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The SORT1 Protective Allele is Associated with Attenuated Postprandial Lipaemia in Young Adults

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The *SORT1* Protective Allele is Associated with Attenuated Postprandial Lipaemia in Young Adults

by

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Abstract

Elevated levels of lipids and lipoproteins have strong genetic determinants, and are key risk factors for cardiovascular disease. Study aims were to determine if young adults, when stratified by genotype at the rs646776 variant of the 1p13 locus, displayed differential postprandial responses to an oral fat tolerance test. Participants received a high fat mixed meal following an overnight fast and a fat-exclusion meal at 8 h postprandially. Blood samples were obtained at t=0, 2, 4, 6, 8 and 24 h for lipoprotein analyses via nuclear magnetic resonance profiling. Carriers of the minor, protective allele (TC/CC) displayed lower concentrations of fasting and postprandial very-low density lipoproteins and chylomicrons, in addition to triglyceride content, compared to major, risk allele homozygotes (TT). We report a novel association between the *SORT1* 1p13 locus and postprandial lipaemia. These results provide evidence of decreased exposure to atherogenic particles in carriers of the minor *SORT1* allele, suggesting relative protection against cardiovascular disease compared to TT homozygotes.

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To my sister

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
AAV	Adeno-associated virus
AIMM Young	Assessing Inherited Markers of Metabolic Syndrome in the Young
Apo	Apolipoprotein
CE	Cholesterol ester
CETP	Cholesterol ester transfer protein
CHD	Coronary heart disease
CM	Chylomicron
CVD	Cardiovascular disease
DNA	Deoxyribose nucleic acid
EDTA	Ethyl diamine tetra-acetic acid
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated protein degradation
FFA	Free fatty acid
GWAS	Genome wide association study
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein cholesterol
HMG-CoA	3-hydroxy-3-methyl-glutaryl coenzyme A reductase
HL	Hepatic lipase
iAUC	Incremental area under the curve
IDL	Intermediate-density lipoprotein
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein cholesterol
LDL-R	Low-density lipoprotein receptor
LPL	Lipoprotein lipase
LRP	Low-density lipoprotein receptor related protein
MAF	Minor allele frequency
MI	Myocardial infarction
mTORC1	Serine/threonine kinase mammalian target rapamycin
NCEP ATP III	National Cholesterol Education Program Adult Treatment Panel III
NMR	Nuclear magnetic resonance
OFTT	Oral fat tolerance test
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilsin/kexin type 9
SNP	Single nucleotide polymorphism
tAUC	Total area under the curve
TG	Triglyceride
VLDL	Very low-density lipoprotein
VLDL-C	Very low-density lipoprotein cholesterol
VLDL-TG	Very low-density lipoprotein triglyceride

Epigraph

"Live as if you were to die tomorrow. *Learn* as if you were to live *forever*."

- Mahatma Gandhi

Chapter One: Introduction

1.1 Background

Cardiovascular diseases (CVD), encompassing diseases of the heart, vasculature and brain, are the primary cause of death worldwide, accounting for 17.3 million deaths in 2008 [1]. Of these deaths, over 13.5 million are attributed specifically to atherosclerotic diseases, such as heart attack and stroke [1]. In Canada, CVD remains a serious health concern, contributing to one-third of deaths nationwide [2, 3]. New advances in prevention and treatment have abated the mortality rate; however as mortality rates subside, the number of Canadians living with chronic CVD rises. These individuals are more likely to experience declines in health-related quality of life, exhibit limitations in functional abilities, and impaired activity participation [4, 5]. As such, CVD is one of the greatest economic burdens on the Canadian healthcare system, with costs surmounting $22 billion per year, or 50% of annual expenditure [6]. With an aging population and increasing rates of obesity, there is fear that the healthcare system may be overwhelmed [6]. To complicate matters, cardiovascular events frequently occur in individuals who lack a high level of risk based on traditional single- or multi-variable risk scores, making it challenging to accurately predict risk [2, 3]. It is critical to investigate novel risk factors and develop cost effective identification and primary prevention strategies to alleviate the burden facing the Canadian healthcare system.

Atherosclerosis, commonly referred to as “hardening of the arteries,” is recognized as the primary pathological precursor to CVD [1, 7]. Extensive research has been dedicated towards characterizing risk factors for atherosclerosis to predict CVD risk. Many risk factors with a genetic basis, including elevated low-density lipoprotein (LDL), elevated very low-density

lipoprotein (VLDL) and decreased high-density lipoprotein (HDL) have been strongly associated with CVD risk through landmark studies [8-11].

The link between fasting lipids and lipoproteins has been long-established; however, lipid profiles in the postprandial state have garnered attention only since the late 20th century. Based on a western-style eating pattern, humans spend the majority of time in the postprandial state, challenging the merits of using fasting values to assess CVD risk [12, 13]. Postprandial lipaemia is characterized by an influx of hepatically- and intestinally-derived lipoproteins in the blood [14, 15]. These particles are atherogenic in their own right, highlighting prolonged exposure to postprandial lipaemia as an integral factor in atherogenesis [16, 17]. The extent and duration of postprandial lipaemia is highly variable, depending on genetic, environmental, physiological and pathological factors [15]. In particular, there is mounting evidence of genetic regulation of postprandial lipaemia. This has been demonstrated through familial and single nucleotide polymorphism (SNP)-based studies; however, much of the genetic variation remains unexplained [18]. As such, it is imperative to investigate untested genetic variants to advance knowledge and develop cost effective identification and primary prevention strategies to halt the progression of CVD. Furthermore, genetic risk stratification in youth based upon deleterious variants offers the potential for interventions prior to the clinical onset of symptoms [19, 20].

The 1p13 locus, encompassing the *SORT1* gene, has received considerable attention due to its high minor allele frequency (MAF) [21, 22], and large effect size [21], especially in younger populations [23, 24]. Carriers of the minor, protective allele exhibit 9-13% decreased risk of CVD and 40% reduced risk of myocardial infarction (MI) compared to major, risk allele homozygotes [22, 25]. Despite these robust associations, genotype at this locus accounts for only 1-5% of the variation in fasting LDL cholesterol (LDL-C), a strong predictor of CVD [21],

suggesting an element of residual CVD risk attributed to alternative factors [23, 24]. This may, in part, be due to the fact that associations between *SORT1* and LDL-C have been investigated almost solely in the fasted state.

1.2 Significance

Despite advances in treatment and prevention, CVD remains a major contributor to mortality and morbidity in Canada and worldwide. Patients require extensive medical resources, creating a considerable, unsustainable burden on the healthcare system. While risk factors have been extensively documented, cardiovascular events frequently occur in individuals who lack a high level of risk based upon traditional single- or multi-variable risk scores [3]. This highlights the need to identify novel techniques for risk stratification and shift efforts towards prevention rather than the much more difficult task of treating and reversing CVD.

Due to the long latent period in the progression from atherosclerosis to clinical manifestation of symptoms, CVD is typically considered a disease of older populations. However, it is increasingly apparent that development begins early in life. Evidence of fatty streaks, the first marker of atherosclerosis, have been reported as early as the first decade of life [26]. In young Canadians, risk factors that are strong predictors of cardiovascular events later in life are increasingly prevalent [5, 26]. This is apparent in that 90% of Canadians >20 years of age exhibit at least one risk factor for CVD, and one third exhibit more than three [6]. Fortunately, interventions targeting younger individuals are more effective than those in older adults, as early stage atherosclerotic lesions are easier to reverse. This highlights the importance of early interventions, prior to clinical manifestation of disease, to alleviate the burden of CVD.

The present study investigated the effect of genotype at the 1p13 locus on postprandial lipaemia in young adults. As genotype is immutable, it represents a superior marker of lifetime

exposure to an intermediate trait [6]. The *SORT1* rs646776 polymorphism accounts for 1-5% of the variation in LDL-C based on genome wide association studies (GWAS) [21, 23], and 3.0% in a cohort of University of Calgary students [24]. Based on this relatively large effect size, it is a valuable target for risk stratification and prevention strategies. By investigating the effect of *SORT1* on postprandial lipaemia, we sought to further elucidate the phenotype associated with this polymorphism, thus providing valuable information for individualized, genetics-based disease prevention.

1.3 Hypothesis

We hypothesized that young adults carrying at least one copy of the minor, protective allele at the rs646776 variant of the 1p13 locus will have attenuated postprandial lipaemia in response to an oral fat tolerance test (OFTT). While this relationship has yet to be investigated, several GWAS have found carriers of the minor allele to have significantly lower fasting concentrations of LDL-C [21, 23, 27-46], LDL particles [29, 47] and very-low density lipoproteins (VLDL) [29, 47]. Given the interrelationships between postprandial lipoprotein metabolism and fasting phenotype, it was expected that these differences would extend into the postprandial state.

1.4 Presentation

The composition of this thesis is as follows: chapter one provides a brief examination of CVD, rationale, significance of research findings, and the hypothesis. Chapter two is a review of relevant literature considered during the conception of this project, and evidence supporting genetic regulation of postprandial lipaemia. Chapter three contains a working-manuscript that has been prepared for submission. Chapter four is an in depth discussion of the primary findings, strengths and limitations, in addition to suggestions for future directions.

Chapter Two: Literature Review

2.1 Atherosclerotic Progression Towards Cardiovascular Disease

2.1.1 Defining Atherosclerosis

Atherosclerosis, commonly referred to as "hardening of the arteries", is the primary pathological precursor to CVD [1, 7]. Early observations were reported in the 19th century when Dr. Julius Vogel described accumulations of microscopic fat vesicles in large arteries that frequented those experiencing a "death-struggle" [48]. Atherosclerosis itself was first termed and associated with CVD early in the 20th century [26]. It has since be extensively studied, with a focus on characterizing its risk factors, mechanism of pathogenesis and implications on health.

Atherosclerosis is a complex, progressive disease primarily affecting arterial vasculature [7]. The arterial wall is composed of three morphologically distinct layers separated from the lumen by the endothelium [49] (Figure 2.1).

Figure 2.1 Basic morphology of the arterial wall

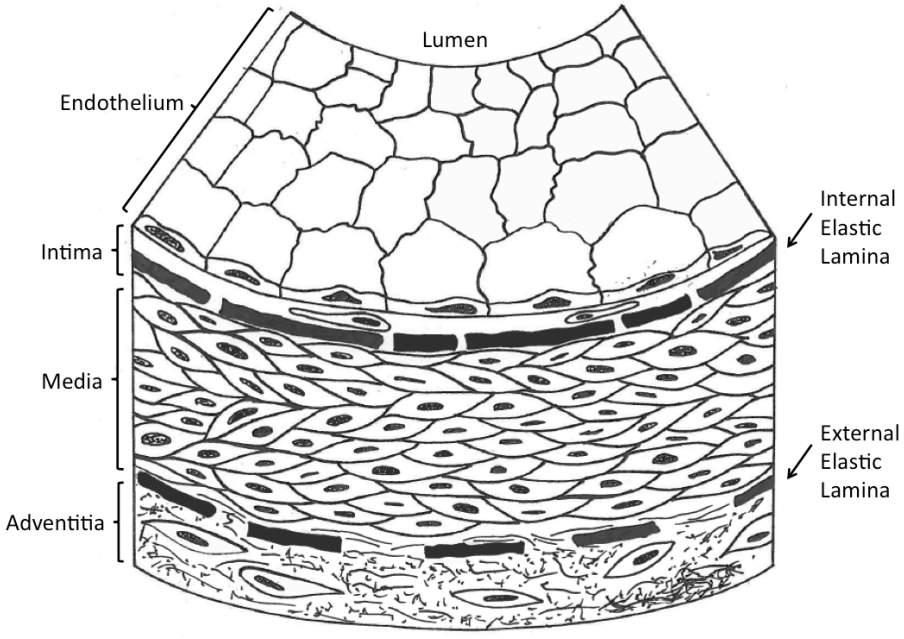

Figure 2.1 (adapted from [49]) The arterial wall is comprised of three distinct layers.

The endothelium is a single layer of cells joined by tight junctional complexes that functions as a selectively permeable barrier separating the blood and the tissues [7]. It actively contributes to arterial physiology through the release of effector molecules involved in regulation of thrombosis, inflammation, vascular tone and remodelling [7]. The innermost layer of the arterial walls, the intima, consists of a matrix of extracellular connective tissue peripherally bound by the internal elastic lamina [49]. Due to its proximity to the lumen, the first changes following exposure to a high-fat, high-cholesterol diet are evident in intima; although secondary changes may be induced in the subsequent layer, the media [7, 49]. The media is formed from a matrix of diagonally arranged smooth muscle cells connected by junctional complexes [49]. The media is separated from the outermost layer, the adventitia, by a layer of elastic tissues, the external elastic lamina [49]. The adventitia is comprised predominantly of fibroblasts mixed with smooth muscle cells arranged between bundles of collagen and surrounded by proteoglycans [49]. Combined, these layers form strong, dynamic vessels integral to normal circulation.

2.1.2 Aetiology of Atherosclerosis

Initiation of atherosclerosis occurs when cholesterol-rich macrophages accumulate in the subendothelial layer of the arteries forming fatty streaks [7]. This process is exacerbated when plasma LDL is elevated, as particles are able to passively diffuse between endothelial cell junctions [7]. Once LDL has penetrated the endothelium, it undergoes a series of modifications including oxidation, lipolysis, proteolysis and aggregation, which contribute to inflammation and the development of foam cells, the hallmark of atherosclerosis [7]. Oxidation of LDL is a pivotal step in the inflammatory process, as oxidized LDL stimulates adhesion molecule proteins, which attract and bind monocytes to the endothelium [7]. Additionally, oxidized LDL inhibits nitric oxide release, thereby limiting intrinsically-stimulated vasodilation [7].

While fatty streaks are not clinically significant, they are precursors to advanced lesions resulting from further accumulation of lipids, necrotic debris and smooth muscle cells [7]. Developing lesions are continually exposed to the sheer stress of blood flow, which may induce calcification, damage to the luminal surface, and haemorrhage of smaller blood vessels [7]. Advanced atherosclerosis is marked by fibrous plaques that extend into the lumen [7, 49]. Fibrous plaques are composed of intimal smooth muscle cells enriched with cholesterol and cholesterol ester (CE), surrounded by lipid, collagen, elastic fibres and proteoglycans [7, 49]. Fibrous plaques may be damaged, inducing haemorrhage, calcification, cell necrosis and thrombosis, forming complicated lesions [49]. Complicated lesions usually result in clinical manifestation of symptoms due to complete occlusion, ultimately ending in MI or stroke [7].

2.1.3 Evidence of Atherosclerosis in Young Populations

Due to the long latent period in the pathogenesis of atherosclerosis, 30-40 years, it is typically considered a disease of older adults [50]. However, strong evidence supports initiation in younger populations [10, 11, 26, 51, 52]. Lipid accumulation in the coronary arteries is first evident in the form of fatty streaks following the first and second decades of life; accumulation of smooth muscle and connective tissue, which marks the development of fibrous plaques, may be observed following the second and third decades of life [7, 26].

Early development of atherosclerosis first gained attention in 1953 following a landmark paper describing the prevalence of gross lesions in the coronary arteries of soldiers killed during the Korean War [51]. Of the 300 young men (mean age=22.1 y) autopsied, a striking 77.3% presented lesions [51]. A systematic investigation of this phenomenon, the Pathobiological Determinants of Atherosclerosis in Youth study, reported that 60% of 2876 autopsied young adults aged 15-35 y exhibited lesions [53]. Fatty and fibrous plaques were most common,

whereas complicated and calcified lesions were rare, however the overall prevalence increased with age [53]. Interestingly, the extent of raised lesions separating high- and low-risk groups was exaggerated during the early twenties, marking this as a critical period in atherogenesis and risk stratification [54]. These findings emphasize the importance of targeting younger populations for interventions to prevent and halt the progression of atherosclerotic lesions.

2.1.4 Risk Factors for Atherosclerosis

In conjunction with characterizing atherogenesis, extensive research investigated risk factors for atherosclerosis and subsequent CVD. The Framingham Heart Study, along with additional large-scale epidemiological studies, provided invaluable information regarding risk factors for CVD. Traditional risk factors include: smoking status [9, 11], age [11], sex [9], blood pressure [9], obesity [9], and perhaps most notably, lipids and lipoproteins [9]. Elevated LDL, elevated VLDL, low HDL, elevated lipoprotein(a), hypertension, family history and metabolic syndrome are considered heritable; whereas high fat diet, smoking, low antioxidant levels and sedentary behaviour are environmental risk factors [7, 11]. These risk factors form the basis of cardiovascular risk assessment employed by primary healthcare providers to target patients for risk modification through therapeutic and lifestyle interventions [55]. However, cardiovascular events frequently occur in individuals who lack a high level of risk based upon traditional single- and multivariable- risk scores, highlighting the need for novel identification strategies [2, 3]. Additionally, with more advanced diagnostic techniques, in depth analyses of lipids and lipoproteins are becoming increasingly relevant.

2.1.5 Heterogeneity of Lipids Lipoproteins

Lipids are large hydrophobic organic molecules essential to physiological function. Three main forms are present in the human body, including: (1) triglycerides (TG), (2) phospholipids,

and (3) sterols [56]. TG, the primary source of dietary lipids, are composed of a glycerol molecule ester-bonded to three fatty acid chains [56]. Phospholipids are similar in structure to TG, however the third fatty acid chain is replaced by a phosphorous group [56]. Sterols, including cholesterol, are formed from four hydrocarbon rings that give them unique physiological properties [56]. Cholesterol forms the basis for endogenous steroid hormone synthesis, maintains membrane integrity and is necessary for the production of bile salts and oxysterols [57]. The majority of cholesterol is endogenously synthesized from acetyl-coA precursors through a series of enzymatically controlled steps; the remaining 20% is derived from dietary sources such as meats, poultry, fish, eggs and dairy products [19, 57]. Within the body, cholesterol is stored in three distinct pools. The rapidly miscible pool includes cholesterol contained within the plasma, blood cells, liver and intestines, and remains in rapid equilibrium with the plasma [57]. Cholesterol stored within the viscera and peripheral tissues represents the intermediate rate pool, whereas cholesterol within adipose, connective tissue, skeletal muscles and arterial walls comprises the pool with the slowest turnover [57].

Due to its hydrophobic nature, circulation of lipids in the aqueous blood is mediated by large, spherical macromolecules called lipoproteins [19]. Lipoproteins are composed of a hydrophobic core containing phospholipids, fat-soluble antioxidants and vitamins, and cholesterol esters (CE), surrounded by a hydrophilic membrane formed from phospholipids, free cholesterol molecules and apolipoproteins (apo) [19, 58]. Apolipoproteins are the primary structural component of lipoproteins. Peripheral apolipoproteins adhere to the membrane and may be exchanged or transferred between particles; whereas integral apolipoproteins embed within the membrane and function as ligands with stimulatory and inhibitory abilities [59].

Blood lipoproteins are heterogeneous based upon their physiochemical properties, such as buoyant size, charge, lipid content and the presence or absence of apolipoproteins (Table 2.1) [59]. As such, lipoproteins are stratified into four discrete categories, from largest to smallest, VLDL, IDL, LDL and HDL; however each category is composed of a continuum of subclasses [59]. Up to seven distinct subclasses of LDL particles have been identified of differing size, density, surface lipid content and apo B-100 conformation [59]. Chylomicrons (CM), which are similar in structure to VLDL, are the primary transporters of dietary TG and cholesterol [58]. CM, CM-remnants, VLDL and VLDL-remnants, are considered TG-rich lipoproteins as they are the primary carriers of plasma TG [19, 60]; whereas smaller particles, LDL and HDL are the major transporters of CE [19, 60]. Based on these factors, lipoprotein composition is highly variable, influencing their function and role in disease pathogenesis [58, 61].

Table 2.1 Summary of important lipoprotein and CM characteristics

	Size (nm)	Density ($g \bullet m^{-3}$)	Composition (% mass)				Apolipoproteins	Origin	Function
			Protein	Chol	TG	PL			
Chylomicrons	>60	<1.006	1	4	90	5	A, B-48, C, E	Intestinal	Transport of dietary TG and CE
VLDL	>27	<1.006	8	25	55	12	B-100, C, E, (a)	Hepatic	Delivery of endogenous TG and CE to the peripheral tissues
IDL	23-27	1.006-1.019	10	45	30	15	B-100, C, E, (a)	Hepatic (via VLDL)	Delivery of endogenous TG and CE to the peripheral tissues
LDL	18-23	1.019-1.063	20	55	5	20	B-100, (a)	Hepatic (via VLDL)	Delivery of endogenous cholesterol to the peripheral tissues
HDL	7.3-13	1.063-1.210	50	20	5	25	A, C, E	Hepatic and intestinal	Reverse cholesterol transport

Chol, cholesterol; TG, triglyceride; PL, phospholipids; CE, cholesterol ester [58, 62, 63].

2.1.6 Lipid and Lipoprotein Metabolism

2.1.6.1 Exogenous Lipid Metabolism

Immediately following food ingestion, neural and hormonal signals trigger transition from the fasted to fed state, ensuring efficient absorption of nutrients [61, 64]. The exogenous cholesterol pathway has a large capacity to respond to lipid fluxes through lipid enrichment of CMs, the primary carriers of dietary lipids [64]. Within the intestine, pancreatic lipase hydrolyzes dietary TG, phospholipids, cholesterol and fat-soluble vitamins into free fatty acids (FFA) and 2-monoacylglycerols, which cross the apical membrane into the enterocyte [61]. Dietary fat absorbed into the enterocyte may be (1) stored within the enterocyte as a lipid droplet, (2) incorporated into CE and phospholipid stores, (3) oxidized or (4) reesterified into diacyl glycerol and TG for repackaging as a CM, which are released into circulation [65].

The initiating step of CM assembly is transcription of apo B-48, which is cotranslationally translocated from the rough endoplasmic reticulum (ER) to the ER lumen [64]. Apo B-48, a truncated form of apo B-100, remains embedded within the CM and CM-remnants throughout circulation [61, 64]. In the ER lumen, diacyl glycerol and TG resynthesized from monoacylglycerols and FFA are incorporated into the core of a nascent CM particle by microsomal TG transfer protein, forming primordial apo B-containing particles [61]. These lipid-enriched particles are transferred to the Golgi complex, where mature CMs are packaged in large transport vesicles that are secreted into the lymphatic system via the basolateral membrane [61, 64].

In circulation, CMs acquire peripheral apo C-I, C-II, C-III and E [61]. Apo C-II interacts with lipoprotein lipase (LPL) initiating TG hydrolysis, such that fatty acids are released to muscles for energy and adipocytes for storage [61]. CMs and CM-remnants are rapidly removed

from circulation, as CM-derived fatty acids are taken up to a greater extent than VLDL-derived fatty acids in the early (0-2 h), mid (2-4 h) and late (4-6 h) postprandial periods in skeletal muscle and adipose tissue [65, 66]. TG hydrolysis occurs concurrently with the transfer of phospholipids, free cholesterol, apo C-II and apo C-III to HDL, ultimately yielding smaller CM-remnant particles enriched with apo E and CE [61]. Apo E, a high-affinity binding ligand, facilitates hepatic clearance via the LDL-receptor (LDL-R) and the LDL-R-related proteins (LRP) [61].

2.1.6.2 Endogenous Lipid Metabolism

Whereas CMs are the primary transporter of dietary lipids in the exogenous pathway, VLDLs are the hallmark transporters of endogenously produced lipids [61]. Apo B-100, the primary apolipoprotein of VLDL particles, is synthesized at the rough ER [67]. When adequate fatty acids are available, apo B-100 is lipidated as it translocates across the ER membrane into the lumen, forming a primordial apo B-100 particle [61]. In a second, discrete step, there is a bulk transfer of core lipids and fatty acids to the particle as it is transferred to the Golgi complex, where it undergoes maturation [61]. These fatty acids are derived primarily from (1) lipolysis of adipocytes, (2) lipoprotein remnants and (3) the intestine by way of the portal vein; however hepatic mobilized storage pools and *de novo* synthesis are also contributing factors [61]. Under fasting conditions, adipocyte lipolysis is upregulated, such that its relative contribution to circulating lipoprotein content is increased [64]. Mature VLDL particles are then secreted by hepatocytes via the hepatic vein into circulation [61, 64].

In circulation, VLDL is hydrolyzed by LPL, yielding progressively smaller, denser VLDL-remnants [61]. In conjunction with hydrolysis, the particles are enriched with HDL-derived apo E, forming IDL particles [61]. Some IDL particles undergo further hydrolysis and

removal of apo E producing LDL particles, which feature a single apo B-100 molecule [61]. Both apo E and apo B-100 are high-affinity ligands for hepatic LDL-R, which facilitate the particles' clearance from circulation [61]. LDL particles bound to receptors are endocytosed in clathrin-coated pits by hepatocytes prior to undergoing lysosomal degradation [19, 61]. Degradation of LDL particles releases amino acids and cholesterol into the cell, which may be either used for cellular processes, recycled and repackaged as VLDL, or degraded [19, 61]. Free cholesterol and amino acids inhibit 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMG-CoA) activity, thus suppressing the rate-limiting step of endogenous cholesterol synthesis [61]. VLDL and IDL particles experience a similar degradation process, although it is complicated due to the presence and recycling of the apo E [61].

Combined with the HDL-governed reverse cholesterol transport pathway, the endogenous and exogenous pathways interact to regulate lipid metabolism in the body. When these processes are disrupted due to genetic abnormalities or environmental factors, clinical manifestation of disease frequently results. As such, further elucidation of these pathways is essential to understanding disease pathogenesis.

Figure 2.2 Summary of endogenous and exogenous cholesterol metabolism

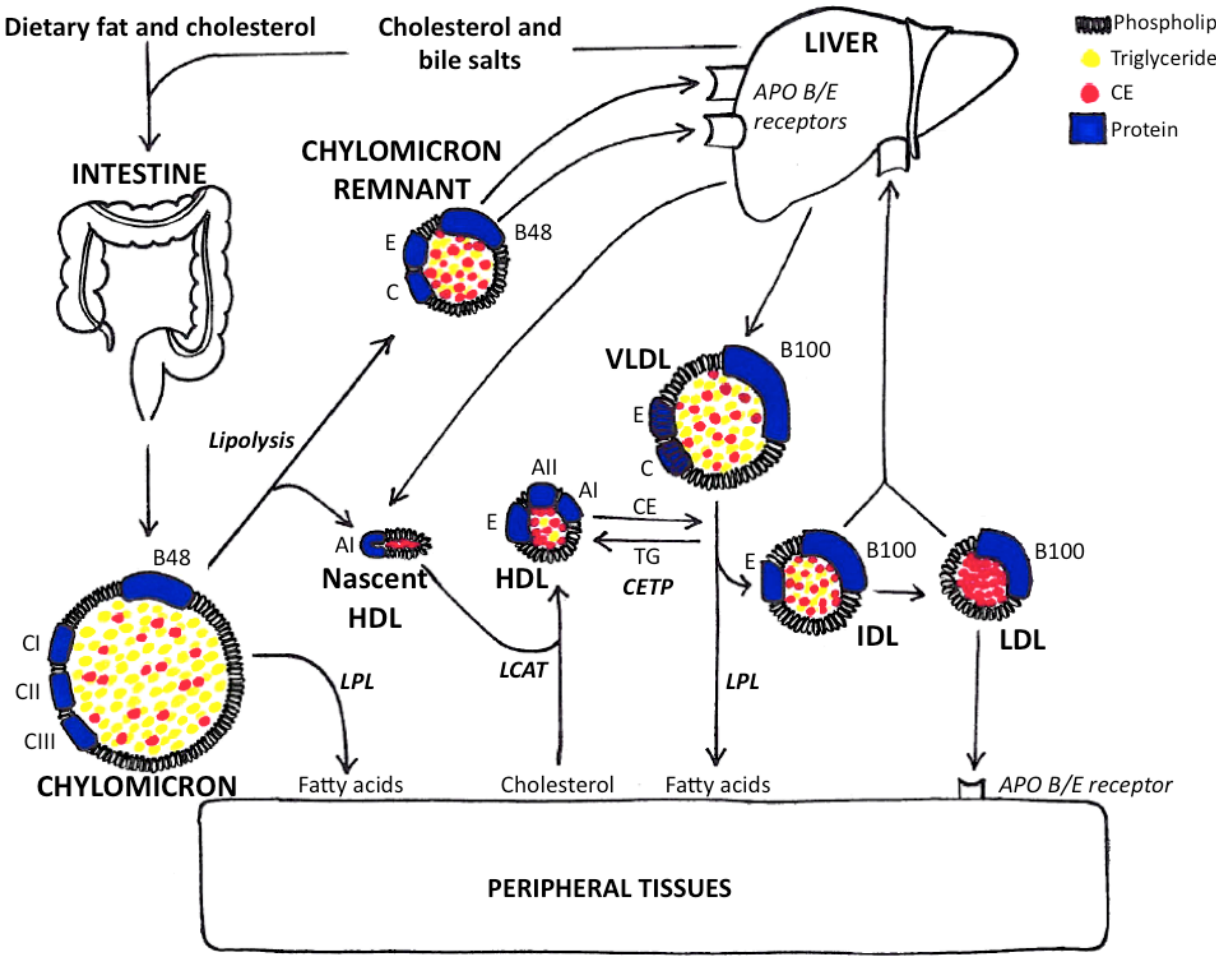

Figure 2.2 (adapted from [68]). Within the small intestine, dietary TG and cholesterol are packaged into CMs that enter circulation via the lymphatic system. CM are hydrolyzed by LPL, yielding CM-remnants, which are removed via hepatic receptor-mediated reuptake. TG derived from remnant lipoproteins, reesterification of circulation FFA, mobilization of lipid pools and *de novo* synthesis are packaged with apo B-100 forming VLDL particles in the liver. VLDL particles released into circulation undergo a delipidation cascade leading to progressively smaller IDL and LDL particles, which are removed via hepatic receptor-mediated clearance.

2.1.7 Lipids and Lipoproteins as Risk Factors for Cardiovascular Disease

Evidence from large epidemiological studies, such as the Framingham Heart Study provided substantial support for associations between lipid profile and CVD risk [9]. Early reports found a stepwise relationship between increasing plasma total cholesterol and risk for coronary heart disease (CHD) [9]. This relationship was replicated in numerous studies, including the Pathobiological Determinants of Atherosclerosis in Youth study, which found VLDL-C and LDL-C to be positively associated, and HDL-C to be negatively associated with atherosclerotic lesions in the aorta and right coronary artery of young adults [11].

LDL-C, or so-called "bad cholesterol," is arguably the most characterized and infamous lipid risk factor. As the primary cholesterol-carrying lipoprotein in the blood, LDL-C accounts for 60-70% of total serum cholesterol [69]. Through investigations of individuals exhibiting familial hypercholesterolaemia, the development of cholesterol-lowering pharmacotherapies such as statins, and evidence of endothelial disruption, LDL-C has been identified as the "single most predictive CVD risk factor" [19, 70, 71]. As such, it is the primary target for therapeutic intervention by all National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) and Canadian reports [55, 72]. This designation is not unwarranted, as for every 1% reduction in LDL-C, there is a 1% reduction in CHD risk, therefore representing a powerful means by which risk management and risk assessment are determined [71, 72].

Despite the apparent strength of LDL-C as a predictor of cardiovascular risk, it is not without criticism. A recent Canadian report on dyslipidaemia and prevention of CVD suggested that LDL-C provides a suboptimal representation of LDL particle number, particularly in hypertriglyceridaemic individuals, in whom LDL-C is replaced by LDL-TG [55]. This is due to variation in the relative amounts of CE and TG contained within the lipid core, and the particle

size [73]. While LDL particle diameter varies <3 nm, or 12% per particle, this translates to 40% variation in volume, such that 70% more small LDL particles than large LDL particles would be necessary to carry an equivalent amount of cholesterol [73]. This evidence, combined with reports that the association between LDL particle number and CVD is twice that of LDL-C, undermines its predictive value [71].

An additional shortcoming of current clinical practice to rely heavily upon cholesterol for assessing cardiovascular risk is the *cholesterol paradox* [50]. To illustrate the cholesterol paradox, consider a total plasma cholesterol concentration of >7.5 mmol/L, which is associated with a 90% risk of CHD [50]. Only 3% of individuals with clinically diagnosed CHD meet or exceed this threshold; whereas 45% of patients exhibit total cholesterol levels ≥5.2 mmol/L, which is associated with only 20% risk of CHD [50]. Based on the NCEP ATP III guidelines, optimal fasting total cholesterol should not exceed 5.17 mmol/L, highlighting the disconnect between clinical guidelines for assessment and cardiovascular events [69]. This phenomenon has been observed, as patients with clinically diagnosed CVD frequently fall within normal ranges of blood cholesterol based upon standardized age- and sex-matched cut-offs, and therefore go unidentified base on this criterion [3, 50, 74].

One potential explanation of the cholesterol paradox is the reliance of practitioners on fasting lipid profiles to assess CVD risk. Fasting lipid profiles are practical and convenient in their ability to reduce day-to-day variability in plasma lipid concentrations and abundance of population norms [75]. However, focusing exclusively on fasting values ignores the variability in postprandial responses, which may be indicative of diseased or high-risk states [76]. Additionally, it highlights the need for novel identification strategies employing genetic testing and comprehensive lipoprotein analysis to ameliorate the current limitations in risk prediction.

2.1.8 Genetic Risk Factors for Cardiovascular Disease

The heritability of atherosclerosis is estimated as >50% [52, 68]. Genetic risk factors for common forms of atherosclerosis are multifactorial; however, investigations of rare monogenic, or Mendelian, forms were instrumental in elucidating its aetiology and fundamental lipoprotein pathways, such as LDL-R mediated endocytosis of apo B-carrying lipoproteins [7, 77].

2.1.8.1 Candidate Gene Studies

Candidate gene studies are founded upon monogenic disorders, wherein a mutation in a single gene elicits an extreme phenotype, such as LDL-C concentrations exceeding the 95th percentile [19]. Rare genotypes inspired the *multiple rare-variant hypothesis*, which posits that CVD risk results from distinct genetic variants with low MAF and large effect sizes (odds ratio > 2.0) [78]. However, wide variation in lipoprotein concentration exists in individuals lacking these mutations, suggesting the presence of other causative genes [19]. This led to formation of the *common disease-common variant* hypothesis, which proposes that CVD risk is resultant from a combination of influence of several common variants with MAF >1% and lower odds ratios (1.5-2) [19, 78, 79]. As such, efforts shifted towards characterizing CVD-related phenotypes under complex inheritance resulting from interactions between several genetic and non-genetic factors [80]. Investigations of these diseased-states require mapping of multiple loci associated with complex traits in individuals lacking monogenic disorders [19, 80].

2.1.8.2 Genome Wide Association Studies

New technological advances, such as the Human Genome Project, HapMap Project and micro-arrays, along with multi-site collaborations with unprecedented sample sizes revolutionized investigations of the genetics of CVD [19]. GWAS search for variation at SNP base pairs, where the nucleotide has shifted from its wild-type form [81]. Approximately 1/300

nucleotide base pairs vary among individuals, switching between one or two natural variants, and account for the majority of human genetic variation [81]. The outcome of such variation is dependent upon the SNP's location, in a coding or non-coding region, and whether it is synonymous or nonsynonymous [81]. Synonymous SNPs are less likely to exert deleterious effects as the protein coding sequence is unchanged; nonsynonymous SNPs may induce changes in protein structure or activity, disrupt metabolic pathways, and ultimately contribute to disease pathogenesis [81].

The first successful GWAS were completed in the mid-2000s, yielding an abundance of genetic information [21, 22, 36]. To date, 157 loci have been associated with lipid traits, of which over two-thirds had no prior recognized role in lipid metabolism [82]. These novel genes often exhibit equally strong associations with lipoproteins [82]. Genetic markers of lipid and cardiovascular phenotypes have considerable potential clinical value. Whereas associations between traditional CVD risk and plasma lipoproteins may be "diluted" by non-genetic factors, genetic determinants of CVD risk offer a more direct association, better suited to withstand confounding non-genetic variables [19]. Furthermore, genotype represents a superior marker of exposure to an intermediate trait over time compared to an acute biochemical test [19, 20]. As such, genetic risk stratification based upon deleterious variants is possible, prior to the onset of clinical evidence of atherosclerosis or dyslipidaemia [19, 20].

2.1.8.3 Missing Heritability of CVD and Nutrigenetics

While GWAS have successfully validated over 12 million different SNPs, only a fraction of the variability of respective traits has been explained, provoking the puzzle of missing heritability [79, 83]. Only 10-12% of lipid trait variability is attributed to the 95 lipid-associated loci originally identified via GWAS [30]. This contrasts results from twin and family studies,

which provide evidence of >50% heritability [52, 68]. Certain methodological limitations have been proposed, such as unidentified loci with MAF too low to detect, structural variants poorly detected using current technology, inadequate study power and inappropriate controls [79]. Others propose that confounders may interrupt the relationship between genotype and phenotype, such as pleiotropy, epistasis, gene-gene interactions, gene-environment interactions and epigenetic mechanisms such as methylation [19, 84].

The field of nutrigenetics examines the effects of genetic polymorphisms on interactions between risk factors, such as diet, on disease with the aim of developing genetics-based dietary recommendations [13]. Nutrigenetics is founded on the investigation of associations between genetic variants and differential dietary responses, such as postprandial lipaemia, to specific nutrients [13]. In contrast, nutrigenomics explores interrelationships between nutrients, gene expression, and the related phenotypes [85]. Further investigation into these nutrient-gene interactions is anticipated to help foster the identification of novel CVD aetiology pathways and improve our understanding of genetic risk factors for CVD [84].

2.2 Interrelationships between Postprandial Lipaemia and Cardiovascular Disease

2.2.1 Defining Postprandial Lipaemia

While lipid profiles are recognized as one of the paramount risk factors for CVD, most research has focused on fasting levels, which are reflective of endogenous metabolism [13]. Based on a western-style eating pattern of three or more meals per day, humans spend the majority of the time in the postprandial state, challenging the merits of employing fasting profiles to assess CVD risk [15, 86]. Postprandial lipaemia is defined as the period between food ingestion and the postabsorptive state, characterized by rapid remodelling of lipoproteins, inducing a change in their concentration and composition in the blood [14, 15]. This

phenomenon was first reported by Asellius in 1622 [87], who described the milky appearance of serum immediately following meal ingestion. Similar sentiments were later expressed by Hewson in 1774 [87], wherein he credited the milky appearance to the accumulation of tiny particles, later named CMs. While postprandial lipaemia is not a novel discovery, its complexity and innumerable modifying factors fuel continual investigation.

Individual capacity to metabolize and clear lipids in response to meal ingestion is highly variable, depending on genetic, environmental, physiological and pathological factors [13, 15]. Environmental factors include meal composition, physical activity and smoking status [13]. Age, gender and menopausal status are considered physiological factors; whereas obesity, insulin resistance and type 2 diabetes are hallmark pathological factors [13]. Family history of disease, as well as individual SNP and haplotype variation are important genetic factors [13]. Based on the high degree of variability and representativeness of resting conditions, non-fasting lipids have been suggested to be more discriminatory in regards to CVD risk compared to fasting levels [15].

2.2.2 Epidemiological Evidence of Non-Fasting Lipids as a Risk Factor for CVD

To meliorate inherent limitations to employing fasting lipid profiles for cardiovascular risk assessment, large-scale epidemiological studies investigated the relationship between non-fasting lipids and clinical manifestation of atherosclerotic disease. The Women's Health Study followed 26,509 initially healthy women for a median of 11.4 years, and found that non-fasting TG concentrations were strongly and independently associated with cardiovascular events when controlled for age, blood pressure, smoking and use of hormone therapy [76]. Compared to the association with fasting TG concentration, which when controlled for HDL-C and total cholesterol was weakened, non-fasting TG maintained a strong correlation after adjustment [76].

Furthermore, TG concentrations obtained between 2-4 h postprandially were the strongest predictors of subsequent cardiovascular events compared to those obtained 4-8 h, 8-12 h or >12 h since the last meal [76]. Upon further investigation, the authors concluded that non-fasting concentrations of HDL-C, TG and apo A-1 had stronger associations with CVD than their non-fasting counterparts; whereas the opposite was true for total cholesterol, LDL-C, apo B-100 and non-HDL-C [88].

Similarly, the Copenhagen City Heart and Copenhagen General Population studies identified non-fasting TG as an independent risk factor for MI, ischemic stroke and total mortality in men and women [89, 90]. When the 33,391 participants of the Copenhagen General Population Study were stratified into tertiles, the highest- versus the lowest-tertile of non-fasting total cholesterol, non-HDL-C, LDL-C, apo B, TG, ratio of total cholesterol to HDL-C and ratio of apo B to apo A1 predicted 1.7 to 2.4-fold increase risk of cardiovascular events [91].

The Norwegian Counties Study followed 86,261 originally CVD-free, 20-50 years olds from 1974-2007 and observed an association between non-fasting TG and CVD risk [92]. However, in contrast to the Women's Health Study and Copenhagen Studies, this association was not independent of traditional risk factors; such that non-fasting TG was unable to predict CVD beyond that of traditional Framingham CHD risk scores [92]. These findings were paralleled in the Multiple Risk Factor Intervention Trial, wherein non-fasting and fasting TG had similar adjusted and unadjusted predictive value for CVD risk [93].

While some controversy remains around the strength and independence of the association between non-fasting lipids and clinical outcomes, it seems clear the relationship is sex-dependent. When stratified by sex, the association between non-fasting TG and MI, ischemic heart disease and CVD was greater in women than men, as determined by multivariable and age-

adjusted analyses [92, 94]. Specifically, non-fasting TG ≥5 mmol/L compared to <1 mmol/L was associated with an age-adjusted 17-fold increase in risk for MI in females, compared to 5-fold in men [89]. In contrast, non-fasting total cholesterol was a superior predictor of cardiovascular events in men [94].

The evidence presented by these studies warrants further investigation to elucidate the mechanisms driving the observed associations between non-fasting lipids and clinical outcomes. Furthermore, given the strength of the associations, non-fasting lipids should be considered as an alternative to fasting lipid profiles, particularly in populations in whom fasting procedures are cumbersome [90, 91].

2.2.3 Postprandial Lipaemia and the Development of Atherosclerosis

2.2.3.1 Atherosclerosis as a "Postprandial Phenomenon"

The role of postprandial lipaemia in atherogenesis was first recognized in the early 1950s. However, it wasn't until 1979 that Dr. Donald Zilversmit [16] popularized the concept of atherosclerosis as a "postprandial phenomenon". Considering the first stage of CM degradation occurs in contact with the vascular endothelium, he hypothesized their atherogenic potential [16]. This hypothesis was supported by a series of *in vivo* experiments. In rabbits, he found that cholesterol content of CM and CM-remnants rose as dietary cholesterol increased, thus supporting a role for CMs in cholesterol regulation [16]. Secondly, Zilversmit concluded that CM-remnants were as atherosclerotic as endogenously produced LDL-C and VLDL-C, and that CMs were the primary contributors to atherosclerosis in rabbits [16]. Finally, he found that patients with type III hyperlipoproteinaemia, a Mendelian lipid disorder [95], had elevated CM-remnant concentrations, further supporting their role in the atherogenesis [16]. Based on these findings, Zilversmit concluded that even in the absence of hypercholesterolaemia, postprandial

elevations in CMs and CM-remnants increase atherogenesis, thus promoting risk of CVD [16]. He further emphasized the need for OFTTs to examine interindividual differences in postprandial lipid metabolism to identify those at elevated risk.

2.2.3.2 Direct Mechanisms of Postprandial Lipaemia on Atherogenesis

Since Zilversmit's pioneering work, several mechanisms citing direct and indirect effects of postprandial lipaemia on atherogenesis have been proposed. Mechanisms proposing direct effects attribute deleterious effects of postprandial lipaemia to TG-rich lipoproteins themselves [96]. Such mechanisms highlight these particles as promoters of vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 expression, thus inducing changes in vascular tone [97]; as well as enhancing inflammatory marker expression [98] and leukocyte activation [99, 100]. TG-rich lipoproteins themselves may also penetrate the endothelium [101, 102], the rate of which increases in direct proportion to the concentration and decreases with increasing particle size [103]. Additionally, transcytotic vesicles facilitate transport of larger TG-rich lipoproteins, up to 70 nm diameter, from the luminal surface to the subendothelial space, therefore permitting the accumulation of larger, unhydrolyzed CMs and VLDL-remnants [104].

2.2.3.3 Indirect Mechanisms of Postprandial Lipaemia on Atherogenesis

Perhaps less intuitive, but no less damaging, are the indirect effects of postprandial lipaemia on atherogenesis. As a result of increased CM and CM-remnants, circulating VLDLs rise due to competition for LPL and cell surface binding sites [50, 66]. Receptor affinity for CMs and CM-remnants is greater than that for VLDL, therefore many CMs exit circulation while quite large [66]. As VLDL is the least effective ligand for the binding sites, it remains in circulation the longest [50], therefore representing an important contributor to postprandial lipaemia [66]. As such, 80% of the remnant TG-rich lipoproteins accumulated during

postprandial lipaemia are attributed to apo B-100-containing particles (VLDL, IDL and LDL subfractions) rather than apo B-48-containing particles (CMs and CM-remnants) [66, 105].

Accumulation of plasma TG and lingering VLDL alters the composition of other lipoprotein subfractions. It promotes CE transfer protein (CETP)-mediated transformation of VLDL into LDL, and overproduction of small, dense LDL particles [50, 66], the most detrimental of the LDL subclasses [71, 73, 106]. During hypertriglyceridaemia CETP catalyzes the exchange of one molecule of TG from VLDL for one molecule of CE from the LDL core, thereby depleting the LDL core of CE and enriching it with TG [58, 59, 73, 107]. These TG-enriched LDL particles are a favourable target for hydrolysis by hepatic lipase to restore the TG-CE balance, resulting in a remodelled core and surface, ultimately producing smaller, denser LDL particles [59, 73, 107]. The rate of this transfer is proportional to TG residence time in circulation, therefore it is presumed that prolonged postprandial lipaemia increases extent of lipid exchange and thus, smaller, denser particles overall [108].

While lipid enrichment of CMs accounts for the majority of dietary TG accumulation [64], the primary alteration in lipoprotein concentration is the accumulation of VLDL particles resulting from increased endogenous secretion and competition for hepatic re-uptake with CMs [109, 110]. Overall, these changes in postprandial TG-rich lipoprotein metabolism and enzyme activity account for ~50% of LDL particle size variability, highlighting the impact on lipoprotein physiology [111]. Other indirect effects of postprandial lipaemia include negative associations with HDL-cholesterol [112] and associations with prothrombotic markers [113-115].

2.2.4 Associations Between Postprandial Lipaemia and Clinical Manifestation of Disease

Due to the deleterious effects of exposure to postprandial lipoproteins, many pathological states have been associated with abnormal postprandial lipaemia. The majority of human-based

research investigating the relationship between impaired postprandial lipaemia and atherosclerosis has been grounded upon cross-sectional studies investigating response to acute fat challenges, OFTTs.

In one of the earliest studies, men with documented MI displayed a more pronounced and prolonged increase in total lipids compared to age- and sex-matched controls [116, 117], thus substantiating the role of postprandial lipaemia in the clinical manifestation of disease. Over the next 40 years, further support for this relationship was presented, wherein elevated response and delayed clearance of lipids were reported to be positively associated with coronary artery disease [118-120], peripheral artery disease [121], intima media thickness [122], hypertriglyceridaemia [87, 105, 123] and carotid artery stenosis [124]. More recently, it was shown that exaggerated postprandial lipaemia persists in CVD patients, despite low fat diet, normal fasting lipid profile and statin-therapy, highlighting it as an independent risk factor [125].

2.2.5 Heritability of Postprandial Lipaemia: Familial Studies

Investigations of the influence of familial risk factors on postprandial lipaemia provided initial support for the influence of genetics. Early evidence was presented wherein young men (23.3 ± 4.6 y) with paternal history of premature MI (<55 y) exhibited prolonged postprandial hypertriglyceridaemia compared to age-matched controls [126]. A similar finding was reported wherein the progeny of patients with diagnosed coronary artery disease had consistently elevated TG response compared to healthy parental spouses [127]. In contrast to these findings, a much larger study that compared young men with paternal history of premature MI (n=407) and without (n=415) found no difference in postprandial TG response between the two groups [128]. However, when these subjects were stratified into tertiles based on fasting TG concentrations, men in the highest tertile with paternal history of premature MI exhibited increased and

prolonged TG response compared to controls [128]. Of interest, many of these studies found differences in non-fasting lipid concentrations at 8, 10 or 12 h after fat ingestion between cases and controls, suggesting that the atherogenic risk of postprandial lipaemia is primarily due to a prolonged, rather than augmented, response [105, 126].

2.2.6 Heritability of Postprandial Lipaemia: Single Nucleotide Polymorphisms

Completion of the Human Genome Project in 2003 allowed for increased cost efficiency and accessibility to genetic testing, resulting in an abundance of genetic information in the form of SNPs [81, 129]. Many of these SNPs were statistically associated with fasting lipids [21, 28, 30, 36], however few have been investigated in the context of postprandial lipaema. In the past 15 years, the European Atherosclerosis Research Study II and the Lipids and Atherosclerosis Research Unit in Spain have commenced investigations into the influence of SNPs on postprandial lipaemia in young males, and represent the majority of research in the field.

2.2.6.1 Apolipoproteins

Genes involved in apolipoprotein synthesis and regulation have been the primary focus of candidate gene studies in young adults. Apolipoproteins are expressed on the surface of lipoproteins and are integral to metabolic processes, making them a popular target for investigation. Apolipoproteins that have been associated with postprandial lipaemia in young adults include: A-I [130-132], A-IV [133], A-V [130, 134-136], B [137, 138]; E [139-142] and C-III [130].

2.2.6.2 Lipid Metabolism Enzymes and Receptors

Extensive testing has also investigated relationships between lipid metabolism enzyme and receptor polymorphisms with postprandial lipaemia. Several variants located within the *LPL* gene, encoding for LPL, have been associated with postprandial lipaemia in young adults [143-

146]. Similarly, variants at genes encoding for hepatic lipase (*HL*) [147, 148], scavenger receptor class B type I (*SCARB1)* [149, 150], peroxisome proliferator-activated receptor 2 (*PPAR)* [151, 152], melanocortin-4 (*MC4R*) [153] and perilipin (*PLIN*) [154] have been identified.

2.2.6.3 Transporter Proteins

Evidence of associations between polymorphisms in transporter proteins, such as intestinal fatty acid binding protein 2 (*FABP2*) and fatty acid transport proteins (*FATP*), and postprandial lipaemia are limited [15]. However, preliminary data has been presented supporting the prediction that genotype at the adenosine triphosphate gene superfamily, including *ABCA1, ABCG1, ABC4* and *ABCG8* may influence postprandial lipaemia in a sex-dependent manner [155-157].

2.2.6.4 Considerations and Limitations to SNP-Based OFTTs

While many studies investigating the effects of individual SNPs on postprandial lipaemia have successfully identified significant associations, some important considerations must be made. Firstly, OFTTs as a method of quantifying postprandial lipaemia lack standardization in terms of pre-testing instructions, test meal quantity and composition, duration of testing and lipid parameters selected. Most test-meals are composed of breakfast-like mixed meals, while many others are dairy-based shakes. As meal size and composition are significant factors in the postprandial lipaemia [13], external validity is often limited, making it very difficult to make comparisons across the literature. Generalizability of the findings is also limited as essentially all of the studies investigating the influence of SNPs on postprandial lipaemia in young adults are performed in males, whose metabolic physiology differs from females [13].

Secondly, statistical integrity is frequently of concern when considering OFTT studies. Due to the rigorous nature of OFTTs, small sample sizes are common, and simple models are

used wherein a single SNP or haplotype is investigated at a time. Furthermore, many studies perform statistical analyses on at least five variables at up to nine time-points, therefore compounding the risk of committing a type 1 error. While some studies utilize multiple testing corrections, stochastic errors are evident in the inconsistent findings throughout the literature. Moreover, postprandial lipaemia is a polygenic trait, such that each gene involved makes a very small contribution to interindividual differences in response. Considering this, in addition to the fact that most gene variants occur in only a small segment of the population, many OFTTs are underpowered to detect significant intergroup differences. Finally, many of these studies report small effect sizes, suggesting that other genetic factors are involved, such as *SORT1*.

2.3 *SORT1* as a Potential Regulator of Postprandial Lipaemia

2.3.1 Genome Wide Association Studies and Identification of the 1p13 Locus

Since its initial identification in 2007, the 1p13 locus has been consistently recognized as one of the loci most strongly associated with LDL-C [21, 23, 27-46] and CVD [22, 37, 158, 159]. As LDL-C is recognized as a causal risk factor for CVD [69], this locus is unique based on its strong association with the intermediate phenotype, in addition to clinical manifestations, such as abdominal aortic aneurism [160], coronary stenoses [161] and MI [22, 25].

The association between the 1p13 locus and CVD was first elucidated following the Wellcome Trust Case Consortium (1,926 CVD patients; 2,938 controls) and its replication cohort, the German Myocardial Infarction Study (875 MI patients; 1,644 controls) [22]. Combined, these studies provided evidence that 29% reduced relative risk of CVD or MI could be attributed per minor, protective allele at the rs599839 polymorphism [22]. Given the MAF of 22%, a relatively large effect size for such a common variant, there was substantial interest for further investigation.

In subsequent GWAS, rs646776 and rs599839, both located within the 1p13 locus, were two of thirty SNPs associated with LDL-C ($p=5x10^{-42}$ combined; effect size= -0.16 ± 0.1 mmol/L per minor allele) [21, 33]. This finding was replicated shortly thereafter, wherein a dose-response of 0.12-0.24 mmol/L decrease in LDL-C per copy of the minor, protective allele [23, 28, 36, 38, 44, 110, 161] was reported, independent of lipid-lowering therapy [39]. Clinically speaking, this was translated into a 9-13% and 40% reduced risk of CVD [37] and MI [21, 22], respectively.

While initial GWAS investigating *SORT1* were founded upon European samples, the associations between the 1p13 locus and both CVD and LDL-C have been reproduced in non-Hispanic blacks [45], Mexican Americans [45], non-Hispanic whites [45], East Asians [30, 38] and South Asians [28, 30, 158]. Additionally, they have been replicated employing Baynesian Variable Selection techniques, a novel strategy proposed to extend beyond single SNP analyses performed by GWAS, providing strong evidence linking the rs629301 variant of the 1p13 locus with LDL-C, apolipoprotein B and TG [162].

2.3.2 Functional Validation Studies of the 1p13 Locus

The success of preliminary GWAS sparked efforts to functionally characterize the 1p13 locus. These studies sought to answer four fundamental questions, which would identify: (1) the causal variant located at the 1p13 locus, (2) the gene regulated by this variant, (3) the mechanism by which the variant modified gene expression, and (4) the mechanism by which the gene altered lipoprotein metabolism [47].

Within the 1p13 locus, six SNPs, including rs646776, rs599839, rs12740374 and rs629301 are in high linkage disequilibrium (Figure 2.3) [47]. The two predominant "major" and "minor" haplotypes are possessed by 68% and 29% of Europeans, respectively [47]. These

variants lie in a non-coding region in close proximity to the *SORT1, PSRC1* and *CELSR2* genes [47]. *SORT1* encodes for the multiligand sorting receptor sortilin, which was first characterized in autopsied human brain samples [47, 163]. *PSRC1* encodes for the proline/serine-rich coiled protein 1, which is believed to play a role in the cell cycle and development of the mitotic spindle [67]; whereas *CELSR2* encodes for cadherin EGF LAG seven-pass G-type receptor 2 a protein involved with neuron migration and adhesion [67, 83].

Figure 2.3 Chromosomal map of the 1p13 locus

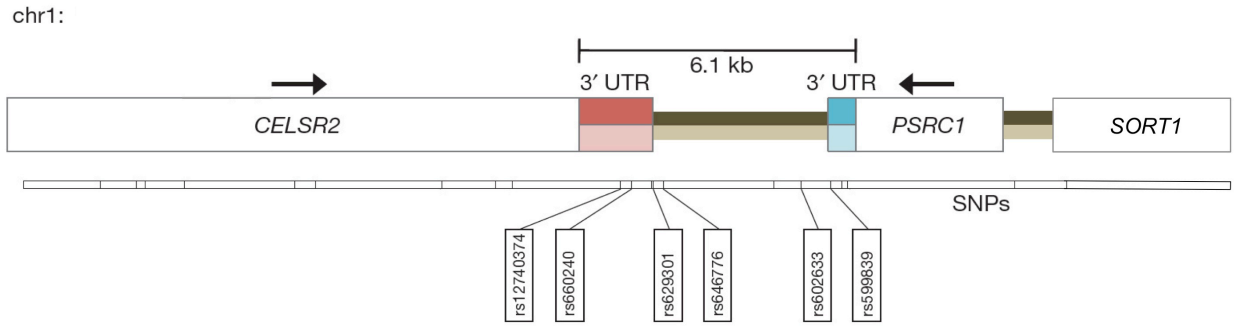

Figure 2.3 (adapted from [47]). The six SNPs of the 1p13 locus most strongly associated with LDL-C in human GWAS lie within a 6.1 kb noncoding region between the stop codons of the *CELSR2* and *PSRC1* genes.

The first comprehensive mechanistic investigation of the 1p13 locus was published by Linsel-Nitschke et al. [37] in 2009. An in depth GWAS was conducted, which found carriers of the minor allele at the rs599839 variant to exhibit sortilin overexpression, as was evident in elevated sortilin mRNA [37]. This prompted *in vitro* investigations of *SORT1* cDNA-transfected HEK293 cells, in which sortilin overexpression enhanced hepatic uptake of LDL particles, thereby lowering plasma LDL-C [37]. Combined, these results formed the foundation for a

proposed mechanism wherein sortilin overexpression, resulting from the presence of one or more minor alleles, facilitates LDL particle re-uptake, hence lowering plasma LDL-C [37].

Approximately one year later, Musunuru et al. [47] published a paper outlining thorough investigations into the 1p13 locus. Through a series of *in vitro* experiments comparing the effect of altering each SNP in the minor haplotype to its major allele, it was determined rs12740374 is the causal variant in the locus [47]. Presence of the minor allele at this variant created a CCAAT/enhancer α binding protein transcription factor binding site, whereas the major allele disrupted it [47]. To identify the gene regulated by the causal variant, rs12740374, *CELSR2, PSRC1* and *SORT1* transcript levels were quantified in human liver, subcutaneous fat and omental fat tissue samples [47]. Compared to major allele homozygotes, individuals homozygous for the minor alleles displayed 12-fold higher *SORT1* and *CELSR2* transcript levels in the liver, but not fat tissue, suggesting a liver-specific mechanism [47]. In subsequent murine studies, Musunuru et al. [47] found adeno-associated virus serotype (AAV) 8-mediated hepatic sortilin expression to be associated with 70% reductions in total plasma cholesterol, 73% reduction in LDL-C and 57% lower rate of VLDL secretion, further supporting the role of sortilin as a regulator of lipid metabolism. Based on these findings, Musunuru et al. [47] proposed a mechanism whereby sortilin overexpression, as is seen in carriers of the minor allele, modulates hepatic VLDL secretion, thereby lowering LDL-C.

Controversy arose when, almost concurrently, Kjolby et al. [164] published a paper detailing observations of double knock-out mice ($Sort^{-/-}$ x $Ldlr^{-/-}$) that exhibited 30% lower plasma cholesterol compared to $Ldlr^{-/-}$ mice, following a six-week western-type diet intervention. $Sort^{-/-}$ x $Ldlr^{-/-}$ mice also exhibited lower apo B-100, but not apo B-48 concentrations, attenuated hepatic lipid accumulation and 60% lower aortic atherosclerotic lesions compared to the $Ldlr^{-/-}$

mice [164]. Furthermore, hepatic sortilin overexpression selectively induced using AAV was observed to promote *Sort*$^{-/-}$ x *Ldlr*$^{-/-}$ mice to revert back to a hypercholesterolaemic phenotype [164]. Based on these findings and the observed high binding capacity for sortilin for apo B-100 in the ER and trans-Golgi compartments, the authors proposed a mechanism by which hepatic sortilin overexpression facilitates VLDL release [164]. Combined, these results suggested that sortilin expression is associated with elevated LDL-C, disputing the results of Linsel-Nitschke et al. [37] and Musunuru et al [47].

Figure 2.4 Proposed mechanisms of sortilin

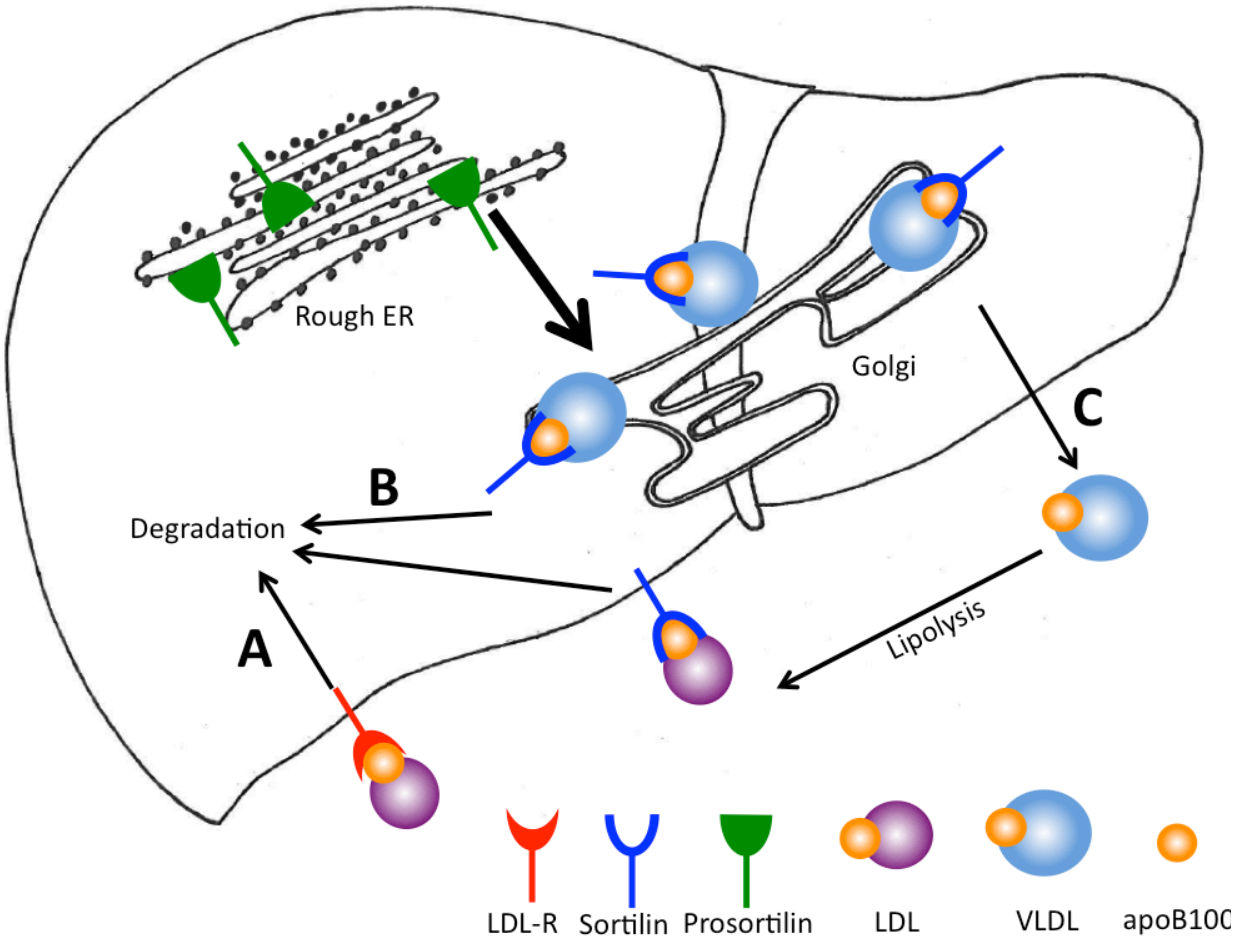

Figure 2.4 (adapted from [67]). Sortilin is synthesized as a proprotein in the rough ER, and must be cleaved by furin in the trans-Golgi to yield a mature sortilin protein. Several mechanisms have been proposed to explain sortilin's function in the cell. (A) Linsel-Nitschke et al. [37] proposed a mechanism wherein sortilin facilitates hepatic re-uptake of LDL particles. (B) Musunuru et al. [47] suggested that sortilin facilitates VLDL degradation, thus limiting its availability for hydrolysis into LDL particles. (C) Kjolby et al. [164] proposed a mechanism wherein sortilin facilitates hepatic release of VLDL.

2.3.3 Recent Advancements in Functional Validation of the 1p13 Locus

Recent investigations have focused on refining approaches to elucidate sortilin's function as a sorting receptor in attempt to form consensus surrounding the mechanism and direction of its influence on lipid metabolism. Sortilin is a multiligand sorting receptor, which localizes to the Golgi apparatus, with small amounts (10%) present in clathrin-coated pits on the cell membrane [47, 165]. It binds a variety of ligands and trafficks them to the lysosome for degradation [166]. This process is facilitated in part by pH-dependent binding, such that sortilin binds apo B-100 with high and low affinities in neutral and acidic environments, respectively [166]. Given that the average pH of the ER, Golgi apparatus and lysosomes are 7.2, 6.5 and 4.7, respectively, this adaptation facilitates efficient binding at the ER and Golgi apparatus, and release into the lysosome [167].

Recent functional validation studies employing "humanized" mouse models demonstrated that AAV-8 mediated hepatic sortilin expression attenuates apo B-100 secretion in a lysosome-dependent manner in *Ldlr*$^{-/-}$ and wild-type mice, a relationship that has also been observed in primary hepatocytes [166]. Hepatic sortilin overexpression has also been associated with increased LDL catabolism [166]. These findings, in addition to the 40% decline in fractional catabolic rate of LDL in *Sort*$^{-/-}$ mice compared to wild-type, suggest a dual role for sortilin wherein it: (1) facilitates LDL catabolism, and (2) mediates apo B-100 secretion, in the form of VLDL particles [166].

Despite the apparent success of recent investigations into sortilin's mechanism of action, they are not without controversy. Whole body sortilin knock-out mice (*Sort*$^{-/-}$) have been observed to exhibit normocholesterolaemic [164, 166] phenotypes, in addition to reduced apo B

secretion [166] and delayed LDL catabolism [166]. These findings contrast reports of liver-specific sortilin-overexpression, which have been associated with lowered total cholesterol, LDL-C, apo B and VLDL [37, 47]. Moreover, a recent publication outlined a novel role for sortilin as a facilitator of hepatocytic secretion of proprotein convertase subtilisin/kexin type 9 (PCSK9), a circulating protein that targets LDL-R for lysosomal degradation, thus increasing LDL-C [168]. These results highlight the importance of distinguishing between models of full-body and liver-specific sortilin expression, and support future investigations to further elucidate the role of sortilin and direction of its effects on lipid metabolism.

Hepatic sortilin expression was recently implicated with diet and obesity or diabetes status [169, 170]. Reduced hepatic sortilin protein expression has been observed in murine models of type 1 and type 2 diabetes, in addition to humans suffering from obesity or liver steatosis [170]. In both diet-induced obese and genetically obese (*ob/ob*) mice, sortilin mRNA and protein expression were reduced between 50-81% following 20 weeks of a high-fat diet [169]. It is believed that elevated FFA, which is commonly observed in diseased populations, induces ER stress, interrupting posttranslational sortilin protein expression [169, 170]. When hepatic sortilin was reconstituted in *ob/ob* mice and upregulated in wild-type mice via *Sort1*-expressing AAV-8 injection, total cholesterol, TG and apo B-100 secretion were reduced [169, 170]. Additionally, insulin affects binding capacity, wherein insulin acutely facilitates apo B-100-sortilin interactions, thereby mitigating release of apo B-100-containing particles [171]. These findings draw attention towards sortilin as a potential therapeutic target for risk modification.

2.3.4 Evidence of a Gene-Age Interaction at the 1p13 Locus

One unique characteristic of the 1p13 locus is evidence of a genotype-age interaction. In initial GWAS of older adults, including those with metabolic disease [21, 33], the *SORT1* locus was reported to explain ~1% of the variation of LDL-C [21]. In contrast, genotype at the rs646776 variant accounted for 3.6% of the variation in LDL-C in 548 young adults (23.86 ± 5.71 y), which was validated in a cohort of 810 sixth graders (effect size= 2.5%) by Devaney et al. [23]. Evidence of larger effect sizes in younger individuals was replicated in the longitudinal Utah Pedigree and NHLBI Family Heart Studies [172], and in our University of Calgary Assessing Inherited Markers of Metabolic Syndrome in the Young (AIMM Young) cohort [24]. The robustness of these associations is due, in part, to the absence of confounders such as age, environmental factors, disease or treatment regimens in younger adults, therefore allowing for a clearer understanding of the genotype [143]. This provides support for genotype-phenotype studies in young adults and may suggest this locus is susceptible to gene-environment interactions, such as that with postprandial lipids and lipoproteins.

2.4 Rationale

There is indisputable evidence supporting lipids and lipoproteins as integral factors in atherogenesis and CVD risk. Heritability of such risk factors is increasingly apparent; however despite the success of GWAS in identifying variants, there is a disconnect between the heritability observed in familial and twin studies [79, 83] and the variance attributed to individual SNPs [52, 68]. Furthermore, while risk factors have been extensively documented, cardiovascular events continue to frequent individuals in whom traditional risk factors are absent [3]. This suggests that other factors, such as genetics and impaired postprandial lipid clearance

may be involved. As such, it is necessary to develop screening techniques that employ novel identification strategies to target those at greatest risk for earlier intervention and prevention.

Advancements in genetic testing and recognition of impaired lipid metabolism as a contributor to atherogenesis make this a valuable area to investigate in order to elucidate specific characteristics of those at greatest risk. Given the substantial evidence marking non-fasting TG concentration as a CVD risk factor [76, 88-92], and the disparate risk for CVD and MI based upon genotype at the 1p13 locus [22, 25], we investigated the relationship between genotype at the rs646776 variant and postprandial lipaemia in young adults. Examining the *SORT1* gene provides an opportunity to uncover valuable genetic targets for therapy, and the potential for personalized, genetics-based interventions. The rs646776 variant at the *SORT1* 1p13 locus is particularly valuable as it has a high MAF and relatively large effect size, especially in young adults [23, 24, 172]. While substantial efforts have been directed towards characterizing this variant, further research may refine proposed mechanisms and clarify the genotype-to-phenotype relationship. Furthermore, evidence has been presented demonstrating a gene-age interaction at this locus, suggesting it may be susceptible to other modifiers, such as diet and environment. Identifying genome-specific relations is important, as certain individuals may be more or less responsive to pharmacological or lifestyle interventions based on their genotype. As an up-and-coming field, genetics-based medicine has enormous potential to expand in the future, revolutionizing clinical practice.

Chapter Three: The *SORT1* Protective Allele is Associated with Attenuated Postprandial Lipaemia in Young Adults[1]

3.1 Introduction

3.1.1 Epidemiology of Cardiovascular Disease

Cardiovascular diseases (CVD) remain the primary cause of morbidity and mortality worldwide [1]. In fact, many cardiovascular events occur in individuals who lack a high level of risk based on traditional single- or multi-variable risk scores, such as Framingham Risk Scores or the European Systematic Coronary Risk Evaluation [3]. Based on this evidence, it is apparent that other factors, such as genetics and unconventional biomarkers contribute to this risk.

3.1.2 SORT1 as a Predictor of Cardiovascular Disease Risk

A group of single nucleotide polymorphisms (SNP) located at the 1p13 locus are consistently associated with CVD, myocardial infarction, coronary artery disease and fasting low-density lipoprotein (LDL) cholesterol (LDL-C) based on genome-wide association studies [21, 28, 30, 158, 159, 173]. Of these, the rs646776 variant near the *SORT1* gene is recognized as one of the most common, strongest LDL-C associated SNPs, an association which persists even after lipid-lowering therapy [21, 39]. This variant is in high linkage disequilibrium with rs12740374, a non-coding variant that creates a new CCAAT/enhancer α binding protein transcription factor-binding site that increases hepatic expression of sortilin, a multiligand sorting receptor, which localizes to the Golgi apparatus, with small amounts present in clathrin-coated pits on the cell membrane [47, 165]. Functional validation studies employing "humanized" mouse models demonstrate that adeno-associated virus serotype 8 mediated hepatic sortilin

[1]A version of this chapter has been submitted for publication in *Circulation: Cardiovascular Genetics*. Connors, K.E., Gnatiuk, E.A, Karlos, A.E., Shearer, J., Reimer, R.A., and Hittel, D.S. SORT1 Protective Allele is Associated with Attenuated Postprandial Lipaemia in Young Adults.

overexpression increases LDL catabolism and decreases rate of very low-density lipoprotein (VLDL) secretion compared to those injected with the null virus [47]. Given that VLDL is a precursor to LDL, these authors suggest a mechanism wherein sortilin serves a dual role in binding and trafficking extracellular LDL, and VLDL at the Golgi apparatus, to the lysosome for degradation, thus eliciting a phenotype characterized by decreased LDL-C as is observed in humans [47, 166].

In humans, presence of the minor allele at the rs646776 variant of the 1p13 locus, which has an average frequency of ~0.212 (0.197-0.437), is associated with 0.1-0.2 mmol/L lower fasting LDL-C compared to individuals who are homozygous for the major, risk allele [21, 37, 39, 46, 47]. Clinically, this translates into a 9-13% decreased risk of CVD and 40% reduced risk of myocardial infarction in carriers of the minor allele [22, 25]. Despite these robust associations, variants at the *SORT1* 1p13 locus account for only 1-5% of the variation in fasting LDL-C levels, suggesting an element of residual CVD risk attributed to alternative factors [23]. We speculate that this may be partially attributed to the fact that associations between *SORT1* and LDL-C have only been investigated in the fasting state [21, 23, 24, 30, 37, 47, 172].

3.1.3 Postprandial Lipaemia as a Modifier of Cardiovascular Disease Risk

Based on a western-style eating pattern, humans spend the majority of the time in the postprandial state; as such, the merits of using fasting levels to assess CVD risk and resting conditions are challenged [12, 13, 130]. Postprandial lipaemia is characterized by an influx of intestinally- and hepatically-derived triglyceride-rich lipoproteins, including VLDL, chylomicrons (CM) and CM-remnants [14, 15, 117]. These particles are atherogenic in their own right, indicating that prolonged exposure to postprandial lipaemia is an important contributor to

atherogenesis [16, 117]. Furthermore, accumulation of CM particles coincides with VLDL production in the liver, exacerbating postprandial lipaemia [66] .

3.1.4 Purpose

The purpose of the present study was to investigate whether presence of the minor allele (C) at the rs646776 SNP near the *SORT1* gene was associated with postprandial lipid and lipoprotein responsiveness in healthy young adults, in whom the association of *SORT1* is stronger compared to that in older adults with diagnosed metabolic disease [21, 33] due to fewer clinical confounding variables [23, 172]. Postprandial lipaemia was characterized using an oral fat tolerance test (OFTT) in combination with lipoprotein profiling via nuclear magnetic resonance (NMR) spectroscopy.

3.2 Materials & Methods

3.2.1 Participants and Recruitment

Subjects who completed the AIMM Young Study at the University of Calgary [24] were recruited via email and invited to participate. The AIMM Young study is a multicentre study investigating associations between risk factors for chronic disease and genetic variants in healthy, young adults. The AIMM Young study employs four testing sites located at Howard University, University of Massachusetts Amherst, East Carolina University, and the University of Calgary. At the time of this study, 197 individuals had completed the AIMM Young Study at the Calgary site, of whom 175 were recruited based upon prior consent for re-contact.

Interested individuals were phone-screened to assess eligibility and included if they: (1) had completed the initial AIMM Young testing protocol in its entirety; (2) were between the ages of 18 and 35 y at the time of testing; (3) post-puberty; (4) were willing and able to provide informed consent; (5) free of any dietary restrictions preventing test meal consumption.

Participants were excluded from study participation if they demonstrated: (1) evidence of clinically relevant systemic disease associated with disorders of glucose metabolism; (2) chronic use of glucocorticoid or appetite suppressants; (3) concomitant use of drugs known to alter glucose or lipid metabolism (i.e. fenofibrates, metformin, thiazolidinediones, xanthine oxidase inhibitors etc.); (4) inability to provide fasting and/or postprandial blood samples; (5) pregnancy; (6) menopause; (7) inability to provide informed consent; (8) previous diagnosis or treatment for any haematologic or oncologic disorder; (9) history or current treatment for an eating disorder; (10) current treatment for weight loss; (11) history of bariatric surgery; (12) history of a neurosurgical procedure; (13) participation in another clinical trial involving an investigational drug; (14) age <18 or >35 y at the time of recruitment; (15) inability to consume the test meal.

Ethical approval for this study was granted by the University of Calgary Conjoint Health Research Ethics Board in accordance with the Declaration of Helsinki. Informed consent was obtained from all subjects prior to participation (Ethics ID# 24813).

3.2.2 Anthropometric and Blood Pressure Measurements

Height and weight were measured using a calibrated balance beam scale. Body mass index (BMI) was calculated by dividing weight (kg) by height squared (m^2). Percent body fat was determined via dual energy x-ray absorptiometry (Hologic QDR 4500A scanner, Hologic Inc., Waltham, MA, United States). Heart rate, systolic and diastolic blood pressure were measured manually from the left arm while the participant was seated.

3.2.3 Diet Analysis

All participants submitted a 3-day dietary record completed over the course of two weekdays and one weekend-day prior to the test day. Three-day averages of macronutrient intake were determined using FoodWorks 14.0 software (The Nutrition Company, Long Valley, NJ).

3.2.4 Oral Fat Tolerance Test

Following an overnight fast (8-12 h), participants were given a high-fat meal composed of two breakfast sandwiches, one hash-brown and orange juice from a major fast-food chain. The meal totalled 91 g fat, 500 mg of cholesterol and 1490 kcal (Appendix A). 55%, 32% and 14% of the meal's energy content was attributed to fat, carbohydrates and protein, respectively. This meal was selected as a very high dose of fat (>80 g) to exaggerate postprandial lipaemia [13], therefore decreasing the chance of committing a type 2 error.

Participants consumed the meal within 30 min; afterwards water was permitted *ad libitum,* no other food or beverages were consumed for the next 8 h. Blood samples were obtained at fasting and 2, 4, 6 and 8 h postprandially, where time=0 was defined as meal initiation (Figure 3.1). This testing duration reflected the consensus that elevated triglycerides persist into the last postprandial phase (5-8 h) [13]. Immediately following the 8 h blood draw, participants received a fat-exclusion meal, which contained 3.9 g fat, 0 mg cholesterol and 603 kcal (Appendix A). 6%, 84% and 10% of the meal's energy content was attributed to fat, carbohydrates and protein, respectively. Participants consumed this meal in its entirety, allowing for a second 8-12 h fast before returning for a 24 h blood draw. Participants remained sedentary for the entire 24 h testing duration, in addition to 48 h prior.

Figure 3.1 Schematic of experimental procedures and timeline

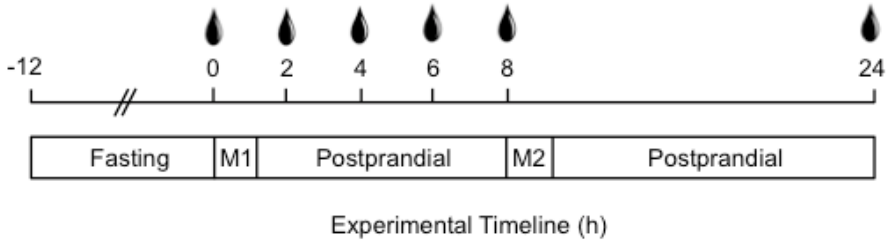

Figure 3.1 M1, high-fat test meal; M2, fat exclusion meal

3.2.5 Blood Sample Collecting and Handling

Approximately 6 ml of venous blood was collected at each time point from the antecubital fossa using LipoScience Lipoprotein Analysis (serum separator) tubes by a trained phlebotomist. Following collection, whole blood samples were rested for 30 min at room temperature. They were then centrifuged for 15 min at 3000 rpm at room temperature to separate the serum. A portion of serum was pipetted off and stored at -80^{O}C for future analyses. Remaining samples were refrigerated (2-8^{O} C) and shipped to LipoScience (Raleigh, NC) via overnight courier, using a unique identification number that did not disclose genotype.

3.2.6 Lipid and Lipoprotein Analysis

3.2.6.1 Nuclear Magnetic Resonance Spectroscopy

Fasting and postprandial blood samples were subject to comprehensive lipoprotein analysis using NMR spectroscopy (LipoScience, Raleigh, NC). Lipoprotein subclass particle concentrations including small- and large- LDL; small-, medium- and large- high-density lipoprotein (HDL); intermediate-density lipoprotein (IDL); and small-, medium-, and large-VLDL were determined using a 400-MHz NMR spectrometer (Bruker BioSpin Corporation, Billerica, MA) and proprietary analysis software (LipoScience, Raleigh, NC).

NMR spectroscopy is a fully automated method to quantify several subclasses of lipoproteins at once, as it does not require physical separation of the subclasses [63]. It is based upon the principle that VLDL, LDL and HDL subclasses of differing sizes emit unique NMR signals (Figure 3.2), whose amplitudes are directly proportional to the number of particles composing that subclass [63]. Methyl signals are produced from the terminal methyl group protons of phospholipid, unesterified cholesterol, CE and TG, which cumulatively contribute to the signal emitted for each lipoprotein subclass [63]. While there is no way to distinguish the

methyl signals from phospholipids, cholesterol or TG, the aggregate signal from each lipoprotein is distinct due to unique magnetic properties [63]. Specifically, compared to smaller lipoproteins, larger lipoproteins emit signals with a distinctive shape and higher frequency [63].

Figure 3.2 NMR signals depicting characteristic methyl shifts of lipoprotein subclasses

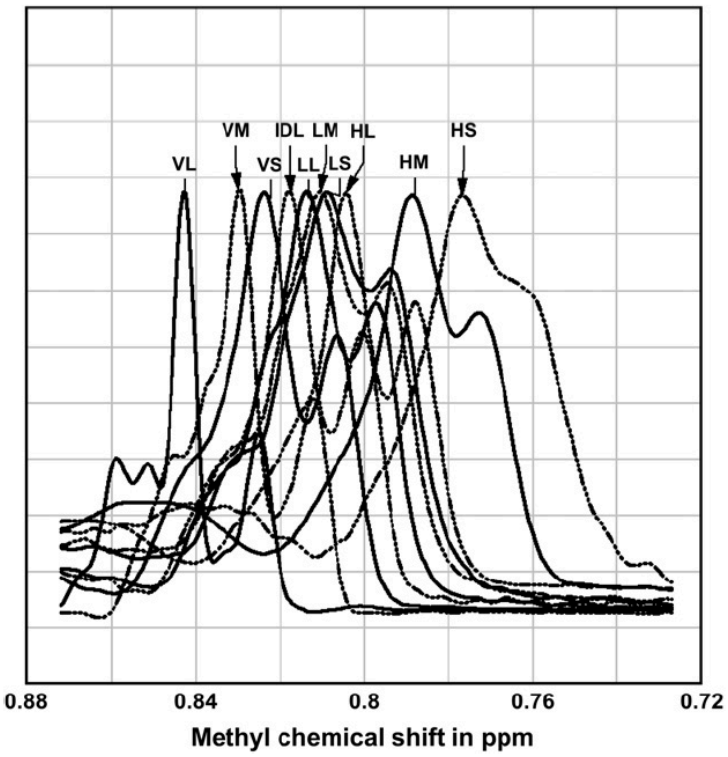

Figure 3.2 [63] VL, large VLDL; VM, medium VLDL; VS, small VLDL; IDL; LL, large LDL; LM, medium LDL; LS, small LDL; HL, large LDL; HM, medium HDL; HS, small HDL.

The amplitude of each subclass signal is determined utilizing computer software that compares the signal to a library of over 30 reference standard methyl signals representing every VLDL, LDL and HDL subclass likely to be observed in normolipidaemic or dyslipidaemic individuals [63]. Given the relative similarity of the frequency and shape of each of the signal amplitudes, subclasses are grouped into a smaller number of categories (i.e. large, medium and small) to minimize the risk of misclassification [63]. Signal amplitudes are then converted to

concentration based upon subclass-specific conversion factors derived from the reference library [63]. This is possible due to the relatively constant lipoprotein structure and associations between particle diameter and total core lipid content [63]. As cholesterol esters and triglycerides each contribute three methyl groups to the signals, NMR is able to reliably quantify particle number despite fluctuations in triglyceride and cholesterol ester content [63].

Weighted average VLDL, LDL and HDL particle sizes, represented as diameter (nm), were calculated based upon each subclass' relative mass percentage and intensity of the NMR signal by LipoScience [63]. Likewise, total triglyceride, VLDL-TG, HDL-C and total cholesterol levels were estimated from lipoprotein particle concentrations and established conversion factors [63], which closely correlate to chemically-derived values ($r > 0.9$) [63]. Finally, nephelometric apolipoprotein B immunoassays were used to determine the concentration of apolipoprotein B (AU680s Beckman Coulter, Brea, CA). The use of NMR spectroscopy for postprandial lipoprotein quantification has been reported previously [174].

3.2.6.2 Quantification of Postprandial Lipaemia

Total area under the curve (tAUC) was calculated using the trapezoidal rule by Microsoft Excel 2007. tAUC represents the entire area subtended by the data points and the x-axis. Given that tAUC is highly correlated with fasting concentration [175], it was considered to be more reflective of overall exposure to a given variable, rather than postprandial changes. tAUC was determined as follows in Figure 3.3.

Figure 3.3 tAUC as estimated by the trapezoidal rule

$$tAUC = [\tfrac{1}{2}(C_1 + C_2)(t_2 - t_1)] + [\tfrac{1}{2}(C_2 + C_3)(t_3 - t_2)] + [\tfrac{1}{2}(C_n + C_{n+1})(t_{n+1} - t_n)] \ldots$$ [2] [176]

[2] Where C= concentration at a given time point, and t= time

Incremental area under the curve (iAUC) was calculated using the trapezoidal rule by MATLAB 8.1. iAUC represents the area bound by the fasting concentration and the curve estimated from the individual data points. In contrast to tAUC, iAUC is relatively independent from fasting concentration, and thus regarded as a superior measurement of postprandial response [175, 177].

3.2.7 Genotype Determination

Genotype at the rs646776 variant was accessed from previous testing as part of the AIMM Young study at the University of Calgary [24]. This variant was selected based upon its relatively large effect size and abundance of lipid-associated phenotypes [29], in addition to the strong association with LDL-C that we previously observed in this cohort [24]. No other variants were considered in the development, execution or analysis of this study.

Peripheral blood samples were collected in ethylene diamine tetra-acetic acid (EDTA) anticoagulant tubes, which were stored at 2-8^{o}C for a maximum of one week prior to analysis at the Children's National Medical Centre in Washington, DC. Genotype at the rs646776 variant was determined using TaqMan allelic discrimination assay [178]. This technique employs two unique fluorogenic probes, each of which bind to an allele sequence during a 5' nuclease assay, allowing for polymerase chain reaction (PCR) amplification and detection to occur simultaneously [178]. By including an oligonucleotide probe in addition to the necessary forward and reverse primers, the probe hybridizes to the target sequence during the PCR annealing step [178]. This probe is subsequently detected and cleaved by *Taq* DNA polymerase, resulting in a cleavage site that is bound by a fluorogenic probe [178]. Probes are labelled with allele-specific fluorescent reporter dyes, VIC or FAM, thus allowing for the detection of alleles in biallelic

systems [178]. Increased fluorescent signal of one dye, but not the other, indicates homozygosity for that specific allele, whereas a combination of signals represents heterozygosity [178].

Genetic testing introduces the risk of misidentification resulting from sample mishandling and errors from the genotyping process [179]. To mitigate this risk, the Hardy-Weinberg Equilibrium is calculated to identify genotyping error within large data sets using a χ^2 test to compare the observed allelic frequencies to those expected from the population [179]. The Hardy-Weinberg Equilibrium for the rs646776 variant was calculated for this population previously and observed to be in concordance with the expected frequency [24]. For analyses, participants were stratified based upon whether they were carriers of the minor, protective allele (TC/CC) or homozygous for the major, risk allele (TT), in accordance with previous reports of this variant [21, 23, 24, 30, 37, 47, 172].

3.2.8 Statistical Analysis

All statistical analyses were performed using SPSS Statistics, version 20 (IBM, Armonk, NY). All data are presented as mean ± SEM. A list of *SORT1* associated metabolites considered for analyses are shown in Table 3.1. All studies listed reported fasting lipoproteins, with the exception of Chasman et al. [29] who reported associations in a combined cohort of fasting (72%) and non-fasting individuals. Normality of distribution was tested by the Shapiro-Wilk test; non-normally distributed data were transformed accordingly. Comparisons were made via two-way ANOVA, using sex and genotype at the rs646776 variant as fixed factors. The Mann Whitney-U test was used to determine differences in 8 h iAUC between genotypes. Statistical significance was considered $p<0.05$ for all analyses.

Table 3.1 *SORT1* 1p13 locus associated phenotypes

Lipid Associated Phenotype	Variant	Design	Reference
VLDL & CM Particles (Total)	rs646776	GWAS	[29, 47]
Large VLDL & CM Particles	rs646776	GWAS	[47]
Medium VLDL Particles	rs646776	GWAS	[47]
Small VLDL Particles	rs646776	GWAS	[29, 47]
VLDL Mean Size	rs646776	GWAS	[29]
IDL Particles (Total)	rs646776	GWAS	[29]
LDL Particles (Total)	rs646776	GWAS	[29]
Large LDL Particles	rs646776	GWAS	[29, 47]
Small LDL Particles	rs646776	GWAS	[29, 47]
LDL Mean Size	rs599839	GWAS	[40]
Triglyceride (by NMR)	rs646776	GWAS	[29]
Cholesterol (Total)	rs646776	GWAS	[44]
	rs629301	GWAS	[30-32]
	rs599839	GWAS	[40, 41]
LDL-Cholesterol	rs646776	GWAS	[21, 23, 29, 44-46]
	rs599839	GWAS	[21, 36-43]
	rs12740374	GWAS	[33-35]
	rs629301	GWAS	[30-32]
	rs4970833	GWAS	[29]
	rs611917	GWAS	[28]
	rs629301	GWAS	[27]
Apolipoprotein B	rs4970833	GWAS	[29]
	rs599839	GWAS	[40, 41]
	rs646776	GWAS	[46]

3.3 Results

3.3.1 Subject Characteristics

Table 3.2 Subject characteristics according to genotype at rs646776

	Major Alleles (TT) n=15	**Minor Allele** (TC/CC) n=15
Age (y)	23.20 ± 0.89	23.93 ± 1.08
Height (m)	171.68 ± 2.33	172.01 ± 1.83
Weight (kg)	69.43 ± 4.08	70.24 ± 3.80
Body Mass Index ($kg \cdot m^{-2}$)	23.43 ± 1.12	23.52 ± 0.83
Body Fat (%)	18.14 ± 1.56	18.97 ± 1.25
Resting Heart Rate ($beats \cdot min^{-1}$)	64 ± 3.07	64 ± 2.76
Systolic Blood Pressure (mmHg)	110 ± 2.84	108 ± 2.60
Diastolic Blood Pressure (mmHg)	69 ± 2.86	65 ± 1.49
VO_2peak ($ml \cdot kg^{-1} \cdot min^{-1}$)	49.31 ± 1.93	47.89 ± 1.51
Grip Strength (kg)	91.47 ± 5.31	86.23 ± 5.16
Food Intake ($kcal \cdot day^{-1}$)	2238 ± 167	2460 ± 221
Carbohydrates (%)	50 ± 2	48 ± 2
Fat (%)	31 ± 1	32 ± 2
Protein (%)	18 ± 1	18 ± 1

Thirty participants completed the OFTT study as outlined. 80% of participants were Caucasian; remaining participants were Asian (16.7%) and Egyptian (3.3%). Males and females were equally stratified by genotype (TT: 8 M, 7 F; TC/CC: 7 M, 8 F) into two groups with comparable subject characteristics ($p>0.05$). No genotype x sex interactions were observed (Appendix B).

Table 3.3 Prevalence of non-optimal body composition and fasting lipids

	All % (n=30)	Males % (n=15)	Females % (n=15)	Criteria
BMI	26.7 (8)	53.3 (8)	0 (0)	> 25 ($kg \bullet m^{-2}$) †
% Body Fat	13.3 (4)	6.7 (1)	20 (3)	Females: > 25.3%† Males: > 19.4%†
Total Cholesterol	6.7 (2)	6.7 (1)	6.7 (1)	> 5.17 mmol/L ‡
Total Triglycerides	6.7 (2)	13.3 (2)	0 (0)	> 1.70 mmol/L ‡

†American College of Sports Medicine [180]

‡ National Cholesterol Education Program: Adult Treatment Panel III Guidelines [69]

Seven participants (7 males) had higher than optimal BMI (>25 $kg \cdot m^2$) and four participants (3 female, 1 male) had higher than optimal percent body fat (>25.3% for females, >19.4% for males) [180]. Two males displayed evidence of fasting hypertriglyceridemia, and 2 participants (1 male, 1 female) had elevated total cholesterol according to the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) Guidelines [69].

3.3.2 Fasting Lipoproteins

Table 3.4 Baseline lipid profiles according to genotype at rs646776

	Major Alleles (TT) n=15	**Minor Allele** (TC/CC) n=15
VLDL & CM Particles (nmol/L)	48.81 ± 5.07	30.05 ± 2.99*
Large VLDL & CM Particles (nmol/L)	2.77 ± 0.65	2.17 ± 0.56
Medium VLDL Particles (nmol/L)	21.94 ± 3.63	14.48 ± 1.75
Small VLDL Particles (nmol/L)	24.10 ± 3.88	13.37 ± 2.34*
Mean VLDL Size (nm)	47.67 ± 1.46	51.26 ± 1.97
IDL Particles (nmol/L)	52.53 ± 10.71	75.67 ± 14.42
LDL particles (nmol/L)	969.87 ± 61.73	957.20 ± 53.42
Small LDL Particles (nmol/L)	332.40 ± 64.27	224.07 ± 50.20
Large LDL Particles (nmol/L)	584.93 ± 40.15	657.67 ± 62.36
Mean LDL Size (nm)	21.20 ± 0.11	21.30 ± 0.12
Cholesterol (mmol/L)	4.31 ± 0.15	4.29 ± 0.16
Triglycerides (mmol/L)	1.12 ± 0.08	0.97 ± 0.07
VLDL & CM Triglyceride (mmol/L)	0.76 ± 0.08	0.59 ± 0.08*
Apolipoprotein B (g/L)	0.82 ± 0.04	0.82 ± 0.03

* $p<0.05$ for TT vs. TC/CC.

Fasting (8-12 h) lipid and lipoprotein concentrations as determined via NMR from serum obtained from antecubital venipuncture immediately prior to OFTT. Data are mean ± SEM. No significant interactions between genotype x sex were observed for baseline lipoprotein measures (Appendix B).

Figure 3.4 Fasting VLDL and CM particles according to genotype at rs646776

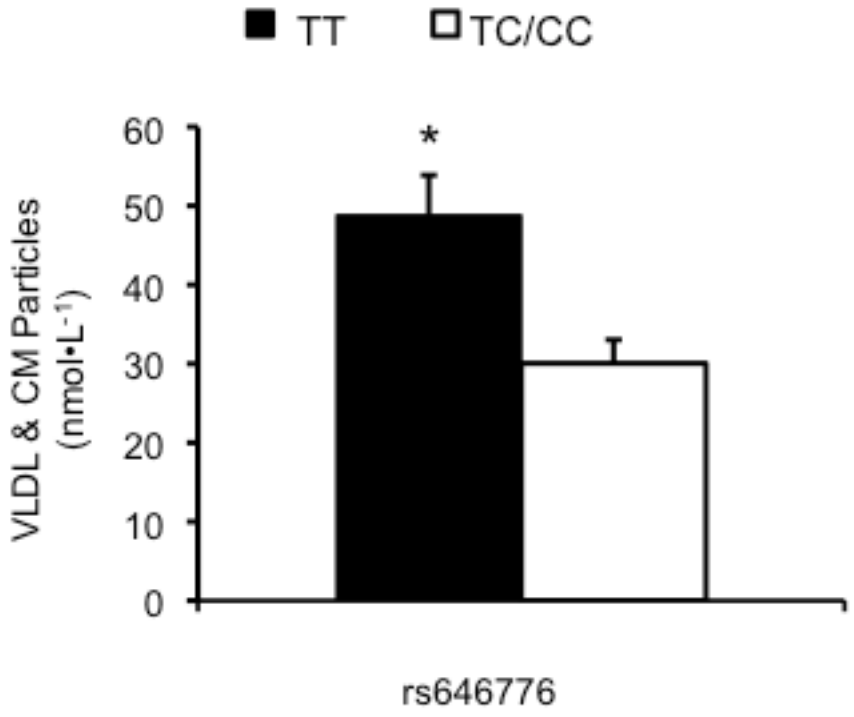

Figure 3.4 Combined fasting VLDL and CM particles concentrations were lower in carriers of the minor allele compared to TT homozygotes (30.1 ± 3.0 nmol/L vs. 48.8 ± 5.1 nmol/L, $p<0.01$). These individuals displayed nominally reduced fasting small VLDL particles (13.4 ± 2.3 nmol/L vs. 24.1 ± 3.9 nmol/L, $p=0.05$), and had comparable large VLDL ($p=0.28$) and medium VLDL ($p=0.33$) (Table 3.4). All values represent n=15 per group. * $p<0.05$ TC/CC vs. TT.

Figure 3.5 Fasting VLDL and CM triglyceride according to genotype at rs646776

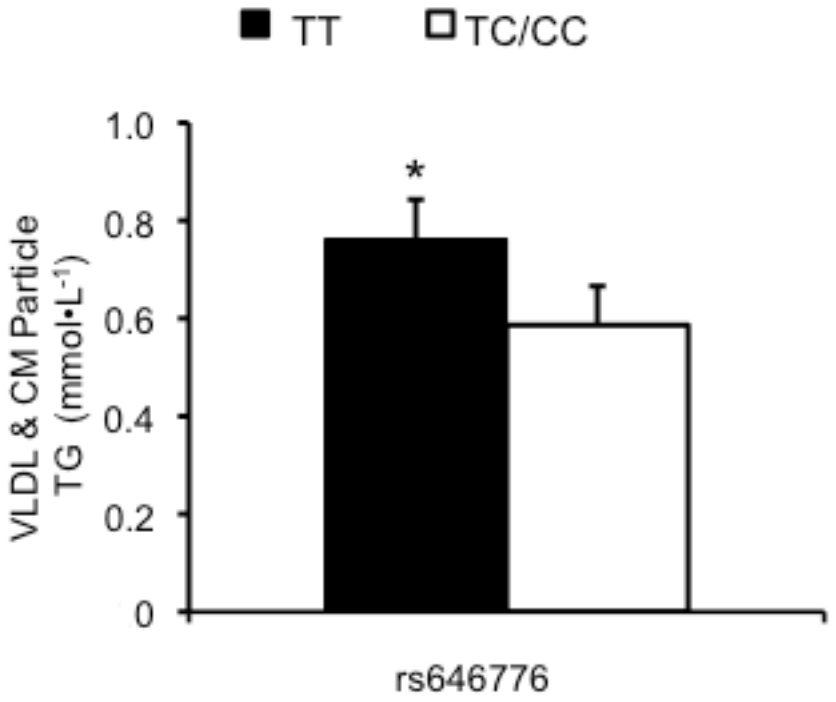

Figure 3.5 Individuals carrying at least one copy of the minor, protective allele demonstrated nominally lower fasting VLDL and CM triglyceride concentration (combined) ($p=0.05$). All values represent n=15 per group. * $p<0.05$ TC/CC vs. TT.

Figure 3.6 Fasting triglyceride according to genotype at rs646776

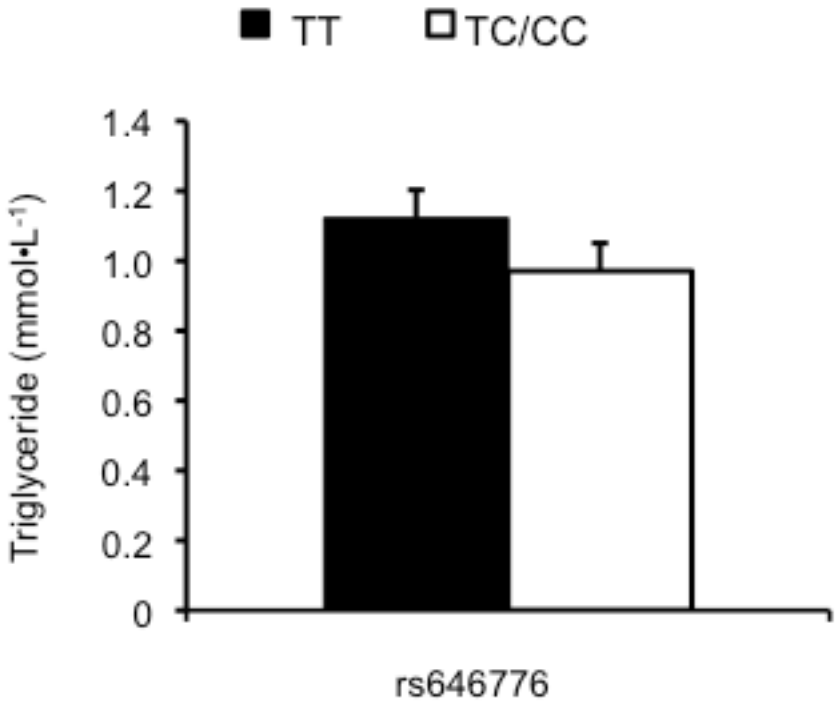

Figure 3.6 No significant difference in fasting triglyceride concentration was observed between carriers of the minor allele and TT homozogytes (p=0.12). All values represent n=15 per group.

3.3.3 Postprandial Lipoproteins

Figure 3.7 Postprandial VLDL and CM particles according to genotype at rs646776.

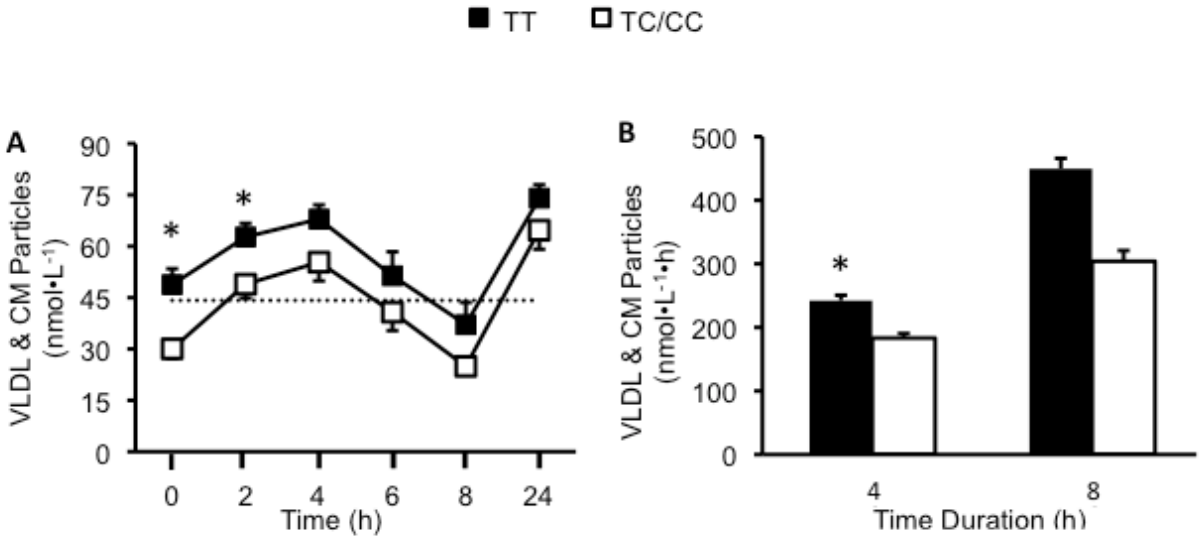

Figure 3.7 (A) VLDL & CM particle concentration over 24 h testing duration. Dashed line denotes the mean VLDL & CM particle concentration for carriers of the minor allele (TC/CC) over the 24 h duration. (B) tAUC for total VLDL & CM particles over 4 h and 8 h durations. All values represent n=14-15 per group. * $p<0.05$ TC/CC vs. TT.

TC/CC genotypes displayed lower 4 h tAUC ($p=0.02$), in addition to reduced mean concentration of VLDL and CM particles over the entire 24 h period (44.2 ± 3.1 nmol/L vs. 57.0 ± 4.5 nmol/L, $p=0.03$) (Appendix B). Carriers of the minor allele (TC/CC) had lower concentrations of circulating VLDL and CM particles at 2 h postprandial ($p=0.04$) compared to TT homozygotes. In contrast to tAUC, there were no significant differences in iAUC when stratified by genotype (Appendix B).

Figure 3.8 Postprandial VLDL and CM triglyceride according to genotype at rs646776.

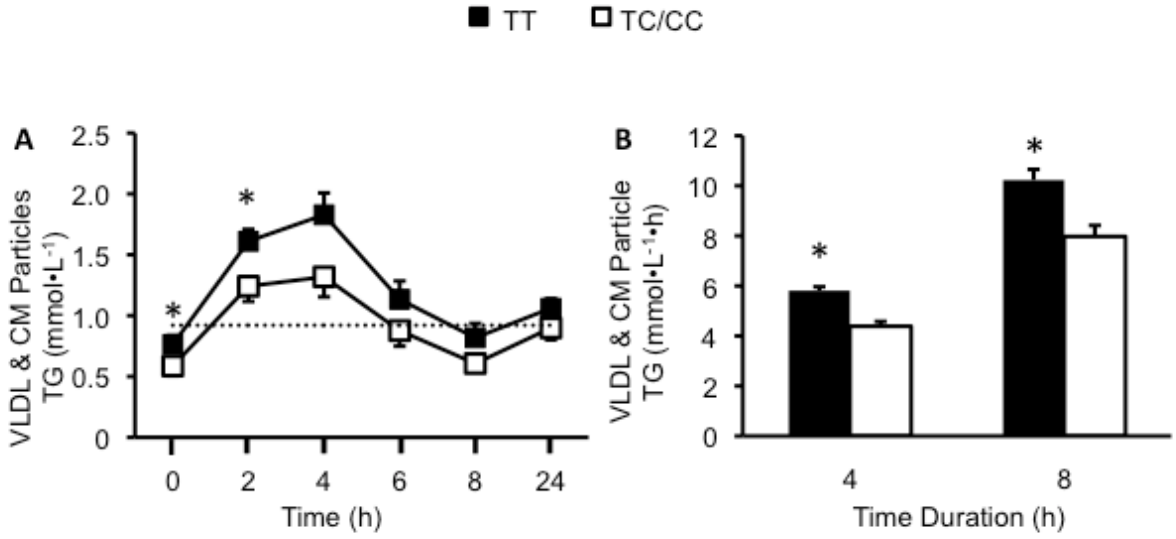

Figure 3.8 (A) Total VLDL & CM triglyceride concentration over 24 h testing duration. Dashed line denotes the mean VLDL & CM triglyceride concentration for carriers of the minor allele (TC/CC) over the 24 h duration. (B) tAUC for VLDL & CM triglyceride over 4 h and 8 hr durations. All values represent n=14-15 per group. * $p<0.05$ TC/CC vs. TT.

Individuals with the TC/CC genotype demonstrated decreased postprandial VLDL and CM triglycerides, with respect to the 4 h and 8 h tAUC ($p=0.03$ and. $p=0.02$, respectively). In contrast to tAUC, there were no significant differences in postprandial iAUC for lipoproteins when stratified by genotype (Appendix B).

Figure 3.9 Postprandial triglycerides according to genotype at rs646776

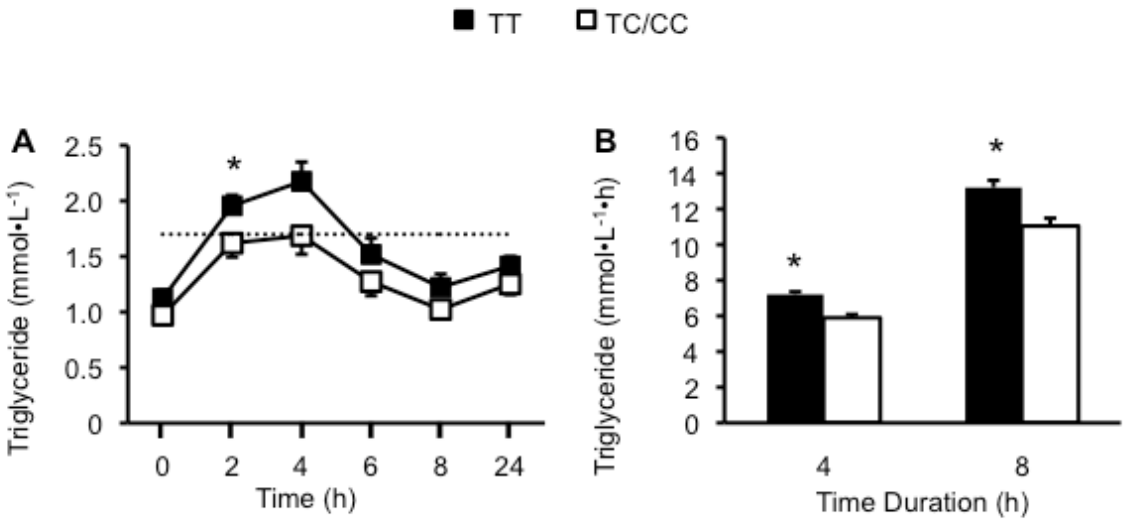

Figure 3.9 (A). Total plasma triglycerides over 24 h testing duration. Dashed line denotes National Cholesterol Education Program Adult Treatment Panel III Guideline for fasting TG [69]. (B). tAUC for total plasma TG over 4 h and 8 h durations. All values represent n=14-15 per group. * $p<0.05$ TC/CC vs. TT.

Carriers of the minor allele exhibited an attenuated postprandial total TG response to the OFTT compared to the TT homozygotes. This was evident in the 20-30% lower 4 h and 8 h tAUC compared to TT homozygotes. Additionally, postprandial TG concentrations displayed by TT homozygotes as a group exceeded the NCEP ATP III clinical guideline for fasting TG of 1.69 mmol/L [69]. Such observations are apparent in the 2 h (1.96 ± 0.10 mmol/L, $p=0.02$) and 4 h (2.18 ± 0.19 mmol/L, $p= 0.02$) postprandial concentrations in TT homozygotes. In contrast, TC/CC individuals did not exceed this clinical threshold (1.69 mmol/L [69]).

3.4 Discussion

3.4.1 Summary of Key Findings

Objectives of the present study were to investigate whether genotype at the rs646776 variant of 1p13 locus is associated with differences in postprandial lipaemia in young adults. Major findings show that individuals heterozygous or homozygous for the minor, protective allele display: (1) reduced fasting levels of VLDL and CM particles (combined); (2) decreased postprandial VLDL and CM particles; (3) decreased postprandial VLDL and CM triglyceride content (combined) and total triglycerides; and (4) no differences in apolipoprotein B or LDL particle subclasses. Overall, carriers of the minor allele exhibited attenuated responses to the OFTT as seen in decreased exposure to atherogenic triglyceride-rich lipoproteins. Combined, these findings provide insight into the disparate risk for CVD and myocardial infarction in carriers of the minor, protective allele compared to homozygotes for the major, risk allele.

3.4.2 Fasting Lipoproteins

The 1p13 locus gained attention following its initial identification and robust association with coronary artery disease, and later LDL-C, through genome wide association studies [22]. Utilizing comprehensive NMR profiling, we show an association between the minor, protective allele at the *SORT1* 1p13 locus and decreased VLDL particles in humans at fasting. This parallels previous findings wherein the 1p13 locus was associated with NMR derived total VLDL particles in a large-scale genome wide association study [29]. Presence of the minor allele at the 1p13 locus is associated with liver-specific increases in sortilin protein and *SORT1* mRNA expression in humans [37, 47]. Our findings provide experimental evidence in humans in support of the mechanism of sortilin action proposed by Musunuru et al. [47], wherein sortilin hepatic overexpression decreases VLDL secretion through enhanced pre-secretory degradation.

3.4.3 Postprandial Lipoproteins

Baseline reductions in VLDL and CM particle numbers persisted into the postprandial state in carriers of the minor allele (TC/CC). These individuals exhibited attenuated tAUC and mean concentration over the duration of the study, however no difference in the iAUC compared to TT homozygotes. Given that tAUC is highly dependent upon fasting lipoprotein concentrations, these results suggest that the differences observed in the total exposure to VLDL and CM particles postprandially are reflective of fasting values, rather than indicative of a unique response. Regardless, elevated fasting and postprandial circulating VLDL and CM particles in major allele homozygotes are detrimental in their own right, as they are associated increased risk for type 2 diabetes, obesity, dyslipidemia and progression of atherosclerotic plaques [169].

Reductions in VLDL and CM particle number were also reflected in the triglyceride content of these particles, as carriers of the minor allele exhibited a decreased postprandial response compared to TT homozygotes. It was expected that elevations in VLDL and CM triglyceride concentration would coincide with increased particle numbers, particularly since particle size was comparable between the two groups. Prolonged exposure to triglyceride-rich lipoproteins promotes triglyceride enrichment of LDL and HDL, which facilitates subsequent hydrolysis and production of small, dense LDL particles, the most detrimental of the LDL subclasses [50, 108]. Furthermore, the inter-lipoprotein exchange of lipids during the postprandial state is primarily propelled by endogenously synthesized VLDL particles secreted from the liver rather than intestinally-derived CMs, providing further evidence pointing towards sortilin as a player in postprandial lipaemia [110].

We found that total triglycerides were lower in carriers of the minor allele genotypes relative to TT homozygotes. Unlike VLDL and CM particles, these differences were not

apparent at fasting, but at two hours postprandially, a time point which has been identified as a decisive predictor of future coronary artery disease risk [76]. Furthermore, carriers of the minor allele contrasted TT homozygotes as they had triglyceride concentrations that exceeded the National Cholesterol Education Program Adult Treatment Panel III clinical fasting guidelines at the 2 and 4 h time points [69]. Given that non-fasting triglyceride is recognized as a strong, independent risk factor for CVD [89, 92, 94], carriers of the minor allele exhibited a less detrimental postprandial triglyceride response compared to TT homozygotes. These findings may, in part, explain the disparate risk of CVD between carriers of the minor allele and TT homozygotes. Furthermore, these findings in addition to a novel association between *SORT1* and triglycerides recently reported using a multi-phenotype Bayesian strategy [162] may represent an additional role of the *SORT1* gene in lipid metabolism.

3.4.4 Reconciling Disparate Findings

No association between *SORT1* and apolipoprotein B or LDL subclasses was observed at fasting or during the postprandial period. As apolipoprotein B-100 is the major structural protein of VLDL and LDL [17], we expected elevations in apolipoprotein B-100 to coincide with those observed in VLDL. Furthermore, these observations were surprising given *SORT1*'s strong associations with LDL-C and total cholesterol. Most previous investigations have been founded upon the Friedewald calculation, wherein LDL-C is calculated as a function of triglyceride concentration, which may provide insight into the results presented [181]. This highlights the value of NMR-based metabolomics analysis [182, 183]. Discrepant findings in LDL particles may also be attributed to the population studied, as younger adults typically exhibit lower variance and fewer subjects with abnormal values, therefore potentially lowering the statistical power of the study [23].

3.5 Conclusion

Using an OFTT, we identified the genetic variant rs646776 in the 1p13 locus, a SNP that has been strongly associated with LDL-C, coronary artery disease and myocardial infarction [21, 28, 30, 158, 159, 173], to be a modifier of fasting lipid profile and postprandial lipaemia in fit, healthy, young adults. These results provide further support for sortilin's role as a regulator of lipid metabolism. This strengthens its potential value as an identification tool for early screening programs, and highlights the merits of future investigation into sortilin as a target for therapeutic intervention in an effort to alleviate the burden of CVD.

Chapter Four: Discussion

The purpose of the present study was to investigate whether presence of the minor, protective allele at the rs646776 variant of the 1p13 locus near the *SORT1* gene is associated with postprandial lipaemia in healthy, young adults. In doing so, we simultaneously sought to gain insight into the mechanism by which genotype at this locus alters phenotype. We identified carriers of the minor allele to exhibit attenuated postprandial lipaemia, providing evidence of decreased exposure to atherogenic lipoproteins and relative protection against CVD compared to TT homozygotes.

4.1 Strengths and Limitations

4.1.1 Study Population

Examining relationships between genotype and phenotype is particularly valuable in young adults for several reasons. Firstly, genetic loci discovered in young adults are associated with early onset disease, rather than clinically manifested disease as is observed in older adults [23]. As such, elucidation of genetic risk factors for CVD in young adults offers the potential for genetics-based identification strategies; allowing for the implementation of preventative interventions prior to clinical onset of disease [23]. Secondly, genetic associations are more robust in young adults due to the absence of confounding variables such as age, exposure to environmental factors, disease or treatment regimens [23]. In fact, evidence of a genotype-age interaction at the 1p13 locus has been presented, such that larger effect sizes are observable in young adults [23, 24, 172] and children [23], compared to older adults [21, 33]. This target population was selected to take advantage of the strength of the association between the 1p13 locus and lipoprotein phenotypes in young adults.

4.1.2 Methodological Considerations

4.1.2.1 Methods of Recruitment

By recruiting participants from those who had completed the AIMM Young study at the University of Calgary, we had the opportunity to access a wealth of previously collected data, including genotype, body composition and cardiorespiratory fitness. Genotype at the rs646776 variant was used to stratify participants for analyses; whereas other variables such as percent body fat and cardiorespiratory fitness were used to confirm homogeneity between the groups.

Although participants were recruited from the AIMM Young Study, not all participants were interested, while others did not respond. At the time of this study, 197 individuals had completed the AIMM Young Study at the University of Calgary, of whom 175 were recruited based upon prior consent for re-contact. Of those contacted, 76 expressed interest in the study, 58 were successfully phone screened, and 42 enrolled based upon interest and eligibility criteria. Therefore, a convenience sample was used wherein all volunteers who qualified based on eligibility criteria were included. Convenience samples are inherently limited due to self-selection bias, such that attributes of those who volunteer may differ from those who do not, undermining the generalizability of the findings [184].This represents a potential for bias, as participants who volunteer for research studies typically present superior motivation, activity level and overall positive attitudes towards health than those who do not enrol [184, 185].

4.1.2.2 Sample Size

Due to the rigorous nature of an OFTT study, a relatively small sample size (n=30) was employed. As such, the statistical power, or the probability that the null hypothesis will be rejected when the polymorphism is truly associated with the given phenotype [186], was weakened. A *post hoc* power calculation revealed that we were, in fact, underpowered to detect

significant differences in fasting lipoprotein values with the exception of VLDL & CM particles (β=0.89). As such, there is a chance that a type II error was committed, wherein we failed to reject a null hypothesis when it was truly false [184]. Similarly, we were underpowered to detect significant differences in postprandial lipoprotein responses. This was not surprising, given that postprandial lipaemia is highly variable [15], and increasingly so over longer time courses. An additional consequence to our relatively small sample size was the inability to control for confounding factors during analyses [187]. To partially amend concerns attributed to sample size, we investigated a SNP with robust associations to a well-characterized lipid phenotype, particularly in young adults. Furthermore, we selected a highly controlled sample of fit, healthy, young adults who were screened for covariates associated with lipid metabolism, such as age, sex, and disease.

4.1.2.3 Addressing Confounding Variables

One of the greatest challenges encountered in studying postprandial lipaemia is the variability of individual capacity to metabolize and clear lipids in response to fat ingestion depending upon environmental, physiological, pathological and genetic factors [15]. Moreover, confounding variables interact, making it extremely difficult to isolate the effects of one factor from those of another. As such, it is imperative to minimize the effects of these confounding variables through study design, analyses and inclusion/exclusion criteria.

Diet is a key environmental modifier of postprandial lipaemia, as certain constituents alter lipoprotein secretion and clearance of hepatically- and intestinally-derived lipoproteins and their remnants [15]. Diets high in saturated fatty acids [188, 189] and refined carbohydrates [190] are associated with exaggerated postprandial lipaemia; whereas those rich in n-3 polyunsaturated fatty acids [188, 189] and complex carbohydrates [190] are associated with

attenuated responses. Three-day dietary records were collected from all participants and electronically analyzed for energy and macronutrient content. This provided a comprehensive reflection of habitual nutrient intake [191, 192], which confirmed dietary homogeneity of carriers of the minor allele and TT homozygotes, thereby allowing comparisons between genotypes to be made.

Physical activity is another environmental determinant of lipid metabolism. An acute bout of physical activity reduces the extent of postprandial lipaemia by 24-35%, depending on intensity and energy expenditure [13, 193]. The mitigating effects of exercise persist up to 24 h, such that lipoprotein clearance from circulation is upregulated, decreasing transit time [13]. However, when individuals refrain from physical activity for two days or more, the beneficial effects are nullified, such that postprandial responses to an OFTT are similar between untrained, endurance trained and sprint trained individuals [194]. As such, participants were advised to refrain from structured physical activity for the 48 h prior, in addition to the 24 h testing duration to minimize the confounding effects of acute exercise and training status on postprandial lipaemia.

Physiological factors are also key determinants of postprandial lipaemia. Compared to young adults, older individuals exhibit augmented postprandial lipaemia [195]. Age was addressed by employing inclusion/exclusion criteria that limited participation to those 18-35 y. A unique feature of this study was the inclusion of males and females. Females typically exhibit attenuated postprandial lipid due to more rapid TG-rich lipoprotein clearance resultant from estradiol- and estrogen-mediated changes in LDL-R binding capacity and redistribution of the lipoprotein subfractions [13, 195]. Due to the relatively small sample size, subjects were not stratified based upon sex for analyses. Males and females were, however, equally distributed

between genotypes, alleviating concerns regarding sex as a confounding factor. Furthermore, no genotype-sex interactions were observed based upon two-way ANOVA.

Several pathological factors, including obesity, hypertriglyceridaemia, insulin resistance and type 2 diabetes modify the extent and duration of postprandial lipaemia [13]. Obesity is associated with three-fold greater postprandial hypertriglyceridaemia, independent of insulin resistance, metabolic syndrome, type 2 diabetes and atherogenic lipid profile [13]. Similarly, fasting hypertriglyceridaemia is associated with exaggerated postprandial lipaemia, marked by a four-fold increase in the half-life of circulating TG-rich lipoproteins [13]. One limitation to this study is that these two potent modifiers of postprandial lipaemia were not directly controlled for; although they were indirectly addressed by selecting a young, healthy population. Two participants exhibited fasting hypertriglyceridaemia and four had non-optimal percent body fat. As such, these factors may have confounded the observations presented. In the future, body composition and hypertriglyceridaemia may be controlled for using exclusion criteria or through multivariable analyses.

Postprandial lipaemia is under polygenic regulation, such that its phenotype is resultant from the cumulative effects of several interacting genes [15]. For the purposes of this study, we investigated the effects of a single SNP on postprandial lipaemia. This is a limitation for two reasons; firstly, it has been reported that individual gene variants account for only a small portion of variance in postprandial lipaemia [18], undermining the statistical power of the study. This limitation was partially amended by choosing the *SORT1* rs646776 polymorphism, as it has a relatively large effect size and high MAF [21, 22, 30]. Secondly, given that postprandial lipaemia is a polygenic trait, investigating a single SNP is a simplified scenario that fails to consider gene-gene interactions [18]. More realistic investigations should consider the effects of haplotypes on

postprandial lipaemia, however this will require extremely large sample sizes that extend beyond the scope of this project.

4.1.2.4 OFTT Composition

Several factors contributed to the decision to employ a mixed meal composed of breakfast sandwiches from a fast-food chain for the OFTT. Meal size and macronutrient composition, particularly fat content, are important determinants of postprandial lipaemia [13, 196]. A fat load of 91 g was used, as acute fat loads of 30-50 g are sufficient to elicit significant elevations in plasma TG in a dose-dependent manner; whereas very high doses (≥80 g) exaggerate postprandial lipaemia without dose-dependence [13]. There are two approaches to determining meal size for OFTTs; the first requires subjects to ingest equal amounts of energy/fat irrespective of body size, whereas the second standardizes fat content received per body weight or body surface area [196]. For this study, meal size was independent of body weight, as this approach is most practical and minimizes experimental errors [128, 196]. A mixed meal was selected, rather than a shake-based meal, as they elicit more representative gastric emptying, digestion, absorption and transit of nutrients [196]. A final consideration in test meal selection was its relative commonplace in western society, thus increasing the generalizability to "real life". In fact, one participant reported consuming the test meal the day prior to testing.

4.1.2.5 Multiple Testing Considerations

With technological advancements increasing the availability of genetic information and comprehensive biochemical analyses, multiple testing has become a major concern for genetic association studies [186, 197]. Millions of SNPs may be assessed simultaneously with respect to hundreds, if not thousands of variables, using state-of-the-art statistical packages. With each

additional test performed, the probability of committing a type I error is compounded, increasing the likelihood that the null hypothesis will be rejected when no true association exists between a SNP and a given phenotype [186, 197].

Multiple testing corrections aim to control for, or at least quantify, the probability of committing a type I error through two approaches [186]. Firstly, the number of tests performed may be limited [186]. For this study, we employed this method by addressing only traits previously associated with the 1p13 locus, and using techniques such as area under the curve to quantify postprandial lipaemia as a whole. Secondly, established corrections may be applied to adjust for the number of tests performed [186]. The Bonferroni correction is the simplest multiple-testing correction technique, however it is extremely conservative when considering correlated tests, such as interrelated lipoprotein subclasses [186]. Alternatively, controlling for the false discovery rate is less conservative, however it undermines the power to detect candidate-gene associations with moderate effect sizes [186]. As such, no multiple testing corrections were applied to the data presented, thus increasing the statistical power of the study. On the contrary, the likelihood of committing a type I error was magnified as a result of this decision.

4.1.2.6 Biochemical Tests

Comprehensive NMR spectroscopy was used to quantify the particle concentration and mean size for lipoprotein subclasses. This in depth approach was a major strength, as studies employing plasma TG analysis alone underestimate differences that may be observed in particle number, particle size and changes in lipid subfractions [13]. One limitation to this approach is that NMR differentiates between lipoprotein subclasses on particle size alone, and is therefore unable to distinguish between intestinally-derived CM and large VLDL particles of hepatic

origin [63, 198]. While we observed significant differences in total VLDL and CM particle concentration, we were unable to conclude whether these findings were driven by CM, VLDL, or both. Given the *SORT1*'s previously suggested role as a regulator of VLDL [47, 166] secretion, in addition to significant differences observed in small VLDL particles, it may be speculated that the disparities are VLDL-driven. However, future investigations should clarify the relationship, as this information could have ramifications in further elucidating the specific mechanism by which sortilin exerts its action during postprandial lipaemia.

4.2 Global Interpretation and Discussion

4.2.1 Summary of Key Findings

The objective of the present study was to investigate whether genotype at the rs646776 variant of the 1p13 locus is associated with postprandial lipaemia in young adults. Major findings indicate that individuals heterozygous or homozygous for the minor, protective allele display: (1) a less atherogenic lipid profile at fasting marked by reduced levels of VLDL and CM particles; (2) decreased exposure to VLDL and CM particles, and TG postprandially; (3) no differences in apo B or LDL particle subclasses at fasting or postprandially. Combined, these findings provide insight into the disparate risk for CVD and MI based upon genotype at the 1p13 locus.

4.2.2 Evidence of Protection Against Atherogenic Dyslipidaemia

Elevated fasting VLDL and CM particles are hallmark features of atherogenic dyslipidaemia, a clustering of lipid traits marking a more detrimental phenotype associated with type 2 diabetes, insulin resistance and atherosclerosis [169, 170]. Additional characteristics include elevated fasting small, dense LDL particles, TG, VLDL, apo B, and decreased HDL-C [199]. In the present study, carriers of the minor allele exhibited reduced concentrations of total

VLDL and CM particles, and tended to have decreased concentrations of small, dense LDL along with decreased TG. This observation is consistent with previous reports, wherein heterozygosity or homozygosity for the minor alleles at this variant was associated with decreased VLDL and LDL particles, particularly smaller subfractions, at fasting [29, 47]. VLDL particles are direct precursors to LDL particles [61], which have been recognized as the "single most predictive CVD risk factor" due to their interference with the endothelial wall and involvement in atherogenesis [19, 71]. Increased exposure to TG-rich lipoproteins, such as VLDL, promotes the transfer of CE from LDL in exchange for TG [108]. TG enriched LDL particles are a favourable target for subsequent hydrolysis by hepatic lipase and LPL, facilitating production of small, dense LDL particles, the most detrimental of the LDL subclasses [50, 108]. Based on these findings, it is apparent that genotype at the 1p13 locus may predispose or protect against CVD based upon exposure to atherogenic lipoproteins.

4.2.3 Exposure to Hypertriglyceridaemia

Substantial evidence exists suggesting that non-fasting TG is as discriminatory, if not more so, than fasting lipid profiles to predict CVD risk [76]. Debate continues as to the independence of non-fasting TG as a risk factor for CVD, and clinical importance based upon the relatively small increase postprandially and variation due to other factors [91]. Despite this controversy, non-fasting TG has recently been proposed to be the primary contributor to exacerbating existing cardiovascular risk [200]. Based on a western-style diet pattern of three or more meals per day, circulating plasma TG concentrations are maintained well above fasting for most of the day [13, 18]. Moreover, with the exception of breakfast, meals are consumed before plasma TG return to fasting values, therefore there is a cumulative effect such that plasma TG at night may be double fasting values [13].

Non-fasting TG is a surrogate marker of remnant cholesterol, the amount of cholesterol contained within VLDL, IDL and CM particles [201]. Both non-fasting TG and remnant cholesterol have been strongly associated with CVD, however remnant cholesterol is believed to be the causal factor based on genetic association studies [201]. Specifically, Varbo et al. [201] found 1 mmol/L genetic elevations in non-fasting remnant cholesterol to be associated with a 2.8-fold increased causal risk for ischemic heart disease, independent of HDL-C. Similarly, genetically lowered non-fasting TG concentrations have been associated with reduced all-cause mortality [202]. Genetically determined lipid profiles represent lifelong exposure from birth, and are therefore less influenced by confounding risk factors [201]. As such, postprandial lipid profiles have been critically examined to determine their role in atherogenesis.

In the present study, we observed no difference in fasting TG concentration based upon genotype; whereas carriers for the minor, protective allele exhibited attenuated postprandial lipaemia. As a group, TT homozygotes exceeded the NCEP ATP III guideline for hypertriglyceridaemia and remained so from two to six hours postprandially. Furthermore, these individuals had significantly higher TG concentrations at 2 h postprandial, compared to carriers of the C allele, a time point that has been suggested to be a stronger predictor of CVD compared to TG concentrations 4-8 h, 8-12 h or >12 h since the last meal [76]. As such, these individuals experience increased exposure to atherogenic lipoprotein particles, promoting risk of CVD despite the absence of fasting hypertriglyceridaemia [16]. This highlights inherent limitations to considering only fasting lipid profiles for assessing cardiovascular risk, and may provide insight into the incidence of cardiovascular events in individuals who lack a high level of risk based on traditional single- or multi-variable risk scores [2, 3].

4.2.3.1 Residual Risk for Cardiovascular Disease

Residual risk represents the risk for major cardiovascular events that persists in statin-treated patients despite achieving pre-determined lipid targets [200]. In other words, it reflects disparities between the predictive value of traditional lipid measures, such as LDL-C, and actual cardiovascular events. Atherogenic TG-rich lipoproteins and their remnants are not captured by traditional lipid profiles, including LDL-C and HDL-C, and therefore represent a significant source of residual risk in patients with obesity, type 2 diabetes and other metabolic diseases [200]. As such, they represent a valuable target for novel risk-stratification strategies.

The 1p13 locus, despite having a relatively large effect size, accounts for only 1-5% of variability in lipid traits, such as LDL-C [23]. GWAS, however, suggest genotype at this locus confers a 9-13% and 40% difference in risk for CVD and MI [22, 25], respectively, suggesting an element of residual CVD risk attributed to alternative factors. In the present study, we observed differences in postprandial TG response despite similar fasting phenotypes. Given that non-fasting TG is recognized as a strong, independent risk factor for CVD [89, 92, 94], carriers of the minor allele exhibited a less detrimental postprandial TG response compared to TT homozygotes. These findings may, in part, explain the disparate risk of CVD between carriers of the minor allele and TT homozygotes by uncovering differential exposure to atherogenic lipoproteins that are unidentifiable based on fasting phenotypes. Furthermore, they provide support for incorporating non-fasting lipid assessment into research and clinical practice to amend current discrepancies between trait variation and disease outcomes that are currently attributed to residual risk.

4.2.4 Insight to the Mechanism of SORT1

While no direct mechanistic experiments were performed as part of this study, tentative conclusions may be extrapolated from the present findings. Our primary findings were significantly lower fasting and postprandial VLDL particle concentration in carriers of the minor, protective allele. Given that the minor, protective allele at the 1p13 locus is associated with liver-specific overexpression of sortilin mRNA and protein, these findings provide support for work by Musunuru et al. [47, 166] wherein sortilin overexpression mitigates VLDL secretion.

Sortilin is a multiligand lysosomal sorting receptor that localizes primarily to the Golgi apparatus, with small amounts (10%) present in clathrin-coated pits on the cell membrane [170, 203]. Intracellularly, sortilin binds apo B with high affinity, mediating its transfer to the lysosome for posttranslational degradation, the primary regulatory mechanism controlling VLDL secretion [166]. Murine loss-of-function studies demonstrate that hepatic sortilin regulation of apo B secretion is mediated in a lysosome-dependent manner [166]. Peripherally, sortilin serves as a “bona fide” LDL-R, facilitating hepatic-reuptake and catabolism of circulating LDL particles [166]. In the present study, carriers of the minor allele tended to exhibit fewer small LDL particles, providing support for the dual-role of sortilin in regulating lipid profiles.

Figure 4.1 Proposed dual role of sortilin in regulating LDL phenotype

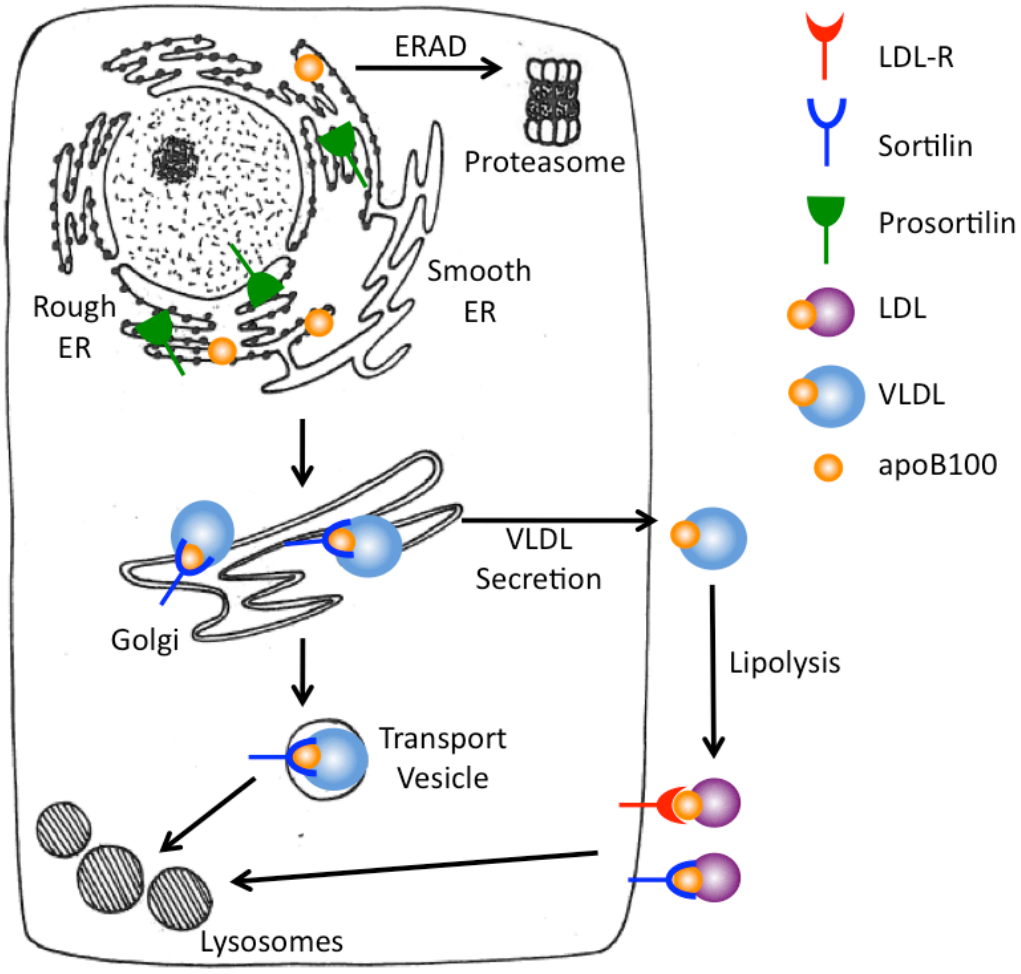

Figure 4.1 (adapted from [166]) Sortilin localizes primarily to the Golgi apparatus, with small amounts (10%) present in clathrin-coated pits on the cell membrane [170, 203]. Based on evidence from Strong et al. [166] and that presented, a dual role for sortilin in lipoprotein regulation is supported wherein it: (1) binds LDL in clathrin-coated pits on the plasma membrane, increasing LDL catabolism; and (2) targets apolipoprotein B for lysosomal and ER-associated degradation (ERAD) in the proteasome, decreasing VLDL secretion.

4.2.5 *Nutrigenetics and Nutrigenomics of SORT1*

Disparities in hepatic sortilin protein and mRNA expression between obese and diabetic states have recently been identified, providing evidence that sortilin-1 expression is regulated not only through genetic variation, but also posttranslational mechanisms [169, 170]. Specifically, hepatic decreases in sortilin-1 protein and mRNA expression have been observed in genetically obese (*ob/ob*) [169] and diet-induced obese mice [169, 170]. Furthermore, *Ldlr*$^{-/-}$ mice fed a western-style diet for 14 weeks exhibited 40% and 54% repression in sortilin protein and mRNA, respectively, providing evidence of nutrient-gene interactions [169].

In the present study, we observed differential postprandial lipaemia depending upon genotype at the *SORT1* rs646776 variant in young adults. Specifically, carriers of the minor, protective allele exhibited attenuated postprandial TG, VLDL and CM responses, marking decreased exposure to atherogenic lipoproteins. Given that postprandial elevations in FFA are associated with postprandial hypertriglyceridaemia [204, 205], it may be inferred that these individuals also experienced decreased exposure to FFA.

Metabolic disorders such as obesity, insulin resistance and type 2 diabetes are associated with increased plasma and tissue FFA, in addition to elevated hepatic VLDL secretion, hallmark features of atherogenic dyslipidaemia [169, 170]. One mechanism through which these changes are induced is through serine/threonine kinase mammalian target of rapamycin (mTORC1), a signalling protein that integrates nutritional stimuli with growth through protein and ribosome biogenesis [169]. Elevated mTORC1 and saturated fatty acids in obese mice induces ER stress, which has implications on *SORT1* expression [169]. ER stress promotes expression of activating transcription factor 3 (*ATF3*), a protein coding gene, which binds to an upstream *SORT1*

promoter, repressing transcription of *SORT1* [169]. Similarly, elevated FFA are associated with the extracellular signal-regulated kinases pathway, such that hepatic sortilin expression is inhibited, inducing VLDL oversecretion [170]. This process has been proposed to function as an adaptation to shunt energy away from the liver to alleviate stress on hepatocytes [85], however it results in detrimental exposure to atherogenic lipoproteins, increasing CVD risk.

Combined with evidence of FFA-induced ER stress downregulating hepatic *SORT1* expression, we speculate the presence of a cyclical pathway wherein increased exposure to FFA, or postprandial lipaemia, inhibits sortilin expression, thus exaggerating subsequent exposure. As such, carriers of the minor, protective allele, in whom hepatic sortilin expression is upregulated, may be protected against these detrimental effects compared to TT homozygotes.

4.2.6 Reconciling Disparate Findings

Human-based research into lipid phenotypes associated with *SORT1* have been founded upon relationships between the 1p13 locus, LDL-C [21, 23, 27-46], and its associated clinical endpoints, such as MI [21, 25, 30, 33, 36, 82]. Fewer studies have identified associations with LDL and VLDL subfractions [29, 47]. In the present study, no association was observed between *SORT1* and LDL subclasses at fasting or postprandially. This observation was surprising given the abundance of literature reporting *SORT1*'s strong association with LDL-C. It is essential, however, to consider the modality of data collection when comparing this study to those in the literature. Whereas we employed NMR-based lipoprotein analysis, the majority of investigations have been founded upon the Friedewald equation, wherein LDL-C is calculated as function of TG concentration, as follows:

Figure 4.2 Friedewald Equation

$$[\text{LDL-C}] = [\text{TC}] - [\text{HDL-C}] - [\text{VLDL-C}] = [\text{TC}] - [\text{HDL-C}] - ([\text{TG}]/2.2)^{3} \quad [181]$$

The Friedewald equation is founded upon three major assumptions positing that: (1) most plasma TG is contained in VLDL; (2) VLDL-C is relatively constant; thus (3) the ratio between VLDL-C and TG is constant [206]. This has been a source of criticism given that the ratio between VLDL-C and TG gradually changes with rising plasma TG, introducing considerable error when plasma TG $\geq$ 4.52 mmol/L [207]. In fact, 62.7% of the variability of LDL-C estimated from the Friedewald equation is attributed to the VLDL-C/TG ratio [206]. As such, it may be speculated that lipid phenotypes associated with the 1p13 locus may be reflective of VLDL concentrations, rather than LDL-C, as observed in the present study. This highlights the value to NMR-based metabolomics analysis and supports the potential clinical utility of VLDL for risk stratification and preventative interventions.

Discrepant findings in LDL particles may also be attributed to the statistical power of the present study. Carriers of the minor allele tended towards decreased small LDL particles at fasting, in accordance with previous NMR-based investigations [29], although these results were not significant. The study was, in fact, underpowered for this trait (β=0.26). This may be partially attributed to the population studied, as younger adults typically exhibit lower variance and fewer subjects with abnormal values, therefore lowering the statistical power of the study [23].

[3] For mmol/L, [TG]/2.2 is used; for mg/dl, [TG]/5 is used

4.3 Future Directions

4.3.1 Investigation of a Sexual Dimorphism

While no genotype x sex interactions were observed, we did not investigate the relationship between genotype at the 1p13 locus and postprandial lipaemia when stratified by sex. Previous investigations of *SORT1* have identified a sexual dimorphism at this locus, such that stronger associations between genotype and phenotype have been observed in both males [24] and females [23, 172]. Based on these discrepant results, it may be inferred that other factors, such as postprandial responses, potentially modify the relationship. As such, elucidating sex-specific differences in postprandial lipaemia when stratified by genotype represents a valuable means by which these inconsistent findings may be reconciled. Furthermore, if a true sexual dimorphism exists at this variant, it will be an important consideration for the direction and efficacy of intervention studies. Future studies should aim to investigate larger cohorts, increasing statistical power allowing for genotype- and sex- stratification, thus clarifying the presence or absence of a sexual dimorphism at this locus.

4.3.2 Longitudinal Studies

While we have identified an association between genotype at the rs646776 variant and postprandial lipaemia, longitudinal investigations should be performed to delineate this relationship. A prospective investigation should aim to quantify postprandial lipaemia in young adults stratified by genotype, and monitor incidence of future cardiovascular events. In doing so, the predictive value of postprandial lipaemia and genetic risk stratification may be determined [3]. This will clarify evidence in support of, or against, similar investigations and implementation into clinical practice as a means of risk stratification and targeting for interventions.

Furthermore, longitudinal investigations will allow for increased confidence in any causal relationships between postprandial lipaemia, genotype, and incidence of cardiovascular events.

4.3.3 Intervention Studies

Substantial evidence exists linking AAV- and cDNA-mediated hepatic *SORT1* overexpression with favourable phenotypes such as reduced LDL-C [37] and attenuated VLDL secretion [47, 169, 170]. Furthermore, reconstitution of hepatic sortilin expression in obese and diabetic mouse models has been shown to revert hypercholesterolaemic and hypertriglyceridaemic phenotypes back to those expressed in wild type mice [169, 170]. Given the strength of associations between hepatic sortilin expression and lipid phenotypes, it represents a valuable target for therapeutic intervention. AAV gene therapy has emerged as a potential means by which extracellularly active proteins may be synthesized and secreted [208]. Phase I and II clinical trials have emerged for AAV therapies, however several barriers, such as vector-immune responses, must be resolved prior to widespread implementation into practice [209]. By extending AAV-mediated hepatic sortilin expression studies to humans, the clinical utility may be assessed as a means by which fasting and postprandial lipid phenotypes may be modified, particularly in at risk groups with obesity, diabetes and fatty liver disease [170].

4.3.4 Mechanistic Investigations

Several sophisticated *in vivo* and *in vitro* studies have been performed that have provided the basis for elegant mechanisms of the *SORT1* gene. However, consensus regarding the magnitude and direction of the locus' phenotypic effects has yet to be achieved. This study provided evidence supporting a dual mechanism proposed by Strong et al. [166], wherein presence of the minor allele is associated with upregulated LDL catabolism and diminished VLDL secretion. However, in employing NMR analyses, we were unable to differentiate

between large lipoproteins of intestinal- versus hepatic- origin [198]. As such, whether these differences were propelled primarily by VLDL or CM particles remains unclear. Future investigations should include direct quantification of hepatically- versus intestinally-derived particles, which may be achieved by employing assays for apo B-100 or apo B-48, respectively. Greater understanding of the mechanism of *SORT1* could ultimately drive novel treatment development of oral therapies targeting intestinally-derived CM, or upregulation of hepatic sortilin expression to target endogenously produced VLDL particles.

Additionally, direct assay of lipids should be performed in conjunction with NMR. While NMR-calculated lipids are highly correlated with chemically derived concentrations ($r=0.96$, $p<0.0001$ for TG [198]), direct assay will validate the present findings. Furthermore, it will allow for more appropriate comparison of the present results to those from other studies, which have predominately employed chemical techniques for lipoprotein analysis. Determining assay-derived TG and HDL-C will also allow for calculation of LDL-C by the Friedewald equation, which will allow for validation of previously reports in a small sample of healthy, young adults.

4.3.5 Incorporation of Nutrigenetics and Nutrigenomics into Clinical Practice

In the present study, we identified genotype at the rs646776 polymorphism as a modifier of postprandial lipaemia in response to an OFTT in young adults. Future intervention studies investigating gene-diet interactions employing repeated sampling and accurate measurement of phenotypes are necessary to validate this novel relationship prior to implementation into clinical practice. Ultimately, nutrigenetics has the potential to be incorporated into multifactoral predictive models for disease risk assessment, thus improving current identification and prevention strategies. Furthermore, genetics-based dietary recommendations may be developed, contributing to the innovative field of “personalized nutrition”.

4.4 Conclusion

Despite advances in treatment and prevention, CVD remains a major contributor to morbidity and mortality in Canada and worldwide. Patients require extensive medical resources, creating a considerable, unsustainable burden on the healthcare system. While risk factors have been extensively documented, cardiovascular events frequently occur in individuals who lack a high level of risk based upon traditional single- or multi-variable risk scores, indicating an element of residual cardiovascular risk. We speculated that this disconnect may be partially attributed to the reliance on fasting lipid profiles to assess cardiovascular risk.

Using an OFTT, we identified the genetic variant rs646776 in the 1p13 locus, a SNP that has been strongly associated with LDL-C, coronary artery disease, MI among other lipid-associated phenotypes, to be a modifier of fasting lipid profile and postprandial lipaemia in fit, healthy, young adults. These results provide support for sortilin's role as a regulator of lipid metabolism and highlight the merits of future investigation into sortilin as a target for therapeutic interventions to modify CVD risk. Furthermore, we found carriers of the minor, protective allele to exhibit attenuated postprandial hypertriglyceridaemia, such that they did not exceed the NCEP ATP III clinical threshold of 1.69 mmol/L. As such, evidence is presented wherein genotype is a determinant of exposure to postprandial TG, modifying risk of CVD.

These results provide support for genotype-based risk stratification in conjunction with assessment of postprandial lipaemia to improve CVD prediction. Through this combination of approaches, individuals may be more accurately targeted for early, personalized preventative interventions, thus alleviating the burden of CVD.

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APPENDIX A: MEAL COMPOSITION

A.1. High-fat meal macronutrient composition

Item	Commercial Provider	Quantity	Calories (kcal)	Fat (g)	Sat. Fat (g)	Trans Fat (g)	Chol. (mg)	Sodium (mg)	CHO (g)	Fiber (g)	Sugar (g)	Protein (g)
Homestyle Ham N' Egger	A&W	2	1190	80	22	0.8	500	2280	74	2	8	50
Hashbrown	A&W	1	180	11	0.5	0	0	330	18	2	0	2
Orange juice	A&W	9 oz	120	0	0	0	0	0	30	0	27	0.5
		TOTAL	1490	91	22.5	0.8	500	2610	122	4	35	52.5

A.2. Fat-exclusion meal macronutrient composition

Item	Commercial Provider	Quantity	Calories (kcal)	Fat (g)	Sat. Fat (g)	Trans Fat (g)	Chol. (mg)	Sodium (mg)	CHO (g)	Fiber (g)	Sugar (g)	Protein (g)
Vegetable Soup	Campbell's Healthy Request	400 ml	208	1.6	0.32	0	0	752	43.2	4.8	12.8	4.8
Mini Bun	Tim Horton's	1	110	0.5	0	0	0	240	21	2	0	4
Veggie Salad	Subway	1	60	1	0	0	0	80	11	4	5	3
Sweet Onion Dressing	Subway	30 ml	35	0	0	0	0	720	7	0	4	1
Fruit Bar	Sunrype	1	120	0	0	0	0	10	32	2	29	0.4
Rice Cakes	Quaker	2	70	0.8	0	0	0	0	14	0	0	2
		TOTAL	603	3.9	0.32	0	0	1802	128.2	12.8	50.8	15.2

APPENDIX B: SUPPLEMENTARY DATA

B.1. Subject characteristics according to sex and genotype at rs646776

	Combined		Males		Females		p-value		
	Major Alleles (TT) n=15	**Minor Allele** (TC/CC) n=15	**Major Alleles** (TT) n=8	**Minor Allele** (TC/CC) n= 7	**Major Alleles** (TT) n= 7	**Minor Allele** (TC/CC) n= 8	**rs646776**	**Sex**	**rs646776 x Sex**
Age (y)	23.20 ± 0.89	23.93 ± 1.08	24.25 ± 0.88	24.00 ± 1.02	22.00 ± 1.57	23.88 ± 1.90	0.57	0.41	0.46
Height (m)	171.68 ± 2.33	172.01 ± 1.83	176.80 ± 2.50	175.21 ± 2.64	165.83 ± 2.85	169.21 ± 2.21	0.73	**<0.01**	0.34
Weight (kg)	69.43 ± 4.08	70.24 ± 3.80	79.63 ± 5.29	80.97 ± 4.74	57.77 ± 1.81	60.85 ± 3.23	0.59	**<0.01**	0.83
Body Mass Index ($kg•m^{-2}$)	23.43 ± 1.12	23.52 ± 0.83	25.47 ± 1.69	26.23 ± 0.75	21.09 ± 0.87	21.15 ± 0.66	0.60	**<0.01**	0.68
Body Fat (%)	18.14 ± 1.56	18.97 ± 1.25	15.51 ± 2.24	14.81 ± 1.00	21.14 ± 1.58	22.61 ± 1.05	0.81	**<0.01**	0.50
Resting Heart Rate ($beats•min^{-1}$)	64 ± 3	64 ± 3	61 ± 3	66 ± 5	68 ± 6	63 ± 3	0.97	0.61	0.21
Systolic Blood Pressure (mmHg)	110.87 ± 2.84	108.53 ± 2.60	118.00 ± 2.73	116.29 ± 2.95	102.71 ± 3.11	101.75 ± 2.15	0.63	**<0.01**	0.89
Diastolic Blood Pressure (mmHg)	69.80 ± 2.86	65.20 ± 1.49	74.75 ± 3.52	68.00 ± 2.24	64.14 ± 3.80	62.75 ± 1.65	0.18	**<0.01**	0.37
VO_2peak ($ml•kg^{-1}•min^{-1}$)	49.31 ± 1.93	47.89 ± 1.51	52.04 ± 2.45	52.26 ± 1.32	46.19 ± 2.76	44.08 ± 1.67	0.66	**<0.01**	0.59
Grip Strength (kg)	91.47 ± 5.31	86.23 ± 5.16	106.19 ± 5.26	102.07 ± 6.88	74.64 ± 3.97	72.38 ± 2.32	0.51	**<0.01**	0.85
Food Intake ($kcal•day^{-1}$)	2238 ± 167	2460 ± 221	2499 ± 235	2985 ± 341	1941 ± 193	2001 ± 175	0.27	**<0.01**	0.38
Carbohydrates (%)	50 ± 2	48 ± 2	48 ± 3	48 ± 4	53 ± 2	47 ± 3	0.38	0.50	0.31
Fat (%)	31 ± 1	32 ± 2	31 ± 2	31 ± 3	31 ± 2	34 ± 2	0.58	0.48	0.68
Protein (%)	18 ± 1	18 ± 1	20 ± 1	20 ± 2	16 ± 1	17 ± 1	0.83	**<0.01**	0.70

Data are mean ± SEM.

B.2. Fasting lipid and lipoprotein concentrations according to sex and genotype at rs646776

	Combined		Males		Females		p-value		
	Major Alleles (TT) n=15	**Minor Allele** (TC/CC) n=15	**Major Alleles** (TT) n=8	**Minor Allele** (TC/CC) n= 7	**Major Alleles** (TT) n= 7	**Minor Allele** (TC/CC) n= 8	**rs646776**	**Sex**	**rs646776 x Sex**
VLDL & CM Particles (nmol/L)	48.81 ± 5.07	30.05 ± 2.99	47.28 ± 5.71	35.41 ± 4.73	50.56 ± 9.18	25.35 ± 3.17	**<0.01**	0.57	0.27
Large VLDL & CM Particles (nmol/L)	2.77 ± 0.65	2.17 ± 0.56	2.86 ± 1.08	2.70 ± 1.14	2.67 ± 0.72	1.71 ± 0.38	0.46	0.58	0.62
Medium VLDL Particles (nmol/L)	21.94 ± 3.63	14.48 ± 1.75	20.96 ± 5.12	19.06 ± 2.10	23.06 ± 5.54	10.48 ± 1.81	0.27	0.42	0.07
Small VLDL Particles (nmol/L)	24.10 ± 3.88	13.37 ± 2.34	23.46 ± 5.23	13.63 ± 3.16	24.83 ± 6.21	13.15 ± 3.60	**0.05**	0.75	0.84
Mean VLDL Size (nm)	47.67 ± 1.46	51.26 ± 1.97	47.88 ± 1.69	52.09 ± 3.40	47.43 ± 2.62	50.30 ± 1.91	0.17	0.66	0.79
IDL Particles (nmol/L)	52.53 ± 10.71	75.67 ± 14.42	39.50 ± 11.02	90.29 ± 21.50	67.43 ± 18.51	62.88 ± 8.98	0.14	0.99	0.08
LDL particles (nmol/L)	969.87 ± 61.73	957.20 ± 53.42	969.00 ± 89.46	930.14 ± 84.81	970.86 ± 91.55	980.88 ± 71.67	0.87	0.76	0.78
Small LDL Particles (nmol/L)	332.40 ± 64.27	224.07 ± 50.20	403.00 ± 102.13	293.86 ± 84.81	251.71 ± 68.67	163.00 ± 54.00	0.23	0.09	0.90
Large LDL Particles (nmol/L)	584.93 ± 40.15	657.67 ± 62.36	526.50 ± 34.94	546.14 ± 78.03	651.71 ± 70.99	755.25 ± 84.28	0.38	**0.02**	0.55
Cholesterol (mmol/L)	4.31 ± 0.15	4.29 ± 0.16	4.23 ± 0.22	4.08 ± 0.26	4.41 ± 0.20	4.46 ± 0.18	0.84	0.20	0.66
Triglycerides (mmol/L)	1.12 ± 0.08	0.97 ± 0.07	1.11 ± 0.13	1.13 ± 0.14	1.13 ± 0.09	0.97 ± 0.07	0.12	0.20	0.09
VLDL & CM Triglyceride (mmol/L)	0.76 ± 0.08	0.59 ± 0.08	0.78 ± 0.13	0.77 ± 0.14	0.74 ± 0.10	0.59 ± 0.08	**0.05**	**0.03**	0.05
Apolipoprotein B (g/L)	0.82 ± 0.04	0.82 ± 0.03	0.79 ± 1.63	0.79 ± 0.05	0.85 ± 0.07	0.83 ± 0.04	0.97	0.46	0.67

Data are mean ± SEM.

B.3. Postprandial lipid and lipoprotein concentrations according to sex and genotype at rs646776

	Combined		Males		Females		p-value		
	Major Alleles (TT) n=15	**Minor Alleles** (TC/CC) n=15	**Major Alleles** (TT) n=8	**Minor Alleles** (TC/CC) n= 7	**Major Alleles** (TT) n=7	**Minor Alleles** (TC/CC) n= 8	**rs646776**	**Sex**	**rs646776 x Sex**
VLDL & Chylomicron Particles (total) (nmol/L)									
8-h tAUC (nmol/L•h)	449.7 ± 41.3	364.0 ± 28.7	497.5 ± 58.9	411.4 ± 43.2	395.0 ± 54.5	316.7 ± 31.0	0.10	**0.04**	0.90
4-h tAUC (nmol/L•h)	242.2 ± 16.4	183.2 ± 16.9	253.5 ± 16.3	218.4 ± 28.1	229.3 ± 30.5	152.5 ± 13.7	**0.02**	0.06	0.36
8-h iAUC (nmol/L•h) †	89.3 ± 17.9	117.1 ± 16.7	129.5 ± 24.3	141.7 ± 27.8	43.4 ± 12.5	92.5 ± 15.3	0.18	-	-
Mean Concentration (nmol/L)	57.0 ± 4.5	44.2 ± 3.1	61.3 ± 6.4	50.5 ± 4.7	52.1 ± 6.4	38.7 ± 3.4	**0.03**	0.06	0.81
Large VLDL Particles (nmol/L)									
8-h tAUC (nmol/L•h)	22.0 ± 4.8	16.4 ± 3.2	24.7 ± 8.3	22.7 ± 5.4	19.0 ± 4.3	10.2 ± 1.5	0.17	**0.04**	0.15
4-h tAUC (nmol/L•h)	13.4 ± 2.8	10.1 ± 2.2	14.7 ± 4.8	14.1 ± 4.2	11.9 ± 2.8	6.6 ± 1.0	0.11	0.11	0.13
8-h iAUC (nmol/L•h) †	4.7 ± 1.2	4.7 ± 1.4	6.3 ± 2.1	7.0 ± 2.3	3.0 ± 0.7	2.4 ± 1.0	0.97	-	-
Mean Concentration (nmol/L)	2.8 ± 0.6	2.1 ± 0.4	3.1 ± 1.0	2.9 ± 0.9	2.4 ± 0.6	1.4 ± 0.2	0.41	0.13	0.53
Medium VLDL Particles (nmol/L)									
8-h tAUC (nmol/L•h)	299.1 ± 37.9	220.5 ± 26.7	349.5 ± 55.1	255.0 ± 42.7	241.4 ± 45.6	186.1 ± 29.7	0.15	0.05	0.66
4-h tAUC (nmol/L•h)	160.1 ± 15.7	117.2 ± 14.2	177.4 ± 21.3	139.4 ± 23.1	140.3 ± 22.4	97.8 ± 15.4	0.06	0.07	0.91
8-h iAUC (nmol/L•h) †	134.4 ± 16.9	109.1 ± 21.9	178.3 ± 17.3	115.0 ± 35.3	84.2 ± 15.6	103.3 ± 28.5	0.28	-	-
Mean Concentration (nmol/L)	35.7 ± 4.3	25.6 ± 2.8	38.4 ± 7.5	28.3 ± 3.9	30.0 ± 5.1	21.1 ± 3.0	0.14	**0.03**	0.96
Small VLDL Particles (nmol/L)									
8-h tAUC (nmol/L•h)	128.6 ± 118.5	127.1 ± 11.4	123.3 ± 30.5	133.8 ± 17.0	134.6 ± 21.5	120.5 ± 16.0	0.94	0.97	0.60
4-h tAUC (nmol/L•h)	68.8 ± 11.0	56.0 ± 6.0	61.5 ± 12.8	64.9 ± 9.5	77.2 ± 19.2	48.2 ± 7.2	0.32	0.97	0.21
8-h iAUC (nmol/L•h) †	5.5 ± 3.0	26.6 ± 7.6	8.3 ± 5.5	34.5 ± 11.9	2.3 ± 1.4	18.7 ± 9.5	**<0.01**	-	-
Mean Concentration (nmol/L)	18.5 ± 2.4	16.5 ± 1.3	17.6 ± 3.7	16.8 ± 1.9	19.6 ± 3.3	16.2 ± 1.8	0.47	0.81	0.64

IDL Particles (nmol/L)									
8-h tAUC (nmol/L•h)	422.5 ± 49.0	500.6 ± 66.5	404.3 ± 64.7	467.6 ± 91.4	443.4 ± 79.2	533.6 ± 102.2	0.37	0.94	0.89
4-h tAUC (nmol/L•h)	178.6 ± 25.5	269.4 ± 37.5	155.1 ± 43.9	219.6 ± 54.8	205.4 ± 21.2	313.0 ± 49.4	0.07	0.12	0.63
8-h iAUC (nmol/L•h) †	99.8 ± 31.8	110.5 ± 45.3	95.8 ± 21.7	59.7 ± 41.5	104.4 ± 66.4	161.2 ± 79.4	0.60	-	-
Mean Concentration (nmol/L)	53.1 ± 5.4	64.1 ± 7.5	49.6 ± 8.2	63.9 ± 11.7	57.1 ± 7.2	64.3 ± 10.3	0.27	0.69	0.71
LDL Particles (nmol/L)									
8-h tAUC (nmol/L•h)	7919.3 ± 551.6	7726.8 ± 400.5	7954.8 ± 844..3	7528.6 ± 541.3	7879.3 ± 755.2	7925.0 ± 623.7	0.79	0.82	0.67
4-h tAUC (nmol/L•h)	3814.5 ± 271.0	3704.9 ± 198.7	3788.5 ± 409.9	3630.9 ± 242.7	3844.1 ± 378.0	3769.6 ± 320.4	0.74	0.78	0.91
8-h iAUC (nmol/L•h) †	316.2 ± 77.6	210.1 ± 74.1	333.6 ± 109.0	264.8 ± 133.2	296.5 ± 119.0	155.4 ± 71.0	0.34	-	-
Mean Concentration (nmol/L)	978.0 ± 66.8	937.6 ± 50.1	985.7 ± 102.6	931.5 ± 69.4	969.3 ± 90.9	943.1 ± 76.2	0.50	0.79	0.83
Small LDL Particles (nmol/L)									
8-hr tAUC (nmol/L*hr)	2516.9 ± 512.1	1653.2 ± 374.8	3105.8 ± 836.3	2310.3 ± 649.2	1843.9 ± 487.0	996.1 ± 207.9	0.20	0.05	0.97
4-hr tAUC (nmol/L*hr)	1252.5 ± 280.1	787.1 ± 155.9	1566.6 ± 471.1	1052.6 ± 270.9	893.6 ± 228.4	554.9 ± 140.0	0.24	0.10	0.89
8-hr iAUC (nmol/L*hr) †	292.4 ± 117.9	113.7 ± 46.6	396.0 ± 94.1	175.4 ± 87.8	174.0 ± 117.6	51.9 ± 20.9	0.58	-	-
Mean Concentration (nmol/L)	315.7 ± 63.7	200.3 ± 43.8	393.1 ± 104.3	285.2 ± 79.1	227.2 ± 58.3	126.0 ± 28.7	0.15	0.15	0.67
Large LDL Particles (nmol/L)									
8-h tAUC (nmol/L•h)	4980.0 ± 477.8	5573.1 ± 547.7	4444.5 ± 557.4	4750.9 ± 434.9	5592.0 ± 781.1	6395.4 ± 702.4	0.39	**0.04**	0.70
4-h tAUC (nmol/L•h)	2383.3 ± 230.9	2648.3 ± 211.1	2067.1 ± 270.6	2358.9 ± 221.0	2744.7 ± 357.8	2901.5 ± 332.9	0.47	0.05	0.83
8-h iAUC (nmol/L•h) †	549.3 ± 151.6	463.3 ± 147.5	524.5 ± 233.0	544.8 ± 202.6	577.5 ± 205.5	381.8 ± 226.0	0.84	-	-
Mean Concentration (nmol/L)	609.1 ± 54.7	673.3 ± 53.3	542.9 ± 62.1	582.4 ± 57.3	684.7 ± 89.4	752.9 ± 79.0	0.47	**0.04**	0.85
Total Cholesterol (mmol/L)									
8-h tAUC (mmol/L•h)	35.1 ± 1.1	34.2 ± 1.2	34.8 ± 1.6	33.7 ± 2.1	35.4 ± 1.7	34.7 ± 1.2	0.59	0.65	0.93
4-h tAUC (mmol/L•hr)	17.3 ± 0.6	17.3 ± 0.6	17.0 ± 0.8	16.5 ± 1.0	17.7 ± 0.9	17.9 ± 0.8	0.90	0.23	0.71
8-h iAUC (mmol/L•h) †	0.8 ± 0.2	0.7 ± 0.2	1.0 ± 0.3	0.9 ± 0.2	0.6 ± 0.3	0.4 ± 0.2	0.90	-	-
Mean Concentration (mmol/L)	4.4 ± 0.1	4.3 ± 0.1	4.3 ± 0.2	4.2 ± 0.3	4.4 ± 0.2	4.4 ± 0.1	0.65	0.52	0.78

Triglycerides (mmol/L)									
8-h tAUC (mmol/L•h)	13.2 ± 1.0	11.0 ± 0.8	14.1 ± 1.5	12.1 ± 1.3	12.2 ± 1.2	10.0 ± 0.8	0.05	0.13	0.73
4-h tAUC (mmol/L•h)	7.2 ± 0.4	5.9 ± 0.4	7.6 ± 0.6	6.8 ± 0.7	6.7 ± 0.5	5.1 ± 0.5	**0.04**	**0.03**	0.54
8-h iAUC (mmol/L•h) †	4.8 ± 0.6	3.6 ± 0.6	5.7 ± 0.8	4.0 ± 1.1	3.7 ± 74.4	3.3 ± 0.7	0.14	-	-
Mean Concentration (mmol/L)	1.6 ± 0.1	1.3 ± 0.1	1.7 ± 0.2	1.5 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	**0.04**	0.05	0.57
VLDL & Chylomicron Triglyceride (mmol/L)									
8-h tAUC (mmol/L•h)	10.2 ± 1.0	8.0 ± 0.8	11.3 ± 1.5	9.3 ± 1.2	9.0 ± 1.2	6.6 ± 0.7	**0.02**	0.05	0.50
4-h tAUC (mmol/L•h)	5.8 ± 0.4	4.4 ± 0.4	6.4 ± 0.6	5.4 ± 0.6	5.2 ± 0.6	3.5 ± 0.4	**0.03**	**<0.01**	0.49
8-h iAUC (mmol/L•h) †	4.7 ± 0.6	3.6 ± 0.6	5.6 ± 0.7	4.0 ± 1.1	3.7 ± 0.7	3.3 ± 0.6	0.15	-	-
Mean Concentration (mmol/L)	1.2 ± 0.1	0.9 ± 0.1	1.3 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	**<0.01**	**0.02**	0.29
Apolipoprotein B (g/L)									
8-h tAUC (g/L•h)	6.6 ± 0.3	6.7 ± 0.2	6.5 ± 0.4	6.7 ± 0.4	6.8 ± 0.5	6.6 ± 0.3	0.97	0.80	0.75
4-h tAUC (g/L•h)	3.2 ± 0.2	3.3 ± 0.1	3.1 ± 0.3	3.3 ± 0.2	3.4 ± 0.4	3.3 ± 0.1	0.99	0.60	0.60
8-h iAUC (gL•h) †	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.34	-	-
Mean Concentration (g/L)	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.91	0.86	0.65

† 8 h iAUC represented by mean ± SEM, p-values for Mann Whitney-U Test. Data are mean ± SEM.

APPENDIX C: PUBLICATIONS

PUBLICATIONS

Connors, KE, Gnatiuk, EA, Karlos, AE, Reimer, RA, Shearer, J, Hittel, DS. SORT1 Protective Allele is Associated with Attenuated Postprandial Lipaemia in Young Adults. *Circulation: Cardiovascular Genetics*, 2014 (revisions submitted).

ABSTRACTS (ORAL PRESENTATIONS)

Connors, KE, Gnatiuk, EA, Shearer, J, Barfield, W, Hittel, DS. The SORT1 risk allele is associated with exaggerated postprandial lipaemia in young adults. Experimental Biology. San Diego, CA; April 26-30, 2014.

Connors, KE, Gnatiuk, EA, Shearer, J, Hittel, DS. Effects of the SORT1 Gene on Postprandial Lipaemia in Young Adults. The Biomedical Basis of Human Performance International Workshop. Calgary, AB; June 4-7, 2013.