles and can be further metabolized into VLDL remnant (VLDL-r) particles[58]. VLDL-r are depleted of TGs, phospholipids, and apoCs and enriched in cholesterol esters as well as apoE.

1.2.1.2 Low-density lipoprotein (LDL)

VLDL-r particles can be further hydrolyzed by LPL to form LDL particles, which are depleted of TG, apoCs and apoE, while still retaining apoB100[54]. Alongside VLDL, LDL is also one of the major apoB-containing lipoproteins found in the plasma[57]. LDL cholesterol is removed from the circulation by the LDL receptor (LDL-r) in the liver. The C-terminus of apoB100 on LDL particles is able to bind to the LDL-r to mediate clearance of LDL from the circulation[12]. Due to its relatively slow turnover (12-24 hours) LDL generally reflects the greatest proportion of plasma cholesterol in the fasted state and thus has been studied extensively in its role in CVD[59].

1.2.1.3 High-density lipoprotein (HDL)

The HDL lipoprotein fraction contains apoA-I, apoA-II, apoA-IV and apoE apolipoproteins. Lipid poor apoA-I is initially secreted from the liver and interacts with ATP binding cassette A1 (ABCA1) to initiate the biogenesis of discoidal HDLs. ABCA1 donates phospholipids from cell membranes to the lipid poor apoA-I particle that further accepts cholesterol from cell membranes. Discoidal HDL particles are secreted from the liver and are converted into mature, spherical HDL by lecithin: cholesterol acyltransferase (LCAT)[60]. The

intestine can also secrete apoA-I into the mesenteric lymph and form HDL[61]. HDL plays an important role in reverse cholesterol transport (RCT), whereby, HDL transports excess cholesterol from peripheral tissues to the liver through scavenger receptor class B type 1 (SR-B1). The cholesterol delivered to the liver by HDL can be redistributed into other tissues or converted into bile acids and excreted in the feces. Additionally, cholesterol esters on HDL particles can be exchanged with the TG on VLDL and chylomicrons to promote the formation of cholesterol dense remnant VLDL and chylomicrons through the action of the enzyme cholesterol ester transport protein (CETP). The TG rich HDL particle can then be catabolized by the kidney[57].

Several studies have demonstrated the inverse relationship between plasma HDL-C and CVD risk. However, genetic studies have provided little evidence to demonstrate as causal association between elevated HDL-C and reduced CVD[62]. Mice naturally lack CETP, and unlike humans, primarily transport cholesterol on HDL particles, which results in mice being protected from developing atherosclerosis[63]. To study CETP in mice, transgenic mice models expressing CETP were generated[64]. Transgenic CETP mice have a shift in cholesterol distribution from HDL towards VLDL/LDL making them a more optimal model to study atherosclerosis[63]. Pharmacological inhibitors of CETP have been developed and are found to significantly reduce LDL-C and increase HDL-C in humans[65]. However, clinical trials with CETP inhibitors failed to demonstrate clinical benefit with pharmacologically raised HDL[65,66]. Indeed, many CETP inhibitors, such as trocetrapid, despite raising HDL-C by more than 70%, resulted in excess death and CVD compared to standard of care. The adverse effects from trocetrapid were attributed to off-target effects on blood hormones and adrenal hormone production[65]. The unexpected clinical results of pharmacologically raising HDL through CETP inhibitors have led researchers to further understand the role of HDL function in the role of CVD. HDL particles are heterogeneous and more research is needed to understand the composition of the HDL particle and its relationship to CVD risk[64].

Novel research from our laboratory has also shown that in a rodent model of insulin resistance, intestinal apoA-I secretion is reduced, and lymphatic HDL had an 85% higher TG content and enrichment in cholesterol compared to controls[67]. It has been proposed that the increased TG content in the HDL fraction may decrease the capacity for effective RCT and potentially enhance CVD risk[68]. Additionally, microRNA (miR)-223 has been shown to be an

important regulator of peripheral cholesterol metabolism by enhancing *Abca1* expression and cholesterol efflux to lipid-depleted apoA-I[69]. MicroRNAs are small, non-protein coding segments of RNA that can regulate gene expression by repressing their target mRNAs. Our laboratory also demonstrated that miR-223 was associated with lymph lipoprotein fractions, enriched in lymphatic-HDL, and significantly decreased during insulin resistance[67]. The following suggests that the intestine and lymphatics, in addition to contributing to non-fasting remnant cholesterol metabolism, also play an important role in HDL biogenesis and RCT.

1.2.1.4 Chylomicrons

Dietary lipids are absorbed by the intestine and packaged into chylomicrons (CM) in the enterocyte. CMs are large TRL particles enriched in TG and cholesterol. In humans, apoB48 is the integral protein associated with CM particles and is necessary for the functioning of the CM. Each CM particles contains one non-exchangeable apoB48 protein and this can be used to quantify the number of CM in plasma[58]. In humans, apoB48 is formed by a posttranscriptional editing of *APOB* mRNA by apoB-editing catalytic component 1 (APOBEC1), resulting in truncation of the protein. CMs also contain the apolipoproteins apoA-I, apoA-IV and apoCI, apoCII, apoCIII and apoE. Animal models (especially rodents) often have the post-transcriptional edit for apoB48 in the liver as well as the intestine[55,56].

1.2.1.4.1 Dietary Lipid Digestion and Absorption

Dietary fats are comprised of several polar and nonpolar lipids including phospholipids, sterols (cholesterol) and other lipids such as fat-soluble vitamins. In the small intestine, bile salts and pancreatic lipases emulsify and metabolize lipids, respectively to aid in their digestion and absorption by the enterocyte. The majority (>95%) of dietary fat is in the form of TG. TG are digested by pancreatic lipases in the upper jejunum into 2-monoacylglycerol (2-MAG) and FFAs. Pancreatic lipases further hydrolyze 2-MAG into glycerol and FFA. FFA are taken up into the intestinal lumen by a number of protein candidates including FAT/CD36 and fatty acid transport proteins (FATP)[70]. FFA are synthesized into TAG in the enterocyte by the concerted actions of monoacylglycerol acyltransferases (MGAT) and diacylglycerol acyltransferase (DGAT) (Figure 1-2). The lipids synthesized in the endoplasmic reticulum (ER) membrane can either become part of cytosolic lipid droplets and stored or transported to the ER for secretion into the plasma[71]. Cholesterol in the small intestine is derived from both the diet (~300-500 mg/day) and from bile

(~800-1200g/day). In humans, cholesterol absorption in the gut lumen varies from 15-75% and the remainder is excreted in the feces[72]. The variance in cholesterol absorption is mainly due to intraindividual differences in metabolic and genetic factors as well as the partitioning of cholesterol into bile micelles[73]. Only free cholesterol can be incorporated into bile acid micelles, while esterified cholesterol needs to be hydrolyzed by cholesterol esterase[74]. Dietary cholesterol exists mainly as free cholesterol (only 8-15% existing as cholesterol esters) and is taken up into the enterocyte through the transporter proteins Niemann-Pick C1 like 1 (NPC1L1) and is re-esterified into a cholesterol-ester[75]. Other transporters, including scavenger receptor class B type 1 (SR-B1), have also been implicated in facilitating cholesterol absorption[76]. Following the absorption of dietary lipids and cholesterol, they are packaged into CM particles (described further below) and released into the plasma. The above is called the postprandial phase of lipid metabolism. The majority of plasma cholesterol is contained within LDL-C particles, which have a very slow turn over (~4 days)[77]. The contribution of non-fasting CM total cholesterol is much less than LDL-C yet has a much more rapid turn over of minutes rather than days[78]. Therefore, the cholesterol carried within CM particles is much more rapidly distributed and may have a much greater influence on the body's cholesterol burden than LDL[59]. The following demonstrates that digestion and absorption of dietary lipids is variable and can have a substantial impact on whole-body cholesterol metabolism.

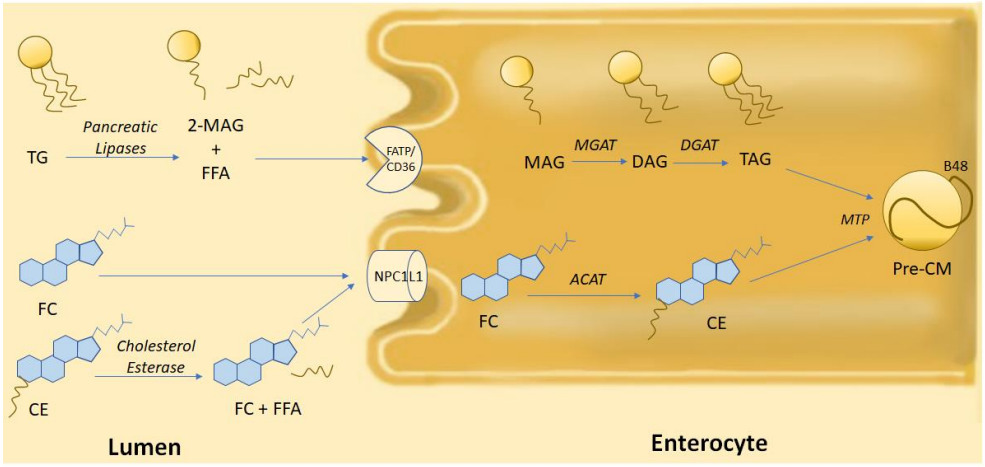

Figure 1-2: *Dietary Lipid Absorption.* Dietary fats include phospholipids, sterols (cholesterol) and other lipids such as fat-soluble vitamins. TG are digested by pancreatic lipases in the upper jejunum into 2-monoacylglycerol (2-MAG) and FFAs. Pancreatic lipases further hydrolyze 2-MAG into glycerol and FFA. FFA are taken up into the intestinal lumen by a number of protein candidates

including FAT/CD36 and fatty acid transport proteins (FATP)[70]. FFA are synthesized into TAG in the enterocyte by the concerted actions of monoacylglycerol acyltransferases (MGAT) and diacylglycerol acyltransferase (DGAT). Dietary cholesterol exists mainly as free cholesterol and is taken up into the enterocyte through the transporter proteins Niemann-Pick C1 like 1 (NPC1L1) and is esterified into a cholesterol-ester. Other transporters, including scavenger receptor class B type 1 (SR-B1) has also been implicated in facilitating cholesterol absorption. Following the absorption of dietary lipids and cholesterol, they are packaged into CM particles and released into the plasma. Figure by Jacqueline Krysa©

1.2.1.4.2 Chylomicron Synthesis

Chylomicron (CM) synthesis in the small intestine is initiated by the truncation of apoB mRNA into apoB48 through the APOBEC1 enzyme[79]. Newly translated apoB48 is chaperoned into the ER by microsomal triacylglycerol transfer protein (MTP). MTP also facilitates the addition of phospholipids, cholesterol and small amounts of TAG and cholesterol ester (CE). There are two proposed models of CM assembly[80]: In the first model small dense CM particles and large TG-rich CM particles are assembled by continuous addition of lipid to the CM[81]. In the second model, CM synthesis is a two-step process: the primordial CM is formed by the lipidation of apoB48 by MTP while concomitantly, TAG, synthesized on the cytosolic side of the ER, is transported to the luminal side of the ER where MTP facilitates TAG to fuse with the primordial CM particle. The result of both of these models is the enlargement and lipidation of the particle to form a pre-chylomicron[82]. The pre-chylomicron is subsequently transported to the *cis*-Golgi through pre-chylomicron transport vesicles (PCTVs). Once in the golgi lumen, pre-chylomicrons acquire apoA-I and apoB48 is glycosylated. Lastly, CM exit the golgi body and are transported to the basolateral side of the enterocyte for secretion into the lymphatics[83] (Figure 1-3).

CM synthesis is mainly regulated by the availability of lipid substrate[84]. In absence of sufficient lipid substrate, intestinal apoB is degraded and CM synthesis is reduced. Accordingly, increases in circulating FFAs stimulate CM synthesis by increasing the amount of intracellular lipid substrate and/or by enhancing the stability of apoB[85,86]. Insulin acutely suppresses CM synthesis either directly or indirectly through decreasing circulating FFAs[86]. Furthermore, recent work by Adeli and colleagues have found that the incretin hormone glucagon-like peptide (GLP)-1 also influences CM production[87]. GLP-1 is a gut peptide secreted by ileal enteroendocrine L cells in response to dietary nutrients, particularly glucose and fatty acids[88]. GLP-1 has insulinotropic activity and has been shown to directly reduce CM production through direct and indirect

mechanisms[87]. CM production is also significantly altered by changes in nutrients, particularly fructose, which has been shown to greatly enhance CM synthesis and secretion[89]. Ultimately, the nutritional availability plays an important role in CM synthesis and secretion and further demonstrates that overnutrition and metabolic disease will enhance CM production.

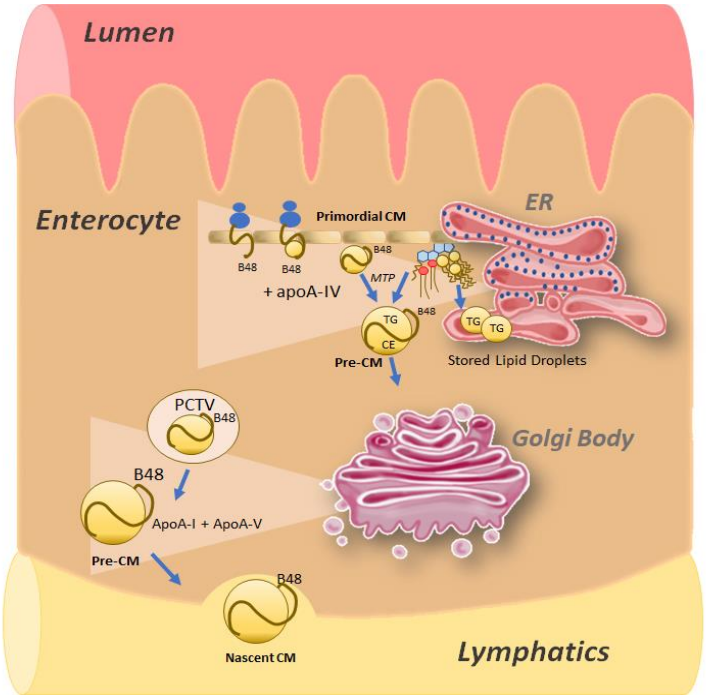

Figure 1-3: *Chylomicron Synthesis*. ApoB is synthesized in the intestine and truncated by APOBEC1 form apoB48. ApoB48 is lipidated by MTP to form a primordial chylomicron particle. TG-rich lipid droplets from the ER fuse with the primordial CM to form a pre-chylomicron particle. Pre-chylomicron transport vesicles transport the pre-chylomicron to the golgi body for further maturation. The nascent chylomicron is secreted from the golgi into the lymphatics and then transported into the circulation. Figure by Jacqueline Krysa©

1.2.1.4.3 Chylomicron Secretion, Lipolysis, and Clearance

CM are secreted into the mesenteric lymphatics and enter the circulation at the thoracic duct. A recent study demonstrated that the basal secretion of apoB48 in adult male volunteers was approximately 50 mg/day and following absorption of a lipid meal was approximately 230 mg/day[90]. Once in circulation, CM are metabolized into CM-remnants by LPL, which is activated by the presence of apoC-II on the particle[58]. Additionally, CM remnants acquire ApoE and cholesterol from HDL through CETP, while exchanging apoC-II and TG, thus reducing the ability of LPL to further breakdown TG. Throughout the metabolism of the CM particle, the apoB48

protein remains an integral component of the CM particle and thus has been used as a marker to differentiate between intestinal derived and hepatic derived particles[91]. CM-remnants are cleared from the circulation through the LDL-r, like LDL. However, unlike LDL, CM remnants binds to the LDL-r through apoE[12]. Additionally, CM-remnants can bind to other hepatic receptors including lipoprotein receptor related protein (LRP) and syndecan-4[55].

1.2.1.5 Lipoprotein Metabolism during the Fed and Fasted State

Fasting and feeding is a fundamental process that all living organisms must adapt to in order to properly utilize and store metabolic fuels for survival and overall metabolic health[92]. Fatty acids, in a normal physiological state, are stored in the adipose tissue and mobilized to fit the metabolic requirements of the organism. The intestine, liver and skeletal muscle also play important roles in handling fatty acids in the post-absorptive period (Figure 4).

1.2.1.5.1 Postprandial Metabolism

Insulin is released from the pancreas in response to elevated plasma glucose and has pleiotropic effects on lipoprotein metabolism. In the adipose tissue, insulin inhibits adipose triglyceride lipase (ATGL) to prevent the adipose from mobilizing stored lipids and in the liver, insulin inhibits the assembly of VLDL particles by increasing the degradation of apoB100 and reducing MTP expression. Accordingly, insulin also acutely inhibits CM particle assembly in the small intestine by promoting the degradation of apoB48[93]. In the plasma, insulin also acts to enhance the lipolysis of TRL particles by activating apoCII and suppressing apoCIII thereby enhancing adipose LPL activity[94]. After consuming a meal containing fat, the small intestine packages and secretes dietary and stored lipids as CM particles into the plasma. The increase in TG postprandially is predominantly due to an increase in TG in the CM particle. Approximately 80% of postprandial circulating TG has originated from CM particles. The increase in CM-TG during the postprandial period is due to the lipid enrichment of the CM particle. However, in the fasted and postprandial phase the majority of circulating TGs are derived from VLDL particles[58]. Fatty acids that are lipolyzed from the CM particle are subsequently shuttled to tissues and organs[95]. During the postprandial period, there is an increase in both CM and VLDL particles resulting in a competition of VLDL and CM for lipolysis and clearance pathways[58]. The potential result of this is that more VLDL remnant can accumulate and delay lipid metabolism, particularly during chronic disesase[96] (Figure 1-4 (a)).

1.2.1.5.2 Fasting State

Conversely, during fasting plasma insulin levels are decreased. ATGL promotes the lipolysis of TG to DAG and hormone sensitive lipase (HSL) converts DAG to MAG. MAGs are then cleaved into a fatty acid and glycerol. Fatty acids are released into the plasma and transverse to the muscle and liver. During fasting, fatty acids are taken up by the muscle and shuttled towards oxidation. The liver is the major site of free fatty acid removal and fatty acids are used by the liver for oxidation, TG synthesis and VLDL formation[94]. During fasting, VLDL-TG is mainly comprised of FFA from adipose tissue lipolysis[80]. Converse to the postprandial phase, intestine lipid absorption drastically decreases during the fasted fate. The basal secretion of CM during the fasted state is comprised of lipid-poor CM in normal, healthy conditions[95] (Figure 1-4 (b)).

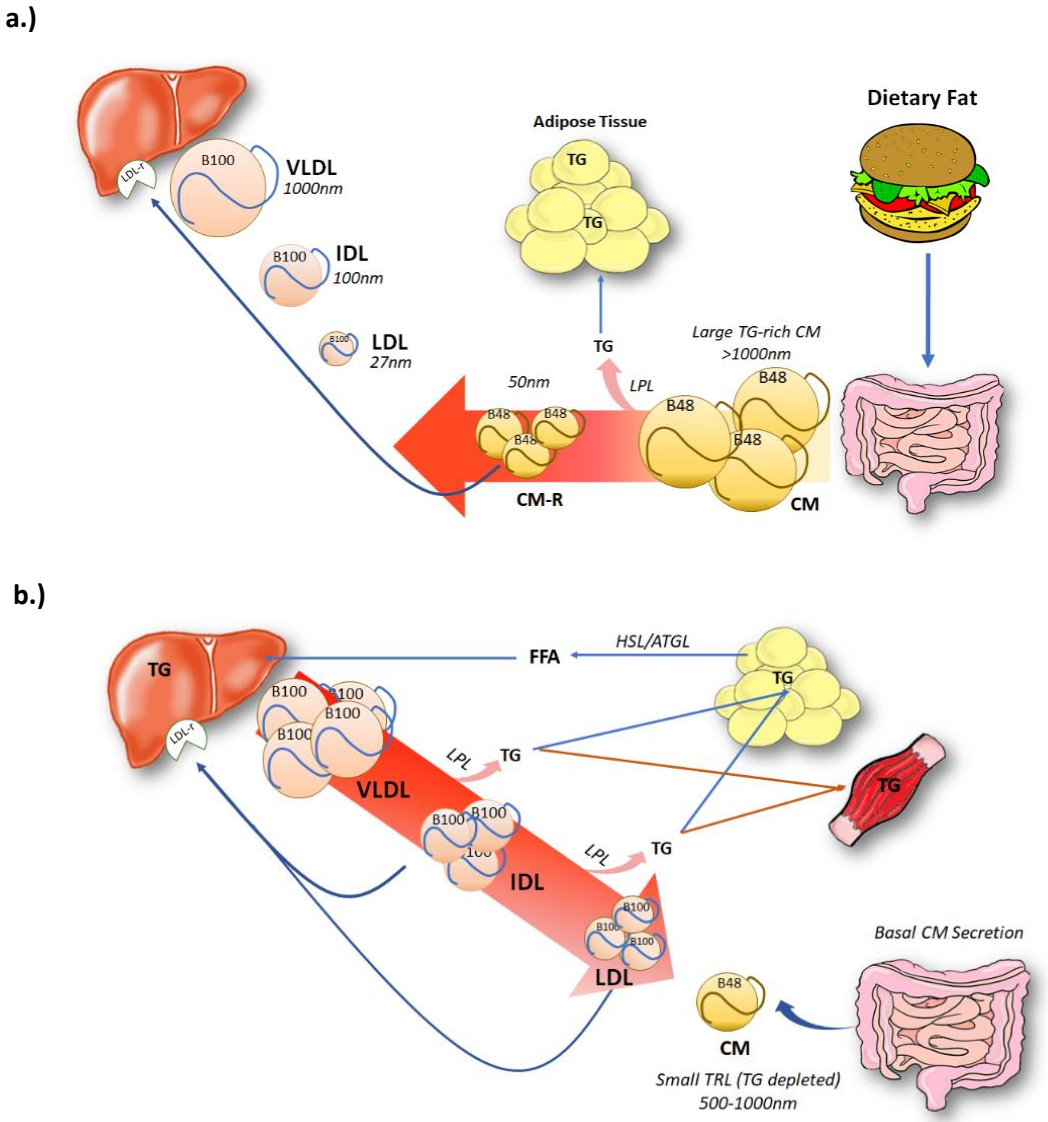

Figure 1-4: *Lipoprotein Metabolism During the Fed and Fasted State.* (a) *Postprandial Metabolism*: Dietary fat is digested and absorbed by the small intestine where it is packaged into chylomicron particles and secreted in the lymphatics and drained into the circulation. Herein, triglycerides are lipolyzed off the chylomicron by lipoprotein lipase (LPL) and stored in the adipose tissue. Remnant chylomicron particles (CM-R) are cleared from the circulation by the hepatic LDL-R. (b) *Fasting State*: Hormone sensitive lipase (HSL) liberates FFA stored in the adipose tissue, which are transported to the liver and reassembled into TG. The liver packages TG and endogenous cholesterol into VLDL particles, which are secreted into the plasma. LPL hydrolyses TG off the VLDL particle, which is used by the adipose and other peripheral tissues including the muscle. Hydrolyzed VLDL is converted into IDL and lastly LDL. Figure by Jacqueline Krysa©

1.2.1.6 Impaired Postprandial Metabolism, Remnant Lipoproteins, and Postprandial Lipemia

Following in the intake of dietary fats, TG and cholesterol are packaged into apoB48 containing CMs that are secreted into the circulation. In a healthy individual, plasma TG and apoB48 levels peak 3-4 hours following a high-fat meal and return to baseline around 6-8 hours. Conversely, individuals with CVD, obesity, and diabetes are found to have an elevated and prolonged peak in both TG and apoB48 that is 2-3 times greater than a healthy individual following the consumption of dietary fat[97]. Furthermore, these individuals also have a delayed clearance of TGs and apoB48 from the circulation[12]. Higher than normal plasma concentrations of apoB48 and TG following a high fat meal is called postprandial lipemia (PPL) and has been shown to associate with increased CVD risk in adults[98,99]. PPL can be measured by quantifying plasma apoB48 following a fatty meal[100]. Elevated non-fasting apoB48 is reflective of remnant cholesterol accumulation in the fed-state. Fasting apoB48 has been shown to strongly correlate with the postprandial apoB48 response (or area under the curve) and therefore is predictive of PPL in adults[98]. Interestingly, it has been observed that normolipidemic subjects with CAD can have PPL, independent of elevated plasma LDL-C levels[101]. CM particles require greater expression of the LDL-R for clearance from the circulation and therefore, elevated CM particles would be a greater metabolic burden for clearance than LDL and would further enhance CVD risk in these individuals[102,103] (Figure 1-5).

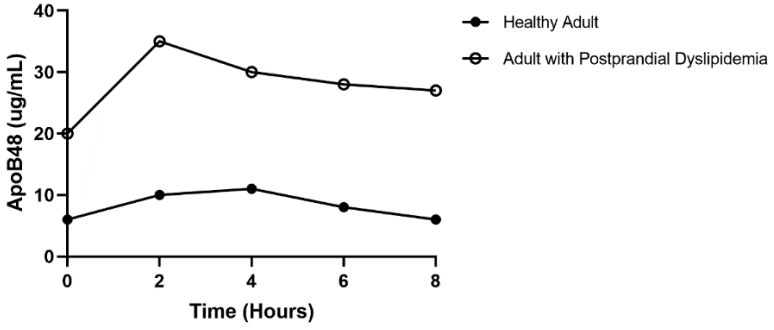

Figure 1-5: Postprandial Dyslipidemia. Following a high-fat meal, an individual with postprandial dyslipidemia has an elevated and prolonged postprandial response in apoB48 compared to a healthy adult (redrawn based on historical values)[98].

1.3 The Role of Chylomicrons in CVD and the Influence of Non-Fasting Lipids on CVD Risk

CM remnants have been shown to have a causal association with CVD in adults and are also found to be directly involved in the pathophysiology of CVD. Similar to LDL, cholesterol within remnant particles is able to enter the arterial intima where they contribute to the formation of foam cells, plaques, and atherosclerosis progression[104].

1.3.1 Arterial Delivery, Efflux, and Retention of Remnants

Both VLDL and CM-remnants are cholesterol dense apoB-lipoprotein remnants and are considered atherogenic lipoproteins[30]. Using novel imaging techniques, our laboratory was the first to show that CMs and VLDL are metabolized to become smaller, cholesterol dense particles that are able to penetrate the arterial wall[7]. Remnants contain significantly more cholesterol per particle than LDL-C and therefore may contribute more cholesterol per particle to the vessel wall than LDL-C[9,12]. Cholesterol particles (in the form of small dense lipoproteins) are retained in the vessel wall by electrostatic binding to proteoglycans in the extracellular matrix of the artery. The positively charged residues associated with apoB100 (LDL-C) or apoE (remnants) bind to the negatively charged residues associated with the glycosaminoglycan chains of arterial proteoglycans. When LDL-C is bound to proteoglycans it stimulates monocytes to differentiate into macrophages, which internalize LDL-C and promote foam cell formation and a pro-inflammatory response[105]. Important experiments by O'Brien and Chait have demonstrated that human atherosclerotic lesions have a high degree of co-localization of apoE with the proteoglycan biglycan, which infers that remnants may be preferentially retained in human arterial tissue[106]. Unlike LDL-C, remnants do not require modification to be taken up by macrophages and can readily induce foam cell formation[8]. Our laboratory has also demonstrated, in both rabbit and rat models using ex vivo perfusion methodology, that there is preferential accumulation of apoB48 particles in the vessel (over time) and this can be explained by a greater egress (efflux out of the arterial vessel) of apoB100 particles from atherosclerotic plaque tissue[7]. More recently, this has been confirmed in human arterial tissue[107].

1.3.2 Role of Non-Fasting Triglycerides at CVD Risk Prediction

Elevated TGs have been shown to be significantly associated with CVD risk[104]. Interestingly, non-fasting TGs are better predictors of CVD risk than fasting TGs[108]. As discussed

earlier, cholesterol derived from LDL-C and remnant lipoproteins is retained in the arterial wall and directly contributes to atherosclerosis. Conversely, TGs alone, are not directly involved in the progression of atherosclerosis[109]. Non-fasting TGs have been shown to be more significantly correlated to postprandial remnant lipoproteins using direct assays compared to fasting TG and remnant lipoproteins. In the postprandial state, there is an approximate 80% increase in plasma TGs, and this is derived from mainly remnant lipoproteins[110]. Therefore, the increased capacity of TGs in the non-fasting state to predict CVD may be due to the increase in postprandial remnant lipoproteins, which are causally associated with CVD and have a direct role in CVD pathophysiology[111–113].

1.3.3 Causal Association of Non-Fasting Remnant Cholesterol to CVD in Europe

Recent community and population studies from Europe have revealed that elevated non-fasting remnant cholesterol is causally associated with major cardiovascular events and in some cases, more so than for fasting lipid parameters[114]. Indeed, an increase of 1mmol/L in apoB remnant molecules was associated with a 2.8-fold causal increase in risk for ischemic heart disease in over 900,000 adults independent of HDL cholesterol[7]. Collectively, this demonstrates that elevated non-fasting remnant cholesterol has a crucial role in the pathophysiology of CVD.

1.3.3.1 Postprandial lipemia as an Independent Predictor of CVD Risk

Postprandial lipemia contributes to atherogenic risk and is an independent predictor of CVD risk. In adults, fasting plasma apoB48 can strongly predict non-fasting or postprandial apoB48 following a high fat-meal challenge, demonstrating that elevated fasting apoB48 can be used as a biomarker of PPL and CVD risk[11,13]. PPL is exacerbated by over-nutrition and central adiposity, leading to an increase in subclinical CVD risk[12]. Fasting apoB48 has been well characterized in several adult populations and has been shown to be higher in males compared to females and elevated in adults with CVD, the metabolic syndrome, and diabetes[33,91,114,115]. Presently, there are no studies demonstrating whether youth with obesity and the metabolic syndrome have PPL to the extent observed in adults.

1.3.4 Enhanced Chylomicron Retention During Type 2 Diabetes and the Metabolic Syndrome

It has also been well established that low density lipoprotein cholesterol (LDL-C) is a prominent contributor to the formation of atherosclerotic plaques[116,117]. However, in certain clinical conditions, including the metabolic syndrome and/or insulin resistance, LDL-C levels are normal. This suggests that atherosclerosis in these individuals may be derived from other cholesterol sources[110,118]. Previous studies from our laboratory and others have shown that normolipidemic subjects with type 1 diabetes and T2D have a higher concentration in both fasting and postprandial intestinal-remnant cholesterol despite having normal LDL-C, HDL-C and total cholesterol[42,91]. Our laboratory has also shown that rodents with type 1 diabetes and T2D have distinct biochemical changes in their arterial proteoglycan expression that promote the binding and retention of remnant cholesterol in the arterial intima[42]. These findings suggest that during insulin resistance and diabetes there is enhanced CM retention in the arterial intima, and this may partly explain the increased risk of CVD in individuals with type 1 and type 2 diabetes.

1.4 Chylomicrons Metabolism During Chronic Disease

In pathological conditions, such as insulin resistance and obesity, remnant cholesterol metabolism is impaired either due to intestinal apoB48-CM overproduction, impaired lipolysis, or impaired lipoprotein uptake pathways leading to delayed clearance of apoB48 remnant particles[58].

1.4.1 Obesity and the Metabolic Syndrome

Obesity is a growing concern worldwide and is associated with health consequences for both adult and pediatric populations[198]. Obesity is associated with various metabolic abnormalities that enhance chronic disease progression. The clustering of metabolic effects that arise from obesity are referred to as the metabolic syndrome. The main features of the metabolic syndrome include: atherogenic dyslipidemia, abdominal obesity, elevated blood pressure, elevated glucose and pro-inflammation[119]. Individuals with the metabolic syndrome have been shown to have a two-fold increased risk for CVD[120].

1.4.1.1 Pathophysiology of Obesity and the Metabolic Syndrome

Obesity is caused by an accumulation of lipids within the adipose tissue resulting in an expansion of the adipose tissue. In men, obesity is often found to occur in the upper body in the subcutaneous and visceral pools, whereas in women upper body obesity is often due to accumulations in subcutaneous adipose tissue. Upper body obesity is more commonly associated with the promotion of the metabolic syndrome than lower body obesity[121]. According to the lipotoxicity hypothesis, obesity is characterized by an excessive storage of TG within the adipose tissue that increases adipose lipolysis. Consequently, there is an enhanced release of FFA into the circulation, resulting in increased circulating FFAs or lipotoxicity. Excess lipids and their metabolites are toxic as they promote oxidative stress in the ER and mitochondria. Lipotoxicity induced oxidative stress occurs in the adipose and other peripheral organs including the liver and the pancreas and promotes metabolic dysfunction of these organs[43]. Furthermore, the excessive circulating FFA are stored as TG in the adipose and inhibit lipogenesis, which prevents adequate clearance of plasma TG and results in hypertriglyceridemia and dysfunction of the insulin receptor. The consequent insulin resistant state enhances hyperglycemia along with compensatory increases in hepatic gluconeogenesis and decreased utilization of insulin stimulated muscle glucose update[43]. Increased lipid supply to the adipose also results in adipose inflammation resulting in an increased production in pro-inflammatory cytokines including TNF-α, and IL-6[122]. These proinflammatory cytokines may then further impair insulin sensitivity by interfering with insulin signalling pathways in the liver and small-intestine promoting an overproduction in both VLDL and CM particles[123]. Consequently, obesity induced lipotoxicity results in an increase in circulating lipids coupled with hyperglycemia that further drives metabolic dysfunction and enhances chronic disease risk[43] (Figure 1-6).

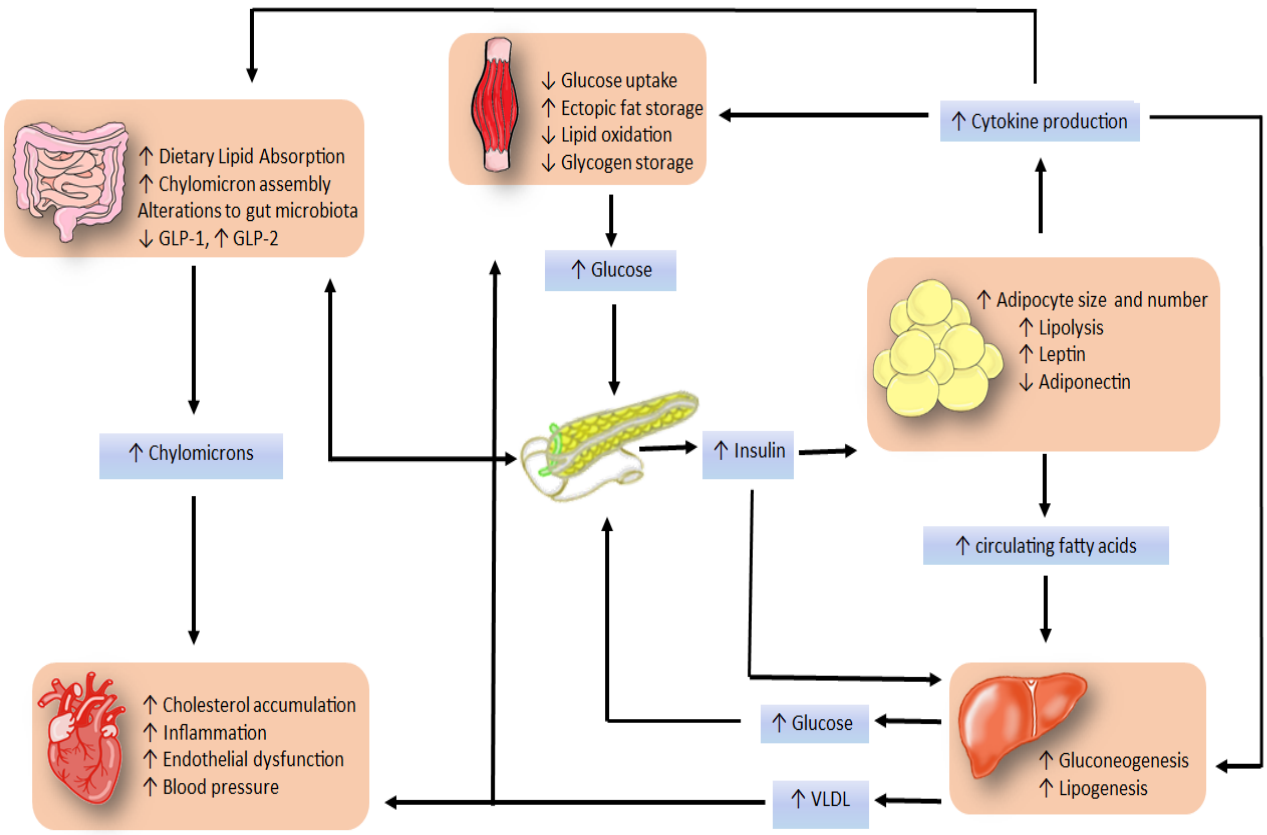

Figure 1-6: *The Pathophysiology of Obesity*. Chronic obesity is associated with decreased peripheral insulin sensitivity resulting in increased insulin secretion from the pancreas. Defective insulin signalling in the adipose tissue enhances the lipolysis of stored fatty acids as well as inflammatory cytokine production. Insulin resistance and inflammation promote ectopic lipid deposition in the muscle and further decrease insulin signalling. Impaired insulin signalling also dysregulates gluconeogenesis in the liver resulting in elevations in circulating glucose. Excess circulating fatty acids are taken up by the liver to increase lipogenesis and VLDL secretion. Additionally, insulin resistance promotes VLDL secretion and chylomicron secretion from the liver. Circulating CM and VLDL can be retained within the arterial wall and contribute to atherosclerosis and enhance CVD progression alongside chronic low-grade inflammation (such as IL-1β and CRP). Figure by Jacqueline Krysa©

1.4.2 Insulin Resistance and Type 2 Diabetes

Insulin resistance (IR) is characterized by an impairment in insulin-stimulated glucose transport and metabolism in the skeletal muscle and impaired fatty acid metabolism in the adipocyte[124]. IR is commonly observed during metabolic conditions such as obesity and T2D. IR

is known to promote a distinct pattern of dyslipidemia termed 'atherogenic dyslipidemia'. Atherogenic dyslipidemia is characterized by elevated small-dense LDL-C particles, reduced HDL-C particles and elevated TG. Overall, this distinct lipid profile is associated with increased CVD risk[125].Both pre-clinical and clinical studies have clearly demonstrated that insulin resistance promotes an accumulation of circulating intestinal-remnant lipoproteins. Insulin resistance can impair remnant cholesterol metabolism either by directly promoting intestinal lipoprotein overproduction or disrupting circulating remnant cholesterol metabolism (impaired lipolysis) and clearance[126,127].

1.4.3 Intestinal Over-Production of Chylomicrons

Intestinal CM overproduction occurs in both the fasting and postprandial state during the metabolic syndrome and diabetes. Hyperinsulinemia promotes enhanced absorption of lipids by the intestine, and excessive CM secretion in rats, hamsters, and humans[126,128,129]. Furthermore, insulin sensitizers, such as Rosiglitazone, have been shown to significantly reduce intestinal over production of apoB48 in an insulin-resistant hamster models[127]. There are several mechanisms that may contribute to the intestinal CM overproduction observed during IR including: intestinal stimulation by excess FFA[130], alterations in incretin hormones (GLP-1 and GLP-2)[87], impaired insulin signalling in the enterocyte, increased MTP mass and activity, increased TNF-α, as well as enhanced enterocyte *de novo* lipogenesis[93,131]. Our laboratory has further elucidated these mechanisms in a rodent model of insulin resistance by demonstrating associations of intestinal over production with the suppression of enterocyte PPARα[132], increased SREBP-1[133], and increased phosphorylation of c-Jun N-terminal kinase (JNK) protein[134]. These changes would promote increased enterocyte *de novo* lipogenesis, and impair insulin signalling, respectively. A recent clinical study by Jan Borén's group demonstrated using an integrated non-steady state model of apoB48 kinetics that during lipid absorption apoB48 is found in not only CM lipid fractions, but also $VLDL_1$ and $VLDL_2$ type particles. Further exploration demonstrated that apoB48 containing VLDL-type particles have a longer residency time and at fasting, were observed more in overweight subjects with high circulating TG than those with normal TG levels[90]. This evidence may further explain why individuals with insulin resistance and obesity have higher fasting plasma apoB48 compared to normal weight individuals[135]. Overall, there are several

mechanisms by which IR may drive intestinal over production (Figure 1-7). Currently, it is not known whether intestinal over production of CM occurs in youth with pre-diabetes

1.4.4 Mechanisms of Delayed CM Clearance

Delayed clearance of CM remnants may be due to increased VLDL secretion in the liver resulting in competition for lipolysis and clearance pathways. LPL activity has also been shown to be impaired during insulin resistance, partly due to dysfunctional regulation of insulin on LPL activity and altered expression of apoCII and apoCIII lipoproteins on TRL particles. Insulin resistance is also associated with a reduction in LDL-r expression which would delay CM clearance resulting in an increase in circulating CM[136]. Interestingly, during IR there are modest increases in LDL-C compared to the aberrant postprandial dyslipidemia. The binding of remnant lipoproteins to the LDL-r requires 4 binding sites whereas with LDL there is only one. Therefore, changes in LDL-r expression would impact remnant lipoprotein metabolism much more than LDL-C metabolism[136].

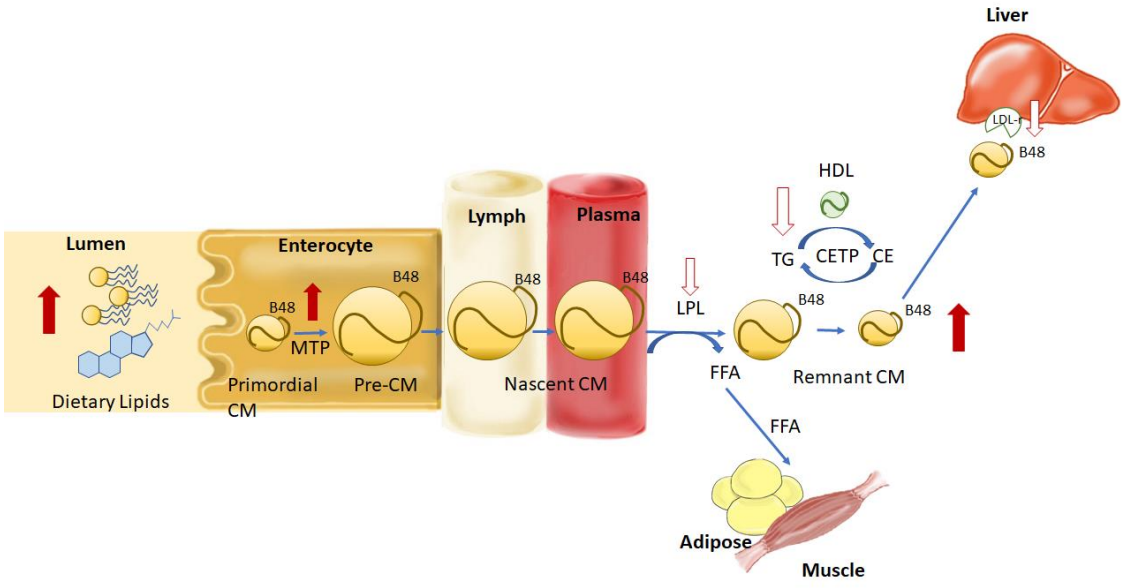

Figure 1-7: *Chylomicron Metabolism During Insulin Resistance*. During obesity and insulin resistance there is an increase in dietary lipid absorption into the enterocyte. MTP mass and activity increases, resulting in increased chylomicron production, assembly, and secretion. Insulin resistance decreases LPL and CETP activity, delaying the lipolysis and removal of TG from the CM particle. LDL-R expression is also decreased during insulin resistance. All the above would result in an increase in circulating remnant CM during insulin resistance. Figure by Jacqueline Krysa©

1.5 Approaches to Modulating PPL Metabolism in Humans

In general, pharmacologic agents that influence lipid metabolism also impact PPL. However, the efficacy of these drugs at lowering PPL differs and there is limited and conflicting clinical evidence. Inconsistencies between studies is likely due to the highly variable study design, test meals and methods by which postprandial lipoproteins are quantified[137]. This is further explored in Chapter 2.

1.5.1 HMG-CoA reductase inhibitors

3-Hydroxy-3-Methyl-Glutaryl-Coeznyme A Reductase (HMGCR) inhibitors or statins are one of the most widely prescribed lipid-lowering agents. Statins primarily target circulating LDL-C and TC by increasing the expression of the hepatic LDL-r. Statins inhibit the rate limiting enzyme in the cholesterol biosynthesis pathway, HMG-CoA reductase. Consequently, cells are depleted of intracellular cholesterol and upregulate pathways in cholesterol clearance from the circulation, mainly the LDL-r. Statins may also influence TRL-remnant metabolism, as remnant particles also use the LDL-r for clearance from the circulation. In one study, statins were found to improve PPL in patients with type 2 diabetes and combined dyslipidemia[138]. In the ACCORD Lipid Study, statin therapy reduced postprandial TG, however this reduction was much greater when statin therapy was in conjunction with fibrates[139]. The effect of statins on PPL is most likely due to the reduction in fasting TG attributed to reductions in VLDL-TG and not CM. Therefore, the modest reduction in PPL from statins is mainly attributed to the reduction in fasting TG, which reduces the competition for lipolysis and clearance pathways during the postprandial period. Importantly, although CM-r are cleared by the LDL-r they are also cleared by the LDL-receptor-related protein, LRP, which is not regulated by statins[137].

1.5.2 Ezetimibe and Fibrates

Ezetimibe selectively inhibits the uptake and absorption of dietary and biliary cholesterol by the enterocyte through inhibition of the protein NPC1L1. Ezetimibe and statin treatment has been shown to significantly reduce fasting and postprandial apoB48 in patients with Type 2 Diabetes[140]. Importantly, coadministration of ezetimibe and statins has also been shown to significantly reduce LDL-C and apoB48 greater than monotherapy. Pre-clinical findings from our laboratory have demonstrated co-administration of ezetimibe and statins also reduces the

atherosclerosis[141]. The above demonstrates that inhibiting dietary cholesterol uptake can have beneficial effects on whole body cholesterol metabolism[142].

Fibrates are agonists of the nuclear transcription factor peroxisomal proliferator activated receptor (PPAR) alpha. PPARα has pleiotropic effects on lipid metabolism including increasing LPL activity and increasing FFA oxidation in the liver. The majority of fibrate studies have been very successful in lowering both fasting and postprandial TG[143,144]. Kinetic studies have demonstrated that fibrates reduce PPL by enhancing the clearance and decreasing the production of VLDL apoB-100[145]. Recently, the Joint International Atherosclerosis Symposium and Residual Risk Reduction Initiative (R3i) released a consensus statement on Selective Peroxisome Proliferator Activated Receptor alpha Modulators (SPPARMα)[146]. It is now recognized that SPPARMα are a distinct therapeutic class from fibrates and may be a promising therapeutic to treat patients with atherogenic dyslipidemia. Currently, the PROMINENT trial is assessing the efficacy and safety of SPPARMα in patients with T2D to understand whether this therapeutic target is beneficial to reduce residual risk[146].

1.5.3 Niacin

Niacin (nicotininc acid; vitamin B3) has found to be effective at PPL and increase HDL cholesterol[147,148] and its effect is even more pronounced when coupled with pravastatin[149]. However, the precise mechanisms by which niacin reduces PPL remain unclear. Niacin is thought to reduce hyperlipidemia by inhibiting hormone-sensitive lipase in adipose tissue, which would decrease FFA flux to the liver and intestine and reduce TG synthesis.[150]. Niacin has also been shown to inhibit DGAT2 in cell culture, which may influence the synthesis of TG and CM secretion[151]. It is also suggested that niacin may increase the catabolic rate of both apoB100 and apoB48 TRL particles[152]. Our laboratory demonstrated that niacin significantly reduces fasting plasma and lymph CM apoB48 while concomitantly increasing lymphatic HDL secretion in an insulin resistant rodent model. The increase in HDL was accompanied by increases in *PPARα*, which would enhance fatty acid oxidation. These results suggest that, by raising lymphatic HDL secretion, niacin supplementation may also enhance reverse cholesterol transport and fatty acid oxidation to reduce plasma cholesterol and postprandial lipemia[67].

1.5.4 Fish oil

Fish oil has been shown to significantly reduce postprandial TG in several clinical trials, while inconsistently influencing apoB48 metabolism. The active components of fish oil are long chain n-3 fatty acids including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Fish oil has been shown to reduce TG synthesis by inhibiting DGAT, fatty acid synthase, and acetyl CoA carboxylase enzymes, while enhancing PPARα to increase fatty acid oxidation. Fish oil has also been shown to reduce TG by supressing the activity of the nuclear transcription factor SREBP1-c, which results in a reduction in *de novo* lipogenesis[153]. Fish oil may also increase the degradation of newly synthesized apoB, thereby decreasing the amount of circulating apoB[154]. In obese rodents with MetS, n-3 polyunsaturated fatty acid (PUFA) supplementation was found to improve both fasting and postprandial TG, cholesterol and apoB48 potentially through downregulation of hepatic and/or adipose expression of lipogenic enzymes[134]. In humans, it has also been demonstrated that n-3 fatty acids can decrease TRL apoB48 by decreasing apoB48 secretion[155]. However, in insulin resistant men with visceral obesity, chronic fish oil supplementation did not resolve elevated postprandial apoB48 but did improve postprandial TG[156]. A recent clinical trial investigated the effects of icosapent ethyl, a highly purified eicosapentaenoic acid ethyl ester (Vascepa), on risk of ischemic events in patients with elevated triglycerides. Individuals that received 4g of Vascepa daily had a significant reduction in fasting TG as well as a significant decrease in ischemic events and death from CVD[157]. The results of this study suggest that by lowering TG, a concentrated EPA isolate can reduce CVD. These findings further underscore the importance in lowering fasting and postprandial TRL, including apoB48 containing lipoproteins, to reduce CVD[157]. Currently, it is not known whether n-3 PUFA from fish oil will reduce fasting and/or non-fasting and/or remnant lipoproteins in children who are overweight or obese.

1.5.5 Novel Pharmacological Therapies

Novel pharmacologic therapies provide insights to mechanisms influencing PPL metabolism and several have shown promising effects at lowering PPL.

1.5.5.1 ApoCIII Inhibitor

ApoCIII is an LPL inhibitor and as such, inhibition of apoCIII increases LPL activity. Individuals deficient in *APOC3* were found to have a blunted postprandial response in plasma TG following a high fat meal compared to those without mutations in the *APOC3* suggesting that apoCIII inhibition would be a promising target to reduce PPL[158].

1.5.5.2 ANGPTL3 Inhibitor

LPL is also regulated by angiopoietin-like (ANGPTL) proteins. Individuals deficient in *ANGPTL3* have reductions in all major lipids and lipoproteins and have significantly reduced PPL due to increased catabolism of CM particles[159].

1.5.5.3 GLP-1 Agonists

GLP-1 is an incretin hormone released postprandially that enhances insulin secretion. Intravenous administration of GLP-1 into healthy subjects has been shown to significantly reduce postprandial TG[160]. GLP-1 analogues are novel pharmacologic agents that have been used to combat obesity and reduce cardiovascular risk in patients with type 2 diabetes. The GLP-1 analogue, Liraglutide, was also found to significantly reduce PPL in patients with T2D following a high fat meal[161].

1.5.5.4 SGLT2 Inhibitors

Inhibition of the sodium-glucose transport protein 2 (SGLT2) has recently become a promising therapeutic target to treat type 2 diabetes. SGLT2 inhibitors inhibit the reabsorption of glucose in the kidneys thereby reducing blood glucose levels. The SGLT2 inhibitor Exenatide was found to significantly reduce PPL following a high fat meal in patients with impaired glucose intolerance and type 2 diabetes[162]. It is hypothesized that SGLT2 inhibitors reduce PPL by slowing gastric emptying, which results in delayed nutrient transport into the small intestine[163].

1.5.5.5 PCSK9 inhibitors

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a protein that promotes the degradation of the LDL-r and has become a novel and successful therapeutic target to reduce LDL-C and CVD. TRL remnant particles also use the LDL-r for clearance and therefore, PCSK9 inhibition may also reduce PPL. Recent findings found that individuals with loss of function

variants (LOF) in *PCSK9* have significantly reduced PPL compared to those without variants[164]. Another study found no differences in apoB48 after an oral fat challenge in healthy normolipidemic individuals following an 8 week monoclonal antibody therapy with evolocumab[165]. The authors speculate that PCSK9 monoclonal antibodies do not influence remnant lipoprotein metabolism because they do not influence lipolytic pathways such as apoCIII and ANGTPL3[165]. However, another recent study determined that patients receiving at least 3 doses PCSK9 inhibitors (evolocumab or alirocumab) had a significant reduction in plasma non-fasting remnant cholesterol and TG following PCSK9 inhibition[166]. Therefore, there is mixed evidence on the efficacy of PCSK9 inhibitors to reduce non-fasting lipids.

Drug Treatment	Mechanism of Action to Reduce PPL
Statins	Inhibits HMG-CoA Reductase
Ezetimibe	Blocks NPC1L1 transporter
Fibrates	PPAR-α agonist
Bile Acid Sequestrants	Binds to Bile Acids to reduce lipid absorption
Niacin	Reduces fractional catabolic rate of apoA-I, DGAT2, and Hormone Sensitive Triglyceride Lipase
Plant Sterols and Stanols	Decreases cholesterol incorporation into mixed micelles
Fish oil	Inhibits DGAT2, fatty acid synthase, acetyl-CoA carboxylase, and SREBP-1. Enhances PPAR-α activity

Table 1-2: *Current Drug Therapies to Target PPL*

1.6 PCSK9 Metabolism and Role in CVD Pathophysiology

1.6.1 PCSK9 Metabolism and Mechanism of Action

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a serine protease involved in the regulation of hepatic apoB-lipoprotein uptake and cholesterol metabolism via the LDL-r. Since the discovery of PCSK9 in 2003, it has been investigated as a target to reduce plasma cholesterol concentrations, particularly LDL-C and associated CVD risk[167,168]. *PCSK9* is primarily expressed and secreted from the liver, but is also expressed in smaller amounts in the small intestine, kidney, and brain[167]. The liver and the small intestine have major inter-dependent roles in whole-body cholesterol homeostasis, particularly in relation to apoB-lipoprotein-cholesterol metabolism[169]. Overall, an upregulation of *PSCK9* mRNA expression has been shown to be associated with an increase in LDL-r degradation, resulting in decreased uptake of cholesterol dense apoB-lipoproteins, and an increase in the plasma concentration of LDL-C[170]. PCSK9 is produced as a zymogen precursor that undergoes 2 consecutive catalytic cleavages in ER resulting in the release of an active PCSK9 protein into the circulation. Circulating PCSK9 binds to the cell surface by attaching to the first epidermal growth factor-like repeat (EGF-A) domain of the LDL-r. The PCSK9-LDL-r complex is internalized into the cell. When PCSK9 is bound to the LDL-r it inhibits the endocytic recycling of the LDL-R back to the cell surface and results in the lysosomal degradation of both proteins[171]. The degradation of the PCSK9-LDL-R complex results in a reduction in hepatic LDL-r on the cell surface and a reciprocal increase in circulating LDL-C and remnant lipoproteins. Accordingly, the reciprocal relationship between the LDL-r and PCSK9 can be viewed as a counter-regulatory mechanism to maintain cholesterol homeostasis and constant LDL-C concentrations. Offsetting this balance in cholesterol homeostasis, for example in individuals overexpressing PCSK9, results in excess LDL-C due to increased LDL-R degradation and reduced apoB-remnant cholesterol clearance and subsequently increases the risk of developing CVD[172–174] (Figure 1-8).

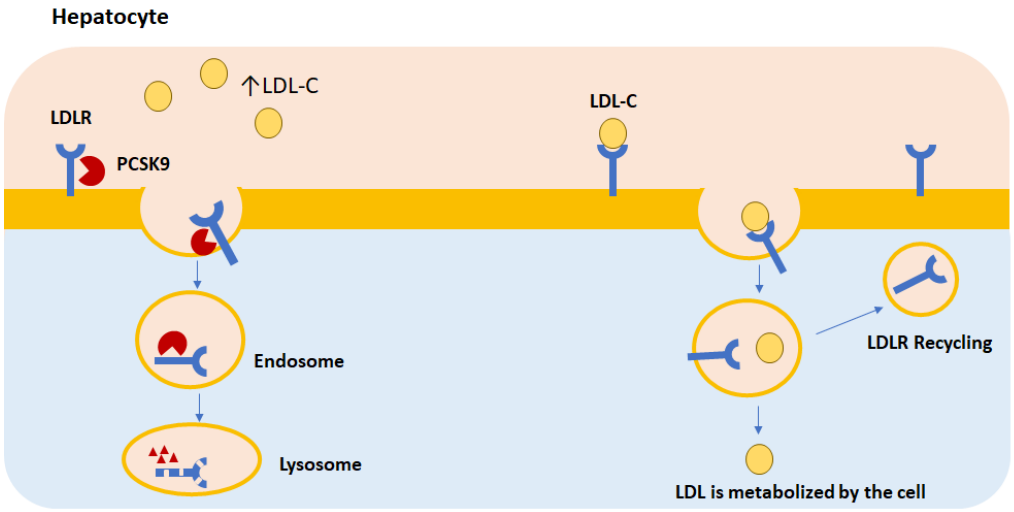

Figure 1-8: *PCSK9 Mechanism of Action*. Circulating PCSK9 binds to the EGF-A domain of the hepatic LDL-R. PCSK9 prevents the recycling of LDL-R in the endosome and results in the shuttling and degradation of the LDL-R in the lysosome thus reducing the overall hepatic surface expression of the LDL-R and increasing circulating LDL-C levels. Figure adapted from MBL International Corporation.

1.6.2 Influence of PCSK9 on Circulating Lipids

PCSK9 activity and secretion is directly involved in cholesterol homeostasis[175]. Plasma PCSK9 concentrations have been positively correlated with diurnal variations in hepatic cholesterol synthesis and plasma LDL-C concentrations as well as the secretion of TRL particles[176]. Indeed full knock out of *PCSK9* in mice results in severe hypocholesterolemia corresponding to an almost 80% reduction in circulating LDL-C and 40% in total cholesterol[177]. Interestingly, a liver specific knockout in *PCSK9* resulted in only a 30% reduction in total cholesterol and an attenuated phenotype of the full body knock out, which suggests that PCSK9 plays an important role in full body lipid metabolism[178].

Alongside its strong influence on LDL-C, PCSK9 also influences other lipoprotein fractions. Individuals with gain of function (GOF) variants in PCSK9 are found to have increased levels in all apoB100 containing lipoproteins (VLDL, and VLDL remnants)[179]. Additionally, monoclonal antibody therapies against PCSK9 are found to modestly reduce plasma TG[180]. The LDL-r has been shown to promote the degradation of presecretory apoB. Accordingly, PCSK9-mediated degradation of hepatic LDL-r can promote hypertriglyceridemia by reducing the degradation of apoB and reducing the update of TRL[181]. PCSK9 is also produced by the small

intestine and has been shown to have a similar effect on plasma TRL concentrations. PCSK9 has been shown to enhance the production and secretion of intestinal TRL through transcriptional and posttranscriptional mechanisms. Indeed, knockout of *PCSK9* in mice resulted in significant reductions in plasma apoB48 and postprandial TGs[182]. However, it is unknown if reductions in PCSK9 would impact intestinal TRL metabolism in humans.

1.6.3 Pharmacologic Inhibition of PCSK9

PCSK9-inhibitors have emerged as treatments for dyslipidemic patients, particularly in those who are statin-resistant or have severe dyslipidemia, such as FH[183,184]. Therefore, PCSK9 inhibitors have been rapidly developed and are currently in clinical use. Thus far, there have been 2 major PCSK9 inhibitor safety and efficacy studies[185,186] as well as studies evaluating the effects of PCSK9 inhibitors on atherosclerosis[187], on cardiovascular disease outcomes[188] and others confirming the lipid lowering effect of PCSK9 inhibitors[189]. Evolocumab and Alirocumab, are monoclonal antibody therapy against PCSK9 developed by Amgen and Sanofi pharmaceuticals, respectively. Evolocumab compared to standard therapy was found to reduce LDL-C by 61% and was additionally found to promote atheroma regression in more than 80% of subjects[187]. The Further Cardiovascular Outcomes Research with PCSK9 Inhibition in Subjects with Elevated Risk (FOURIER) study examined cardiovascular outcomes in patients on the PCSK9 inhibitor Evolocumab and found that after 48 weeks, LDL-C was reduced by 59% and reduced the risk of adverse cardiovascular events by 15%[188]. Similarly, Alirocumab was found to significantly reduce LDL-C by approximately 60% and also significantly decreased the risk of occurring ischemic CV-events in patients who have had previous acute coronary syndrome[190]. Current recommendations state that PCSK9 inhibitors should be used in patients with ASCVD with elevated LDL-C, additional ASCVD risk factors, and on maximum tolerated statin therapy as well as individuals with heterozygous hypercholesterolemia on maximum tolerated statin therapy[191]. Overall, PCSK9 long term safety trials are still being investigated and optimal treatment plans for patients are still unclear. At the moment, it is recommended that physicians deal with PCSK9 therapy on a per-patient basis and consider the number needed to treat to benefit the patient, the cost of the therapy, the need for subcutaneous injection, and important of follow-up lipid monitoring[191].

1.6.4 Genetic Variants in PCSK9 and Influence on CVD Risk

Genetic studies of *PCSK9* (including genome-wide association studies and Mendelian randomization trials) have identified polymorphisms of *PCSK9* that significantly impact CVD risk[192,193]. The gene for PCSK9 is highly polymorphic[6]. Populations have variants that result in a more functional or increase in circulating PCSK9 (GOF) or, conversely, a less functional or decrease in circulating PCSK9 (loss-of-function (LOF))[7]. LOF variants are more common than GOF variants. GOF mutations often result in FH and exacerbated CVD risk[172]. PCSK9-LOF results in greater expression of hepatic LDL-r, lower concentrations of LDL-C, and protection from CVD[8,9]. These studies have provided direct evidence that inhibition of PCSK9 to reduce the degradation of the LDL-r may be beneficial in regulating cholesterol metabolism and prevention of CVD. It is not known if gene variants in PCSK9 are related to PPL. The 4th chapter of my thesis was designed to demonstrate that LOF variants in PCSK9, alongside reduced LDL-C, have lowered PPL, which may contribute to the overall reduction in CVD risk seen in these individuals.

1.7 Postprandial Lipemia in Youth

1.7.1 Evidence for the Role of apoB-Remnants in Childhood Obesity

Despite the convincing evidence in adults, it is unclear whether apoB48-remnant cholesterol contributes to subclinical CVD risk in youth. Recent studies have demonstrated fasting plasma apoB48 concentrations are elevated two-fold in obese pre-pubertal children (case-control study) and is more strongly associated with central adiposity indices compared to LDL-C or TC at this age[47,48]. Our results to date suggest fasting plasma apoB48 may be a representative biomarker of enhanced subclinical CVD risk in youth, however it is unclear if this relationship remains relevant in the larger general population. The focus of chapter 5 in this thesis aims to determine the association between fasting apoB48 and cardiometabolic risk in a large, adolescent population.

1.7.2 Targeting Postprandial Lipemia in Youth with the Metabolic Syndrome

The adult definition of MetS cannot be applied to the pediatric population due to the large variations in blood pressure, lipids and body size during development. Puberty results in large changes in circulating lipids and body fat distribution, making a universal definition of the MetS in this population difficult (described further below). The current definition of MetS in the pediatric population according to the International Diabetes Federation divides youth into three

age categories 6-<10, 10-<16 and >16 years, with those over 16 years of age being defined by adult definitions of the metabolic syndrome. For those youth age 10-<16 years the metabolic syndrome can be diagnosed by abdominal obesity measures, elevated fasting triglycerides, low plasma HDL-C, elevated blood pressure, and elevated fasting plasma glucose. However, in youth ages 6-<10 years only waist circumference is included in the definition of the MetS as other metabolic measures cannot be accurately diagnosed as high-risk[194]. Because of the complex nature of the MetS in youth, several novel biomarkers have been proposed to help detect and predict this complex clustering of metabolic conditions including: adipokines such as adiponectin and leptin, as well as microalbumin, gut peptides (including GLP-1 and GLP-2), and lipoprotein markers such as apoB48[195]. Remnant lipoproteins (RLP) are comprised of VLDL and CM remnants and have been shown to be significantly elevated in obese youth and strongly correlate with insulin resistance[196]. Our laboratory has also demonstrated that fasting apoB48 is significantly elevated in pre-pubertal youth with obesity[47], and tracks with central obesity in youth[48]. The above evidence suggests that youth with characteristics of the metabolic syndrome may exhibit elevated remnants lipoproteins. We know in adults that elevated remnant chylomicrons predict PPL, therefore it is likely that youth with characteristics of the MetS also display PPL and further, we can potentially target this in to lessen their cardiometabolic burden in early adulthood[98,197]. Chapter 5 and 6 in this thesis will explore whether youth with obesity have elevated CM-remnants and have PPL. The findings from these studies will help further elucidate the mechanisms behind early MetS and may provide more precise biomarkers to target for MetS risk reduction at this age.

1.8 Childhood Obesity

1.8.1 Definition and Prevalence

In children and adolescents the prevalence of overweight-obesity continues to remain at high levels in developed countries[198]. In Canada, the prevalence of overweight and obesity in children is nearly 27%, with obesity prevalence stabilizing at about 13% from 2004-2013[199]. Childhood obesity according to the World Health Organization is defined as a body mass index (BMI) ≥95 percentile[200,201].

1.8.2 Adverse Adult Health Outcomes

Early adiposity, particularly central adiposity, is a significant public health concern as it often progresses into adulthood and tracks with a cluster of cardiometabolic abnormalities that

contribute to premature CVD risk[198,201]. Obesity persists from childhood to adulthood, such that the likelihood of obese children becoming obese adults increases from 20% at age 4 to 80% by adolescence[201]. Childhood obesity drives a clustering of metabolic disturbances that promote complications associated with obesity including MetS. The metabolic syndrome includes insulin resistance, dyslipidemia, hypertension, and central obesity. Several of the metabolic complications of chronic diseases have their origins in childhood[202]. Importantly, early alterations in glucose metabolism and fat infiltration of the liver can lead to the development of non-alcoholic fatty liver disease (NAFLD). As discussed earlier, atherosclerosis has its origins in childhood and during childhood obesity the atherosclerosis process is accelerated. Despite this, the clinical manifestations of atherosclerosis do not appear in youth and impaired glucose tolerance and NAFLD have no clinical manifestations[203]. Therefore, it is critical to develop appropriate screening and diagnostic measures in children with obesity to detect the presence of these adverse metabolic complications (Figure 1-9)

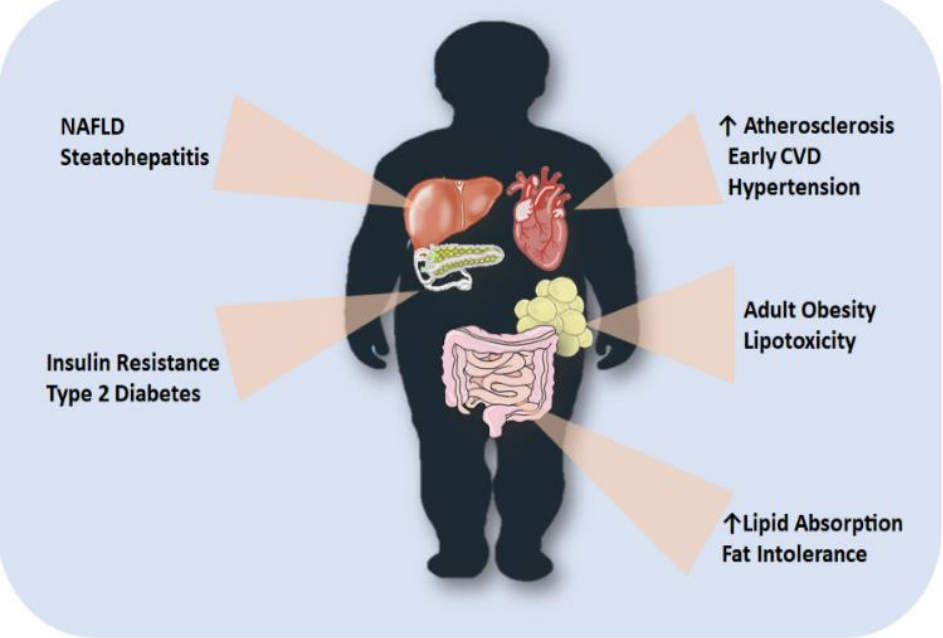

Figure 1-9: *Childhood Obesity and Adverse Adult Metabolic Health Outcomes.* Obesity during childhood can promote adverse changes to metabolism that can enhance the risk of develop metabolic disorders in adulthood. Early obesity can promote early insulin resistance, which can lead to the development of T2D and NAFLD. Additionally, enhanced adiposity in youth has been associated with enhanced chylomicron metabolism, which may increase atherosclerosis and promote early CVD. Figure by Jacqueline Krysa©.

1.8.3 Proposed Pathophysiology of Childhood Obesity

The pathophysiology of childhood obesity is multifactorial and is influenced by genetic and non-genetic factors[204]. The combination of these genetic and non-genetic factors influences energy consumption and expenditure[92]. Lifestyle factors that contribute to early obesity including lack of physical activity and unhealthy eating patterns that promote an excess energy balance contributing to weight gain. Genetic and social factors are also important determinants of weight in children[200]. Due to the complex nature of the etiology of childhood obesity, it is difficult to isolate a single-risk factor[204]. There are few monogenic conditions in which obesity is the specific outcome. Monogenic conditions that arise in obesity are detectable at very young ages in children. Single gene defects in the melanocortin-4 receptor are responsible for 5-6% of early-onset pediatric obesity, others include Prader-Willi Syndrome, Bardet-Biedl and Alstrom Syndrome[205]. Recent research also demonstrates the important of epigenetic risk factors on childhood obesity. Intrauterine exposures to gestational diabetes and maternal adiposity are found to result in higher or lower birth weight of the child, which can ultimately promote obesity due to adverse epigenetic programming[206]. Childhood obesity can also be promoted or enhanced by endocrine disorders including hypothyroidism, growth hormone deficiency, and polycystic ovarian syndrome[200,207].

1.8.4 Lipoprotein Metabolism during Pubertal Development

Lipoprotein metabolism has been shown to be significantly altered during puberty. A longitudinal study by Eissa and colleagues examined the changes in plasma lipid levels during puberty. Results from this study demonstrated that total cholesterol, LDL-C, and non-HDL were all significantly decreased during puberty while changes in HDL-C and TG varied between males and females[208]. These results are consistent with other studies that also demonstrate a decline in TC, LDL-C and non-HDL-C during puberty[209–211]. However, the mechanisms by which lipids decline during puberty is still not well understood and for my thesis, is the part of premise for chapters 5 and 6 in this thesis. Puberty is well known to be a transient period of insulin resistance characterized by decreased peripheral insulin sensitivity and enhanced insulin secretion, which is found to resolve itself by the end of the pubertal period[212]. Increased insulin secretion during puberty is thought to maintain glucose homeostasis during times of rapid growth and development[213]. Pubertal IR is also characterized by decrease glucose oxidation and a reduction in the insulin-suppression of free fatty acid oxidation. Due to this, increased fat oxidation will

compete with glucose oxidation to reduce glucose uptake and enhance insulin resistance[213]. Interestingly, the IR during puberty is not associated with sex hormones or percent body fat, but may possibly be related to the growth hormone and insulin-growth-factor-I axis[214]. Growth hormone administration has been shown to significantly reduce LDL-C and total cholesterol in part by enhancing hepatic LDL-r activity[215].

1.8.5 Universal Lipid Screening in Children

Currently, blood lipid screening is only recommended for selected high-risk children or adolescents with a family history of CVD of FH[216]. Consequently, an adverse blood lipid profile may go undiagnosed or may be underestimated in a significant portion of this population, particularly in youth with overweight-obesity[216]. Furthermore, there are limited international clinical practice guidelines for assessment of blood lipids and lipid lowering strategies for youth[217]. The National Cholesterol Education Program recently added decision limits for fasting plasma non-HDL-C ($\geq$ 2.8 mmol/L), total apoB ($\geq$ 2.8 mmol/L) and TG (0-9 years: $\geq$ 2.6 mmol/L; 10-19 years: $\geq$ 3.4 mmol/L) relevant to youth[216]. However, diagnostic and treatment cut-offs primarily use fasted plasma LDL-C concentrations[216,218]. Statin therapy can be used in youth $\geq$ 10 years if LDL-C is $\geq$ 4.9 mmol/L or LDL-C is $\geq$ 4.1 mmol/L and is accompanied by 1 or more additional risk factors and/or the presence of heterozygous FH[216]. However, LDL-C and TC have been shown to remain unchanged or decrease during adolescence[208]. Therefore, other lipid biomarkers may be more representative of subclinical CVD risk during this age[216]. Novel data collected during the course of this thesis contributes to this hypothesis and aims to provide evidence that non-fasting apoB-remnant cholesterol may be a promising early CVD-risk marker for this younger population.

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Chapter 2: Rationale, Objectives, and Hypothesis

2.1 Introduction to Research Hypothesis

Cardiovascular disease (CVD) remains a leading cause of morbidity and mortality worldwide. An important contributor to the pathogenesis of CVD is an increase circulating lipids (dyslipidemia) inclusive of traditional lipid risk markers (low density lipoprotein cholesterol (LDL-C) and total cholesterol (TC)), as well as novel lipid risk markers (remnant cholesterol)[1]. It is well established that both LDL-C and remnant cholesterol contribute to the formation of atherosclerotic plaques[2]. Fasting LDL-C is the primary screening tool and treatment target for dyslipidemia, yet, substantial proportions of the population (including those with insulin resistance and diabetes) still suffer residual CVD risk despite normal fasting LDL-C values[3]. Community and population studies from Europe have recently demonstrated that elevated non-fasting remnant cholesterol is causally associated with major cardiovascular events (MCEs)[4]. Interestingly, in some cases, the relationship between remnant cholesterol and MCE is greater than that of fasting lipid parameters[5]. Due the increased awareness of remnant cholesterol, non-fasting lipid assessment has been added to lipid screening guidelines in Europe[6] and more recently, to Canadian guidelines[7]. The recent Canadian Health Measures Survey (CHMS) developed reference intervals for several non-fasting lipids in approximately 12,000 Canadians ages 3-79 years[8]. Despite this, there remains no decision limits of non-fasting lipids in Canadian adults and limited research investigating remnant cholesterol metabolism in youth and its relationship to early cardiometabolic risk. While adverse CVD events typically manifests in adulthood, there is strong evidence that the etiology of heart and vascular diseases begin in childhood[8]. Childhood obesity is a growing concern in most developed countries and often persists into adulthood and enhances CVD risk[9]. Fasting total cholesterol and LDL-C are often used by pediatricians to identity children at risk of developing CVD[10]. However, these parameters do not accurately reflect the subclinical progression of CVD in pediatrics and are often normal in overweight/obese children, suggesting that other lipid pathways may be involved[11,12]. We know that adults with CVD have elevated remnant particles (assessed as fasting and non-fasting apoB48), which may be caused by an impairment in postprandial dyslipidemia (higher than normal blood concentrations of remnants and lipids following a lipid-rich meal) and/or intestinal overproduction[13]. Our laboratory also has data demonstrated that fasting apoB48 is elevated in obese pre-pubertal children[11], obese female

adolescents[14], and tracks with early increases in central adiposity in children[12]. Collectively, our current data suggests children and adolescents have altered intestinal lipid metabolism and postprandial dyslipidemia, which may be an early subclinical CVD risk marker.

2.2 'Residual risk' of CVD after LDL Cholesterol Lowering

Most clinical trials that target lipid management for CVD reduction focus primarily on lowering fasting plasma LDL-C[15]. LDL-C lowering has consistently demonstrated to improve most CVD morbidity and mortality in the general population[16]. However, even with low LDL-C, sub-sets of high-risk patients continue to be at a high risk for CVD, which is termed as residual risk. Indeed, almost two-thirds of adverse CVD events occur despite a lowering of LDL-C and is most commonly observed in individuals with diabetes or the metabolic syndrome[17]. It is now recognized that lowering LDL-C (via statin-therapy) is not an optimal treatment target for patients with diabetes and/or the metabolic syndrome and suggests that alternative lipid targets must also be lowered to prevent residual-risk[18].

2.3 Role Non-Fasting Remnant Cholesterol in CVD Pathogenesis and CVD risk prediction

Approximately 60% of individuals with type 2 diabetes have elevated TG accompanied by LDL-C levels below 100 mg/dL (2.59 mmol/L), which is within the normal range[19]. While TGs themselves are not atherogenic *per se*, plasma TGs have been shown to independently predict CVD mortality independent of other lipid fractions[20]. Non-fasting TGs, in particular, have been demonstrated to predict CVD mortality better than fasting LDL-C[21]. Non-fasting TGs are representative of triglyceride rich lipoproteins (TRLs) and their remnants[22]. Remnant cholesterol is the cholesterol derived from TRL particles (chylomicrons (CM) and very low-density lipoprotein (VLDL)) that have been lipolyzed in the plasma to small, cholesterol dense particles. Like LDL-C, remnant cholesterol can be taken up and retained within the arterial wall and contribute to atherogenesis[2]. Unlike, LDL-C, remnant cholesterol does not need to be oxidized to become incorporated into foam cells[23]. Recent data from Europe has demonstrated that circulating remnant cholesterol can independently predict CVD risk and appears to be a better predictor of CVD risk than LDL-C[4]. Indeed, for every 1 mmol/L increase in non-fasting remnant cholesterol, there was found to be a 2.8 fold increase for ischemic heart disease[24]. The above demonstrates that

remnant cholesterol is directly involved in CVD pathophysiology and is a very strong predictor of CVD morbidity and mortality.

Critical Note: *Although non-fasting remnant cholesterol has been shown to be a significant and independent predictor of CVD in Europe, it is not known whether this relationship will be maintained in a Canadian population. Furthermore, there are no decision limits of remnant cholesterol or non-fasting lipids in North America, particularly in Canada.*

2.4 PCSK9 as a Novel CVD Risk Marker

Recently, PCSK9 has become a promising lipid lowering target. Circulating PCSK9 reduces LDL-Rr expression on the hepatic cell surface, which in turn increases circulating LDL-C[25]. Individuals with almost complete LOF mutations in *PCSK9* have an observed 80% reduction in CVD over the life time, accompanied by a significant reduction in circulating LDL-C[26]. *PCSK9*-LOF have no apparent adverse health effects, which has spurred the rapid development of PCSK9 inhibitors. Pharmacological blockade of PCSK9 has been very successful in significantly reducing both circulating LDL-C and the occurrence of adverse CV events in patients[27]. Overall, PCSK9 remains a promising, novel therapeutic target to treat dyslipidemia and CVD.

Critical Note: *The substantial reduction in CVD in PCSK9-LOF are not likely due to reduced circulating LDL-C alone. Therefore, other lipid targets may also potentially be reduced by PCSK9-LOF. Remnant lipoproteins also utilize the LDL-r for clearance, and therefore may be affected by modulating circulating PCSK9. To date, no one has analyzed postprandial lipid metabolism in PCSK9-LOF.*

2.5 Postprandial Lipemia During Obesity and Diabetes

The transient increase of postprandial TGs and apoB48 containing CMs following a lipid-meal is a normal physiological process. However, in individuals with obesity[28], diabetes[29], the metabolic syndrome[30] and/or normolipidemic individuals with CAD[31], there is an exaggerated and prolonged increase in TG and apoB-remnant lipoproteins following a high-fat meal. The above physiological phenomena is termed postprandial dyslipidemia (PPL) and is positively correlated with the progression of CAD and coronary atherosclerosis in adults[32,33]. Interestingly, fasting apoB48

strongly correlates with PPL following a lipid-rich meal in adults, which suggests that there is an accumulation of CM-remnants particles during these metabolic disorders[28]. There are several mechanisms proposed to explain PPL during conditions of metabolic dysfunction including: delayed particle clearance, increased particle production, and delayed particle lipolysis, which are explained in further detail in Chapter 1. The above demonstrates that adults with obesity and diabetes have altered CM metabolism due to metabolic complications of obesity and/or insulin resistance, which may promote PPL in these individuals and further enhance their atherogenic risk[34]. Recently, our laboratory has also shown that adolescent girls with obesity, as well as those with polycystic ovary syndrome (PCOS) may have PPL[14]. The following suggests that the pathophysiological phenomena of fat intolerance may have its origins early in life.

2.6 Modifying Known CVD Risk Factors Influences Postprandial Lipemia

Well established CVD risk factors including age, sex, menopausal status, and lifestyle factors are all known to influence PPL. Moreover, most therapeutics that influence lipid metabolism also reduce PPL including omega-3 fatty acids[35], niacin, fibrates[36], and ezetimibe[37]. Lifestyle interventions such as diet and exercise[38], reductions in alcohol intake[39,40], and smoking cessation also reduce PPL[41,42]. Overall, this demonstrates that changing modifiable risk factors for CVD also changes PPL, underscoring the role of PPL in CVD pathophysiology[13]. Further research needs to investigate whether novel CVD risk factors also influence PPL.

2.7 Methods to Assess Postprandial Lipemia, and Clinical Challenges

PPL can be determined by calculating the area under the curve (AUC) of the postprandial response following a lipid-rich meal[43]. The calculated AUC of a postprandial lipid curve allows one to measure the overall residency time of atherogenic particles and assess the extent of PPL following a lipid-rich meal. However, assessing PPL in the clinic is difficult and time consuming. Historically, there have been several technical challenges to assess PPL in humans[29]. Researchers have used many different methodologies in the past to quantify postprandial lipid metabolism including remnant lipoprotein cholesterol (RLP-C)[44], retinyl esters[45], and emulsions[46]. However, these methods are complex, time consuming and are indirect measures intestinal derived chylomicrons, which can be confounded by other lipid fractions. Recently, direct measures of chylomicron metabolism have become available for commercial use including the development of human monoclonal against apoB48. A human apoB48 antibody allows one to specifically measure

the amount of chylomicron particles in plasma[47]. Furthermore, a measurement of fasting apoB48 in the fasted state has been shown to strongly correlate to the postprandial apoB48 response following a high fat meal and can therefore be used to predict PPL in a timelier manner than previous methods[28]. More clinically revenant approaches to assess PPL have also been developed including C^{13} breath test[48] and the capillary finger prick method[49]. Cardiochek® PA is a handheld monitor developed by Roxon (PTS Diagnositcs). Cardiocheck can perform instant read-outs of TG and lipid panels from small amounts of plasma[50]. Non-fasting TG levels, although not a direct measurement of CM particles, could be applied to out of hospital situations, in the clinic, and for self monitoring of PPL[51].

2.7.1 Fat Tolerance Testing and Meal Volume Standardization

To assess PPL a standardized fat tolerance test (FTT) is provided and the postprandial TG and response is determined 8 hours following the consumption of the meal[53]. TG concentrations at 4 hours following an FTT is considered the best representation of PPL[54]. The postprandial TG response is dependent on the amount of dietary fat consumed, and thus, standardization of the test meal is paramount to study PPL[53]. According to an expert panel on postprandial TGs, it is recommended that a standardized FTT is a single fat load after an 8 hour fast. Additionally, the expert panel suggests that the FTT contains approximately 75g of fat (or adjusting by body weight as 1g fat/kg body weight[55]) and should be a mixed meal containing 25g of carbohydrates and 10g of proteins as they aid in the digestion of dietary fat[54]. The meal should also contain both unsaturated and saturated fatty acids[54]. Despite this, there remains uncertainty whether to adjust the standardized meal test by body weight or body surface area (BSA) or to not adjust the meal volume at all[54]. It has been proposed to adjust meals by body weight or BSA because in animal models, obesity is associated with increased intestinal absorption and permeability[56]. Furthermore, in individuals with obesity there is increased gastric emptying, however, this was not correlated with BSA[57]. It has also been demonstrated in individuals without obesity that intestinal transit time of nutrients was directly proportional to their body weight[57]. Therefore, adjusting meal tests by body weight or BSA may compensate for increased gastric emptying in individuals with a higher body weight and consider the distribution of ingested fat[58]. However, calculations in BSA have recently been challenged, as they often rely of formulas that may not accurately reflect the actual BSA on the individual which may result in over-or under dosing[59]. It has been also suggested that

adjusting meal volume for body weight may confound study results as one would expect a higher postprandial lipid response when providing more dietary fat, as the majority of dietary TGs are absorbed by the small intestine[60]. Therefore, more research is needed to understand whether adjusting an FTT for body weight or BSA is necessary.

Critical Note: *PPL has been shown to be a significant CVD risk factor in adults. The recent development of human monoclonal against apoB48 allows high-throughput measurement of apoB48 in large populations, which can be used to evaluate plasma concentrations of apoB48 containing CM remnants and PPL. To date, no study has measured apoB48 remnants in a large cohort of youth, nor is it not known if whether high concentrations of apoB48 remnants cluster with aspects of the metabolic syndrome in obese adolescents.*

2.8 The Role of Chylomicron Metabolism in Early Cardiometabolic Risk

Despite the growing importance of targeting PPL in adults at high CVD risk, there is little research on the role of PPL in early cardiometabolic risk. Childhood obesity is a growing concern worldwide and greatly increases a child's risk of developing cardiometabolic complications later in life[52]. Currently, there is limited knowledge on atherosclerosis pathophysiology in children as well as few metabolic markers that reflect the obese condition in children[53]. Indeed, fasting TC and LDL-C, which are commonly used to assess CVD-risk in adults, are often normal in obese children and do not track with obesity at this age[11]. Recent studies from our laboratory have demonstrated that apoB48 remnants are significantly elevated in obese children compared to normal weight peers independent to elevations in fasting LDL-C or TC[11]. Additionally, we have shown that following a 2 year-follow up period in youth, apoB48 was the only fasting lipid risk marker to increase with age, and significantly tracked with positive changes in central adiposity[12]. Our laboratory has also demonstrated that adolescent girls with PCOS have elevated postprandial remnant lipoproteins compared to their normal weight peers[14]. Therefore, chylomicron metabolism may be altered during childhood obesity, independent of liver lipid metabolism, and may be a promising target for screening and therapeutic interventions for early cardiometabolic risk.

Critical Note: *Although PPL is a known CVD risk factor in adults with obesity and diabetes, it is not known whether youth with obesity have PPL. Furthermore, it is also unclear whether apoB48 remnants are predictive of PPL at a younger age.*

2.9 General Working Hypothesis

Elevations in apopB48 containing CM-remnants are predictive of metabolic dysfunction and cardiovascular disease risk. Importantly, fasting CM-remnants significantly predict PPL, which is an integral part of early and late cardiovascular disease risk progression. Therefore, we can utilize non-fasting lipid measurements to better capture cardiovascular disease risk in children and adults.

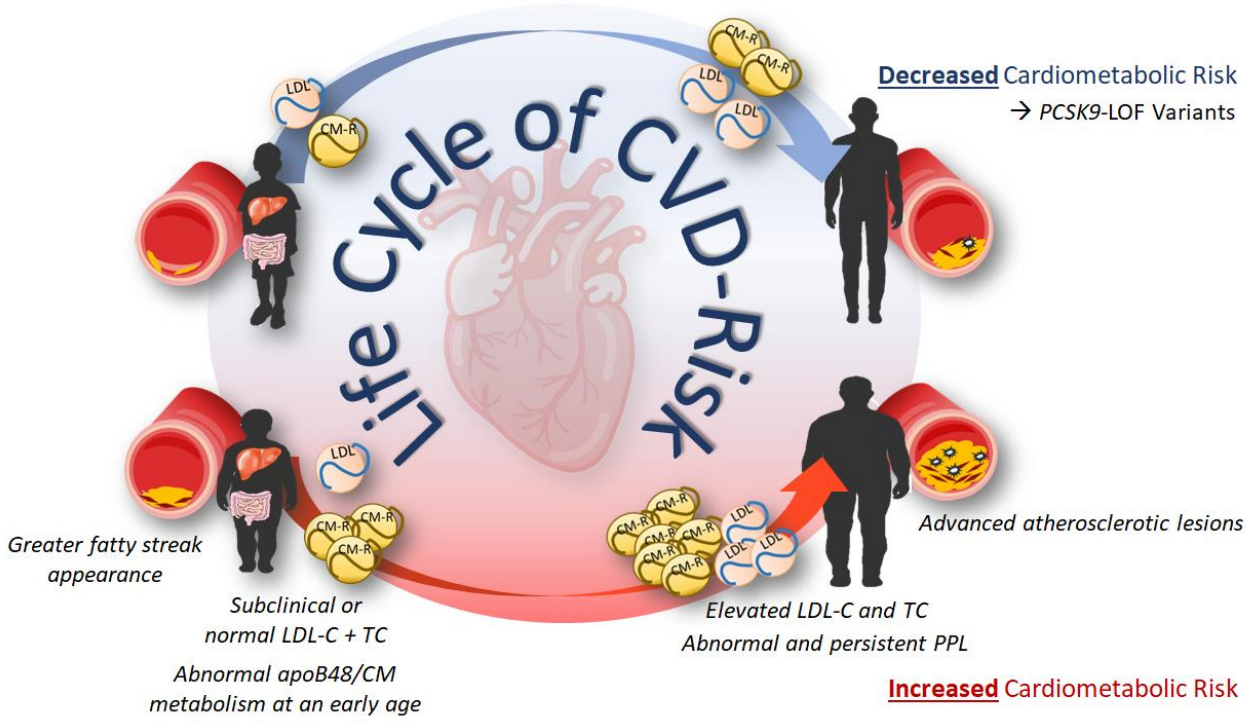

Figure 2-1: *CVD Progression Over the Lifespan.* Childhood obesity is associated with an increase in circulating CM remnants, independent to changes in LDL-C. Obesity during youth often tracks into adulthood and results in a further increase in circulating CM remnants accompanied by increases in LDL-C. Overall, early increases in CM remnants during childhood obesity promote an early atherogenic phenotype, which increases CVD risk in adulthood. Figure by Jacqueline Krysa©

2.10 Thesis Objectives and Hypothesis

Study 1: Non-Fasting Remnant Cholesterol in Alberta's Tomorrow Project: Implications for CVD Risk Prediction

Aim 1: To conduct a preliminary analysis to examine whether non-fasting remnant cholesterol is associated with CVD in a large Canadian population.

Hypothesis: As found with other non-Canadian groups, we predict that non-fasting remnant cholesterol will be significantly and independently associated with CVD prevalence and incidence.

Specific Objectives:

i. To determine the concentration of remnant cholesterol in non-fasting plasma samples collected from participants in the Alberta Tomorrow Project (ATP) cohort
ii. To associate non-fasting lipid parameters with prevalence and incidence of CVD
    a. To compare the risk prediction of non-fasting remnant cholesterol and LDL-C to CVD prevalence and incidence

Expected Outcome: Non-fasting remnant cholesterol will be significantly elevated in individuals with CVD in ATP and will have a positive and independent association to both CVD prevalence and incidence.

Study 2: The Effect of PCSK9 Loss-of-Function Variants on the Postprandial Lipid and ApoB Lipoprotein Response

Aim 2: To determine whether individuals with LOF variants in the *PCSK9* gene have altered postprandial lipid metabolism.

Hypothesis: We hypothesized that individuals with *PCSK9*-LOF variants will have lower fasting and postprandial remnant lipoproteins than control subjects

Specific Objectives: To determine the concentrations of fasting and postprandial TG, total-apoB and apoB48 as indicators of remnant lipoprotein metabolism in *PCSK9*-LOF carriers and controls with no PCSK9 variants

Expected Results: *PCSK9*-LOF carriers will have lower postprandial TG, total-apoB and apoB48 compared to non-variants.

Study 3: ApoB48-Lipoprotein Remnants are Associated with Increased Cardiometabolic Risk in Adolescents

Aim 3: To examine whether fasting apoB48 lipoproteins are positively associated with early cardiometabolic risk in a large-cross sectional adolescent population from the Raine study.

Specific Objectives: To measure fasting plasma apoB48 as a marker of intestinal remnant concentration in youth and to establish the relationship of apoB48 with other plasma lipids; as well as cardiometabolic risk factors

Hypothesis: Fasting apoB48 will have a significant positive association with cardiometabolic risk factors in adolescents from the Raine study.

Expected Results: Fasting plasma apoB48 will be positively associated with triglycerides, the metabolic syndrome, measures of adiposity, and other cardiometabolic risk factors in adolescents.

Study 4: ApoB-Lipoprotein Remnant Dyslipidemia and High-Fat Meal Intolerance is Associated with Increased Cardiometabolic Risk in Youth

Aim 4: To determine whether youth with obesity have impaired metabolism of plasma apoB48-lipoproteins and TG following a high-fat meal compared to healthy-weight youth, and to determine if fasting apoB48-lipoprotein remnants correlate with the postprandial response in apoB48-lipoproteins

Hypothesis: We hypothesize that youth with obesity will have elevated apoB48 and TG following a lipid-rich meal compared to healthy-weight youth and that fasting apoB48 will be predictive of PPL at this age.

Specific Objectives: To quantify fasting and non-fasting plasma apoB48 and TG following a high-fat meal challenge in normal weight and obese youth and to correlate fasting apoB48 to the postprandial apoB48 response in lean and obese youth to determine whether fasting apoB48 is predictive of PPL.

Expected Results: Youth with obesity will have an elevated postprandial response in apoB48 and TG following a lipid-rich meal. Fasting apoB48 will also be elevated in obese youth and will have a strong positive correlation with PPL.

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Chapter 3: Non-Fasting Remnant Cholesterol in Alberta's Tomorrow Project: Implications for CVD Risk Prediction

3.1 INTRODUCTION

An integral pathology associated with CVD is the formation of atherosclerotic plaques in the major vessels of the heart[1]. The circulating cholesterol that contributes to this process is derived from both fasting and non-fasting lipoproteins and therefore, lowering circulating cholesterol is considered a critical CVD risk reduction strategy[2–4]. In Canada, the primary lipid screening measure for CVD is fasting low-density lipoprotein cholesterol (LDL-C)[4]. Despite fasting LDL-C being a central screening and treatment target for dyslipidemia, substantial proportions of the population (including those with insulin resistance and diabetes) still suffer residual CVD risk with normal fasting LDL-C values[5]. Recently, non-fasting remnant cholesterol (RC) (inclusive of chylomicrons and/or very low-density lipoprotein remnants) has emerged as a novel CVD risk marker[6]. Non-fasting RC originates from the both the liver (very-low density lipoprotein remnant lipoproteins) and intestine (chylomicron remnant lipoproteins)[7]. RC can be calculated as: RC = TC – [LDL-C + HDL-C][8]. Recent data from Europe has demonstrated that circulating non-fasting RC can independently predict CVD risk and appears to be a better predictor of CVD risk than LDL-C[9]. Indeed, for every 1 mmol/L increase in non-fasting RC, there was found to be a 2.8 fold increase for ischemic heart disease[8]. The following demonstrates that RC is directly involved in CVD pathophysiology and is a robust predictor of CVD morbidity and mortality. Due the increased awareness of RC, non-fasting lipid assessment has been added to lipid screening guidelines in Europe and more recently, to Canadian guidelines[4]. However, key data on non-fasting RC has been published in Europe. In Canada, a recent study determined non-fasting lipid values in a large community-based cohort[10]. However, to date, no Canadian cohort has determined non-fasting remnant cholesterol nor assessed its association to CVD, until now.

To conduct initial analysis on non-fasting lipids in Canada we propose to utilize Alberta's Tomorrow Project (ATP). ATP is a province-wide longitudinal cohort study of cancer and chronic diseases[11]. Additionally, ATP participants consented to data linkage to provincial health care databases and other health records to investigate the association between health care utilization and chronic disease[12]. Recruitment for ATP consists of two phases. Phase I: 2000-2008 and Phase

II: 2009-2015. Both Phase I and II participants completed baseline questionnaires and provided consent to data linkage and follow-ups.

Individuals recruited for phase I into the ATP were Alberta residents aged 35-69 years with no history of cancer, other than melanoma skin cancer[13]. Starting in 2008 (Phase II ATP), ATP began a collaboration with the Canadian Partnership Against Cancer and created the Canadian Partnership of Tomorrow Project (CPTP). CPTP is a collection of five independent cohorts across Canada[14]. Existing Phase I participants were invited to join the CPTP cohort Study Centres to provide physical measurements as well as biospecimen donations (including non-fasting blood and urine samples); approximately 60% of phase I participants visited Study Centers (n=15,162). Phase II recruited n=22,932 new ATP-CPTP participants that also completed ATP questionnaires and surveys. Key methodological differences include the following: Phase I used stratified telephone random sampling by digit dialing and had different baseline questionnaires and follow-up periods compared to Phase II. Phase I has three follow-ups: 2004, 2008, 2017 and Phase II has one follow-up in 2017. The total ATP/CPTP cohort from 2000-2015 is n=52,810 and includes participants that have completed baseline questionnaires and consented to data linkage to health care databases and other health care records. Questionnaires from ATP include information on health and lifestyle, diet history, and physical activity[13]. Collectively, the ATP cohort is a robust source of data-sources and exposure measures with follow-up and is an excellent cohort to study chronic disease in Canada. Recent findings published using ATP data demonstrate low adherence to regular colorectal cancer screening[15], associations between better diet quality and reduced chronic disease[16], lack of association between atopic dermatitis and CVD[17], as well as no association between systematic antibiotic use and risk of diabetes[18]. A complete list of ATP publications can be found at [www.myATP.ca]. Therefore, we can use the data provided from ATP as a unique opportunity to explore the relationship between non-fasting lipids and CVD and other chronic diseases in a Canadian population.

CVD *prevalence* is the number of CVD cases reported in the ATP cohort, whereas *incidence* refers to the number of new CVD cases developed following recruitment. The purpose of the proposed study was to generate non-fasting RC and other lipid risk indices in ATP cohort and to compare the levels of non-fasting RC and LDL-C in those with and without CVD *prevalence* and *incidence.* We predicted that non-fasting RC would be elevated in participants with both

prevalence and *incidence* of CVD and would have greater odds of association to CVD compared to LDL-C.

3.2 METHODS

3.2.1 *Study Population*

The present thesis chapter is a cross-sectional analysis of the Alberta's Tomorrow Project (ATP) (n=52,810). ATP is a longitudinal research cohort that began in October 2000 in Alberta, Canada and has been in partnership with the expanded Canadian Partnership for Tomorrow Projects (CPTP) since 2008. The purpose of ATP is to facilitate research to study the etiology of chronic disease. Recruitment and enrollment data for ATP is described in further detail elsewhere[11,19]. Almost all (>99.0%) ATP participants provided their Personal Health Number (PHN) and consented to data linkage with Alberta Health administrative databases and other health records including Physician Claim Data and Hospital Discharge Abstract Data. Ethical approval for ATP recruitment and data collection was obtained through the former Alberta Cancer Board Research Ethics Committee and the University of Calgary Conjoint Health Research Ethics Board[19]. The present analysis of non-fasting lipids in the ATP cohort was approved by the University of Alberta Research Ethics Board (Pro00073641) and will focus on participants aged 35-69 recruited between 2000 and 2015 with complete lipid-panel biochemical data. Participants were excluded from the subsequent analysis if they did not have a complete lipid panel and/or their calculated LDL-C and RC value were equal to, or less than 0. After applying these exclusion criteria, n=16,251 participants were used for the present study.

3.2.2 ATP Database Approval Process

Our approval process to access the ATP database started in October 2017. To access ATP data a feasibility inquiry form was submitted to ensure that the databases within ATP support the proposed research question. Following initial approval, completion of a study application is required and includes providing a research proposal, ethics approval, appropriate research funding, and selecting study variables from ATP data dictionaries. Research funding was secured in July 2017 through the Alberta Diabetes Institute, and University of Alberta Research Ethics Board approval was received in April 2018. The ATP data dictionaries include survey questionnaires and biochemical data (please see appendix table 3-A1 for the full list of ATP surveys and data

dictionaries). For the present analysis we selected key variables from the following databases: Biospecimens (BIO), Canadian Diet History Questionnaire (CDHQ1), CORE, Health and Lifestyle Questionnaire (HLQ), Physical Activity & Nutrition Survey (PANS), Past-Year Total Physical activity Questionnaire (PYTPAQ), Updated Health & Lifestyle Questionnaire ((UHLQ), and externally linked data from Alberta Health Services. ATP approval for the present study was received in January 2019. Data linkage with Alberta Health administrative databases was conducted in collaboration with the Alliance for Canadian Health Outcomes Research in Diabetes (ACHORD) by using a 'cross-walk' file to merge ATP participant IDs with a data linkage ID for access to administrative data. Data linkage with ATP was completed in April 2019 and analysis for the present chapter was conducted from April to June 2019. Further details to access ATP data is available from [www.myATP.ca] or by emailing [ATP.Research@ahs.ca].

3.2.3 Anthropometry

Phase I participants, including those invited to CPTP, provided self-assessed anthropometric measures included body mass index (BMI) and waist circumference. Participants in Phase II of the ATP/CPTP visited designated Study Centres to complete physical examinations including height, weight, percentage body fat, hip and waist circumference, and blood pressure. BMI was calculated as weight/height and is derived from self-reported height and weight.

3.2.3 *Biochemistry and Metabolism*

Phase I participants of ATP donated blood samples at their time of enrollment into ATP. Blood sample collection for ATP/CPTP began in December 2008 and ended July 2015. Approximately 50 mL of non-fasting blood was collected into SST and EDTA tubes from n=29,183 consenting ATP participants at designated Study Centres and include post-collected separation into plasma and serum for lipid analysis. Samples were stored at -80°C within 24 hours following collection. Plasma HDL-C, TG, and TC were measured. LDL-C was calculated by the Friedewald Formula as TC-HDL-(TG/2.19), non-HDL-C was calculated as TC-HDL-C, and non-fasting remnant cholesterol concentration was calculated as RC = TC- (LDL-C +HDL-C).

3.2.4 Cardiovascular Disease

Individual level information on CVD (or related medical procedures) were obtained from ATP personal linked Hospital Discharge Abstract data. CVD events included *prevalence* and

incidence of ischemic heart disease (IHD), myocardial infarction (MI), angina, heart failure (HF), transient ischemic attack (TIA), acute ischemic stroke (AIS). Procedures and death variables were only available as *prevalence* and included percutaneous coronary intervention (PCI), coronary artery bypass graft (CABG), and CVD death. Participants who reported a *prevalence* of CVD or had an *incidence* of CVD following study recruitment were included in the study. The 'CVD composite' variable is inclusive of any CVD events included in this study.

3.2.5 Statin Use

Systematic statin use in the ATP cohort was obtained from a linked Alberta Blue Cross dataset from 2000-2015 and from Alberta's Pharmaceutical Information Network (2008-2015). The Alberta Blue Cross dataset only provides medication information on seniors and other individuals that require social assistance in Alberta. The cost of their prescribed medications is covered by Government of Alberta-sponsored plans, and from Alberta's Pharmaceutical Information Network. Alberta's Pharmaceutical Information Network is a web-enabled repository of medication profiles of Alberta patients. Both Alberta Blue Cross and Alberta's Pharmaceutical Information Network use the Anatomical Therapeutic Chemical Classification coding system (C10AA)[18]. For the present study, statin use was taken from both Alberta Blue Cross data, and Alberta's Pharmaceutical Information Network and linked to ATP study ID. For the present analysis, we examined the number of participants in ATP that had been prescribed a statin to represent overall statin use in the cohort.

3.2.6 *Comorbidity Indices*

Comorbidity burden in the ATP cohort was assessed by the Elixhauser and Charlson comorbidity indices. Briefly, the Charlson index is a weighted score of 17 comorbidities as an indicator of disease burden[20]. Elixhauser identifies 30 categories of comorbidities using ICD-9 CM codes[21]. For the present study, the Charlson and Elixhauser comorbidity index was calculated using the ICD-9/10 coding algorithm for administrative medical data[18].

3.2.7 *Statistical Analysis*

Data were analysed using Stata/IC version 14 (StataCorp LP, College Station, Texas). Means and standard deviation (SD) were calculated for descriptive statistics of continuous variables. A p value <0.05 was considered statistically significant. The number of coded CVD *prevalence* and

incidence events was grouped by non-fasting lipid quartiles. Univariate logistic regression was used to determine the odds ratio and 95% confidence intervals (CIs) for the association between non-fasting remnant cholesterol and CVD. Differences in Statin use was assessed by chi-square analysis. Sex differences between groups was determined by a one-way analysis of variance (ANOVA) followed by Bonferroni post-tests for multiple comparisons.

3.3 RESULTS

3.3.1 Study Population and Non-Fasting Remnant Cholesterol

A subset of the ATP cohort was analyzed and included participants who had consented to providing a blood sample and had available plasma lipid data. The cohort subset contained n=16,251 individuals and was approximately 67% female that were, in general, postmenopausal. The mean age was 62.3 ± 9.7 years and the males in the cohort were significantly older than the females (64.1 years ± 9.4 versus 61.5 years ± 9.7, $p<0.0001$). Initial analysis examined the relationship between quartiles of RC and LDL-C with CVD and statin use within quartiles of non-fasting RC and LDL-C. All subsequent analyses were divided into CVD *prevalence* and *incidence* and by males and females. Herein we aimed to determine the mean concentration of non-fasting RC and LDL-C in individuals with and without CVD *prevalence* and *incidence* and determine the odds ratio between RC and LDL-C with CVD *prevalence* and *incidence* by logistic regression. Our flow diagram of participant selection and analysis is presented below (Figure 3-1).

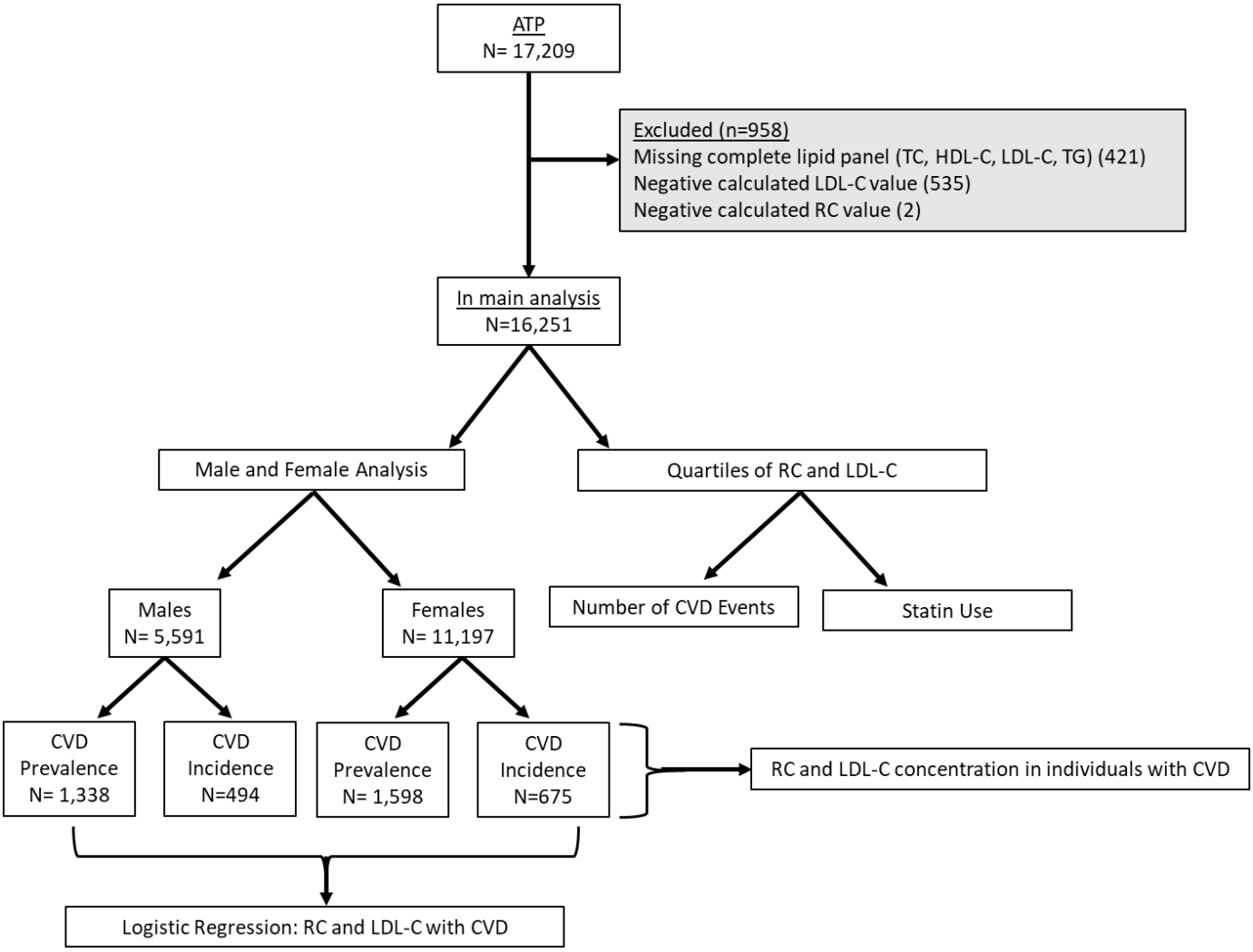

Figure 3-1: Flow Diagram of ATP Analysis.

The histogram below represents the distribution of non-fasting RC and the log transformation of remnant cholesterol in mmol/L in ATP (Figure 3-2). The distribution of non-fasting RC is slightly skewed to the right and has a limited population with values greater than 2. The mean non-fasting remnant cholesterol value was 0.79 ± 0.39 mmol/L with a minimum value of 0.06 mmol/L and a maximum value of 4.83 mmol/L (Table 3-1).

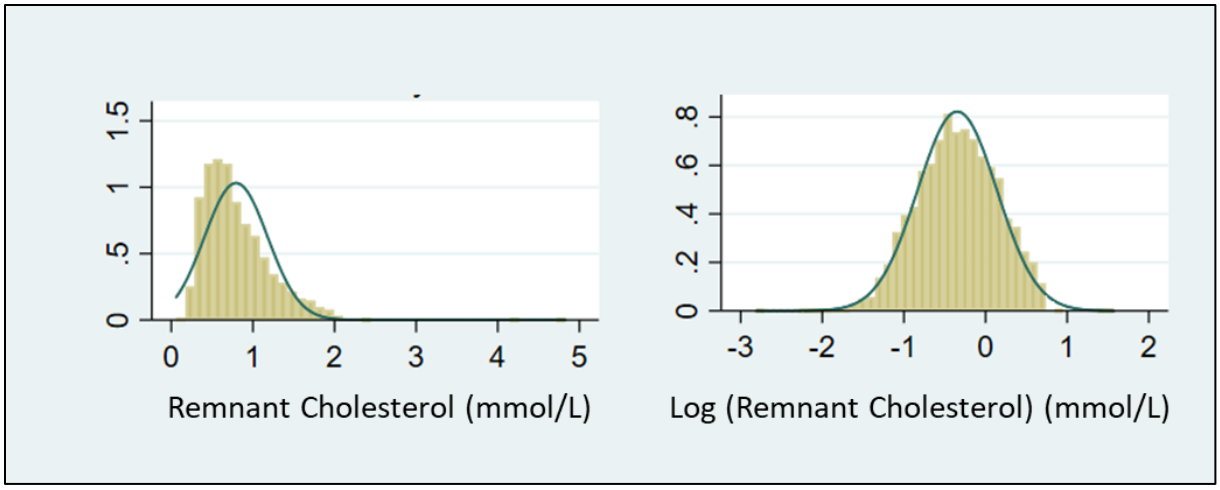

Figure 3-2: Histogram of non-fasting RC from Alberta's Tomorrow Project

3.3.2 Non-Fasting Plasma Lipids

The non-fasting lipid panel of the ATP cohort is presented below (Table 3-1). Non-fasting LDL-C, HDL-C, and TC were significantly higher in females in the cohort compared to males (LDL: 2.73 mmol/L ± 0.89 versus 2.88 mmol/L ± 0.85; HDL-C: 1.25 mmol/L ± 0.35 versus 1.61 mmol/L ± 0.44; TC: 4.87 mmol/L ± 0.98 versus 5.24 mmol/L ± 0.96, $p<0.0001$), while non-fasting RC and TG were significantly higher in males compared to females ((RC: 0.89 mmol/L ± 0.41 versus 0.75 mmol/L ± 0.36) (TG: 1.97 mmol/L ± 0.90 versus 1.64 mmol/L ± 0.80), $p<0.0001$). There were no significant differences in non-fasting non-HDL-C between sexes (3.62 mmol/L ± 0.96 versus 3.63 mmol/L ± 0.96; $p=0.52$). Additionally, statin use was significantly higher in males than females (35.1% versus 18.6%, $p<0.0001$) (Table 3-1).

	Total (n=16,251)	**Males** (n=5,294)	**Females** (n=10,957)	**P Value (M v. F)**
Age (Years)	62.3 ± 9.7	64.1 ± 9.4	61.5 ± 9.7	**<0.0001**
LDL-C (mmol/L)	2.83 ± 0.87	2.73 ± 0.89	2.88 ± 0.85	**<0.0001**
HDL-C (mmol/L)	1.49 ± 0.44	1.25 ± 0.35	1.61 ± 0.44	**<0.0001**
Total Cholesterol (mmol/L)	5.11 ± 0.98	4.87 ± 0.98	5.24 ± 0.96	**<0.0001**
Non-HDL-C (mmol/L)	3.63 ± 0.95	3.62 ± 0.96	3.63 ± 0.96	0.52
Remnant Cholesterol (mmol/L)	0.79 ± 0.39	0.89 ± 0.41	0.75 ± 0.36	**<0.0001**
Triglycerides (mmol/L)	1.75 ± 0.85	1.97 ± 0.90	1.64 ± 0.80	**<0.0001**
Statin Use n (%)	3916 (24.1)	1,858 (35.1)	2,038 (18.6)	**<0.0001**

Table 3-1. Non-fasting lipid panel in ATP cohort. Data presented as mean ± SD.

To further explore the distribution of non-fasting RC and LDL-C in the ATP cohort, RC and LDL-C were separated into quartiles (Table 3-2).

Quartile	**N**	**Remnant cholesterol (mmol/L)**	**N**	**LDL-C (mmol/L)**
Q1	4157	0.39 ± 0.08	4129	1.77 ± 0.39
Q2	4093	0.61 ± 0.06	4065	2.54 ± 0.16
Q3	4002	0.85 ± 0.09	4012	3.08 ± 0.16
Q4	3999	1.35 ± 0.27	4045	4.00 ± 0.51

Table 3-2. Quartiles of non-fasting RC and LDL-C in the ATP cohort. Data presented as mean ± SD.

Subsequent analysis explores the relationship between non-fasting RC and LDL-C with CVD by examining relationships with both *prevalence* and *incidence* of CVD as well as between males and females. The results below are separated by *prevalence* and *incidence* analysis.

The Relationship Between Non-Fasting RC and LDL-C with Composite CVD *Prevalence*

3.3.3 *CVD Prevalence Case Numbers by Quartiles of Non-Fasting RC and LDL-C*

The relationship between quartiles of non-fasting RC and LDL-C with the case number of CVD composite *prevalence* is shown in Figure 3-3. The case numbers for *prevalence* of adverse CV events increased parallel to increasing quartiles of non-fasting RC for composite *prevalence*, IHD, MI, angina, HF, TIA, AIS, PCIS, and CABP events/procedures. Conversely, case numbers declined with increasing quartiles of non-fasting LDL-C including MI, angina, TIA, AIS, and PCI (Table 3-3).

	RC (mmol/L)					**LDL-C (mmol/L)**			
	Q1 n=4157	**Q2** n=4093	**Q3** n=4002	**Q4** n=3999		**Q1** n=4129	**Q2** n=4065	**Q3** n=4012	**Q4** n=4045
CVD Composite	544 (13.0)	694 (17.0)	794 (19.8)	904 (22.6)		1080 (26.2)	640 (15.7)	595 (14.8)	621 (15.4)
IHD	501 (12.0)	636 (15.5)	735 (18.4)	845 (21.1)		1014 (24.6)	586 (14.4)	547 (13.6)	571 (14.1)
MI	46 (1.1)	73 (1.8)	83 (2.1)	89 (2.2)		178 (4.3)	43 (1.1)	37 (0.92)	33 (0.82)
Angina	40 (0.9)	62 (1.5)	60 (1.5)	73 (1.8)		131 (3.2)	41 (1.0)	34 (0.85)	29 (0.72)
HF	80 (1.92)	105 (2.6)	116 (2.9)	158 (4.0)		181 (4.4)	94 (2.3)	100 (2.50)	84 (2.1)
TIA	5 (0.12)	10 (0.2)	9 (0.22)	15 (0.38)		19 (0.46)	9 (0.22)	7 (0.17)	4 (0.10)
AIS	19 (0.46)	20 (0.49)	21 (0.52)	26 (0.65)		44 (1.07)	14 (0.34)	16 (0.40)	12 (0.30)
PCI	45 (1.1)	69 (1.7)	74 (1.8)	92 (2.3)		184 (4.5)	39 (0.96)	30 (0.75)	27 (0.67)
CABG	14	17	27	28		4	51	16	8

	(0.34)	(0.42)	(0.67)	(0.70)		(0.10)	(1.3)	(0.40)	(0.20)
Death	1	21	13	10		5	2	1	4
CVD	(0.02)	(0.51)	(0.32)	(0.25)		(0.12)	(0.05)	(0.02)	(0.10)

Table 3-3. *Prevalence* case numbers for CVD events by quartile of non-fasting RC and LDL-C. Data presented as n (%). ISH (ischemic heart disease), MI (myocardial infarction), angina, HF (heart failure), TIA (transient ischemic attack), AIS (acute ischemic stroke), PCI (percutaneous coronary intervention), and CABG (coronary artery bypass graft).

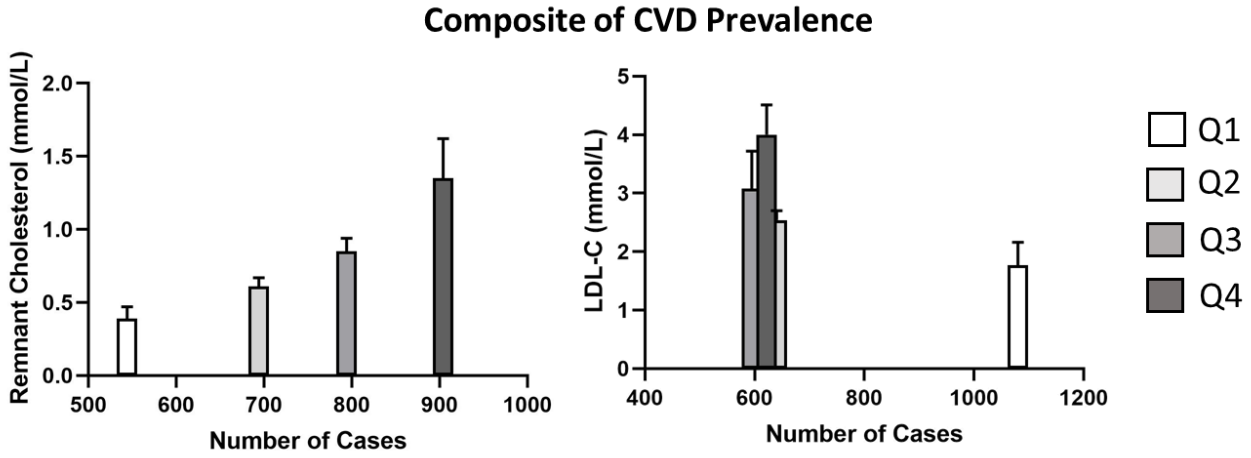

Figure 3-3. Composite of CVD *prevalence* cases by quartiles of non-fasting RC and LDL-C. Data is presented as mean ± SD.

3.3.4 Statin Use

The *prevalence* of statin use in the ATP cohort was approximately 24% of the ATP cohort used for the present analysis and was recorded prior to blood lipid analysis. Over 35% of male participants and 18% of female participants had been prescribed a statin. The number of statin users per quartile of non-fasting RC and LDL-C was determined and is shown is Table 3-5. Statin use increases with increasing quartiles of non-fasting RC, where as statin use is highest in the lowest quartile of non-fasting LDL-C. In figure 3-4 the number of statin users per quartile of non-fasting RC and LDL-C is plotted over the composite of CVD *prevalence* events per quartile of non-fasting RC and LDL-C (Table 3-4; Figure 3-4).

	RC (mmol/L)					LDL-C (mmol/L)			
	Q1 n=4157	**Q2** n=4093	**Q3** n=4002	**Q4** n=3999		**Q1** n=4129	**Q2** n=4065	**Q3** n=4012	**Q4** n=4045
Statin Use	553 (13.3)	852 (20.8)	1095 (27.4)	1408 (35.2)		1741 (42.2)	817 (20.1)	549 (13.7)	801 (19.8)

Table 3-4. The number of statin users per quartile of non-fasting RC and LDL-C. Data is presented as n (%).

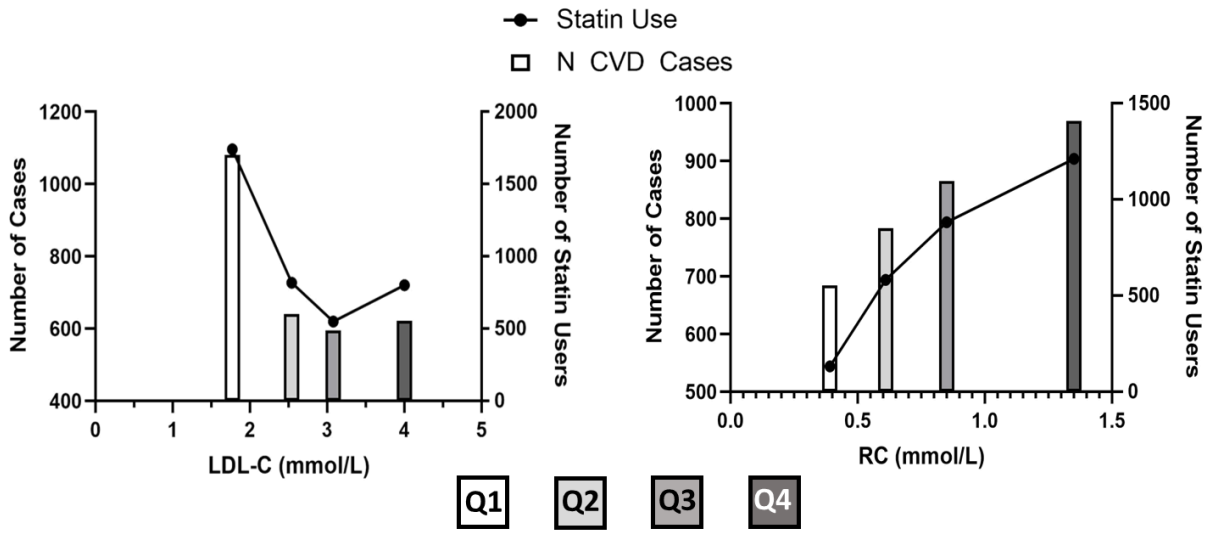

Figure 3-4. The number of statin users and composite of CVD *prevalence* case numbers by quartile of non-fasting RC and LDL-C. Systematic statin data was collected from the Alberta Blue Cross dataset and Alberta's Pharmaceutical Information Network between 2000-2015.

3.3.5 *Non-Fasting RC and LDL-C Values in Individuals with **Prevalence** of CVD*

The mean concentration of non-fasting RC and LDL-C in individuals with and without *prevalence* of an adverse cardiovascular event or procedure are shown below (Figure 3-5, Table 3-5). On average, non-fasting RC concentrations were approximately 10% higher in those individuals with *prevalence* of an adverse cardiovascular event (composite of CVD *prevalence*: 0.78 mmol/L ± 0.38 versus 0.87 mmol/L ± 0.40, $p<0.0001$). Conversely, non-fasting LDL-C tended to be approximately 8% lower in individuals with *prevalence* of an adverse cardiovascular event (CVD composite: 2.87 ± 0.84 versus 2.63 ± 0.95, $p<0.0001$). Both non-fasting RC and LDL-

C were significantly different in individuals with *prevalence* of an IHD, MI, angina, HF, TIA, AIS, PCI, and CABG as well as the composite CVD variable ($p<0.05$) (Table 3-5).

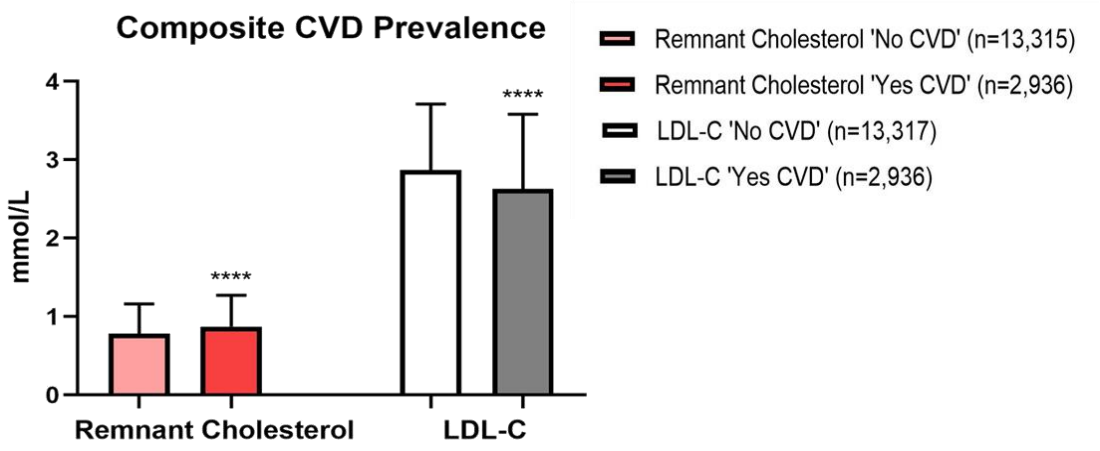

Figure 3-5. Mean non-fasting RC and LDL-C in individuals with and without composite of CVD *prevalence.* Data is presented as mean ± SD.

		Remnant Cholesterol (mmol/L)			**LDL-C (mmol/L)**		
		CVD *Prevalence*					
CVD Event	**N Cases**	**No**	**Yes**	**P Value**	**No**	**Yes**	**P Value**
CVD Composite	2,936	0.78 ± 0.38	0.87 ± 0.40	**<0.0001**	2.87 ± 0.84	2.63 ± 0.95	**<0.0001**
IHD	2717	0.78 ± 0.38	0.87 ± 0.40	**<0.0001**	2.88 ± 0.84	2.62 ± 0.95	**<0.0001**
MI	291	0.79 ± 0.39	0.87 ± 0.39	**0.0005**	2.84 ± 0.86	2.18 ± 0.91	**<0.0001**
Angina	235	0.79 ± 0.69	0.87 ± 0.39	**0.0018**	2.84 ± 0.86	2.28 ± 1.02	**<0.0001**
HF	459	0.79 ± 0.39	0.89 ± 0.42	**<0.0001**	2.84 ± 0.86	2.54 ± 0.93	**<0.0001**
TIA	39	0.79 ± 0.38	0.94 ± 0.41	**0.0165**	2.83 ± 0.86	2.33 ± 0.91	**<0.0001**
AIS	86	0.79 ± 0.39	0.88 ± 0.52	**0.033**	2.84 ± 0.86	2.32 ± 0.89	**<0.0001**
PCI	280	0.79 ± 0.39	0.88 ± 0.40	**<0.0001**	2.84 ± 0.86	2.09 ± 0.92	**<0.0001**
CABG	86	0.79 ± 0.39	0.88 ± 0.36	**0.034**	2.83 ± 0.86	2.21 ± 0.94	**<0.0001**
Death CVD	12	0.71 ± 0.29	0.90 ± 0.41	0.078	2.63 ± 0.81	2.46 ± 1.04	0.61

Table 3-5. Mean non-fasting remnant cholesterol and LDL-C in individuals with and without *prevalence* of CVD. Data is presented as mean ± SD.

The concentration of non-fasting RC was significantly elevated in female patients with composite of CVD *prevalence*, IHD, MI, HF, PCI ($p<0.05$), whereas RC was not significantly different for males with and without CVD *prevalence* ($p>0.05$). The concentration of non-fasting LDL-C was significantly decreased in male patients with composite of CVD *prevalence*, IHD, MI, HF, TIA, AIS, PCI, and CABG ($p<0.05$). LDL-C was significantly lower in females with composite of CVD *prevalence*, IHD, MI, HF, and AIS (Table 3-7). The decrease in LDL-C with *prevalence* of CVD was much greater than females (composite of CVD *prevalence*: (males: 2.82 mmol/L ± 0.85 versus 2.42 mmol/L ± 0.92, $p<0.05$ (14%); females: 2.90 mmol/L ± 0.83 versus 2.80 mmol/L ± 0.94, $p<0.05$ (3%) (Table 3-6).

	CVD *Prevalence*			
	No		**Yes**	
RC (mmol/L)	**Males**	**Females**	**Males**	**Females**
CVD Composite	0.89 ± 0.41^{a}	0.73 ± 0.36^{b}	0.91 ± 0.42^{a}	**0.83 ± 0.38^{c}**
IHD	0.89 ± 0.41^{a}	0.73 ± 0.36^{b}	0.91 ± 0.41^{a}	**0.84 ± 0.39^{c}**
MI	0.89 ± 0.41^{ac}	0.74 ± 0.36^{b}	0.87 ± 0.39^{c}	**0.89 ± 0.39^{c}**
Angina	0.89 ± 0.41^{a}	0.75 ± 0.36^{b}	0.89 ± 0.41^{a}	0.84 ± 0.34^{ab}
HF	0.89 ± 0.41^{a}	0.74 ± 0.36^{b}	0.94 ± 0.48^{a}	**0.85 ± 0.37^{a}**
TIA	0.89 ± 0.41^{a}	0.75 ± 0.36^{b}	0.90 ± 0.43^{ab}	0.97 ± 0.41^{ab}
AIS	0.89 ± 0.41^{a}	0.74 ± 0.36^{b}	0.86 ± 0.40^{a}	0.76 ± 0.32^{ab}
PCI	0.89 ± 0.41^{a}	0.75 ± 0.36^{b}	0.88 ± 0.40^{a}	**0.89 ± 0.39^{a}**
CABG	0.89 ± 0.41^{a}	0.75 ± 0.36^{b}	0.87 ± 0.37^{a}	**0.94 ± 0.29^{a}**
Death CVD	0.82 ± 0.32^{a}	0.66 ± 0.26^{a}	0.89 ± 0.45^{a}	0.93 ± 0.38^{a}

	CVD *Prevalence*			
	No		**Yes**	
LDL-C (mmol/L)	**Males**	**Females**	**Males**	**Females**
CVD Composite	2.82 ± 0.85^{a}	2.90 ± 0.83^{b}	**2.42 ± 0.92^{c}**	**2.80 ± 0.94^{a}**
IHD	2.83 ± 0.85^{a}	2.90 ± 0.83^{b}	**2.41 ± 0.93^{c}**	**2.80 ± 0.94^{a}**
MI	2.75 ± 0.88^{a}	2.89 ± 0.85^{b}	**2.10 ± 0.83^{c}**	**2.40 ± 1.08^{d}**

Angina	2.75 ± 0.88[a]	2.89 ± 0.85[b]	**2.12 ± 0.92[c]**	**2.61 ± 1.18[a]**
HF	2.74 ± 0.88[a]	2.89 ± 0.85[b]	**2.36 ± 0.92[c]**	**2.66 ± 0.93[a]**
TIA	2.73 ± 0.89[a]	2.88 ± 0.85[b]	**2.23 ± 0.77[c]**	2.44 ± 1.04[abc]
AIS	2.73 ± 0.89[a]	2.89 ± 0.85[b]	**2.22 ± 0.86[c]**	**2.46 ± 0.92[ac]**
PCI	2.76 ± 0.88[a]	2.89 ± 0.85[b]	**2.05 ± 0.88[c]**	**2.25 ± 1.07[d]**
CABG	2.74 ± 0.89[a]	2.88 ± 0.85[b]	**2.07 ± 0.83[c]**	2.80 ± 1.40[ab]
Death CVD	2.50 ± 0.75[a]	2.70 ± 0.84[a]	2.60 ± 1.06[a]	2.19 ± 1.09[a]

Table 3-6. Mean non-fasting remnant cholesterol and LDL-C in males and females with and without CVD *prevalence* and event numbers by sex. Data is presented as mean ± SD. Values with a different letter are significantly different ($p<0.05$).

3.3.6 *Logistic Regression Between RC and LDL-C with CVD **Prevalence***

Table 3-7 provides the unadjusted odds ratios for non-fasting RC and LDL-C and their relationship to the *prevalence* of an adverse cardiovascular event. Non-fasting RC was significantly related to increased odds for CVD for composite of CVD *prevalence* (OR:1.14 (CI: 1.08-1.18)), IHD (OR:1.14 (CI: 1.09-1.19)) and angina (OR:1.15 (CI: 1.02-1.30), $p<0.0001$). Conversely, LDL-C was associated with significantly decreased odds for CVD in all adverse cardiovascular conditions (composite of CVD *prevalence*: (OR:0.71 (CI: 0.67-0.74), $p<0.0001$)) except for death from CVD (Table 3-7).

CVD *Prevalence*	**N Cases**	**Odds Ratio per Unit Increase of RC (mmol/L)**	**P Value**	**Odds Ratio per Unit Increase of LDL-C (mmol/L)**	**P Value**
CVD Composite	2,936	1.14 (1.08-1.18)	**<0.0001**	0.71 (0.67-0.74)	**<0.0001**
IHD	2717	1.14 (1.09-1.19)	**<0.0001**	0.70 (0.67-0.74)	**<0.0001**
MI	291	1.09 (0.97-1.23)	0.14	0.37 (0.32-0.43)	**<0.0001**
Angina	235	1.15 (1.02-1.30)	**<0.0001**	0.43 (0.36-0.51)	**<0.0001**
HF	459	1.18 (0.92-1.51)	0.18	0.65 (0.58-0.72)	**<0.0001**
TIA	39	1.20 (0.92-1.57)	0.18	0.48 (0.33-0.72)	**<0.0001**
AIS	86	1.19 (0.99-1.43)	0.06	0.47 (0.36-0.62)	**<0.0001**
PCI	280	1.02 (0.89-1.17)	0.73	0.31 (0.27-0.36)	**<0.0001**

CABG	86	1.17 (0.97-1.41)	0.100	0.38 (0.29-0.49)	**<0.0001**
Death CVD	12	1.66 (0.82-3.34)	0.16	0.79 (0.36-1.71)	0.55

Table 3-7. Logistic regression non-fasting RC and LDL-C for CVD *prevalence.* Data presented as odds ratio (CI).

Unadjusted odds ratio for the relationship between non-fasting remnant cholesterol and *prevalence* of CVD in males and females is shown in Table 3-8. The odds ratio for females and composite of CVD *prevalence* was (OR:2.05 (CI:1.79 to 2.35) (p<0.0001)) for increasing non-fasting RC. The odds ratios in females were also significant for increasing non-fasting remnant cholesterol and *prevalence* of IHD (OR:2.06 (CI: 1.79 to 2.37)), MI (OR:2.52 (CI: 1.50 to 4.24), angina (OR:1.93 (CI: 1.08 to 3.42), HF (OR:2.01 (CI: 1.48 to 2.74)), TIA (OR:2.76 (CI: 1.42 to 9.92)), and PCI (OR:2.52 (CI: 1.32 to 4.91)). No odds ratios were significant between non-fasting RC and CVD *prevalence* in males. Decreasing LDL-C was significantly associated with *prevalence* of CVD in males (OR:0.68 (CI: 0.54 to 0.63)) and females (OR:0.87 (CI: 0.92 to 0.93)). Comparable odds ratios were observed between LDL-C and CVD *prevalence* in males and females except for CABG (males (OR:0.40 (CI: 0.30 to 0.53), p<0.0001); females (OR:0.89 (CI: 0.46 to 1.70) p=0.72) (Table 3-8).

	Odds Ratio per Unit Increase of Remnant Cholesterol (mmol/L)			
CVD *Prevalence*	**Males**	**P Value**	**Females**	**P Value**
CVD Composite	1.14 (0.98-1.32)	0.09	2.05 (1.79-2.35)	**<0.0001**
IHD	1.15 (0.99-1.34)	0.07	2.06 (1.79-2.37)	**<0.0001**
MI	0.85 (0.6-1.19)	0.34	2.52 (1.50-4.24)	**<0.0001**
Angina	0.95 (0.65-1.39)	0.80	1.93 (1.08-3.42)	**0.03**
HF	1.31 (0.95-1.79)	0.10	2.01 (1.48-2.74)	**<0.001**
TIA	1.06 (0.36-3.10)	0.10	3.76 (1.42-9.92)	**0.008**
AIS	1.41 (0.76-2.64)	0.28	1.14 (0.48-2.75)	0.78
PCI	0.92 (0.67-1.28)	0.62	2.52 (1.32-4.81)	**0.005**
CABG	0.84 (0.48-1.50)	0.57	2.01 (0.94-10.93)	0.07
Death CVD	1.74 (0.16-19.39)	0.65	21.80 (0.56-852.90)	0.10

Odds Ratio per Unit Increase of LDL-C (mmol/L)				
CVD *Prevalence*	**Males**	**P Value**	**Females**	**P Value**
CVD Composite	0.58 (0.54-0.63)	**<0.0001**	0.87 (0.82-0.93)	**<0.0001**
IHD	0.57 (0.53-0.62)	**0.001**	0.87 (0.82-0.94)	**<0.0001**
MI	0.40 (0.34-0.48)	**<0.0001**	0.47 (0.35-0.63)	**<0.0001**
Angina	0.42 (0.35-0.51)	**<0.0001**	0.67 (0.50-0.90)	**0.008**
HF	0.60 (0.51-0.70)	**<0.0001**	0.75 (0.64-0.87)	**<0.0001**
TIA	0.51 (0.30-0.87)	**0.003**	0.50 (0.28-0.89)	**0.014**
AIS	0.50 (0.36-0.70)	**<0.0001**	0.53 (0.34-0.80)	**0.003**
PCI	0.37 (0.31-0.44)	**<0.0001**	0.36 (0.25-0.52)	**<0.0001**
CABG	0.40 (0.30-0.53)	**<0.0001**	0.89 (0.46-1.70)	0.72
Death CVD	1.15 (0.40-3.31)	0.79	0.45 (0.11-1.90)	0.28

Table 3-8. Logistic regression of RC and LDL-C with CVD *prevalence* in males and females. Data presented as odds ratio (CI).

The odds ratio for non-fasting RC and LDL-C with adverse cardiovascular events were determined by quartiles of non-fasting RC and LDL-C (Table 3-9). Quartiles 1, 2 and 3 of non-fasting RC were significantly associated with increased odds for composite of CVD *prevalence* (Q1: (OR: 3.26 (CI: 1.00 to 10.59),p=0.049))); Q2: (OR:6.41 (CI: 1.63 to 25.18), p=0.008))); Q3: (OR:2.94 (1.33 to 8.68) p=0.007))), while Q1 was trending towards a positive association (p=0.06). Only Q1 of non-fasting LDL-C was significantly associated with odds of lower CVD events.

	Odds Ratio per Unit Increase of CVD Prevalence			
Quartile	**RC (mmol/L)**	**P**	**LDL-C (mmol/L)**	**P**
Q1	3.26 (1.00-10.59)	**0.049**	0.36 (0.30-0.43)	**<0.0001**
Q2	6.41 (2.52-25.18)	**0.0001**	0.85 (0.50-1.44)	0.54
Q3	2.51 (1.43-8.68)	**0.007**	0.80 (0.48-1.37)	0.43
Q4	1.17 (0.90-1.53)	0.24	1.04 (0.89-1.23)	0.60

3-9. Logistic regression of quartiles of RC and LDL-C with composite of CVD *prevalence.* Data presented as odds ratio (CI)

The Relationship Between Non-Fasting RC and LDL-C with *Incidence of* CVD

3.3.7 *CVD Incidence Case Numbers by Quartiles of Non-Fasting RC and LDL-C*

The case numbers for *incidence* of adverse CVD case numbers also increased parallel to increasing quartiles of non-fasting RC for composite of CVD *incidence*, IHD, MI, angina, HF, TIA, AIS, PCIS, and CABG events/procedures. Conversely, *incidence* case numbers declined with increasing quartiles of non-fasting LDL-C including MI, angina, TIA, AIS, and PCI. The relationship between quartiles of non-fasting RC and LDL-C with the number of composite CVD *incidence* cases is shown in Table 3-10 and Figure 3-6.

	RC (mmol/L)					**LDL-C (mmol/L)**			
	Q1 n=4157	**Q2** n=4093	**Q3** n=4002	**Q4** n=3999		**Q1** n=4129	**Q2** n=4065	**Q3** n=4012	**Q4** n=4045
CVD Composite	220 (5.3)	252 (6.2)	330 (8.2)	367 (9.2)		387 (9.4)	276 (6.8)	244 (6.1)	263 (6.5)
IHD	298 (7.2)	227 (5.5)	296 (7.4)	342 (8.6)		357 (8.6)	248 (6.1)	221 (5.5)	243 (6.0)
MI	29 (0.70)	21 (0.51)	38 (0.95)	36 (0.90)		57 (1.4)	16 (0.39)	20 (0.50)	19 (0.47)
Angina	9 (0.22)	16 (0.39)	19 (0.47)	18 (0.45)		27 (0.65)	15 (0.37)	10 (0.25)	10 (0.25)
HF	29 (0.70)	36 (0.88)	46 (1.15)	59 (1.50)		61 (1.5)	42 (1.0)	35 (0.87)	32 (0.79)
TIA	2 (0.05)	5 (0.12)	4 (0.10)	7 (0.18)		6 (0.14)	7 (0.17)	3 (0.07)	2 (0.05)
AIS	6 (0.14)	9 (0.22)	12 (0.30)	13 (0.33)		19 (0.46)	6 (0.15)	7 (0.17)	8 (0.20)

Table 3-10. *Incidence* case numbers for CVD events by quartile of non-fasting RC and LDL-C. Data presented as n (%).

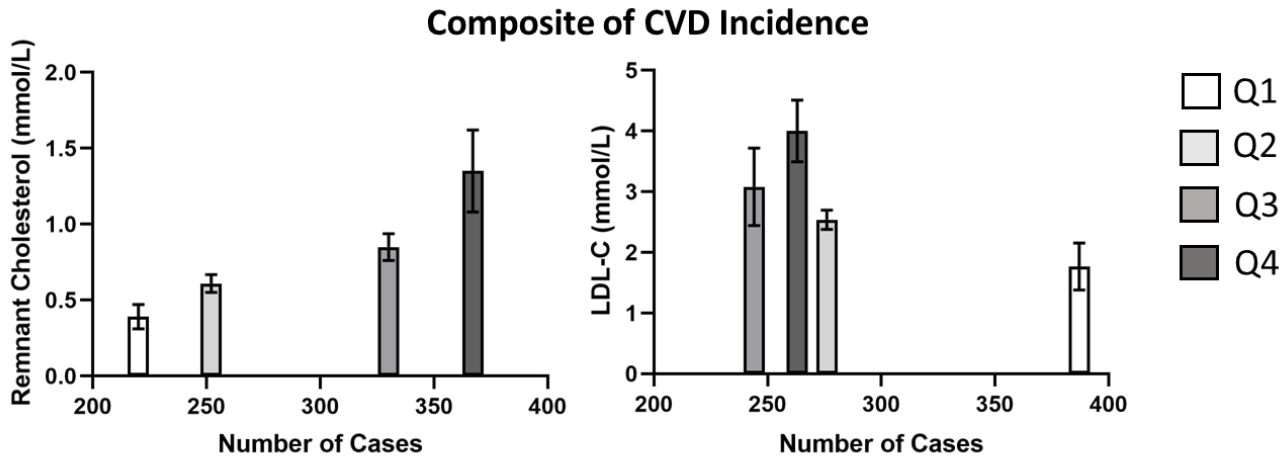

Figure 3-6. Composite of CVD *incidence* case numbers by quartile of non-fasting RC and LDL-C. Data is presented as mean ± SD.

3.3.8 *Non-Fasting RC and LDL-C Values in Individuals with and without CVD* ***Incidence***

In the Table 3-12, the mean concentration of non-fasting RC and LDL-C was compared in individuals with and without *incidence* of an adverse cardiovascular event or procedure since their enrollment in ATP (Figure 3-7, Table 3-11). Non-fasting LDL-C was significantly higher (3%) in individuals with an *incidence* of IHD, MI, and composite of CVD *incidence* compared to those without. Non-fasting RC was not significantly different in individuals with an adverse cardiovascular event (Figure 3-7, Table 3-11).

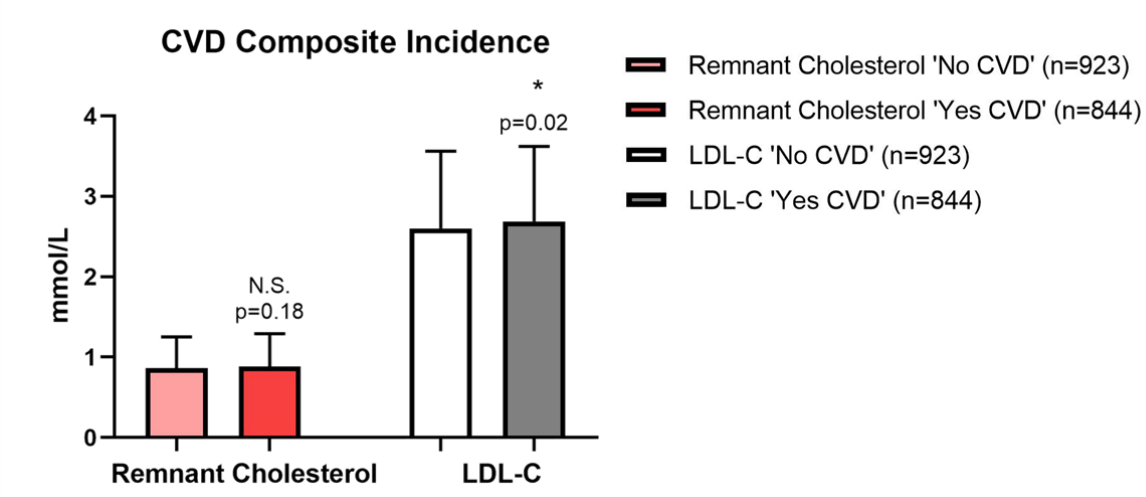

Figure 3-7. Mean non-fasting RC and LDL-C in individuals with and without composite of CVD *incidence*.

		CVD *Incidence*					
		RC (mmol/L)			LDL-C (mmol/L)		
CVD Event	**N Cases**	**No**	**Yes**	**P Value**	**No**	**Yes**	**P Value**
CVD Composite	1,169	0.86 ± 0.39	0.88 ± 0.41	0.18	2.60 ± 0.96	2.69 ± 0.93	**0.02**
IHD	1,068	0.87 ± 0.39	0.88 ± 0.41	0.55	2.58 ± 0.96	2.69 ± 0.94	**0.003**
MI	112	0.85 ± 0.37	0.92 ± 0.41	0.13	2.06 ± 0.81	2.38 ± 1.03	**0.004**
Angina	62	0.86 ± 0.39	0.90 ± 0.40	0.49	2.22 ± 1.05	2.45 ± 0.93	0.06
HF	170	0.88 ± 0.39	0.91 ± 0.48	0.47	2.53 ± 0.93	2.56 ± 0.96	0.74
TIA	18	0.94 ± 0.44	0.93 ± 0.39	0.94	2.27 ± 0.97	2.41 ± 0.86	0.64
AIS	40	0.79 ± 0.36	0.98 ± 0.65	0.09	2.26 ± 0.82	2.39 ± 0.96	0.66

Table 3-11. Mean non-fasting RC and LDL-C in individuals with and without CVD *incidence.* Data presented as mean ± SD.

Non-Fasting RC was not significantly different in males or females with an *incidence* of an adverse CVD event compared to those without (Composite of CVD *incidence*: (males: 0.91 mmol/L ± 0.40 versus 0.91 mmol/L ± 0.44, $p>0.05$; females: 0.82 mmol/L ± 0.38 versus 0.85 mmol/L ± 0.39, $p<0.05$)) (Table 3-12). Non-Fasting LDL-C was significantly elevated in males with composite CVD *incidence* (2.37 mmol/L ± 0.94 versus 2.52 mmol/L ± 0.89, $p<0.05$ (6%)), IHD, and MI, whereas females did not have any significant differences in non-fasting LDL-C between those with and without CVD (Table 3-12).

	***Incidence* of CVD**			
	No		**Yes**	
RC (mmol/L)	**Males**	**Females**	**Males**	**Females**
CVD Composite	0.91 ± 0.40[a]	0.82 ± 0.38[b]	0.91 ± 0.44[ac]	0.85 ± 0.39[bc]
IHD	0.92 ± 0.40[a]	0.82 ± 0.38[b]	0.91 ± 0.44[ac]	0.85 ± 0.39[bc]
MI	0.85 ± 0.37[a]	0.83 ± 0.38[a]	0.90 ± 0.42[a]	0.95 ± 0.39[a]
Angina	0.89 ± 0.42[a]	0.81 ± 0.31[a]	0.89 ± 0.41[a]	0.92 ± 0.41[a]
HF	0.92 ± 0.40[a]	0.84 ± 0.37[a]	0.98 ± 0.62[a]	0.87 ± 0.36[a]

TIA	0.79 ± 0.36[a]	1.03 ± 0.47[a]	0.99 ± 0.47[a]	0.85 ± 0.25[a]
AIS	0.87 ± 0.38[a]	0.70 ± 0.32[a]	1.04 ± 0.78[a]	0.87 ± 0.30[a]

	***Incidence* of CVD**			
	No		**Yes**	
LDL-C (mmol/L)	**Males**	**Females**	**Males**	**Females**
CVD Composite	2.37 ± 0.94[a]	2.79 ± 0.94[b]	**2.52 ± 0.89[c]**	2.81 ± 0.94[b]
IHD	2.35 ± 0.94[a]	2.79 ± 0.94[b]	**2.51 ± 0.90[c]**	2.83 ± 0.95[b]
MI	1.98 ± 0.74[a]	2.33 ± 0.96[ab]	**2.33 ± 0.94[b]**	2.47 ± 1.20[b]
Angina	2.05 ± 0.95[a]	2.57 ± 1.21[b]	2.32 ± 0.82[ab]	2.73 ± 1.11[b]
HF	2.30 ± 0.91[a]	2.74 ± 0.93[b]	2.49 ± 0.94[a]	2.61 ± 0.94[b]
TIA	2.32 ± 0.68[a]	2.24 ± 1.14[a]	2.15 ± 0.86[a]	2.81 ± 0.75[a]
AIS	2.02 ± 0.77[a]	2.51 ± 0.81[a]	2.40 ± 0.90[a]	2.38 ± 1.09[a]

Table 3-12. Mean non-fasting RC and LDL-C in individuals with and without CVD *incidence* by sex. Data is presented as mean ± SD. Means with a different letter are significantly different ($p<0.05$).

3.3.9 *Logistic Regression Between RC and LDL-C with CVD* ***Incidence***

The unadjusted odds ratios were determined between non-fasting RC and LDL-C with of CVD *incidence* events. Non-Fasting RC had no significant associations with odds for CVD *incidence*, whereas the odds ratio between non-fasting LDL-C and CVD *incidence* was significant for composite of CVD *incidence* (OR:1.11 (CI: 1.03 to 1.20)), IHD (OR:1.13 (CI: 1.04 to 1.22)), and MI (OR:1.47 (CI: 1.13 to 1.91)) (Table 3-1).

CVD *Incidence*	**N Cases**	**Odds Ratio per Unit Increase of RC (mmol/L)**	**P Value**	**Odds Ratio per Unit Increase of LDL-C (mmol/L)**	**P Value**
CVD Composite	2,936	1.08 (0.91-1.31)	0.36	1.11 (1.03-1.20)	**0.008**
IHD	2,717	1.08 (0.89-1.31)	0.42	1.13 (1.04-1.22)	**0.004**
MI	291	1.63 (0.89-3.0)	0.12	1.47 (1.13-1.91)	**0.004**

Angina	235	1.24 (0.60-2.58)	0.56	1.24 (0.95-1.63)	0.12
HF	459	1.19 (0.76-1.85)	0.45	1.05 (0.86-1.28)	0.65
TIA	39	0.98 (0.21-4.56)	0.98	1.18 (0.56-2.39)	0.64
AIS	86	2.37 (0.83-6.76)	0.11	1.20 (0.74-1.94)	0.47

Table 3-13. Unadjusted odds ratio for non-fasting RC and LDL-C with CVD *Incidence*. Data presented as odds ratio (CI).

There were no significant odds ratio between non-fasting RC and CVD *incidence* in males and females. Conversely. Males had significant associations between non-fasting LDL-C and odds for composite of CVD *incidence* (OR:1.19 CI:1.06-1.34)), IHD (OR:1.20(1.06-1.36)), and MI (OR:1.67 (1.18-2.37)) (Table 3-14).

	Odds Ratio per Unit Increase of Remnant Cholesterol (mmol/L)			
CVD *Incidence*	**Males**	**P Value**	**Females**	**P Value**
CVD Composite	1.04 (0.79-1.35)	0.79	1.20 (0.93-1.56)	0.16
IHD	1.01 (0.77-1.33)	0.94	1.23 (0.94-1.61)	0.13
MI	1.43 (0.70-2.95)	0.33	2.22 (0.66-7.26)	0.19
Angina	1.00 (0.43-2.34)	0.99	2.50 (0.55-11.31)	0.23
HF	1.26 (0.70-2.28)	0.44	1.26 (0.63-2.52)	0.51
TIA	3.52 (0.33-37.69)	0.30	0.27 (0.02-3.94)	0.34
AIS	1.66 (0.57-4.83)	0.35	6.22 (0.63-60.62)	0.12

	Odds Ratio per Unit Increase of LDL-C (mmol/L)			
CVD *Incidence*	**Males**	**P Value**	**Females**	**P Value**
CVD Composite	1.19 (1.06-1.34)	**0.005**	1.02 (0.92-1.13)	0.74
IHD	1.20 (1.06-1.36)	**0.004**	1.03 (0.92-1.15)	0.59
MI	1.67 (1.18-2.37)	**0.004**	1.13 (0.75-1.70)	0.57
Angina	1.36 (0.94-2.00)	0.10	1.11 (0.72-1.72)	0.63
HF	1.25 (0.91-1.72)	0.16	0.85 (0.64-1.13)	0.26

TIA	0.74 (0.22-2.52)	0.63	1.74 (0.67-4.55)	0.26
AIS	1.75 (0.88-3.51)	0.11	0.84 (0.39-1.78)	0.64

Table 3-14. Odds ratio non-fasting RC and LDL-C with CVD event *incidence* in males and females. Data presented as odds ratio (CI).

In Table 3-15 the unadjusted odds ratio between non-fasting RC and LDL-C quartiles with composite of CVD *incidence* is presented. There were no significant odds ratios amongst quartiles of non-fasting RC or LDL-C.

	Odds Ratio per Unit Increase of CVD Composite *Incidence*			
Quartile	**RC (mmol/L)**	**P**	**LDL-C (mmol/L)**	**P**
Q1	0.22 (0.02-1.98)	0.18	1.18 (0.87-1.61)	0.28
Q2	0.46 (0.03-5.99)	0.55	0.90 (0.33-2.46)	0.85
Q3	2.26 (0.43-11.95)	0.37	1.23 (0.45-3.37)	0.69
Q4	1.13 (0.72-1.80)	0.58	0.95 (0.72-1.26)	0.71

Table 3-15. Logistic Regression by individual quartiles of RC and LDL-C with composite of CVD *incidence* in males in females. Data presented as odds ratio (CI).

3.3.10 *Relationship of Non-Fasting RC and LDL-C with Comorbidity Indices*

As part of a further exploratory analysis, we had the opportunity to assess concentrations of non-fasting RC and LDL-C in the two comorbidity indices Elixhauser and Charlson. The Elixhauser and Carlson indices provide weighted scores as indicators of disease burden[20,21]. Univariate regression analysis demonstrated a positive and significant association between non-fasting RC and both Elixhauser and Charlson indices (Exlihauser: coefficient=0.22, R^2=0.006, p<0.0001; Charlson: coefficient=0.53, R^2=0.002, p<0.0001). Conversely, LDL-C had a significant negative association between both Elixhauser and Charlson indices (Exlihauser: coefficient=-0.13, R^2=0.01, p<0.0001; Charlson: coefficient=-0.06, R^2=0.10, p<0.0001). In Figure 3-8 it is shown that non-fasting RC tends to increase with increasing comorbidities indices, while LDL-C declines.

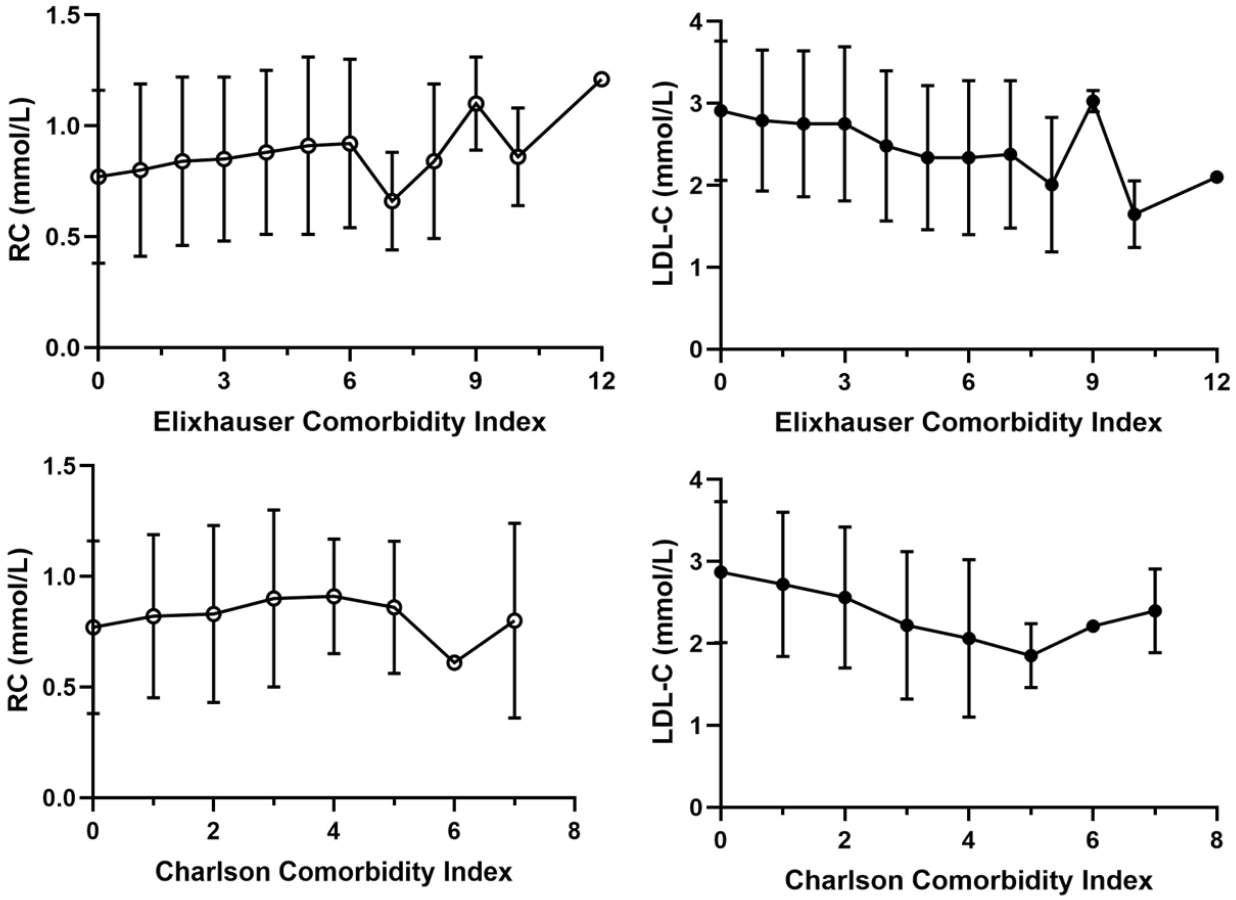

Figure 3-8. Non-fasting RC and LDL-C plotted against the Elixhauser and Charlson comorbidity indices. Data presented as mean ± SD.

3.4 DISCUSSION

The present thesis chapter is the first to our knowledge to establish values of non-fasting RC in a large Canadian population. These analyses demonstrate a positive relationship between non-fasting RC and *prevalence* of CVD, specifically in females, which was not observed for non-fasting LDL-C. In contrast, for CVD *incidence* we observed significant increases in non-fasting LDL-C, specifically in males, which was not observed for non-fasting RC.

3.4.1 Non-Fasting Lipid Values in the ATP

In our present analysis of non-fasting lipid data within ATP, female participants had significantly higher non-fasting LDL-C, HDL-C, and TC compared to male participants. Conversely, male participants had significantly elevated non-fasting RC and TG, and non-HDL-C was not significantly different between groups. Often, fasting LDL-C, TC, TG and RC are higher in males than females[22,23]. It is unclear why in the present study females had significantly higher non-fasting LDL-C and TC compared to males. However, our mean non-fasting lipid values are comparable to another Canadian study that observed similar non-fasting lipid values and differences between males and females[10]. Recently, the Canadian Laboratory Initiative on Pediatric Reference Intervals (CALIPER) cohort, also assessed non-fasting lipid intervals in youth and determined non-fasting RC[24]. It was found that in children and adolescents ages 1- <19 years old non-fasting RC ranged from 0.23-1.01 mmol/L (n=1722), there were similar values observed between males and females at this age[24].

3.4.2 The ATP Cohort Compared to the CCHS

The Copenhagen General Population Study (CGPS), the Copenhagen City Heart Study (CCHS) and the Copenhagen Ischemic Heart Disease Study (CIHDS) were combined for analysis and will be herein referred to as the combined CCHS. Briefly, CGPS is prospective study of Copenhagen that began in 2003 and has ongoing enrollment (n=47,351). The CCHS is also a prospective study of the Copenhagen population that began in 1976-1978 with follow-up examinations from 1981 to 1983, 1991 to 1994, and 2001 to 2003 (n=10,609). Lastly, the CIHDS is comprised for n=5,185 patients with IHD from Copenhagen University Hospital from 1991 to 2009[8,25]. The ATP subset utilized for the present analysis was 67% female. The CCHS and the CPGS were also majority female (55% and 61%, respectively). The subset of ATP used for the

present analysis was slightly older than the full ATP cohort (62.32 ± 9.69 years versus full ATP cohort (phase I 51.2 ± 9.4 years; phase II 52.7 ± 9.2 years)) and the combined CCHS (CCHS 59 (46-59) years, CGPS 55 (46-65) years)[8]. Statin use was also higher in ATP compared to the CCHS (24% in ATP versus 10% in the CPGS).

3.4.3 The ATP Cohort has Comparable Non-Fasting RC and LDL-C Values to the Combined CCHS

The mean non-fasting RC values in ATP were comparable to RC values in the combined CCHS (CCHS: 0.7 mmol/L versus ATP: 0.8 mmol/L)[8]. Furthermore, when we split non-fasting RC into quartiles the cut points were comparable to the quintiles established from the combined CCHS data (CCHS: Q1 <0.4, Q2 0.4-0.6, Q3 0.6-0.7, Q4 0.7-1.1, Q5>1.1 versus ATP: Q1 <0.4 , Q2 0.41-0.84, Q3 0.85-1.34, Q4 >1.35)[8]. The quartiles generated of non-fasting LDL-C from ATP were slightly lower in Q1 compared to the combined CCHS but comparable for Q2, 3 and 4. Within the ATP cohort there was a composite CVD *prevalence* rate of n=2,936 and n=2717 of ischemic heart disease. The percentage of ischemic heart disease *prevalence* per individual is comparable with the combined CCHS (n=11,984 IHD events, total: n=75,513).

3.4.4 Non-Fasting Remnant Cholesterol is Positively Associated with Increased CVD Case Numbers

When RC and LDL-C were split into quartiles, only RC tracked with increasing rates of *prevalence* and *incidence* of CVD events, while LDL-C had no linear relationship. The combined CCHS did not see the same inverse relationship of LDL-C with CVD as we did in the present study. We hypothesized that these incongruent findings could be due to statin use in the population. To understand this relationship between statins and non-fasting lipids in ATP, we examined the number of individuals using statins in quartiles of non-fasting RC and LDL-C. It was observed that statin use was linear with increasing quartiles of non-fasting RC but highest in the lowest quartile of LDL-C. These findings suggest that the lowest quartile of LDL-C is a subpopulation of individuals where the majority have been treated with statins. It has been previously demonstrated that certain high-risk populations, including those with diabetes and the metabolic syndrome, are at elevated CVD-risk despite lowering of LDL-C[26]. Currently, there are few standard pharmacological therapies that would reduce non-fasting RC. Statins tend to have mixed or null effect on TG and therefore may not influence non-fasting RC[27]. Future work can further explore

this quartile of LDL-C to elucidate whether this population has a high *prevalence* of diabetes and comorbidity rates. These findings could help explain the inverse relationship of LDL-C with CVD observed in the present analysis.

3.4.5 Non-Fasting Remnant Cholesterol is Positively Associated with Prevalence of CVD, Independent of LDL-C

We observed a significant elevation in non-fasting RC in individuals with a *prevalent* CVD event or procedure (10%). Interestingly, non-fasting LDL-C was found to be significantly lower in individuals with *prevalence* of CVD (8%). However, when we compare RC and LDL-C for *incidence* of CVD following recruitment into ATP the opposite was seen. There was significant change in non-fasting RC between those with and without an *incidence* of CVD, while there was a small but significant increase in LDL-C (3%).

The current Canadian Cardiovascular Guidelines consider those individuals with fasting LDL-C $\geq$ 3.5 mmol/L or non-HDL $\geq$ 4.3 mmol/L at elevated cardiovascular risk and are recommended to be treated with lipid lowering therapies such as a statin[28]. LDL-C, HDL-C, and TC change minimally during the postprandial period, and therefore the values observed in our study should be comparable to fasted lipid levels. Neither non-fasting LDL-C nor non-HDL-C was, on average, higher than the reference ranges values. The high prevalence of statin use in this population may explain the observed 'normal' ranges of LDL-C and non-HDL-C. Furthermore, the absolute percent change in both non-fasting LDL-C and RC between individuals with and without CVD *prevalence* is minimal (8% versus 10%, respectively) and may not be considered a clinically significant difference.

Individuals with CVD *prevalence* had a mean RC value of 0.87 mmol/L, which is slightly less than the $\geq$ 0.9 mmol/L cut point for non-fasting remnant cholesterol in Europe[29]. Interestingly, RC was higher in both 'yes' and 'no' *incidence* groups than the average non-fasting RC in the general ATP population. Herein, the *incidence* subset of population may more prone to CVD but may have not yet had an event due to the time course of the study. The ATP study was completed between 2000-2015, therefore some participants may have not had a long enough follow-up time to experience an adverse CVD event. In the CALIPER study, approximately 11% of healthy children and adolescents had non-fasting RC values above the European adult RC cut-off[24].

Therefore, future research is needed to clarify appropriate reference ranges for non-fasting RC in both Canadian adults and children.

The change in relationship between non-fasting in LDL-C in patients with and without *prevalence* or *incidence* of CVD could also be explained by the time course of the condition. Individuals with *prevalence* of a CVD event or elevated blood cholesterol earlier in life may have been put on a statin to prevent additional or future CVD events. Statin use may explain why individuals with a *prevalence* of CVD have lower LDL-C than those without. Furthermore, this may also explain why LDL-C is slightly elevated in individuals with an *incidence* of CVD, as they may not have been previously screened for blood cholesterol before having an event. It is well understood that elevated LDL-C and TC cholesterol increases CVD burden[2], therefore, it is important to further explore this inverse relationship with CVD *prevalence* .

Non-fasting RC was also shown to have a significant increased odd for *prevalence* of a CVD event (OR:1.14 (CI: 1.08 to 1.18)), whereas LDL-C had significant decreased odds for CVD *prevalence* (OR:0.71 (CI: 0.67 to 0.74)). However, when we split apart RC and LDL-C into quartiles and perform an additional logistic regression our results change. RC has a trending positive odds ratio at Q1 as well as a significant positive relationship at Q2 and Q3 whereas LDL-C, only Q1 is significantly associated with CVD *prevalence*. These findings are partly in line with the above analysis. However, it is unclear why we do not observe a linear relationship with CVD quartiles as we increase quartiles of RC. *Incidence* of CVD was found to not have significant odds with either non-fasting RC or LDL-C. The histogram of RC as shown in Figure 1 demonstrates that the mean non-fasting RC is slightly skewed right. Therefore, when we split the data into quartiles this may cause high confidence interval upper limits in odds ratio calculations due to differences in sample size between quartiles and distribution within the quartiles. To mitigate these confounders, we could create quartiles with a log-transformed variable or perform a sensitivity analysis based on clinically meaningful reference ranges.

3.4.6 *Non-Fasting Remnant Cholesterol Better Predicts CVD in Females*

We found that non-fasting RC was significantly elevated in females with CVD composite *prevalence* (12%), but not males. Interestingly, LDL-C was significantly lower in both males and females with *prevalence* of CVD composite (14% versus 3%, respectively). Conversely, there were no significant changes in RC amongst sexes with and without CVD *incidence*, while LDL-C

was significantly elevated in males with CVD *incidence,* but not females. These differences are further emphasized when we conduct a logistic regression separated by sex. We observed similar trends whereby non-fasting RC is significantly associated with CVD *prevalence* in females and not males, and not associated with CVD *incidence*. Furthermore, odds ratio analysis in males in females demonstrated reduced LDL-C is significantly associated with CVD *prevalence* in males and females and elevated LDL-C is significantly associated with increased odds for CVD *incidence* in males only. Since ATP has more female than male participants it would be interesting to create a matched group to compare CVD *prevalence* and *incidence* event rates as well as LDL-C and RC values to further understand sex differences in this cohort.

3.4.7 *Limitations and Future Directions*

The presented thesis chapter is an initial analysis exploring the concentration of non-fasting RC within the ATP cohort and its relationship to CVD. Due to the preliminary nature of this work there are key limitations to be considered when interpreting this data. The current findings may be a result of statin use in the population, adjusting for this variable may provide us further insight to the relationships we observe with LDL-C and CVD. Furthermore, we pooled all non-fasting RC. The sampling time in ATP was recorded and provides a time since last meal. Previous work in non-fasting RC has found that RC peaks at 3 to 4 hours after the last meal and has its highest levels at 1pm[6]. It would be useful to also explore these values to confirm whether RC changes significantly amongst the cohort depending on their sampling time. Importantly, other conditions also contribute to the pathophysiology of CVD such as genetics, obesity, and lifestyle factors, all of which, can influence the relationship between non-fasting RC and CVD. Future directions include adjusting for these covariates in regression analysis between RC and CVD.

There are several future directions that will result from this project. A critical objective will be to perform a Cox proportional hazards regression model with 'time to event' as the time scale to determine a hazard ratio between non-fasting RC and LDL-C with CVD *incidence*. We are also interested to examine the anthropometrics of the participants, especially measurements of weight status including body mass index (BMI), and waist circumference. Additionally, we can explore the relationship between RC and these parameters. It has previously been shown that non-fasting RC has a positive relationship with BMI and this may further exacerbate CVD risk in these individuals[30]. Future analysis could determine values of non-fasting lipids at BMI cut points and

determine their relationship to CVD risk. We hypothesize that individuals with higher BMI values would also have elevated RC and be at increased risk of developing CVD.

Additionally, it will be interesting to compare concentrations of non-fasting lipids in patients with diabetes and determine whether these patients have a stronger relationship to CVD with RC than LDL-C. Initial analysis of *prevalence* of type I and type II diabetes shows that patients *prevalence* with type I or type II diabetes have significantly elevated non-fasting RC (0.77 ± 0.40 mmol/L versus 0.97 ± 0.41 mmol/L) and significant lower LDL-C (2.90 ± 0.83 mmol/L versus 2.26 ± 0.93 mmol/L). We plan to further explore this by comparing the values of RC and LDL-C in patients with *prevalence* and *incidence* of type 2 diabetes and compare CVD risk in these individuals, along with statin use, in this subpopulation. We also plan to utilize the two comorbidity indices (Elixhauser and Charlson) defined within the ATP cohort. Initial analysis of these indices demonstrates that non-fasting RC tends to increase with increasing comorbidities indices, while LDL-C slightly declines (Figure 3-7). Future analysis can explore these relationships by creating age stratified analysis and further, comparing CVD risk at higher comorbidity indices

Lastly, it is imperative to determine normative reference values of non-fasting lipids that can be used by physicians in Canada. In Europe the recommended flagged values for non-fasting RC are $\geq$ 0.9 mmol/L and $\geq$ 3 mmol/L for LDL-C[29]. We can evaluate the robustness of these values to predict CVD in the ATP to confirm their utility for practice in Canada.

3.5 CONCLUSION

Our initial data suggests that non-fasting RC is significantly associated with CVD in females and may be a better CVD risk predictor than LDL-C. Future work will further delineate the predictive power of non-fasting RC and explore its utility as a novel CVD risk marker in Canada.

3.6 APPENDICIES

Survey/Source	Description
Alberta Cancer Registry (ACR)	Linked data from Alberta's Cancer Registry
Alberta Health Derived Administrative Data	Data returned from previous researchers
Baseline	Participant baseline characteristics
Biospecimens	Biomarker analysis from participants that donated 50 mL of non-fasting blood
Canadian Diet History Questionnaire (CDHQ-I)	Assess food and nutrient intakes in the year prior to questionnaire completion
Canadian Urban Environmental Health Research Consortium (CANUE)	Compiled and derived standardized environmental exposure data
CORE	Refined version of UHLQ and PANS
Heath and Lifestyle Questionnaire (HLQ)	Personal and family health history
Past-Year Total Physical Activity Questionnaire (PYTPAQ)	Type and amount of physical activity undertaken by participants in the past twelve months
Physical Activity & Nutrition Survey (PANS)	Physical activity and dietary habits
Physical Measurements	Including height, weight, waist circumference etc.
Survey 2004	Survey distributed to patients that joined ATP between 2000 and 2003 that contained questions on personal healthy history, cancer screening tests, body measurements etc.
Survey 2008	Survey distributed to patients that joined ATP between 2001 and 2007 that contained questions on lifetime residential history, personal and family healthy history, cancer screening tests etc.
Updated Health & Lifestyle Questionnaire (UHLQ)	Based on original HLQ completed by participants that joined ATP between 2001 and 2008.

Appendix Table 3-A1. Survey Descriptions and Data Dictionaries from Alberta's Tomorrow Project. Adapted from Alberta's Tomorrow Project (2019) [www.myATP.ca].

Quartile LDL-C (mmol/L)	N	Remnant cholesterol (mmol/L)
Q1	4129	0.80 ± 0.43
Q2	4065	0.75 ± 0.38
Q3	4012	0.79 ± 0.37
Q4	4045	0.83 ± 0.35

Quartile RC (mmol/L)	N	LDL-C (mmol/L)
Q1	4157	2.65 ± 0.75
Q2	4093	2.90 ± 0.83
Q3	4002	3.00 ± 0.90
Q4	3999	2.81 ± 1.00

Appendix Table 3-A2. Concentration of non-fasting RC and LDL-C by LDL-C quartile and RC quartile.

	Remnant Cholesterol (mmol/L)			
Quartile	**Males**	**P Value**	**Females**	**P Value**
Q1	0.49 (0.07-3.5)	0.47	5.72 (1.27-25.7)	**0.02**
Q2	3.28 (0.36-30.17)	0.29	17.34 (3.0 – 100.1)	**0.001**
Q3	3.45 (0.87-13.74)	0.08	2.53 (0.75-8.53)	0.13
Q4	1.02 (0.71-1.47)	0.89	1.25 (0.84-1.87)	0.27

	LDL-C (mmol/L)			
Quartile	**Males**	**P Value**	**Females**	**P Value**
Q1	0.43 (0.33-0.55)	**<0.0001**	0.41 (0.31-0.55)	**<0.0001**
Q2	0.46 (0.20-1.06)	0.07	0.43 (0.65-2.69)	0.43
Q3	0.53 (0.22-1.29)	0.16	1.09 (0.56-2.13)	0.79
Q4	0.97 (0.70-1.32)	0.83	1.09 (0.90-1.32)	0.89

Appendix Table 3-A3. Logistic regression LDL-C and RC quartiles with CVD composite prevalence variable in males and females

	Remnant Cholesterol (mmol/L)			
Quartile	**Males**	**P Value**	**Females**	**P Value**
Q1	1.86 (0.05-67.8)	0.73	0.06 (0.00 – 0.96)	0.05
Q2	0.04 (0.00 – 2.5)	0.12	1.97 (0.07-51.5)	0.67
Q3	0.29 (0.03 – 3.32)	0.32	17.00 (1.65-174.7)	**0.02**
Q4	1.45 (0.78-2.69)	0.24	0.90 (0.45-1.80)	0.78

	LDL-C (mmol/L)			
Quartile	**Males**	**P Value**	**Females**	**P Value**
Q1	1.13 (0.83-1.53)	0.44	1.10 (0.82-1.46)	0.52
Q2	1.42 (1.05-1.91)	**0.02**	1.02 (0.81-1.28)	0.87
Q3	1.37 (1.09-1.72)	**0.007**	1.08 (0.89-1.32)	0.42
Q4	1.02 (0.84-1.24)	0.84	0.94 (0.79-1.13)	0.52

Appendix Table 3-A4. Logistic regression LDL-C and RC quartiles with CVD composite incidence variable in males and females

CVD *Prevalence*	Males	Females
CVD Composite	1,338	1,598
IHD	1,260	1,457
MI	212	79
Angina	165	70
HF	214	245
TIA	19	20
AIS	50	36
PCI	229	51
CABG	73	13
Death CVD	8	4

CVD *Incidence*	Males	Females
CVD Composite	494	675
IHD	454	614
MI	74	38
Angina	43	19
HF	68	102
TIA	11	7
AIS	26	14

Appendix Table 3-A5. CVD *incidence* and *prevalence* case numbers in males and females

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Chapter 4: The Effect of PCSK9 Loss-of-Function Variants on the Postprandial Lipid and ApoB-Lipoprotein Response

This chapter has been published as a manuscript (Please see Appendix B)

4.1 INTRODUCTION

Proprotein convertase subtilisin kexin 9 (PCSK9) is a secreted protein expressed primarily in the liver and small intestine[1]. It is the ninth member of the family of proprotein convertases (PC)[2]. Different from other PCs, it has only itself as substrate and it escorts the low-density lipoprotein receptor (LDLR) to the lysosome for degradation[3]. By reducing the amount of cell surface LDLR, the low-density lipoprotein cholesterol (LDL-C) concentration is increased[4]. Consequently, PCSK9 inhibitors have been rapidly developed and are currently in clinical use. One of them, evolocumab, has recently been shown to further reduce the risk of cardiovascular events in patients who are already on statin therapy[5].

The gene for PCSK9 is highly polymorphic[6]. Populations have variants that result in a more functional or increase in circulating PCSK9 (gain-of-function (GOF)) or, conversely, a less functional or decrease in circulating PCSK9 (loss-of-function (LOF))[7]. LOF variants are more common than GOF variants. *PCSK9*-LOF results in greater expression of hepatic LDL-r, lower concentrations of LDL-C, and protection from cardiovascular disease (CVD)[8,9]. LDL-C is a well-established risk marker of CVD and is used to assess and manage CVD risk[10]. Despite lowering LDL-C to recommended levels by statin therapy, there is significant residual risk for cardiovascular events, especially in high risk patients with diabetes and the metabolic syndrome[11].

Recent data indicate that triglyceride-rich lipoproteins (TRL) have a significant and independent causal association with CVD risk[12]. Remnants of chylomicrons (CM) and very low density lipoproteins (VLDL) contain more cholesterol per particle than LDL and therefore have enhanced atherogenic potential[13]. In chapter 3, we demonstrated that increasing quartiles of non-fasting remnant cholesterol were associating with increases in adverse CV events. Remnant lipoproteins in plasma are assessed by measuring fasting plasma apoB48 and apoB100, the functional proteins present on each CM and VLDL remnant particle respectively[14]. The clearance of remnant lipoproteins is mediated by the liver through the LDLR and the low-density lipoprotein receptor-related protein 1 (LRP1)[15]. Remnant lipoproteins bind to the LDLR through apoE related

mechanisms[15], while LDL binds through interactions with apoB100[16]. Delayed clearance of TRL, especially in the postprandial period, results in accumulation of remnant lipoproteins in plasma manifesting as hypertriglyceridemia and enhanced postprandial lipemia[17,18].

PCSK9 could influence TRL clearance by reducing the amount of cell surface LDL-r. *In vitro*, PCSK9 has also been shown to stimulate the production of TRL[19]. *PCSK9* null mice have reduced postprandial TG excursion as well as intestinal apoB48 expression following an olive-oil bolus[20]. Collectively, the above evidence suggests that PCSK9 could modulate both intestinal secretion and hepatic clearance of TRL. Presently, it is not known whether PCSK9 impacts TRL metabolism in humans, particularly during the postprandial period. We hypothesized that subjects with *PCSK9*-LOF variants will not only have lower fasting LDL-C but also lower fasting and postprandial remnant lipoproteins. The aim of the present study was to compare the fasting and postprandial concentrations of TG, total-apoB (as a surrogate of apoB100) and apoB48 as indicators of remnant lipoprotein metabolism in *PCSK9*-LOF carriers.

4.2 METHODS AND MATERIALS

4.2.1 *Population and Study Participants*

Subjects were recruited from a previously identified Caucasian and African Canadian pool (the Ottawa PCSK9 Cohort) that underwent full exonic sequencing of the *PCSK9* gene[21]. Of this cohort, subjects with 1 or more copies of the *PCSK9* L10ins/A53V and/or I474V and/or R46L variants (n=22), and no *PCSK9* variants at all (n=23), were identified and approached. Males and females, aged 30-80 years with a BMI of 20-40 kg/m^2, and fasting serum LDL-C >2.0 and <7 mmol/L, total cholesterol (TC)/HDL-C ratio >4.0, TG < 4.5 mmol/L and HDL-C > 0.6 mmol/L were eligible. Exclusion criteria included cognitive impairment, being on any medication for dyslipidemia for 6 weeks prior to the oral fat tolerance test (OFTT) or unknown supplements, nutraceuticals, herbal medications and sex hormone therapy (including oral contraceptive pills and testosterone therapy), active or chronic hepatic or renal disease, presence of tendon xanthomas, type 1 or type 2 diabetes mellitus, un-treated hypo- or hyper-thyroidism, major illness such as cancer, pulmonary and gastrointestinal disorders, acute illness or surgical procedure within the previous 3 months, alcohol consumption > 2 drinks per day (1 drink defined as 12 fl. oz of beer or

5 fl. oz of wine or 1.5 oz. of liquor), and apoE2/2, 3/2, 4/2 genotype to avoid the apoE2 allele (which is known to enhance postprandial lipemia). The 22 *PCSK9*-LOF variant subjects had the following variants: I474V (n=9), L10ins/A53V (n=4), R46L (n=2), L10ins/A53V and I474V (n=3), L10ins/A53V and R46L (1), and homozygous I474V (n=3).

4.2.2 *Study Design*

The present study was a case-control, metabolic study. The study protocol was approved by the Research Ethics Board of the Ottawa Hospital Research Institute (OHRI). The study was conducted in the Clinical Research Center (CRC) of the Ottawa Hospital. The OFTT was conducted by experienced staff in a Dynamic Function Testing Facility within the CRC. Samples were processed, stored at -80°C, and subsequently analysed in an adjoining Clinical Research Laboratory and in Edmonton, Alberta. Subjects from our previous study pool were invited to attend an interview. Those who agreed to participate provided written informed consent and underwent further history taking, examination and laboratory screening to establish their eligibility. Tests done included serum glucose, creatinine, alanine transaminase (ALT), thyroid stimulating hormone (TSH), apoE genotyping, and serum lipids, including total cholesterol (TC), TG, high density lipoprotein cholesterol (HDL-C), LDL-C and TC/HDL-C. These results were used to determine eligibility for the OFTT.

4.2.3 *Postprandial Study (Oral Fat Tolerance Test)*

Eligible participants received dietary counseling from a registered dietitian and underwent a 6-week run-in dietary period to standardize the background diet. Subjects were advised to refrain from physical exercise for 60 hours and abstain from alcohol for 36 hours before the OFTT as these are known to acutely affect postprandial lipemia (PPL). A fat load was given at 08:00 h after a 12-hour fast. The test drink consisted of 350 ml whipping cream (35% fat), two tablespoons of chocolate-flavored syrup, one tablespoon of granulated sugar, and one tablespoon of instant non-fat dry milk. Of the total 880 kcal, 5.0% were from protein, 26% from carbohydrate, and 69% from fat. The fat load contained 453 mg cholesterol and a polyunsaturated to saturated fatty acid ratio of 0.06. The meal (69% fat, 1,298 Kcal/350mL) volume was calculated per body surface area (BSA) using the following formula: Volume = 350 X BSA/2, which has been used by our group previously[22]. The fat load was consumed within 10 minutes. Blood was drawn before and 2, 4, and

6 hours after the fat load. The 0 and 4 hour samples had their lipoprotein sub-fractions separated by cumulative floatation ultracentrifugation and their cholesterol and triglyceride contents were determined at each time point. Blood was collected into EDTA-vacutainer tubes and centrifuged at 3,000 rpm for 10 minutes at 22ºC to obtain plasma and leukocytes. To obtain serum for lipid measurements, blood was collected into SST-vacutainer tubes, allowed to clot at room temperature for 20 min and centrifuged at 3,000 rpm for 10 min at 22ºC.

4.2.4 *ApoE Genotyping*

Genomic DNA was available for a*poE* exon sequencing from our previous study[21]. Primers used to encompass the *ApoE2* and *ApoE4* gene variations were forward 5' TAAGCTTGGCACGGCTGTCCAAGGA3' and reverse 5' TAAGCTTGGCACGGCTGTCCAAGGA 3'[23]. Fifty nanograms of gDNA were used in the polymerase chain reaction (PCR) for amplification using platinum Taq DNA polymerase (Invitrogen). Cycle conditions were denaturation 2 min at 94ºC, annealing for 2 min at 64ºC, and extension for 3 min at 72ºC with final extension of 5 min at 72ºC for a total of 35 cycles. Amplified DNA were analysed by standard DNA-sequencing reactions were carried out as a service by BioBasic Sequencing (Markham, ON, Canada)

4.2.5 *Measurement of Serum Lipid and Lipoproteins*

Fasting plasma apoB48, total-apoB, apoCII, and apoCIII as well as subsequent postprandial (2, 4, and 6 hour) measures of apoB48, and total-apoB concentrations were analyzed using a highly sensitive chromogenic sandwich ELISA (Shibayagi, Co., Ltd., Japan; Alercheck., Inc; Abcam., plc, respectively). All samples were quantified 2x and absorbance was measured spectrophotometrically with an intra-assay coefficient of variability (CV) of 4.8-5% and inter-assay CV of 7.5-9.7%. Serum PCSK9 was quantified using a human PCSK9 ELISA (CyClex Co., Japan). Serum PCSK9 was quantified 4x with an intra-assay CV of 1.5-2.6% and an inter-assay CV of 2.9-7.1%.

4.2.6 *Statistical Analysis*

GraphPad Prism 5 software (GraphPad Software Inc) and SPSS version 20 (SPSS Inc., Chicago, USA) were used for statistical analysis. The data are presented as mean (SD) or frequency and percentage. All continuous variables were tested for normal distribution using the Shapiro-Wilks test; normally distributed data were analyzed using Student's t-test (unpaired and paired),

and non-normally distributed data using Mann-Whitney U test for unpaired samples and Wilcoxon Signed-Rank test for paired samples. Categorical variables were tested using chi-squared or Fisher's exact test. Area under the curve (AUC) was calculated through Graph Pad Prism 5 software and corresponds to the total plasma concentration over the 6 hour postprandial measured. The fasting concentration of the respective parameter is further subtracted from the total AUC to generate the incremental area under the curve (iAUC). The iAUC represents the rate of change in the postprandial response adjusted for the initial concentration of the parameter. Outliers were identified using ROUT" Q=1% method recommended in GraphPad Prism. Three outliers were removed from the TG AUC and 2 outliers were removed from calculated apoB48 AUC using this method. Differences in groups were established using Student's t-test and a p-value <0.05 was considered statistically significant.

4.3 RESULTS

4.3.1 Fasting Biochemical Profile of PCSK9-LOF Variant Subjects

Anthropometry and fasting biochemistry of *PCSK9*-LOF variant subjects and non-variant control subjects were summarized in Table 1. The reduction in baseline TG is similar to that seen with other LOF variants[8,9]. Subjects were similar in age, gender, weight, height, BMI, waist circumference and apoE genotype. LOF variants had significantly reduced fasting LDL-C (-13.6%), TC (-7.1%), TG (-21.3%), apoB48 (-25.7%) and VLDL-C (-34%). Additionally, LOF variants had non-significantly lower levels of total-apoB (-17.6%, p=0.059) and PCSK9 (-16.8%, p=0.077) compared to those without variants. There was no significant difference in fasting HDL-C (p=0.46), apoCII (p=0.13), and apoCIII (p=0.66) between the groups (Table 4-1).

	Non-Variant	LOF Variant	*p*-value
n	23	22	
Age (yrs)	63.2 (9.1)	59.0 (12.9)	0.214
Sex			
Male	13	9	
Female	10	13	
apoE (n)			
3/3	17	18	
4/3	6	4	
Height (cm)	165.2 (12.3)	166.7 (6.7)	0.627
Weight (Kg)	76.9 (15.3)	73.9 (12.8)	0.487
BMI (Kg/m²)	28.4 (3.2)	26.7 (4.1)	0.075
Waist Circumference (cm)	94.6 (10.0)	91.4 (15.6)	0.446
BSA (m²)	1.9 (0.2)	1.8 (0.2)	0.543
PCSK9 (ng/mL)	290 (78.0)	241.3 (67.5)	0.077
Cholesterol (mmol/L)	5.6 (0.8)	5.2 (1.1)	**0.046**
TG (mmol/L)	1.59 (0.57)	1.25 (0.53)	**0.032**
VLDL-C (mmol/L)	0.53 (0.33)	0.35 (0.20)	**0.040**
LDL-C (mmol/L)	3.53 (0.72)	3.05 (0.79)	**0.015**
HDL-C (mmol/L)	1.41 (0.32)	1.50 (0.44)	0.460
Total-apoB (µg/mL)	126.7 (38.97)	107.4 (26.64)	0.059
apoB48 (µg/mL)	8.17 (3.29)	6.07 (2.82)	**0.028**
apoCIII (µg/mL)	209.4 (87.1)	198.7 (73.8)	0.661
apoCII (µg/mL)	69.6 (23.2)	58.1 (23.6)	0.125

Table 4-1: Baseline Characteristics of Non-Variant and *PCSK9*-LOF Variant Subjects

Data presented as mean (SD). *Comparisons between PCSK9 non-variant and PCSK9 variant subjects, apoB48, and TG by Mann Whitney U or Student's T test**.** Bolded p-values are statistically significant.

4.3.2 Postprandial Response of Triglycerides, ApoB, and PCSK9

The postprandial response and quantified AUC and iAUC of TG, apoB (total-apoB and apoB48), and PCSK9 are shown in Figure 4-1. Carriers of PCSK9-LOF variants had reduced postprandial response in all parameters examined. The total-apoB AUC was significantly lower for LOF variants (-17%), while the iAUC was almost identical between groups. The triglyceride AUC and iAUC were significantly lower for LOF variants than non-variants (AUC LOF -18% AUC non-variant; iAUC LOF -37% non-variant). The apoB48 AUC and iAUC were significantly reduced for LOF variants (-23% and -35%, respectively). We have examined the largest sub-group with the I474V variant alone (9 heterozygotes plus 3 homozygotes) and the differences in TG AUC and iAUC compared to non-variants were no longer significant ($p=0.0791$ and 0.2245 respectively). The same was true for total apoB and apoB48 ($p = 0.0586$ and 0.9249, and 0.0528 and 0.5896, respectively). At 4 hours post fat load, PCSK9 values drastically declined in both LOF and non-variant groups. Interestingly, the 4-hour postprandial decline was much greater for the LOF group than the non-variant (-24% versus -16% ($p=0.06$), respectively).

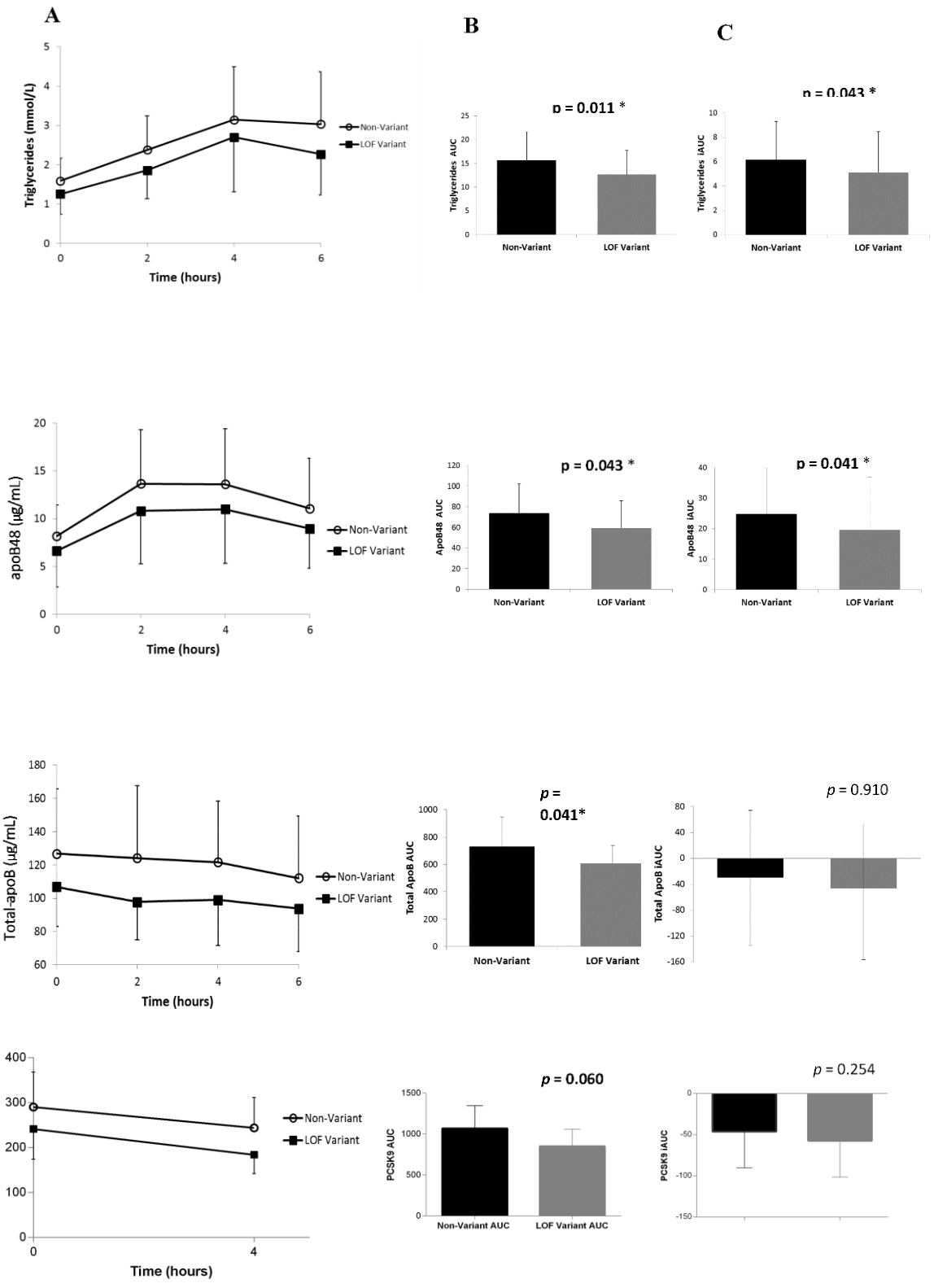
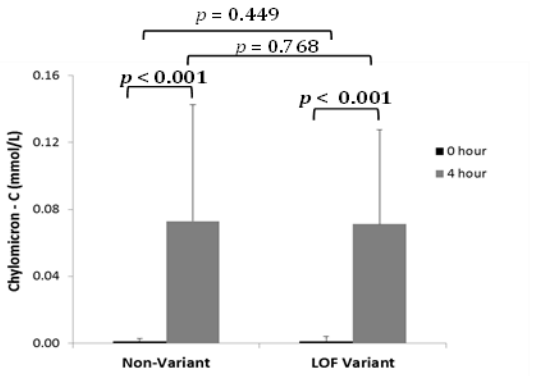

A
Triglycerides (mmol/L)
Time (hours)
Non-Variant
LOF Variant
B
p = 0.011 *
Triglycerides AUC
Non-Variant
LOF Variant
C
p = 0.043 *
Triglycerides iAUC
Non-Variant
LOF Variant
apoB48 (µg/mL)
Time (hours)
Non-Variant
LOF Variant
p = 0.043 *
ApoB48 AUC
Non-Variant
LOF Variant
p = 0.041 *
ApoB48 iAUC
Non-Variant
LOF Variant
Total-apoB (µg/mL)
Time (hours)
Non-Variant
LOF Variant
p = 0.041*
Total ApoB AUC
Non-Variant
LOF Variant
p = 0.910
Total ApoB iAUC
PCSK9 (ng/mL)
Time (hours)
Non-Variant
LOF Variant
p = 0.060
PCSK9 AUC
Non-Variant AUC
LOF Variant AUC
p = 0.254
PCSK9 iAUC

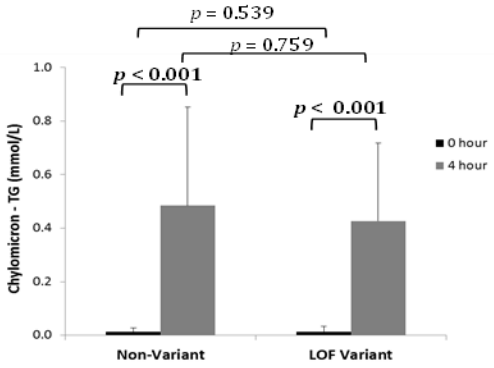
Figure 4-1: *The postprandial response of plasma TG, apoB48, total-apoB, and PCSK9 in LOF variants and non-variant control subjects.* Participants were given a fat load to consume after a 12-hour fast. Blood was drawn before, 2, 4, and 6 hours after consumption. Data are shown as mean ± SD (A), as area under the curve (AUC) values (± SD) (B), and incremental AUC (iAUC) (C). N=23 and 22 for total-apoB and PCSK9, N=23 and 19 for TG data and N=23 and 20 for apoB48 data for non-variant and PCSK9 LOF variant groups.

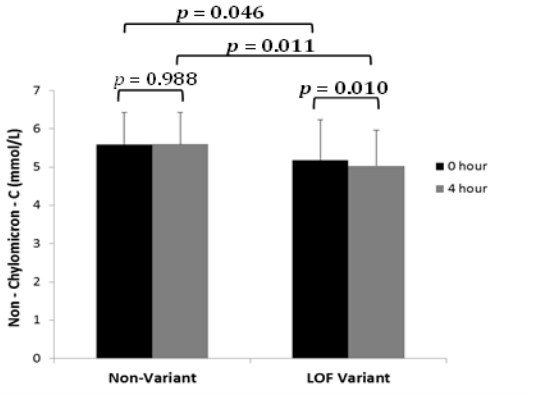
4.3.3 Lipoprotein Sub-fraction Analysis

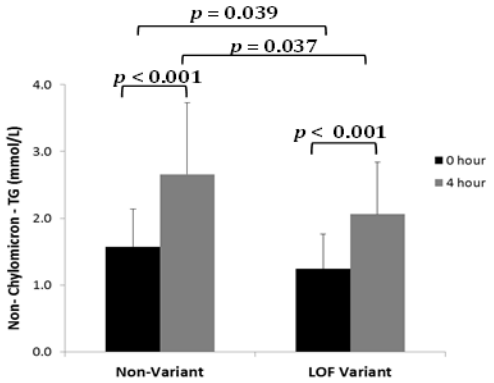
The TG and cholesterol concentrations in lipoprotein sub-fractions are shown in Figure 4-2. There was no difference in fasting and 4-hour postprandial TG and cholesterol levels in the CM fraction between *PCSK9*-LOF and non-variant subjects. However, the concentrations of non-CM TG and cholesterol at both time points were significantly lower in *PCSK9*-LOF subjects than non-variant individuals (Figure 4-2). Likewise, in the non-CM lipoprotein subfractions (VLDL1, VLDL2, IDL, LDL1 and LDL2) the TG and cholesterol concentrations were mostly decreased in *PCSK9*-LOF subjects at both time points as shown in Figure 4-2. However, there was no apparent preferential enrichment or depletion of cholesterol or TG among lipoprotein sub-fractions between groups.

A

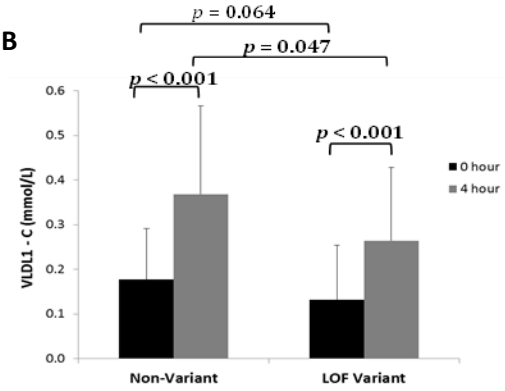

p = 0.449
p = 0.768
p < 0.001
p < 0.001
Chylomicron - C (mmol/L)
0.16
0.12
0.08
0.04
0.00
0 hour
4 hour
Non-Variant
LOF Variant

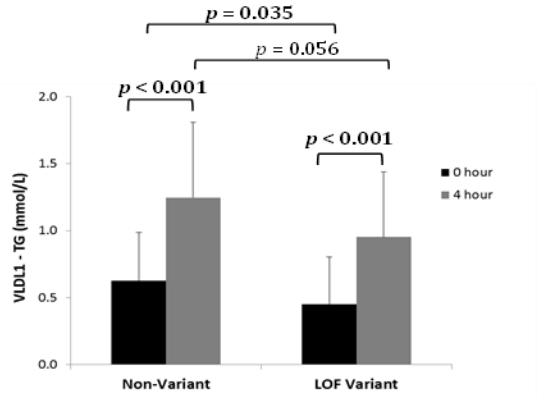

p = 0.539
p = 0.759
p < 0.001
p < 0.001
Chylomicron - TG (mmol/L)
1.0
0.8
0.6
0.4
0.2
0.0
0 hour
4 hour
Non-Variant
LOF Variant


p = 0.046
p = 0.011
p = 0.988
p = 0.010
Non - Chylomicron - C (mmol/L)
7
6
5
4
3
2
1
0
0 hour
4 hour
Non-Variant
LOF Variant

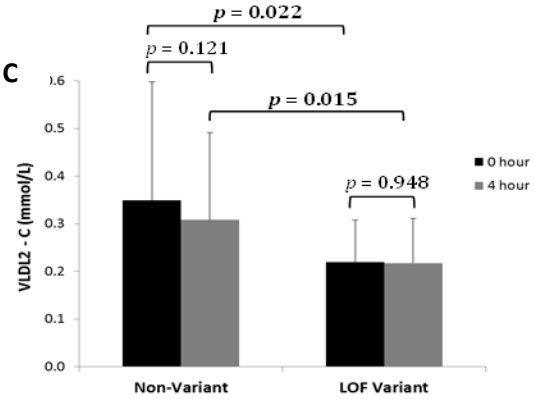

p = 0.039
p = 0.037
p < 0.001
p < 0.001
Non- Chylomicron - TG (mmol/L)
4.0
3.0
2.0
1.0
0.0
0 hour
4 hour
Non-Variant
LOF Variant

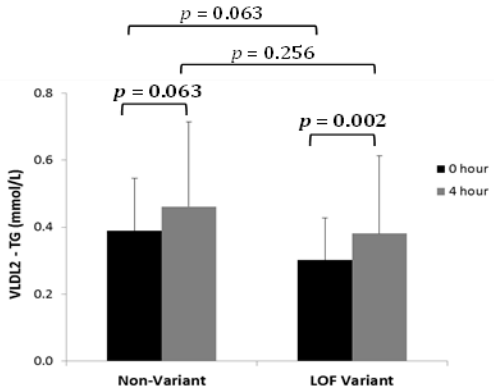
B


p = 0.064
p = 0.047
p < 0.001
p < 0.001
VLDL1 - C (mmol/L)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 hour
4 hour
Non-Variant
LOF Variant

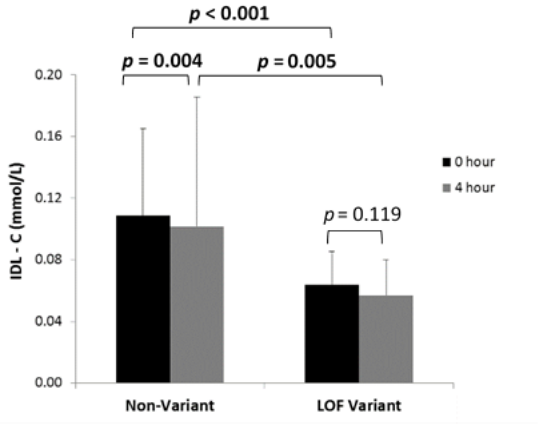

p = 0.035
p = 0.056
p < 0.001
p < 0.001
VLDL1 - TG (mmol/L)
2.0
1.5
1.0
0.5
0.0
0 hour
4 hour
Non-Variant
LOF Variant

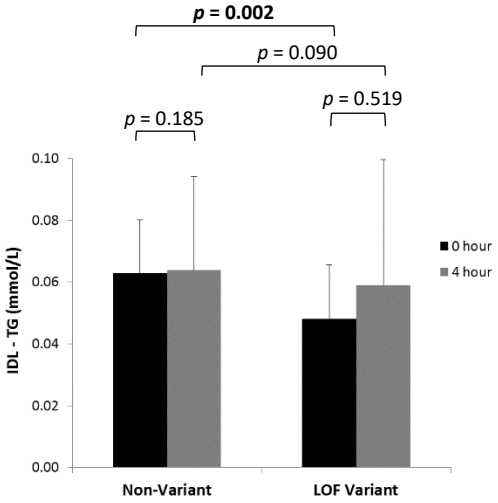
C

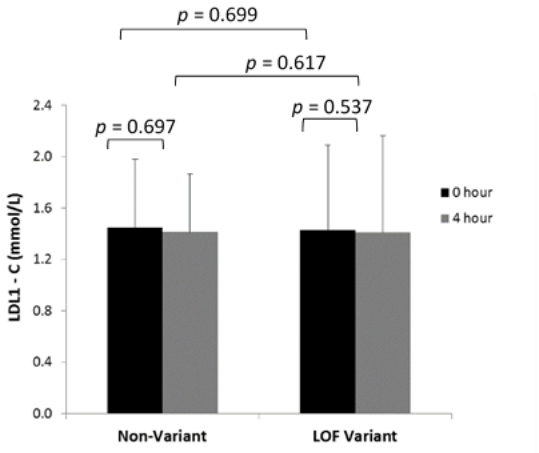

p = 0.022
p = 0.121
p = 0.015
p = 0.948
VLDL2 - C (mmol/L)
0.5
0.4
0.3
0.2
0.1
0.0
0 hour
4 hour
Non-Variant
LOF Variant

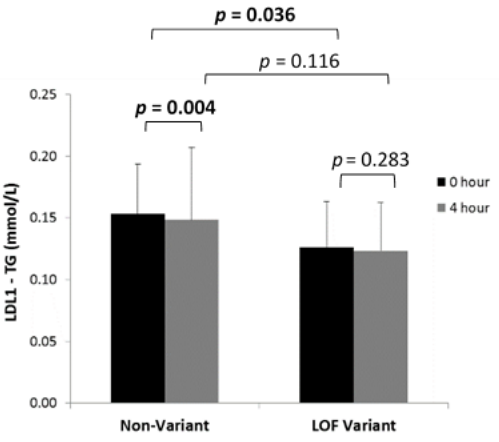

p = 0.063
p = 0.256
p = 0.063
p = 0.002
VLDL2 - TG (mmol/L)
0.8
0.6
0.4
0.2
0.0
0 hour
4 hour
Non-Variant
LOF Variant

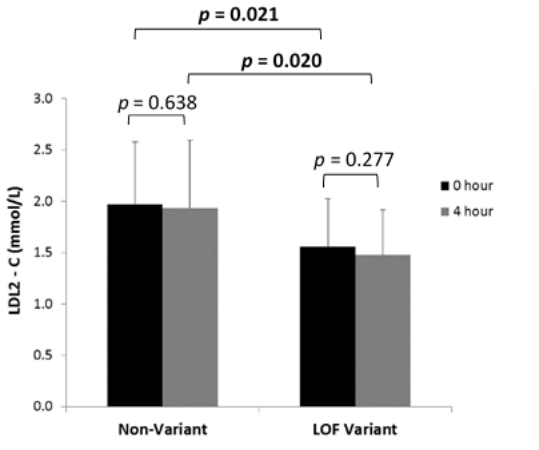
D


p < 0.001
p = 0.004
p = 0.005
p = 0.119
IDL - C (mmol/L)
0.20
0.16
0.12
0.08
0.04
0.00
0 hour
4 hour
Non-Variant
LOF Variant


p = 0.002
p = 0.090
p = 0.519
p = 0.185
IDL - TG (mmol/L)
0.10
0.08
0.06
0.04
0.02
0.00
0 hour
4 hour
Non-Variant
LOF Variant


p = 0.699
p = 0.617
p = 0.697
p = 0.537
LDL1 - C (mmol/L)
2.4
2.0
1.6
1.2
0.8
0.4
0.0
0 hour
4 hour
Non-Variant
LOF Variant


p = 0.036
p = 0.116
p = 0.004
p = 0.283
LDL1 - TG (mmol/L)
0.25
0.20
0.15
0.10
0.05
0.00
0 hour
4 hour
Non-Variant
LOF Variant


p = 0.021
p = 0.020
p = 0.638
p = 0.277
LDL2 - C (mmol/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 hour
4 hour
Non-Variant
LOF Variant

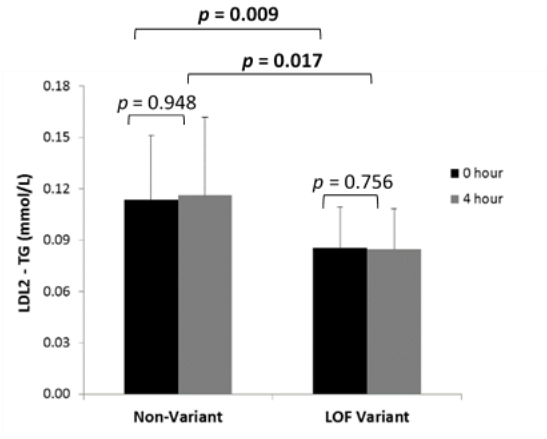

p = 0.009
p = 0.017
p = 0.948
p = 0.756
LDL2 - TG (mmol/L)
0.18
0.15
0.12
0.09
0.06
0.03
0.00
0 hour
4 hour
Non-Variant
LOF Variant

Figure 4-2: *The postprandial cholesterol and TG response in chylomicron and non-chylomicron sub-fractions* (A), as well as the postprandial cholesterol and TG response in VLDL1-C, VLDL-2-C (B), IDL-1-C (C), and LDL1-C and LDL2-C sub-fractions (D). Data are shown as mean ± SD. N=23 and 22 for non-variant and PCSK9 LOF variant groups, receptively.

4.4 **DISCUSSION**

4.4.1 *PCSK9 Loss-of-function variants have reduced triglyceride-rich lipoproteins*

The major finding of this study is that humans with *PCSK9*-LOF variants have lower circulating TRL remnants than those with no *PCSK9*-LOF variants. The impact of circulating PCSK9 on LDL metabolism has been well established, but our findings demonstrate that PCSK9 also influences TRL metabolism. Specifically, we observed that individuals carrying LOF variants in PCSK9 have reduced circulating postprandial lipoproteins, which consist primarily of CM, VLDL, and their remnants. A second important finding in our study is the decline in circulating PCSK9 after an oral fat load. The postprandial decline in PCSK9 was observed in both LOF and non-variant individuals. These novel findings add to the evidence for a role of PCSK9 in TRL metabolism[24-26]. Cholesterol-rich apoB-containing lipoproteins are known to be causally involved in the etiology of atherogenesis[27]. We can consider that the reduction in CAD risk associated with *PCSK9*-LOF variants[8,9] may not only be attributed to lifelong reductions in plasma LDL, but also TRL remnant lipoproteins. In the FOURIER study, with 27,564 participants, TG decreased by 16.2% and 0.7% in the evolocumab-treated subjects and placebo-treated groups respectively[5]. Thus, the magnitude of decrease in baseline TG level in our study (21%), is close to that seen in response to anti-PCSK9 therapy, which was associated with reduced risk of cardiovascular events[5]. This highlights the importance of looking for a role of PCSK9 in TRL metabolism.

4.4.2 *PCSK9 Loss-of-function variants are protected against postprandial lipemia*

We demonstrated *PCSK9*-LOF variants have lower TG and apoB48 not only during fasting, but also during the fed or postprandial state. Elevated fasting apoB48 predicts fat intolerance (as measured by postprandial apoB48 and TG)[15]. The present findings demonstrate that LOF variants are protected from fat intolerance. The mechanisms responsible for attenuation of PPL in our subjects with *PCSK9*-LOF variants remain to be clarified. A possible mechanism is the well-known association of diminished *PCSK9* activity with an increase in the LDL-r, which in addition to binding apoB, can also bind apoE, resulting in greater clearance of apoE-containing

CM remnants (CM-R) and VLDL remnants[7,15,28]. Another possibility is that PCSK9 may affect TRL clearance through its effects on other receptors in the LDLR family, including LRP1, very low density lipoprotein receptor (VLDLR), and apoE receptor 2 (apoER2)[29,30]. In particular, LRP1 has been shown in pre-clinical studies to be an important factor in the clearance of CM-R and VLDLR. It has been shown that PCSK9 can mediate degradation of LRP-1 and that LRP-1 competes with the LDLR for PCSK9 activity[30]. While it has also been shown that PCSK9 induces degradation of VLDLR and apoER2, it is less clear whether these receptors play a significant role in TRL clearance in humans[30]. Beyond an alteration in receptor-mediated TRL clearance, it is also possible that PCSK9 may affect the lipolysis of CM and VLDL and their remnants. To test this, we measured the fasting concentration of apoCII and CIII, which are apolipoproteins involved in the regulation of lipoprotein lipase. We did not find any differences in fasting apoCII, apoCIII or the CII/CIII ratio between groups, suggesting that alterations in lipolysis are a less likely mechanism for PCSK9's action on TRL clearance in this population.

Our non-CM and sub-fraction data indicate a reduction in fasting and postprandial lipoprotein remnants and LDL. TG and cholesterol concentrations were most strikingly reduced in the LDL2 sub-fraction, but least so in the LDL1 sub-fraction in the *PCSK9*-LOF variants. A reduction in the LDL2 sub-fraction is consistent with the well-recognized increase in LDL-r associated with having PCSK9-LOF variant(s)[31]. PCSK9 is highly expressed in the intestine, where it possibly has a role in CM production and transintestinal cholesterol excretion[32]. In the present study, there were no differences between fasting and 4-hour postprandial CM-TG and cholesterol levels in *PCSK9*-LOF variant and non-variant subjects. Kinetic data are necessary to determine whether CM production rate is diminished in the presence of *PCSK9*-LOF variants.

The relationship between PPL and PCSK9 is not well studied. Le May *et al* showed an attenuated postprandial TG response to an oral olive oil load in *PCSK9* knockout mice compared to their wild-type littermates[20], consistent with the findings of the present study. Conversely, Cariou *et al* demonstrated that an oral fat load in 2 heterozygous carriers of the R104C-V114A *PCSK9*-LOF mutation did not alter postprandial plasma TG concentrations compared to non-variant subjects. The authors concluded that plasma PCSK9 was not associated with PPL in humans[33]. The discordance between our studies may be attributed to the phenotype of the variants studied. The LOF variants in Cariou and colleagues' study had very low to undetectable plasma

PCSK9 and very-low plasma LDL-C. In the present study, the *PCSK9*-LOF variants studied (L10ins/A53V and/or I474V and/or R46L) had modest lowering of circulating PCSK9 and lower but normal plasma LDL-C concentrations. Different *PCSK9* variants appear to result in different biochemical characteristics[34,35] and therefore, may explain the differences between these two postprandial studies. Additionally, Chan and colleagues conducted a postprandial study on 17 obese subjects and demonstrated a positive association between plasma PCSK9 and the AUC and iAUC for apoB48 and an inverse association with TRL-apoB48 fractional clearance rate[36]. In the present study, we similarly showed a reduction in fasting apoB48 and apoB48 AUC and iAUC. However, the study by Chan and colleagues was based on plasma PCSK9 concentration, whereas the present study is based on *PCSK9* genotype.

4.4.3 *Plasma PCSK9 significantly declines following a high fat meal*

Plasma PCSK9 was found to decline four hours after an acute oral fat load in individuals with and without LOF PCSK9 variants. Cariou *et al* studied 2 LOF *PCSK9* subjects and also observed significant decreases in serum postprandial PCSK9, consistent with our results[33]. However, the authors further reported postprandial PCSK9 was unchanged after an acute fat load in 10 healthy volunteers[33]. The discrepancy in the postprandial response of plasma PCSK9 between studies is unclear, but could be due to the composition of the meals given. A decrease in plasma PCSK9 after an acute oral fat load may be a result of *PCSK9* expression being under the control of the nuclear transcription factors sterol response element binding protein (SREBP)-2[37] and SREBP-1c[38], both of which act through the same response element on the promotor of *PCSK9*[38]. Increased postprandial cholesterol and fatty acids would act to reduce *PCSK9* expression through a suppression of hepatic SREBP-2 and SREBP-1c, respectively. PCSK9-LOF variants could also act to lower circulating PCSK9 by reducing hepatic LDLR degradation[7]. Circulating PCSK9 is primarily cleared from the circulation by hepatic LDLR[39]. Therefore, increased LDLR expression in subjects carrying LOF variants may enhance the clearance of circulating PCSK9 resulting in a more acute postprandial decline in PCSK9.

4.4.4 *Strengths and Limitations*

In this study, we examined fasting and postprandial lipoproteins from a subset of individuals with *PCSK9*-LOF variants from our Ottawa cohort[21]. The use of full exonic sequencing to identify well-defined variant and control populations was a major strength of the present study. This genotyping approach allowed us to ensure that variant subjects had no other *PCSK9* variants, and non-variant control subjects had no *PCSK9* variants. The exclusion of participants carrying an apoE2 allele added to the robustness of our genotyping approach.

Additionally, our study subjects had *PCSK9* variants associated with a relatively small LOF effect on LDL-C levels. No subject had LOF mutations Q152H[40], Y142X and C679X[8,9] that are associated with major reductions in LDL-C levels. We speculate that any effects on fasting and postprandial concentrations of remnant lipoproteins seen with our "subtle" LOF variants may be even more pronounced with stronger LOF PCSK9 variants. None of our subjects had a GOF variant. It is currently not known whether GOF variants are associated with enhanced PPL. We recognize that studying PPL in individuals with major LOF or GOF variants might potentially provide information of greater clinical relevance but these variants are uncommon. We chose LOF *PCSK9* variants that are common in the Canadian population that have been shown to affect lipid metabolism significantly, even in the heterozygous state[21]. Thus, our results apply to a larger portion of the population than had the effects been shown in subjects with rare but stronger LOF and/or GOF variants.

Since different LOF variants may have different effects on lipoprotein metabolism, it would be informative to compare the effects of different LOF variants. However, sub-group analysis was not possible in our study as the number of subjects in the sub-groups would be too small. Several subjects had an overlap of LOF variants, leaving the number of subjects with a single LOF variant even smaller. The largest sub-group of 12 subjects with the I474V variant did not show any significant difference in fasting and postprandial TG, total apoB and apoB48 in comparison to controls but this was likely because sample size was too small for such comparison.

4.5 CONCLUSION

In this study, we demonstrated that *PCSK9*-LOF variants are associated with attenuated fasting and postprandial response of plasma TG, apoB48 and total apoB. This may confer protection from CVD and further supports the use of PCSK9 inhibitors to lower CVD risk.

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Chapter 5: Remnant Lipoproteins Are Associated with Increased Cardiometabolic Risk in Adolescents

This chapter has been submitted for publication (Please see Appendix C)

5.1 INTRODUCTION

Over the past three decades the prevalence of overweight or obese youth has almost doubled in developed countries[1]. Adolescent adiposity, particularly central adiposity, is a significant public health concern as it often progresses into adulthood and tracks with a cluster of cardiometabolic abnormalities that contribute to premature cardiovascular disease (CVD) risk[1,2]. At present, there is limited understanding of the potential biomarkers to detect or predict premature CVD risk and the relationship to cardiometabolic risk factors in the adolescent population. Establishing early cardiometabolic risk markers would aid prevention strategies and may also provide insight to the early lipid pathogenesis of metabolic and vascular disorders. Currently, blood lipid screening for overweight youth is either inconsistent or absent. For example, some countries only recommend lipid screening for select 'high-risk' children or adolescents with a family history of CVD[3]. Consequently, an adverse blood lipid profile may go undiagnosed or may be underestimated in a significant portion of this population, particularly in obese youth (3). While advancements in lipid guidelines for adults in Europe and North America now include recommendations to measure non-fasting remnant cholesterol[3–5], no data is yet available in large younger populations. Furthermore, international clinical practice guidelines for assessment of blood lipids and lipid lowering strategies for the youth population are currently limited[6]. The National Cholesterol Education Program (NCEP) recently added reference ranges for fasting plasma non-high density lipoprotein cholesterol (Non-HDL-C), total apolipoprotein (apo) B and triglyceride (TG) concentrations relevant to youth and adolescent population[3]. However, diagnostic and treatment cut-offs primarily use fasted plasma low-density lipoprotein cholesterol (LDL-C) concentrations[3,7]. Furthermore, LDL-C and total cholesterol (TC) are often unchanged in obese youth and these lipids have been shown to remain unchanged or decrease during adolescence suggesting that other lipid biomarkers may be more representative of premature CVD risk in this population[3].

Recent studies have shown a causal association between fasting and non-fasting apoB-remnant cholesterol and CVD[8–10]. ApoB-remnant cholesterol is derived from hepatic very low-density lipoprotein (VLDL-apoB100), intermediate density lipoproteins (IDL-apoB100) and intestinal chylomicron (CM-apoB48) triglyceride rich lipoproteins. In chapters 3 and 4 of this thesis, we demonstrated that non-fasting remnant cholesterol is associated with CVD in a Canadian population and is significantly reduced in *PCSK9*-LOF variants that are protected from CVD.

Quantification of fasting or non-fasting plasma apoB48 can be used as a surrogate marker for intestinal remnant concentration. In humans, the apolipoprotein apoB48 is unique to intestinal chylomicron-derived remnant cholesterol particles[11]. Elevated fasting apoB48-remnant cholesterol has been demonstrated to predict non-fasting lipemia following a high-fat meal in adults[12,13]. Dietary lipid intolerance (or non-fasting lipemia) is exacerbated by over-nutrition and central adiposity, leading to an increase in CVD risk[14]. ApoB48 metabolism has been well characterized in adult populations and disease states predisposed to CVD risk[12]. Fasting apoB48 has been shown to be higher in males compared to females, and elevated in adults with CVD, the metabolic syndrome, and diabetes[15–17]. Despite the convincing evidence in adults, it is unclear whether the relationship of apoB48-remnant cholesterol to CVD risk is maintained in youth. Recent studies have demonstrated that fasting plasma apoB48 concentrations are elevated two-fold in obese pre-pubertal children (case-control study) and is more strongly associated with central adiposity indices compared to LDL-C or TC at this age in a French-Canadian cohort[18,19]. Our results to date suggest that fasting plasma apoB48 may be a representative biomarker of early CVD in youth, however it is unclear if this relationship remains relevant in a larger, general population.

The aim of the present study was to measure fasting plasma apoB48 as a marker of intestinal remnant concentration in youth; to establish the relationship of apoB48 with other plasma lipids; as well as cardiometabolic risk factors in a large sample of adolescents from the Western Australian Pregnancy Cohort (Raine) Study at 17 years of age. It was hypothesized that fasting plasma apoB48 would be positively associated with adiposity, fasting plasma triglycerides and associated cardiometabolic risk factors.

5.2 METHODS

5.2.1 *Study Population*

The Western Australian Pregnancy Cohort (Raine) Study is a prospective population cohort that has been described in detail[20,21]. Briefly, 2900 pregnant women between 16 to 20 weeks of gestation were recruited from hospitals in Perth, Western Australia between 1989 and 1992. The recruited women gave birth to 2868 live infants. The offspring have had follow-up examinations at age 1, 2, 3, 5, 8, 10, 14 and 17 years. The 17-year follow-up involved 1754 adolescents. The study was approved by the Human Ethics Committee at King Edward Memorial Hospital and Princess Margaret Hospital for Children in Perth, Western Australia. Complementary approval was also obtained from the University of Alberta (Pro00001941). Adolescents and their primary caregiver provided informed written consent for participation. Participants were excluded from this study if they did not have complete biochemical and anthropometric data sets.

5.2.2 *Anthropometry*

Calibrated measurements of height, weight, and anthropometry were measured by trained research assistants as previously described[22]. In brief, total body weight was measured using a Wedderburn Chair Scale (nearest 100g) and height with a Holtain Stadiometer (nearest 0.1cm). BMI was derived from body weight (kg)/height (m^2). Waist circumference was measured at the umbilicus using a steal tape measure and skinfold thickness (abdominal, subscapular, suprailiac, and tricep) measures with callipers (Holtain) following standardized protocols[23].

5.2.3 *Biochemistry and Metabolism*

Blood samples were obtained following an overnight fast, serum and plasma prepared and stored at -80°C for subsequent analysis as described previously[22,23]. Standard plasma biochemistry analyses was performed including, high density lipoprotein cholesterol (HDL-C), total cholesterol (TC), triglycerides (TG), insulin, glucose, leptin and adiponectin (PathWest Laboratory at the Royal Perth Hospital), as previously described[23]. LDL-C was calculated using the Friedewald formula, non-HDL-C cholesterol was calculated as TC (mmol/L) – HDL-C (mmol/L) and homeostasis model assessment index of insulin resistance (HOMA-IR) was calculated as insulin (μU/mL) x glucose (mmol/L)/22.5, as previously described[23].

5.2.4 *Blood Pressure*

Systolic and diastolic blood pressure was obtained using an oscillometric sphygmomanometer (DINAMAP vital signs monitor 8100, DINAMAP XL vital signs monitor or DINAMAP ProCare 100; GE Healthcare). After resting in the supine position for 5 minutes, six blood pressure readings were taken every two minutes over a 15 minute period and the average blood pressure was calculated using the last 5 readings, as previously described[24].

5.2.5 *Quantification of Plasma ApolipoproteinB48*

ApoB48 was quantified using an established and validated highly sensitive chromogenic sandwich ELISA method (Shibayagi Co., Ltd., Japan), as previously described[19]. In brief, plasma samples were incubated in 96 well monoclonal antibody-coated plates and then bound with an anti-apoB48 antibody. The final reaction results in a colour change proportional to the concentration of apoB48 in the samples, which is quantified by measuring absorbance at 450nm spectrophotometrically[19].

5.2.6 Other Measures

Alcohol consumption information was obtained from a validated questionnaire regarding alcohol intake type (beer, spirits, or wine) and amount consumed during the week, as previously described[25]. For the present analysis, alcohol consumption was treated as a dichotomous variable (drinker or non-drinker). Smoking was assessed by the number of cigarettes smoked each day, within the last 7 days. Oral contraceptive pill use in females was obtained by answering yes or no to the question, 'In the last 6 months, have you taken any prescription medication(s) e.g. the Pill?' (if yes, 'which medication(s), are you still taking it?')[25,26].

5.2.7 Statistical Analysis

Statistical analyses were performed using Stata/IC 13.1 software (StataCorp LP). Distributions of outcomes were assessed for normality and log transformations were applied to insulin, HOMA-IR, leptin, subscapular, suprailiac, and triceps skinfolds where departures from normality were detected following assessment of normality curves. Insulin, and leptin values below the lowest detectable level of the assay were assigned a lower limit (1.9mU/L, 0.14mg/L, 1.4ng/mL, respectively) and for skinfold measurements an upper limit (41mm) as described

previously[22,23]. Means for censored variables (variables with defined upper and lower limits) were determined by Tobit analysis, which takes into account the probability of being above the censored value. Tabulated values are presented as proportions, arithmetic or geometric (for log transformed variables) means and 95% confidence interval (CI)).

Adult cut-offs for diagnosis of the metabolic syndrome are not applicable in children and adolescents, therefore cardiometabolic cluster analysis was developed in this cohort, as previously described[22,23]. The high-risk and low-risk metabolic cluster were formed separately by sex and included fasting plasma TG, BMI, HOMA-IR and systolic blood pressure. Individuals in the high-risk metabolic cluster were deemed to be at increased cardiometabolic risk.

Differences between sex and the metabolic cluster variable were examined by linear regression, and Tobit regression. Regression analysis was undertaken to determine the relationship between fasting plasma apoB48 concentration, metabolic cluster variable and other cardiometabolic risk factors. Linearity of the association was assessed by multiple adaptive regression spline analysis and fractional polynomials. When non-linearity was detected, quadratic terms identified by fractional polynomial analysis were generated and included in the regression producing two coefficients for the associations with fasting plasma apoB48 concentration. Regression models were adjusted for sex, oral contraceptive use in females and BMI (skinfold thickness, and waist circumference were not adjusted for BMI). Further adjustment for smoking and alcohol use was also applied. Interaction analysis was performed to determine potential sex modification on the association of fasting plasma apoB48 with other cardiometabolic outcome variables. All regression analyses included a robust variance adjustment to account for potential correlations between a small number of siblings (familial bias). Significance was determined at $p < 0.05$, which provided 95% power to detect a difference in the fasting plasma apoB48 concentration in the low-risk and high-risk metabolic cluster variable.

5.3 RESULTS

5.3.1 *Subject characteristics*

For the present cohort study, we analysed adolescents (n=1045) aged 17 years with complete biochemistry and anthropometric data sets. In this population cohort, 52% were male,

85% had both parents that were Caucasian, 50% had consumed alcohol in the past 7 days, 21% were defined as smokers, and 31% of females were using an oral contraceptive.

	Total (n=1045)	**Males (n=545)**	**Females (n=500)**	***p*-value (Males/Females)**
Height (cm)	1.72 (1.72-1.73)	1.78 (1.78-1.79)	1.66 (1.66-1.67)	**<0.001**
Weight (kg)	68.51 (67.62-69.40)	72.64 (71.40-73.89)	64.00 (62.86-65.15)	**<0.001**
BMI (kg/m^2) *Overweight (%)* *Obese (%)*	22.99 (22.73-23.26) 13.5 8.2	22.82 (22.46-23.19) 13.0 7.7	23.18 (22.79-23.57) 14.2 8.8	0.187
Waist Circumference (cm)	79.29 (78.59-79.98)	80.72 (79.78-81.66)	77.69 (76.68-78.71)	**<0.001**
Abdominal Skin Fold (mm)* **	20.44 (19.81-21.06)	17.04 (16.19-17.89)	24.37 (23.60-25.13)	**<0.001**
Subscapular Skin Fold (mm)**	12.62 (12.28-12.97)	11.01 (10.62-11.41)	14.75 (14.22-15.30)	**<0.001**
Suprailiac Skin Fold (mm)**	13.20 (12.73-13.69)	10.76 (10.23-11.31)	16.72 (16.02-17.44)	**<0.001**
Triceps Skin Fold (mm)**	13.28 (12.87-13.71)	9.93 (9.55-10.32)	18.44 (17.90-19.00)	**<0.001**
High Risk Metabolic Cluster (%)	17.8	16.3	19.4	
ApoB48 (ug/mL) *High Risk Metabolic Cluster* *Low Risk Metabolic Cluster*	13.93 (13.54-14.41) 16.90 (15.62-18.18) 13.31 (12.94-13.68)	15.28 (14.71-15.86) 20.43 (18.39-22.45) 14.28 (13.76-14.81)	12.45 (11.97-12.92) 13.66 (12.33-14.98) 12.20 (11.70-12.71)	**<0.001** **<0.001** **<0.001**

LDL-C (mmol/L)	2.34 (2.30-2.38)	2.24 (2.19-2.30)	2.44 (2.39-2.50)	**<0.001**
High Risk Metabolic Cluster	2.51 (2.40-2.62)	2.46 (2.29-2.63)	2.55 (2.41-2.70)	**<0.001**
Low Risk Metabolic Cluster	2.29 (2.25-2.33)	2.19 (2.13-2.25)	2.41 (2.35-2.47)	**<0.001**
HDL-C (mmol/L)	1.30 (1.28-1.31)	1.21 (1.19-1.23)	1.39 (1.37-1.42)	**<0.001**
TC (mmol/L)	4.12 (4.07-4.16)	3.94 (3.88-4.00)	4.31 (4.25-4.38)	**<0.001**
High Risk Metabolic Cluster	4.41 (04.29-4.53)	4.37 (4.21-4.53)	4.44 (4.27-4.62)	**<0.001**
Low Risk Metabolic Cluster	4.04 (4.00-4.09)	3.84 (3.78-3.91)	4.27 (4.20-4.34)	**<0.001**
TG (mmol/L)	1.05 (1.02-1.08)	1.06 (1.01-1.10)	1.04 (1.00-1.09)	0.684
Non-HDL-C (mmol/L)	1.04 (1.00-1.09)	1.03 (0.97-1.09)	1.05 (0.99-1.12)	0.691
Systolic Blood Pressure (mmHg)	113.6 (112.9-114.2)	118.0 (117.2-118.8)	108.7 (107.9-109.5)	**<0.001**
Diastolic Blood Pressure (mmHg)	58.72 (58.33-59.11)	58.13 (57.58-58.68)	59.36 (58.81-59.92)	**<0.001**
Glucose (mmol/L)	4.75 (4.72-4.78)	4.85 (4.80-4.91)	4.64 (4.60-4.67)	**<0.001**
Insulin (mU/L)**	7.24 (6.94-7.55)	6.82 (6.42-7.22)	7.73 (7.27-8.21)	**0.003**
HOMA-IR*	1.64 (1.59-1.70)	1.64 (1.56-1.73)	1.65 (1.58-1.73)	0.144
Leptin (ng/mL)**	7.82 (7.11-8.60)	2.66 (2.35-3.02)	24.20 (22.70-25.81)	**<0.001**

Total Adiponectin (ug/mL)	9.66 (9.31-10.01)	8.19 (7.78-8.61)	11.25 (10.71-11.79)	**<0.001**
CRP (mg/L)**	0.57 (0.52-0.63)	0.43 (0.38-0.49)	0.78 (0.69-0.89)	**<0.001**

Table 5.1. Lipid and metabolic markers of cardiometabolic risk in adolescents aged 17. Values expressed as mean (95% CI). * Geometric mean is presented for log transformed normal data ** Geometric mean from Tobit analysis *** Tobit analysis was applied to censored variable. Bolded p-values are statistically significant.

5.3.2 Plasma lipid biochemistry

The mean fasting plasma apoB48 concentration was significantly higher, 19% or 2.8 ug/ml, in males compared to females (Figure 5-1). Conversely, females had significantly higher LDL-C, HDL-C, and TC compared to males, but both had similar TG and non-HDL-C concentrations (Figure 1). Females also had significantly higher fasting plasma insulin, leptin, and adiponectin compared to males. BMI was similar between sexes, although waist circumference was 4% or 3.0 cm higher in males, and abdominal skin fold thickness was 30% or 7.3 cm higher in females (Table 5-1). Interestingly, LDL-C, TC, TG and non-HDL-C were significantly higher in females using oral contraceptives by 11%, 10%, 22%, and 23%, respectively. In contrast, fasting plasma apoB48 concentrations did not differ between oral contraceptive and non-oral contraceptive use, therefore all females were combined in subsequent analyses.

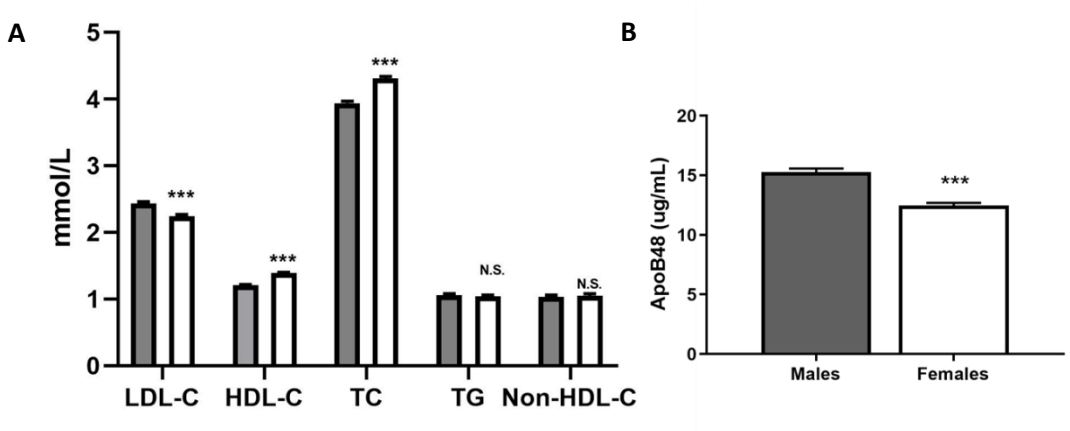

Figure 5-1. Fasting plasma lipid profile (A) and apoB48-remnant lipoproteins (B) in male and female adolescents from the RAINE cohort. Data are expressed as mean ± SEM; * P<0.001. Grey bars indicate male participants and white bars indicate female participants.

Using established metabolic cluster groupings, 17.8% (males and females) of the cohort fell into the high-risk metabolic cluster. Fasting plasma apoB48 concentration was significantly correlated with the high-risk metabolic cluster (OR (95% CI): 1.09 (1.06-1.11)). Fasting plasma apoB48 concentration was elevated 21% (3.6 ug/ml) in males and females in the high-risk metabolic cluster, and this difference was more pronounced in males (31% or 6.8 ug/ml higher) compared to females (11% or 1.5 ug/ml higher) (Figure 5-2).

Fasting plasma LDL-C was mildly elevated in both males and females (8.5%) in the high-risk metabolic cluster (Figure 5-3). Interestingly, this difference was more pronounced in males (11%) compared to females (6%), however the magnitude of difference in the high-risk group (LDL-C 8.5%) was not as pronounced as that reported for plasma apoB48 (21%).

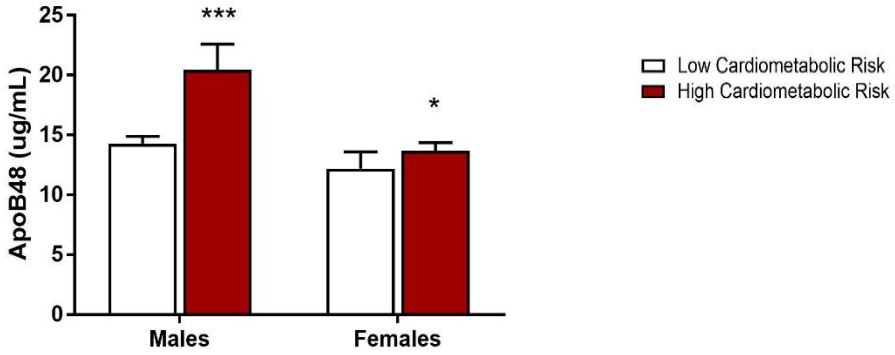

Figure 5-2. Fasting plasma apoB48 concentration in high and low-risk metabolic cluster male and female adolescents from the RAINE cohort. Data are expressed as mean ± SEM; ** P<0.001 * P<0.05.

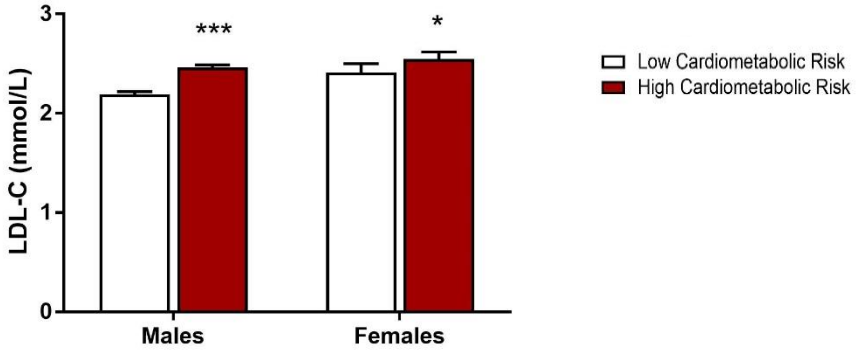

Figure 5-3. Fasting plasma LDL-C concentration in high and low-risk metabolic cluster male and female adolescents from the RAINE cohort. Data are expressed as mean ± SEM; ** P<0.001 * P<0.05

5.3.3 *Regression analysis for apoB48 and cardiometabolic risk variables*

Multivariable regression analysis, adjusted for sex, oral contraceptive use in females and BMI, showed fasting plasma apoB48 concentration was positively associated with TG, TC, insulin, HOMA-IR, leptin, abdominal and subscapular skinfolds and waist circumference (Table 5-2). Plasma apoB48 concentration was inversely associated with HDL-C, and adiponectin (Table 5-2). Interestingly, plasma apoB48 concentration showed a non-linear 'U-shaped' association with BMI, indicating a negative relationship between BMI and apoB48 at lower concentrations, as well as a positive association between these variables at higher concentrations of apoB48. Alcohol and smoking had no effect on outcomes and consequently were not included in the final model. Furthermore, there was no significant effect of sex on the association between fasting plasma apoB48 concentration and outcome variables.

Variable	**Coef. ApoB48 (ug/mL) (95% CI)**	**R^2**	**P**
LDL-C (mmol/L)	-0.003 (-0.0102 – 0.0050)	0.058	0.5
Waist Circumference (cm)	0.123 (0.01062 – 0.2351)	0.023	0.03
Log(Subscapular Skin Fold) (mm)	0.005 (0.0005-0.0096)	0.106	0.03
Abdominal Skin Fold (mm)	0.126 (0.0197 – 0.2317)	0.021	0.02
Log(Leptin) (ng/mL)	0.009 (0.0091 – 0.0354)	0.391	0.010
BMI (kg/m^2)	-0.803#/0.214^ (-1.354 – (-0.251))/(0.0723 – 0.3559)	0.014	0.004#/0.003^
Adiponectin (ug/mL)	-0.071 (-0.113 – (-0.0281))	0.114	0.001
TG (mmol/L)	0.045 (0.0375 - 0.0528)	0.363	<0.001
TC (mmol/L)	0.014 (0.0060 - 0.0214)	0.116	<0.001
HDL-C (mmol/L)	-0.005 (-0.0073 – (-0.0021))	0.169	<0.001
Log(Insulin) (nmol/L)	0.023 (0.0164 – 0.0290)	0.100	<0.001
Log(HOMA-IR) (U)	0.020 (0.0132 – 0.0260)	0.186	<0.001

Coef, 95%, R^2 and P value of linear apoB48
^ Coef, 95% CI, R^2 and P value of apoB48*log(apoB48)

Table 5-2. Regression analysis between apoB48 and outcome variables. Multivariable analysis is adjusted for BMI, sex, correlates amongst siblings, and females using oral contraceptives (BMI, skinfolds, and waist circumference are not adjusted for BMI)

5.4 DISCUSSION

5.4.1 Fasting plasma apoB48 concentration is elevated in conditions of high cardiometabolic risk in adolescents

Our data demonstrates that fasting plasma apoB48 concentration is higher in males compared to females in an adolescent population (17 years of age). Importantly, we also observe that fasting plasma apoB48 was elevated in individuals in the high-risk metabolic cluster, and significantly higher in high-risk males compared to females. Higher concentrations of plasma apoB48 (as a measure of intestinal remnant concentration) in adolescent males in the high-risk cluster may exacerbate their premature CVD risk profile, consistent with observations in adults[27]. In males compared to females, the length of the whole intestine and its different parts is significantly greater[28] while chylomicron clearance is less efficient[29], both of which could contribute to elevated apoB48 concentrations in adolescent males. In adults, fasting plasma apoB48 is frequently higher in males compared to females, and is elevated in adults with the metabolic syndrome, diabetes and CVD[16]. However, this sex difference was not observed in a younger pre-pubertal study (< age 14), suggesting that with increasing age in adolescents, fasting plasma apoB48 concentration may be a suitable predictor of sex differences for CVD risk[18,19]. Interestingly, mean fasting plasma apoB48 concentration in this study was approximately 6.0ug/mL higher compared to our previous reports in pre-pubertal children[18,19]. During adolescence there are distinct changes in body fat distribution and endocrine hormones, as well as decreased insulin sensitivity, and these changes have been reported to result in alterations in plasma lipid profile, notably decreased LDL-C and TC[3]. Our findings suggest that pubertal alterations in endocrine hormones (such as androgens), increased insulin sensitivity and/or body fat distribution may impair apoB48-remnant cholesterol metabolism. Factors that may further contribute to increased fasting plasma apoB48-remnants, particularly in the high-risk metabolic cluster, would also include increased intestinal lipid absorption, chylomicron-apoB48 production and secretion, altered lipolysis and high-affinity or other clearance/uptake pathways of apoB48-remnants from the circulation[30–32].

While we did not measure total apoB in this study, the magnitude of change observed for apoB is often tightly associated with corresponding changes in LDL-C. Despite the fact that all LDL-C values would be considered with the normal range (<2.6mmoL), we did observe percentage increase for LDL-C between low and high risk groups (Figure 3). Percent increases for LDL-C were +12.3% and +5.8% for boys and girls respectively in low versus high groups. In comparison, percent increases for apoB48 between low and high groups were +43.0% and +11.9% for boys and girls respectively. The relative increase for apoB48 was more than 3-fold that for LDL-C in boys and 2-fold for that in girls. The challenge in the interpretation with comparisons such as these, is the understanding of the corresponding clinical relevance of changes in LDL-C (13-6%) versus apoB48 (43-12%), particularly in youth of this age.

Collectively, these factors may result in increased fasting plasma apoB-remnants and may represent increased early CVD risk in adolescents. In adults, elevated fasting plasma apoB48 has been shown to be positively associated with carotid intima-media thickness (cIMT)[12]. Furthermore, young adults with the metabolic syndrome have significantly elevated cIMT compared to control[33]. Although, CVD outcomes beyond the clustering were not measured in this study, we would speculate that elevated fasting plasma apoB48 concentrations in adolescents in the high-risk group may be associated with early and increased premature CVD risk[12,33]. Further studies are required to measure subclinical CVD, such as carotid intimal medial thickness (cIMT) or endothelial function or ankle-brachial blood pressure, in order to determine the association with apoB48/remnant concentrations in this age group[8,33–35].

The use of oral contraceptives in adolescent females did not influence fasting plasma apoB48 concentrations, despite the positive association of oral contraceptive use with LDL-C and TC. Other studies have shown similar elevations in these traditional lipid markers, such as plasma TG, in females taking oral contraceptives[36]. To our knowledge, this is the first study to report no apparent effect of oral contraceptive use on fasting plasma apoB48 concentration.

5.4.2 Fasting apoB48 associates with features of the metabolic syndrome

Multivariable regression analysis demonstrated that in adolescent males and females there was a positive association between fasting plasma apoB48 and TG, TC (but not LDL-C), insulin,

HOMA-IR, leptin, abdominal and subscapular skinfold thickness, and waist circumference. In contrast, there was a negative association between fasting plasma apoB48 and HDL-C and adiponectin. Given there is no sex effect on the relationship between apoB48 and other cardiometabolic variables, the findings suggest other factors may play a greater role, such as adiposity, in determining apoB48 metabolism[19,37]. Interestingly, the relationship between fasting plasma apoB48 and BMI was non-linear ("U-shaped"), suggesting high and low BMI may track with higher concentrations of this CVD risk biomarker. Consistent with this theme, the literature does suggest a U-shaped association between traditional CVD risk factors such as BMI, hypertension and diabetes with CVD mortality[38–40]. However, because BMI is not reflective of total body fat or lean muscle composition *per se*, the relationship between apoB48 and BMI is likely to be more representative of markers of adiposity and metabolic risk in adolescence[19]. Waist circumference has been shown to be a good estimate of central or visceral adiposity compared to other anthropometric measures, particularly BMI[41]. Visceral adipose tissue is strongly associated with the metabolic syndrome, insulin resistance, hypertension, and dyslipidaemia[42]. In a French-Canadian cohort, we have reported that fasting plasma apoB48 concentrations are highly associated with central adiposity in pre-pubertal children[19]. Similarly, fasting plasma apoB48 has been positively associated with visceral adiposity in obese adults[14]. Consistent with these findings, our current study shows fasting plasma apoB48 concentration was positively associated with waist circumference, and abdominal and subscapular skin fold thickness, supporting the relationship between apoB48 and central adiposity in this adolescent population.

5.4.3 Fasting apoB48 is associated with impaired insulin sensitivity in adolescents

The associations between fasting plasma apoB48 concentration and features of the metabolic syndrome (waist circumference, TG, HDL-C, and insulin) suggest that apoB48 concentration is representative of an adverse metabolic profile. We have also shown that fasting plasma apoB48 is associated positively with insulin, HOMA-IR, and leptin, and inversely correlated with adiponectin, consistent with findings in adults[27,43]. These findings suggest that fasting plasma apoB48 is associated with impaired insulin sensitivity and adipokine markers in adolescents. The intestine is particularly responsive to changes in insulin sensitivity, and aberrant changes in insulin metabolism are often associated with postprandial or non-fasting hypertriglyceridemia and apoB48-remnant hyperlipoproteinemia in adults[30,31]. An increase in fasting plasma apoB48-

remnants during insulin resistance has been attributed to the delayed clearance of apoB48-remnant particles from the circulation[44]. However, the insulin resistance state is also known to modulate intestinal apoB48-remnant metabolism by increasing enterocyte *de novo* lipogenesis, including cholesterol ester and TG synthesis, as well as lipidation of primordial chylomicron particles[30,32]. These aberrations promote conditions of overproduction of intestinal chylomicron-apoB48 and may explain the elevated fasting plasma apoB48-remnants we have observed in adolescents in the high-risk metabolic cluster group.

5.4.4 Strengths and Limitations of the Study

The study of an adolescent cohort aged 17 years provides a population representing a predominately post-pubertal stage of development, with established biochemical profiles, and subjects who are likely to have adopted a number of early adult lifestyle choices such as smoking, alcohol consumption, and oral contraceptive use in females. However, the Raine cohort consists predominantly of Caucasians and has shown the potential for retention bias of families that are more likely to be well-educated, health conscious, and having a relatively higher income[45]. Consequently, our findings may limit the generalizability to adolescents of other ethnicities or of lower socio-economic status. Although not measured in this study, it would also be interesting to consider how endocrine metabolism, in particular androgens, play an important role in regulating apoB-lipoprotein and lipid metabolism in different phases of puberty and adolescence. This in turn may lead to a better understanding of sex specific lipid profiles and early CVD risk during adolescence, particularly in those with the metabolic syndrome. Future studies could investigate the longitudinal effects of elevated fasting plasma apoB48-remnant concentrations (and/or non-fasting remnant cholesterol) in youth. If indeed these novel values track with other adverse cardiometabolic outcomes and measures of cardiovascular health into early and late adulthood it could offer improvements to the pediatric lipid guidelines. Specific, experimentally controlled postprandial lipid metabolism studies in youth are also lacking and there is currently no data to indicate if fasting plasma apoB48-remnant concentrations predict non-fasting lipemia in these age groups. It is noteworthy that there are ongoing studies to establish reference ranges for apoB and apoB48 in children and adolescents[46].

5.5 CONCLUSION

In conclusion, the findings from this study have demonstrated that fasting plasma apoB48 concentrations in adolescents are associated with measures of adiposity and features of the metabolic syndrome. The data further supports previous findings in pre-pubertal children and suggests that fasting plasma apoB48 may be a potential biomarker of early premature CVD risk in adolescents. The measurement of fasting plasma apoB48 and apoB-remnant cholesterol in older youth, particularly those at high cardiometabolic risk, may support a recommendation to assess non-fasting lipid parameters and to further implement preventative strategies to reduce long term CVD risk this population. Importantly, the data supports the need for longitudinal prospective studies to delineate if increased apoB48/remnant concentrations during adolescence is a marker of a severe, inflammatory or premature onset of CVD during adulthood

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Chapter 6: ApoB-Lipoprotein Remnant Dyslipidemia and High-Fat Meal Intolerance is Associated with Increased Cardiometabolic Risk in Youth

6.1 INTRODUCTION

According to the World Health Organization Growth Charts approximately one in seven children in developed countries are considered obese[1,2]. Childhood obesity is a major public health concern as obesity often persists into adulthood and becomes a large burden on the health care system[3,4]. Adults with obesity are at a significant increased risk of developing early chronic diseases including cardiovascular disease (CVD) and type 2 diabetes[4]. Therefore, identifying youth with obesity who are at risk of developing CVD and associated comorbidities from an early age is critical in prevention of chronic disease in adulthood[5,6]. However, the mechanisms explaining the etiologic link between adiposity and the pathogenesis of atherosclerotic cardiovascular disease in youth remains unclear[7].

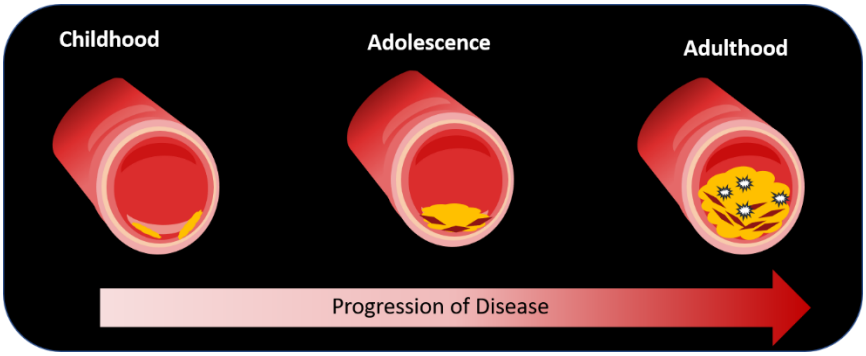


Figure 6-1: The lifelong progression of CVD. CVD pathophysiology arises during childhood and is enhanced by obesity and other metabolic conditions[8,9]. Youth with obesity have a high tendency to track into adolescence and adulthood, which may further enhance CVD and promote adverse cardiovascular events later in life[4].

Fasting total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) are often used by pediatricians to identity children at risk of developing CVD[10]. However, these parameters do not accurately reflect early risk or subclinical progression of CVD in pediatric populations[11]. Indeed, traditional lipid markers (TC, LDL-C, high density lipoprotein cholesterol (HDL-C), and triglycerides (TG)) are often within normal ranges in youth with overweight-obesity, suggesting that other lipid metabolism pathways may be involved[12]. Recent studies from Europe have

demonstrated a causal association between non-fasting remnant cholesterol (RC) and CVD. A 1mmol/L increase in non-fasting remnant cholesterol was found to be associated with a 2.8-fold causal increase in risk for ischemic heart disease in adults[13]. Intestinally derived apoB48-containing lipoproteins, chylomicrons (CM) and their remnants (CM-R) have been shown to have an integral role in atherogenesis and CVD-risk[13,15]. It has been previously demonstrated that CM-R are cholesterol dense particles that are able to penetrate and be retained within the arterial wall resulting in cholesterol accumulation[16]. Additionally, a recent study demonstrated that gene variants associated with lower plasma TG and LDL-C are also correlated with reductions in coronary heart disease and increased concentrations of plasma total apolipoprotein (apo) B[14] It has also been shown that adults with CVD have elevated CM-R particles (assessed as fasting apoB48-lipoprotein remnants). Fasting plasma apoB48 is positively correlated with postprandial dyslipidemia inclusive of higher plasma concentrations of apoB48 and TG following a high-fat meal in adults[17,18].

Chapter 3 of this thesis explored the relationship between non-fasting lipids in CVD and in chapter 4, we demonstrated that individuals with *PCSK9*-LOF variants (that are significantly protected from CVD) have reduced PPL. These findings further emphasize the critical need to assess whether youth with obesity have postprandial dyslipidemia (PPL) and whether this predisposes them to early CVD risk. Data suggests that obese pre-pubertal children have elevated fasting apoB48 remnant lipoproteins and TG compared to healthy weight-aged matched controls[12]. A larger cross-sectional study in children demonstrated that apoB48 remnants have a stronger relationship with indices of adiposity compared to traditional lipid risk markers[19]. Furthermore, in chapter 5 it was found that adolescents at increased cardiometabolic risk have elevated fasting plasma apoB48. Overall, this emerging data demonstrates that there is an accumulation of apoB48 remnant lipoproteins in youth with obesity at high cardiometabolic risk, which strongly suggests that these youth have PPL and increased susceptibility to subclinical CVD. The primary aim of the study was to determine whether youth with obesity have impaired metabolism of plasma apoB48-lipoproteins and TG following a high-fat meal compared to healthy-weight youth, and to determine if fasting apoB48-lipoprotein remnants correlate with the postprandial response in apoB48-lipoproteins[17].

6.2 METHODS

6.2.1 *Participants*

A cross-sectional study was undertaken in male and female youth aged 8-14 years. Participants were recruited from the Pediatric Centre for Weight and Health at the Stollery Children's Hospital and from the greater community in Edmonton, Alberta, Canada from 2011-2018. Obesity in our participants was defined as a body mass index (BMI) $\geq 95^{th}$ percentile (BMI percentile >99 was cut off as 99 for calculation purposes). Participants were excluded from the study if they had underlying conditions that may impact lipid metabolism such as Type 1 or Type 2 Diabetes, and Prader-Willi Syndrome, or taking medications that may alter lipid metabolism. All measurements and tests were completed within 2 months of recruitment of participants. Baseline assessments including anthropometric measurements and the postprandial high-fat meal test were completed on the same day. The study protocol was approved by the Human Research Ethics Board at the University of Alberta (Pro00001941).

6.2.2 *Assessment of Plasma Lipid, Biochemical, and Endocrine Parameters*

Fasting and non-fasting plasma total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), triglycerides (TG) and non-esterified fatty acid (NEFA) levels were measured using enzymatic colorimetric assays (Wako Co. Ltd., Richmond, VA). Non-fasting remnant cholesterol was calculated as non-fasting TC – (HDL-C + LDL-C)[13], and non-HDL-C was calculated as fasting TC-HDL-C. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by fasting insulin (mU/mL) x fasting glucose (mmol/L)/22.5, and insulin resistance was defined by a HOMA-IR reading >4[20]. Inflammatory markers including C-reactive protein, interleukin (IL)-10, IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α, Plasma estradiol, progesterone, and testosterone, and insulin and leptin were measured by multi-plex array analysis through Eve Technologies, AB, Canada.

6.2.3 *Anthropometric, and Body Composition Measurements*

Anthropometric assessments were performed by trained staff using standardized protocols, as previously described[12]. Briefly, height was measured without shoes to the nearest 0.1 cm

using a wall-mounted stadiometer (Holtain Limited, Crymych, UK). Waist circumference was measured to the nearest 1 mm at the levels of the umbilicus using a non-stretch measuring tape. All anthropometric measures were assessed in swimwear, without shoes. Body composition, including total body weight, fat mass, fat-free mass, percentage fat mass, and percentage fat-free mass, were determined by Dual-energy X-ray absorptiometry and air displacement plethysmography technology using the BOD POD system (COSMED/Life Measurement, Inc., Chicago, IL).

6.2.4 *Postprandial Lipid and ApoB-Lipoprotein Metabolism Following a High-Fat Meal Challenge*

The high-fat meal challenge is an established protocol in our laboratory[12,21]. In brief, following a 12-hour overnight fast, a blood sample was taken, and subjects then consumed a high-fat milkshake meal providing 0.61g lipid/kg of body weight to assess non-fasting postprandial lipid metabolism[12,22]. The high fat meal volume was calculated based off of body weight because body weight has been shown to be significantly correlated with the length of the small intestine in humans[23]. Accordingly, obese individuals have been shown to have a greater small intestine length compared to healthy-weight individuals, which may be due to increased nutrient exposure promoting enterocyte proliferation and differentiation[24,25]. Therefore, adjusting meal volume by body weight will correct for differences in intestine length and potential confounders that could arise from these physiological differences. The meal contained cream (35% milk fat) and Ensure® Calorie Plus Meal Replacement Drink (Abbott Laboratories, Saint-Laurent, Quebec, Canada) to provide approximately 62.5% energy as fat, 30% energy as carbohydrate, and 7.5% energy as protein. Blood samples following consumption of the meal were collected in EDTA-coated vacutainers at 2, 4, 6, and 8 hours (Figure 6-2). Plasma was prepared by centrifugation (2500 rpm, 4°C for 15 minutes) and stored at −80°C for lipid and apoB-lipoprotein analysis.

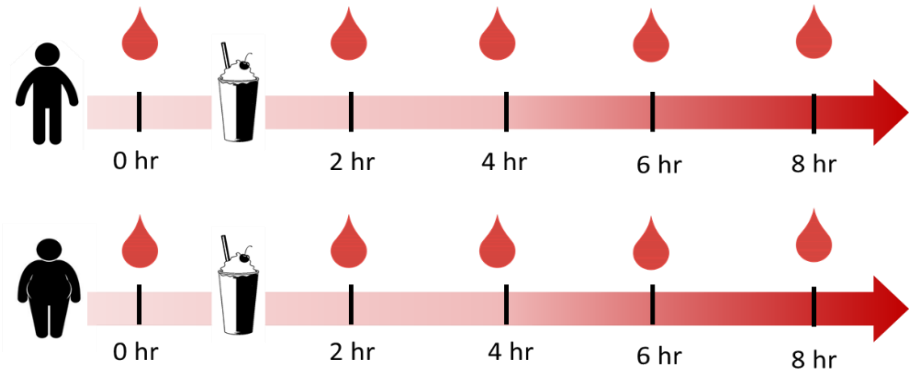


Figure 6-2. High-Fat Meal Challenge. Participants arrive at the clinic following a 12-hour fast and have a baseline plasma sample. A high-fat meal is provided (62.5% energy as fat) and following consumption, additional plasma samples are taken at 2, 4, 6, and 8 hours.

6.2.5 *Quantification of Plasma ApoB-Lipoprotein Levels*

ApoB48 and ApoB100 lipoproteins were quantified using an adapted Western blot method as previously described[26]. Briefly, total plasma proteins were separated on a 3% to 8% NUPAGE Tris-acetate polyacrylamide gel (Invitrogen, Carlsbad, CA) and then transferred onto a polyvinylidene difluoride membrane (0.45 µm, Immobilon PTM; Millipore, Billercia, MA). Membranes were incubated with a primary polyclonal antibody specific for ApoB (Santa Cruz Biotechnology, Dallas, TX) and a secondary antibody tagged with horseradish peroxidase (Santa Cruz Biotechnology). ApoB48 and B100 bands were visualized by enhanced chemiluminescence (ECL Advance; Amersham Biosciences, Little Chalfont, UK) and quantified using linear densitometric comparison with a known mass of purified human ApoB48 and ApoB100 standards[12].

6.2.6 *Statistical Analysis*

All statistical and graphical analysis were performed using GraphPad™ Prism 8.0 (GraphPad Software, Inc. USA). Distributions of outcomes were assessed for normality using the D'Agostino & Pearson omnibus normality test. Differences between groups with respect to anthropometric and biochemical parameters were examined using Student's T-test (unpaired) when data assumed a normal Gaussian distribution. A Mann-Whitney U-test was

used for unpaired samples when data assumed a non-normal Gaussian distribution. To determine sex differences between groups, a one-way analysis of variance (ANOVA) followed by Bonferroni posttests for multiple comparisons was used when data assumed a normal distribution. Kruskal-Wallis test was used followed by a Dunns posttest for multiple comparisons when data assumed a non-normal distribution. Area under the curve (AUC) and incremental area under the curve (iAUC) were calculated as previously described[27]. Briefly, AUC was calculated through GraphPad Prism 8 software and corresponds to the total plasma concentration over the 8-hour postprandial measured period. The fasting concentration of the respective parameter is further subtracted from the total AUC to generate the iAUC. The iAUC represents the rate of change in the postprandial response adjusted for the initial concentration of the parameter. Significance was determined at a $p<0.05$. The study was powered to achieve a significant correlation between fasting apoB48 and apoB48AUC with $\alpha=0.001$[17]. The univariate relationship between fasting lipids and metabolic outcomes was determined by using Pearson correlation coefficients for normally distributed data and Spearman correlation coefficients for non-normally distributed data.

6.3 RESULTS

6.3.1 *Anthropometry and Biochemical Parameters*

Compared to healthy-weight peers, body weight, BMI, waist circumference, waist-to-hip ratio, percent body fat and fat free mass were significantly higher in youth with obesity (Table 6-1). Fasting plasma glucose was not different between groups ($p=0.53$). Youth with obesity had significantly elevated insulin (372.1 mU/L ± 179.3 versus 1271 mU/L ± 77.2; $p<0.0001$), calculated HOMA-IR (1.70 ± 0.87 versus 5.82 ± 3.49; $p<0.0001$), C-peptide (631.4 pg/mL ± 222.8 versus 1435 pg/mL ± 402.3; $p<0.0001$) and leptin (2574 ng/mL ± 1835 versus 20265 ng/mL ± 7467; $p<0.0001$) compared to their healthy-weight peers. There were no significant differences between sex-hormones or inflammatory markers between the two groups (Table 6-1). Additionally, no significant gender differences were observed in anthropometric and biochemical data, therefore, both genders are pooled for subsequent analysis. For complete gender analysis see Appendix Table 6-A1 and 6-A2.

	Healthy-Weight	Obese	P
N	22	13	
Age (years)	12.1 ± 1.75	13.3 ± 0.58	0.61
Sex (M/F)	10/12 (45% male)	5/8 (38% male)	0.74
Height (cm)	156.5 ± 11.92	158.7 ± 15.23	0.83
Weight (kg)	44.6 ± 9.35	88.72 ± 13.58	**0.03**
BMI (kg/m2)	19.04 ± 1.74	33.2 ± 4.30	**0.0007**
BMI Percentile	57.1 ± 21.89	98.2 ± 1.57	0.17
BMI Z-Score	0.19 ± 0.70	2.25 ± 0.31	**0.04**
Body Fat Percentage (%)	17.1 ± 8.05	43.9 ± 4.89	**<0.0001**
Glucose (mmol/L)	4.13 ± 0.60	4.27 ± 0.57	0.53
Insulin (mU/L)	372.1 ± 179.3	1271 ± 779.2	**<0.0001**
HOMA-IR	1.70 ± 0.87	5.82 ± 3.49	**<0.0001**
C-peptide (pg/mL)	631.4 ± 222.8	1435 ± 402.3	**<0.0001**
Leptin (ng/mL)	2574 ± 1835	20265 ± 7467	**<0.0001**
MCP-1 (pg/mL)	67.3 ± 38.8	66.9 ± 50.0	0.88
GIP (pg/mL)	29.3 ± 11.5	39.1 ± 28.5	0.18
Glucagon (pg/mL)	15.7 ± 6.65	20.2 ± 7.91	0.10
Cortisol (pg/mL)	129.0 ± 33.57	135.7 ± 54.84	0.87
Estradiol (pg/mL)	0.119 ± 0.059	0.157 ± 0.065	0.09
Progesterone (ng/mL)	5.16 ± 3.56	6.92 ± 3.39	0.07
Testosterone (ng/dL)	1.76 ± 2.98	0.94 ± 0.743	0.16
T3 (ng/mL)	1.64 ± 0.38	1.68 ± 0.25	0.72
T4 (ng/mL)	4856 ± 3391	3756 ± 1951	0.50
IL-1β (pg/mL)	1.25 0.57	1.27 ± 0.40	0.54
IFN-γ (pg/mL)	17.44 ± 6.43	18.7 ± 4.90	0.55
GMF-CSF (pg/mL)	104.8 ± 105.5	84.2 ± 41.7	0.78
IL-2 (pg/mL)	3.78 ± 1.52	3.63 ± 1.01	0.73
IL-4 (pg/mL)	15.3 ± 8.70	13.8 ± 4.26	0.86
IL-5 (pg/mL)	1.31 ± 0.57	1.74 ± 0.82	0.07

IL-6 (pg/mL)	1.06 ± 0.43	1.43 ± 0.63	0.10
IL-8 (pg/mL)	5.32 ± 2.29	5.20 ± 1.78	0.67
IL-10 (pg/mL)	5.20 ± 1.95	4.72 ± 0.90	0.42
IL-12 (pg/mL)	2.24 ± 1.10	2.45 ± 0.95	0.57
IL-13 (pg/mL)	3.60 ± 1.63	4.56 ± 1.90	0.12
IL-17A (pg/mL)	10.3 ± 3.79	11.6 ± 3.14	0.32
IL-23 (pg/mL)	345.6 ± 233.5	304.3 ± 174.9	0.94
TNF-α (pg/mL)	7.47 ± 2.81	6.63 ± 1.65	0.34

Table 6-1: Anthropometric and biochemical data. Data is presented as mean ± SD. Highlighted p-values are statistically significant.

6.3.2 *Fasting Plasma Lipids and ApoB Lipoproteins*

Fasting total plasma TC, LDL-C, HDL-C, non-HDL-C, TC: HDL-C, and apoB100 were not significantly different between healthy-weight and youth with obesity. Conversely fasting plasma TG (30%, $p=0.03$), and apoB48 (55%, $p<0.0001$) were significantly elevated in youth with obesity (Table 6-2). Total cholesterol AUC was significantly elevated in obese male compared to female youth (22.89 mmol/L ± 4.85 versus 14.90 mmol/L ± 2.78, respectively), with no additional differences between gender (Appendix Table 6-A2). Therefore, all subsequent lipoprotein analysis was pooled (Table 6-2).

6.3.3 *Postprandial Plasma Lipids and ApoB Lipoproteins*

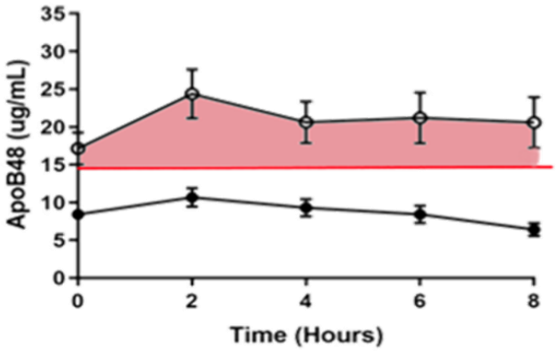
The TC, TG, and apoB48 area under the curve (AUC) following a lipid-rich meal was significantly elevated in youth with obesity compared to their healthy-weight peers, whereas apoB100 AUC was not significantly different ($p=0.34$). The incremental area under the curve (iAUC) response was significantly different between healthy-weight and youth with obesity ($p=0.007$) but was not different for TG ($p=0.149$) (Table 6-2, Figure 6-A2).

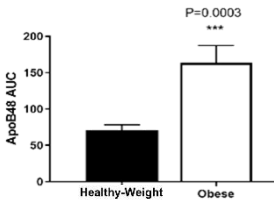
	Healthy-Weight	Obese	P
Total Cholesterol (mmol/L)	4.55 ± 0.69	4.74 ± 0.86	0.47
LDL-C (mmol/L)	2.48 ± 0.43	2.69 ± 0.58	0.21
HDL-C (mmol/L)	1.19 ± 0.234	1.06 ± 0.20	0.10
Non-HDL-C (mmol/L)	3.36 ± 0.82	3.72 ± 0.93	0.24
TC/HDL-C Ratio (mmol/L)	4.04 ± 1.27	4.83 ± 1.48	0.11
Remnant Cholesterol (mmol/L)	1.72 ± 1.10	2.53 ± 2.00	0.13
Triglycerides (mmol/L)	0.86 ± 0.42	1.23 ± 0.54	**0.03**
ApoB48 (ug/mL)	8.09 ± 2.70	18.04 ± 7.07	**<0.0001**
TG/apoB48 ratio	0.11 ± 0.08	0.06 ± 0.05	0.06
ApoB100 (ug/mL)	6436 ± 2970	5113 ± 2556	0.27
TC_{AUC} (mmol/L.h)	33.9 ± 5.90	42.0 ± 8.61	**0.002**
TG_{AUC} (mmol/L. h)	8.32 ± 3.37	12.1 ± 4.06	**0.005**
TG_{iAUC} (mmol/L .h)	2.29 ± 2.15	3.32 ± 2.50	0.149
$ApoB48_{AUC}$ (ug/mL .h)	62.0 ± 16.1	173.0 ± 75.23	**<0.0001**
$ApoB48_{iAUC}$ (ug/mL .h)	10.3 ± 14.9	38.7 ± 33.7	**0.007**
$ApoB100_{AUC}$ (ug/mL.h)	24455 ± 14567	19881 ± 12592	0.34

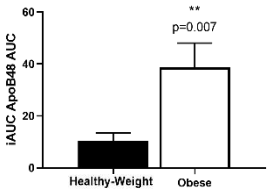
Table 6-2: Fasting and non-Fasting Plasma Lipids and apoB Lipoproteins. Data is presented as mean ± SD. Highlighted p-values are statistically significant.

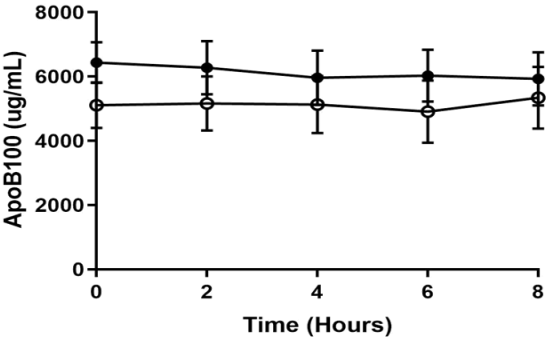
6.3.4 *Postprandial Response in Plasma ApoB Lipoproteins and Lipids Following a High-Fat Meal*

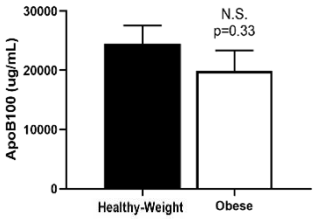
The apoB-lipoprotein postprandial response to the high-fat meal is shown in Figure 6-3. $ApoB48_{AUC}$ was significantly elevated (65%, $p<0.0001$) in youth with obesity compared to healthy-weight peers (Figure 6-3). The $apoB48_{iAUC}$ was significantly different between healthy-weight and youth with obesity ($p=0.007$). Unlike the healthy-weight youth, plasma apoB48 in youth with obesity stayed elevated above fasting levels at 8 hours following a high fat meal, which suggests that obese you have an impairment in intestinal apoB48-remnant cholesterol metabolism in the postprandial state. The TG_{AUC} was also significantly elevated (31%, $p=0.005$) in youth with obesity, however the TG_{iAUC} was not significantly different. There was also a significant increase in postprandial TC in youth with obesity (20%, $p=0.002$) (Figure 6-3). There was no difference in postprandial apoB100 between groups nor a significant change following a high-fat meal as this value does not change significantly from fasting during the postprandial period (Figure 6-3).

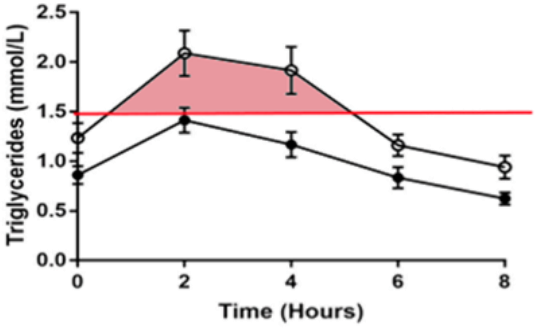

ApoB48 (ug/mL)
Time (Hours)

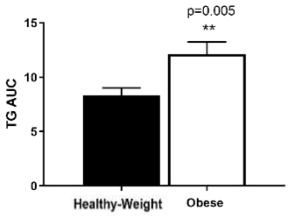

P=0.0003
***
ApoB48 AUC
Healthy-Weight
Obese

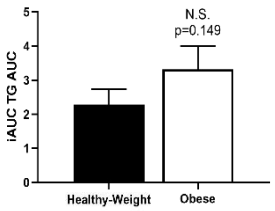

**
p=0.007
iAUC ApoB48 AUC
Healthy-Weight
Obese

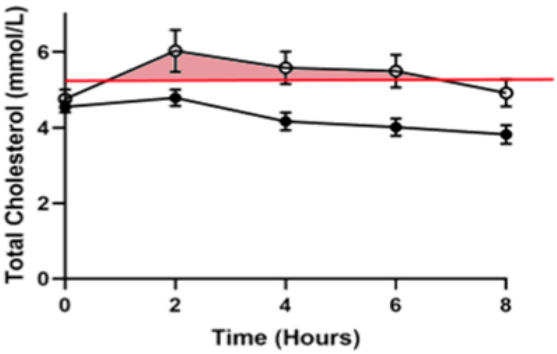

ApoB100 (ug/mL)
Time (Hours)


N.S.
p=0.33
ApoB100 (ug/mL)
Healthy-Weight
Obese


Triglycerides (mmol/L)
Time (Hours)


p=0.005
**
TG AUC
Healthy-Weight
Obese


N.S.
p=0.149
iAUC TG AUC
Healthy-Weight
Obese


Total Cholesterol (mmol/L)
Time (Hours)

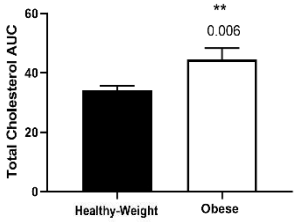

**
0.006
Total Cholesterol AUC
Healthy-Weight
Obese

Figure 6-3. Postprandial response in plasma TG, TC, and apoB48 and apoB100 lipoproteins following a high-fat meal, corresponding area under the curve (AUC) and incremental area under the curve (iAUC) for TG and apoB48. Red line denotes pediatric reference ranges for fasting TG and TC as per National Heart, Lung, and Blood Institute Cardiovascular health and Risk Reduction Guidelines[10]. Cut-offs for fasting apoB48 were estimated on previous studies in youth and adults[12,17,19,28]. Data is presented as mean ± standard error of the mean. ** indicates $p<0.01$, *** indicates $p<0.001$, N.S. = non-significant.

6.3.5 *Range of apoB48 and TG at fasting and following a high fat meal response and individual response*

The individual response to a high fat meal was variable amongst healthy-weight and youth with obesity. The range of fasting apoB48 and response after a high fat meal was more variable in youth with obesity compared to healthy-weight youth. Conversely, fasting TG and TG AUC ranges were comparable amongst groups (Table 6-3, Figure 6-4).

	Healthy-Weight	**Obese**
Fasting TG (mmol/L)	0.34 – 1.99 (0.79)	0.72 – 2.71 (1.15)
TG AUC (mmol/L. h)	3.94 – 15.44 (11.50)	5.79 – 19.76 (13.97)
Fasting apoB48 (ug/mL)	4.68 – 16.82 (12.15)	10.26 – 34.11 (23.85)
apoB48 AUC (ug/mL .h)	38.41 – 97.86 (59.45)	84.96 – 332.1 (247.1)

Table 6-3. The range of fasting apoB48 and TG and apoB48 and TG AUC in healthy-weight and youth with obesity

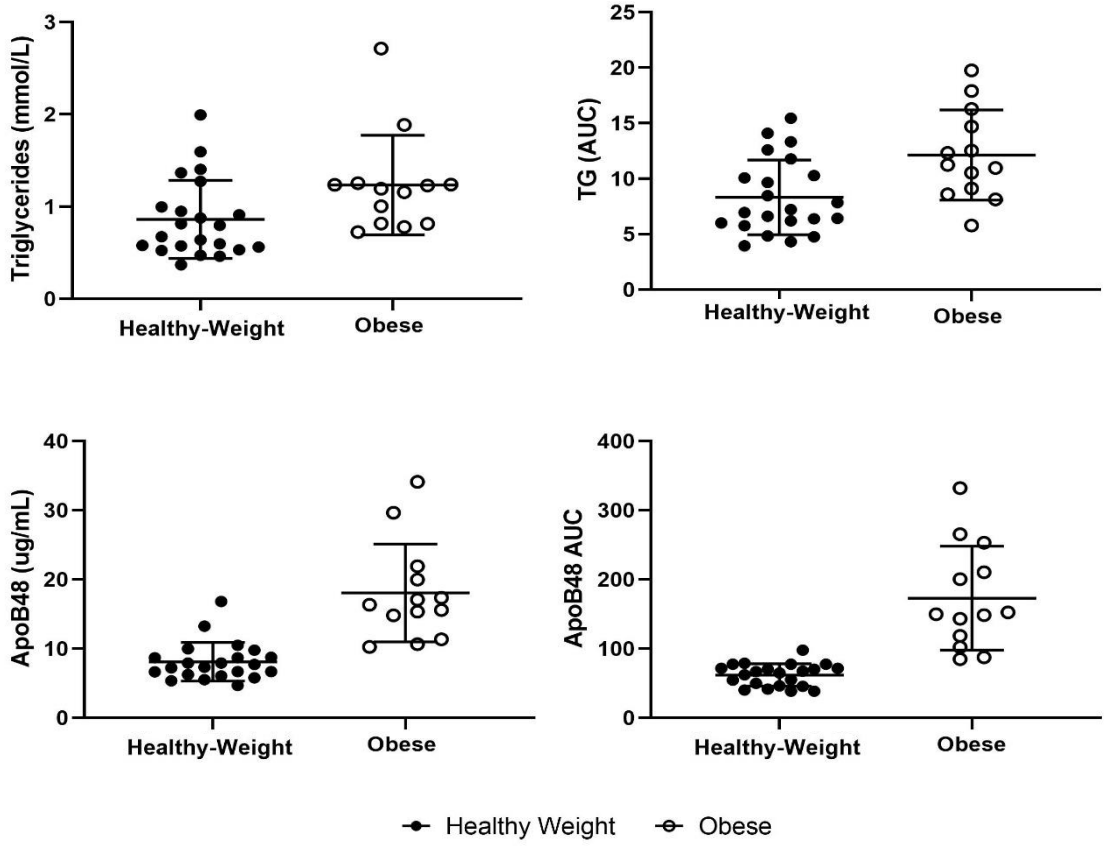


Figure 6-4. Plotted individual fasting TG, apoB48 and TG and apoB48 AUC response following a high fat meal

6.3.6 *Regression Analysis of apoB48 with the Postprandial Lipid Response*

Fasting apoB48 was strongly correlated with the total apoB48 postprandial response ($apoB48_{AUC}$) ($r=0.89$, $p<0.0001$). The following was observed in both healthy-weight and obese youth (Appendix Figure 6-A1). These results are consistent with findings in adult males[17]. The following demonstrates that an elevated fasting measurement of apoB48 is predictive of impaired postprandial lipid metabolism or postprandial lipemia. Fasting apoB48 was not significantly correlated to the postprandial TG response ($r = 0.286$, $p=0.09$). In adults, fasting apoB48 has been shown to strongly predict postprandial TG[17] (Figure 6-5).

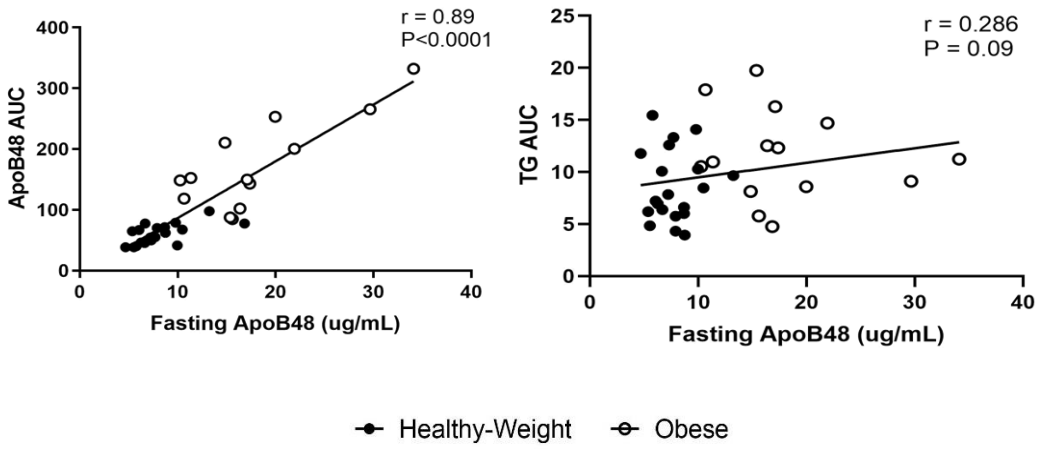


Figure 6-5. Correlation between fasting apoB48 and postprandial apoB48 (AUC), and TG (AUC) in healthy-weight and youth with obesity aged 8-14 years.

6.3.7 *Univariate Regression Analysis of Fasting Lipids with Metabolic Variables*

Multiple correlations of fasting lipids (apoB48, TG, LDL-C, TC, and HDL-C) with metabolic variables are compared below (Table 6-4). Fasting apoB48 and TG are significantly associated with leptin ($r=0.56$ $p<0.0001$ and 0.52 $p=0.002$, respectively) and C-peptide ($r=0.62$, $p<0.0001$ and 0.44, $p=0.02$, respectively), whereas apoB48 also has a significant positive association with insulin ($r=0.58$, $p<0.0001$), HOMA-IR ($r=0.61$, $p<0.0001$), BMI ($r=0.36$, $p=0.03$) and estradiol ($r=0.21$, $p<0.0001$). Fasting LDL-C was positively associated with TC ($R^2=0.16$, $p=0.02$) and negatively associated with HDL-C ($R^2=-0.40$, p.04). TC was negatively associated with estradiol ($R^2=-0.42$, $p=0.01$).

Fasting Lipid and Metabolic Parameters	apoB48	TG	LDL-C	TC	HDL-C
apoB48 (ug/mL)		#0.16	#0.18	#0.04	#-0.30
TG (mmol/L)	#0.13		#0.27	#0.25	#0.06
LDL (mmol/L)	0.02	0.07		**0.39***	**-0.35***
TC (mmol/L)	#0.0004	**0.14***	**0.16***		-0.25
HDL-C (mmol/L)	#-0.21	0.0002	**-0.40***	-0.25	
RC (mmol/L)	#0.0001	#0.03	#0.13	#0.31	#0.04
BMI (kg/m^2)	**#0.36***	#0.26	#0.02	#-0.04	#0.07
Insulin (mU/L)	**#0.58*****	#0.29	#0.17	#0.07	#-0.003
HOMA-IR	**#0.61*****	**#0.32 (p=0.06)**	#0.13	#0.02	#0.04
Leptin (ng/mL)	**#0.56*****	**#0.52****	#0.15	#-0.03	#-0.11
C-peptide (pg/mL)	**#0.62*****	**#0.44****	#0.18	#0.16	#-0.14
Estradiol (pg/mL)	**0.21*****	0.001	-0.001	**-0.42***	0.12
Testosterone (ng/dL)	#0.16	#0.02	#-0.09	#0.01	#0.04
Progesterone (ng/mL)	#0.12	#-0.04	#-0.06	#-0.31	#-0.07
IL-6 (pg/mL)	**#0.38***	#0.08	#0.08	#-0.06	#0.08
IL-1β (pg/mL)	**#0.33 (p=0.05)**	#-0.23	#-0.25	#-0.22	#0.11
TNF-α (pg/mL)	0.34	0.007	0.0008	-0.006	-0.06

Table 6-4: Correlations between fasting lipids and biochemical outcomes. *p<0.05; **p<0.01; ***p<0.001; #=spearman correlation. Highlighted correlates are statistically significant.

6.4 DISCUSSION

Early obesity in youth and adolescents remains a critical health concern worldwide. The strong association between early obesity and adverse metabolic outcomes in adulthood emphasizes the importance in understanding the early pathophysiology of critical metabolic risk factors[4]. It is also paramount to elucidate early metabolic risk markers that can be utilized to detect and manage risk at this early age[29].

The results of this study demonstrate that youth with obesity have postprandial dyslipidemia compared to healthy-weight youth despite other classic lipids risk markers being 'normal'. We observed an elevated fasting apoB48 comparable to adults with obesity and importantly, an over two-fold increase in the apoB48 response following a high fat meal. It is well understood that non-fasting remnant cholesterol is causally associated with CVD risk in adults[30] and our current data also demonstrates an important role in intestinal non-fasting remnant cholesterol in early CVD risk.

Recent research has demonstrated the integral contribution of intestinal derived lipids to CVD and metabolic risk. Remnant lipoproteins have been shown to penetrate the arterial wall and be retained within the sub-endothelial space and thus play a direct role in the atherosclerotic process[16]. Obesity, insulin resistance, type 2 diabetes and other adverse metabolic conditions have been found to significantly upregulate intestinal chylomicron metabolism[18,31]. Importantly, conditions of insulin resistance have been shown to significantly enhance chylomicron production and impair clearance of apoB48-continaing remnant lipoproteins in adults[31]. Obesity and insulin resistance are also well known to upregulate lipogenic pathways including apoB48 and hepatic apoB100-lipoprotein secretion. Insulin resistance has also been shown to increase lipoprotein metabolism through increased activity of SREBP-1c, a nuclear transcription factor which impacts downstream protein expression of genes involved in lipoprotein assembly (MTP), TG synthesis (fatty acid synthase (FAS), acetyl coA carboxylase (ACC)), as well as cholesterol biosynthesis[32]. In our current study, many of the youth with obesity were insulin resistant as defined by a HOMA-IR>4. Therefore, in those individuals, the enhanced postprandial apoB48 and TG response may in part, be due to insulin resistant driven upregulation of intestinal lipogenic pathways.

The results of this study are in line with previous data from our laboratory which demonstrated that pre-pubertal children with obesity as well as adolescent girls (with obesity and PCOS) have elevated apoB48-remnant lipoproteins[12,33]. The data presented in this study further adds to this understanding by also demonstrating that fasting apoB48 is strongly correlated to $apoB48_{AUC}$ in youth. The strong association between fasting apoB48 and $apoB48_{AUC}$ demonstrates that an accumulation of apoB48 remnants in the fasting state are indicative of postprandial lipemia in youth, which is also observed in adulthood[17]. The youth in the present study have a 65% increase in circulating postprandial apoB48-containing chylomicron particles, which greatly enhances the

cholesterol carrying burden and CVD-risk profile in these youth. Furthermore, the significant increase in apoB48$_{iAUC}$ between both groups suggests that beyond a larger amount of CM particles present at fasting, there is also a difference in the kinetics of the postprandial curve. The most prevalent difference in the apoB48$_{iAUC}$ appears to be 2 hours following the high fat meal, which could indicate a significant increase CM production in youth with obesity compared to their healthy-weight peers (Appendix Figure A2). It has been demonstrated in adults, using kinetic modelling techniques, that CM production is greatly increased in adults with obesity[34].

According to the National Cholesterol Education Program Expert Panel on Children, the cut-off for high TG in youth ages 10-19 years is approximately 1.5 mmol/L and for TC is approximately 5.2 mmol/L[10]. The findings presented in this chapter demonstrate that children with obesity have fasting TG and TC that is, on average, within the acceptable cut-off. However, following a high fat meal challenge, both TG and TC in youth with obesity increases beyond these cut-points and would be considered out of range. Furthermore, based off previous data from our laboratory and others, we suggest that fasting apoB48 $\geq$ 15 ug/mL is elevated and predictive of metabolic risk in both youth and adults[12,17,19,28]. Youth with obesity were found to have both fasting and non-fasting apoB48 greater than this cut-off. These results suggest that fasting lipid screening using traditional lipid risk markers would not distinguish youth with obesity from their healthy-weight peers despite their prevalent postprandial dyslipidemia following a high fat meal. These findings provide further rationale to utilize non-fasting lipid testing in youth. Recent research from the CALIPER cohort determined non-fasting LDL-C, non-HDL-C and remnant cholesterol values in youth and developed pediatric reference intervals[35]. Therefore, these intervals can be used to provide Canadian physicians with references for non-fasting lipids in younger populations.

Unlike in adults, fasting apoB48 did not significantly correlate with postprandial TG[17]. Although trending ($p=0.09$) to a significant correlation, our study was not powered to test this association. Furthermore, the differences in fasting and postprandial TG were not as significant as changes to apoB48 metabolism suggesting that this pathway may not play as critical of a role in early risk compared to apoB48. It is possible that the major perturbations in lipid metabolism at this age may impair pathways that influence chylomicron particle assembly and secretion. Upregulating these pathways in the intestine would result in increased chylomicron production, as opposed to changes to pathways in lipid absorption and TG assembly. These potential metabolic

aberrations would package TG into more chylomicron particles resulting in smaller, more cholesterol dense particles. It has been shown that smaller chylomicron particles are more atherogenic as they are not lipolyzed or cleared as well compared to large chylomicron particles[36].

The lack of difference between fasting and postprandial apoB100 as well as fasting LDL-C and TC between healthy-weight and youth with obesity indicates that there are no significant changes in hepatic apoB100 metabolism in youth with obesity. We speculate that the liver is more robust to early adverse changes in adiposity whereas the intestinal exposure of chronic over-nutrition may promote a more rapid upregulation of genes involved in intestinal lipid metabolism[37].

It is not known if elevated apoB48 tracks into adulthood, however, data from previous independent clinical studies has demonstrated that apoB48 is elevated in obese conditions during pre-puberty[12], puberty[19,21], and post-puberty[28]. Collectively, these studies demonstrate that elevated fasting apoB48 exists in multiple cohorts of early obesity and metabolic risk and may be an important early contributor to CVD risk in youth. Additionally, a recent study in youth found that apoB48 was the only fasting lipid marker that significantly tracked with increasing adiposity over a 2-year period[19]. Optimally, a longitudinal cohort study in youth would provide information as to whether early increases in apoB48 track into adulthood and whether these are directly associated with adverse metabolic outcomes[38].

Previous work from our laboratory has proposed that early obesity in youth arises from the chronic imbalance of overnutrition coupled with decreased energy expenditure[12]. Early obesity will promote increased lipid storage in adipocytes and may also result in anatomical and physiological adaptations to the small intestine that would upregulate intestinal remnant cholesterol metabolism[37,39]. We suspect that unlike adults, alterations to hepatic lipoproteins are not influenced by early obesity due to early exposure of overnutrition to the intestine, which would further promote alterations to hepatic lipid metabolism as obesity tracks into adulthood[37].

Interestingly, we observed minimal changes in fasting plasma inflammatory markers between healthy-weight and youth with obesity. In adults obesity is associated with chronic low-grade inflammation[40]. In a previous study in pre-pubertal children, there were slight but significant increases in fasting CRP, and IL-1β with no differences in IL-6, IL-8 and TNF-α[12]. In obesity, adipose hypertrophy will promote lipid spill-over into the plasma and concurrent adipose

macrophage infiltration, which will promote a local adipose immune response in cytokines such as TNF-α and IL-6[41]. However, preliminary data in a subset of participants (n=27) found no significant differences in fasting non-esterified fatty acids. Therefore, the youth with obesity in this study have excess adiposity but may not have yet reached adipose hypertrophy and associated macrophage infiltration and inflammatory response. Additionally, we observed no significant changes in sex hormones between groups. However, both estradiol and progesterone were trending towards being higher in youth with obesity (p=0.09, p=0.07, respectively). The group with obesity was, on average, slightly older than the healthy-weight group, which could influence the pubertal stage these youth were in. Furthermore, in our gender-split analysis obese girls had slightly (but not significantly) higher estradiol than healthy-weight girls (appendix table 3A-1). It has been demonstrated that girls with obesity have an early pubertal onset[42]. Additionally, we observed a significant positive correlation between fasting apoB48 and estradiol in this study. These findings suggest that obesity induced changes in apoB48 may also be associated with changes to the sex hormone estradiol in pubertal youth. Regression analysis between fasting lipids and metabolic parameters in this study also found that apoB48 was significantly and positively associated with BMI, insulin, HOMA-IR, leptin, C-peptide, IL-6 and trending towards a positive association with IL-1β. These findings are similar to our results in chapter 5. In adolescents from the Raine cohort, fasting apoB48 was also significantly associated with BMI, insulin, HOMA-IR, and leptin as well as the low-grade inflammatory marker C-reactive protein. Overall, associations between fasting apoB48 and metabolic outcomes are comparable between pubertal and adolescent youth suggesting that apoB48 is a reliable metabolic risk marker in youth.

We observed a significant individual response to the high fat meal challenge in youth. Intestinal lipid metabolism is variable between individuals and the absorption and secretion of lipids can be affected by various genetic variants, the sex of the individual, the health of the individual and the diet and lifestyle of the individual[43,44]. Interestingly, youth with obesity had more variance in the postprandial apoB48 response than their healthy-weight peers. Firstly, the obese group is smaller, and this can influence the spread of data. Secondly, obesity in youth is multifactorial and some youth in our study may have had genetic mutations to enhance postprandial lipemia while others did not[44]. Because our study did not investigate genotyping or dietary patterns of the youth in this study it is difficult to make assumptions on the individual response to the high fat meal challenge. The individual response to a high fat meal, independent of weight status, also highlights the

importance of implementing non-fasting lipid screening programs in youth as some healthy-weight youth may exhibit postprandial lipemia comparable to an obese individual that would normally not be screened.

We selected our age range for the present study to be in line with the pubertal period where it is known that both LDL-C and TC significantly decline due to transient insulin resistance[45]. The decline in these traditional lipids has resulted in screening programs to avoid the pubertal period to screen youth for cholesterol[10]. However, puberty is a critical time to develop and mitigate metabolic risk. Elucidating novel lipid risk markers that can assess metabolic risk at this period, independent of puberty, are valuable to identify youth at increased metabolic risk. We propose that utilizing non-fasting lipids as a primary screening measure in youth during the pubertal period can provide physicians with a critical window to detect early metabolic risk and allow for early interventions.

The use of both sexes was a major strength of this study. Furthermore, we utilized a weight adjusted high fat meal, which allows us to examine the effect of lipid metabolism independent of weight. We also used a Western blot methodology to directly measure apoB lipoproteins, which allows us to directly compare apoB48 to apoB100 lipoproteins. The smaller numbers of this current study limit the applicability of this study to larger populations. Our study population was primarily comprised of upper-middle class, Caucasian youth and therefore, may not apply to all children. We also performed no formal puberty testing, however, there were no significant differences in sex hormones were observed between groups or between genders (Table 6-1, Appendix 6-A1).

6.5 CONCLUSION

In conclusion, this study has provided evidence that youth with obesity has elevated fasting and non-fasting TG and apoB48-remnant lipoproteins and that fasting apoB48 was strongly predictive of postprandial lipemia. The changes to non-fasting remnant lipoproteins was independent to changes in hepatic-apoB100 derived lipoproteins suggested that intestinal derived lipoproteins may be an important role in early CVD risk in youth and spurs the need to assess lipids in the non-fasting state in youth. The present findings suggest that traditional fasting lipid measures are not adequate to predict cardiometabolic risk at this age, nor encompass the aberrant changes to intestinal lipid metabolism. Future work needs to address the efficacy of diet-lifestyle and/or pharmaceutical therapies to target postprandial lipemia in youth.

6.6 APPENDICIES

	NW Male	NW Female	Obese Male	Obese Female
N	10	12	5	8
Age (years)	12.01 ± 1.94[a]	12.33 ± 1.64[a]	13.28 ± 0.70[a]	13.36 ± 0.54[a]
Height (cm)	151.7 ± 14.2[a]	157.5 ± 13.46[a]	164.7 ± 10.54[a]	162.5 ± 7.83[a]
Weight (kg)	43.37 ± 9.97[a]	47.15 ± 7.53[a]	85.4 ± 16.84[b]	90.89 ± 11.84[b]
BMI (kg/m2)	18.97 ± 2.27[a]	19.09 ± 1.23[a]	31.06 ± 3.91[b]	34.54 ± 4.20[b]
BMI Percentile	60.7 ± 26.6[a]	56.5 ± 17.0[a]	97.8 ± 1.30[b]	98.4 ± 1.77[b]
BMI Z-Score	0.17 ± 0.88[a]	0.21 ± 0.53[a]	2.18 ± 0.32[b]	2.29 ± 0.32[b]
Body Fat Percentage (%)	16.4 ± 10.25[a]	17.64 ± 6.09[a]	42.62 ± 4.59[b]	44.74 ± 5.20[b]
Glucose (mmol/L)	3.88 ± 0.67[a]	4.34 ± 0.45[a]	4.19 ± 0.31[a]	4.31 ± 0.71[a]
Insulin (mU/L)	290.3 ± 122.3[a]	440.3 ± 194.9[ac]	1676 ± 971.7[b]	1018 ± 557.6[bc]
HOMA-IR	2.00 ± 0.43[a]	2.11 ± 0.94[ac]	7.75 ± 4.6[b]	4.61 ± 2.12[bc]
C-peptide (pg/mL)	589.0 ± 154.6[a]	666.7 ± 268.8[a]	1678 ± 451.4[b]	1283 ± 304.4[b]
Leptin (ng/mL)	2323 ± 1932[a]	3113 ± 1751[a]	17618 ± 3471[b]	21918 ± 8977[b]
MCP-1 (pg/mL)	72.42 ± 49.45[a]	63.07 ± 28.83[a]	88.81 ± 72.84[a]	72.42 ± 49.45[a]
GIP (pg/mL)	27.23 ± 8.09[a]	31.02 ± 13.85[a]	51.91 ± 44.84[a]	31.04 ± 7.045[a]
Glucagon (pg/mL)	13.61 ± 6.48[a]	17.02 ± 6.72[a]	21.62 ± 4.77[a]	19.18 ± 9.83[a]
Cortisol (pg/mL)	125.5 ± 30.88[ab]	132.0 ± 36.76[ab]	93.63 ± 35.85[a]	162.0 ± 48.65[b]
Estradiol (pg/mL)	0.08 ± 0.04[a]	0.16 ± 0.05[bc]	0.10 ± 0.03[ab]	0.19 ± 0.05[c]
Progesterone (ng/mL)	4.81 ± 1.94[a]	5.45 ± 4.57[a]	6.88 ± 3.15[a]	6.95 ± 3.74[a]
Testosterone (ng/dL)	3.47 ± 3.83[a]	0.34 ± 0.17[a]	1.56 ± 0.90[a]	0.56 ± 1.64[a]
T3 (ng/mL)	1.69 ± 0.34[a]	1.57 ± 0.41[a]	1.64 ± 0.20[a]	1.71 ± 0.28[a]
T4 (ng/mL)	4585 ± 2791[a]	5082 ± 3932[a]	2662 ± 1566[a]	4439 ± 1933[a]
IL-1β (pg/mL)	0.90 ± 0.24[a]	1.54 ± 0.60[b]	1.15 ± 0.52[ab]	1.34 ± 0.60[ab]
IFN-γ (pg/mL)	14.30 ± 5.07[a]	20.06 ± 6.44[a]	15.95 ± 3.93[a]	20.42 ± 4.85[a]
GMF-CSF (pg/mL)	84.96 ± 133.0[a]	121.3 ± 78.16[a]	90.54 ± 57.69[a]	80.17 ± 32.15[a]
IL-2 (pg/mL)	3.16 ± 0.81[a]	4.30 ± 1.80[a]	3.40 ± 1.31[a]	3.78 ± 0.98[a]
IL-4 (pg/mL)	11.43 ± 5.82[a]	18.51 ± 9.59[a]	14.93 ± 4.40[a]	13.11 ± 4.30[a]
IL-5 (pg/mL)	0.99 ± 0.37[a]	1.58 ± 0.57[a]	1.60 ± 0.23[a]	1.82 ± 1.04[a]

IL-6 (pg/mL)	0.81 ± 0.24[a]	1.27 ± 0.45[a]	1.66 ± 0.79[a]	1.29 ± 0.52[a]
IL-8 (pg/mL)	4.78 ± 1.53[a]	5.78 ± 2.76[a]	5.78 ± 1.70[a]	4.84 ± 1.83[a]
IL-10 (pg/mL)	4.35 ± 1.59[a]	5.91 ± 2.00[a]	4.84 ± 0.91[a]	4.65 ± 0.94[a]
IL-12 (pg/mL)	1.75 ± 0.69[a]	2.65 ± 1.22[a]	2.20 ± 1.02[a]	2.61 ± 0.93[a]
IL-13 (pg/mL)	2.60 ± 0.92[a]	4.43 ± 1.65[a]	4.82 ± 2.30[a]	4.39 ± 1.77[a]
IL-17A (pg/mL)	8.26 ± 3.40[a]	12.04 ± 3.30[a]	10.53 ± 4.02[a]	12.27 ± 2.52[a]
IL-23 (pg/mL)	213.6 ± 255.2[a]	372.3 ± 221.7[a]	277.9 ± 134.9[a]	320.8 ± 203.1[a]
TNF-α (pg/mL)	7.74 ± 2.67[a]	7.24 ± 3.03[a]	6.68 ± 1.35[a]	6.60 ± 1.90[a]

Table 6-A1. Anthropometric and Biochemical Sex Differences Between Healthy-weight and Youth with Obesity. Data presented as mean ± SD.

	HW Male	**HW Female**	**Obese Male**	**Obese Female**
Total Cholesterol (mmol/L)	4.82 ± 0.61[ab]	4.32 ± 0.69[a]	5.41 ± 0.75[b]	4.35 ± 0.73[ab]
TC_{AUC} (mmol/L.h)	16.18 ± 2.63[a]	17.58 ± 3.16[a]	22.89 ± 4.85[b]	14.90 ± 2.78[a]
LDL-C (mmol/L)	2.53 ± 0.28[a]	2.44 ± 0.53[a]	3.01 ± 0.56[a]	2.48 ± 0.56[a]
HDL-C (mmol/L)	1.10 ± 0.239[a]	1.26 ± 0.210[a]	0.94 ± 0.23[a]	1.10 ± 0.215[a]
Non-HDL-C (mmol/L)	3.72 ± 0.628[ab]	3.06 ± 0.866[a]	4.47 ± 0.70[b]	3.25 ± 0.75[a]
TC/HDL-C Ratio (mmol/L)	4.58 ± 1.18[ab]	3.60 ± 1.20[a]	5.98 ± 1.37 [b]	4.11 ± 1.08[b]
RC (mmol/L)	1.39 ± 0.91[a]	2.00 ± 1.22[a]	4.18 ± 2.08[b]	1.50 ± 1.00[a]
Triglycerides (mmol/L)	1.02 ± 0.54[a]	0.73 ± 0.25[a]	1.15 ± 0.457[a]	1.28 ± 0.610[a]
TG_{AUC} (mmol/L. h)	4.27 ± 2.03[a]	4.10 ± 1.38[a]	6.29 ± 2.82[a]	5.93 ± 1.57[a]
TG_{iAUC} (mmol/L .h)	3.10 ± 2.92[a]	1.64 ± 0.90[a]	1.73 ± 1.13[a]	4.31 ± 2.56[a]
ApoB48 (ug/mL)	7.61 ± 1.05[a]	8.97 ± 3.83[ab]	14.94 ± 2.70[bc]	19.98 ± 8.38[c]
$ApoB48_{AUC}$ (ug/mL .h)	27.82 ± 6.64[a]	37.79 ± 17.07[ab]	57.29 ± 16.10[bc]	104.7 ± 35.87[c]
$ApoB48_{iAUC}$ (ug/mL .h)	2.30 ± 3.23[a]	7.07 ± 9.13[a]	6.71 ± 11.22[ab]	21.47 ± 14.34[b]
TG/apoB48 ratio	0.14 ± 0.09[a]	0.07 ± 0.04[ab]	0.06 ± 0.03[ab]	0.06 ± 0.06[b]
ApoB100 (ug/mL)	5912± 2203[a]	5984 ±2884[a]	6384 ±3115[a]	4319± 1944[a]
$ApoB100_{AUC}$ (ug/mL.h)	22684 ± 8642[a]	22599 ± 14077[a]	26418 ± 17282[a]	15796 ± 7180[a]

Table 6-A2. Lipid and Lipoprotein Sex Differences Between Healthy-weight and Youth with obesity. Data presented as mean ± SD. (HW = healthy-weight).

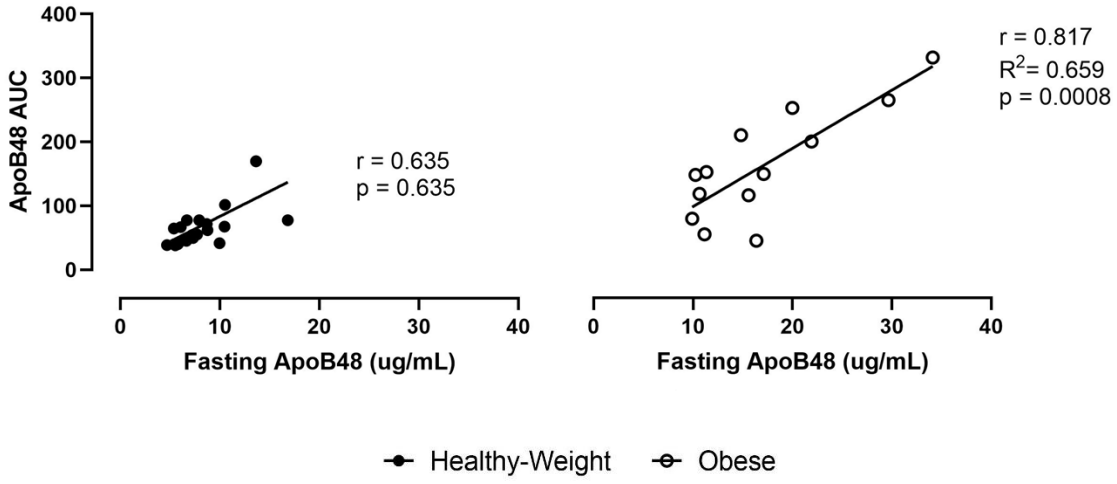


Figure 6-A1. Correlation of fasting apoB48 with postprandial apoB48AUC in healthy-weight and youth with obesity. Spearman correlation was used for healthy-weight youth and Pearson for youth with obeisty (both r and R^2 are presented for comparison).

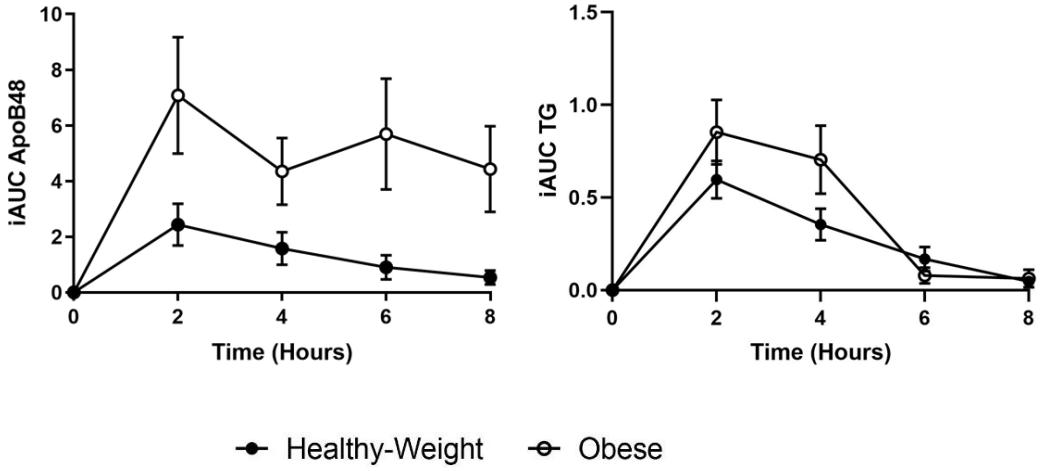


Figure 6-A2. Postprandial apoB48$_{iAUC}$ and TG$_{iAUC}$ following a high fat meal. Data presented as mean ± SEM.

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Chapter 7: Overall Discussion

Parts of this chapter are published or submitted for publication (Please see appendix A, and C)

7.1 Executive Summary of Findings

7.1.1 Non-Fasting Remnant Cholesterol is Elevated in Cardiovascular Disease

In chapter 3 (the *first* study) of this thesis determined the concentration of non-fasting lipids in Alberta's Tomorrow Project and examined the relationship between non-fasting remnant cholesterol (RC) and LDL-C with CVD. The results from this preliminary analysis suggest that non-fasting remnant cholesterol is elevated in females with prevalence of CVD, independent of LDL-C. Furthermore, it was shown that increasing quartiles of RC tracked better with case numbers of CVD prevalence and incidence than LDL-C. The findings from chapter 3 suggest that non-fasting RC may be a better CVD risk predictor than LDL-C in a Canadian cohort. Future work will further delineate the relationship between non-fasting RC and CVD as well as explore the association between non-fasting RC and CVD in patients with type 2 diabetes. In order to explain the mechanisms behind subpopulations with high RC. The following chapters investigated the relevance and utility of fasting and non-fasting apoB48.

7.1.2 PCSK9 LOF variants have Reduced Non-Fasting Triglyceride-Rich Lipoproteins

In the *second* study (chapter 4) it was found that human *PCSK9*-LOF variants have reduced fasting and postprandial TG and apoB48 following a high-fat meal compared to those without variants. These findings demonstrate that *PCSK9*-LOF are protected from postprandial dyslipidemia (PPL) and may provide further insight to the mechanisms by which lifelong reductions in circulating PCSK9 is protective against CVD. These findings suggest that indices of RC can be manipulated by both genetic and lifestyle factors.

7.1.3 Fasting apoB48 is Elevated in Conditions of High Cardiometabolic Risk in Adolescents

The *third* study (chapter 5) of this thesis determined the concentrations of fasting apoB48 and its association with cardiometabolic risk in a large cross-sectional adolescent cohort. We demonstrated a significant association between fasting apoB48 and cardiometabolic risk markers including TG, TC, insulin, HOMA-IR, HDL-C, and waist circumference. Specifically, we found that fasting apoB48 was significantly elevated by 21% in adolescents at high cardiometabolic risk

compared to those at lower risk. Interestingly, the elevation in fasting apoB48 in was much greater in males at high cardiometabolic risk than females. When compared to other traditional lipid risk markers, the elevation in fasting apoB48 at high metabolic risk is much greater than LDL-C or TC, suggesting fasting apoB48 may play an important role in early cardiometabolic risk.

7.1.4 Youth with Obesity have Postprandial Dyslipidemia Compared to Normal Weight Peers

In the *fourth* study (chapter 6) we demonstrated that youth with obesity have overt PPL compared to their normal weight peers. Unlike adults with obesity, fasting LDL-C, TC and HDL-C were not significantly different between normal weight and obese youth. These findings are consistent with previous results in children and demonstrates that traditional, fasting lipid risk markers may not be able to detect early CVD-risk in children[1,2]. Importantly, we also found that fasting apoB48 was significantly and strongly associated with PPL in youth, which demonstrates that we can predict PPL by a single measurement of fasting apoB48 in children. These data imply that intestinal remnants may represent an important CVD risk factor for youth.

7.2 Non-fasting lipids play an important role in CVD risk in adults and youth.

The results from my thesis have demonstrated that non-fasting remnant cholesterol plays an important part in CVD risk across the lifespan. Chapter 3 of this thesis explored the relationship between non-fasting RC and LDL-C with CVD. We demonstrated that increasing quartiles of non-fasting RC track better with CVD case numbers than LDL-C, suggesting non-fasting RC may have better utility as a CVD risk predictor. Interestingly, non-fasting RC is significantly elevated in females with prevalence of CVD in the cohort and not males. These results may provide further insight to the pathophysiology of CVD in postmenopausal women. Women are often protected from CVD until menopause, when their risk of CVD greatly increases[9]. Women are also found to be less likely to receive preventative CVD treatment compared to men at similar ASCVD risk[10,11]. Therefore, exploring potential novel therapeutic lipid targets that predict CVD in women may provide an exciting opportunity to decrease the CVD burden in this population.

In chapter 4 we demonstrated that *PCSK9*-LOF variants had a significant reduction in postprandial lipoproteins compared to non-variant controls. These results suggest that PCSK9-LOF may have had a significant life-long reduction in postprandial remnant lipoproteins alongside reductions in circulating LDL-C. Individuals with more 'extreme' (missense or nonsense) LOF

variants in *PCSK9* have been shown to have an 88% reduction in coronary heart disease compared to non-variants[12]. The *PCSK9*-LOF variants in our study had mild LOF variants compare to the previous study mentioned, however, they still had a significant reduction in circulating PCSK9, LDL-C, TG, and apoB48. Therefore, we hypothesize that these individuals will also experience lifelong cardioprotection compared to non-variants. The results from chapter 4 provide further insight into the role of PCSK9 in non-fasting lipid metabolism and underscore the important of lowering non-fasting lipids early in life to confer protection from CVD. Chapter 4 also supports future directions of genetic profiling and personalized medicine. These would allow physicians to identify specific polymorphisms involved in chronic disease progression and individual pharmacotherapies to mitigate these unique genetic alterations.

Chapter 5 and 6 in this thesis have provided further evidence that apoB48-remnant cholesterol is elevated in youth at increased cardiometabolic risk. These observations were independent to elevations in LDL-C and total cholesterol. We also found that fasting apoB48 is significantly correlated with the non-fasting apoB48 response following a high fat meal. Overall, these findings suggest that non-fasting remnant cholesterol may be an important and independent early CVD-risk marker in youth. Early elevations in non-fasting remnant cholesterol may help explain the presence of advanced fatty streak formation in children with obesity as seen in autopsy studies[13,14]. We further demonstrated that fasting apoB48 remnant lipoproteins are elevated significantly more in male adolescents at high cardiometabolic risk compared to females. In adults, it has been well studied that adult males have an increased risk of CVD[15]. Premenopausal females appear to be relatively protected from CVD, however, following menopause, the risk gap narrows between sexes[10]. The preliminary findings in adolescents in chapter 5 demonstrate an early sexual dimorphism in CVD risk in males at high cardiometabolic risk. Our laboratories previous study in prepubertal youth found no differences in fasting apoB48 between sexes[1], suggesting that adolescence may be a critical period for sexual dimorphic changes in CVD risk to occur. To further elucidate the role of non-fasting remnant cholesterol in early CVD risk there is a need for longitudinal studies to demonstrate whether early elevations in apoB48-remnants track into adulthood and if they are associated with adverse CV-outcomes later in life.

7.3 Non-Fasting Lipid Reference Ranges and Lipid Screening for Adults and Youth in Canada

The studies conducted in the current thesis highlight the importance of developing and validating reference ranges of non-fasting lipids and screening for both adults and youth in Canada. Presently, there are no recommendations for universal lipid screening for youth in Canadian guidelines. In the US, physicians are recommended to universally screen for lipids in youth between ages 9-11 years and again between ages 17-21 years. The primary lipid screening outcome measure is non-fasting non-HDL-C[16]. In Canada, physicians recommend that patients begin lipid screening after the age of 40. However, targeting high cholesterol at a younger age would result in implementing early lifestyle interventions and possibly decrease the risk of developing complications from high blood cholesterol later in life. High blood cholesterol is asymptomatic, and therefore, most patients would not have their blood lipids measured until prompted by a physician[17]. Consequently, at 40 years old, some individuals may have had elevated blood cholesterol for over 20 years and may have already developed complications that would require more intensive interventions.

In chapter 3 we explored concentrations of non-fasting lipids in Canada and their association with CVD risk. Future work will validate the non-fasting lipids values from ATP to European non-fasting lipid ranges[18]. Optimally, we aim to use this data to establish normative reference ranges that can be used by Canadian physicians. Canada has recently allowed for physicians to measure lipids in the non-fasting state in adults. In order to utilize a non-fasting lipid screening approach lab requisition sheets with an option for non-fasting lipid testing, which are already present in British Columbia, is needed in other provinces to allow for physicians to send patients for laboratory. In youth, fasting plasma LDL-C and TC naturally decline during puberty, however, changes in non-fasting lipids are not well understood at this age. Consequently, a standard fasting lipid panel would not accurately reflect their metabolic risk at this age. In chapter 6 we demonstrated that fasting levels of TG and TC are within recommended limits in youth with obesity but rise beyond the normal range during the postprandial phase. The studies presented in this thesis highlight the importance of utilizing non-fasting lipids to detect cardiometabolic risk in youth. To implement a non-fasting lipid screening program in youth, large pediatric cohorts, such as the CALIPER cohort, can be used to determine pediatric reference ranges of non-fasting lipids and appropriate decision limits to indicate metabolic risk[13].

7.4 Clinical Translation of Research Findings

Providing physicians and patients with tools to increase adherence and ease for non-fasting lipid testing is paramount to implementing successful screening strategies in Canada. We propose the use of a Health Canada approved hand-held lipid monitor, Cardiochek® PA (PTS Diagnostics). Cardiochek measures TC, TG, HDL-C and calculates LDL-C and non-HDL-C from a capillary blood sample and could also be used to calculated RC with a non-fasting blood sample[19]. Cardiochek has been validated to accurately measure fasting lipids compared to standard laboratory tests[19] and would allow for physicians to measure ambulatory lipids without the time delay of referring patients to an off-site laboratory. A handheld monitor would also increase patient adherence to lipid screening recommendations[20]. Patients would receive non-fasting lipid results immediately from the doctor without the need for an overnight fast or to make a secondary appointment to receive a blood test. The rapid readout from the Cardiochek would enable the physician to provide immediate medical advice, including ordering further lipid testing at a laboratory. After deriving adequate reference ranges of non-fasting remnant cholesterol in Canada, physicians could then make decisions about whether these patients need to undergo lifestyle or pharmacological lipid lowering therapies. Additionally, Cardiocheck could also allow physicians and/or patients to monitor non-fasting lipid values following certain diet interventions or medications to monitor their efficacy at reducing postprandial dyslipidemia in between doctor visits, tailor medical interventions, and accurately titrate drug doses. Recently, we have generated preliminary data that validate the use of the Cardiochek monitor to accurately measure postprandial TG capillary samples compared to standard laboratory measurements in two young adults (see Appendix D). Research is still needed to validate Cardiochek non-fasting lipid readouts in youth compared to standard laboratory measures.

7.5 What are the Approaches to Target PPL in Youth?

We have demonstrated that youth with obesity have postprandial dyslipidemia (PPL). PPL is a known cardiometabolic risk factor and can enhance the risk of metabolic complications in youth[21,22]. Although PPL can be lowered in adults[23–26], developing approaches to reducing PPL in youth are challenging. In adults, PPL can be targeted with pharmacological therapies such as Ezetimibe[23]. However, pharmacological interventions are not applicable in youth as the adverse

effects that may arise from long-term use of these agents is not well understood[27]. Promising alternatives to pharmacological therapies in youth include nutraceuticals such as fish oil. A recent study demonstrated that Vascepa, an EPA concentrate, was able to significantly lower plasma triglycerides by 18.3% in adult patients with elevated TG on a statin background and was found to significantly reduce adverse-CV events[28]. Additionally, nutrition and exercise interventions may also be promising in lowering PPL in youth[29]. However, more research is needed to test the efficacy of nutraceuticals such as fish oil at reducing PPL in youth as well as the long-term effects of lifestyle modification on PPL in youth. Other considerations include educating physicians on how to detect PPL in youth and what are the optimal interventions for this age group. The above discussion overall highlights the need to develop reference ranges of non-fasting lipids to determine if a youth has PPL.

7.6 Future Directions

Further research is needed to demonstrate whether life-long elevations in apoB48 are associated with CVD risk. Currently, it is not known if elevated apoB48 tracks into adulthood and whether elevated apoB48 in youth is predictive of obesity or CVD in adulthood. To achieve this, large, prospective cohorts, such as he RAINE cohort study could be utilized. Furthermore, the mechanisms behind the observed elevation in apoB48 but not LDL-C and TC in youth are not well understood. Our work provides a rationale that universal lipid screening in the non-fasting fasted state in youth may be beneficial to capture early metabolic risk. Future studies are needed to elucidate changes in intestinal gene and/or protein expression that may enhance apoB48-CM production and/or secretion in obese youth. Understanding changes in protein and gene expression can be challenging in the clinical setting, especially in youth. Opportunities in large animal models that more closely resemble the human gastrointestinal tract, such as swine, may help in providing further insight towards early changes in lipid metabolism in the obese intestine[30]. Despite the wealth of evidence that non-fasting remnant cholesterol is associated with CVD-risk, developing specific measurements of remnant cholesterol has been challenging (see Chapter 2, section 2.7). During my PhD program I have designed and received ethics approval for a pilot study to test the efficacy of a handheld lipid monitor that can test ambulatory capillary lipids. Pilot data from our laboratory suggests that Cardiochek can accurately measure non-fasting lipids relative to

laboratory measures and may be a viable option to conveniently test non-fasting lipids and calculate non-fasting remnant cholesterol (Appendix D).

7.7 Limitations

Chapter 3 was a preliminary analysis of Alberta's Tomorrow Project and additional analysis is needed to further explain the relationship between non-fasting RC and CVD. Future analysis will create a sex and age matched group to compare males and females as well as conduct a survival analysis to determine the hazard ratio between non-fasting RC and CVD.

In chapter 6, the sample size for the number of obese youths was less than the number of healthy weight controls (n=13 versus n=22, respectively). However, both groups were statistically age and sex matched and the numbers in both groups exceeded the power calculation n=12 to achieve a significant correction between fasting apoB48 and apoB48 AUC ($r=0.91$ and $\alpha=0.001$[3]). Furthermore, we achieved significant differences between groups despite a small sample size suggesting that our sample size was effective to observe our primary research objectives.

Chapter 6 also did not have a dietary run in period prior to the testing date. The 'second meal effect' can cause higher CM-TG concentrations in the plasma than what would occur after only a single meal, which may be due to stored fat in the enterocyte, or increased intestinal lymph flow[4,5]. By standardizing the meals leading up to the experimental day, we can reduce the influence of the second meal effect in future studies. Despite the lack of dietary run in period, we achieved relative homogeneity of results within groups in response to a high fat meal. Additionally, chapter 6 was conducted between the ages of 8-14 years old with the assumption that these youth would be around the pubertal period yet, no formal puberty staging data was collected during this study. We observed no significant differences between fasting sex hormones between groups and this suggests that any differences in pubertal status are not influencing fasting circulating sex hormones.

Chapter 5 also did not include any sex hormone data from 17-year-old adolescents from the Raine cohort. Although less likely to be in puberty, sex hormone data at this age could help further explain the relationship between apoB48 and metabolic parameters as well as the sex-specific trends in apoB48. We know from other studies in adolescents that metabolic conditions which alter sex-hormones, such as PCOS, can alter non-fasting lipids[6]. Therefore, sex-hormone measurements may provide additional insight into the data trends we observed in this thesis chapter.

Additionally, neither chapter 5 nor 6 had no genotyping data relevant to lipid metabolism. It is well known that common mutations in the LDL-r and apoE can enhance PPL[7]. However, we observed no significant differences in LDL-C or postprandial apoB100 in chapter 6, which suggests that these youth did not have any significant mutations in genes involved in hepatic lipid metabolism. Furthermore, neither chapter 5 or 6 had any subclinical CVD measurements such as carotid intima medial thickness or ankle brachial index. Although body composition and anthropometric measurements, such as waist circumference, are strong indicators of subclinical CVD risk, they do not directly measure early CV-risk as a subclinical measure would[8]. Therefore, future studies should examine these measurements to validate the association between apoB48-remnants and early sub-clinical CVD risk. Despite this, apoB48-remnant lipoproteins has shown to be integral to CVD pathophysiology in adults[32,33]. Furthermore, in this thesis and elsewhere[1,2], we have provided consistent evidence in children and adolescents that elevated apoB48-remnants are indicative of cardiometabolic risk.

7.8 Conclusion

Collectively, the studies in this thesis support the hypothesis that intestinal-derived remnant lipoproteins play an important role in CVD-risk over the lifespan. In this thesis, we have demonstrated that non-fasting remnant cholesterol is associated with adverse CVD events in a Canadian population. We further demonstrated that intestinal-remnant lipoproteins are reduced in individuals with genetic variants protected against CVD and is elevated in youth at increased risk of CVD. The studies presented in this thesis further add to the rationale to implement non-fasting lipid screening measures in Canada and to assess non-fasting lipids in youth with obesity and other adverse metabolic conditions.

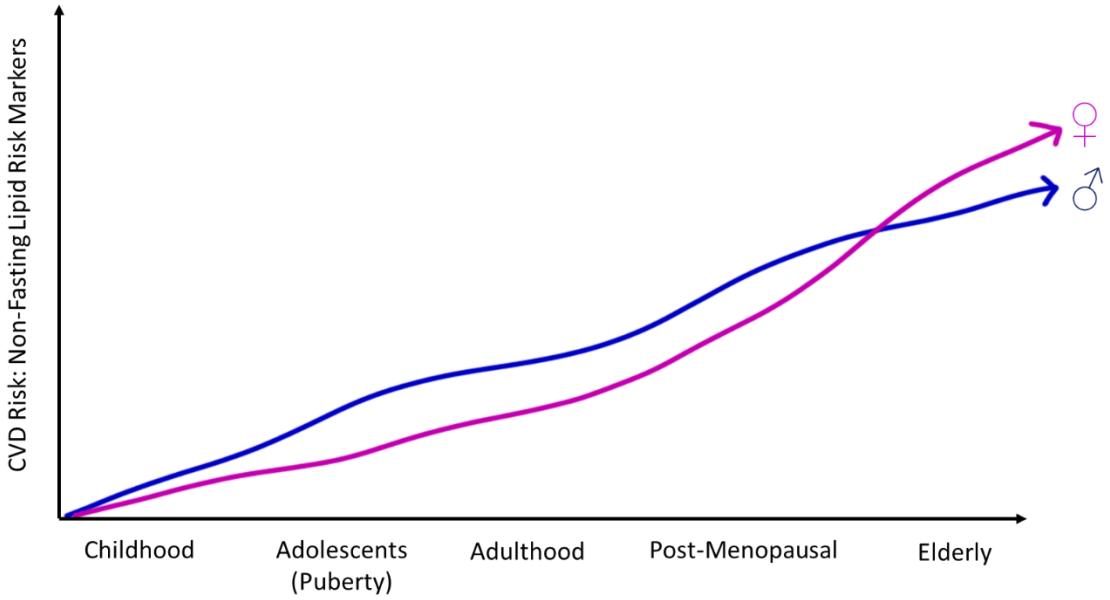

Figure 7-1: *Sexual Dichotomy of CVD-Risk Over the Lifespan.* The collective studies in this thesis demonstrate that non-fasting lipids and relative CVD risk in males and females changes over the lifespan. Non-fasting lipids are comparable in pre-pubertal and pubescent children. However, adolescent males have significantly elevated apoB48-remnants compared to females and continue to experience heightened CVD risk into adulthood. Following menopause, CVD risk in women increases. We demonstrated significant elevations in non-fasting remnant cholesterol in post-menopausal females with CVD, which suggests increased CVD risk compared to males at this age.

7.9 Literature Cited

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Appendix ‘A’

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The Journal of Nutrition
Critical Review

Nutritional and Lipid Modulation of PCSK9: Effects on Cardiometabolic Risk Factors[1,2]

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Abstract

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a serine protease involved in the regulation of LDL receptor (LDLR) expression and apolipoprotein B lipoprotein cholesterol metabolism. Hepatic PCSK9 protein expression, activity, and secretion have been shown to affect cholesterol homeostasis. An upregulation of hepatic PSCK9 protein leads to increased LDLR degradation, resulting in decreased uptake of apoB lipoproteins and a consequent increase in the plasma concentration of these lipoproteins, including LDL and chylomicron remnants. Hence, PCSK9 has become a novel target for lipid-lowering therapies. The aim of this review is to outline current findings on the metabolic and dietary regulation of PCSK9 and effects on cholesterol, apoB lipoprotein metabolism, and cardiovascular disease (CVD) risk. *PCSK9* gene and protein expression have been shown to be regulated by metabolic status and the diurnal pattern. In the fasting state, plasma PCSK9 is reduced via modulation of the nuclear transcriptional factors, including sterol regulatory element–binding protein (SREBP) 1c, SREBP2, and hepatocyte nuclear factor 1α. Plasma PCSK9 concentrations are also known to be positively associated with plasma insulin and homeostasis model assessment of insulin resistance, and appear to be regulated by SREBP1c independently of glucose status. Plasma PCSK9 concentrations are stable in response to high-fat or high-protein diets in healthy individuals; however, this response may differ in altered metabolic conditions. Dietary n–3 polyunsaturated fatty acids have been shown to reduce plasma PCSK9 concentration and hepatic *PCSK9* mRNA expression, consistent with their lipid-lowering effects, whereas dietary fructose appears to upregulate *PCSK9* mRNA expression and plasma PCSK9 concentrations. Further studies are needed to elucidate the mechanisms of how dietary components regulate PCSK9 and effects on cholesterol and apoB lipoprotein metabolism, as well as to delineate the clinical impact of diet on PCSK9 in terms of CVD risk. *J Nutr* 2017;147:473–81.

Keywords: PCSK9, diet, nutrients, cholesterol, apoB lipoproteins, plasma lipids, cardiovascular disease

Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9)[6] is a serine protease involved in the regulation of hepatic apoB lipoprotein uptake and cholesterol metabolism via the LDL receptor (LDLR). Since the discovery of PCSK9 in 2003, it has been investigated as a target to reduce plasma cholesterol concentrations, particularly LDL cholesterol and associated cardiovascular disease (CVD) risk (1, 2). Genetic studies of *PCSK9* (including genomewide association studies and Mendelian randomization trials) have identified polymorphisms of *PCSK9* that significantly affect CVD risk (3, 4). In general, loss-of-function (LOF) mutations appear to be protective against ischemic heart disease (4), whereas gain-of-function (GOF) mutations often result in familial hypercholesterolemia (FH) and exacerbated CVD risk (5). These studies have provided direct evidence that the inhibition of PCSK9 to reduce the degradation of LDLR may be beneficial in regulating cholesterol metabolism and prevention of CVD. PCSK9 inhibitors have emerged as treatments for dyslipidemic patients, particularly in those who are statin-resistant or have severe dyslipidemia, such as FH (6, 7); therefore, PCSK9 has a substantial impact on dyslipidemia and FH, and the metabolic and nutritional regulation of PCSK9 has become of increasing interest in the modulation of cholesterol metabolism and CVD risk (8). The aim of this review is to outline current findings on the metabolic and dietary regulation of PCSK9, and the directions for future research in the role of diet in modulating PCSK9, apoB lipoprotein cholesterol metabolism, and CVD risk.

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[6] Abbreviations used: AMPK, AMP-activated protein kinase; CVD, cardiovascular disease; FH, familial hypercholesterolemia; GOF, gain of function; HNF1α, hepatocyte nuclear factor 1α; LDLR, LDL receptor; LOF, loss of function; LRP, LDL-related protein; mTOR, mechanistic target of rapamycin; PCSK9, proprotein convertase subtilisin/kexin type 9; SREBP, sterol regulatory element–binding protein; TRL, TG-rich lipoprotein.

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PCSK9, apoB Lipoproteins, and Cholesterol Metabolism

PCSK9 activity and secretion is directly involved in cholesterol homeostasis (9). Plasma PCSK9 concentrations have been positively correlated with diurnal variations in hepatic cholesterol synthesis and plasma LDL cholesterol concentrations (10). PCSK9 is primarily synthesized and secreted from the liver, but is also synthesized in smaller amounts in the small intestine, kidney, and brain (1). The liver and the small intestine have major interdependent roles in whole-body cholesterol homeostasis, particularly in relation to apoB lipoprotein–cholesterol metabolism (11). The liver secretes VLDL that contains apoB100, and these are hydrolyzed by lipoprotein lipase and hepatic lipase to form VLDL remnants. In the intestine, chylomicrons that contain apoB48 are secreted into the mesenteric lymphatics and enter the circulation at the thoracic duct, where they are lipolyzed to form chylomicron remnants. Both VLDL and chylomicron remnants are cholesterol-dense apoB lipoprotein remnants, and are considered to be atherogenic lipoproteins (12).

The hepatic uptake of VLDL and chylomicron remnants is dependent on LDLR; therefore, a reduction in LDLR is expected to result in a reduction in the uptake or clearance of these apoB lipoproteins, and a consequent increase in their plasma concentrations (13). Despite this, little is known about the impact of modulation of PCSK9 and apoB remnant cholesterol metabolism compared with the effects on LDL cholesterol. The uptake of apoB remnants is also mediated by LDL-related protein (LRP) 1 (14). PCSK9 has been shown to be associated with the degradation of LRP-1 in the absence of LDLR, at least in melanoma cell lines (14). This may have implications in apoB remnant cholesterol metabolism in FH patients, in whom LRP may play a compensatory role in uptake pathways, but further studies are needed to determine the significance of this pathway under normal clinical conditions. Overall, an upregulation of *PSCK9* mRNA expression has been shown to be associated with an increase in LDLR degradation, resulting in decreased uptake of cholesterol-dense apoB lipoproteins and an increase in the plasma concentration of LDL cholesterol (15). To date, the effect of hepatic *PCSK9* mRNA expression and activity on the metabolism of intestinal chylomicron remnants has not been investigated in the clinical setting.

The synthesis of PCSK9 is modulated by the nuclear transcription factors sterol regulatory-element binding protein (*SREBP*) *2* and *SREBP1c*. These genes activate proteins involved in cholesterol biosynthesis and FA metabolism, respectively (16–18). The proximal promotor of the *PCSK9* gene contains a functional sterol regulatory element that is responsive to changes in intracellular cholesterol concentrations. LDLR protein is also regulated by SREBP2, and a reduction in intracellular cholesterol paradoxically upregulates the transcription of both *PCSK9* and *LDLR*. This results in an increased hepatic uptake of LDL and apoB lipoprotein remnants while simultaneously promoting the degradation of LDLR by PCSK9 (19, 20). Importantly, LDLR is also the primary route of clearance of plasma PCSK9 protein, resulting in a reciprocal relation that contributes to stable plasma LDL cholesterol concentrations. Accordingly, the reciprocal relation between LDLR and PCSK9 can be viewed as a counterregulatory mechanism to maintain cholesterol homeostasis and constant LDL cholesterol concentrations. Offsetting this balance in cholesterol homeostasis, e.g., in individuals overexpressing PCSK9, results in excess LDL cholesterol because of increased LDLR degradation and reduced apoB remnant cholesterol clearance, and subsequently increases the risk of developing CVD (5, 21, 22).

There remains debate on the relation between the plasma concentration of PCSK9 and the activity of the protein. In the fasting state, ~30% of plasma PCSK9 is bound to apoB100 LDL particles. LDL-bound PCSK9 has been shown to have a diminished ability to bind to the LDLR epidermal growth factor A domain (23, 24). Another confounding factor is that PCSK9 circulates in its parent form and in a furin-cleaved form (25). At this point, it is unclear if the furin-cleaved form of PCSK9 reduces the capacity of PCSK9 to degrade LDLR. Furthermore, the majority of studies do not distinguish between these 2 forms of circulating PCSK9 (26, 27). Therefore, when PCSK9 is measured in the plasma, it may not necessarily be reflective of the activity of the protein to degrade LDLR, and it is unknown whether this directly affects LDLR expression.

PCSK9 and TG-Rich Lipoprotein Metabolism

LDL cholesterol is the most common lipoprotein associated with dyslipidemia, and it has been the primary target in the prevention of CVD risk (28). More recently, TG-rich lipoproteins (TRLs) and apoB lipoprotein remnants, particularly chylomicron remnants, have been identified as independent risk factors for atherosclerosis and CVD risk. Clinical and epidemiologic data have shown a causal association between elevated TRL remnants and increased risk of an ischemic event, independent of HDL cholesterol (29). Furthermore, elevated apoB lipoprotein remnants are positively associated with elevated nonfasting plasma TG. PCSK9 may have a role in increasing plasma concentrations of TRLs. For example, increased plasma PCSK9 protein and hepatic and intestinal mRNA have been shown to stimulate the production of TRLs, including both hepatic VLDL and intestinal chylomicron, in *PCSK9*$^{-/-}$ and transgenic mice with a human *PCSK9* GOF mutation (D374Y) and cell-culture (CaCo-2 cells with recombinant human *PCSK9*) studies (30–32). The effect of PCSK9 on hepatic VLDL and intestinal chylomicron metabolism appears to be associated with enhanced transcriptional (apoB lipoprotein and lipid biosynthesis) and posttranscriptional (lipoprotein assembly) pathways (32, 33). In large population cohorts, the concentration of plasma PCSK9 has been shown to be positively associated with LDL cholesterol, total cholesterol, and plasma TG (34–37). At present, there remains limited understanding of the direct or coregulatory role of PCSK9 on whole-body cholesterol homeostasis, postprandial and nonfasting lipoprotein metabolism, and atherosclerotic risk (33, 38). Given the potential role of PCSK9 in apoB lipoprotein degradation and cholesterol metabolism, it has become a novel target for lowering plasma concentrations of apoB lipoproteins (39). Indeed, recent clinical studies that used PCSK9 inhibitors have reported substantial reductions in plasma LDL cholesterol, total apoB, non-HDL cholesterol, and total cholesterol (40).

Metabolic State and PCSK9 Metabolism

PCSK9 in the feed-deprived and non–feed-deprived state. Plasma protein and tissue gene expression of PCSK9 have been shown to be regulated by metabolic conditions in the feed-deprived and postprandial states. Several animal models and clinical studies have reported that plasma PCSK9 concentrations (measured by ELISA) are lower in the feed-deprived state, and steadily decline over a longer feed-deprivation period (**Table 1**). After a 16- to 18-h period of feed deprivation, plasma *PCSK9*

and hepatic mRNA levels decline progressively (10, 45). In the extended feed-deprivation state (≤7 d), plasma PCSK9 concentrations have been shown to be significantly reduced by 70–80% (10, 45–47). Refeeding after a 24-h feed deprivation restores hepatic PCSK9 protein and mRNA expression in a rodent model (the refeeding diet was high in carbohydrate: 48% sucrose and 16% corn starch) (48) (Table 1). Collectively, these studies suggest that the metabolic and nutritional state regulates plasma and hepatic *PCSK9* expression, with the acute feed-deprivation state (24 h) lowering PCSK9 concentrations. The reduction in PCSK9 in the feed-deprivation state is likely to be associated with reduced degradation of LDLR and increased uptake or clearance of apoB remnant lipoproteins, consistent with a lowering of plasma cholesterol, total apoB, and TG in the feed-deprivation state (49, 50).

Impact of the diurnal pattern on PCSK9. During regular feeding intervals, plasma PCSK9 concentrations follow a diurnal pattern, and this appears to be similar to cholesterol biosynthesis (as measured by the lathosterol-to-cholesterol ratio) (10). Plasma PCSK9 concentration (detected by ELISA) is lowest between 1500 and 2100 and peaks at ~0430 (45). It was demonstrated that short-term fasting (≤18 h) does not affect the diurnal variation in PCSK9, but it is unclear whether extended fasting alters this pattern (49). The diurnal pattern of plasma PCSK9 was shown to be negated with the use of cholestyramine, resulting in an elevation in plasma PCSK9 concentrations (45). Cholestyramine treatment depletes hepatic cholesterol concentration by promoting bile acid secretion. Cholesterol biosynthesis and PCSK9 are mutually regulated by intracellular cholesterol pools and SREBP2 (10). In the fasted state, SREBP2 protein expression has been shown to be reduced, resulting in a decline in cholesterol biosynthesis and a decrease in the transcription of *PCSK9* (10, 45). This process results in a subsequent decrease in LDLR degradation to favor the uptake of cholesterol-containing apoB lipoproteins. Despite the similar diurnal pattern of PCSK9 and cholesterol biosynthesis, plasma PCSK9 concentrations are not predictive of an individual's rate of cholesterol biosynthesis or plasma cholesterol concentrations because of the variability in plasma PCSK9 observed between individuals (10). Because plasma PCSK9 does not directly associate with the rate of cholesterol biosynthesis, it does not appear to be solely regulated by SREBP2, but may be codependent on other hepatocellular or metabolic factors.

Other factors influencing PCSK9 function. The overexpression of *PCSK9* (via adenoviral transfection) results in a reduction in hepatic LDLR and an accumulation of LDL cholesterol in the plasma of mice (51). Likewise, LDL cholesterol clearance is reduced in patients with *PCSK9* GOF mutation S127R, and results in increased plasma LDL cholesterol concentrations. Feed deprivation for 24 h in mice that overexpressed *PCSK9* resulted in increased plasma TGs and total cholesterol, which was attributed to decreased hepatic LDLR protein expression and increased VLDL secretion, without changes in lipoprotein lipase activity (52). The reduction in LDLR protein expression after the feed-deprivation period was more pronounced than in the fed state in these animals (−90% compared with −55%, respectively). It has been proposed that the dramatic reduction in hepatic LDLR protein during the feed-deprived state triggers an increase in VLDL secretion by enhancing the mobilization of intrahepatic lipid stores and decreasing the intracellular degradation of apoB. Interestingly, there was a trend toward a decrease in hepatic *SREBP2* mRNA, and no difference in SREBP2 activity (measured by immunoblot analysis of nuclear extracts), suggesting that in the feed-deprived state (at least in this animal model), decreased LDLR protein expression may not be regulated by intracellular cholesterol and SREBP2-related mechanisms (51) (**Figure 1**).

Fasting regulation of PCSK9 by nuclear transcription factors. Fasting PCSK9 concentrations have been shown to be positively correlated with plasma insulin concentrations (35–37). The fasting state also results in a decrease in insulin, with a concurrent rise in plasma glucagon concentrations (Figure 1). At the same time, administration of glucagon results in a decrease in hepatic *SREBP2* expression and *PCSK9* mRNA expression. The reduction in *PCSK9* mRNA expression (−75%) by glucagon treatment has been shown to be 2-fold lower than the reduction in *SREBP2* mRNA expression (25% to 30%), implying that *PCSK9* regulation by glucagon (or other related factors) may be independent of the regulation of SREBP2 (53).

Nutritional relation with hepatocyte nuclear factor 1α and PCSK9. The hepatic nuclear transcription factor hepatocyte nuclear factor 1α (HNF1α) is known to regulate pancreatic insulin secretion. In the extended fasting state (48 h) hepatic HNF1α protein expression, but not mRNA expression, was shown to be reduced (54). Interestingly, the *PCSK9* gene has a conserved HNF1α binding motif, located 28 base pairs

TABLE 1 The effect of fasting on hepatic *PCSK9* expression and serum PCSK9[1]

Fasting time, h	Effect on PCSK9	Model	Method
Refed after >24-h fast	Hepatic mRNA and protein restored	C57BL/6 mice (41)	Western blot, RT PCR
0–8	Serum protein unchanged	Healthy male and female subjects aged 29 ± 13 y, with weight = 78 ± 16 kg, BMI (in kg/m^2) = 26 ± 5, total cholesterol = 173 ± 31 mg/dL, HDL cholesterol = 52 ± 18 mg/dL, LDL cholesterol = 104 ± 25 mg/dL, and TGs = 84 ± 59 mg/dL (7)	ELISA
0–24	Hepatic mRNA or protein unchanged	Syrian golden hamster (42)	RT PCR, ELISA
18	↓ 35% and 38% serum protein	Healthy male and female subjects (43)	ELISA
24	↓ 225% hepatic protein, ↓ 40% hepatic mRNA	C57BL/6 mice (41); Syrian golden hamsters (42)	RT PCR, ELISA
36	↓ 58% serum protein	Healthy participants (7)	ELISA
48	↓ 70% serum protein, ↓ 12% mRNA	Syrian golden hamsters (42)	ELISA, RT PCR
66	↓ 64–97% serum protein	Healthy male and female participants (43)	ELISA
168	↓ 70–80% serum protein	Healthy male and female participants (43, 44)	ELISA

[1] PCSK9, proprotein convertase subtilisin/kexin type 9; RT PCR, real-time PCR.

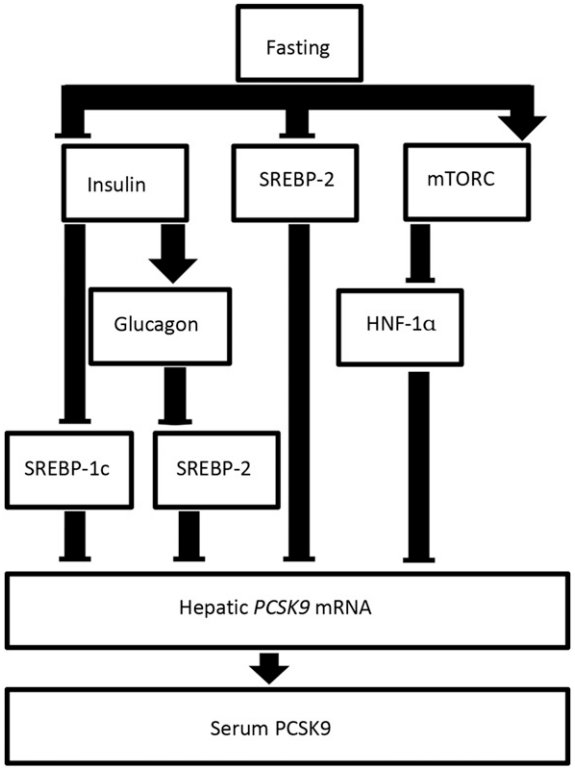

FIGURE 1 Mechanisms that regulate hepatic *PCSK9* expression and serum PCSK9 in the fasting state. *PCSK9* expression appears to be regulated in the fasting state by a reduction in plasma insulin concentration and lower *SREBP2* expression. The lower plasma insulin concentration during the fasting state inhibits SREBP1c signaling, which may decrease *PCSK9* expression. A lowered plasma insulin concentration results in a concurrent rise in plasma glucagon concentration, which may also inhibit SREBP2 activity and decrease *PCSK9* expression. In addition, in the fasting state, *mTORC* expression is upregulated. mTORC has been shown to inhibit HNF1α, which may decrease *PCSK9* expression. HNF1α, hepatocyte nuclear factor 1α; mTORC, mechanistic target of rapamycin complex; PCSK9, proprotein convertase subtilisin/kexin type 9; SREBP, sterol regulatory element–binding protein.

upstream of the sterol regulatory element site of the *PCSK9* promoter, and a reduction in HNF1α protein decreases the amount of PCSK9 protein transcribed (55). This may also involve cell signaling pathways that use the serine-threonine kinase mechanistic target of rapamycin (mTOR) complex 1 (56). Studies in mice with increased mTOR complex 1 activity (via a knockout of the upstream inhibitor tuberous sclerosis complex) resulted in a reduction in HNF1α and *PCSK9* expression, with concurrent increases in hepatic LDLR protein concentrations. In addition, rapamycin (an inhibitor of mTOR complex 1) increases *PCSK9* mRNA expression (57). Therefore, the fasting response of reduced *PCSK9* expression may involve pathways related to HNF1α and upstream mTOR complex signaling pathways. The nuclear transcription factor PPARα is also known to be upregulated in the fasting state and mediates pathways involved in FA catabolism. Fenofibrate (a PPARα agonist) was shown to decrease *PCSK9* mRNA expression by repressing *PCSK9* promotor activity in human hepatocytes (58). The feed-deprived state (48 h) was shown to increase hepatic PPARα mRNA expression in hamsters; however, upon isolation of primary hepatocytes, PPARα agonist treatment did not reduce PCSK9 protein or mRNA expression (54). Collectively, it appears that SREBP2 is the predominant nuclear transcription factor involved in the regulation of PCSK9 in the feed-deprived state. However, SREBP1c and HNF1α also appear to contribute to reduced PCSK9 expression in the feed-deprived state (Figure 1).

PCSK9 Modulation by Glucose Metabolism, Insulin Resistance, or Diabetes

Several studies in healthy participants have demonstrated a positive association between fasting plasma PCSK9, insulin and glucose concentrations, and HOMA-IR (35–37). Serum PCSK9 has also been shown to be positively correlated with fasting plasma HOMA-IR, insulin, and glucose concentrations in large population cohorts, including in Quebec Child and Adolescent Health and Social Survey (36) and the Dallas Heart study (35). Serum PCSK9 concentrations have also been positively correlated with BMI and C-reactive protein in these cohorts, indicating that there is a relation between PCSK9 and metabolic aberrations associated with cardiometabolic risk. Certain studies have shown increased concentrations of circulating PCSK9 in diabetic individuals (35), but others have not (59). Therefore, it is still unclear whether there is an association of PCSK9 with plasma glucose homeostasis.

Insulin treatment analogous to the fed state and overexpression of SREBP1c have been shown to increase *PCSK9* mRNA expression in cultured mouse hepatocytes (48). In contrast, in studies that used a hyperinsulinemic-euglycemic clamp, plasma PCSK9 concentrations are not associated with whole-body and hepatic insulin resistance in healthy volunteers (60). However, in overweight or obese postmenopausal women, there was an observed inverse relation between plasma PCSK9 and insulin sensitivity as measured by hyperinsulinemic-euglycemic clamp. This study also demonstrated that patients in the highest quartile for fasting insulin and HOMA-IR had the highest plasma PCSK9 concentrations, suggesting that hepatic insulin resistance may be associated with higher baseline plasma PCSK9 concentrations (61). Insulin resistance and impaired insulin signaling pathways may alter the normal positive association of plasma insulin and PCSK9 concentrations; however, the mechanisms remain unclear. In human hepatocytes, in vitro studies have shown that insulin stimulates an increase in *SREBP1c* mRNA levels and a decline in PCSK9 protein concentrations (61). This is in discordance with studies in mice that have demonstrated an increase in hepatic *PCSK9* mRNA expression under hyperinsulinemic-euglycemic clamp conditions (48). Furthermore, insulin and overexpression of *SREBP1c* have been shown to increase *PCSK9* mRNA expression in primary hepatocytes isolated from rodents (48). Therefore, there appears to be conflicting data on SREBP1c regulation of PCSK9 between humans and rodents, and this needs clarification to understand the regulation of PCSK9 and its effects on LDLR and apoB lipoprotein clearance in response to insulin under different metabolic conditions (Figure 1).

It is interesting to note that PCSK9 appears to be regulated by insulin via the SREB1c pathway, but evidence suggests that it is not significantly altered by plasma glucose status. This is supported by several studies, including those in isolated hepatocytes, in which *PCSK9* mRNA expression was not altered by glucose and the $PCSK9^{-/-}$ murine model had unaltered glucose homeostasis (48, 62). However, in another study, the $PCSK9^{-/-}$ murine model was observed to have glucose intolerance after an oral glucose tolerance test. In this model, $PCSK9^{-/-}$ mice had a lower postprandial rise in plasma insulin and higher plasma

glucose after oral glucose administration (63). Conversely, in patients with the *PCSK9* LOF variant p.R46L, glucose homeostasis appears to be normal (64). In the ODYESSEY MONO study, increased incidence of elevated fasting plasma glucose was found in response to alirocumab compared with ezetimibe. However, all of these patients had elevated fasting plasma glucose concentrations at baseline and had no changes in glucose or glycated hemoglobin over the course of the 24-wk study (65). Further studies are needed to fully elucidate the impact of PCSK9 inhibitors on long-term insulin-glucose homeostasis.

PCSK9 Modulation by Dietary and Postprandial Lipids

Postprandial plasma PCSK9 concentrations have been shown to remain constant after a high-fat meal (85% fat, containing 35 g SFAs, 30 g MUFAs, 15 g PUFAs, and 88 mg cholesterol) in healthy individuals (60). In addition, a high-fat diet (containing 51.6% SFAs, 27.6% MUFAs, and 8.8% PUFAs, with 376 mg cholesterol/d), a high-fat and high-protein diet (containing 52.6% SFAs, 26.5% MUFAs, and 7.9% PUFAs, with 653 mg cholesterol/d), or buttermilk consumption (5–10% fat with daily energy intake based on a 2500-kcal/d diet; mean total protein 6.4 g, total fat 2.0 g, 1.04 g SFAs, 0.42 g MUFAs, and 0.06 g PUFAs/d) in healthy subjects for 4 wk was shown to have no effect on plasma PCSK9 concentrations (60, 66). However, in *PCSK9* LOF variants (n = 2), plasma PCSK9 concentrations were observed to decrease to very low or undetectable concentrations after a high-fat meal (60). Furthermore, recent data have demonstrated that *PCSK9* LOF carriers (R46L, A53V, and I474V) and nonvariant individuals have a decline in serum PCSK9 4 h after a high-fat meal (69% fat, 0.06:1 PUFA:SFA ratio, and 453 mg cholesterol) (Chronic Disease Program, Ottawa Hospital Research Institute, unpublished results, 2015). In addition, serum PCSK9 appears to decrease to a greater extent in LOF carriers than in nonvariants (−24% compared with −16%, respectively). This suggests that a lipid-rich meal in LOF variants may lower PCSK9 secretion to a greater extent, reflecting an increased expression of LDLR and improved clearance of plasma PCSK9 and apoB lipoproteins. Overall, a high-fat meal or diet appears to have a neutral-to-lowering effect on plasma PCSK9, at least in healthy individuals; however, the postprandial or dietary regulation of PCSK9 activity and LDLR expression in different metabolic conditions requires further investigation. The lipid composition of the high-fat meal may also play a role in the regulation of PCSK9 activity and plasma concentrations, because cholesterol and different FAs may have varying effects on these pathways (**Figure 2**).

Impact of Feeding a Western Diet on PCSK9

A Western-style diet is typified by high total fat, particularly high SFAs and n–6 PUFAs, and high simple carbohydrates. The Western-style diet has been linked to hypercholesterolemia and dyslipidemia, as well as an increase in rates of obesity in Western countries and countries transitioning to a Western-type diet (67). *PCSK9*$^{-/-}$ mice consuming an unpurified diet have been shown to have decreased plasma concentrations of a large variety of molecular lipid species, including cholesterol esters, phosphatidylcholine, sphingomyelin, ceramides, glucosyl/galactosylceramide and phosphatidylinositol. Interestingly, when these mice were fed a diet high in simple carbohydrates (34% sugar, 21% fat, and 0.2% cholesterol) the molecular changes in the sphingolipid and ceramide lipid species were reversed or elevated. These findings demonstrate that diet may modulate molecular lipid changes under *PCSK9* inhibition or LOF conditions (43). In another study, a high-fat Western-style diet fed to mice (60% fat and 21% carbohydrate, with 290 mg cholesterol $\cdot$ kg^{-1} $\cdot$ d^{-1}) was shown to decrease hepatocyte and enterocyte intracellular cholesterol concentrations, resulting in an upregulation of intestinal *SREBP2* and *PCSK9* mRNA expression (68). The addition of cholesterol to high-fat diets appears to alter hepatic *SREBP2* and *PCSK9* mRNA expression and activity in rodent models. In studies that used dietary cholesterol (2–3%), an increase in hepatic total cholesterol and a resultant decrease in SREBP2 mRNA and *PCSK9* mRNA and protein expression has been observed (41, 44, 53). These results presumably translate to a decrease in LDLR degradation and increased uptake of apoB lipoproteins. Consistently, *PCSK9* mRNA and protein expression in intestinal epithelial cells was decreased after incubation with cholesterol and 25-hydroxycholesterol (69). Conversely, a high-fat diet (47% calories) containing 3% cholesterol was reported to have no significant effect on plasma PCSK9 concentrations or hepatic *LDLR* mRNA expression. However, 2–3% dietary cholesterol is a very high amount and is >10-fold that of human dietary consumption of cholesterol; therefore, these results may not be translatable to normal dietary conditions. It is possible that the amount of fat in the diet may mask the effects of increased cholesterol, resulting in a neutral effect on *PCSK9* expression (70). This is shown in high-fat diets that have a neutral to lowering effect on *SREBP2* and *PCSK9* expression (60, 66, 68). Furthermore, a ketogenic diet (SFAs 25%, MUFAs 35%, and PUFAs 17% of total energy) was shown not to alter plasma PCSK9 concentrations, demonstrating that PCSK9 is not responsive to increased ketones and that, during the fasting state, ketones may not be involved in the observed decrease in *PCSK9* expression (71).

Bioactivity of MUFAs and n–6 and n–3 PUFAs and Influence on PCSK9

Recent studies have demonstrated that n–3 PUFAs are able to decrease plasma and hepatic *PCSK9* expression. The n–3 PUFAs EPA (20:5n–3) and DHA (22:6n–3) are well-established lipid-lowering agents, and are particularly promising in reducing plasma TG (72). Dietary supplementation with n–3 PUFAs has been shown to significantly reduce plasma PCSK9 in both healthy women [38.5% EPA, 25.9% DHA, and 6% docosapentaenoic acid (22:5n–3)] (73) and individuals with metabolic syndrome (6% DHA) (42). In rodents, a Western-style diet containing n–3 PUFAs (45% kcal fat, 2% cholesterol, and 10% fish oil) and n–3 PUFA supplementation (2% EPA + DHA) with estrogen administration was found to decrease hepatic *PCSK9* mRNA (74, 75). These rodents were also found to have a reduction in hepatic SREBP2 mRNA and an increase in the ratio of hepatic phosphorylated AMP-activated protein kinase (AMPK) to AMPK mRNA (75). AMPK is an important energy sensing enzyme that, when phosphorylated, inhibits the processing of SREBP and subsequently decreases the transcription of its downstream target genes (76). Therefore, it is possible that n–3 PUFAs, by modulating the phosphorylation of AMPK, are influencing SREBP2 nuclear translocation and thus reducing the transcription of SREBP2 target genes, notably, *PCSK9* (Figure 2).

Likewise, diets enriched with n–6 PUFAs, predominantly linoleic acid (18:2) and MUFAs (18:1n–9; Mediterranean-type diet), decrease serum PCSK9 concentrations in healthy overweight individuals, and this was associated with a reduction

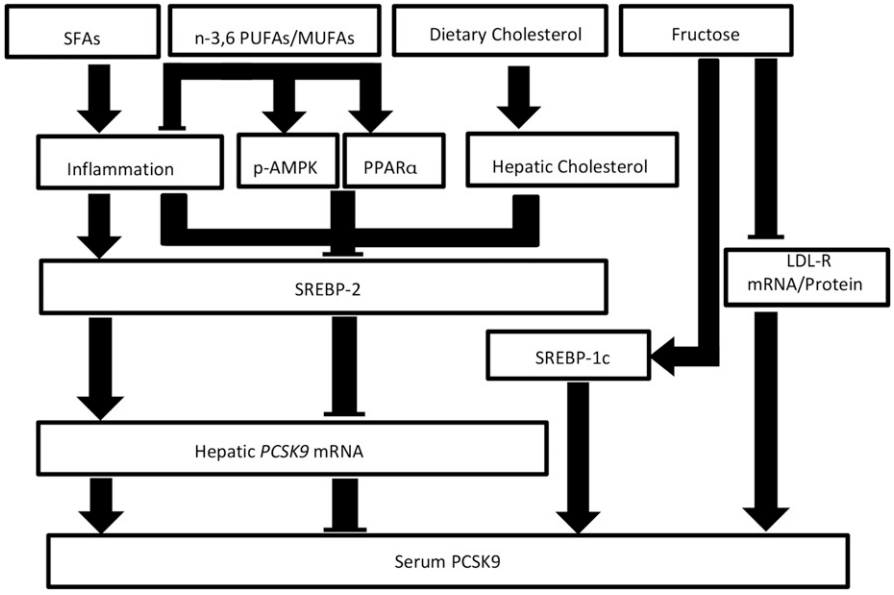

FIGURE 2 Mechanisms associated with nutrient regulation of hepatic and serum PCSK9. Serum PCSK9 concentrations appear to be influenced by dietary nutrients. Both n–3 and n–6 PUFA– and MUFA-enriched diets decrease serum PCSK9 concentrations. These dietary FAs (particularly n–3 PUFAs and MUFAs) decrease inflammation, activate PPARα, and increase p-AMPK, which may activate SREBP2 and downregulate hepatic *PCSK9* mRNA expression. Increased dietary cholesterol increases the hepatic cholesterol pool, decreasing SREBP2 protein and consequently downregulating hepatic *PCSK9* mRNA expression. SFAs increase hepatic *PCSK9* mRNA expression through inflammatory-mediated processes (including TNF receptor 2 and IL1 receptor A) that upregulate SREBP2. Dietary fructose may increase the concentration of serum PCSK9 because of decreased clearance via reduced LDLR expression. Fructose may also act through SREBP1c-mediated signaling pathways to reduce hepatic *PCSK9* mRNA and serum PCSK9 concentration. LDLR, LDL receptor; p-AMPK, phosphorylated AMP-activated protein kinase; PCSK9, proprotein convertase subtilisin/kexin type 9; SREBP, sterol regulatory element–binding protein.

in the inflammatory markers TNF receptor 2 and IL1 receptor A (77, 78). Inflammation has also been shown to be related to an increase in SREBP2, as demonstrated in several in vivo and in vitro studies (79). The administration of LPS has been shown to increase *PCSK9* mRNA expression in mice (80). This demonstrates that inflammation possibly upregulates PCSK9 through SREBP2-related pathways. It is well established that MUFAs and PUFAs play a role in anti-inflammatory pathways (81), and these long-chain unsaturated FAs are ligands for the nuclear transcriptional targets SREBP1c and PPARα (82). Indeed, PUFAs (n–618:2, n–318:3, and n–620:4) have been shown to activate PPARα, and this appears to downregulate *PCSK9* promoter activity and *SREBP1c* expression through the liver X receptor in human hepatocytes. Liver X receptor agonists have been shown to decrease *PCSK9* transcription in rat hepatocytes (58, 83). An anti-inflammatory environment may result in decreased SREBP2, liver-X-nuclear receptor, or PPARα, which subsequently would reduce *PCSK9* expression and protein concentrations. In contrast, increased dietary SFAs have been shown to increase serum PCSK9 concentrations in obese patients (77). SFAs may promote a proinflammatory state, which may upregulate *PCSK9* and *SREBP2* expression, and this may result in an increase in PCSK9 protein transcription. Therefore, it is possible that changes in dietary lipid content can alter inflammatory signaling, and this may affect plasma PCSK9 concentrations; however, further studies are required to investigate the effects of specific FAs (n–3, n–6, and n–9) and the potential mechanisms of PCSK9 regulation (Figure 2). α-Lipoic acid is an endogenous SCFA that acts as a cofactor in enzymes involved in mitochondrial respiration and has been shown to attenuate plasma lipids and body weight (84). Supplementing a high-fat diet (40%) with α-lipoic acid (0.25%) has been shown to decrease hepatic *PCSK9* mRNA levels and serum PCSK9 concentrations in a rodent model (84). Furthermore, analysis of clinical *PCSK9* LOF carriers (R46L) showed that these individuals have significantly reduced lipid species compared with controls (16:0- and 18:0-containing lipid species, including palmitic and stearic acid–containing cholesterol esters, glucosyl/galactosylceramide, lactosylceramide, and ceramide species). These observations were also shown in the mouse $PCSK9^{-/-}$ model after consumption of a diet high in simple carbohydrates (43). Interestingly, the clinical LOF carriers had significantly reduced cholesteryl ester species, and this was not observed in the $PCSK9^{-/-}$ murine model. Collectively, dietary fat and FAs may affect the transcription or translation of PCSK9 and alter plasma PCSK9 concentrations. As mentioned above, in $PCSK9^{-/-}$ mice, dramatic changes occur in the lipidome, which infers that PCSK9 may be able to modulate specific molecular lipid species, including cholesterol esters, ceramides, and sphingomyelin species, and that it is possibly under negative-feedback regulation by these lipids (43). Further studies are needed to elucidate the clinical and metabolic significance of these lipidomic PCSK9 alterations and how these may affect CVD risk or other conditions.

Fructose Effects on PCSK9

High dietary fructose intake has been associated with the development of obesity and metabolic syndrome, including dyslipidemia and insulin resistance (67). High-fructose diets have also been shown to increase serum PCSK9 concentrations in healthy individuals (60). This effect was shown to be independent of changes in plasma total cholesterol, LDL cholesterol, or HDL cholesterol. Moreover, in these subjects, serum PCSK9 concentrations were positively associated with whole-body and hepatic insulin resistance indexes after a 2-step hyperinsulinemic-euglycemic clamp (60). These results are consistent with studies in a hamster model that demonstrate that a high-fructose diet increases serum PCSK9 concentrations; however, this was not accompanied by increases in hepatic *PCSK9* mRNA expression. Despite the fact that the consumption of high fructose increased serum PCSK9 concentrations, there was a nonsignificant decrease in both hepatic *LDLR* mRNA levels and protein concentrations. The authors speculated that the increase in serum PCSK9 may be attributed to a less functional LDLR induced from the high-fructose feeding,

leading to an accumulation of plasma PCSK9, which modulates further degradation of LDLR, thereby increasing plasma apoB lipoproteins (85). In addition, hamsters fed a high-fructose diet and injected with human PCSK9 (tagged human PCSK9 was added to a HuH7 cell line medium and injected into hamster plasma) were observed to have a decreased clearance rate of injected human PCSK9 (quantified by calculated half-life of tagged PCSK9) (85). Importantly, high-fructose feeding also significantly increased hepatic *SREBP1c* mRNA and reduced *SREBP2* mRNA expression. Therefore, fructose may increase serum PCSK9, possibly through alterations to the integrity of hepatic LDLR protein– or SREBP1c-related mechanisms. Fructose is known to activate SREBP1c, resulting in an increase in de novo lipogenesis (86). *PCSK9* mRNA and protein expression (in most models) is associated with the induction of SREBP1c via insulin (44). Therefore, it is quite possible that high-fructose feeding enhances plasma PCSK9 concentrations by a combination of *SREBP1c* transcriptional modulation and reductions in LDLR, thereby decreasing the clearance of PCSK9 from plasma (Figure 2). It has been shown that high amounts of dietary fructose can result in altered insulin sensitivity (87–89). In hyperinsulinemic mouse models, the liver remains sensitive to lipogenesis; therefore, a high-fructose diet may result in elevated plasma insulin, which mediates increases in hepatic *SREBP1c* expression and consequent upregulation in *PCSK9* expression and activity (90). Fructose is also associated with a decrease in extrahepatic lipoprotein clearance (91). One can speculate that this reduction in clearance may be due to a less-functional hepatic LDLR and a reduction in PCSK9 clearance rates, which will further degrade LDLR and decrease apoB lipoprotein clearance (85) (Figure 2). It remains unclear from the present studies whether high dietary fructose alters hepatic *PCSK9* transcription or whether the effects on PCSK9 observed are solely posttranslational and can be attributed to functional changes in LDLR.

Conclusions

PCSK9 has become a promising lipid-lowering target. The use of PCSK9 inhibitors provides an alternative for statin-intolerant individuals and may be prescribed as an add-on to statin therapy in individuals with severe hypercholesterolemia. PCSK9 is modulated by metabolic status and specific dietary interventions. In the fasting state, plasma PCSK9 is significantly reduced, and this appears attributable to reductions in insulin and SREBP1 and SREBP2 or a decline in HNF1α protein. In addition, dietary interventions and nutrients appear to have varying effects on plasma PCSK9 concentrations. Interestingly, interventions that decrease plasma PCSK9 are also well established in lowering plasma lipid concentrations, including in the Mediterranean diet, which is high in MUFAs and n–3 PUFAs. These FAs may decrease PCSK9 through their anti-inflammatory properties, or through phosphorylated AMPK– and PPARα-mediated signaling pathways. Furthermore, diets that are associated with negative effects on lipid and glucose metabolism, including the Western-style diet and high-fructose diets, result in elevated plasma PCSK9, which may perturb the clearance of apoB lipoproteins and increase the CVD risk associated with these diets (60, 68, 85). Moreover, it has been proposed that changes in PCSK9 regulation may promote or be associated with further metabolic perturbations, such as inflammation, which may further aggravate diet-associated increases in cardiometabolic risk.

At this time, there are still gaps in the literature with regard to the specific nutritional regulation of PCSK9. In particular, there is still limited knowledge about the PCSK9-mediated effects on glucose homeostasis and the long-term impact of PCSK9 inhibitors on insulin-glucose metabolism. How nutritional factors affect the action of PCSK9 inhibitors also remains unknown. Moreover, the physiologic role of plasma PCSK9 is not well understood, particularly how it regulates extrahepatic organs and metabolic or lipid metabolism pathways, and whether it acts as a biomarker of signaling molecules. In particular, the impact of plasma PCSK9 on intestinal apoB lipoprotein metabolism and cholesterol homeostasis needs further investigation. Interestingly, the diurnal rhythm of PCSK9 is accompanied by stable serum LDL cholesterol concentrations, and this may explain why the correlation between plasma PCSK9 and LDL cholesterol is modest. It may also suggest that a given concentration of plasma PCSK9 may not be indicative of *PCSK9* activity or LDL clearance (35, 45). Therefore, it is important to clarify these relations in order to understand the physiologic relevance of plasma PCSK9 as a marker of its activity, LDLR expression, and subsequent clearance of atherogenic cholesterol-dense apoB lipoprotein remnants. Moreover, it is imperative to further understand the impact of diet on the regulation of PCSK9 metabolism and the potential impact on apoB lipoprotein metabolism, and the relation to the metabolic milieu in the modulation of CVD risk.

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Appendix ‘B’

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The Effect of PCSK9 Loss-of-Function Variants on the Postprandial Lipid and ApoB-Lipoprotein Response

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Context: Proprotein convertase subtilisin kexin 9 (PCSK9) mediates degradation of the low-density lipoprotein receptor (LDLR), thereby increasing plasma low-density lipoprotein cholesterol (LDL-C). Variations in the *PCSK9* gene associated with loss of function (LOF) of PCSK9 result in greater expression of hepatic LDLR, lower concentrations of LDL-C, and protection from cardiovascular disease (CVD). Apolipoprotein-B (apoB) remnants also contribute to CVD risk and are similarly cleared by the LDLR. We hypothesized that PCSK9-LOF carriers would have lower fasting and postprandial remnant lipoproteins on top of lower LDL-C.

Objective: To compare fasting and postprandial concentrations of triglycerides (TGs), total apoB, and apoB48 as indicators of remnant lipoprotein metabolism in *PCSK9*-LOF carriers with those with no PCSK9 variants.

Design: Case–control, metabolic study.

Setting: Clinical Research Center of The Ottawa Hospital.

Participants: Persons with one or more copies of the L10ins/A53V and/or I474V and/or R46L PCSK9 variant and persons with no PCSK9 variants.

Intervention: Oral fat tolerance test.

Main Outcomes Measures: Fasting and postprandial plasma TG, apoB48, total apoB, total cholesterol, and PCSK9 were measured at 0, 2, 4, and 6 hours after an oral fat load.

Results: Participants with PCSK9-LOF variants (n = 22) had reduced fasting LDL-C (−14%) as well as lower fasting TG (−21%) compared with noncarrier controls (n = 23). LOF variants also had reduced postprandial total apoB (−17%), apoB48 (−23%), and TG (−18%). Postprandial PCSK9 declined in both groups (−24% vs −16%, respectively).

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Abbreviations: apoB, apolipoprotein-B; apoE, apolipoprotein-E; AUC, area under the curve; BMI, body mass index; CM, chylomicron; CV, coefficient of variability; CVD, cardiovascular disease; GOF, gain of function; HDL-C, high-density lipoprotein cholesterol; iAUC, incremental area under the curve; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; LOF, loss of function; LRP1, low-density lipoprotein receptor–related protein 1; OFTT, oral fat tolerance test; PCSK9, proprotein convertase subtilisin kexin 9; PPL, postprandial lipemia; SREBP, sterol response element binding protein; TG, triglyceride; TRL, triglyceride-rich lipoprotein; VLDL, very-low-density lipoprotein; VLDLR, very-low-density lipoprotein receptor.

Conclusions: Participants carrying PCSK9-LOF variants had attenuated levels of fasting and postprandial TG, apoB48, and total apoB. This may confer protection from CVD and further validate the use of PCSK9 inhibitors to lower CVD risk. (***J Clin Endocrinol Metab*** **102: 3452–3460, 2017)**

Proprotein convertase subtilisin kexin 9 (PCSK9) is a secreted protein expressed primarily in the liver and small intestine (1). It is the ninth member of the family of proprotein convertases (2). Unlike other proprotein convertases, it has only itself as substrate, and it escorts the low-density lipoprotein receptor (LDLR) to the lysosome for degradation (3). By reducing the amount of cell surface LDLR, the low-density lipoprotein cholesterol (LDL-C) concentration is increased (4). Consequently, PCSK9 inhibitors have been rapidly developed and are currently in clinical use. One of them, evolocumab, has recently been shown to further reduce the risk for cardiovascular events in patients who are already receiving statin therapy (5).

The gene for PCSK9 is highly polymorphic (6). Populations have variants that result in a more functional or increase in circulating PCSK9 [gain of function (GOF)] or, conversely, a less functional or decrease in circulating PCSK9 [loss of function (LOF)] (7). LOF variants are more common than GOF variants. PCSK9-LOF results in greater expression of hepatic LDLR, lower concentrations of LDL-C, and protection from cardiovascular disease (CVD) (8, 9). LDL-C is a well-established risk marker of CVD and is used to assess and manage CVD risk (10). Despite lowering LDL-C to recommended levels by statin therapy, there is substantial residual risk for cardiovascular events, especially in high-risk patients with diabetes and the metabolic syndrome (11).

Recent data indicate that triglyceride (TG)-rich lipoproteins (TRLs) have a substantial and independent causal association with CVD risk (12). Remnants of chylomicrons (CMs) and very-low-density lipoproteins (VLDLs) contain more cholesterol per particle than LDL and therefore have enhanced atherogenic potential (13). Remnant lipoproteins in plasma are assessed by measuring fasting plasma apolipoprotein-B (apoB) 48 and apoB100, the functional proteins present on each CM and VLDL remnant particle, respectively (14). The clearance of remnant lipoproteins is mediated by the liver through the LDLR and the LDLR-related protein 1 (LRP1) (15). Remnant lipoproteins bind to the LDLR through apolipoprotein-E (apoE)-related mechanisms (15), whereas LDL binds through interactions with apoB100 (16). Delayed clearance of TRL, especially in the postprandial period, results in accumulation of remnant lipoproteins in plasma, manifesting as hypertriglyceridemia and enhanced postprandial lipemia (PPL) (17, 18).

PCSK9 could influence TRL clearance by reducing the amount of cell surface LDLR. *In vitro*, PCSK9 has also been shown to stimulate the production of TRL (19). PCSK9 null mice have reduced postprandial TGs excursion as well as intestinal apoB48 expression following an olive oil bolus (20). Collectively, the evidence presented suggests that PCSK9 could modulate both intestinal secretion and hepatic clearance of TRL. It is unknown whether PCSK9 affects TRL metabolism in humans, particularly during the postprandial period. We hypothesized that persons with PCSK9-LOF variants will have not only lower fasting LDL-C but also lower fasting and postprandial remnant lipoproteins. The aim of the current study was to compare the fasting and postprandial concentrations of TG, total apoB (as a surrogate of apoB100), and apoB48 as indicators of remnant lipoprotein metabolism in PCSK9-LOF carriers.

Materials and Methods

Population and study participants

Participants were recruited from a previously identified white and African Canadian pool (the Ottawa PCSK9 Cohort) that underwent full exonic sequencing of the *PCSK9* gene (21). Of this cohort, participants with one or more copies of the PCSK9 L10ins/A53V and/or I474V and/or R46L variants (n = 22) and no PCSK9 variants at all (n = 23) were identified and approached. Men and women aged 30 to 80 years with a body mass index (BMI) of 20 to 40 kg/m^2, a fasting serum LDL-C level $>$ 2.0 and $<$7 mmol/L, a total cholesterol (TC)/high-density lipoprotein cholesterol (HDL-C) ratio $>$ 4.0, a TG level $<$ 4.5 mmol/L, and an HDL-C level $>$ 0.6 mmol/L were eligible.

Exclusion criteria included cognitive impairment, receipt of any medication for dyslipidemia for 6 weeks before the oral fat tolerance test (OFTT) or unknown supplements, nutraceuticals, herbal medications, and sex hormone therapy (including oral contraceptive pills or testosterone therapy), active or chronic hepatic or renal disease, presence of tendon xanthomas, type 1 or type 2 diabetes mellitus, untreated hypo- or hyperthyroidism, major illness (such as cancer and pulmonary and gastrointestinal disorders), acute illness or surgical procedure within the previous 3 months, alcohol consumption $>$ 2 drinks per day (1 drink defined as 12 fl. oz of beer or 5 fl. oz of wine or 1.5 oz. of liquor), and apoE2/2, 3/2, 4/2 genotype to avoid the apoE2 allele (which is known to enhance PPL).

The 22 PCSK9-LOF variant participants had the following variants: I474V (9), L10ins/A53V (4), R46L (2), L10ins/A53V and I474V (3), L10ins/A53V and R46L (1), and homozygous I474V (3).

Study design

The current study was a case–control, metabolic study. The Research Ethics Board of the Ottawa Hospital Research Institute approved the study protocol. The study was conducted in the Clinical Research Center of the Ottawa Hospital. The OFTT

was conducted by experienced staff in a dynamic function testing facility within the Clinical Research Center. Samples were processed, stored at −80°C, and subsequently analyzed in an adjoining clinical research laboratory and in Edmonton, Alberta. Participants from our previous study pool were invited to attend an interview. Those who agreed to participate provided written informed consent and underwent further history taking, examination, and laboratory screening to establish their eligibility. Tests done included serum glucose, creatinine, alanine aminotransferase, thyroid-stimulating hormone, apoE genotyping, and serum lipids, including total cholesterol, TG, HDL-C, LDL-C, and TC/HDL-C ratio. These results were used to determine eligibility for the OFTT.

Postprandial study (OFTT)

Eligible participants received dietary counseling from a certified dietitian and underwent a 6-week run-in dietary period to standardize the background diet. Participants were advised to refrain from physical exercise for 60 hours and abstain from alcohol for 36 hours before the OFTT because these are known to acutely affect PPL. A fat load was given at 08:00 hours after a 12-hour fast. The test drink consisted of 350 mL of whipping cream (35% fat), 2 tablespoons of chocolate-flavored syrup, 1 tablespoon of granulated sugar, and 1 tablespoon of instant nonfat dry milk. Of the total 880 kcal, 5.0% were from protein, 26% were from carbohydrate, and 69% were from fat. The fat load contained 453 mg of cholesterol and a polyunsaturated/saturated fatty acid ratio of 0.06. The meal (69% fat, 1298 kcal/350 mL) volume was calculated per body surface area by using the following formula: volume = 350 × body surface area/2, which has been used by our group previously (22). The fat load was consumed within 10 minutes. Blood was drawn before and 2, 4, and 6 hours after the fat load. The 0- and 4-hour samples had their lipoprotein subfractions separated by cumulative floatation ultracentrifugation, and their cholesterol and TG contents were determined at each time point. Blood was collected into EDTA-Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 3000 rpm for 10 minutes at 22°C to obtain plasma and leukocytes. To obtain serum for lipid measurements, blood was collected into serum separation tubes/Vacutainer tubes, allowed to clot at room temperature for 20 minutes, and centrifuged at 3000 rpm for 10 minutes at 22°C.

ApoE genotyping

Genomic DNA was available for *apoE* exon sequencing from our previous study (21). Primers used to encompass the *ApoE2* and *ApoE4* gene variations were forward 5′ TAAGCTTGGCACGGCTGTCCAAGGA3′ and reverse 5′ TAAGCTTGGCACGGCTGTCCAAGGA 3′ (23). Fifty nanograms of genomic DNA was used in the polymerase chain reaction for amplification by using platinum Taq DNA polymerase (Invitrogen, Burlington, ON, Canada). Cycle conditions were denaturation for 2 minutes at 94°C, annealing for 2 minutes at 64°C, and extension for 3 minutes at 72°C with final extension of 5 minutes at 72°C for a total of 35 cycles. Amplified DNA were analyzed by standard DNA-sequencing reactions carried out as a service by BioBasic Sequencing (Markham, ON, Canada)

Measurement of serum lipid and lipoproteins

Fasting plasma apoB48, total apoB, apoCII, and apoCIII, as well as subsequent postprandial (2-, 4-, and 6-hour) measures of apoB48 and total apoB concentrations, were analyzed by using a highly sensitive chromogenic sandwich enzyme-linked immunosorbent assay (Shibayagi Co. Ltd., Japan; Alercheck Inc; and Abcam, respectively). All samples were quantified two times, and absorbance was measured spectrophotometrically with an intra-assay coefficient of variability (CV) of 4.8% to 5% and interassay CV of 7.5% to 9.7%. Serum PCSK9 was quantified by using a human PCSK9 enzyme-linked immunosorbent assay (CyClex Co., Japan). Serum PCSK9 was quantified four times with an intra-assay CV of 1.5% to 2.6% and an interassay CV of 2.9% to 7.1%.

Statistical analysis

GraphPad Prism 5 software (GraphPad Software Inc.) and SPSS software, version 20 (IBM, Chicago, IL), were used for statistical analysis. The data are presented as mean (standard deviation) or frequency and percentage. All continuous variables were tested for normal distribution by using the Shapiro–Wilk test; normally distributed data were analyzed by using the Student *t* test (unpaired and paired), and non–normally distributed data were evaluated by using the Mann–Whitney *U* test for unpaired samples and Wilcoxon signed-rank test for paired samples. Categorical variables were tested by using χ^2 or Fisher exact test. Area under the curve (AUC) was calculated through GraphPad Prism 5 software and corresponds to the total plasma concentration over the 6-hour postprandial measured. The fasting concentration of the respective parameter is further subtracted from the total AUC to generate the incremental AUC (iAUC). The iAUC represents the rate of change in the postprandial response adjusted for the initial concentration of the parameter. Outliers were identified by using the ROUT" Q = 1% method recommended in GraphPad Prism. Three outliers were removed from the TG AUC, and two outliers were removed from calculated apoB48 AUC by using this method. Differences in groups were established by using the Student *t* test, and a P value $<$ 0.05 was considered to represent a statistically significant difference.

Results

Fasting biochemical profile of PCSK9-LOF variant participants

Anthropometry and fasting biochemistry of PCSK9-LOF variant participants and nonvariant control participants are summarized in Table 1. The reduction in baseline TG is similar to that seen with other LOF variants (8, 9). Participants were similar in age, sex, weight, height, BMI, waist circumference, and apoE genotype.

LOF variants had significantly reduced fasting LDL-C (−13.6%), total cholesterol (−7.1%), TG (−21.3%), apoB48 (−25.7%), and VLDL-C (−34%). Additionally, LOF variants had nonsignificantly lower levels of total apoB (−17.6%; P = 0.059) and PCSK9 (−16.8%; P = 0.077) compared with those without variants. Fasting HDL-C (P = 0.46), apoCII (P = 0.13), and apoCIII (P = 0.66) did not significantly differ between the groups (Table 1).

Table 1. Baseline Characteristics of Nonvariant and PCSK9-LOF Variant Participants

Characteristic	Nonvariant (n = 23)	LOF Variant (n = 22)	*P* Value[a]
Age, y	63.2 (9.1)	59.0 (12.9)	0.214
Sex			
Male	13	9	
Female	10	13	
apoE, n			
3/3	17	18	
4/3	6	4	
Height, cm	165.2 (12.3)	166.7 (6.7)	0.627
Weight, kg	76.9 (15.3)	73.9 (12.8)	0.487
BMI, kg/m^2	28.4 (3.2)	26.7 (4.1)	0.075
Waist circumference, cm	94.6 (10.0)	91.4 (15.6)	0.446
BSA, m^2	1.9 (0.2)	1.8 (0.2)	0.543
PCSK9, ng/mL	290 (78.0)	241.3 (67.5)	0.077
Cholesterol, mmol/L	5.6 (0.8)	5.2 (1.1)	0.046
TG, mmol/L	1.59 (0.57)	1.25 (0.53)	0.032
VLDL-C, mmol/L	0.53 (0.33)	0.35 (0.20)	0.040
LDL-C, mmol/L	3.53 (0.72)	3.05 (0.79)	0.015
HDL-C, mmol/L	1.41 (0.32)	1.50 (0.44)	0.460
Total-apoB, μg/mL	126.7 (38.97)	107.4 (26.64)	0.059
apoB48, μg/mL	8.17 (3.29)	6.07 (2.82)	0.028
apoCIII, μg/mL	209.4 (87.1)	198.7 (73.8)	0.661
apoCII, μg/mL	69.6 (23.2)	58.1 (23.6)	0.125

Unless otherwise noted, data are presented as mean (standard deviation).

Abbreviation: BSA, body surface area.

[a]Comparisons between PCSK9 nonvariant and PCSK9 variant participants by Mann–Whitney *U* test or Student *t* test.

Postprandial response of TGs, ApoB, and PCSK9

The postprandial response and quantified AUC and iAUC of TG, apoB (total apoB and apoB48), and PCSK9 are shown in Fig. 1. Carriers of PCSK9-LOF variants had reduced postprandial response in all parameters examined. The total apoB AUC was significantly lower for LOF variants (−17%), whereas the iAUC was almost identical between groups. The TG AUC and iAUC were significantly lower for LOF variants than nonvariants (AUC LOF −18% AUC nonvariant; iAUC LOF −37% nonvariant). The apoB48 AUC and iAUC were significantly reduced for LOF variants (−23% and −35%, respectively).

At 4 hours after fat load, PCSK9 values drastically declined in both LOF and nonvariant groups. Interestingly, the 4-hour postprandial decline was much greater for the LOF group than the nonvariant (−24% vs −16%, respectively; $P = 0.06$).

Lipoprotein subfraction analysis

The TG and cholesterol concentrations in lipoprotein subfractions are shown in Fig. 2. There was no difference in fasting and 4-hour postprandial TG and cholesterol levels in the CM fraction between PCSK9-LOF and nonvariant participants. However, the concentrations of non-CM TG and cholesterol at both time points were significantly lower in PCSK9-LOF participants than nonvariant individuals (Fig. 2). Likewise, in the non-CM lipoprotein subfractions (VLDL1, VLDL2, intermediate-density lipoprotein, LDL1, and LDL2), the TG and cholesterol concentrations were mostly decreased in PCSK9-LOF participants at both time points, as shown in Fig. 2. However, there was no apparent preferential enrichment or depletion of cholesterol or TG among lipoprotein subfractions between groups.

Discussion

PCSK9 LOF variants have reduced TRL

The major finding of this study is that humans with PCSK9-LOF variants have lower circulating TRL remnants than those with no PCSK9-LOF variants. The effect of circulating PCSK9 on LDL metabolism has been well established, but our findings demonstrate that PCSK9 also influences TRL metabolism. Specifically, we observed that individuals carrying LOF variants in PCSK9 have reduced circulating postprandial lipoproteins, which consist primarily of CM, VLDL, and their remnants. A second important finding in our study is the decline in circulating PCSK9 after an oral fat load. The postprandial decline in PCSK9 was observed in both LOF and nonvariant individuals. These novel findings add to the evidence for a role of PCSK9 in TRL metabolism (24–26). Cholesterol-rich apoB-containing lipoproteins are known to be causally involved in atherogenesis (27). We can consider that the reduction in CAD risk associated with PCSK9-LOF variants (8, 9) may be attributed to

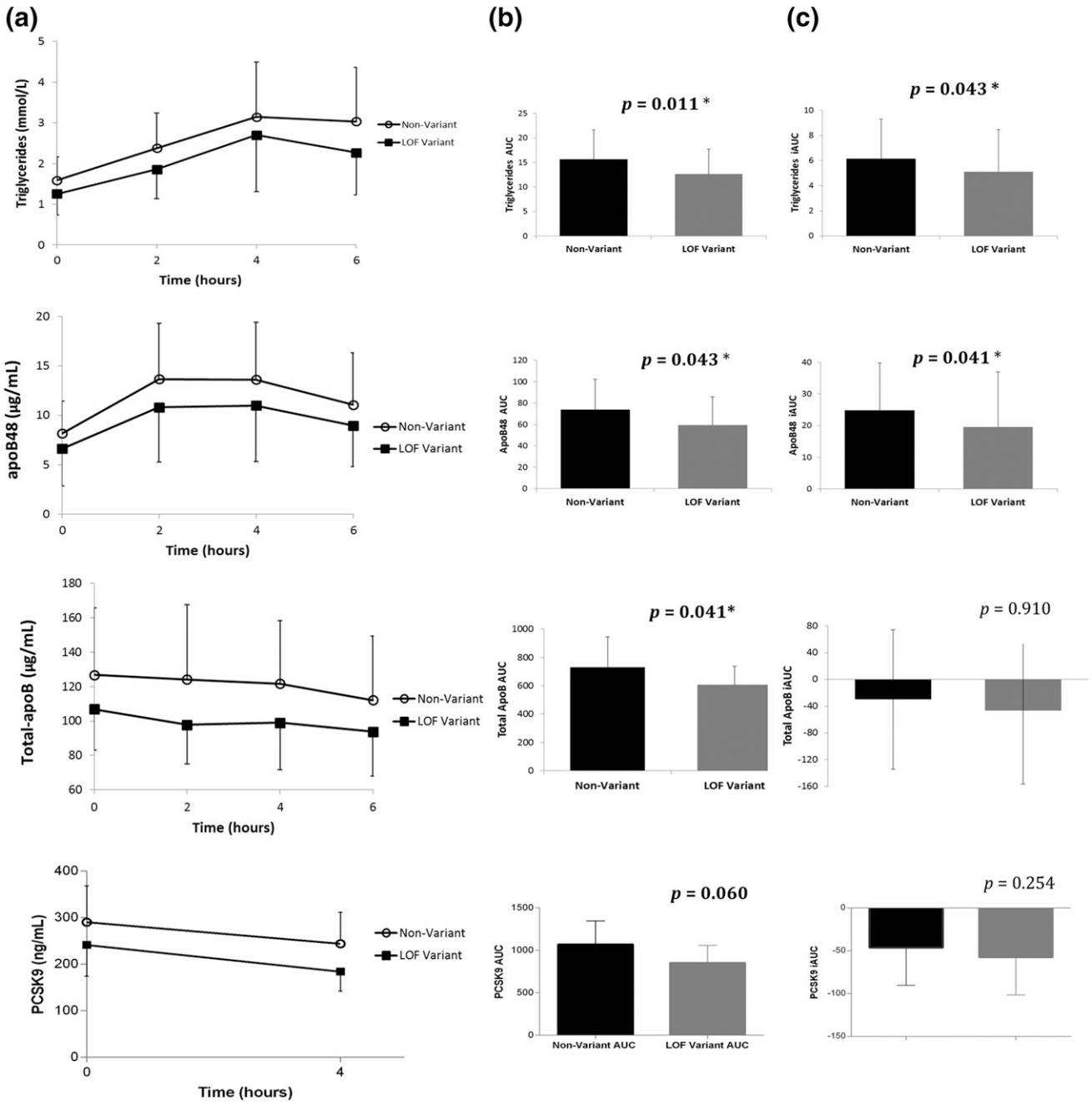

Figure 1. The postprandial response of plasma TG, apoB48, total apoB, and PCSK9 in PCSK9-LOF variant and nonvariant control participants. Participants were given a fat load to consume after a 12-hour fast. Blood was drawn before and 2, 4, and 6 hours after consumption. Data are shown as (a) the mean ± standard deviation, (b) as AUC values (± standard deviation), and (c) iAUC values (± standard deviation). For nonvariant and PCSK9 LOF variant groups, respectively, n = 23 and 22 for total-apoB and PCSK9, n = 23 and 19 for TG data, and n = 23 and 20 for apoB48 data. *$P < 0.05$.

lifelong reductions in not only plasma LDL but also TRL remnant lipoproteins. In the Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Subjects With Elevated Risk (FOURIER) study, with 27,564 participants, TG decreased by 16.2% and 0.7% in the evolocumab-treated participants and placebo-treated groups, respectively (5). Thus, the magnitude of decrease in baseline TG level in our study (21%) is close to that seen in response to anti-PCSK9 therapy, which was associated with reduced risk for cardiovascular events (5). This highlights the importance of a potential role of PCSK9 in TRL metabolism.

PCSK9 LOF variants are protected against postprandial lipemia

We demonstrated that PCSK9-LOF variants have lower TG and apoB48 not only during fasting but also during the fed or postprandial state. Elevated fasting apoB48 predicts fat intolerance (as measured by postprandial apoB48 and TG) (15). The present findings

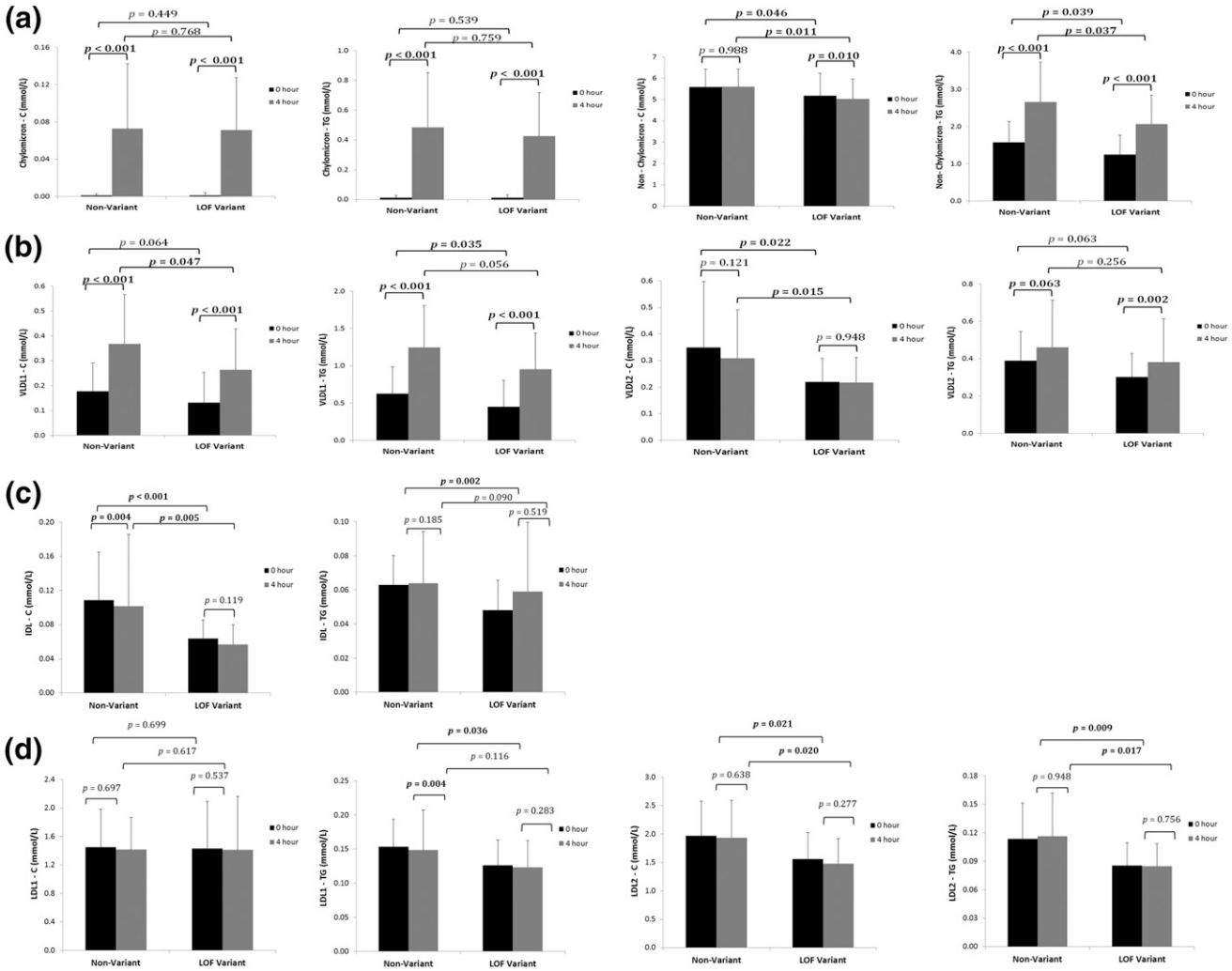

Figure 2. The postprandial cholesterol and TG response in (a) CM and non-CM subfractions and the postprandial cholesterol and TG response in (b) VLDL1-C and VLDL2-C, (c) IDL1-C, and (d) LDL1-C and LDL2-C subfractions. Data are shown as mean ± standard deviation. For nonvariant and PCSK9 LOF variant groups, respectively, n = 23 and 22.

demonstrate that LOF variants are protected from fat intolerance. The mechanisms responsible for attenuation of PPL in our participants with PCSK9-LOF variants remain to be clarified. A possible mechanism is the well-known association of diminished PCSK9 activity with an increase in the LDLR, which in addition to binding apoB can also bind apoE, resulting in greater clearance of apoE-containing CM remnants and VLDL remnants (7, 15, 28). Another possibility is that PCSK9 may affect TRL clearance through its effects on other receptors in the LDLR family, including LRP1, VLDL receptor (VLDLR), and apoE receptor 2 (29, 30). In particular, LRP1 has been shown in preclinical studies to be an important factor in the clearance of CM remnants and VLDLR. PCSK9 can mediate degradation of LRP-1, and LRP-1 competes with the LDLR for PCSK9 activity (30). Although research has shown that PCSK9 induces degradation of VLDLR and apoE receptor 2, it is less clear whether these receptors play an important role in TRL clearance in humans (30). Beyond an alteration in receptor-mediated TRL clearance, it is also possible that PCSK9 may affect the lipolysis of CM and VLDL and their remnants. To test this, we measured the fasting concentration of apoCII and CIII, which are apolipoproteins involved in the regulation of lipoprotein lipase. We did not find any differences in fasting apoCII, apoCIII, or CII/CIII ratio between groups, suggesting that alterations in lipolysis are a less likely mechanism for PCSK9's action on TRL clearance in this population.

Our non-CM and subfraction data indicate a reduction in fasting and postprandial lipoprotein remnants and LDL. TG and cholesterol concentrations were most strikingly reduced in the LDL2 subfraction, but least so in the LDL1 subfraction in the PCSK9-LOF variants. A reduction in the LDL2 subfraction is consistent with the well-recognized increase in LDLR associated with having PCSK9-LOF variants (31). PCSK9 is highly expressed in the intestine, where it possibly has a role in CM

production and transintestinal cholesterol excretion (32). In the current study, there were no differences between fasting and 4-hour postprandial CM-TG and cholesterol levels in PCSK9 LOF variant and nonvariant participants. Kinetic data are necessary to determine whether CM production rate is diminished in the presence of PCSK9-LOF variants.

The relationship between PPL and PCSK9 is not well studied. Le May *et al.* (20) showed an attenuated postprandial TG response to an oral olive oil load in PCSK9 knockout mice compared with their wild-type littermates, consistent with the findings of the current study. Conversely, Cariou *et al.* (33) demonstrated that an oral fat load in two heterozygous carriers of the R104C-V114A PCSK9-LOF mutation did not alter postprandial plasma TG concentrations compared with nonvariant participants. The authors concluded that plasma PCSK9 was not associated with PPL in humans. The discordance between our studies may be attributed to the phenotype of the variants studied. The LOF variants in Cariou and colleagues' study had very low to undetectable plasma PCSK9 and very low plasma LDL-C. In the current study, the PCSK9-LOF variants studied (L10ins/A53V and/or I474V and/or R46L) had modest lowering of circulating PCSK9 and lower but normal plasma LDL-C concentrations. Different PCSK9 variants appear to result in different biochemical characteristics (34, 35) and therefore may explain the differences between these two postprandial studies. Additionally, Chan and colleagues (36) conducted a postprandial study on 17 obese participants and demonstrated a positive association between plasma PCSK9 and the AUC and iAUC for apoB48 and an inverse association with TRL-apoB48 fractional clearance rate. In the current study, we similarly showed a reduction in fasting apoB48 and apoB48 AUC and iAUC. However, the study by Chan and colleagues was based on plasma PCSK9 concentration, whereas the current study is based on *PCSK9* genotype.

Plasma PCSK9 significantly declines following a high-fat meal

Plasma PCSK9 was found to decline 4 hours after an acute oral fat load in individuals with and without LOF PCSK9 variants. Cariou *et al.* (33) studied two LOF PCSK9 participants and also observed significant decreases in serum postprandial PCSK9, consistent with our results. However, the authors further reported postprandial PCSK9 was unchanged after an acute fat load in 10 healthy volunteers (33). The discrepancy in the postprandial response of plasma PCSK9 between studies is unclear but could be due to the composition of the meals given. A decrease in plasma PCSK9 after an acute oral fat load may be a result of *PCSK9* expression being under the control of the nuclear transcription factors sterol response element binding protein (SREBP)-2 (37) and SREBP-1c (38), both of which act through the same response element on the promotor of *PCSK9* (38). Increased postprandial cholesterol and fatty acids would reduce *PCSK9* expression through a suppression of hepatic SREBP-2 and SREBP-1c, respectively. PCSK9-LOF variants could also lower circulating PCSK9 by reducing hepatic LDLR degradation (7). Circulating PCSK9 is primarily cleared from the circulation by hepatic LDLR (39). Therefore, increased LDLR expression in participants carrying LOF variants may enhance the clearance of circulating PCSK9, resulting in a more acute postprandial decline in PCSK9.

Strengths and limitations

This study examined fasting and postprandial lipoproteins from a subset of individuals with *PCSK9*-LOF variants from our Ottawa cohort (21). The use of full exonic sequencing to identify well-defined variant and control populations was a major strength of the current study. This genotyping approach allowed us to ensure that variant participants had no other PCSK9 variants, and nonvariant control participants had no PCSK9 variants. The exclusion of participants carrying an apoE2 allele added to the robustness of our genotyping approach.

Additionally, our study participants had PCSK9 variants associated with a relatively small but significant LOF effect on LDL-C levels. No participant had LOF mutations Q152H (40), Y142X, and C679X (8, 9), which are typically associated with major reductions in LDL-C levels. We speculate that any effects on fasting and postprandial concentrations of remnant lipoproteins seen with our "subtle" LOF variants may be even more pronounced with stronger LOF PCSK9 variants. None of our participants had a GOF variant. It is not known whether GOF variants are associated with enhanced PPL. We recognize that studying PPL in individuals with major LOF or GOF variants might provide information of greater clinical relevance, but these variants are uncommon. We chose LOF PCSK9 variants that are common in the Canadian population and have been shown to affect lipid metabolism significantly, even in the heterozygous state (21). Thus, our results apply to a larger portion of the population than had the effects been shown in participants with rare but stronger LOF and/or GOF variants.

Because different LOF variants may have different effects on lipoprotein metabolism, it would be informative to compare the effects of different LOF variants. However, subgroup analysis was not possible in our study because the number of participants in the subgroups would be too small. Several participants were

LOF compound heterozygotes, making the number of participants with a single LOF variant even smaller.

Conclusion

This study demonstrated that PCSK9-LOF variants are associated with attenuated fasting and postprandial response of plasma TG, apoB48, and total apoB. This may protect against CVD and further validate the use of PCSK9 inhibitors to lower CVD risk.

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Appendix ‘C’

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Remnant Lipoproteins Are Associated with Increased Cardiometabolic Risk in Adolescents

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ABSTRACT

Background. Cardiovascular disease (CVD) is proposed to begin in youth, and may be exacerbated by obesity and the metabolic syndrome. Apolipoprotein(Apo)B-remnant cholesterol is considered a primary contributor to CVD risk. Fasting plasma apoB48 can be used as a biomarker of intestinal remnant cholesterol. In adults, elevated fasting plasma apoB48 is strongly associated with cardiometabolic risk factors and obesity, whereas in adolescents there is limited knowledge of these relationships.

Objective. To measure fasting plasma apoB48 as a marker of remnant lipoprotein concentration and determine the relationship with cardiometabolic risk factors in adolescents from a large cross-sectional population.

Design. A cross-sectional population analysis of fasting plasma apoB48 in participants from the Western Australian Pregnancy Cohort (Raine) Study.

Participants. Adolescent males and females aged 17 years with complete fasting plasma apoB48, biochemical, and anthropometry data (n=1045).

Main Outcome Measure(s). The relationship between fasting plasma apoB48 and other cardiometabolic risk factors. The high-risk metabolic cluster variable was defined using elevated BMI, HOMA-IR, fasting plasma triglycerides, and systolic blood pressure.

Results. Fasting plasma apoB48 was significantly higher in male (15.28±2.95ug/mL) compared to female (12.45±2.43ug/mL) adolescents (p=0.0003), and was increased by 21% (3.60ug/mL; p=0.0000) in the high-risk metabolic cluster group and even more pronounced in males (31%, 6.15ug/mL; p=0.0000). Fasting plasma apoB48 was positively associated with fasting plasma

triglycerides, total-cholesterol (but not LDL-C), insulin, leptin, HOMA-IR, and the anthropometric parameters waist-circumference and skinfold-thickness. Fasting plasma apoB48 was inversely associated with fasting plasma HDL-C, and adiponectin.

Conclusions. Fasting plasma apoB48-lipoprotein remnant concentration is associated with cardiometabolic risk factors and is a potential biomarker to evaluate premature CVD risk in youth.

INTRODUCTION

Over the past three decades the prevalence of overweight or obese youth has almost doubled in developed countries (1). Adolescent adiposity, particularly central adiposity, is a significant public health concern as it often progresses into adulthood and tracks with a cluster of cardiometabolic abnormalities that contribute to premature cardiovascular disease (CVD) risk (1, 2). At present, there is limited understanding of the potential biomarkers to detect or predict premature CVD risk and the relationship to cardiometabolic risk factors in the adolescent population. Establishing early cardiometabolic risk markers would aid prevention strategies and may also provide insight to the early lipid pathogenesis of metabolic and vascular disorders. Currently, blood lipid screening for overweight youth is either inconsistent or absent. For example, some countries only recommend lipid screening for select 'high-risk' children or adolescents with a family history of CVD (3). Consequently, an adverse blood lipid profile may go undiagnosed or may be underestimated in a significant portion of this population, particularly in obese youth (3). While advancements in lipid guidelines for adults in Europe and North America now include recommendations to measure non-fasting remnant cholesterol (3-5), no data is yet available in large younger populations. Furthermore, international clinical practice guidelines for assessment of blood lipids and lipid lowering strategies for the youth population are currently limited (6). The National Cholesterol Education Program (NCEP) recently added reference ranges for fasting plasma non-high density lipoprotein cholesterol (Non-HDL-C), total apolipoprotein (apo) B and triglyceride (TG) concentrations relevant to youth and adolescent population (3). However, diagnostic and treatment cut-offs primarily use fasted plasma low-density lipoprotein cholesterol (LDL-C) concentrations (3, 7). Furthermore, LDL-C and total cholesterol (TC) are often unchanged in obese youth and these lipids have been shown to remain

unchanged or decrease during adolescence suggesting that other lipid biomarkers may be more representative of premature CVD risk in this population (3).

Recent studies have shown a causal association between fasting and non-fasting apoB-remnant cholesterol and CVD (8-10). ApoB-remnant cholesterol is derived from hepatic very low-density lipoprotein (VLDL-apoB100), intermediate density lipoproteins (IDL-apoB100) and intestinal chylomicron (CM-apoB48) triglyceride rich lipoproteins.

Quantification of fasting or non-fasting plasma apoB48 can be used as a surrogate marker for intestinal remnant concentration. In humans, the apolipoprotein apoB48 is unique to intestinal chylomicron-derived remnant cholesterol particles (11). Elevated fasting apoB48-remnant cholesterol has been demonstrated to predict non-fasting lipemia following a high-fat meal in adults (12, 13). Dietary lipid intolerance (or non-fasting lipemia) is exacerbated by over-nutrition and central adiposity, leading to an increase in CVD risk (14). ApoB48 metabolism has been well characterized in adult populations and disease states predisposed to CVD risk (12). Fasting apoB48 has been shown to be higher in males compared to females, and elevated in adults with CVD, the metabolic syndrome, and diabetes (15-17). Despite the convincing evidence in adults, it is unclear whether the relationship of apoB48-remnant cholesterol to CVD risk is maintained in youth. Recent studies have demonstrated that fasting plasma apoB48 concentrations are elevated two-fold in obese pre-pubertal children (case-control study) and is more strongly associated with central adiposity indices compared to LDL-C or TC at this age in a French-Canadian cohort (18, 19). Our results to date suggest that fasting plasma apoB48 may be a representative biomarker of early CVD in youth, however it is unclear if this relationship remains relevant in a larger, general population.

The aim of the present study was to measure fasting plasma apoB48 as a marker of intestinal remnant concentration in youth; to establish the relationship of apoB48 with other plasma lipids; as well as cardiometabolic risk factors in a large sample of adolescents from the Western Australian Pregnancy Cohort (Raine) Study at 17 years of age. It was hypothesized that fasting plasma apoB48 would be positively associated with adiposity, fasting plasma triglycerides and associated cardiometabolic risk factors.

METHODS

Study Population

The Western Australian Pregnancy Cohort (Raine) Study is a prospective population cohort that has been described in detail (20, 21). Briefly, 2900 pregnant women between 16 to 20 weeks of gestation were recruited from hospitals in Perth, Western Australia between 1989 and 1992. The recruited women gave birth to 2868 live infants. The offspring have had follow-up examinations at age 1, 2, 3, 5, 8, 10, 14 and 17 years. The 17-year follow-up involved 1754 adolescents. The study was approved by the Human Ethics Committee at King Edward Memorial Hospital and Princess Margaret Hospital for Children in Perth, Western Australia. Complementary approval was also obtained from the University of Alberta (Pro00001941). Adolescents and their primary caregiver provided informed written consent for participation. Participants were excluded from this study if they did not have complete biochemical and anthropometric data sets.

Anthropometry

Calibrated measurements of height, weight, and anthropometry were measured by trained research assistants as previously described (22). In brief, total body weight was measured using a

Wedderburn Chair Scale (nearest 100g) and height with a Holtain Stadiometer (nearest 0.1cm). BMI was derived from body weight (kg)/height (m^2). Waist circumference was measured at the umbilicus using a steal tape measure and skinfold thickness (abdominal, subscapular, suprailiac, and tricep) measures with callipers (Holtain) following standardized protocols (23).

Biochemistry and Metabolism

Blood samples were obtained following an overnight fast, serum and plasma prepared and stored at -80°C for subsequent analysis as described previously (22, 23). Standard plasma biochemistry analyses was performed including, high density lipoprotein cholesterol (HDL-C), total cholesterol (TC), triglycerides (TG), insulin, glucose, leptin and adiponectin (PathWest Laboratory at the Royal Perth Hospital), as previously described (23). LDL-C was calculated using the Friedewald formula, non-HDL-C cholesterol was calculated as TC (mmol/L) – HDL-C (mmol/L) and homeostasis model assessment index of insulin resistance (HOMA-IR) was calculated as insulin (μU/mL) x glucose (mmol/L)/22.5, as previously described (23).

Blood Pressure

Systolic and diastolic blood pressure was obtained using an oscillometric sphygmomanometer (DINAMAP vital signs monitor 8100, DINAMAP XL vital signs monitor or DINAMAP ProCare 100; GE Healthcare). After resting in the supine position for 5 minutes, six blood pressure readings were taken every two minutes over a 15 minute period and the average blood pressure was calculated using the last 5 readings, as previously described (24).

Quantification of Plasma ApolipoproteinB48

ApoB48 was quantified using an established and validated highly sensitive chromogenic sandwich ELISA method (Shibayagi Co., Ltd., Japan), as previously described (19). In brief, plasma samples were incubated in 96 well monoclonal antibody-coated plates and then bound with an anti-apoB48 antibody. The final reaction results in a colour change proportional to the concentration of apoB48 in the samples, which is quantified by measuring absorbance at 450nm spectrophotometrically (19).

Other Measures

Alcohol consumption information was obtained from a validated questionnaire regarding alcohol intake type (beer, spirits, or wine) and amount consumed during the week, as previously described (25). For the present analysis, alcohol consumption was treated as a dichotomous variable (drinker or non-drinker). Smoking was assessed by the number of cigarettes smoked each day, within the last 7 days. Oral contraceptive pill use in females was obtained by answering yes or no to the question, 'In the last 6 months, have you taken any prescription medication(s) e.g. the Pill?' (if yes, 'which medication(s), are you still taking it?') (25, 26).

Statistical Analysis

Statistical analyses were performed using Stata/IC 13.1 software (StataCorp LP). Distributions of outcomes were assessed for normality and log transformations were applied to insulin, HOMA-IR, leptin, subscapular, suprailiac, and triceps skinfolds where departures from normality were detected following assessment of normality curves. Insulin, and leptin values below the lowest detectable level of the assay were assigned a lower limit (1.9mU/L, 0.14mg/L, 1.4ng/mL, respectively) and for skinfold measurements an upper limit (41mm) as described previously (22, 23). Means for censored variables (variables with defined upper and lower limits)

were determined by Tobit analysis, which takes into account the probability of being above the censored value. Tabulated values are presented as proportions, arithmetic or geometric (for log transformed variables) means and 95% confidence interval (CI)).

Adult cut-offs for diagnosis of the metabolic syndrome are not applicable in children and adolescents, therefore cardiometabolic cluster analysis was developed in this cohort, as previously described (22). The high-risk and low-risk metabolic cluster were formed separately by gender and included fasting plasma TG, BMI, HOMA-IR and systolic blood pressure. Individuals in the high-risk metabolic cluster were deemed to be at increased cardiometabolic risk.

Differences between gender and the metabolic cluster variable were examined by linear regression, and Tobit regression. Regression analysis was undertaken to determine the relationship between fasting plasma apoB48 concentration, metabolic cluster variable and other cardiometabolic risk factors. Linearity of the association was assessed by multiple adaptive regression spline analysis and fractional polynomials. When non-linearity was detected, quadratic terms identified by fractional polynomial analysis were generated and included in the regression producing two coefficients for the associations with fasting plasma apoB48 concentration. Regression models were adjusted for gender, oral contraceptive use in females and BMI (skinfold thickness, and waist circumference were not adjusted for BMI). Further adjustment for smoking and alcohol use was also applied. Interaction analysis was performed to determine potential gender modification on the association of fasting plasma apoB48 with other cardiometabolic outcome variables. All regression analyses included a robust variance adjustment to account for potential correlations between a small number of siblings (familial bias). Significance was determined at $p < 0.05$, which provided 95% power to detect a difference

in the fasting plasma apoB48 concentration in the low-risk and high-risk metabolic cluster variable.

RESULTS

Subject characteristics

For the present cohort study, we analysed adolescents (n=1045) aged 17 years with complete biochemistry and anthropometric data sets. In this population cohort, 52% were male, 85% had both parents that were Caucasian, 50% had consumed alcohol in the past 7 days, 21% were defined as smokers, and 31% of females were using an oral contraceptive.

Plasma lipid biochemistry

The mean fasting plasma apoB48 concentration was significantly higher, 19% or 2.8 ug/ml, in males compared to females (Figure 1). Conversely, females had significantly higher LDL-C, HDL-C, and TC compared to males, but both had similar TG and non-HDL-C concentrations (Figure 1). Females also had significantly higher fasting plasma insulin, leptin, and adiponectin compared to males. BMI was similar between genders, although waist circumference was 4% or 3.0 cm higher in males, and abdominal skin fold thickness was 30% or 7.3 cm higher in females (Table 1). Interestingly, LDL-C, TC, TG and non-HDL-C were significantly higher in females using oral contraceptives by 11%, 10%, 22%, and 23%, respectively. In contrast, fasting plasma apoB48 concentrations did not differ between oral contraceptive and non-oral contraceptive use, therefore all females were combined in subsequent analyses.

Using established metabolic cluster groupings, 17.8% (males and females) of the cohort fell into the high-risk metabolic cluster. Fasting plasma apoB48 concentration was significantly correlated with the high-risk metabolic cluster (OR (95% CI): 1.09 (1.06-1.11)). Fasting plasma apoB48 concentration was elevated 21% (3.6 ug/ml) in males and females in the high-risk metabolic cluster, and this difference was more pronounced in males (31% or 6.8 ug/ml higher) compared to females (11% or 1.5 ug/ml higher) (Figure 2).

Fasting plasma LDL-C was mildly elevated in both males and females (8.5%) in the high-risk metabolic cluster (Figure 3). Interestingly, this difference was more pronounced in males (11%) compared to females (6%), however the magnitude of difference in the high-risk group (LDL-C 8.5%) was not as pronounced as that reported for plasma apoB48 (21%).

Regression analysis for apoB48 and cardiometabolic risk variables

Multivariable regression analysis, adjusted for gender, oral contraceptive use in females and BMI, showed fasting plasma apoB48 concentration was positively associated with TG, TC, insulin, HOMA-IR, leptin, abdominal and subscapular skinfolds and waist circumference (Table 2). Plasma apoB48 concentration was inversely associated with HDL-C, and adiponectin (Table 2). Interestingly, plasma apoB48 concentration showed a non-linear 'U-shaped' association with BMI, indicating a negative relationship between BMI and apoB48 at lower concentrations, as well as a positive association between these variables at higher concentrations of apoB48. Alcohol and smoking had no effect on outcomes and consequently were not included in the final model. Furthermore, there was no significant effect of gender on the association between fasting plasma apoB48 concentration and outcome variables.

DISCUSSION

Fasting plasma apoB48 concentration is elevated in conditions of high cardiometabolic risk in adolescents

Our data demonstrates that fasting plasma apoB48 concentration is higher in males compared to females in an adolescent population (17 years of age). Importantly, we also observe that fasting plasma apoB48 was elevated in individuals in the high-risk metabolic cluster, and significantly higher in high-risk males compared to females. Higher concentrations of plasma apoB48 (as a measure of intestinal remnant concentration) in adolescent males in the high-risk cluster may exacerbate their premature CVD risk profile, consistent with observations in adults (27). In males compared to females, the length of the whole intestine and its different parts is significantly greater (28) while chylomicron clearance is less efficient (29), both of which could contribute to elevated apoB48 concentrations in adolescent males. In adults, fasting plasma apoB48 is frequently higher in males compared to females, and is elevated in adults with the metabolic syndrome, diabetes and CVD (16). However, this gender difference was not observed in a younger pre-pubertal study (< age 14), suggesting that with increasing age in adolescents, fasting plasma apoB48 concentration may be a suitable predictor of gender differences for CVD risk (18, 19). Interestingly, mean fasting plasma apoB48 concentration in this study was approximately 6.0ug/mL higher compared to our previous reports in pre-pubertal children (18, 19). During adolescence there are distinct changes in body fat distribution and endocrine hormones, as well as decreased insulin sensitivity, and these changes have been reported to result in alterations in plasma lipid profile, notably decreased LDL-C and TC[3]. Our findings suggest that pubertal alterations in endocrine hormones (such as androgens), increased insulin sensitivity and/or body fat distribution may impair apoB48-remnant cholesterol metabolism. Factors that

may further contribute to increased fasting plasma apoB48-remnants, particularly in the high-risk metabolic cluster, would also include increased intestinal lipid absorption, chylomicron-apoB48 production and secretion, altered lipolysis and high-affinity or other clearance/uptake pathways of apoB48-remnants from the circulation (30-32).

While we did not measure total apoB in this study, the magnitude of change observed for apoB is often tightly associated with corresponding changes in LDL-C. Despite the fact that all LDL-C values would be considered with the normal range (<2.6mmoL), we did observe percentage increase for LDL-C between low and high risk groups (Figure 3). Percent increases for LDL-C were +12.3% and +5.8% for boys and girls respectively in low versus high groups. In comparison, percent increases for apoB48 between low and high groups were +43.0% and +11.9% for boys and girls respectively. The relative increase for apoB48 was more than 3-fold that for LDL-C in boys and 2-fold for that in girls. The challenge in the interpretation with comparisons such as these, is the understanding of the corresponding clinical relevance of changes in LDL-C (13-6%) versus apoB48 (43-12%), particularly in youth of this age.

Collectively, these factors may result in increased fasting plasma apoB-remnants and may represent increased early CVD risk in adolescents. In adults, elevated fasting plasma apoB48 has been shown to be positively associated with carotid intima-media thickness (cIMT) (12). Furthermore, young adults with the metabolic syndrome have significantly elevated cIMT compared to control (33). Although, CVD outcomes beyond the clustering were not measured in this study, we would speculate that elevated fasting plasma apoB48 concentrations in adolescents in the high-risk group may be associated with early and increased premature CVD risk (12, 33). Further studies are required to measure subclinical CVD, such as carotid intimal medial

thickness (cIMT) or endothelial function or ankle-brachial blood pressure, in order to determine the association with apoB48/remnant concentrations in this age group (8, 33-35).

The use of oral contraceptives in adolescent females did not influence fasting plasma apoB48 concentrations, despite the positive association of oral contraceptive use with LDL-C and TC. Other studies have shown similar elevations in these traditional lipid markers, such as plasma TG, in females taking oral contraceptives (36). To our knowledge, this is the first study to report no apparent effect of oral contraceptive use on fasting plasma apoB48 concentration.

Fasting apoB48 associates with features of the metabolic syndrome

Multivariable regression analysis demonstrated that in adolescent males and females there was a positive association between fasting plasma apoB48 and TG, TC (but not LDL-C), insulin, HOMA-IR, leptin, abdominal and subscapular skinfold thickness, and waist circumference. In contrast, there was a negative association between fasting plasma apoB48 and HDL-C and adiponectin. Given there is no gender effect on the relationship between apoB48 and other cardiometabolic variables, the findings suggest other factors may play a greater role, such as adiposity, in determining apoB48 metabolism (19, 37). Interestingly, the relationship between fasting plasma apoB48 and BMI was non-linear ("U-shaped"), suggesting high and low BMI may track with higher concentrations of this CVD risk biomarker. Consistent with this theme, the literature does suggest a U-shaped association between traditional CVD risk factors such as BMI, hypertension and diabetes with CVD mortality (38-40). However, because BMI is not reflective of total body fat or lean muscle composition *per se*, the relationship between apoB48 and BMI is likely to be more representative of markers of adiposity and metabolic risk in adolescence (19). Waist circumference has been shown to be a good estimate of central or

visceral adiposity compared to other anthropometric measures, particularly BMI (41). Visceral adipose tissue is strongly associated with the metabolic syndrome, insulin resistance, hypertension, and dyslipidaemia (42). In a French-Canadian cohort, we have reported that fasting plasma apoB48 concentrations are highly associated with central adiposity in pre-pubertal children (19). Similarly, fasting plasma apoB48 has been positively associated with visceral adiposity in obese adults (14). Consistent with these findings, our current study shows fasting plasma apoB48 concentration was positively associated with waist circumference, and abdominal and subscapular skin fold thickness, supporting the relationship between apoB48 and central adiposity in this adolescent population.

Fasting apoB48 is associated with impaired insulin sensitivity in adolescents

The associations between fasting plasma apoB48 concentration and features of the metabolic syndrome (waist circumference, TG, HDL-C, and insulin) suggest that apoB48 concentration is representative of an adverse metabolic profile. We have also shown that fasting plasma apoB48 is associated positively with insulin, HOMA-IR, and leptin, and inversely correlated with adiponectin, consistent with findings in adults (27, 43). These findings suggest that fasting plasma apoB48 is associated with impaired insulin sensitivity and adipokine markers in adolescents. The intestine is particularly responsive to changes in insulin sensitivity, and aberrant changes in insulin metabolism are often associated with postprandial or non-fasting hypertriglyceridemia and apoB48-remnant hyperlipoproteinemia in adults (30, 31). An increase in fasting plasma apoB48-remnants during insulin resistance has been attributed to the delayed clearance of apoB48-remnant particles from the circulation (44). However, the insulin resistance state is also known to modulate intestinal apoB48-remnant metabolism by increasing enterocyte *de novo* lipogenesis, including cholesterol ester and TG synthesis, as well as lipidation of

primordial chylomicron particles (30, 32). These aberrations promote conditions of overproduction of intestinal chylomicron-apoB48 and may explain the elevated fasting plasma apoB48-remnants we have observed in adolescents in the high-risk metabolic cluster group.

Strengths and Limitations of the Study

The study of an adolescent cohort aged 17 years provides a population representing a predominately post-pubertal stage of development, with established biochemical profiles, and subjects who are likely to have adopted a number of early adult lifestyle choices such as smoking, alcohol consumption, and oral contraceptive use in females. However, the Raine cohort consists predominantly of Caucasians and has shown the potential for retention bias of families that are more likely to be well-educated, health conscious, and having a relatively higher income (45). Consequently, our findings may limit the generalizability to adolescents of other ethnicities or of lower socio-economic status. Although not measured in this study, it would also be interesting to consider how endocrine metabolism, in particular androgens, play an important role in regulating apoB-lipoprotein and lipid metabolism in different phases of puberty and adolescence. This in turn may lead to a better understanding of gender specific lipid profiles and early CVD risk during adolescence, particularly in those with the metabolic syndrome. Future studies could investigate the longitudinal effects of elevated fasting plasma apoB48-remnant concentrations (and/or non-fasting remnant cholesterol) in youth. If indeed these novel values track with other adverse cardiometabolic outcomes and measures of cardiovascular health into early and late adulthood it could offer improvements to the pediatric lipid guidelines. Specific, experimentally-controlled postprandial lipid metabolism studies in youth are also lacking and there is currently no data to indicate if fasting plasma apoB48-remnant concentrations predict

non-fasting lipemia in these age groups. It is noteworthy that there are ongoing studies to establish reference ranges for apoB and apoB48 in children and adolescents (46).

In conclusion, the findings from this study have demonstrated that fasting plasma apoB48 concentrations in adolescents are associated with measures of adiposity and features of the metabolic syndrome. The data further supports previous findings in pre-pubertal children and suggests that fasting plasma apoB48 may be a potential biomarker of early premature CVD risk in adolescents. The measurement of fasting plasma apoB48 and apoB-remnant cholesterol in older youth, particularly those at high cardiometabolic risk, may support a recommendation to assess non-fasting lipid parameters and to further implement preventative strategies to reduce long term CVD risk this population. Importantly, the data supports the need for longitudinal prospective studies to delineate if increased apoB48/remnant concentrations during adolescence is a marker of a severe, inflammatory or premature onset of CVD during adulthood.

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Figure Legends

Figure 1. Fasting plasma lipid profile and apoB48-remnants in male and female adolescents from the RAINE cohort. Data are expressed as mean ± SEM; * P<0.001

Figure 2. Fasting plasma apoB48 concentration in high and low-risk metabolic cluster male and female adolescents from the RAINE cohort. Data are expressed as mean ± SEM; ** P<0.001 * P<0.05.

Figure 3. Fasting plasma LDL-C concentration in high and low-risk metabolic cluster male and female adolescents from the RAINE cohort. Data are expressed as mean ± SEM; ** P<0.001 * P<0.05.

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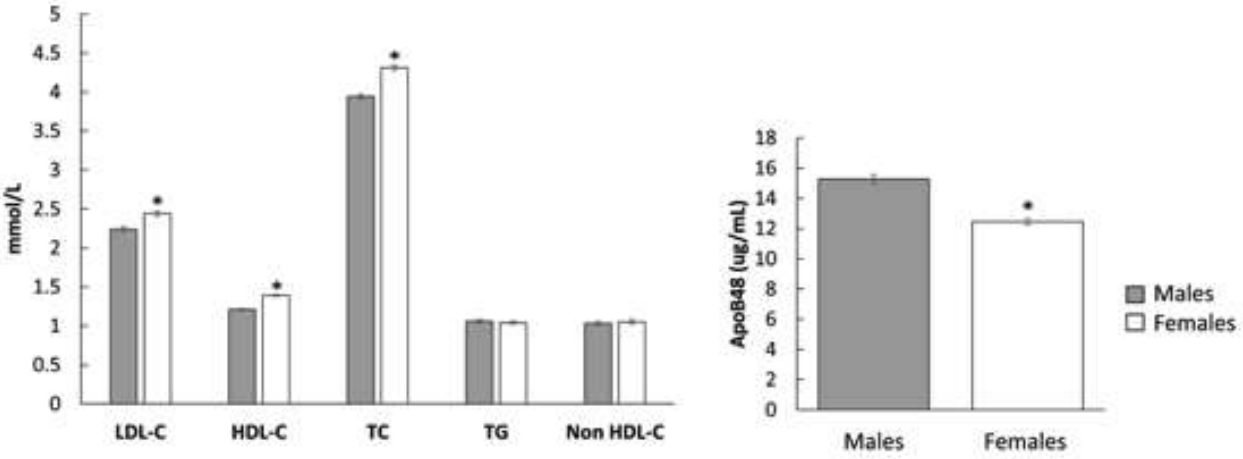
Figure 1


mmol/L
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4.5
4
3.5
3
2.5
2
1.5
1
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0
LDL-C
HDL-C
TC
TG
Non HDL-C
ApoB48 (ug/mL)
18
16
14
12
10
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6
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2
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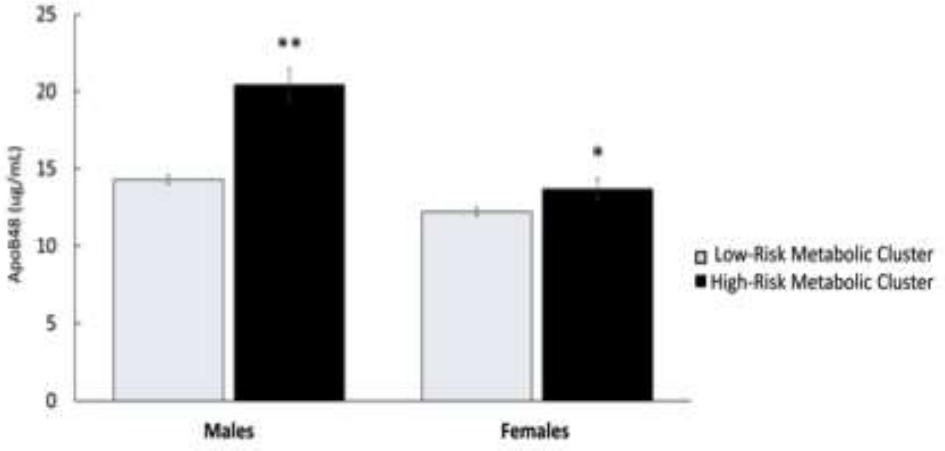
Males
Females
Males
Females

Figure 2


25
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15
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ApoB48 (ug/mL)
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Males
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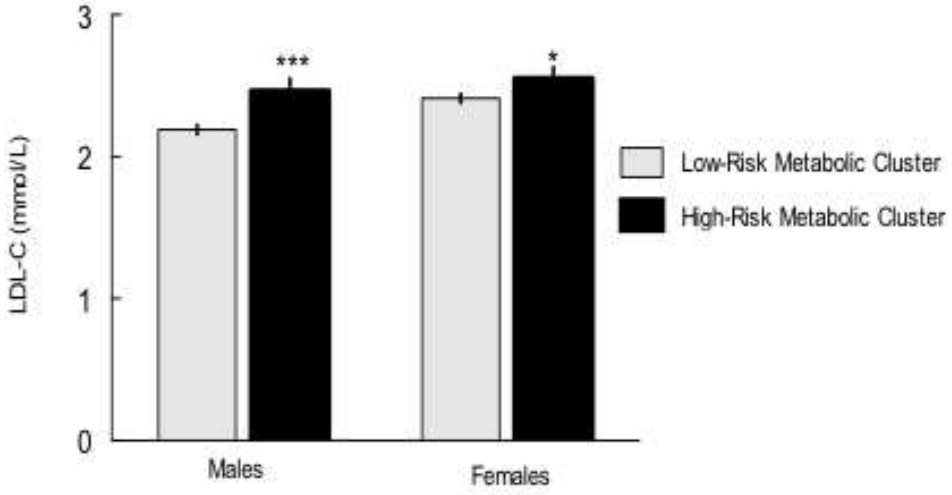
Low-Risk Metabolic Cluster
High-Risk Metabolic Cluster

Figure 3.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LDL-C (mmol/L)
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Males
Females
Low-Risk Metabolic Cluster
High-Risk Metabolic Cluster

Table 1 Lipid and metabolic markers of cardiometabolic risk in adolescents aged 17

	Total (n=1045)	Males (n=545)	Females (n=500)	P value (Males/Females)
Height (m)	1.72 (1.72-1.73)	1.78 (1.78-1.79)	1.66 (1.66-1.67)	<0.001
Weight (kg)	68.51 (67.62-69.40)	72.64 (71.40-73.89)	64.00 (62.86-65.15)	<0.001
BMI (kg/m^2)	22.99 (22.73-23.26)	22.82 (22.46-23.19)	23.18 (22.79-23.57)	0.187
Overweight (%)	13.5	13.0	14.2	
Obese (%)	8.2	7.7	8.8	
Waist Circumference (cm)	79.29 (78.59-79.98)	80.72 (79.78-81.66)	77.69 (76.68-78.71)	<0.001
Abdominal Skin Fold (mm)***	20.44 (19.81-21.06)	17.04 (16.19-17.89)	24.37 (23.60-25.13)	<0.001
Subscapular Skin Fold (mm)**	12.62 (12.28-12.97)	11.01 (10.62-11.41)	14.75 (14.22-15.30)	<0.001
Suprailiac Skin Fold (mm)**	13.20 (12.73-13.69)	10.76 (10.23-11.31)	16.72 (16.02-17.44)	<0.001
Triceps Skin Fold (mm)**	13.28 (12.87-13.71)	9.93 (9.55-10.32)	18.44 (17.90-19.00)	<0.001
High Risk Metabolic Cluster (%)	17.8	16.3	19.4	
ApoB48 (ug/mL)	13.93 (13.54-14.41)	15.28 (14.71-15.86)	12.45 (11.97-12.92)	<0.001
High Risk Metabolic Cluster	16.90 (15.62-18.18)	20.43 (18.39-22.45)	13.66 (12.33-14.98)	<0.001
Low Risk Metabolic Cluster	13.31 (12.94-13.68)	14.28 (13.76-14.81)	12.20 (11.70-12.71)	<0.001
LDL-C (mmol/L)	2.34 (2.30-2.38)	2.24 (2.19-2.30)	2.44 (2.39-2.50)	<0.001
High Risk Metabolic Cluster	2.51 (2.40-2.62)	2.46 (2.29-2.63)	2.55 (2.41-2.70)	<0.001
Low Risk Metabolic Cluster	2.29 (2.25-2.33)	2.19 (2.13-2.25)	2.41 (2.35-2.47)	<0.001
HDL-C (mmol/L)	1.30 (1.28-1.31)	1.21 (1.19-1.23)	1.39 (1.37-1.42)	<0.001
TC (mmol/L)	4.12 (4.07-4.16)	3.94 (3.88-4.00)	4.31 (4.25-4.38)	<0.001
High Risk Metabolic Cluster	4.41 (04.29-4.53)	4.37 (4.21-4.53)	4.44 (4.27-4.62)	<0.001
Low Risk Metabolic Cluster	4.04 (4.00-4.09)	3.84 (3.78-3.91)	4.27 (4.20-4.34)	<0.001
TG (mmol/L)	1.05 (1.02-1.08)	1.06 (1.01-1.10)	1.04 (1.00-1.09)	0.684
Non-HDL-C (mmol/L)	1.04 (1.00-1.09)	1.03 (0.97-1.09)	1.05 (0.99-1.12)	0.691
Systolic Blood Pressure (mmHg)	113.6 (112.9-114.2)	118.0 (117.2-118.8)	108.7 (107.9-109.5)	<0.001
Diastolic Blood Pressure (mmHg)	58.72 (58.33-59.11)	58.13 (57.58-58.68)	59.36 (58.81-59.92)	<0.001
Glucose (mmol/L)	4.75 (4.72-4.78)	4.85 (4.80-4.91)	4.64 (4.60-4.67)	<0.001
Insulin (mU/L)**	7.24 (6.94-7.55)	6.82 (6.42-7.22)	7.73 (7.27-8.21)	0.003
HOMA-IR*	1.64 (1.59-1.70)	1.64 (1.56-1.73)	1.65 (1.58-1.73)	0.144
Leptin (ng/mL)**	7.82 (7.11-8.60)	2.66 (2.35-3.02)	24.20 (22.70-25.81)	<0.001
Total Adiponectin (ug/mL)	9.66 (9.31-10.01)	8.19 (7.78-8.61)	11.25 (10.71-11.79)	<0.001
hsCRP (mg/L)**	0.57 (0.52-0.63)	0.43 (0.38-0.49)	0.78 (0.69-0.89)	<0.001

Values expressed as mean (95% CI).

* Geometric mean is presented for log transformed normal data

** Geometric mean from Tobit analysis

*** Tobit analysis was applied to censored variables

Table 2 Regression analysis between apoB48 and outcome variables

Multivariable analysis is adjusted for BMI, gender, correlates amongst siblings, and females using oral contraceptives (BMI, skinfolds, and waist circumference are not adjusted for BMI)

Variable	Coef. ApoB48 (ug/mL) (95% CI)	R^2	P
Anthropometry			
BMI (kg/m^2)	-0.803$^{\#}$/0.214$^{\wedge}$ (-1.354 – (-0.251))/(0.0723 – 0.3559)	0.014	0.004$^{\#}$/0.003$^{\wedge}$
Waist Circumference (cm)	0.123 (0.01062 – 0.2351)	0.023	0.03
Log(Subscapular Skin Fold) (mm)	0.005 (0.0005-0.0096)	0.106	0.03
Abdominal Skin Fold (mm)	0.126 (0.0197 – 0.2317)	0.021	0.02
Plasma Lipids			
LDL-C (mmol/L)	-0.003 (-0.0102 – 0.0050)	0.058	0.5
TG (mmol/L)	0.045 (0.0375 - 0.0528)	0.363	<0.001
TC (mmol/L)	0.014 (0.0060 - 0.0214)	0.116	<0.001
HDL-C (mmol/L)	-0.005 (-0.0073 – (-0.0021))	0.169	<0.001
Metabolic Markers			
Log(Insulin) (nmol/L)	0.023 (0.0164 – 0.0290)	0.100	<0.001
Log(HOMA-IR) (U)	0.020 (0.0132 – 0.0260)	0.186	<0.001
Log(CRP)(mg/L)	-0.026 (-0.039 – (-0.0126))	0.087	<0.001
Log(Leptin) (ng/mL)	0.009 (0.0091 – 0.0354)	0.391	0.010
Adiponectin (ug/mL)	-0.071 (-0.113 – (-0.0281))	0.114	0.001

Coef, 95%, R^2 and P value of linear apoB48

^ Coef, 95% CI, R^2 and P value of apoB48*log(apoB48)

Appendix ‘D’

Cardiochek TG AUC Measurement

Appendix D

TG AUC

	CardioChek	**Laboratory**	**%Difference**
Participant 1	5.54	5.36	3.2
Participant 2	7.57	8.33	9.1

Appendix D. TG area under the curve (AUC) following a high fat meal challenge in two female participants (P1: 30 years, BMI 21.5k kg/m^2; P2: 22 years, BMI 22 kg/m^2) measured by laboratory enzymatic colormetric assay (Wako Chemicals USA, INC) and by Cardiochek PA (Bieleny and Chappell (2019); NUTR450 honours project, unpublished).