GENETIC VARIANTS AFFECTING RESPONSES OF PLASMA LIPIDS AND CHOLESTEROL KINETICS TO DIETARY CHOLESTEROL VERSUS PLANT STEROL CONSUMPTION IN A FOUNDER POPULATION

by

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ABSTRACT

Lowering plasma LDL-cholesterol (LDL-C) and increasing HDL-cholesterol (HDL-C) concentrations remain the primary targets in cardiovascular disease (CVD) risk reduction. Dietary cholesterol and plant sterols differentially modulate cholesterol kinetics and lipoprotein distribution. Inter-individual variations in the rates of cholesterol absorption and synthesis, and the reciprocal interaction between them affect the responses to dietary sterols. Genetic heterogeneity profoundly influences such responsiveness. However, limited research exists on the genetic determinants of dietary cholesterol versus plant sterols responsiveness in healthy individuals, especially in a founder population, such as the Hutterites in Manitoba of European descent who practice a communal living system. Our study examined the differential effects of dietary cholesterol versus plant sterol consumption on plasma lipoprotein levels, subclasses, and cholesterol kinetics and assessed how genetic variants influenced these responses. A double-blind, randomized, crossover study with three interventional periods of 4 wk duration each was conducted. Healthy Hutterite individuals (n=49) from Manitoba consumed daily either 2 g of plant sterols or 600 mg of cholesterol incorporated into milkshakes, or a placebo during each period. Plasma lipid profile and lipoprotein subclass distribution were determined. Cholesterol absorption and synthesis were assessed by stable isotopic tracer techniques. Participants were genotyped for 38 candidate single nucleotide polymorphisms across 25 genes involved in cholesterol and lipoprotein metabolism. Dietary cholesterol consumption increased plasma TC, HDL-C concentrations and large HDL subclasses with no changes in cholesterol absorption or synthesis. In contrast, plant sterol intake failed to reduce LDL-C concentrations, with a modest reduction in cholesterol absorption, and did not affect lipoprotein subclasses. However, a large non-compensatory increase in cholesterol synthesis was observed due to plant sterol

consumption. Gender and common genetic variants affected plasma HDL-C and HDL subclass distribution to dietary cholesterol and plant sterol consumption. *ACAT2* and *NPC1L1* gene variants affected plasma campesterol and β-sitosterol concentrations respectively, to plant sterol intake by modifying cholesterol absorption. In summary, our results demonstrate that dietary cholesterol and plant sterol intake differentially modulate cholesterol trafficking in a manner dependent on common genetic variants and gender in healthy individuals. Such knowledge facilitates the development of effective cholesterol lowering strategies for the alleviation of CVD burden.

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DEDICATION

Dedicated to my parents, my support and strength… you are with me always. To my sister who stood by my side all along… your gifts have brought me here. To the ONE who always watches over me.

To all the people who I owe this journey to. An action, a kind word, an even a good thought go a long way. Thank you

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ABBREVIATIONS

ABCA1	Adenosine triphosphate protein binding cassette transporter A1
ABCG5	ATP-binding cassette, sub-family G (WHITE), member 5
ABCG8	ATP-binding cassette, sub-family G (WHITE), member 8
ACAT2	Acetyl-CoA acetyltransferase
ALKP	Alkaline phosphatase
ALP	Atherogenic lipoprotein phenotype
ALT	Alanine aminotransferase
APOA1	Apolipoprotein A1
APOB48	Apolipoprotein B48
APOE	Apolipoprotein E
ASR	Absolute synthesis rate
AST	Asparagine aminotransferase
5AZA	5-Aza-2′-deoxycytidine
CBP/p300	Histone acetylase cyclic adenosine monophosphate responsive element binding protein1-binding protein
CE	Cholesterol ester(s)
CEL	Carboxyl ester lipase
CHD	Coronary heart disease
CVD	Cardiovascular disease
CYP7A1	Cholesterol 7α-hydroxylase
CYP8B	Sterol 12α-hydroxylase
DHCR7	7-dehydrocholesterol reductase

DI	Deuterium incorporation
DNA	Deoxyribonucleic acid
FSR	Fractional synthesis rate
FXR	Farnesoid X-receptor
GC-FID	Gas chromatography- flame ionization detector
GGT	Gamma-glutamyltransferase
GWAS	Genome-wide association study
HDAC3	Histone deacetylase 3
HDL-C	High density lipoprotein cholesterol
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzymeA
HMGCS	3-hydroxy- 3-methylglutaryl-Co-enzymeA synthase
HMGCR	3-hydroxy-3-methylglutaryl-Co-enzymeA reductase
IRMS	Isotope ratio mass spectrometry
LDH	Lactate dehydrogenase
LDL-C	Low density lipoprotein cholesterol
LDLr	Low density lipoprotein receptor
LIPA	Lysosomal acid lipase
LIPC	Hepatic lipase
LIPF	Gastric lipase
LIPG	Endothelial lipase
LPL	Lipoprotein lipase
LSS	Lanosterol synthase
LXR	Liver X receptor(s)

MAF	Minor allele frequency
MIDA	Mass isotopomer distribution analysis
miRNA	Micro-riboxy nucleic acid(s)
MTTP	Microsomal triglyceride transfer protein
NCBI	National Center for Biotechnology Information
NCEP ATP III	National Cholesterol Education Program Adult Treatment Program III
NPC1L1	Niemann-Pick C1 Like 1 protein
NRU	Nutritional Research Unit
PARK2	Parkin ring between ring family E3 ubiquitin protein ligase
PCSK9	Protein convertase subtilisin kexin-9
PDB	Pee Dee belemnite
PPARA	Peroxisome proliferator-activated receptor alpha
PUFA	Polyunsaturated fatty acids
RBC	Red blood cells
RCFFN	Richardson Center for Functional Foods and Nutraceuticals
RCT	Reverse cholesterol transport
RNA	Ribonucleic acid
SCAP	SREBP cleavage–activating protein
SCF	Skp, Cullin, F-box containing complex
SCD	Stearoyl-CoA desaturase
SCFA	Short chain fatty acid(s)
SFA	Saturated fatty acid(s)
SEM	Standard error of the mean

SIRT	Sirtuin
SNP	Single nucleotide polymorphisms
SMOW	Standard mean ocean water
SR-BI	Scavenger receptor class B member 1
SRE	Sterol response element
SREBP	Sterol regulatory binding protein
SREBF2	Sterol regulatory element-binding protein 2
TC	Total cholesterol
TG	Triglycerides
VLDL	Very low density lipoprotein

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Cardiovascular disease (CVD) persists as the leading cause of morbidity and mortality

worldwide, and the number of CVD associated deaths has been estimated to reach 23.3 million

by 2030 (1). Lowering total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) is

still held as the mainstay in primary prevention of CVD risk (2). Dietary cholesterol compared to

plant sterol consumption differentially affect plasma lipid responses (3, 4).

1.1.1 Influence of dietary cholesterol on plasma lipids

Dietary cholesterol consumption raises plasma cholesterol concentrations, and variability in such

responsiveness is usually seen, where hyper-responders alone increase plasma total cholesterol

concentrations (5, 3). The majority of the population have a normal plasma cholesterol response

to dietary cholesterol consumption, with the proportion of hyper-responders being about only

one fourth of the whole population (6, 3). Besides, hyper-responders exhibit an increase in both

plasma LDL-C and HDL-C usually with no changes in LDL-C/HDL-C ratio (6, 7, 8, 9). The

United States dietary recommendation of <300 mg/d of dietary cholesterol for healthy

individuals and its relation with CVD risk had been heavily questioned and the current dietary

guidelines does not include an upper limit for cholesterol (10, 11).

1.1.2 Influence of plant sterol supplementation on plasma lipids

Plant sterol consumption on the other hand significantly lowers plasma LDL-C and TC concentrations, resulting in a substantial reduction in CVD risk (12, 13). Plant sterols wield this cholesterol reduction by inhibiting small intestinal cholesterol absorption, however, the precise mechanisms of action remain unresolved (12). The North American diet provides plants sterols ranging between 300-400 mg/d (14). In spite of having minor structural variations, plant sterols are absorbed to a low degree in the small intestine ($< 2\%$) compared to the 50% absorption efficiency of dietary cholesterol (15). Clinical studies with plant sterol interventions have demonstrated large variability among individuals in the degree of LDL-C lowering response to plant sterol intake (16, 17, 18). Various inherent physiological factors have been ascribed for such variability and lack of LDL-C lowering efficacy (16). LDL-C lowering was found to be greater in individuals with high baseline plasma LDL-C concentrations than those with normal or borderline concentrations (19). Frequency and time of consumption have been shown to affect LDL-C lowering efficacy of plant sterol formulations, although, wide disagreement in this aspect prevails amongst studies (16, 20, 21).

1.1.3 Effects of cholesterol and plant sterol intake on endogenous cholesterol absorption and synthesis

Maintenance of cholesterol homeostasis in humans in response to dietary, therapeutic or environmental stimuli is achieved by maintenance of the reciprocal interaction between key control processes of cholesterol absorption and synthesis (22, 23, 24, 25). Such reciprocal relation between cholesterol absorption and synthesis affects the efficacy of any cholesterol management strategy (5, 24, 25). However, the degree of change in synthesis corresponding to a

change in absorption and vice versa in response to the external perturbations has been found to be highly variable (5, 25). For instance, hyper-responders to dietary cholesterol elevate their plasma TC, which stems from their inability to regulate plasma cholesterol homeostasis either by decreasing intestinal cholesterol absorption or by reducing synthesis (5, 26, 3). Cholesterol supplementation in humans has been shown to reduce (27), have a modest effect (28), or have no effect on cholesterol synthesis (22, 23, 29). Jones et al. (28) studied the response of cholesterol biosynthesis to various levels of dietary cholesterol and observed a small decrease in cholesterol synthesis by dietary cholesterol independent of plasma cholesterol levels. Similarly, plant sterol consumption along with considerable lowering of cholesterol absorption, is known to incite a compensatory rise in cholesterol synthesis (30, 31). Nevertheless, an increase in cholesterol synthesis in response to a reduction in cholesterol absorption does not necessarily always correlate with each other in terms of the magnitude of change (30, 31, 32). Rudkowska et al. (32) reported that non-responders to plant sterols display lower reductions in cholesterol absorption compared to the responders, but with similar magnitudes of reciprocal upregulation of cholesterol synthesis. Inter-individual variations in endogenous cholesterol synthesis have been suggested to affect the LDL-C lowering efficacy of plant sterols (17). Studies investigating the effects of inherent basal cholesterol synthesis status on LDL-C lowering efficacy of plant sterols have shown that individuals with high basal cholesterol synthesis exhibit poor LDL-C lowering response to plant sterol intake (17, 18). These findings reinforce the notion that plant sterols could induce an over-compensatory increase in cholesterol synthesis corresponding to a minimal reduction in absorption, in a proportion of the population. Such hyper-reactive increases in synthesis in these individuals may affect the cholesterol lowering efficacy of plant sterols.

1.1.4 Genetic factors affecting cholesterol metabolism

Genetic heterogeneity profoundly contributes to the inter-individual variability observed in plasma lipid responsiveness to dietary cholesterol and plant sterols (33, 34, 35, 36). For instance, *ABCG5/8* transporter polymorphisms have been shown to affect plasma lipid responses to dietary cholesterol (33). Similarly, the role of *APOE* polymorphisms in altering cholesterol synthesis and plasma lipid responses to cholesterol feeding have also been previously studied (37, 38). Mackay et al. (36) showed that *CYP7A1 rs3808607* and *APOE* polymorphisms work in concert to determine plasma LDL-C responses to plant sterol interventions. Variations in *ABCG5/8* and *NPC1L1* polymorphisms are linked to plasma LDL-C responses to plant sterol intake (39, 32). In one study, plant sterol responses were found to vary according to ABCG8 gene polymorphism and the basal plant sterol levels. Individuals possessing the A allele with high basal plant sterol concentrations displayed 3.9-fold LDL-C reduction compared to their counterparts with low basal plant sterol concentrations (39). The effect of NPC1L1 polymorphisms were investigated in the same study. Individuals carrying minor alleles for the NPC1L1 haplotype of 872 C > G (L272L) and 3929 G > A (Y1291Y), exhibited a 2.4-fold reduction in LDL-C concentrations compared to the common allele carriers. These findings support the role of genetic polymorphisms in modulating cholesterol metabolism, resulting in phenotypic variations in responsiveness to cholesterol lowering interventions.

1.1.5 Lipoprotein metabolism and CVD risk

The importance of analyzing plasma lipoprotein particle size and distribution rather than just the levels in estimating CVD risk is gaining momentum. Individuals have been recognized to possess either pattern A, comprising of large buoyant LDL particles or pattern B composed

largely of small, dense LDL with respect to LDL particle size profile (40, 41). The latter profile

is associated with higher CVD risk due to the atherogenic nature of the small dense LDL

particles (40). Small LDL particles possess a greater ability to penetrate the arterial wall, and are

more suscepible to oxidation, thereby attracting macrophages creating a conducive environment

for atherosclerotic plaque progression (40, 3). Similarly, HDL can be classified as large, medium

and small particles (42). Large HDL particles are inversely associated with CVD risk because of

their functional role in in reverse cholesterol transport (43, 44, 42). Hence, the relative

enrichment of large HDL particles is now being heralded as more important than HDL-C

concentrations *per se* (43, 45).

1.1.6 Effects of cholesterol and plant sterol supplementation on lipoprotein subclasses

The functionality, absorption and metabolism of dietary cholesterol differs largely from that of

plant sterols, and this is reflected in the manner in which these dietary sterols differentially alter

lipoprotein metabolism and subclass distribution (3, 4). Dietary cholesterol consumption

considerably affects lipoprotein subclasses, resulting in an increase in large LDL subclasses with

an accompanying increase in large HDL particles as well (10, 46, 47, 48, 49). Studies in

overweight, obese, normocholesterolemic and hypercholesterolemic participants have shown that

high intake of dietary cholesterol results in the formation of large HDL particles (50, 46, 6, 47,

3), especially, increases large HDL 2a subclass particle concentrations (51). The effects of plant

sterol consumption on plasma LDL subclass distribution have been investigated in a number of

studies (4). Studies conducted in type 2 diabetic (52), overweight and moderately

hypercholesterolemic individuals (53) did not observe any changes in LDL particle size after

plant sterol supplementation, despite significant reductions in plasma LDL-C concentrations.

However, reductions in small dense LDL-C concentrations have been seen following plant sterol consumption in individuals with type 2 diabetes (52); metabolic syndrome (54) and in hypercholesterolemic children (55). In contrast, De Smet et al. (56) observed reductions in large LDL particles with no changes in small LDL particle concentrations after plant sterol consumption in healthy participants. Plant sterol consumption in general does not exhibit any effect on plasma HDL-C concentrations or HDL subclass distribution (57, 58, 59, 60).

1.1.7 Genetic factors influencing lipoprotein subclasses

In addition, gene-diet interactions can possibly modify the responsiveness of lipoprotein subclass distribution to dietary sterols. A number of different studies with a candidate gene approach have investigated the linkage of single nucleotide polymorphisms (SNPs) in candidate genes involved in lipoprotein metabolism and subclass distribution (61, 62, 63, 64, 65). Results from sib-pair linkage analyses suggest the existence of mutilocus genetic determinants of LDL phenotypes (63, 64). Common polymorphisms in the promoter region of hepatic lipase gene (*LIPC*) have also been shown to modify LDL particle distribution (66, 65). Notwithstanding this, there is a lack of research on the role of common polymorphisms in candidate genes related to lipoprotein metabolism, especially various other genes involved in cholesterol metabolism, and their modulatory effects on plasma LDL and HDL subclass distribution in response to dietary cholesterol and plant sterols consumption in healthy humans. To date, only one study has investigated the association between *APOB* variants and HDL2 cholesterol in response to a low cholesterol, low fat diet (67). For instance, polymorphisms in the intestinal sterol transporters, endothelial, lipoprotein and lysosomal acid lipases and LDL regulators could influence

lipoprotein subclass responses to dietary sterols (68, 69, 25). Hence, it is essential to understand the impact of common gene variants affecting lipoprotein subclasses to dietary sterols.

1.1.8 Founder populations

Genetic isolates and founder populations have been known to provide a unique opportunity for genetic mapping studies with regards to Mendelian diseases (70). A founder population is established by a very small number of individuals from a larger population, who are not gentically represantative of the parent population (70). More recently, great interest has been shown on founder populations towards understanding common diseases, due to their reduced genetic and environmental heterogeneity (70). Amongst founder populations such as the Finnish, Old Order Amish, Sardinian and Jewish communities, the Hutterites are a genetically isolated population of European descent practicing a unique community lifestyle (71). Examining the effects of common genetic variants and diet interactions affecting plasma lipid responses in founder populations such as the Hutterites would offer better understanding of common and complex diseases, such as CVD (71). Common polymorphisms known to modulate plasma lipid and plant sterol responses to dietary sterols in the general population remain uninvestigated in the Hutterites. Only one study exists which evaluated the effects of plant sterol intervention in healthy Hutterites (31). Hence, a knowledge gap exists as to whether common genetic variants known to influence plasma lipid responses to dietary sterols in the general population, also affect plasma lipid responses in Hutterites in a similar manner.

1.1.9 Conclusion

Dietary cholesterol and plant sterols differentially modulate plasma lipids by influencing cholesterol absorption and synthetic rates in humans. Also, these dietary sterols modify lipoprotein subclass distribution in humans. However, huge inter-individual variability exists in the biomarker responses and lipoprotein subclass distribution to dietary sterols. Genetic heterogenity is suggested to largely contribute to these inter-individual variations. Hence, genetically propelled human interventional studies investigating whether dietary cholesterol and plant sterols follow similar plasma lipid modulatory mechanisms are essential. Conducting such studies in a founder population helps deciphering the crucial role of genetics in the complex etiology of CVD. Advances in knowledge gained from these experimental studies will eventually lead to improved dietary recommendations for cholesterol management and mitigation of CVD risk.

1.2 RATIONALE

The functionality, absorption and metabolism of plant sterols in humans differ largely from that of dietary cholesterol, and these govern the differential modulation of plasma lipids and lipoprotein subclasses to dietary sterol intake. Dietary cholesterol affects plasma cholesterol concentrations and modifies lipoprotein subclass distribution. Similarly, the plasma LDL-C lowering responsiveness of plant sterols and its effects on lipoprotein subclass distribution. A large inter-individual variability in responsiveness to these dietary sterols is usually seen. Such inter-individual variability is predominantly governed by the inherent differences in an individual's cholesterol metabolism. The reciprocal interaction between cholesterol absorption and synthesis in regulating cholesterol homeostasis affects the efficacy of any cholesterol

management strategy. Variability among individuals exists in the magnitude to which endogenous cholesterol synthesis changes corresponding to changes in absorption and vice versa, to cholesterol versus plant sterol supplementation. Emerging evidence shows that genetic heterogeneity may play a major role in contributing to such inter-individual variability in responsiveness to dietary sterols. However, a lack of research exists on the role of common genetic variants in modifying the responses of lipid biomarkers to cholesterol versus plant sterol consumption. As such, a pronounced need exists to examine how dietary cholesterol differentially modulates plasma lipids and lipoprotein subclasses compared to plant sterols, by interacting with cholesterol absorption and synthesis in healthy individuals. Noteworthy, the effects of common genetic variants associated with plasma lipid responsiveness may be different in a founder population such as the Hutterites. Hence, investigating such associations is essential in broadening our understanding of the pathophysiological processes governing CVD.

Based on the need to understand the genetically oriented effects of cholesterol versus plant sterol supplementation, and using a controlled human intervention design, the primary focus of this research is to delineate the impact of dietary cholesterol versus plant sterol consumption on plasma lipid biomarkers in healthy Hutterite individuals. Stable isotopic tracer techniques used in this research permit discerning the effects of cholesterol versus plant sterol intake on endogenous cholesterol absorption and synthesis rates. The secondary focus of this research is to investigate the associations between the lipid biomarker and cholesterol kinetic responses to cholesterol versus plant sterol consumption and common genetic variants within cholesterol metabolic pathways. Healthy individuals from two Hutterite colonies in Manitoba consumed for 4 weeks a single serving of milk shake/d containing either 600 mg of cholesterol or 2 g of plant sterols or a

placebo milkshake along with breakfast. The outcomes of this research will provide useful insights on the functional role of dietary sterols in cardiovascular health by advancing our understanding of genetic heterogeneity of plasma lipid responsiveness. Such knowledge will aid in the deployment of strategies targeted towards improving the efficacy of cholesterol lowering interventions for the alleviation of CVD risk.

1.3 HYPOTHESES

The hypotheses tested are as follows:

1. Consumption of 600 mg of dietary cholesterol will differentially modulate plasma lipid responses compared to the consumption of 2 g of plant sterols in healthy individuals.

2. Dietary cholesterol supplementation will increase cholesterol absorption and decrease synthesis, whereas plant sterol consumption will result in decreased cholesterol absorption and correspondingly increased synthesis, and these changes will affect plasma LDL-C responses in a manner dependent on the degree of change in cholesterol synthesis.

3. Consumption of dietary cholesterol and plant sterols will substantially increase LDL and HDL particle size and will differentially alter lipoprotein subclass distribution.

4. Common variants in candidate genes in lipoprotein and cholesterol metabolic pathways will modify the responses of LDL and HDL subclass distribution to dietary cholesterol in a different manner compared to plant sterol consumption.

5. Common polymorphisms in candidate genes will influence plasma lipid and plant sterol responses to cholesterol and plant sterol intake, by modulating cholesterol absorption and

synthesis rates in Hutterites, in a similar way consistent with that of the general

population.

1.4 OBJECTIVES

The overarching objective of this study was to investigate how dietary cholesterol consumption

compared to plant sterol consumption differentially modulates plasma lipid responses and

cholesterol trafficking mechanisms, and identify whether and how common genetic variants

influence the responsiveness of CVD biomarkers to consumption of these different sterols.

The specific objectives include:

1. Assess the plasma lipid responsiveness to 600 mg of cholesterol versus 2 g of plant sterol
    supplementation in healthy individuals.
2. Investigate the influence of dietary cholesterol versus plant sterol consumption on rates of
    cholesterol absorption and synthesis using stable isotope tracer techniques.
3. Evaluate the effects of dietary cholesterol versus plant sterol consumption on LDL and
    HDL subclass distribution and changes in particle size.
4. Examine how common polymorphisms in candidate genes in lipoprotein and cholesterol
    metabolic pathways associate with changes in LDL and HDL subclass distribution in
    response to dietary cholesterol and plant sterol consumption in a Hutterite founder
    population.
5. Evaluate the effects of common candidate gene variants involved in cholesterol
    metabolism on plasma lipid and plant sterol responses to dietary cholesterol and plant
    sterol intake, in relation to changes in cholesterol absorption and synthesis rates.

1.5 OUTLINE OF THIS THESIS

This thesis is structured and written using the manuscript style and comprises of four manuscripts. The first manuscript (Chapter II) follows the General Introduction (Chapter I) and is followed by a Mini-Literature Review (Chapter III), after which the remaining three manuscripts follow. Chapter II (Manuscript 1) reviews the literature on cholesterol metabolism, especially focusing on the factors governing reciprocal relationship between cholesterol absorption and synthesis and delineates the role of genetics, epigenetics, gut microbiome, dietary and therapeutic factors affecting the relationship. The Mini-Literature Review (Chapter III) summarizes the literature surrounding the role of lipoprotein subclasses in CVD and the effect of dietary cholesterol and plant sterols on LDL and HDL subclass distribution. The review also discusses the genetic linkage studies in genes affecting lipoprotein metabolism. Thereafter, research manuscripts addressing the specific objective and findings are presented. Chapter IV presents the effect of dietary cholesterol and plant sterols on the plasma lipid responsiveness and rates of cholesterol absorption and synthesis measured by stable isotope tracer techniques. Chapter V summarizes the effects of the dietary sterols on lipoprotein subclass distribution and the common genetic variants that associate with the responsiveness. Chapter VI addresses the genetic heterogeneity in candidate genes in cholesterol metabolism and their associations with plasma lipid and plant sterol responsiveness to dietary sterols in relation to cholesterol absorption and synthesis. Manuscript 1 has been published and Manuscript 2, 3 and 4 are under submission. Bridges in between chapters facilitate a consistent flow of the thesis. The final chapter (Chapter VII) provides an overall summary of the research findings, strengths and limitations, future directions, implications and concluding remarks.

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BRIDGE TO CHAPTER II

Chapter II provides an overview of human cholesterol metabolism with a special focus on the reciprocal relationship between cholesterol synthesis and absorption and its clinical implications. This literature review presents various factors including physiological, dietary, therapeutic and gene-diet interactions that potentially affect this relationship, especially, delineating the impact of dietary cholesterol and plant sterol consumption. The effects of dietary cholesterol versus plant sterols on the reciprocal interaction between cholesterol synthesis and gene-diet interactions affecting cholesterol metabolism dealt with in this review, in essence, form the basis that leads to the research investigation and project presented in Chapters IV and VI. The following manuscript has been published in Lipids 2015 (online); 51(5):519-536. 10.1007/s11745-015-4096-7.

CHAPTER II

MANSUCRIPT 1: LITERATURE REVIEW

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REVISITING HUMAN CHOLESTEROL SYNTHESIS AND ABSORPTION: THE RECIPROCITY PARADIGM AND ITS KEY REGULATORS

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2.1 ABSTRACT

Hypercholesterolemia is a major risk factor for cardiovascular disease. Cholesterol homeostasis in the body is governed by the interplay between absorption, synthesis, and excretion or conversion into bile acids. A reciprocal relationship between cholesterol synthesis and absorption is known to regulate circulating cholesterol in response to dietary or therapeutic interventions. However, the degree to which these factors affect synthesis and absorption and the extent to which one vector shifts in response to the other are not thoroughly understood. Also, huge inter-individual variability exists in the manner in which the two systems act in response to any cholesterol lowering treatment. Various factors are known to account for this variability and in light of recent experimental advances new players such as gene-gene interactions, gene-environmental effects and gut microbiome hold immense potential in offering an explanation to the complex traits of inter-individual variability in human cholesterol metabolism. In this context, the objective of the present review is to provide an overview on cholesterol metabolism and discuss the role of potential factors such as genetics, epigenetics, epistasis, and gut microbiome, as well as other regulators in modulating cholesterol metabolism, especially, emphasizing the reciprocal relationship between cholesterol synthesis and absorption. Furthermore, an evaluation of the implications of this push-pull mechanism on cholesterol lowering strategies is presented.

2.2 INTRODUCTION

Cardiovascular disease (CVD) remains the number one cause of mortality worldwide, with the number of people who die from CVD projected to reach 23.3 million by 2030 (1, 2). Hypercholesterolemia promotes atherosclerosis and is a classic risk factor for CVD (3). Lowering serum total (TC) and low density lipoprotein cholesterol (LDL-C) levels thus represents the golden target for primary and secondary prevention of CVD. Cholesterol, an essential and ubiquitous 27-carbon steroid, is a major structural part of cellular membranes of vertebrates (4, 5). Bile salts, steroid hormones and oxysterols are some important cholesterol metabolites controlling specific and significant biological functions (6). Serious clinical conditions such as Smith–Lemli–Opitz syndrome and desmosterolosis result from a defective in the ability to synthesize cholesterol, thereby underscoring its importance (7).

Whole-body cholesterol metabolism is an intricate system with a plethora of non-linear interactive networks traversing across its various constitutive components. Of noteworthy importance are the positive and negative feedback mechanisms and the complicated crosstalk between vectors such as cholesterol synthesis and absorption (8). Whole body cholesterol trafficking is thought to be majorly regulated by the interaction of three factors: absorption, synthesis, and excretion or conversion into bile acids (9).The regulation of cell cholesterol is facilitated by sensing cellular cholesterol content followed by the onset of homeostatic feedback mechanisms of the intracellular membranes (10, 11). Grundy et al. (1969) demonstrated the relationship between total daily synthesis of cholesterol and the amount of cholesterol absorbed, thereby emphasizing the interaction of cholesterol absorption and synthesis. This group also showed that an interruption of the enterohepatic circulation of cholesterol by inhibiting sterol

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absorption resulted in a diminution of plasma cholesterol (12). However, a compensatory increase in cholesterol synthesis was also observed. Such feedback regulation of cholesterol synthesis by dietary cholesterol is partly determined by the amount of dietary cholesterol that can be absorbed and the degree to which it can curb synthesis (12). Quintao et al. (1971) suggested that the intestinal cholesterol reabsorption originating from dietary and endogenous sources may be affected by different levels of exogenous cholesterol influencing the net cholesterol absorption (9). Also, great inter-individual variability in the magnitude of reciprocal inhibition of cholesterol synthesis by absorption is present. These variations determine the degree to which different individuals compensate by feedback suppression of synthesis of cholesterol in response to a dietary cholesterol burden (9).

Cholesterol homeostasis is known to be controlled by important internal factors such as genetics, body weight, endocrine factors, circadian rhythm and other key external therapeutic and nutritional factors like statin, ezetimibe, plant sterol/stanol treatment and weight loss (13, 14). These factors majorly influence either cholesterol absorption or synthesis. However, the degree to which these factors affect synthesis and absorption and the extent to which one vector shifts in response to the other are not thoroughly understood. As mentioned earlier, huge inter-individual variability also exists in the manner the two systems act in response to any cholesterol lowering treatment. The review by Santosa et al. (2007) attempted to resolve whether a reciprocal relationship exists between cholesterol absorption and synthesis when the homeostatic system is perturbed, and if it does to what magnitude. The authors in their review identified various internal and therapeutic factors that affect cholesterol synthesis and absorption and how the reciprocal effects responded to one another (13). However, in light of recent advances in the

areas of genetics, especially gene-gene interactions, epigenetic effects and the role of gut

microbiome, it is plausible in the near future to address the complexity surrounding this

paradigm. Hence, the objective of the present review is to provide an overview on cholesterol

metabolism in the context of discussing the role of potential factors including genetics,

epigenetics, epistasis, and gut microbiome on cholesterol metabolism, especially emphasizing

the reciprocal relationship between human cholesterol synthesis and absorption. Furthermore, the

future implications of these reciprocal effects on the cholesterol lowering therapeutic strategies

are discussed in detail. Factors governing the cholesterol synthesis and absorption are presented

in Figure 1 and a summary of the factors and its implication in cholesterol synthesis and

absorption is summarized in Table 1.

2.3 CHOLESTEROL SYNTHESIS

The cholesterol biosynthetic pathway is a complex biochemical process that involves more than

30 different reactions utilizing over 15 enzymes from many different subcellular compartments

(5, 15). Generally, this pathway can be categorized into two stages (i) condensation of isoprenoid

units yielding the 30-carbon molecule squalene and (ii) cyclization of squalene to produce

lanosterol, which is eventually converted to cholesterol (4, 5, 16). The pathway begins with the

formation of the 6- carbon 3-hydroxy-3-methylglutaryl-coenzymeA (HMG-CoA) from one

molecule of acetyl-CoA (2C) and one of acetoacetyl-CoA catalyzed by the enzyme 3-hydroxy-

3-methylglutaryl-Co-enzymeA synthase (HMGCS). The membrane bound enzyme 3-hydroxy-3-

methylglutaryl-Co-enzymeA reductase (HMGCR) converts HMG-CoA to mevalonate. This

reaction is often considered to be the principal flux governing step in the biosynthesis of

cholesterol and serves as an important target of the statin drugs (5). However, recently, squalene

monooxygenase, known to catalyze the first oxygenation step in cholesterol synthesis is also considered to be a potential secondary target in inhibiting cholesterol synthesis (17).

Cholesterol biosynthesis is regulated through a system of feedback inhibition where the intracellular cholesterol levels are sensed resulting in the modulation of the expression of key proteins that control cholesterol homeostasis (18). Sterol regulatory binding protein (SREBP, mainly type 2) is the most important element in this process. SREBP2 is usually located in the endoplasmic reticulum in the form of a complex with SREBP2 cleavage–activating protein (SCAP) under conditions when the cell cholesterol levels are high. However, when depleted of sterols, SCAP facilitates the transfer of SREBP2 from the endoplasmic reticulum to the Golgi apparatus. At this stage, the complex is cleaved resulting in the release of a part of the protein from the membrane. This enables SREBP2 to enter the nucleus, and activate the transcription of multiple genes regulating cholesterol synthesis by binding to a sterol response element (SRE) in their enhancer/promoter regions (19).

2.3.1 Measurement of cholesterol synthesis

In vivo cholesterol synthesis can be determined by various methods such as sterol balance, plasma cholesterol precursor measurement and tracer incorporation techniques including deuterium incorporation (DI) and mass isotopomer distribution analysis (MIDA) (20). The DI and MIDA methods yield comparable results to that of the gold standard cholesterol balance method (cholesterol intake + synthesis = fecal excretion of bile salts + neutral sterols) (21, 22). The DI method determines cholesterol synthesis as the rate of incorporation of deuterium from body water into red cell membrane free cholesterol over a 24 h period (23, 24).

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2.4 FACTORS AFFECTING CHOLESTEROL SYNTHESIS

2.4.1 Dietary quantity and quality

The rates of cholesterol synthesis are affected by various dietary factors. Suppression of cholesterol biosynthesis by dietary restriction is one of the major dietary strategies in humans (20). Increasing the meal frequency reduces cholesterogenesis (20, 25, 26). Dietary fats play a predominant role in regulating cholesterol synthesis and homeostasis. Saturated fatty acids (SFA) reduce LDL receptor (LDLr) activity in the liver, which elevates circulating LDL-C and lowers cholesterol synthesis (27). Higher synthesis rates have been observed in mildly hypercholesterolemic individuals as well as normolipdemic individuals consuming poly unsaturated fatty acids (PUFA) enriched diets (28) but not consistently in all the studies (29, 30, 31, 32).

2.4.2 Dietary cholesterol

Dietary cholesterol is thought to modulate the cholesterol synthesis, and in humans, dietary cholesterol provided at different levels in various studies either modestly altered or suppressed (31, 33, 34, 35, 36) or did not have any effect at all (12, 37, 38, 39) on synthesis. However, in some studies, an almost equal decrease in synthesis per increase in absorbed cholesterol were observed (40, 41). Jones et al. (1996) measured deuterium incorporation and mevalonic acid excretion in individuals and found modest decreases in synthesis when dietary cholesterol was provided in a stepwise incremental fashion from 50 to 650 mg/d (36). However, the cholesterol absorbing and synthesizing ability of each individual could heavily influence this push-pull

mechanism accounting for a wide inter-individual variability in responsiveness to dietary cholesterol.

2.4.3 Body weight

One of the key factors that must be taken into account is overweight and especially abdominal obesity (42, 43, 44) which have larger effects on cholesterol synthesis. The rate of synthesis is estimated to range between 11–13 mg/kg/d at an ideal weight, whereas with increasing adiposity and weight gain, the synthesis rates could rise up to 20 mg/kg/d (45). Such an increase is often associated with a suppression of cholesterol absorption (13, 46). Conversely, weight loss of 3 to 8 kg in overweight and obese men resulted in a decline in whole body cholesterol synthesis (47), which corroborates the existence of body weight dependent reciprocity between cholesterol synthesis and cholesterol absorption.

2.4.4 Circadian rhythm

In humans, circadian rhythm is thought to play an important role in cholesterol synthesis. Fractional synthesis rates (FSR) were found to peak at 06:00 h and reach a nadir at 14:00 to 18:00 h (48). Delaying meal times altered maximum and minimum cholesterol synthesis rates (49). Gene expression of enzymes controlling cholesterol metabolism such as HMGCR (50) and sterol 12α-hydroxylase (CYP8B) (51) has also been found to exhibit a circadian rhythm. CYP8B enzyme determines the synthesis of cholic acid, (52) and in knockout mice devoid of the *CYP8B* gene, suppression of cholesterol absorption with a consequent upregulation of cholesterol synthesis was seen (53). In humans, cholic acid supplementation enhances cholesterol

absorption, suppresses cholesterol synthesis, indicating the influence of circadian rhythm (54, 55).

2.4.5 Endocrine factors

Cholesterol synthesis is known to be regulated by various hormones, and especially the hormonal regulation of HMGCR has been well studied (14). Thyroid hormones affect cholesterol synthesis, which act at a transcriptional level by regulating *HMGCR* gene expression (56). Shin and Osborne, (2003) using a hypothyroid animal model demonstrated that thyroid hormone regulation and cholesterol metabolism are connected through SREBP-2 (57). Glucocorticoids affect the post-translational modifications of the enzyme (56). However, the effects of thyroid and glucocorticoids with respect to their influence on the reciprocal relationship between cholesterol synthesis and absorption needs to be further determined. Insulin is another major hormone known to modulate HMGCR at both transcriptional and post-translational levels (58, 59). High cholesterol synthesis and low cholesterol absorption were related to insulin resistance in a study with 72 healthy normoglycemic participants suggesting the interrelation of insulin and cholesterol metabolism (60, 61). Interestingly, patients with diabetes mellitus exhibited significantly higher cholesterol synthesis and a correspondingly lower absorption compared to their counterparts who were healthy (62). This emphasizes that insulin affects the reciprocal relationship between cholesterol synthesis and absorption. Messa et al. (2005) reported that 17-β-estradiol regulated LDLr and HMGCR, which confirmed to an earlier report which suggested that estrogens are capable of modulating LDLr transcription (63, 64).

2.4.6 Genetic factors

Genetic factors have a major effect on cholesterol metabolism and affect absorption and synthesis of cholesterol. The huge inter-individual variability observed in response to cholesterol lowering treatments such as statin therapy adds evidence to the role of genetic polymorphisms in modulating cholesterol metabolism (13). The membrane bound transcription factor SREBP1 regulates cholesterol synthesis and uptake from circulating LDL-C. Shimano et al. (1997), in their study with mice lacking *SREBP1* gene, demonstrated the upregulation of mRNAs for *HMGCS*, *HMGCR* and squalene synthase genes, with a concomitant increase in cholesterol synthesis (65). In a study evaluating the effect of 148 single nucleotide polymorphisms (SNP) across 10 candidate genes to pravastatin therapy in 1536 participants, 2 tightly linked SNP in the *HMGCR* gene (SNP 12 and SNP 29) were found (66). These SNP were significantly associated with a difference in the change in lipid response. However, neither cholesterol synthesis nor absorption assessments were carried out. Future investigation on the effect of these SNP on cholesterol synthesis will provide valuable insights. Genetic polymorphisms in *SREBP 1 & 2* genes associated with cholesterol synthesis are also implicated in the ezetimibe induced cholesterol lowering responsiveness by modulating cholesterol absorption (67). The above finding further asserts the notion that cholesterol synthesis and absorption are tightly linked and generally move in opposition to each other.

2.4.7 Epigenetic factors affecting cholesterol synthesis

Despite the recent progress in the number of newly identified gene loci, presently known genetic factors linked to cholesterol explain only 5–8% of the inter-individual variation in circulating

lipid levels (68). Most of the novel variants in candidate loci that modulate plasma cholesterol levels identified through genome-wide association studies (GWAS) lack a functional explanation (69, 70). However, more recently, some of these associations have been suggested to be modulated by interactions between variants and environmental factors, such as early feeding patterns, revealing the role of epigenetic factors (71). Epigenetic mechanisms play an important role in regulating cholesterol synthesis. Evidence from recent studies has brought into spotlight the role of microRNA (*miRNA*) in cholesterol metabolism and homeostasis (16). *SREBP1* and *SREBP2* genes possess two intron encoded *miRNA*, *miR-33a* and *miR-33b*, respectively. Low cholesterol levels induce the increase in *miR-33a* together with *SREBP2*, whereas high sterol levels promoting the opposite (72). Also the expression of *miR-33a* and *miR-33b*, results in a decrease in the expression of *SIRT6* suggesting the involvement of SIRT (Sirtuin) family of protein deacylases in cholesterol homeostasis (73). A potential role of a *miRNA* response element for *miR-185* in the promoter of *SREBP2* was highlighted when lentiviral overexpression of this *miRNA* led to a decrease in hepatic *HMGCR* and *LDLr* was demonstrated (74). Additionally, it was found that both *miR-520d* and *miR-224* significantly inhibit the mRNA expression of *HMGCR*, indicating that these both play a key role in the coordination of cholesterol homeostasis. Acetylation of the SREBP proteins at the conserved residues by the histone acetylase cAMP responsive element binding protein1-binding protein (CBP)/p300 is known to impede the ubiquitination and degradation of *SREBPs*, thereby enhancing its transcriptional activity (75). Another important finding indicates the ability of *SIRT1* in counteracting this action (76). SREBPs are also thought to undergo phosphorylation enabling the multiprotein Skp, Cullin, F-box containing (SCF) complex to ubiquitinate it (77). These findings suggest that the homeostasis of transcriptionally active *SREBP* is under complex regulation at multiple levels.

Findings from various studies have suggested epigenetic regulation of genes involved in cholesterol biosynthesis. A 10-fold decrease of the rate of cholesterol synthesis due to overexpression of histone deacetylase 3 (HDAC3) in CHO cells has been demonstrated (78). Interestingly, the HDAC3 effect did not primarily affect HMGCR, instead, lanosterol synthase (LSS) catalyzing the cyclisation of the isoprenoid 2, 3-oxidosqualene to the steroid lanosterol, was significantly down regulated. In another study by Knutson et al. (2008) in a *HDAC3* knockout mouse model, liver cholesterol synthesis along with other cholesterogenic genes were markedly increased (79). Therefore, an increase in HDAC3 leads to a decrease in cholesterol synthesis and content (16). However, these studies failed to include the measurement of any markers for cholesterol absorption, and hence future studies are needed to assess whether the epigenetic modulation of cholesterol synthesis causes an equivocal effect on the absorption vector too. It is also important to note that, cholesterol synthesis rates are variable between different organs and tissues, and even between the cells of the same organ (16, 80) accounting for the age related variability of cells involved in cholesterol synthesis (81).

2.4.8 Early nutrition, epigenetic changes and cholesterol metabolism

Intriguing evidence suggests that adult blood cholesterol, and thus CVD risk, may be influenced by feeding practices in early life (82, 83, 84). Importance of cholesterol content of early nutrition and its possible imprinting of later cholesterol metabolism was first hypothesized by Reiser and Sidelmen (85). These authors concluded that serum cholesterol homeostasis undergoes canalization based on the cholesterol content of milk (85). Cholesterol content of human milk (90-150 mg/L) is greater than that of regular cow's milk formulas (10–40 mg/L) or soy milk-based formulas which contain no cholesterol (86). The lower plasma cholesterol concentrations

observed in adults who had been breast-fed in infancy raise the possibility that exposure to high

cholesterol containing breast milk may have long term effects on processes controlling sterol

trafficking and thus plasma cholesterol concentrations in later life (87, 88, 89). Long term

metabolic responses to high intake of dietary cholesterol in infants potentially include elevated

serum cholesterol, increased cholesterol absorption, reduced endogenous synthesis, and

augmented fecal cholesterol excretion. Early exposure to high cholesterol containing breast milk

could reduce endogenous synthesis of cholesterol through down-regulation of the HMGCR and

also as a result of an expanding cholesterol central pool (90). It can be hypothesized that high

cholesterol intake from breast milk may inhibit endogenous cholesterol synthesis, while low

cholesterol intake in formula-fed infants stimulates synthesis. These alterations in

cholesterogenesis rates potentially remain for prolonged periods, providing a benefit later in life

when the individual is challenged with dietary cholesterol (87, 91, 92, 93). Evidence is

accumulating that environmental factors are able to alter gene expression and change the

phenotype through the modification of epigenome (94, 95). The 'developmental origins of adult

disease' hypothesis, often called the 'Barker hypothesis' supports this early programming of

genes and its role in controlling adult life physiology and metabolism (96).

The existence of a maternal-fetal cholesterol transport mechanism in early fetal life has been well

established and also the importance of maternal supply of cholesterol to the fetus in pregnancy

has been emphasized (97). Total cholesterol concentrations in pregnant women increase by

30%–50% due to elevated synthesis in the liver, which is pronounced in the third trimester of

pregnancy (98). Low maternal serum cholesterol concentrations during pregnancy have been

previously linked to microcephaly, preterm birth, growth impairment as well as other adverse

birth events (99). The finding was confirmed by studies which reported intrauterine growth

restriction in pregnancies of mothers who had low circulatory levels of cholesterol (100, 101).

Baardman et al. (2013) in their review underscored the importance of maternal supply of

cholesterol to the fetus during early pregnancy and suggested that disruption of maternal

cholesterol levels due to diseases or metabolic conditions could bear an untoward effect on fetal

development (98). For example, infants born to diabetic mothers are prone to neonatal

macrosomia (102), fetal hyperglycemia and hyperinsulinemia (103). In a study in streptozotocin-

induced mildly diabetic rats, the progenies suffered from fetal macrosomia, which deranged their

cholesterol metabolism from birth through adulthood (104). The mature macrosomic rats

exhibited elevated liver cholesterol synthesis resulting in hypercholesterolemia.

Maternal nutrition during pregnancy plays a major role in determining the health of the offspring

as well as lipid metabolism through fetal programming (105). Protein and folic acid content of

the diet are found to impart a programming effect on the offspring's lipid metabolism which was

also dependent on the fat content of the diet (105). These findings suggest that maternal

undernutrition could result in long term dysregulation in the offspring through epigenetic

mechanisms. However, more research attention is needed in understanding whether such

epigenetic shifts due to early dietary exposure affects the long term configuration of cholesterol-

regulating pathways.

2.4.9 Gut microbiome and cholesterol synthesis

The human intestine harbors ~10^{14} bacteria, a number that surpasses the total number of cells in

the whole body. These bacteria contain 100 fold more genes that the human genome (106, 107).

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This symbiotic community, now more of a microbial organ, may affect energy homeostasis, metabolism and insulin sensitivity (108). Gut microbiome is undoubtedly one of the major determinants of inter-individual variability to dietary interventions. Gut microbiota are known to metabolize cholesterol and bile acids, but their influence on cholesterol synthesis and absorption is an unexplored area (109). Understanding the complex microbiota with different cholesterol and bile acids metabolizing activities will aid in evaluating the real impact of these metabolisms on host's physiology, and the explain the inter-individual variability to cholesterol lowering strategies (109).

In a study with hamsters, supplementation of plant sterol esters resulted in a reduction of cholesterol absorption, accompanied by a shift in the microbial communities (110). A compensatory increase in cholesterol synthesis was also observed, which resulted in a further increase of bile-excreted cholesterol. The authors hypothesized that, the combined effects of high biliary cholesterol excretion and reduced cholesterol absorption increased the concentrations of free and esterified cholesterol in the gastrointestinal tract (110). These cholesterol derivatives were reported to exert an antibacterial effect on specific members of the gastrointestinal microbiota, causing alterations in the microbial community. Assuming that this hypothesis is true, the long term effects of plant sterol treatments can be expected to result in an altered gut microbiome with a differential cholesterol metabolizing capacity. The implication of this change on the reciprocal relationship between cholesterol absorption and synthesis needs to be determined by future investigations. In humans too the same effect could be expected. Also, of noteworthy importance is the short chain fatty acid (SCFA) production and metabolizing functions of gut microbiome (111). SCFA are known to modulate various physiological

functions in the host and serve as substrates in the cholesterogenesis pathways (111). Hence, the role of SCFA enhancing substrates such as dietary fibres and their contribution towards cholesterol homeostasis requires further research.   A clinical trial conducted in 944 individuals taking simvastatin at 40mg/d for 6 weeks used a targeted gas chromatography-mass spectrometry metabolomics platform to measure a panel of metabolites within cholesterol synthesis, dietary sterol absorption, and bile acid formation (112). The authors attempted to determine the metabolite signatures that may aid in the prediction of variation in statin therapy with regards to LDL-C lowering. It was observed that the pretreatment levels of bile acids derived from gut bacteria and nutrient inputs correlated well with response to simvastatin (112). This finding further supports the hypothesis that gut microbiota may play a key role in modulating cholesterol metabolism and determine an individual's response towards cholesterol lowering treatments.

2.4.10 Pharmacological factors

Statins are a known class of drugs which aid in a 20–30% reduction of plasma cholesterol levels by inhibiting HMGCR (113).When the response to statin therapy was evaluated in participants with high initial cholesterol absorption versus those with low absorption, a greater suppression in synthesis in those with low baseline cholesterol absorption levels was found following the treatment (41). Interestingly, no difference in plasma total cholesterol levels were observed between the two groups. In addition, increased cholesterol absorption was observed in those who exhibited larger reductions in synthesis. This study suggests that cholesterol absorption responds reciprocally to changes in cholesterol synthesis (41). Another study found a similar relationship too when comparing the supplementation of atorvastatin and simvastatin, (114) where atorvastatin significantly lowered cholesterol synthesis rates compared to simvastatin.

Unsurprisingly, cholesterol absorption was found to be higher in the atorvastatin supplemented group compared to the simvastatin group (114). The hypothesis that cholesterol absorption behaves reciprocally to changes in cholesterol synthesis is corroborated by the above findings.

2.4.11 Environmental pollutants

One of the major emerging factors that result in significant negative health outcomes are environmental pollutants because of their purported disruptive effects on endocrinal physiology and metabolism of humans (115). Chronic exposure to organic pollutants has been established to result in insulin resistance (116). It is also shown that pollutants such as phthalates, organophosphate and fibrates modulate liver X receptors (LXR) which play an essential role in cholesterol homeostasis, bile acid, triglyceride and carbohydrate metabolism (117, 118). But, how such chronic exposure of the pollutants affect the reciprocal relationship between cholesterol synthesis and absorption needs to be further explored.

2.5 THE CHOLESTEROL ABSORPTION PROCESS

Cholesterol entering the small intestinal lumen, and subsequently absorbed by the enterocytes, is derived from three major sources: diet, bile, and intestinal epithelial sloughing (119). The Western diet contributes ~ 300−500 mg of cholesterol/day (120). Bile contributes about 800−

1200 mg/day and the intestinal mucosal epithelium turnover provides nearly 300 mg/ day (120). The duodenum and proximal jejunum are the major sites of absorption (121, 122). In healthy humans, approximately 50% of intestinal cholesterol is absorbed (123). For the cholesterol to be absorbed, micellar solubilization must take place (124). Dietary cholesterol is partly esterified

(<15%) in comparison to biliary cholesterol which is predominantly unesterified (125). For example, egg yolk cholesterol is 8 to 19% esterified (126). This requires the dietary cholesteryl esters (CE) to be de-esterified by pancreatic carboxyl ester lipase (CEL), before cholesterol can be packed and transported to the brush border of enterocytes (125). It must be noted that, since the biliary pool of unesterified cholesterol is larger than the esterified dietary pool of cholesterol, targeted inhibition of the pancreatic lipolytic enzymes becomes ineffective in reducing cholesterol absorption (119). This finding can explain why disruption of the *CEL* gene has a very modest effect on intestinal cholesterol absorption as demonstrated in mice (127, 128). Two enzymes mainly control the intestinal uptake and transport process of sterols: acetyl-CoA acetyltransferase (ACAT2), which facilitates intracellular cholesterol esterification, and the microsomal triglyceride transfer protein (MTTP), which is involved in intestinal chylomicron assembly (119).

The absorption of biliary cholesterol is affected by its physical chemistry, but it is quite unclear whether dietary cholesterol is absorbed to the same degree as biliary cholesterol (125). Animal studies suggest that dietary cholesterol is ineffectively absorbed relative to biliary cholesterol, when provided as part of a high cholesterol diet, whereas on low and moderate cholesterol the phenomenon does not exist (129). The Niemann-Pick C1 Like 1 (NPC1L1) transporter facilitates the uptake of cholesterol and plant sterols/stanols (130). NPC1L1 is located in the brush border membrane of enterocytes in the proximal jejunum of the small intestine. Mice lacking NPC1L1 gene in comparison to control mice exhibit a 70% and 90% suppression in cholesterol and plant sterol/stanol absorption, respectively (131, 132).Two important half-transporters, adenosine triphosphate protein binding cassette transporters G5/G8 (ABCG5 and ABCG8) are found in the

proximal small intestine, where NPC1L1 co-localizes with them. The primary function of these transporters is to promote the efflux of unesterified cholesterol and plant sterols/ stanols from the enterocyte back into the intestinal lumen (133). The non-effluxed intracellular cholesterol translocates to the endoplasmatic reticulum and subsequently gets esterified by ACAT2 (134). The esterified cholesterol is then incorporated into chylomicron together with triacylglycerols (TAG), phospholipids and to apolipoprotein (apo) B48 by MTTP to be delivered to the lymph (135). In addition, enterocytic cholesterol can be transferred to apolipoprotein A1(ApoAI) high density lipoprotein (HDL) particles via ATP-binding cassette transporter A1 (ABCA1) (136).

2.5.1 Measurement of cholesterol absorption

Sterol balance method is the gold standard method for measuring cholesterol absorption (38, 137). The plasma isotope ratio technique estimates cholesterol absorption by measuring the fraction of tracer labeled cholesterol absorbed over a fixed period of time (138, 139, 140). In this method, cholesterol absorption is derived from the ratio of oral cholesterol tracer enrichment to intravenous tracer enrichment in the blood (140). Another approach to assess cholesterol absorption is to measure plasma surrogate markers such as the ratio of plant sterols (campesterol and sitosterol) or cholestanol (cholesterol metabolite) to cholesterol in plasma (141).

2.6 FACTORS AFFECTING CHOLESTEROL ABSORPTION

2.6.1 Biliary factors and luminal factors

Decreased size of biliary bile salt pool and output and biliary phospholipid output reduces the percent cholesterol absorbed (119, 142, 143, 144). Also, factors such as increased cholesterol

content of bile, hydrophilic-hydrophobic index of bile salt pool and biliary cholesterol output suppress absorption (145, 146). Such a suppression of absorption can be expected to result in a corresponding elevation in synthesis. Conversely, when dietary cholesterol intake is increased, the push-pull mechanism sets in suppressing cholesterol synthesis through SREBP-1 pathway triggering an enhanced secretion of cholesterol into bile and bile acids synthesis from cholesterol (147). Luminal factors such as increased gastric emptying and small intestinal transit times are known to increase absorption (119, 148, 149). Reduced mucin production, especially, Mucin1 has been identified to reduce absorption as well (119, 148). Knock out studies in mice with deficient activities of enzymes CEL and pancreatic triglyceride lipase have shown reduced cholesterol absorption in the intestine (119, 128, 150). These factors could potentially induce the cholesterol synthetic machinery and compensate for the reduced absorption. However, there exists a lack of studies in this area.

2.6.2 Dietary and pharmacological factors

2.6.2.1 Dietary cholesterol

It is known that a difference of 800 mg/d in dietary cholesterol results in only a 6% variation in total plasma cholesterol (42, 151). It is hence widely questioned whether dietary cholesterol independent of plasma cholesterol concentrations is associated with the risk of CVD (42). This is counterpointed by other findings, which indicate a causal relationship between dietary cholesterol and CVD (152, 153, 154). However, lowering dietary cholesterol intake in individuals hyper-responding to dietary cholesterol levels could still be beneficial in reducing CVD risk (155). It has been observed that some individuals hypo-respond to dietary cholesterol,

while others hyper-respond (156) and interestingly, variability in the ability to synthesize cholesterol is also quite common. Such variability becomes utmost important in defining the therapeutic recommendations of hypolipidemic agents for their efficacious action: ezetimibe for hyper-absorbers and statins for strong synthesizers (42).

2.6.2.2 Dietary fibre

Dietary fibres are known to have an impact on cholesterol absorption, because of their ability to sequester bile acids affecting the enterohepatic bile acid cycle (157). Another potential possibility by which dietary fibres could directly affect cholesterol synthesis is by lowering postprandial glucose levels resulting in a decline in insulin levels, consequently suppressing hepatic cholesterol synthesis by inhibition of HMGCR (158). However, these suggestions need further exploration in the direction as to what degree the push-pull function is altered. Also, a bi-directional physiological association between dietary fibres (prebiotics) and their metabolizing gut microbiome is known. Hence, in the context of cholesterol management , it would be interesting to evaluate the impact of prebiotic-probiotic combination on the sterol push-pull mechanism.

2.6.2.3 Phospholipids

The normal dietary intake of phospholipids is 2–8 g/day from foods such as eggs, organ and lean meats, fish, shellfish, cereal grains and oilseeds (159). Higher amounts of dietary phospholipids lead to decreased cholesterol absorption as demonstrated consistently in animal and human

studies (160, 161). However, a lack of information exists as to whether these changes in cholesterol absorption also lead to a concurring change in synthesis.

2.6.2.4 Plant sterols

Plant sterols are naturally occurring sterols with very similar chemical structures to that of cholesterol (162). The North American diet supplies plant sterols at a level of around 300-400 mg/d (162, 163). Campesterol and sitosterol are the most abundant plant sterols, however, less efficiently absorbed than cholesterol and found at low concentrations in human plasma (119, 163). Plant sterols significantly inhibit intestinal cholesterol absorption with an efficacy of 5% to 15% from dietary and biliary sources (140, 164). The exact mechanism by which plant sterols/stanols lower blood cholesterol levels by inhibiting absorption remains unresolved. The poor solubility and the crystalline nature of plant sterols/stanols is thought to interfere in their dissolution in the small intestine and as a result compete with the absorption of cholesterol (164). More than 200 clinical trials have consistently confirmed the effect of plant sterols/stanols on circulating cholesterol levels. Some of the major factors affecting LDL-C lowering efficacy of plant sterols/stanols are subject characteristics, food matrix, frequency, and time of intake (162) as well as dosage responses (165, 166). Plant sterols and stanols are poor substrates for ACAT2 compared to cholesterol, a major limiting factor of their absorption (167). In addition to lowering cholesterol absorption, plant sterols and stanols are reported to induce a compensatory rise in cholesterol synthesis (140, 168, 169, 170). Mackay et al. (2015) conducted a dual center, randomized crossover clinical intervention, where 63 mildly hypercholesterolemic adults who were segregated as low and high cholesterol synthesizers based on the baseline lathosterol to cholesterol ratio consumed 2 g plant sterols/day for 28 days (171).

The authors demonstrated that low cholesterol synthesizing individuals responded to plant sterol treatment with a reduction in TC and LDL-C, whereas the high cholesterol synthesizing individuals failed to show such a change (171). It is quite clear from this study that the cholesterol absorption inhibitory effect of plant sterols is tied to endogenous cholesterol synthesis.

Several polymorphisms in the *ABCG5* and *ABCG8* genes that may moderately influence plasma sterol levels have been identified (172). A pathophysiological condition called sitosterolemia exists, which is a rare autosomal recessive disorder characterized by high absorption and retention of sterols, caused by mutations in the *ABCG5/G8* genes (133, 173). Patients with sitosterolemia absorb 20–30% of dietary sitosterol intake, in contrast to the typical <5% sitosterol absorption rate in normal individuals (174, 175, 176, 177).

A variable LDL-C lowering efficacy in response to plant sterol treatment has always been known (170, 178). A retrospective study conducted by Rideout et al. (2010) evaluated the LDL-C lowering efficacy of plant sterol interventions, by measuring FSR of cholesterol in 135 individuals. The non-responders to plant sterol therapy had significantly higher basal cholesterol FSR, when compared to the responders (179). This means that individuals exhibiting high cholesterol absorptive efficiency, respond to plant sterol treatment well, which is also supported by other studies (180, 181).

Table 2.1 Factors influencing cholesterol synthesis and absorption

Factors	Implication in cholesterol synthesis and absorption	References
Body weight	Weight gain increases synthesis rates	(45)
Endocrine factors	Major hormones: thyroid, insulin, glucocorticoids, estrogens (affects synthesis)	(14, 56, 58)
Circadian rhythm	Delaying meal times modulates synthetic rates	(49, 145)
Genetics	Gene polymorphisms: *HMGCR*, *SREBP*1&2 (synthesis) & *APOE*, *ABCG5&8*, *NPC1L1*, *ACAT2* (absorption)	(66, 67, 187, 195, 197, 201)
Gut microbiome	Roles in SCFA production & composition of bile acid pool. Alters absorption and synthesis	(110,111, 211)
Biliary & luminal factors	Biliary bile salt & phosholipid output regulates cholesterol absorption. Increased gastric emptying increases absorption	(119)
Early nutrition & Epigenetics	Maternal nutrition, maternal-fetal cholesterol input and metabolic state controls fetal programming and cholesterol metabolism in offspring	(98, 100, 104)
Dietary factors	Dietary fat quality and quantity, dietary cholesterol affects synthesis and absorption. Plant sterols, dietary fibres and phospholipids intake reduces absorption	(20, 27, 36, 140, 157,160)
Pharmacological factors	Statins (synthesis), Ezetimibe (absorption)	(113, 182)
Environmental pollutants	Endocrine disruption, modulation of LXR and synthesis	(115, 118)

HMGCR-3-hydroxy-3-methylglutaryl-Co-enzymeA reductase gene; *SREBP1&2*-sterol regulatory element binding protein 1&2 gene; *APOE*-apolipoprotein E gene; *ABCG5&8*-ATP-binding cassette, subfamily G, member 5&8 genes; *NPC1L1*- Niemann-Pick C1 Like 1 transporter gene; *ACAT2*- acetyl-CoA acetyltransferase gene; SCFA-short chain fatty acids; LXR-Liver X receptors
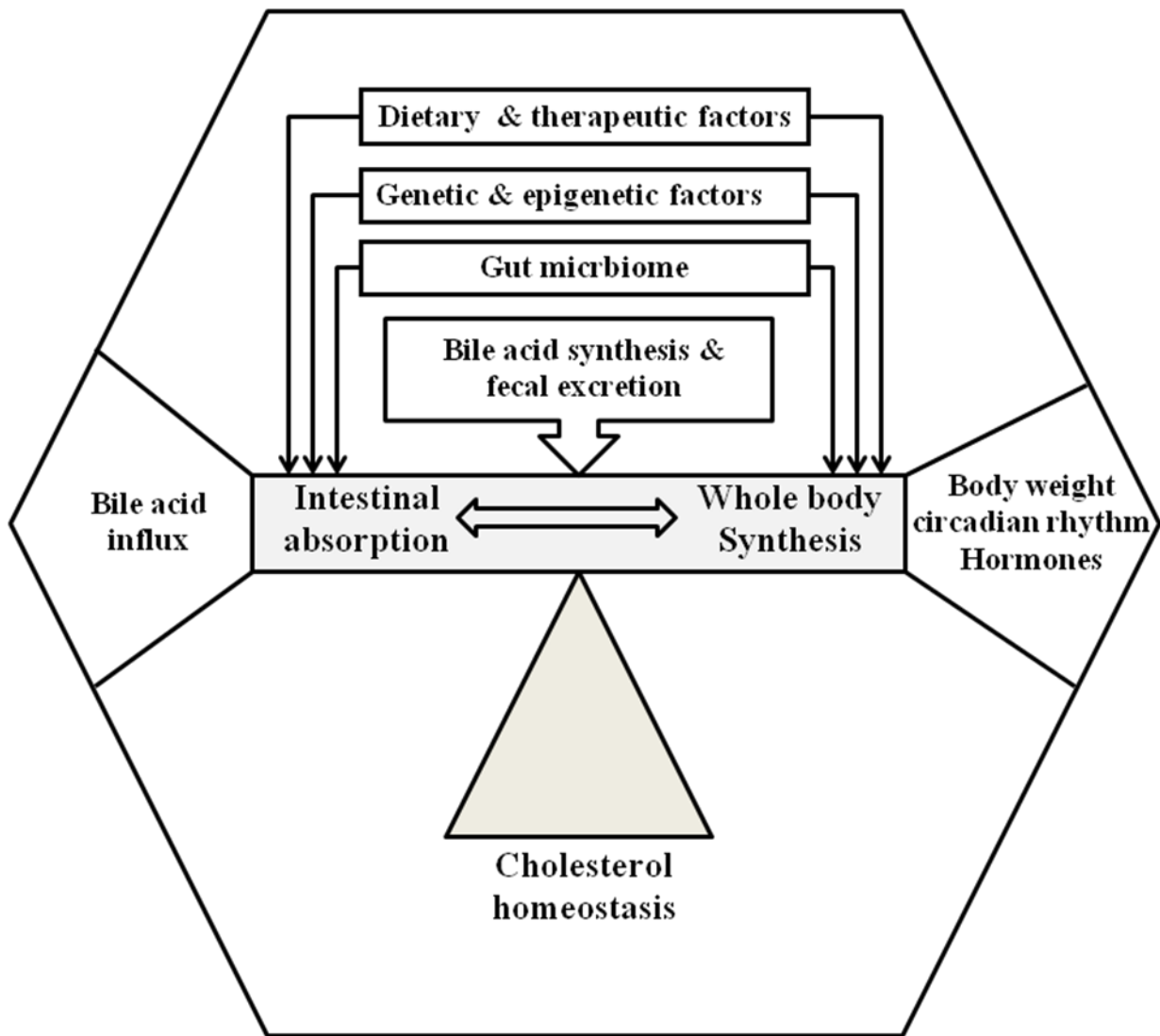
Figure 2.1 Factors governing cholesterol absorption and synthesis

2.6.2.5 Therapeutic factors affecting cholesterol absorption

Ezetimibe (SCH 58235) is a pharmaceutical drug, developed to reduce cholesterol absorption (182), which exerts its action by interfering with the function of the NPC1L1 transporter (132, 183). A dose of 10 mg/d of ezetimibe is found to lower TC up to 15% and LDL-C up to 20% (182). The effect of ezetimibe on cholesterol absorption and synthesis was evaluated in a randomized, double-blind, placebo-controlled cross-over trial, in individuals with mild to moderate hypercholesterolemia (123).The authors reported a 54% decrease in cholesterol absorption due to ezetimibe compared to placebo and also found a corresponding rise in cholesterol synthesis. Thereby, ezetimibe also results in a compensatory increase in cholesterol synthesis while inhibiting absorption. But, the inter-individual variability in the reciprocal effects have been less explored.

2.6.3 Genetic factors influencing cholesterol absorption

Genetic factors are heavily involved in regulating cholesterol absorption and also attest to the existence of many gene–nutrient interactions explaining the variability of individual responses (184, 185, 186). The most notable polymorphisms studied were in Apolipoprotein E (*APOE*), which is responsible for 7% of the variation in cholesterol levels (187). Among the polymorphisms, carriers of the *APOE2* allele are known to have lower rates of cholesterol absorption and higher rates of cholesterol synthesis compared to carriers of the *APOE3/E4* allele (187, 188, 189). For instance, *APOE4* individuals tend to be more sensitive to dietary cholesterol exhibiting nearly a 10% increase in TC for additional 300 mg/d cholesterol (190). However, the effect is suggested to be subdued by a high PUFA: SFA ratio (191). Plant sterols or ezetimibe would work well with these individuals. Interestingly, the *APOE* polymorphism seems to be

poorly correlated with cholesterol metabolism at low cholesterol intakes (26,189). Hence, it could be expected that the apparent effect of *APOE* polymorphisms on the reciprocal relationship between cholesterol absorption and synthesis could possibly be exhibited only at higher levels of dietary cholesterol intakes.

A common variant in the promoter region (-204A > C) of the cholesterol 7α-hydroxylase gene (*CYP7A1*) is shown to be associated with enhanced CYP7A1 activity (192, 193). C-allele carriers due to increased intestinal bile acids and more efficient cholesterol absorption show enhanced cholesterol lowering in response to plant sterol consumption. Also, an increased feedback cholesterol synthesis to the plant sterol intervention was observed confirming the existence of the push pull mechanism between the two vectors of cholesterol trafficking (192). The allele *D19H* (exon 1 mutation) of the *ABCG8* gene was found to be associated with low cholesterol absorption markers and high cholesterol synthesis markers (61). Also on the Island of Kosrae, an *ABCG8* exon 2 mutation was identified and the carriers displayed elevated plasma plant sterols levels and decreased lathosterol levels suggesting an heightened absorption, and a concomitant reciprocal suppression of synthesis (194).

Mutations in the *NPC1L1* gene have been reported in individuals displaying low cholesterol absorption and an associated lower levels of plasma LDL-C concentrations (195). Hegele et al. (2005), studied a haplotype found to be common to 1 in 8 subjects, and associated with the inter-individual variation in the response of plasma LDL-C to ezetimibe treatment (196). Also, feeding a cholesterol/cholate diet to wild type mice results in down regulation of intestinal mRNA expression of *NPC1Ll* (131). Mice lacking *NPC1L1* however displayed upregulated intestinal

HMGCS mRNA, and as a result, increased cholesterol synthesis (131). All these findings point to

the possibility that *NPC1L1* polymorphisms may control cholesterol synthesis by affecting

cholesterol absorption. Buhman et al. (2000) showed that, *ACAT2* deficient mice possessed

reduced dietary cholesterol absorption ability on a high cholesterol diet, but not when fed a chow

diet (197). The nuclear hormone receptor Farnesoid X-receptor (FXR) is involved in the

regulation of many aspects of cholesterol metabolism, including reverse cholesterol transport,

intestinal cholesterol absorption and enterohepatic circulation of cholesterol. *FXR*-deficient mice

display increased intestinal cholesterol absorption (198). Disruption of *Mucin1* gene (which

regulates mucin secretion in the small intestine) reduced the intestinal cholesterol absorption by

50% in mice (148). Recently, a novel variant within the gene locus of *PARK2* (parkin RBR E3

ubiquitin protein ligase), *rs9364628*, was identified in two Finnish cohorts of healthy children

using a large scale genotyping (199). The *PARK2* variant was found to be associated with dietary

fat quality and serum LDL-C concentrations. However, the effect of this polymorphism on

cholesterol trafficking and the push-pull mechanism needs to be studied.

A number of studies have explored the effect of genetic polymorphisms on the LDL-C

cholesterol lowering effects of plant sterols. In one study, the association between genetic

variation in *HMGCR* and LDL-C lowering response to plant sterols was examined using a

retrospective clinical evaluation of 112 subjects consuming 3.8 g plant stanol esters/d for 8

weeks (200). The study, however, failed to find any associations. In another investigation, where

82 hypercholesterolemic individuals fed plant sterols were categorized according to low vs. high

basal plasma plant sterol concentrations, individuals with mutant alleles of the *NPC1L1*

haplotype of 872 C > G (L272L) and 3929 G > A (Y1291Y) showed marked LDL-C reductions

compared with their wild type counterparts (201). These findings were contradicted by the results of Rudkowska et al. (202) who found no association for the same variants in *NPC1L1* and responsiveness to cholesterol. Also, the study by Zhao et al. (201) revealed that the carriers of the A allele of the *T400 K ABCG8* polymorphisms responded greatly to LDL-C lowering action of plant sterols. However, it has to be understood that responsiveness to any dietary intervention is controlled by multiple genes (186, 203). This suggestion implies that the combination and interaction of different gene polymorphisms may affect the way an individual responds to a dietary intervention.

2.6.4 Epigenetic factors in cholesterol absorption

Limited data exists on the epigenetic regulation of key genes controlling cholesterol absorption. Methylation of the promoter region of *NPC1L1* gene in a mouse model was found to regulate its expression in the gastrointestinal tract, especially, resulting in a low colonic expression (204). Interestingly, the methylation was not present in the ileum or jejunum, and 5AZA (Decitabine; 5-Aza-2′-deoxycytidine) treatment resulted in the release of this suppression in NPC1L1 expression in the colon (204). This seems to explain the redundancy in the regulatory process (16). Also, very limited data exists on the epigenetic regulation of intestinal *ABCG5/G8*. Future studies are required to examine the role of epigenetic mechanisms on these intestinal transporters involved in cholesterol absorption and the way in which they modulate cholesterol synthesis and impact the overall cholesterol metabolism.

2.6.5 Role of epistatic mechanisms in regulating cholesterol metabolism

Most of the SNPs associated with complex diseases bear a small effect size and only contribute to a miniscule portion of the variability caused by their additive effects (205, 206). The case of missing heritability is thought to result from structural variants, rare variants, gene-environment interactions, epigenetics, and complex inheritance (207). Epistasis (gene-gene interactions) could explain heritability to a certain level (208).The role of epistasis in understanding the relationship between genotype and phenotype is gaining more attention recently. However, the application of the same is at its inception, and is limited by the requirement of elaborate and complex series of analyses (209). Ma et al. (2012) assessed the effect of gene–gene interactions of 125 SNPs from 95 loci on the levels of TC, LDL-C, HDL-C, and TAG based on prior knowledge of established GWAS hits, protein– protein interactions, and pathway information. The researchers identified an interaction between *HMGCR* and a locus near *LIPC* (hepatic triglyceride lipase) and its effect on HDL-C levels (210). Interestingly, the authors also revealed that the effect of the gene-gene interaction on HDL-C was more pronounced than that predicted by the sum of the marginal effects of the two loci independently (210). Hence, the application of the epistasis becomes important to reveal the effects of a group of genes interacting together to result in a phenotype. Cholesterol metabolism, especially synthesis and absorption, is governed in an individual by complex interplay between genes and their resultant metabolic end products. Such molecular approaches will help in a comprehensive understanding of the mechanisms orchestrating these vectors and will eventually help in designing better strategies of lowering cholesterol.

2.6.6 Gut microbiome and cholesterol absorption

The role of gut microbiome in cholesterol absorption originates from the fact that the bile acid metabolism is affected by the gut microbiome. Recent studies show that microbiome not only controls the composition of the bile pool but also affects the expression of genes controlled by *FXR* (211). In the small intestine, bile salt hydrolase-active bacteria in the gut microbiota perform bile acids deconjugation and aid in the maintenance of normal levels of circulating deconjugated bile acids and cholesterol (211, 212).These studies therefore suggest that gut microbiome could potentially affect intestinal cholesterol absorption and also result in a corresponding alteration of cholesterol synthesis.

2.7 CONCLUSION AND FUTURE PERSPECTIVES

In this review, various factors affecting cholesterol synthesis and absorption and how perturbation of one vector leads to a reciprocal change in another has been discussed. Cholesterol lowering studies with statins, ezetemibe and dietary constituents such as dietary cholesterol and plant sterols have unequivocally established the existence of a push-pull mechanism between cholesterol synthesis and absorption. However, it is not uncommon to see large inter-individual variability affecting the magnitude to which these two vectors change in relation to one another. In order to develop effective and personalized cholesterol lowering strategies specific towards hyper-absorbers and high synthesizers, an in-depth understanding of the various unexplored aspects affecting this push-pull mechanism becomes essential. In this context, this is the first review to suggest the potential role of multifarious elements such as epigenetic & epistatic mechanisms and gut microbiome in controlling this system beyond their conventionally

established functions. Future research in these directions could unearth the mechanisms responsible for the less understood reciprocal relationship between cholesterol synthesis and absorption in response to therapeutic and dietary interventions. Understanding such mechanisms would serve as better tools for prediction of the clinical outcomes of an intervention in an individual with respect to his/her genotype and environmental interaction. This would certainly enable the design and implementation of effective clinical management of hypercholesterolemia and alleviate the morbidity associated with CVD.

Conflict of interest: None

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BRIDGE TO CHAPTER III

The following chapter presents the importance of analyzing LDL and HDL subclass distribution in enhancing the predictive power of plasma LDL-C and HDL-C measurements for the estimation of an individual's CVD risk. The review summarizes the effects of dietary cholesterol versus plant sterol consumption on lipoprotein subclass distribution and its implications in cardiovascular health. Specifically, the research gap with regards to the role of common genetic polymorphisms within cholesterol metabolic pathways and their potential modulatory effects on lipoprotein distribution responses to dietary cholesterol and plant sterol consumption is highlighted. In general, this chapter serves as the basis for the research interest and project presented in Chapter V.

CHAPTER III

MINI-LITERATURE REVIEW

SIGNIFICANCE OF LIPOPROTEIN SUBCLASS ANALYSIS: IMPACT OF DIETARY CHOLESTEROL AND PLANT STEROL CONSUMPTION ON LIPOPROTEIN SUBCLASS DISTRIBUTION

3.1 INTRODUCTION

The burden of cardiovascular disease (CVD) still remains the foremost cause of disability and mortality worldwide. Elevated levels of plasma low density lipoprotein cholesterol (LDL-C) and low levels of high density lipoprotein cholesterol (HDL-C) are identified as some of the key risk factors for CVD by the Adult Treatment Panel III of the National Cholesterol Education Program (1, 2). Thus dietary and therapeutic interventional strategies that lower LDL and improve HDL remain the major focal point for CVD risk alleviation among health practitioners. Recently, the adequacy of using plasma LDL-C and HDL-C concentrations in diagnosis and treatment administration for individuals with high CVD risk has been questioned (3, 4, 5). As a result, a need for additional measures of estimating CVD risk has risen. Determining LDL particle size distribution has been suggested to confer greater predictive power to LDL-C measurements in CVD risk estimation (1). Lipoproteins constitute a broad and diverse array of particles with distinct differences in size, shape, density, and composition especially with respect to lipid and apolipoprotein content (6). The 'lipid triad" or "atherogenic lipoprotein phenotype" (ALP) commonly seen in individuals with cardiovascular diseases is characterized by the prevalence of

small, dense LDL particles, and usually related with increased concentrations of triglyceride (TG) and very low density lipoprotein (VLDL) particles, along with reduced concentrations of HDL-C and large HDL particles (7, 8, 6). The aim of this literature review, therefore, is to provide an outlook on the characteristics of LDL and HDL lipoproteins, their respective subclasses and their clinical significance. Also, this review serves to delineate the effect of dietary sterols, namely dietary cholesterol and plant sterols on lipoprotein particle size distribution and the role of genetic polymorphisms modulating the responsiveness to these sterols.

3.2 LIPOPROTEIN SUBCLASSES AND HETEROGENEITY

3.2.1 LDL subclasses

LDL is the major carrier of cholesterol in circulation (9) with a molecular mass varying between 2.4-3.9 MDa (10). The density of human LDL particles ranges between 1.019–1.063 g/ml, made up of approximately 50% free and esterified cholesterol, 25% protein, 20% phospholipids and 5% TG (11, 12). Over 95% of the LDL protein mass is apolipoprotein B-100 (apo B-100), which has a molecular mass of 549 kDa is the major (95%) protein component of LDL, with each LDL particle containing only one molecule of apo B-100 (11, 12). LDL particles are usually spherical with a central core packed with cholesteryl esters and TG (13). Free cholesterol provides rigidity to the LDL outer coat which is in the form of a phospholipid monolayer, and is found intercalated between the fatty acid chains of the phospholipids (13).

LDL subclasses manifest distinct heterogeneity, varying in size, density and composition and other properties such as isoelectric point, electrical charge and immunoreactivity (13, 14). Also,

the sub populations of LDL exhibit a large inter-individual variability with respect to size and composition (14). All the LDL subclasses have apoB-100 as their major protein component and cholesteryl ester as their primary lipid moiety (15). The density determines the free cholesterol content and usually free cholesterol decreases with increasing density (15). Sialic acid content is also suggested to determine the atherogenicity of the LDL particles (16, 17). Small LDL particles have lower sialic acid content and are thought to possess greater affinity towards arterial wall proteoglycans (16, 17, 18). The small LDL particles are therefore considered more atherogenic due to their ability to attract macrophages by penetrating the arterial wall and being more vulnerable for becoming oxidized (6). Such attraction of macrophages and their conversion to foam cells eventually result in the induction of the atherosclerotic process (19). Some of the other atherogenic attributes of small dense LDL particles suggested are its low affinity for LDL receptor (20, 21, 22) and stimulation of vasoconstrictor and pro-aggregatory intermediates (23). A bimodal distribution of particle diameter of the LDL major peak has been used to classify individuals (24, 25). Two major patterns of LDL are observed in individuals, pattern A with a mean particle diameter 25.5 nm or greater and pattern B with less than 25.5 nm mean particle diameter (24, 25, 14).

3.2.2 HDL subclasses

Plasma HDL constitute a diverse spectrum of lipoprotein particles, characterized by a small size (5-12 nm) and density >1.063 g/mL (26, 27). ApoA-I is the major apolipoprotein (60%) of HDL particles, followed by apoA-II (20%), and minor quantities of other apolipoproteins such as apo C, E, A-IV, D and J (26, 27). The outer layer which interfaces with the plasma is composed of free cholesterol and phospholipids together with few apolipoproteins, whereas TG and

cholesteryl esters constitute the central core of hydrophobic portion (28, 26). Similar to LDL particles, the subclasses of HDL also vary in size and composition and exhibit variations in shape, charge and density (28). The continuous intravascular remodeling of HDL particles throughout the process of reverse cholesterol transport (RCT) adds to the HDL subclass heterogeneity (29). RCT involves the selective uptake of cholesterol from peripheral tissues, and subsequent transportation to the liver, where the cholesterol is directed for biliary excretion (30). Nascent discoidal HDL particles undergo successive lipidation by lecithin-cholesterol acyltransferase enzyme during the process of RCT, increasing in size from small to large HDL particles (29).

The relative concentrations of HDL subclasses are considered more important than the HDL-cholesterol concentrations per se in determining the anti-atherogenic potential of HDL due to enhanced RCT (31, 32, 33). Large HDL subclass particles have been negatively associated with CVD risk (6, 34). In a case-control study, where 59 type 1 diabetic controls were compared with incident coronary artery disease cases, a decrease in large HDL particles was reported in coronary artery disease patients with Type 1 diabetes (35). The notion that larger HDL particles are more athero-protective is supported by most studies and therapeutic interventions are targeted to improve large HDL subpopulations (36, 37, 38, 39, 40, 41, 42, 43). Cholesteryl ester transfer protein (CETP) plays a major role in remodeling of HDL by mediating the equimolar exchange of cholesteryl esters (CE) from HDL particles for TG in apoB100-containing lipoproteins (44, 32). Enrichment of apoB100-containing lipoproteins with CE occurs under conditions of elevated CETP activity, resulting in reduced plasma HDL-C concentrations, which is undesirable (44, 45). However, it has been suggested that when increases in CETP activity are

simultaneously observed with elevated levels of HDL-C, reverse cholesterol transport is activated with enhanced CE catabolism, conferring greater cardio-protection (45, 46).

3.3 MEASUREMENT OF LDL AND HDL SUBFRACTIONS

LDL and HDL subclasses are fractionated by taking advantage of their differences in physico-chemical properties including density, size and mobility (47, 48). Ultracentrifugation is one of the earliest applications in the attempt of fractionating and quantification of LDL and HDL subclass particles (49, 50). Based on the flotation rate, LDL can be fractionated into three subclasses LDLI, II and III (50), while for HDL two density fractions could be obtained utilizing this method, HDL-2 and HDL-3 (49, 51). However, this method is often marred by technical demands making it impractical for clinical use (52). Vertical auto profiling is another derivation of ultracentrifugation method used for lipoprotein subfractionation (53, 54). Non-denaturing gel electrophoresis is another technique by which LDL and HDL subfractionation is performed (55, 56). Using this method, 7 LDL subclasses (LDL-I, LDL IIa, LDL IIb, LDL IIIa, LDL IIIb, LDL IVa, LDL IVb) can be separated (57) and with respect to HDL particles, HDL-2 and HDL-3 subfractions can be further separated into five subfractions: HDL-2b, HDL-2a, HDL-3a, HDL-3b and HDL-3c (55). Two dimensional electrophoresis offers improved resolution of lipoprotein particles (58). However, the electrophoretic methods despite wide adaptability and accuracy have certain limitations in terms of variability in measures and need for strict quality control and custom made gels (52).

A method of analysis using double dextran precipitation exists where HDL particles can be fractionated into HDL-2C an HDL-3C using double precipitation (59). Despite its cost-

effectiveness and ease of use, the validity of the results obtained using this method questions its use (52). HDL lipoproteins can also be separated by immuno-affinity chromatographic method (60), however, the robustness of this method has not been studied thoroughly. Recently, a gas-phase, ion mobility, electrophoretic method has been described, which fractionates and directly estimates large HDL2b particles from smaller HDL particles on the basis of macromolecular mobility (61). The LipoPrint system® is a 3% linear polyacrylamide gel tube electrophoresis method, based on electrophoresis, which can separate LDL into 7 subclasses and HDL into 10 main subclasses (62). This method classifies HDL subclasses into three main groups; large (HDL1-3; >88 Å), intermediate (HDL4-7; 73-88 Å) and small (HDL8-10; <73 Å) relative to particle size (62). Studies have shown that high concentrations of small HDL subfractions (HDL8-10) are linked to the atherogenic lipid profile, suggesting deleterious functionality for these subfractions (63, 64). Nuclear magnetic resonance spectroscopy is a valuable technique for the determination of lipoprotein particle concentrations, based on emitted signals from frequencies of the terminal fatty acyl methyl group proton by the lipids in lipoprotein particles, using a library of reference spectra of lipoprotein subclasses (65, 36). Other methods of analysis of lipoprotein particle size include ion mobility (66) and high-performance liquid chromatography (67).

3.4 EFFECTS OF DIETARY CHOLESTEROL CONSUMPTION ON LIPOPROTEIN SUBCLASSES

3.4.1 Dietary cholesterol and LDL subclasses

Various studies have shown that the size and distribution of plasma LDL subclasses are profoundly modified by dietary cholesterol consumption (68, 46, 69, 2). For instance, in one

such study conducted by Mutungi et al. (70), providing an additional 640 mg of cholesterol/d along with a carbohydrate restricted diet resulted in larger plasma LDL diameter, elevated concentrations of plasma large LDL subclass particles and reduced number of small LDL particles. All these cholesterol challenge studies evince that the lipoprotein profile changes resulting from dietary cholesterol consumption do not increase the risk for CVD.

A majority of the population do not display any increase in plasma LDL-C even at high cholesterol intakes of 640 mg/day, while hyper-responding individuals exist, who increase their plasma TC and LDL-C levels to cholesterol intake (71, 45, 72, 73, 74). In a cross-over study with 25 men and 27 premenopausal women, consumption of 640 mg/d of cholesterol for 30 days resulted in an increase in large LDL-1 subclass particles in hyper-responders (46). The authors indicated that high cholesterol intake even in hyper-responders does not seem to negatively affect the atherogenicity of LDL particles. Therefore, regardless of responsiveness, reductions in plasma small LDL subfractions (69, 70) and corresponding increases in large LDL (46, 68), are always seen in these cholesterol feeding studies. Noteworthy, some studies have observed plasma LDL profile shifts from a more atherogenic pattern B to the desirable pattern A after cholesterol supplementation, although only in some individuals (75, 70).

3.4.2 Dietary cholesterol and HDL subclasses

Studies which have reported increases in LDL-C levels as a result of cholesterol feeding have also observed concomitant increases in HDL-C concentrations (76, 77, 74, 78, 79). Increased enrichments of large HDL subclass particles are always associated with such increases in HDL-C levels (69, 70). Importantly, regardless of the responsiveness to dietary cholesterol intake, HDL

particle size increases are frequently observed, with hyper-responders showing relatively higher concentrations of the large HDL particles compared to hypo-responders (69). This formation of large HDL particles following dietary cholesterol consumption has been suggested to occur as a result of the enrichment of HDL particles with cholesterol (80). Clifton et al. (80) administered 700 mg/day of dietary cholesterol to hyper and normocholesterolemic men and women, and found significant increases in HDL2 concentrations. Mutungi et al. (70) reported that 640 mg/day of dietary cholesterol consumption increased large HDL particles in overweight and obese men. However, in insulin-dependent diabetic patients, cholesterol consumption of 800 mg/day for 3 weeks failed to alter HDL subclass distribution or total HDL concentrations (81). However, in the same study, control participants showed a substantial increase in large HDL2a concentrations (81). Also, control participants alone displayed a significant enrichment of HDL particles with cholesterol following cholesterol intake (81). This indicates impaired HDL metabolism in diabetic patients and hence cholesterol consumption may not necessarily reduce CVD risk by increasing the anti-atherogenic large HDL subclass particle concentrations.

Gender has been showed to significantly influence the HDL-C and HDL subclass distribution in response to dietary cholesterol consumption (82, 83). In a randomized controlled study, where men and women matched for age, BMI, plasma LDL-C and TG consumed 650 mg cholesterol and 31 g of fat/day for 3 weeks, women exhibited relatively higher increases in HDL2 cholesterol than did men (82). This observation was replicated in a further study by Clifton et al. (83) with 120 men and women participants. Such variations in responses among men and women in HDL subclass distribution are thought to be partly because of the differences in fat distribution between the men and women (83). Also, it has been suggested that compared to men, women

have greater efficiency in incorporating cholesterol from diet into large HDL subclasses by facilitating the transport of cholesterol from TG-rich particles (82).

3.5 EFFECTS OF PLANT STEROL CONSUMPTION ON LIPOPROTEIN SUBCLASSES

3.5.1 LDL subclass distribution in response to plant sterol and stanol consumption

Plant sterols are structurally similar to cholesterol (84), and similar to cholesterol, plant sterols are also carried in the circulation in lipoproteins, with LDL particles carrying 70-80% of the plant sterols and the rest 20-30% carried by HDL lipoprotein particles (85). The efficacy of plant sterol intake in lowering plasma LDL-C concentrations has been well-established (86, 87) and consumption of 2 g/d of plant sterols results in plasma LDL-C reductions of 5-15% (86). The typical North American diet provides approximately 300-400 mg of plant sterols per day (84). Small intestinal absorption of plant sterols is less (<2%) compared to that of dietary cholesterol, which is absorbed with ~50% efficiency (88). Plant sterols exert their plasma LDL-C lowering effect by inhibiting cholesterol absorption, however, the exact mechanism of action remains unclear (89).

Studies conducted in type 2 diabetic, overweight and moderately hypercholesterolemic individuals failed to observe any changes in LDL particle size after plant sterol intake despite significant reductions in plasma LDL-C concentrations (90, 91, 85). However, a decrease in small dense LDL cholesterol is usually seen in many studies following plant sterol consumption (90, 92, 93, 94). Studies in type 2 diabetic patients with plant sterol supplementation revealed reductions in small dense LDL cholesterol measured by ultracentrifugation (90, 92). In adults

with metabolic syndrome, a plant sterol supplementation of 4 g/day for a period of 2 months also showed significant reductions in both total and small dense LDL cholesterol concentrations (93). Also, a similar effect was found in hypercholesterolemic children, who consumed 2 g/d of plant sterols along with the Step II diet for a long duration of 6-12 months (94).

Studies in individuals with metabolic syndrome, moderate hypertriglyceridemia and in normolipidemic individuals showed that plant stanol consumption of 2 g/day significantly lowered large and medium VLDL particle concentrations, however, was relatively less effective in normolipidemic individuals (95). In another study, where individuals with metabolic syndrome consumed either 2.5 g/day of plant sterols alone or in combination with different doses of eicosapentaenoic + docosahexaenoic acids, no changes in LDL particle distribution were observed with plant sterols alone (96). However, plant sterols alone induced a reduction of cholesterol content in total LDL in four LDL subclasses. De Smet et al. (97) evaluated the effects of plant stanol ester consumption for 3 weeks on lipoprotein particle concentrations of healthy participants and found reduced concentrations of total VLDL, small VLDL and large LDL with no changes in levels of small LDL. Overall, results from studies evaluating the effects plant sterols on lipoprotein subclasses are inconclusive and warrant further research investigation.

3.5.2 HDL subclass distribution in response to plant sterol and stanol consumption

Plant sterol consumption in general does not have any effect on HDL-C concentrations (98, 85). Also, no changes in HDL particle concentrations are typically reported. Plat and Mensink (95) failed to find any changes in HDL particle concentrations in individuals with metabolic syndrome and normolipidemic individuals in response to plant stanol consumption. Jacobs et al.

(96) reported that plant sterol consumption alone did not change HDL subclass distribution, but when combined with eicosapentaenoic + docosahexaenoic acids, reduced cholesterol and TG levels in the large VLDL and small HDL particles and increased the large HDL particles. However, this effect on HDL particle distribution could only be attributed to lipoprotein modifying role of omega 3 fatty acids (99).

3.6 GENETIC POLYMORPHISMS AND RESPONSES OF LIPOPROTEIN PARTICLE DISTRIBUTION

Various factors have been suggested as affecting LDL and HDL lipoprotein heterogeneity including age (12), gender (50, 100, 82, 83) and plasma lipid and lipoprotein levels (12, 50, 101). The heterogeneity in lipoprotein distribution based on gender has been linked to the male-female differences in hepatic lipase (HL) activity (12, 102, 100). Also, emerging associations indicate that lipoprotein lipase (LPL) and CETP participate in the remodeling of lipoproteins and subclass allocation, contributing to the observed inter-individual variability in lipoprotein particle size distribution (103, 104, 100). However, the role of genetics has been increasingly suggested to account for the heterogeneity in lipoprotein subclass responsiveness to dietary and therapeutic interventions (16, 105).

One of the earliest observations on the effect of a genetic trait in LDL subclass patterns was reported by Austin et al. (106). The authors, by performing segregation analysis of 61 nuclear families, showed that phenotype B (ALP phenotype) with small dense LDL particles could be inherited as a single-gene trait with a dominant mode of inheritance (24). In a further study, the authors demonstrated an association between phenotype B and increased concentrations of

plasma TG, VLDL, IDL, and apo B along with reduced concentrations of HDL cholesterol, HDL2 mass, and apo A-I, suggesting that ALP could be a genetic marker for increased CVD risk (7). In an attempt to investigate the association of specific genes involved in ALP phenotype, Austin et al. (107) investigated the genetic linkage between LDL size, TG, and atherogenic lipoproteins and 8 candidate genes within lipid metabolic pathways using a quantitative sib-pair linkage analysis approach in a sample of 126 dizygotic women twin pairs. Their analysis revealed the possible role of *APOB* gene in controlling LDL heterogeneity (107). Following this, other attempts focused on finding associations between apolipoprotein B (*APOB*) loci and LDL subclass phenotypes under the strong assumption that *APOB* loci may be responsible for the LDL heterogeneity (16, 108, 109). However, all these studies failed to see any linkage indicating that *APOB* locus may not control LDL subclass phenotypes. Overall, the relationship between *APOB* variants and lipoprotein subclass distribution remains unclear.

Zambon et al. (105) studied the association between common polymorphisms in the *LIPC* promoter region and LDL buoyancy, HDL2 cholesterol concentrations and HL activity in a case control study, comparing coronary artery disease patients with controls. It was found that the genotype of the *LIPC* promoter polymorphism at position -250(G$\rightarrow$A) accounted for 20 to 32% of the HL activity and possibly mediated LDL particle size, buoyancy and HDL-2C levels (105). Couture et al. (110) also reported an association between a promoter polymorphism in *LIPC* promoter (C to T substitution at the -514 position) and lipoprotein subclass profiles in the Framingham Offspring study participants. All these studies confirm the multilocus control of the LDL phenotypes, to which other studies add support using sib-pair linkage analyses (107, 111). Genetic polymorphisms were also thought to influence the lipoprotein subclass distribution and

responsiveness to dietary and therapeutic interventions. Also, the effects of such polymorphisms on dietary cholesterol and plant sterol induced responsiveness need to be better understood. The aforementioned studies did not investigate the role of common genetic variants on lipoprotein subclasses to cholesterol and plant sterol supplementation. Hence, a paucity of research exists on the role of common polymorphisms on lipoprotein subclass responsiveness to dietary sterols.

3.7 CONCLUSION

From this review, it is evident that analyzing LDL and HDL subclasses and their distribution reinforces the predictive power of plasma LDL-C and HDL-C measurements in estimating an individual's CVD risk. Also, examination of subclasses enables monitoring the effectiveness of dietary or therapeutic interventions in CVD risk alleviation. It is clear that dietary cholesterol and plant sterols significantly impact lipoprotein subclass distribution by differentially modulating LDL and HDL subclasses, where dietary cholesterol appears to influence both LDL and HDL subclasses, while plant sterol intake affects only LDL subclasses. However, genetic polymorphisms may also influence the lipoprotein subclass responsiveness to such dietary sterols. Research gaps exist as to how common gene polymorphisms within lipoprotein and cholesterol metabolic pathways affect the responses to dietary sterol induced particle size distribution. Acquiring such knowledge will enhance our understanding as to whether dietary cholesterol and plant sterols follow similar mechanisms through which they modulate lipoprotein particle distribution and will improve interventions aimed at reducing CVD risk through cholesterol management.

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BRIDGE TO CHAPTER IV

Cholesterol absorption and synthesis reciprocally interact with each other to maintain cholesterol homeostasis in response to dietary perturbations. Huge inter-individual variability exists in the degree to which cholesterol synthesis changes in response to changes in absorption and vice versa. Dietary cholesterol and plant sterols differentially modulate cholesterol kinetics and circulating cholesterol. Hence, it is important to ascertain how plasma lipid responsiveness of individuals shift with cholesterol and plant sterol intake, driven by the changes in endogenous cholesterol absorption and synthesis. Such assessment will help in improving the recommendations pertaining to dietary cholesterol and plant sterol consumption in relation to alleviation of CVD risk. Data in Chapter IV examine these implications by substantiating the impact of dietary cholesterol and plant sterol intake on plasma lipid responses and cholesterol trafficking in the healthy population of this research. This chapter includes discussion of results related to non-compensatory reciprocal variations in cholesterol synthesis in response to changes in absorption after plant sterol intake leading to large inter-individual variability in responsiveness, ultimately affecting the cholesterol lowering efficacy of plant sterols.

CHAPTER IV

MANUSCRIPT 2

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EFFECT OF DIETARY CHOLESTEROL AND PLANT STEROL CONSUMPTION ON PLASMA LIPID RESPONSIVENESS AND CHOLESTEROL TRAFFICKING IN HEALTHY INDIVIDUALS

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4.1 ABSTRACT

Dietary cholesterol and plant sterols differentially modulate cholesterol kinetics and circulating cholesterol. Understanding how healthy individuals with their inherent variabilities in cholesterol trafficking respond to such dietary sterols will aid in improving strategies for effective cholesterol lowering and alleviation of cardiovascular disease risk. The objectives of this study were to assess plasma lipid responsiveness to dietary cholesterol versus plant sterol consumption and to determine the response in rates of cholesterol absorption and synthesis to each sterol using stable isotope approaches in healthy individuals. A randomized, double-blind, crossover, placebo-controlled clinical trial (n=49) with 3 treatment phases of 4-wk duration was conducted in a Manitoba Hutterite population. During each phase, participants consumed one of the three treatments as beverages containing 600 mg/d dietary cholesterol, 2 g/d plant sterols or a placebo after breakfast meal. Plasma lipid profile was determined and cholesterol absorption and synthesis were measured by oral administration of [3, 4-^{13}C]-labeled cholesterol and ^2H_2O, respectively. Dietary cholesterol consumption increased total (0.16±0.06 mmol/L, p=0.0179) and HDL cholesterol (0.08±0.03 mmol/L, p=0.0216) concentrations with no changes in cholesterol absorption or synthesis. Plant sterol consumption failed to reduce LDL-C concentrations despite showing a reduction (6%, p=0.0004) in cholesterol absorption. An over-compensatory reciprocal increase in cholesterol synthesis (36%, p=0.0026) corresponding to a small reduction in absorption was observed with plant sterol consumption, possibly resulting in reduced LDL-C lowering efficacy of plant sterols. These data suggest that inter-individual variability in cholesterol trafficking mechanisms may profoundly impact plasma lipid responses to dietary sterols in healthy individuals.

4.2 INTRODUCTION

Lowering plasma total (TC) and low density lipoprotein cholesterol (LDL-C) levels continue to represent the prime target for cardiovascular disease (CVD) risk reduction. Cholesterol absorption and synthesis reciprocally interact with each other to maintain cholesterol homeostasis in response to dietary perturbations (1, 2, 3). However, the magnitude of change in synthesis in response to a change in absorption and vice versa has been shown to be highly variable (4, 3). The LDL-C lowering efficacy of any drug treatment or dietary intervention is affected by this interaction between cholesterol absorption and synthesis (3). Dietary cholesterol is known to raise plasma cholesterol, and variability in such responsiveness exists, where hyper-responders increase plasma TC, being unable to regulate plasma cholesterol homeostasis either by decreasing intestinal cholesterol absorption or by reducing synthesis (4, 5, 6). However, hyper-responders represent only about one fourth of the entire population, while the rest have a normal response to cholesterol intake (7, 6). Also, importantly, in these hyper-responders, an increase in both plasma LDL-C and HDL-C is usually seen with no changes in LDL-C/HDL-C ratio (8, 9, 10, 7). Hence, the recommendation of <300 mg/d of dietary cholesterol for healthy individuals is no longer included in the US dietary guidelines (11). Different levels of dietary cholesterol intake have been shown to either modulate (12), or have no effect on cholesterol synthesis (13, 1, 14). Jones et al. (14) measured the response of cholesterol biosynthesis in participants to different levels of dietary cholesterol, and observed a modest suppression of cholesterol synthesis by dietary cholesterol independent of plasma cholesterol levels.

In contrast to cholesterol, intake of plant sterols has been repeatedly shown to exert favorable modifications on circulating lipid profile and potential subsequent reduction in risk of CVD (15,

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16). Typical intakes of plant sterols in the North American diet ranges between 300-400 mg/d (17). Plant sterols are absorbed less ($< 2\%$) within the intestine compared to cholesterol which is absorbed at ~50% efficiency (18). Plant sterol consumption of about 2 g/d has generally been reported to reduce LDL-C levels by 5-15% (15). Plant sterols are known to lower blood cholesterol levels by inhibiting cholesterol absorption, however, the exact mechanism remains to be clarified (19). Clinical trials have however shown, similar as seen with cholesterol, large variability among individuals in the degree of LDL-C lowering response to plant sterol intake (20, 21, 22). LDL-C lowering was found to be greater in individuals with high baseline plasma LDL-C concentrations compared to those with normal or borderline concentrations (23). Also, wide inconsistency in results exists among studies regarding the effect of frequency and time of consumption of plant sterol servings on LDL-C lowering (20). In addition to lowering cholesterol absorption, plant sterols and stanols are reported to induce a compensatory rise in cholesterol synthesis (15). Inter-individual variations in cholesterol synthesizing ability affect the response to LDL-C lowering efficacy of plant sterols (21). Studies investigating the effects of basal cholesterol synthesis status on LDL-C lowering efficacy of plant sterols have shown that individuals with high basal cholesterol synthesis exhibit poor LDL-C lowering response upon plant sterol consumption (21, 22). Also, genetic variations have been linked to variability in responsiveness to dietary cholesterol and plant sterols (24, 25, 26).

The functionality, absorption and metabolism of cholesterol differs from that of plant sterols, despite, only minor structural variances existing between these entities (27). Hence, it is important to understand how individuals respond to dietary cholesterol versus plant sterols, given the inherent variabilities in the control and kinetics of cholesterol absorption and synthesis

pathways. Such knowledge will aid in developing strategies targeted towards improving the efficacy of cholesterol lowering treatments and thereby mitigating CVD risk. Hence, the present study aims to assess the plasma lipid responsiveness to dietary cholesterol and plant sterols and to determine the differences in rates of cholesterol absorption and synthesis using stable isotope approaches in healthy individuals.

4.3 EXPERIMENTAL METHODS

4.3.1 Study participants

Normocholesterolemic and slightly hypercholesterolemic, but otherwise healthy individuals (n=55), aged 18-50 yrs were recruited from two Hutterite colonies in Manitoba. The two colonies were Barrickman colony located west of Winnipeg city near Cartier, and the Pineland colony located in southeastern Manitoba. Potential confounders such as diet differences, ethnicity and physical activity among the Hutterites are reduced due to the homogenous lifestyle and community living system, providing an opportunity to investigate the study hypotheses in a controlled setting. Otherwise healthy was defined as the absence of a known chronic or infectious disease and supported by the fasting blood sampling. Information sessions were conducted in the colonies and potential study participants were initially screened with a questionnaire regarding personal health information, medical conditions and disease history. Anthropometric parameters such as age, weight, height, blood pressure, hip and waist circumferences were recorded. Individuals determined as potentially eligible underwent a blood screening at the first visit, where a 10 ml fasting blood sample was taken to test for lipid profile measurements such as TC, HDL-C, LDL-C and triglycerides (TG). Inclusion criteria were

fasting plasma TG <3.0 mmol/L and a body mass index (BMI) between 20 and 40 kg/m^2.

Exclusion criteria included history of recent or chronic use of oral hypolipidemic therapy, probucol, systemic antibodies, corticosteroids, androgens, or phenytoin and natural health products including fish oils known to affect lipid metabolism; history of CVD, kidney disease, liver disease, sitosterolemia, recent onset of angina, congestive heart failure, inflammatory bowel disease, pancreatitis, diabetes, or significant gastrointestinal, pulmonary and cancers. Also individuals with a history of chronic use of alcohol (>2 drinks/d), smoking, exercise with energy expenditure > 4,000 kcal/wk and female individuals who were pregnant or planning for pregnancy during the study period were excluded. A total of 55 participants (men=25; women=30) were finally enrolled into the study.

4.3.2 Ethics approval and clinical trial registration

This study was conducted according to the guidelines laid down in the Declaration of Helsinki (28).The study procedures were evaluated and approved by the biomedical research ethics board (BREB) of the University of Manitoba (Protocol no: B2013:019). All participants accepted into the study provided written informed consent. The study is registered in the clinicaltrials.gov registry (NCT01825668; www.clinicalTrials.gov).

4.3.3 Study design and protocol

The study was a free-living, double-blinded, randomized, crossover, placebo-controlled trial conducted at the Nutritional Research Unit of the Richardson Center for Functional Foods and Nutraceuticals (RCFFN), University of Manitoba. An outline of the study design is presented in

Figure 4.1. The study was comprised of three treatment phases of 4-wk duration each, separated by 4-wk washout intervals, where the test products were not consumed. Participants were randomized to receive three experimental products in the form of milkshakes during the study. All the three experimental milkshake products were isoenergetic (~304 Kcal; total fat: 18.3 g; saturated fat: 5.85 g; carbohydrates: 22.46 g and protein: 13.47 g) and were prepared in the metabolic kitchen at RCFFN and were then delivered to the two Hutterite colonies twice a week. During the three intervention phases, participants consumed each day a single serving (240 ml) of a milk shake which contained either 600 mg of cholesterol (egg yolk cholesterol) or a milkshake with 2 g of plant sterols (from soy containing ~ 55% β-sitosterol, 25% campesterol and 20% stigmasterol) or a milkshake without plant sterols/cholesterol (placebo), along with breakfast depending on the phase and assigned treatment. Supervisors were delegated in each colony to monitor the consumption of milkshakes and compliance to the study protocols. In addition, weekly checklists for milkshake consumption were recorded and collected. Participants were advised to maintain consistency in their typical diet and physical activity levels and refrain from excessive caffeinated beverages or alcohol during the study phases. Participants completed and returned 3-d food records during the start and end of each phase and were asked to report any major inconsistencies in diet and physical patterns during the intervention period. Also, adverse events due to treatments were monitored and participants were advised to communicate to the study personnel regarding any health conditions, symptoms and or change in medications arising during the study period. Post-treatment plasma plant sterol concentrations were used as an indicator of compliance.
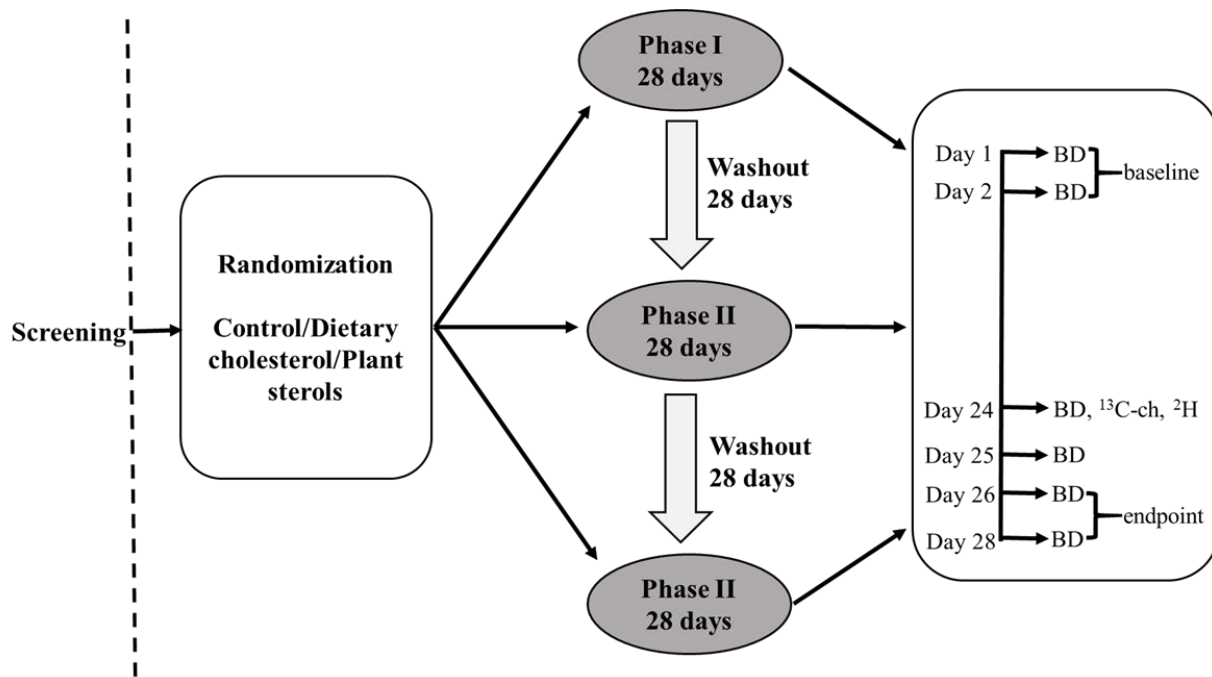
Figure 4.1 Study design. BD, blood draws; ^{13}C-ch, [3,4-^{13}C] cholesterol; ^{2}H, ^{2}H$_2$O, deuterium oxide
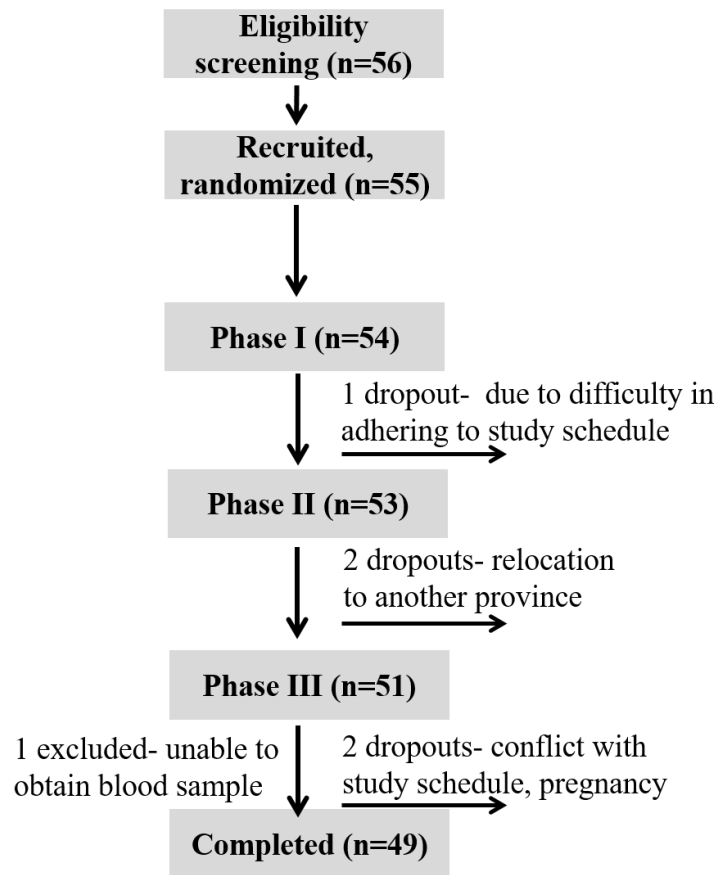
Figure 4.2 Participant flow through the study

4.3.4 Blood collection, sample storage and plasma lipid analyses

Fasting blood samples were collected in heparin containing tubes on days 1, 2, 26 & 28 of each intervention period of the trial. The blood samples after collection were stored at 4°C until brought to the laboratory and plasma, buffy coat and erythrocyte fractions were then separated by centrifugation at 3000 rpm for 20 min at 4°C. Aliquots of the separated fractions were distributed into different tubes and stored at -80°C until further use. Plasma TC, HDL-C, TG and glucose levels were determined by automated enzymatic methods on a Vitros-350 chemistry analyzer (Ortho-Clinical Diagnostics, Markham, ON, Canada). Low density lipoprotein cholesterol (LDL-C) levels were calculated by using the Friedewald equation (29).

4.3.5 Stable isotope tracer intake

On day 24 of each treatment phase, participants consumed 75 mg of the stable carbon isotope [3, 4-^{13}C] cholesterol dissolved in 5 g of margarine and spread on half of an English muffin to measure cholesterol absorption. In addition, participants also consumed 0.7 g/kg estimated body water (estimated at 60% of total body weight) of deuterium oxide (^{2}H$_2$O) as a tracer to measure cholesterol synthesis. Blood samples were obtained at baseline on day 24, as well as fasting samples on days 25, 26 and 28 to monitor isotopic enrichment/decay levels.

4.3.6 Determination of cholesterol absorption

Cholesterol absorption was determined using the stable-isotope single tracer method (30). Red blood cell (RBC) fraction of blood collected on days 24, 25, 26 & 28 were used for the free cholesterol extraction (31). Briefly, about 0.5 g of RBC fraction was weighed and extracted with

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8 ml of methanol at 55°C for about 15 min and to this hexane: chloroform (4:1) mixture was added and vortexed. After vortexing, deionized water was added, vortexed and centrifuged at 1500 rpm for 15 min at 4°C. The supernatants were collected and the procedure was repeated with fresh methanol and hexane:chloroform mixture. The pooled supernatants were then dried under nitrogen. The dried supernatants were re-extracted with 0.5 ml of hexane and transferred into GC vials for analysis. These extracts were then used to determine ^{13}C-cholesterol enrichment using on-line gas chromatography/combustion/isotope ratio mass spectrometry (IRMS, Agilent 6890N chromatograph interfaced with a Finnigan Delta V Pulse isotope ratio mass spectrometer; Bremen, Germany). Isotopic abundances were expressed in delta (δ) per mil (‰), and were calculated using carbon dioxide as a reference gas and further correction was performed against the Pee Dee Belemnite (PDB) international standard. From 24 to 96 h (72 h post-^{13}C-cholesterol ingestion), cholesterol absorption is presented as area under the [3, 4]-^{13}C cholesterol RBC enrichment curve by using the trapezoidal rule and values corrected for baseline values.

4.3.7 Cholesterol synthesis determination

Cholesterol fractional synthetic rates (FSR) (% per d) were calculated as the rate of incorporation of ^{2}H within the body water pool into RBC cholesterol pools by measuring the ^{2}H RBC enrichment over 24 h (32). Free cholesterol was extracted as described above for cholesterol absorption (31) and ^{2}H enrichment was measured in both RBC free cholesterol and plasma water. RBC free cholesterol enrichment was measured using online gas chromatography–pyrolysis-IRMS (Finnigan Delta V plus Isotope ratio mass spectrometer, Bremen, Germany) using previously established methods. Isotope abundances, expressed in δ ‰, were calculated by

using H_2 as a reference gas. Cholesterol ^2H enrichment was expressed relative to standard mean

ocean water (SMOW) and a series of standards of known enrichment. Cholesterol FSRs

represent RBC free cholesterol ^2H enrichment values relative to the corresponding mean plasma

water sample enrichment after correcting for the free cholesterol pool. Cholesterol FSR was

calculated as shown in the following equation adopted from Jones et al. (33) and Myrie et al.

(34):

$$FSR \ (\%/d) = [\delta \text{ cholesterol}/ \ (\delta \text{ plasma water} \times 0.478)] \times 24 \text{ h} \times 100 \quad (1)$$

where δ is the ^2H enrichment of cholesterol or plasma water above baseline in a 24 h period. The

factor 0.478 stands for the fraction of hydrogen atoms per cholesterol molecule that may become

enriched by ^2H during in vivo cholesterol biosynthesis (33).

The absolute synthesis rate (ASR) of rapidly exchanging free cholesterol was calculated based

on previously established models (33, 34, 35):

$$ASR \ (g/d) = FSR \ (\%/d)/100\% \times M1 \times 0.33 \quad (2)$$

M1 represents the size of the rapidly exchanging free cholesterol pool and was calculated

according to the model of Goodman et al. (35) and the factor 0.33 accounts for the proportion of

free cholesterol in the overall plasma total cholesterol pool.

4.3.8 Liver function parameters analysis

Analyses of the liver enzymes such alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALKP), gamma-glutamyltransferase (GGT), lactate dehydrogenase (LDH) and total bilirubin (TBIL) as safety biomarkers were performed by autoanalyzer (VITROS 350), with enzymatic reagents.

4.3.9 Statistical analysis

Sample size was calculated to detect LDL-C reduction to plant sterol treatment in participants. The sample size was determined to detect an anticipated difference in LDL-C levels due to plant sterol treatment of 10% (0.54 mmol/L) using a standard deviation of 0.732 mmol/L (36). The alpha and power were 0.05 and 0.7 respectively. A sample size of 35 participants was calculated to meet the requirement for both the objectives, with a target of 30-31 subjects, taking into account the block size and an estimated 10% premature participant withdrawal rate. However, to avoid any situation where drop-out rates could be higher, and to increase the statistical power of detecting differences in the secondary endpoints, a sample size of 45 was calculated. Statistical analysis was performed using the statistical software, SAS version 9.2 (SAS Inc). Endpoint measurements of treatment and control phases were compared. Values were expressed as least square means and standard error of the mean (SEM), unless stated. Effects of dietary treatments were examined using a mixed model ANOVA procedure for repeated measures with participant ID as a random factor and treatment as an independent factor. The effect of treatment, sequence, and gender were included in the model as fixed factors. Statistical significance was set at $p < 0.05$

for all analyses. Associations between variables were tested using Pearson correlation coefficient analyses.

4.4 RESULTS

4.4.1 Baseline characteristics

Baseline characteristics of the participants who completed the study are presented in **Table 4.1**. A total of 49 (Male=21; Female=28) participants completed all the three phases and study requirements (**Figure 4.2**). No change in physical activity was reported by the study participants, and no significant changes in bodyweight were noticed during the study period. Majority of the participants were normocholesterolemic as indicated by TC levels (≤5.03 mmol/) and had near optimal LDL-C levels (≤3.02 mmol/L). A large proportion of the participants were overweight (BMI: 27-29 kg/m^2), and men had higher body weights (p=0.0013), lower plasma HDL-C (p=0.0133) and higher TG (p=0.0392) concentrations compared to women.

4.4.2 Plasma lipid responses to dietary cholesterol and plant sterols

Dietary cholesterol consumption of 600 mg/d for 4 weeks resulted in an increase in the concentration of plasma TC (0.16±0.06 mmol/L, p=0.0179) compared to the control (**Table 4.2**). An increase in HDL-C concentrations (0.08±0.03 mmol/L, p=0.0216) was found after cholesterol consumption. No changes in TG (p=0.9523), non-HDL-cholesterol (p=0.4080)

Table 4.1 Baseline characteristics of study participants who completed the study

Variable	Overall (n=49)		Men (n=21)		Women (n=28)		P values Men vs. Women
	Means	SEM	Means	SEM	Means	SEM	
Age (yrs)	33.04	1.54	32.05	2.51	33.72	1.95	p=0.6028
Weight (kg)	75.88	2.33	85.17	3.82	69.48	2.32	p=0.0017
BMI (kg/m^2)	27.81	0.75	29.21	1.29	26.83	0.87	p=0.1383
Waist circumference (cm)	86.54	1.22	94.25	2.86	80.25	2.70	p=0.0042
Hip circumference (cm)	97.54	1.73	99.92	1.99	95.77	2.63	p=0.03297
Waist/Hip ratio	0.88	0.01	0.94	0.01	0.85	0.01	p<0.0001
Triglycerides (mmol/L)	1.33	0.09	1.57	0.17	1.18	0.10	p=0.0392
Total cholesterol (mmol/L)	5.03	0.13	5.03	0.16	5.04	0.22	p=0.5208
LDL-cholesterol (mmol/L)	3.02	0.13	3.06	0.15	2.99	0.19	p=0.7596
HDL-cholesterol (mmol/L)	1.40	0.06	1.26	0.08	1.49	0.08	p=0.0133
Glucose (mmol/L)	5.38	0.19	5.75	0.44	5.13	0.09	p=0.1822

All values are means ± SEM (n=49). P values for Men vs. Women by unpaired t-test

BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein.

concentrations and LDL-C to HDL-C ratio (p=0.9993) were found following cholesterol consumption. Significant heterogeneity in LDL-C responsiveness to cholesterol consumption was seen in the study population (**Figure 4.3A**). Plant sterol consumption of 2 g/d did not show any reductions in plasma TC (p=0.3327), LDL-C (p=0.3525) and non-HDL-cholesterol (p=0.1040) concentrations compared to the control. Also, no changes were seen in plasma TG (p=0.6525), HDL-C (p=0.9801) concentrations and LDL-C to HDL-C ratio (p=0.4375) following plant sterol consumption when compared to the control. Similar to cholesterol consumption, a large inter-individual variability in LDL-C responsiveness was found in the study population following plant sterol consumption (**Figure 4.3B**). Effects of gender on plasma lipid responses following cholesterol and plant sterol supplementation were analyzed (data not shown). Gender significantly influenced the plasma HDL-C increasing effect of cholesterol consumption in the study population (p=0.0028). Women had a greater increase (0.12±0.04 mmol/L, p=0.0424) in plasma HDL-C concentrations in response to cholesterol consumption compared to men (0.05±0.05 mmol/L, p=0.9076). No changes in plasma HDL-C concentrations were seen after plant sterol consumption in either genders. Also, no other significant gender effects were observed with any other plasma lipid variables in response to cholesterol and plant sterol consumption.

4.4.3 Effect of dietary cholesterol and plant sterols on cholesterol absorption and biosynthesis rates

Consumption of 600 mg/d of cholesterol for 4 weeks did not result in any changes in cholesterol absorption (p=0.1192) as measured by the area under the [3, 4]-^{13}C cholesterol RBC enrichment curve compared to the control (**Figure 4.4A**). However, plant sterol consumption reduced

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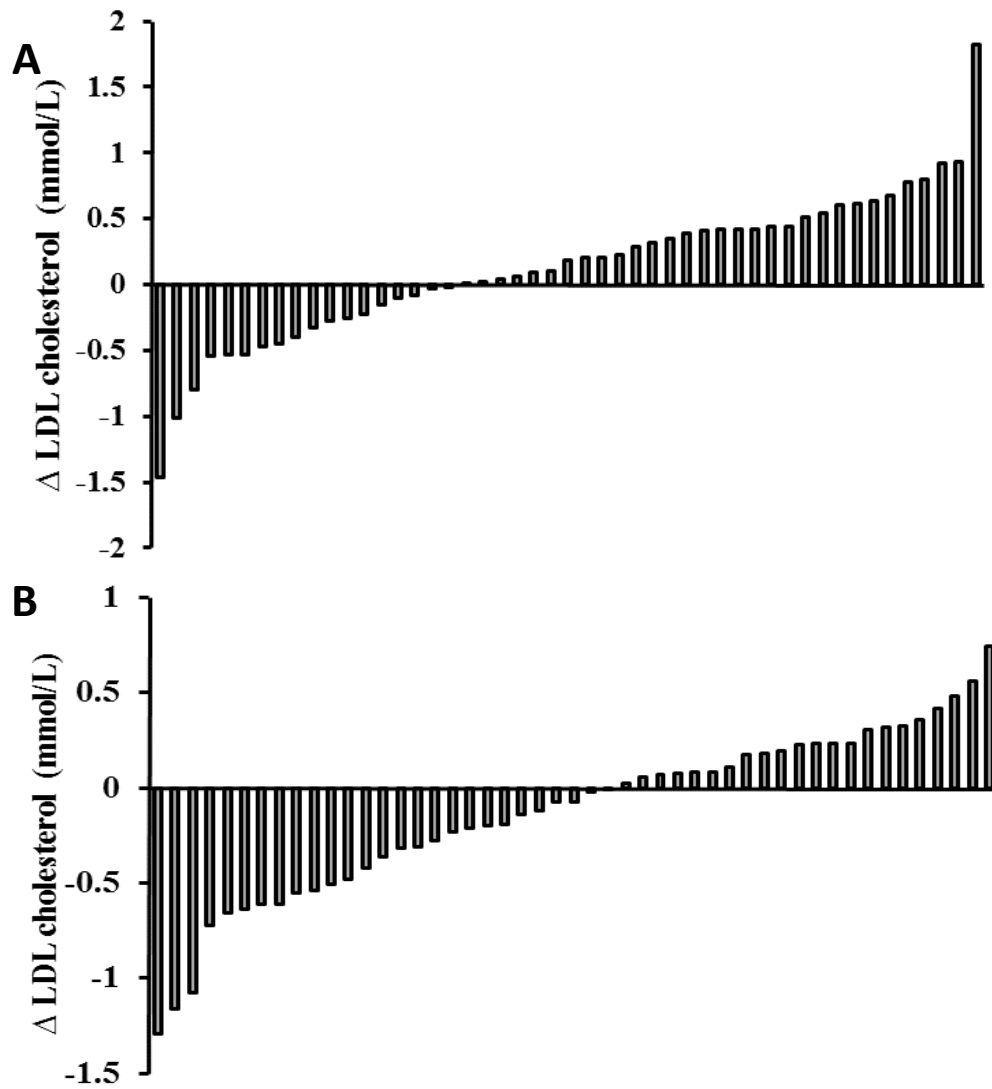
Figure 4.3 (A), Individual changes in LDL-cholesterol in response to dietary cholesterol consumption and (B), plant sterol consumption. Δ, absolute change in LDL-cholesterol (mmol/L) of endpoints of dietary cholesterol and plant sterol phases from endpoint of control phase. Order of responses of individual participants in panel A are not the same from panel B (participants not matched across the two panels)

Table 4.2 Plasma lipid responses to dietary cholesterol and plants sterol treatment after 4 weeks

Lipid variables	Control		Cholesterol		Plant sterols	
	Means	**SEM**	**Means**	**SEM**	**Means**	**SEM**
Total cholesterol (mmol/L)	4.87[a]	0.14	5.03[b]	0.13	4.78[a]	0.14
Absolute change from control			0.16	0.06	-0.09	0.07
Change from control (%)			4.76	1.42	-1.58	1.43
LDL-cholesterol (mmol/L)	2.85[ab]	0.13	2.96[a]	0.13	2.73[b]	0.13
Absolute change from control			0.11	0.06	-0.12	0.06
Change from control (%)			5.40	2.61	-2.86	2.39
HDL-cholesterol (mmol/L)	1.44[a]	0.06	1.53[b]	0.06	1.45[a]	0.06
Absolute change from control			0.09	0.03	0.01	0.03
Change from control (%)			6.64	2.06	1.28	1.85
Non-HDL cholesterol (mmol/L)	3.43[ab]	0.14	3.51[a]	0.14	3.32[b]	0.14
Absolute change from control			0.08	0.06	-0.10	0.06
Change from control (%)			4.17	1.99	-2.54	1.89
Triglycerides (mmol/L)	1.25	0.11	1.27	0.11	1.29	0.11
Absolute change from control			0.01	0.06	0.04	0.06
Change from control (%)			1.55	3.12	7.95	4.97
LDL-C/HDL-C	2.13	0.13	2.13	0.13	2.05	0.13
Absolute change from control			-0.002	0.06	-0.08	0.06
Change from control (%)			0.90	3.37	-8.63	4.95

All values are least square means ± SEM (n=49). Values with different superscript letters are significantly different from each other; $p < 0.05$ is considered significant. Differences between treatments were assessed by SAS mixed procedure ANOVA with Tukey-Kramer adjustments. LDL, low density lipoprotein; HDL, high density lipoprotein.
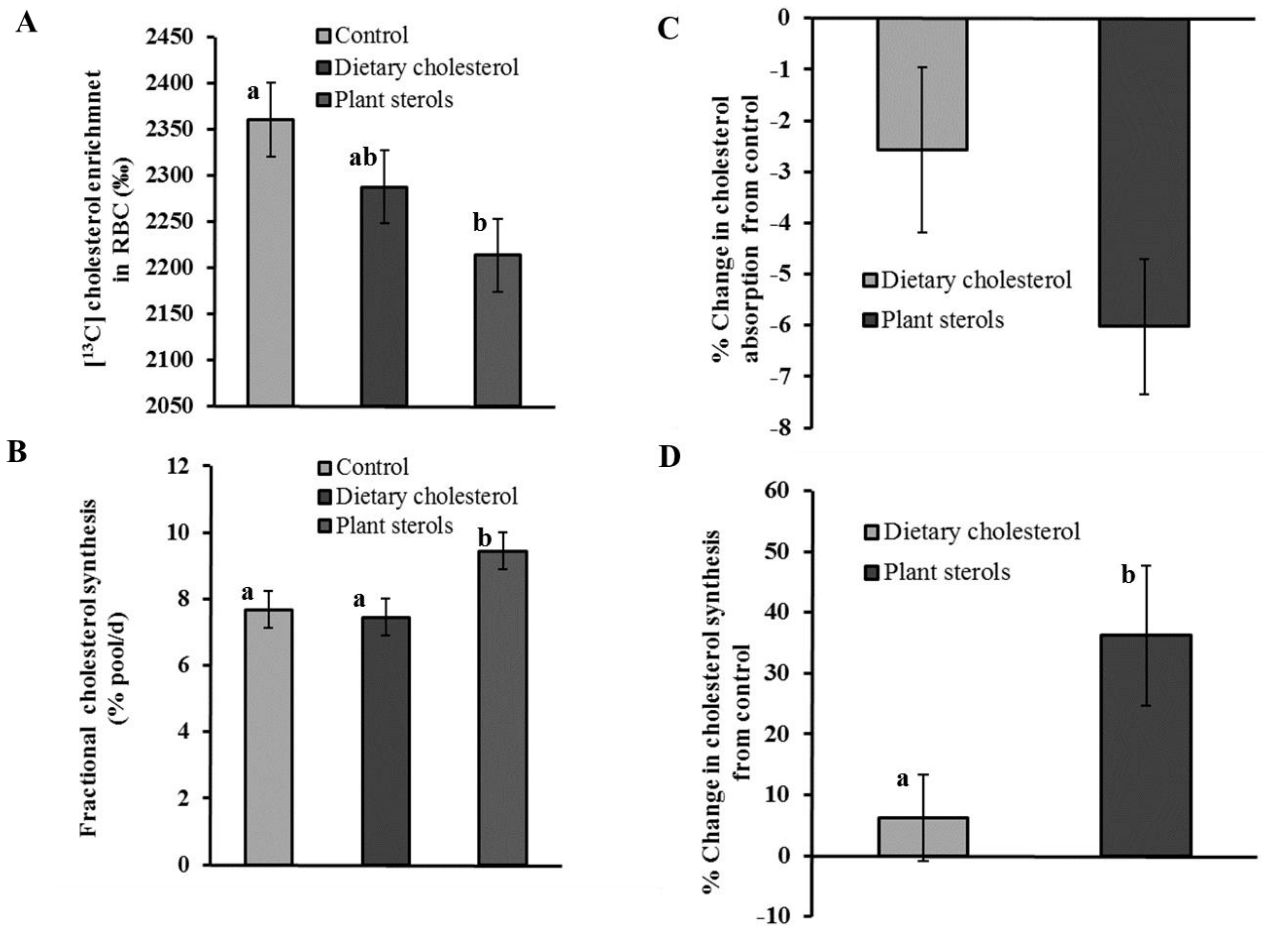
Figure 4.4 (A), Cholesterol absorption measured by area under the [^{13}C] cholesterol red blood cell enrichment curve over 24, 48 and 96 h post administration of 75 mg of [3, 4-^{13}C] cholesterol in response to control, dietary cholesterol and plant sterol supplementation (B), cholesterol fractional synthesis rate measured as the rate of ^{2}H incorporation within the body water pool into RBC cholesterol pools over 24 h post ^{2}H$_2$O administration in response to control, dietary cholesterol and plant sterol supplementation. (C), percentage changes in cholesterol synthesis and (D), cholesterol absorption in response to dietary cholesterol and plant sterol consumption compared to control. All values are least square means ± SEM. Differences between treatments assessed by SAS mixed procedure ANOVA with Tukey-Kramer adjustments; Labeled means with a different superscript letter are significantly different, p<0.05.

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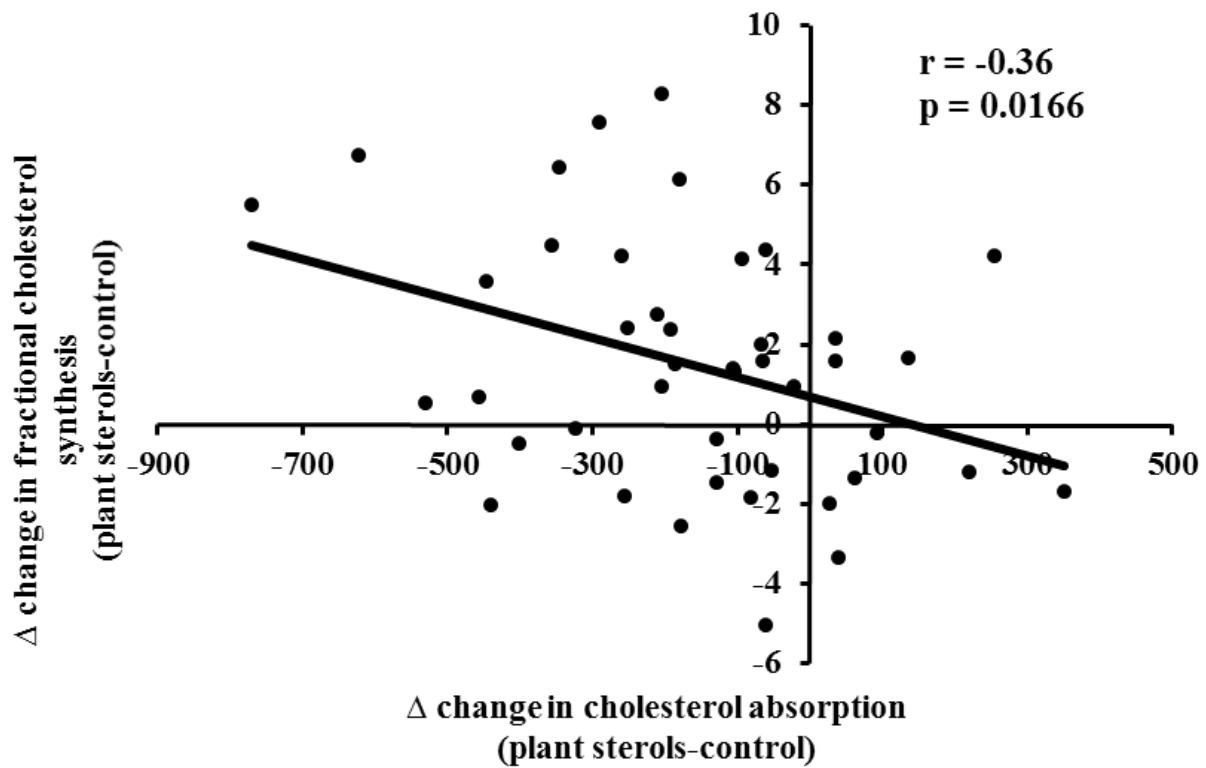
Figure 4.5 Pearson's product-moment correlation (r) between change in cholesterol absorption measured by area under the [3,4-^{13}C] cholesterol enrichment curve in red blood cells (‰) from control and change in fractional cholesterol synthesis (% pool/d) from control in response to plant sterol consumption. Values in least square means ± SEM. p<0.05 considered significant. Δ, absolute change.

Table 4.3 Liver function test parameters in response to dietary cholesterol and plant sterols after 4 weeks

Parameters	Control		Cholesterol		Plant sterols	
	Means	**SEM**	**Means**	**SEM**	**Means**	**SEM**
AST (U/L)	27.53	1.30	27.37	1.27	27.18	1.28
ALT (U/L)	31.06	2.50	30.55	2.47	32.31	2.50
ALKP (U/L)	63.30	2.74	62.15	2.71	63.81	2.74
GGT (U/L)	21.52	4.31	23.25	4.30	22.82	4.31
LDH (U/L)	396.85	9.57	385.09	9.51	391.70	9.57
TBIL (μmol/L)	8.98	0.33	9.19	0.33	8.91	0.33

AST, asparagine aminotransferase; ALT, alanine aminotransferase; ALKP, alkaline phosphatase; GGT, gamma-glutamyltransferase; LDH, lactate dehydrogenase; TBIL, total bilirubin

All values are least square means ± SEM (n=49). Treatment effects were examined by SAS mixed procedure ANOVA, *p<0.05 is considered significant.

cholesterol absorption (p=0.0004) compared to control (**Figure 4.4A**). Compared to control,

dietary cholesterol feeding did not affect cholesterol FSR (p=0.8438), which is representative of

the proportion of the rapidly turning over cholesterol pool synthesized per day, measured by

estimating ^{2}H incorporation after ^{2}H$_2$O consumption (**Figure 4.4B**). A similar trend was found

where plant sterol consumption increased cholesterol ASR (p=0.0010) compared to the control,

however no change was seen after cholesterol consumption (p=0.9131). Cholesterol absorption

and FSR in response to plant sterols were negatively correlated (r=-0.36, p=0.0166) as shown in

Figure 4.5.

4.4.4 Liver function test parameters in response to dietary cholesterol and plant sterols

Liver enzyme activities measured are presented in **Table 4.3**. No changes in any of the plasma

liver enzymes were found due to treatment during the study period.

4.5 DISCUSSION

Increased dietary cholesterol absorption usually suppresses endogenous cholesterol synthesis as

opposed to the reduction of cholesterol absorption and reciprocal elevation of endogenous

synthesis caused by plant sterol consumption (2, 3, 14, 15). The present study using stable

isotopes techniques, has shown that a cholesterol challenge of 600 mg/d did not affect

cholesterol absorption or synthesis, but resulted in increased plasma total cholesterol and HDL-C

concentrations, whereas, plant sterol consumption of 2 g/d induced an over-compensatory

increase in cholesterol synthesis corresponding to a small decrease in cholesterol absorption, and

possibly subdued the plasma LDL-C lowering efficacy of plant sterols in healthy Hutterite individuals.

Dietary supplementation of 600 mg/d of cholesterol for 4 weeks failed to produce any change in plasma LDL-C concentrations in the study population, however did increase TC concentrations. The same finding has been reported earlier by various studies, where it has been shown that the majority of individuals do not exhibit any increase in plasma LDL-C concentrations after cholesterol consumption (37, 38, 6, 8, 9, 10). These studies support the existence of inter-individual differences leading to hypo- and hyper responsiveness to dietary cholesterol. Hypo-responders to dietary cholesterol usually do not experience any increase in plasma LDL-C concentrations, while hyper-responders, despite increasing LDL-C concentrations, are found to maintain the LDL-C/HDL-C ratio (8,9). These findings corroborate the possibility that the increase in plasma LDL-C concentrations in response to a cholesterol challenge in our study could have been obfuscated by the presence of both hyper and hypo-responders to dietary cholesterol in study population, resulting in an overall small increment in plasma LDL-C concentrations. On the other hand, cholesterol supplementation resulted in a significant elevation in plasma HDL-C concentrations in study participants. A similar effect where an increase in plasma HDL-C concentrations in response to dietary cholesterol consumption has been demonstrated by various studies as well (38, 39, 40, 41). These studies also found no increases in plasma LDL-C concentrations along with a rise in HDL-C concentrations similar to what we observed in our present study. Concurring with previous findings from other authors (8,9), we found that the overall LDL-C/HDL-C ratio remain unchanged after cholesterol consumption compared to control. One interesting finding was that women alone experienced an increase in

plasma HDL-C concentrations to cholesterol consumption regardless of baseline differences in

HDL-C concentrations between men and women. Clifton and Nestel (42) have reported a similar

finding where a cholesterol challenge of 650 mg/d for 3 weeks together with a low fat diet

resulted in substantial elevation in plasma HDL-C and large HDL-subclass concentrations in

women compared to men (42, 43). Such differences have been ascribed to the differential fat

distribution between men and women (43). It can be inferred that dietary cholesterol

consumption does not unfavorably modify plasma lipid profiles in healthy individuals and may

endow women with anti-atherogenic benefits by increasing plasma HDL-C concentrations in

comparison to men. Hence, development of gender specific dietary cholesterol recommendations

may prove to be useful.

Surprisingly, plant sterol consumption of 2 g/d did not induce a significant reduction in plasma

LDL-C concentrations and TC concentrations in this study population. Contrary to the well-

established plasma LDL-C lowering effect induced by plant sterol intake in numerous studies

(18, 44, 45, 34, 16), our study did not detect a prominent reduction. Some studies have

previously reported the lack of efficacy of plant sterol treatment in lowering cholesterol (46, 47,

48). The lack of effects in some of these studies appears to be due to poor solubility of the plant

sterol formulations tested (46,47). However, we believe that the format in which plant sterols

were adminstered in our study could have not contributed to the lack of effects, since many

studies have attested the efficacious plasma LDL-C lowering and bioavailability of similar plant

sterol preparations (49, 50, 51, 34). Frequency and time of administration have also been

suggested as possibly affecting the efficacy of plant sterol formulations (48,52, 20). Our study

used a single dose administration of plant sterol treatment accompanied with breakfast, which led

us to speculate whether the lack of efficacy stems from dosage and time of administration. But, single dosage studies of plant sterols have shown equivalent efficacies in plasma LDL-C reduction (53, 54, 34) comparable to studies with distributed dosage regimens (55, 45, 56, 44, 57). Hence, it does not appear that the lack of efficacy could be due to the single dosage regimen of plant sterols in our study. AbuMweis et al. (48) reported ineffectiveness of different plant sterol preparations in reducing plasma LDL-C, where the treatments were administered as a single morning dose 1.0-1.7 g/d for 4 weeks. The authors suggested that the lack of effects may have been due to the fact that the plant sterol treatments were administered as a single dose along with breakfast meal, where a circadian variation in cholesterol metabolism could have contributed to the reduced efficacy (48). Cholesterol biosynthesis is known to be affected by circadian rhythm as shown by the modulatory effects of meal time lags on cholesterol synthesis (58, 59, 60, 48, 3). Intuitively, AbuMweis et al. (48) speculated that lower cholesterol absorption in morning due to reciprocal relation between absorption and synthesis could potentially render plant sterols ineffective in furthering cholesterol inhibition leading to sub-optimal plasma LDL-C reduction. Likewise, Doornbos et al. (52) showed a single dose consumption of plant sterol formulation with lunch to be more effective in lowering plasma LDL-C concentrations than the same dose with breakfast (52). These findings were further corroborated by the study of AbuMweis et al. (19), where a three-time daily dose of plant sterols proved to be more efficacious than a single breakfast dose. In contrast, some studies demonstrated optimal plasma LDL-C lowering with single dose regimen of plant sterols accompanied with breakfast (61, 62, 63, 64, 22). Also, unavailability of data on the circadian rhythm of cholesterol absorption makes it difficult to ascribe lack of efficacy to plant sterol products based on the time of ingestion.

Various other factors could also have contributed to the low efficacy of plant sterols in plasma LDL-C reduction in our study. Large inter-individual variability in plasma LDL-C responsiveness to plant sterols was present in the population akin to hypo and hyper-responsiveness to dietary cholesterol. Noteworthy, is that our study population was predominantly normocholesterolemic with most individuals exhibiting near-optimal baseline plasma LDL-C concentrations. It has been shown that individuals with high baseline plasma LDL-C concentrations experience greater reductions in LDL-C due to plant sterol or stanol consumption compared to individuals with optimal or borderline LDL-C concentrations (65, 66, 67, 68). Another factor to be considered is the cholesterol synthesizing ability of the study population. Rideout et al. (21) and Mackay et al. (22) have demonstrated the influence of basal cholesterol synthesis status on the plasma LDL-C lowering efficacy of plant sterols. These authors have postulated that high basal cholesterol synthesizing individuals exhibit poor LDL-C lowering response upon plant sterol consumption and have assigned a genetic basis for the non-responsiveness (21, 22). Mackay et al. (26) proposed that individuals having a combination of TT allele for *CYP7A1 rs3808607* SNP and *APOE* ε3 isoform tend to be non-responders to plant sterol consumption. The present study was conducted in a genetically isolated Hutterite population. It is possible that genetic makeup of the Hutterite participants could have resulted in the non-responsiveness phenotype, imparting low efficacy to plant sterol treatment. The genotype frequency and distribution of various gene polymorphisms affecting cholesterol metabolism is different in the Hutterites compared to the general population (69, 70). Therefore, genotyping the study population and testing for associations of candidate SNP affecting lipid and cholesterol metabolism are both crucial in unravelling the observed huge variability in responsiveness to dietary sterols.

Dietary cholesterol consumption did not affect cholesterol absorption as measured by the area under the [3, 4]-^{13}C cholesterol RBC enrichment curve using the stable isotope single-tracer method. It is important to consider that the single-tracer method does not measure the absolute amount of cholesterol absorbed, but rather represents only the efficiency of cholesterol absorption (18, 19). Also, the stable-isotope tracer administered could have been diluted by the dietary cholesterol influx of 600 mg/d, and since only fractional cholesterol absorption happens in the intestine (~50%), the fraction of the tracer absorbed might have been less than that usually absorbed during the control phase. An increase in plasma TC concentrations was seen following cholesterol challenge, which indicates that the cholesterol absorption was indeed more compared to the control. Using a dual-isotope method specifically for measuring the effect of dietary cholesterol on cholesterol absorption would likely have been more precise in estimating absolute cholesterol absorption. On the other hand, plant sterol administration for 4 weeks reduced cholesterol absorption compared to the control, however, did not reduce plasma LDL-C concentrations as expected. Similar observation has been previously reported by AbumWeis et al. (19), where the authors found a significant reduction in cholesterol absorption due to plant sterol consumption, but did not see any plasma LDL-C reduction. The authors indicated that a reduction in cholesterol absorption efficiency may not always be associated with a reduction in the magnitude of plasma cholesterol concentrations (19).

In our study, the dietary cholesterol challenge failed to affect cholesterol FSR, measured by estimating ^{2}H incorporation after ^{2}H$_2$O consumption. Jones et al. (14) did find a modest decrease in synthesis in response to different levels of dietary cholesterol feeding. However, unlike their findings, we did not find any changes in cholesterol synthesis. Concomitantly, we found a

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marked rise in plasma cholesterol. Nestel and Poyser (12) observed that moderate intakes of dietary cholesterol induced the suppression of synthesis and prevented the rise of plasma cholesterol. This finding possibly explains the rise in plasma cholesterol concentrations following the cholesterol challenge in our study population and is also indicative of the presence of a large proportion of hyper-responding individuals to dietary cholesterol, as discussed previously. Plant sterol consumption dramatically increased cholesterol FSR and ASR compared to the control presently. A reciprocal upregulation of cholesterol synthesis in response to a decrease in cholesterol absorption is usually seen (15, 19, 34). In our study, the increase in synthesis was 36% (FSR; **Figure 4.4D**) and 39% (ASR) higher compared to the control corresponding to only a 6% reduction in cholesterol absorption (**Figure 4.4C**). Previous studies have reported a larger reduction (~25%) in cholesterol absorption with a compensatory increase in synthesis (15, 19, 34). An over-compensatory increase in cholesterol synthesis in tandem with a small reduction in absorption in our study could have most likely contributed to the reduced effect of plant sterol induced LDL-C lowering. As previously discussed, the works of Rideout et al. (21) and Mackay et al. (22) on the association between basal cholesterol synthesis status and LDL-C responsiveness to plant sterols support this observation. Hence, the role of gene polymorphisms affecting cholesterol kinetics and gene-diet interactions determining responsiveness to dietary sterols needs to be better understood (26).

Some of the major strengths of the study include the following. This is the first undertaking to our knowledge where the first direct comparison between the effects of cholesterol versus plant sterol intake for 4 weeks on plasma lipids and cholesterol kinetics using stable isotope approaches were examined in a single study. The study was a double-blind randomized crossover

placebo-controlled clinical trial. Another unique aspect was the study population, which was a genetically isolated founder population of Hutterites with minimal environmental and lifestyle related confounders. However, our study did suffer from some limitations. The study was free-living with less control over dietary intake. However, to our advantage the Hutterites have a community dining system with all individuals in the population consuming typically the same diet, except for individual variations in caloric intake. Another limitation of the study is that challenges exist in extrapolating the findings to the general population.

In summary, using stable isotopic assessment in healthy Hutterite individuals, we found that dietary cholesterol supplementation did not affect cholesterol absorption or synthesis, but increased plasma total cholesterol and HDL-C concentrations, whereas, plant sterol consumption resulted in an over-compensatory elevation in cholesterol synthesis pertaining to a small reduction in cholesterol absorption. Such hyper-reactive increase in cholesterol synthesis corresponding to a miniscule reduction could have possibly hampered the plasma LDL-C lowering efficiency of plant sterol administration. Our results thereby affirm the existence of inter-individual variability in endogenous cholesterol synthesis, potentially inflecting the reciprocal relationship between cholesterol absorption and synthesis, and the extent to which they are correspondingly offset due to dietary perturbations. Our findings also show that dietary cholesterol consumption did not unfavorably alter the plasma lipid profile of our study participants. Notably, large inter-individual variability in plasma lipid responsiveness to the consumption of dietary sterols was found in the study population. Hence, scrutinizing genetic heterogeneity and phenotype associations will facilitate understanding gene-diet interactions that modulate the reciprocal relationship between cholesterol absorption and synthesis, and thereby

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determine an individual's responsiveness to cholesterol versus plant sterol consumption. Development of personalized dietary intervention strategies will therefore become possible which can be directed towards regulating plasma cholesterol levels and prevention of CVD risk.

4.6 ACKNOWLEDGMENTS & AUTHORS CONTRIBUTIONS

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BRIDGE TO CHAPTER V

Chapter IV demonstrated that dietary cholesterol and plant sterols variably modulate cholesterol absorption and synthesis, affecting plasma cholesterol levels. Monitoring the impact of dietary interventions on lipoprotein distribution enhances the capability of LDL and HDL cholesterol in CVD risk prognosis. However, the genetic basis of how dietary cholesterol and plant sterols modify lipoprotein subclass distribution remains to be understood. The following chapter addresses these questions, and to our knowledge, for the first time reports the potential role of gender and common genetic variants within lipoprotein and cholesterol metabolic pathways on lipoprotein subclass responsiveness and distribution to dietary cholesterol and plant sterol consumption. The findings of this chapter suggest that plant sterol consumption does not alter plasma LDL or HDL subclass distribution, while cholesterol consumption increases plasma HDL-C levels and large HDL subclass concentrations dependent on the influences of gender and common genetic variants.

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GENDER AND COMMON POLYMORPHISMS IN CANDIDATE GENES AFFECT HDL SUBCLASS DISTRIBUTION IN RESPONSE TO DIETARY CHOLESTEROL AND PLANT STEROLS IN A FOUNDER POPULATION

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5.1 ABSTRACT

Dietary cholesterol and plant sterols affect LDL and HDL lipoprotein subclasses. However, limited information exists on the influence of common gene polymorphisms in the variability of LDL and HDL subclass distribution in response to dietary cholesterol and plant sterols. Our study examined the differential effects of dietary cholesterol versus plant sterol consumption on lipoprotein subclasses and how common polymorphisms in candidate genes influence lipoprotein subclass distribution responses to these dietary sterols in a Hutterite founder population. A double-blind, randomized, crossover, placebo-controlled study with three intervention phases of 4 weeks each separated by a 4-wk washout was conducted in 49 healthy individuals. Participants consumed daily either 2 g of plant sterols or 600 mg of cholesterol, or placebo, incorporated into milkshakes for 4 wks during each interventional period. LDL and HDL subclasses were fractionated and quantified. All the participants were genotyped for 38 candidate SNPs across 25 genes. Neither dietary cholesterol nor plant sterols changed plasma LDL-cholesterol or LDL-subclasses. Dietary cholesterol induced an increase in plasma large HDL subclasses (0.12 ± 0.04 mmol/L, p=0.0167). Females, but not males responded to dietary cholesterol by increasing plasma HDL-2 (0.06 ± 0.02 mmol/L, p=0.0253) and HDL-3 (0.03 ± 0.01mmol/L p=0.0383) subclasses. Plant sterols failed to alter plasma HDL subclass distribution. Genotypes of *ABCA1 rs2066714*, *LIPC rs6083*, *LIPF rs814628*, *SCD rs2234970* & *SREBF1 rs2297508* influenced plasma HDL subclass distribution in response to dietary cholesterol consumption. Only *ABCA1 rs2066714* genotype was associated with plasma HDL subclass responses to plant sterol consumption. Results suggest that plant sterol consumption does not alter plasma LDL or HDL subclass distribution, whereas cholesterol consumption increased plasma HDL-C and large HDL subclasses in healthy individuals, and the responses were affected by gender and common

genetic variants, particularly, those within lipoprotein and cholesterol metabolic pathways. This trial was registered at clinicaltrials.gov (NCT01825668).

5.2 INTRODUCTION

It is well established that elevated levels of plasma LDL cholesterol (LDL-C) and low levels of HDL cholesterol (HDL-C) associate with increased cardiovascular disease (CVD) risk (1). However, renewed interest in the role of lipoproteins in atherosclerosis disease progression has focused on not just the levels but the size and distribution of these particles as well. The pattern B plasma LDL profile composed largely of small, dense LDL particles is associated with three times higher risk for CVD compared to pattern A (large, buoyant LDL particles) due to the atherogenic nature of the small dense LDL particles (1, 2). Likewise, large HDL particles are inversely associated with CVD risk (3, 4) due their functional role in reverse cholesterol transport (RCT) and are now being widely recognized as more important than HDL-C concentrations *per se* (5, 6). Recently, the association of dietary cholesterol and eggs in CVD risk has been heavily debated (7). Lipoprotein subclasses are considerably affected by dietary cholesterol consumption, where an increase in large LDL subclasses with a concomitant increase in large HDL particles is usually observed (8, 9, 10, 11, 12). These data call into question the atherogenic role of dietary cholesterol even in populations at risk.

Plant sterol consumption effectively lowers plasma LDL-C levels by inhibiting cholesterol absorption (13). Despite having minor structural variations, the functionality, absorption and metabolism of plant sterols are different from that of cholesterol (14). Such differences may potentially dictate their effects on lipoprotein subclass distribution. Mixed data exist on the effect of plant sterol consumption on LDL subclass distribution. Plant sterol intake has been observed to either decrease plasma small LDL particles (15, 16, 17), or decrease large LDL particles, but not small LDL particles (18), or have no effect on LDL particle size or subclasses (19),

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regardless of significant LDL-C reduction in all these studies. Generally, plasma HDL-C and HDL subclasses are not affected by plant sterol consumption (14).

Gene-diet interactions may predominantly influence the responsiveness of lipoprotein subclass distribution to dietary cholesterol and plant sterols. Previous attempts have majorly focused on linkage between apolipoprotein B (*APOB*) loci and LDL subclass phenotypes, since ApoB is the major protein component of LDL (20, 21). These studies have failed to show any linkage, indicating that the *APOB* locus may not control LDL subclass phenotypes. However, multilocus control of the LDL phenotypes has been suggested by others using sib-pair linkage analyses (22, 23). For instance, polymorphic variants in hepatic lipase gene (*LIPC*) have been suggested to alter LDL particle distribution (24, 25). Likewise, various common polymorphisms in other genes involved in cholesterol and lipoprotein metabolism may potentially influence lipoprotein subclass distribution in response to dietary sterols (26, 27). To our knowledge, however, no studies have been conducted previously to assess the effects of such common variants on plasma lipoprotein subclass distribution in response to dietary cholesterol or plant sterols consumption in healthy humans. Only one study exists, which evaluated the influence of *APOB ins/de1* and Xbal polymorphisms on HDL2 subclass responses to low cholesterol, low saturated fat diet in 87 participants (28). Individuals who were *ins/ins* and X - /X – homozygotes, exhibited a large decrease in HDL2 concentrations compared to *del/del* and X + /X + groups, following consumption of the low fat, low-cholesterol diet (28). Exploring the associations of common polymorphisms can be anticipated to help in revealing underlying mechanisms that could orchestrate the differential response of plasma lipoprotein subclasses to dietary cholesterol versus plant sterol consumption. Investigating whether dietary cholesterol or plant sterols induce

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similar mechanisms through which they modulate plasma lipoprotein subclasses could provide useful insights into the functional role of these sterols in cardiovascular health.

Accordingly, we hypothesized that dietary cholesterol compared to plant sterol consumption, will differentially alter plasma low and high density lipoprotein subclass distribution in healthy humans and such changes will be linked to specific genetic polymorphisms in candidate genes. Genetic isolates and founder populations have always been of interest for genetic mapping studies with respect to Mendelian diseases, but have also been recognized increasingly towards understanding common diseases due to reduced genetic and environmental heterogeneity (29). Hence, the objective of our study was to the evaluate effects of dietary cholesterol versus plant sterol consumption on lipoprotein subclasses and how common polymorphisms in candidate genes influence subclass distribution in a Hutterite founder population.

5.3 STUDY PARTICIPANTS AND METHODS

5.3.1 Study participants

The study participant characteristics are discussed in Chapter IV, section 4.3.1 and table 4.1

5.3.2 Study design and protocol

The study design and protocol are elaborated in Chapter IV, section 4.3.3.

5.3.3 Blood sampling and plasma lipid analyses

Twelve-hour fasting blood samples collected in EDTA containing tubes obtained on days 26 and 28 were used for the LDL and HDL subfractionation analyses. Blood samples collected in heparin containing tubes on day 26 and 28 were used for determination of plasma total cholesterol (TC), LDL-C and HDL-C levels. Plasma TC and HDL-C levels were determined by automated enzymatic methods on a Vitros-350 chemistry analyzer (Ortho-Clinical Diagnostics, Markham, ON, Canada). LDL-C levels were calculated by using the Friedewald equation (30).

5.3.4 LDL and HDL subfractions analysis

LDL and HDL particle sizes were analyzed using the Quantimetrix Lipoprint LDL and HDL system. This system separates LDL and HDL subfractions by a linear polyacrylamide gel based capillary electrophoresis technique (31). Lipoproteins are separated on the basis of net surface charge and size, and the technique separates LDL into seven LDL subclasses and HDL into 10 subclasses. High-resolution 3% polyacrylamide gel tubes were used for electrophoresis. LDL and HDL subfractionation were performed separately as described in the manufacturer's kit. Plasma samples collected in EDTA tubes were used for the analysis. Briefly, 25 uL of plasma sample followed by 200 uL (LDL subfractionation) and 300 uL (HDL subfractionation) of liquid loading gel containing sudan black dye were added to the top of the precast gel tubes. The gel tubes were then subjected to photopolymerization for 30 min, followed by electrophoresis. Gels were scanned with a ArtixScan M2 digital scanner (Mikrotek, Co, Hsinchu, Taiwan) and analyzed using Lipoware software (Quantimetrix, Redondo Beach, CA, USA) (31, 32). LDL1 & 2 bands correspond to large LDL subclasses, whereas bands LDL3-7 correspond to small LDL

subclasses. In case of HDL subfractionation, HDL 1-3 correspond to large HDL, whereas HDL 4-7 represent intermediate HDL and HDL 8-10 small HDL subclasses (31, 33). The relative area under the curve for each LDL subclass band was determined and multiplied by the total cholesterol concentration of the sample to obtain the amount of cholesterol for each band. For HDL subclasses, each band's relative area under the curve was multiplied by the total HDL cholesterol concentration of the sample (31, 33).

5.3.5 SNP genotyping

The study participants were genotyped for a set of 38 candidate SNPs across 25 different genes previously known to be associated with lipoprotein and cholesterol metabolism to evaluate the influence of these SNPs on the responsiveness to plant sterols and dietary cholesterol (**Table 5.1**). The SNPs genotyped included *ABCA1* (*rs2230808*, *rs2066714*), *ABCG5* (*rs6720173*, *rs6756629*, *rs2278356*, *rs11887534*), *ABCG8* (*rs4148211*, *rs4148217*, *rs6544718*, *rs41360247*), *ACAT2* (*rs25683*), *APOA4* (rs5104), *APOB* (*rs676210*), *APOE* (*rs7412, rs429358*), *CETP* (*rs5882*), *CYP7A1* (*rs3808607*), *DHCR7* (*rs760241, rs1044482*), *LDLR* (*rs688*), *LIPA* (*rs1051338*), *LIPC* (*rs6083*), *LIPF* (*rs814628*), *LIPG* (*rs2000813*), *LPL* (*rs13702, rs3200218*), *LSS* (rs2839158, rs34115287), *MTTP* (*rs2306986*), *NPC1L1* (*rs79519744, rs79803700, rs2073547*), *PCSK9* (rs562556), *PPAR* (*rs6008259*), *SCAP* (*rs12487736*), *SCD* (*rs2234970*), *SREBF1* (*rs2297508, rs11868035*) and *SREBF2* (*rs2228313, rs2228314*). Among the selected SNPs, 37 SNPs across 24 genes were primarily non-synonymous missense mutations (27 SNPs), with some in the near gene 5´ region (3 SNPs), some in the 3´untranslated region (5 SNPs), one

intronic variant SNP and one synonymous mutation (found in the LDL-receptor). *APOE* variants (ε2, ε3, ε4) were also assessed by genotyping SNPs *rs7412* and *rs429358* in the APOE gene.

Genomic DNA was extracted from buffy coat by using a commercial spin-column based DNA extraction and purification kit (DNeasy Blood and Tissue Kit) according to the manufacturer's instructions (QIAGEN Sciences, Germantown, MD, USA). The concentration and integrity of the extracted genomic DNA were assessed by micro-volume spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific Inc., Waltham, MA, USA). DNA samples were genotyped by TaqMan SNP genotyping assay, using TaqMan® GTXpress™ Master Mix with allele-specific probes (Life Technologies, Burlington, ON) on a StepOnePlus Real-Time PCR System (Applied Biosystems®, Life Technologies, Burlington, ON).

5.3.6 Statistical analysis

Statistical analysis was performed using the statistical software, SAS version 9.2 (SAS Inc). Endpoint measurements of the treatment and control phases were compared. Values were expressed as least square means and standard error of the mean (SEM), unless otherwise stated. Effects of dietary treatments were examined using a mixed model ANOVA procedure

Table 5.1 Candidate single nucleotide polymorphisms in genes related to cholesterol and lipoprotein metabolism

Gene SNP	Type of SNP	Variation	Minor allelic frequency†	Gene function
ABCA1				Cholesterol efflux pump in the cellular lipid
rs2230808	NS - missense	G to A	A=0.410	removal pathway.
rs2066714	NS - missense	T to C	C=0.366	
ABCG5				Half-transporter (with ABCG8) that
rs6720173	NS - missense	G to C	C=0.211	promotes intestinal and biliary excretion of
rs6756629	NS - missense	G to A	A=0.074	sterols.
rs2278356	3'-UTR	A to C	C=0.403	
rs11887534	Near gene-5'	G to C	C=0.065	
ABCG8				Half-transporter (with ABCG5) that
rs4148211	NS – missense	A to G	G=0.441	promotes intestinal and biliary excretion of
rs4148217	NS - missense	C to A	A=0.218	sterols.
rs6544718	NS - missense	C to T	T=0.106	
rs41360247	Intron	C to T	C=0.075	
ACAT2				Catalyzes the conversion of free cholesterol
rs25683	NS - missense	A to G	G=0.365	into cholesterol ester
APOA4				Controls lecithin-cholesterol acyltransferase
rs5104	NS - missense	C to T	C=0.241	and cholesterol ester transfer protein activity.
APOB				Primary apolipoprotein of chylomicrons,
rs676210	NS - missense	G to A	G=0.366	VLDL, IDL, and LDL particles.

Table 5.1 continued

Gene SNP	Type of SNP	Variation	Minor allelic frequency†	Gene function
APOE variant *Typical frequency**	E2-7.9%	E3-78.6%	E4-13.5%	Associated with triglyceride-rich lipoproteins (chylomicrons and VLDLs) and HDL.
CETP *rs5882*	NS-missense	A to G	G=0.448	Facilitates transport of cholesteryl esters and triglycerides between the lipoproteins
CYP7A1 *rs3808607*	Near gene-5'	T to G	G=0.450	The rate-limiting enzyme in the synthesis of bile acid in the classic pathway.
DHCR7 *rs760241*	NS - missense	G to A	A=0.179	Enzyme which catalyzes the conversion of 7-dehydrocholesterol to cholesterol.
LDLR *rs688*	Synonymous	C to T	T=0.276	A cell surface protein involved in receptor-mediated endocytosis.
LIPA *rs1051338*	NS - missense	A to C	C=0.286	Catalyzes the hydrolysis of cholesterol esters
LIPC *rs6083*	NS - missense	A to G	A=0.394	Regulation of plasma triglycerides. Function of the enzyme is controlled by HDL.
LIPF *rs814628*	NS - missense	A to G	G=0.168	Digestion of dietary fats
LIPG *rs2000813*	NS - missense	C to T	T=0.2406	Involved in lipoprotein and possibly HDL metabolism

Table 5.1 continued

Gene SNP	Type of SNP	Variation	Minor allelic frequency†	Gene function
LPL				Dual functions as triglyceride hydrolase and
rs13702	3'-UTR	C to T	C=0.335	ligand/bridging factor for receptor-mediated
rs3200218	3'-UTR	A to G	G=0.148	lipoprotein uptake.
LSS				Catalyzes the conversion of (S)-2,3
rs2839158	NS - missense	C to T	T=0.137	oxidosqualene to lanosterol.
rs34115287	NS - missense	T to C	C=0.136	
MTTP				A protein that plays a central role in
rs2306986	NS - missense	G to C	C=0.118	lipoprotein assembly
NPC1L1				Controls influx of free cholesterol into cells
rs79519744	NS - missense	G to C	G=0.009	and intestinal cholesterol absorption.
rs79803700	NS - missense	A to C	A=0.015	
rs2073547	Near gene-5'	T to C	C=0.268	
PCSK9				A convertase belonging to the proteinase K
rs562556	NS - missense	A to G	G=0.148	subfamily which induces LDLR degradation.
PPAR				Nuclear transcription factor that regulates
rs6008259	3'-UTR	A to G	A=0.271	lipid metabolism
SCAP				A protein with a sterol sensing domain which
rs12487736	NS - missense	C to T	T=0.476	is involved in SREBFs regulation.
SCD				Rate limiting enzyme in the synthesis of
rs2234970	NS-missense	A to C	C=0.389	unsaturated fatty acids.

Table 5.1 continued

Gene SNP	Type of SNP	Variation	Minor allelic frequency†	Gene function
SREBF1				Transcription factor that regulates genes
rs2297508	NS-missense	A to G	G=0.487	involved in cholesterol production and lipid
rs11868035	3'-UTR	C to G	G=0.415	metabolism.
SREBF2				Transcription factor that controls cholesterol
rs2228313	NS - missense	G to C	C=0.366	homeostasis by stimulating transcription of
rs2228314	NS - missense	G to C	C=0.066	sterol-regulated genes.

†Frequency according dbSNP in January 2016 *frequency from (n=1209) Ordovas et al. 1987 (34).

ABCA1, ATP-binding cassette, sub-family A, member 1; *ABCG5*, ATP-binding cassette, sub-family G (WHITE), member 5; ABCG8, ATP-binding cassette, sub-family G (WHITE), member 8; *ACAT2*, acetyl-Coenzyme A acetyltransferase 2; *APOA4*, apolipoprotein A-IV; *APOB*, apolipoprotein B; *APOE*, apolipoprotein E epsilon variations; *CETP*, cholesteryl ester transfer protein; *CYP7A1*, cholesterol 7 alpha-hydroxylase; *DHCR7*, 7-dehydrocholesterol reductase; *LDLR*, low density lipoprotein receptor; *LIPA*, lysosomal acid lipase gene; *LIPC*, hepatic lipase; *LIPF*, gastric lipase; *LIPG*, endothelial lipase; *LPL*, lipoprotein lipase; *LSS*, lanosterol synthase; *MTTP* microsomal triglyceride transfer protein; *NPC1L1*, Niemann-pick C1-like 1 protein; *PCSK9*, proprotein convertase subtilisin/kexin type 9; *PPAR*, peroxisome proliferator-activated receptor alpha; *SCAP*, SREBF chaperone; *SCD*, stearoyl-CoA desaturase; *SREBF1*, sterol regulatory element binding transcription factor 1; *SREBF2*, sterol regulatory element binding transcription factor 2.

with participant ID as a random factor and treatment as an independent factor followed by Tukey-Kramer adjustments for multiple comparisons. The effect of treatment, sequence, and gender were included in the model as fixed factors. Genotype and treatment by genotype were included as fixed factors. When the number of the minor allele homozygotes failed to exceed 5% of the total population within a given SNP, they were combined with the heterozygotes of that SNP and compared to the major allele homozygotes. X^2 test for distribution was used to test for Hardy-Weinberg equilibrium of the population for a given candidate SNP with two alleles. Gene-diet interactions were assessed using mixed model ANOVA procedures, with genotype and gender as fixed effects, followed by Tukey-Kramer adjustments for multiple comparisons. These data were expressed as changes from endpoint of control phase in response to endpoint of dietary cholesterol and plant sterol treatment (Δ values) phases. Statistical significance was set at $p < 0.05$ for all analyses.

5.4 RESULTS

5.4.1 Effects of dietary cholesterol versus plant sterols on LDL lipoprotein subclasses

LDL subclass distribution in response to dietary cholesterol versus plant sterols is presented in **Table 5.3**. Overall, dietary cholesterol or plant sterol consumption did not result in any significant changes in LDL-C concentrations or LDL subclass distribution. All participants had a Type A profile (predominantly large buoyant LDL subclass) in the control phase. None of the study participants displayed a propensity towards a type B profile, typical of increased number of small dense LDL subclasses (LDL:4-7) after the dietary cholesterol or plant sterol intervention period.

5.4.2 Effects of dietary cholesterol versus plant sterols on HDL lipoprotein subclass concentrations

Effects of dietary cholesterol versus plant sterols on the distribution of HDL subclasses are presented in **Table 5.3**. Consumption of 600 mg of dietary cholesterol resulted in an increase in HDL-C (0.08 ± 0.03 mmol/L, p=0.0216) and large HDL subclasses (0.12 ± 0.04 mmol/L, p=0.0167), whereas, plant sterol consumption did not alter HDL-C or HDL subclass distribution. Dietary cholesterol supplementation resulted in an increase in HDL-1 (0.04 ± 0.01 mmol/L, p=0.0222), HDL-2 (0.05 ± 0.02 mmol/L, p=0.0220) and HDL-3 (0.03 ± 0.01mmol/L, p=0.0444) subclass concentrations. The individual changes in large HDL subclasses to dietary cholesterol versus plant sterols compared to the control are shown in **Figures 5.1 A & B**. No changes in intermediate (HDL 4-7) and small (HDL 8-10) subclasses were observed in response to dietary cholesterol consumption.

5.4.3 Gender effects on dietary cholesterol and plants sterol induced HDL subclass distribution

Strong gender influenced differences were observed in HDL subclass distribution in response to dietary cholesterol and plant sterol consumption (**Figure 5.**). Women participants displayed an increase in large HDL subclasses (0.15 ± 0.04 mmol/L, p=0.0026) in response to dietary cholesterol intake, whereas no such effect was observed in men (0.02 ± 0.05 mmol/L, p=0.9981).
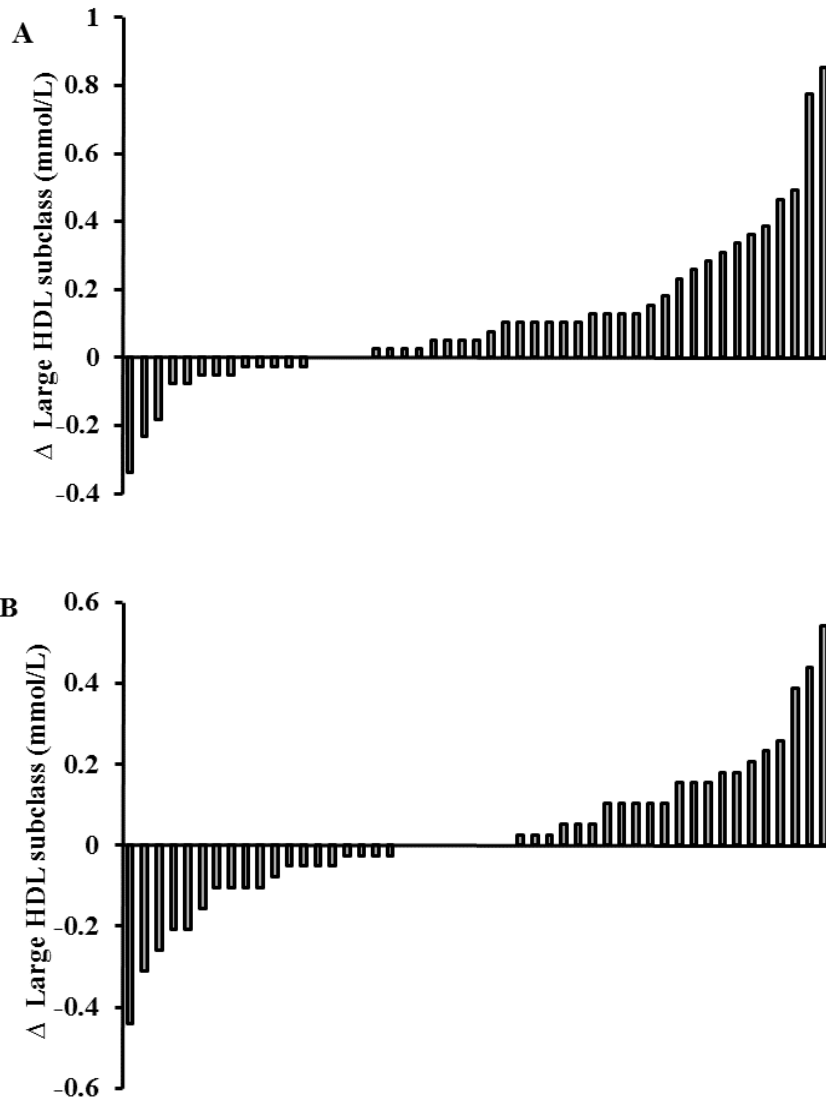
Figures 5.1 (A), Individual changes in large HDL-subclass in response to 4-week dietary cholesterol consumption and (B), 4-week plant sterol consumption. Δ, absolute change in large HDL-subclass (mmol/L) (endpoint of each dietary phase minus control phase).

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Specifically, women participants alone showed an increase in HDL-1 particles (p=0.0085) in response to dietary cholesterol consumption. No such gender related effect was seen following plant sterol consumption. Likewise, dietary cholesterol induced an increase in HDL-2 subclass particles (0.06±0.02 mmol/L, p=0.0253) in women and not in men, and the magnitude of such change was greater in women (0.13±0.03 mmol/L, p=0.0005) than men. Women also showed an increase in HDL-2 subclass particles (0.11±0.03 mmol/L, p=0.0067) after plant sterol consumption compared to men. Similarly, with respect to HDL3, women alone exhibited an increase in HDL-3 concentrations (0.03±0.01 mmol/L, p=0.0383) in response to dietary cholesterol intake, and the difference in response was greater (0.06±0.02 mmol/L, p=0.0042) when compared to men. HDL-3 responses were not different between men and women following plant sterol consumption. No gender related changes were seen in intermediate (HDL 4-7) and small (HDL 8-10) subclasses due to dietary cholesterol or plant sterol supplementation.

5.4.4 Impact of genotype on response of LDL and HDL subclass distribution to dietary cholesterol versus plant sterol consumption

The genotype distribution of all candidate SNPs are presented in **Table 5.4.** Out of 38 SNPs across 25 genes, only 30 SNPs were tested for their responsiveness associations with the treatments. The remaining 8 SNPs in genes *ABCG5* (*rs6756629, rs11887534*), *ABCG8* (*rs41360247*), *DHCR7* (*rs760241*), *MTTP* (*rs2306986), NPC1L1* (*rs79519744, rs79803700*) and *SREBF2* (*rs2228313*) were excluded because of the presence of only one genotype or insufficient individuals (<5%) in two of the three possible genotypes for the candidate SNP in the study population. No significant associations were found between any of the candidate SNPs and LDL subclass distribution in response to dietary cholesterol or plant sterol consumption.

Table 5.2 Effects of dietary cholesterol versus plant sterols on LDL lipoprotein subclasses

LDL subclasses (mmol/L)	Control	Cholesterol	Plant sterols
Total LDL-C			
All	2.85 ± 0.13^{ab}	2.96 ± 0.13^{a}	2.73 ± 0.13^{b}
Male	2.80 ± 0.20	3.05 ± 0.20	2.78 ± 0.20
Female	3.00 ± 0.17	3.04 ± 0.17	2.82 ± 0.17
IDL-C			
All	0.37 ± 0.02	0.39 ± 0.02	0.36 ± 0.02
Male	0.34 ± 0.03	0.37 ± 0.03	0.35 ± 0.03
Female	0.39 ± 0.02	0.40 ± 0.02	0.36 ± 0.02
IDL-B			
All	0.44 ± 0.02	0.44 ± 0.02	0.43 ± 0.02
Male	0.39 ± 0.03	0.42 ± 0.03	0.41 ± 0.03
Female	0.48 ± 0.03	0.43 ± 0.03	0.44 ± 0.03
IDL-A			
All	0.78 ± 0.01	0.76 ± 0.01	0.79 ± 0.01
Male	0.65 ± 0.06	0.70 ± 0.06	0.70 ± 0.06
Female	0.87 ± 0.05	0.82 ± 0.05	0.85 ± 0.05
LDL-1			
All	0.77 ± 0.05	0.81 ± 0.05	0.76 ± 0.05
Male	0.85 ± 0.07	0.84 ± 0.07	0.81 ± 0.07
Female	0.76 ± 0.06	0.85 ± 0.06	0.76 ± 0.06
LDL-2			
All	0.14 ± 0.04	0.18 ± 0.04	0.13 ± 0.04
Male	0.27 ± 0.04	0.31 ± 0.04	0.24 ± 0.04
Female	0.07 ± 0.04	0.11 ± 0.04	0.08 ± 0.04

Table 5.2 continued

LDL subclasses (mmol/L)	Control	Cholesterol	Plant sterols
LDL-3			
All	0.004±0.01	0.009±0.01	0.006±0.01
Male	0.007±0.008	0.02±0.008	0.01±0.007
Female	0.002±0.007	0.004±0.007	0.001±0.007
Mean particle size (nm)			
All	274.09±0.35	274.60±0.34	275.16±0.35
Male	273.55±0.46	272.90±0.45	273.57±0.46
Female	276.02±0.38	275.56±0.38	276.09±0.38

All values are least square means ± SEM (n=49). Differences between treatments were assessed by SAS mixed procedure ANOVA with Tukey-Kramer adjustments; $p<0.05$ is considered significant. Values with different superscript letters are significantly different from each other. IDL, intermediate density lipoprotein; LDL, low density lipoprotein. LDL1 & 2 corresponds to large LDL subclasses and LDL3 correspond to small LDL subclass.

Table 5.3 Effects of dietary cholesterol versus plant sterols on HDL lipoprotein subclasses

HDL subclasses (mmol/L)	Control	Cholesterol	Plant sterols
Total HDL-C			
All	1.44 ± 0.06^a	1.53 ± 0.06^b	1.45 ± 0.06^a
Male	1.27 ± 0.09	1.32 ± 0.09	1.26 ± 0.09
Female	1.58 ± 0.08^a	1.69 ± 0.08^b	1.60 ± 0.08^{ab}
HDL-1			
All	0.14 ± 0.01^a	0.18 ± 0.01^b	0.15 ± 0.01^{ab}
Male	0.12 ± 0.02	0.14 ± 0.02	0.12 ± 0.02
Female	0.15 ± 0.02^a	0.21 ± 0.02^b	0.18 ± 0.02^{ab}
HDL-2			
All	0.19 ± 0.02^a	0.23 ± 0.02^b	0.21 ± 0.02^{ab}
Male	0.16 ± 0.02	0.15 ± 0.02	0.15 ± 0.02
Female	0.23 ± 0.02^a	0.29 ± 0.02^b	0.26 ± 0.02^{ab}
HDL-3			
All	0.11 ± 0.01^a	0.13 ± 0.01^b	0.12 ± 0.01^{ab}
Male	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01
Female	0.13 ± 0.01^a	0.16 ± 0.01^b	0.15 ± 0.01^{ab}
HDL-4			
All	0.14 ± 0.01	0.15 ± 0.01	0.14 ± 0.01
Male	0.13 ± 0.01	0.14 ± 0.01	0.13 ± 0.01
Female	0.14 ± 0.01	0.16 ± 0.01	0.16 ± 0.01
HDL-5			
All	0.15 ± 0.01	0.16 ± 0.01	0.15 ± 0.01
Male	0.14 ± 0.01	0.15 ± 0.01	0.14 ± 0.01
Female	0.15 ± 0.01	0.16 ± 0.01	0.17 ± 0.01

Table 5.3 continued

HDL subclasses (mmol/L)	Control	Cholesterol	Plant sterols
HDL-6			
All	0.27±0.01	0.26±0.01	0.26±0.01
Male	0.24±0.02	0.24±0.02	0.24±0.02
Female	0.30±0.01	0.27±0.01	0.28±0.01
HDL-7			
All	0.09±0.004	0.09±0.004	0.09±0.004
Male	0.08±0.006	0.08±0.006	0.09±0.006
Female	0.10±0.004	0.09±0.004	0.10±0.004
HDL-8			
All	0.09±0.004	0.08±0.004	0.09±0.004
Male	0.08±0.008	0.07±0.008	0.08±0.008
Female	0.10±0.007	0.08±0.007	0.09±0.007
HDL-9			
All	0.06±0.004	0.05±0.004	0.06±0.004
Male	0.06±0.007	0.06±0.007	0.06±0.007
Female	0.06±0.006	0.04±0.006	0.05±0.006
HDL-10			
All	0.09±0.01	0.08±0.38	0.09±0.38
Male	0.09±0.01	0.09±0.01	0.11±0.01
Female	0.09±0.01	0.07±0.01	0.06±0.01
Large			
All	0.45±0.04[a]	0.55±0.04[b]	0.48±0.04[ab]
Male	0.38±0.05	0.40±0.05	0.36±0.05
Female	0.51±0.04[a]	0.65±0.04[b]	0.58±0.04[ab]

Table 5.3 continued

HDL subclasses (mmol/L)	Control	Cholesterol	Plant sterols
Intermediate			
All	0.66±0.02	0.66±0.02	0.66±0.02
Male	0.61±0.04	0.61±0.04	0.59±0.04
Female	0.70±0.03	0.70±0.03	0.70±0.03
Small			
All	0.25±0.02	0.22±0.02	0.23±0.02
Male	0.23±0.03	0.23±0.03	0.25±0.03
Female	0.26±0.03	0.21±0.03	0.21±0.03

All values are least square means ± SEM (n=49). Differences between treatments were assessed by SAS mixed procedure ANOVA with Tukey-Kramer adjustments; $p<0.05$ is considered significant. Values with different superscript letters are significantly different from each other. HDL, high density lipoprotein. HDL 1-3 correspond to large HDL, whereas HDL 4-7 represent intermediate HDL and HDL 8-10 small HDL subclasses.
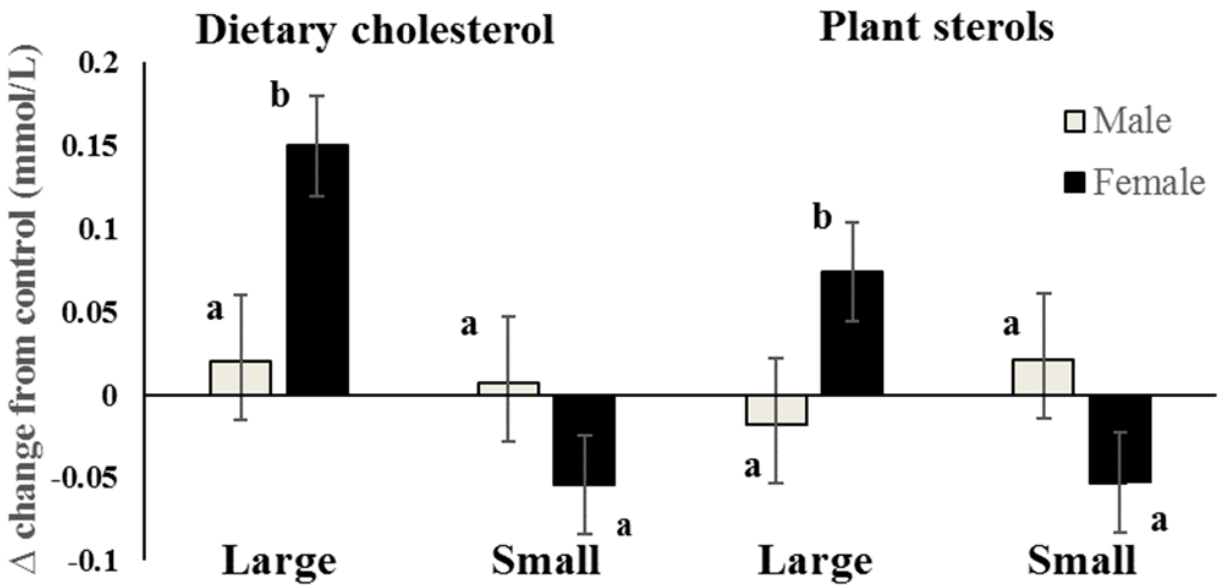
Figure 5.2 Changes in large and small HDL-subclass responsiveness to dietary cholesterol and plant sterol consumption from control in male and female participants. All values are least square means ± SEM. Differences between the groups and treatments effects assessed by SAS mixed procedure ANOVA with Tukey-Kramer adjustments. Labeled means with a different superscript letter are significantly different, $p<0.05$. Δ, absolute changes in large and small HDL subclasses (mmol/L) of endpoints of dietary cholesterol and plant sterol intervention phases from endpoint of control phase.

Amongst 31 SNPs tested, 5 candidate SNPs (*ABCA1 rs2066714*, *LIPC rs6083*, *LIPF rs814628*, *SCD rs2234970* & *SREBF1 rs2297508*) were associated with HDL subclass distribution in response to dietary cholesterol consumption (**Tables 5.5 & 5.6**). Only *ABCA1 rs2066714* was associated with changes in HDL-2 subclass particle concentrations in response to plant sterol consumption (**Table 5.5**).

In *ABCA1* SNP *rs2066714* G/A heterozygotes, HDL-2 increased after dietary cholesterol consumption, while G-homozygotes did not respond (ΔHDL-2 (mmol/L): 0.107±0.04 for G/A vs. 0.018±0.01 for A/A). An identical association was seen for plant sterol consumption (ΔHDL-2 (mmol/L): 0.105±0.04 in G/A vs. -0.005±0.01in A/A) (**Table 5.5**). Moreover, *ABCA1* SNP *rs2066714* G/A heterozygosity associated with an HDL-3 increase after cholesterol consumption, while A-homozygosity did not (ΔHDL-3 (mmol/L): 0.069±0.01 for G/A vs. 0.012±0.008 for G/A) (**Table 5.6**). Since no SNP *rs2066714* G homozygotes were present in the participants, the observed increases in HDL2 and HDL3 might be associated with the G allele.

LIPF SNP *rs814628* A/G heterozygotes increased HDL-2 after cholesterol consumption, while A-homozygotes did not respond (ΔHDL-2 (mmol/L): 0.112±0.04 in A/G vs. 0.017±0.01 in A/A) (**Table 5.5**). No effects of *rs814628* genotypes on large HDL subclass particles were observed due to plant sterol supplementation. However, greater reductions in small HDL particle concentrations were seen in A/G heterozygotes (-0.178±0.05 mmol/L, p=0.0142) compared to A/A homozygotes (0.007±0.02 mmol/L, p=0.9998) in response to plant sterol consumption but not with dietary cholesterol consumption. Since no G-homozygotes were present in the study cohort, the observed effects might be associated with the SNP *rs814628* G allele.

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For *SCD* SNP *rs2234970* an allelic dose effect for increased HDL-2 in response to dietary cholesterol was observed, where increases were associated with the C allele (ΔHDL-2 (mmol/L): 0.112$\pm$0.04 in C/C vs. 0.047$\pm$0.03 in A/C vs. -0.012$\pm$0.02 in A/A) (**Table 5.5**). *SREBF1* SNP *rs11868035* A/G heterozygotes increased HDL-2 after cholesterol consumption, while G homozygotes showed no change (ΔHDL-2 (mmol/L): 0.058$\pm$0.02 in A/G vs. -0.012$\pm$0.02 in G/G) (**Table 5.5**). In contrast to *LIPC* SNP *rs6083* G and A homozygotes, A/G heterozygotes did increase HDL-3 after dietary cholesterol consumption (ΔHDL-3 (mmol/L): 0.006$\pm$0.02 in G/G vs. -0.012$\pm$0.01 in A/A vs. 0.039$\pm$0.01 in A/G) (**Table 5.6**).

Table 5.4 Genotype distribution of candidate single nucleotide polymorphisms in the study population

Gene	Genotypes			Allele frequency		P values*
SNP				%		
ABCA1						
rs2230808	G/G= 44	G/A=5	A/A=0	G=94.9	A=5.1	p=0.7067
rs2066714	A/A=42	A/G=7	G/G=0	A=92.86	G=7.14	p=0.5903
ABCG5						
rs6720173	G/G=38	G/C=8	C/C=3	G=85.71	C=14.29	p=0.0196
rs6756629	G/G=42	A/G=7	G/G=0	G=92.86	A= 7.14	p=0.5903
rs2278356	A/A=29	A/C=13	C/C=7	A= 72.45	C=27.55	p=0.0189
rs11887534	G/G=47	C/G=2	C/C=0	G= 97.96	C= 2.04	p= 0.8841
ABCG8						
rs4148211	A/A=20	A/G=25	G/G=4	A= 66.33	G=33.67	p=0.3196
rs4148217	A/A=41	A/C=8	C/C=0	A= 91.84	C=8.16	p=0.5338
rs6544718	C/C=36	C/T=13	T/T=0	C= 86.73	T=13.27	p=0.2844
rs41360247	T/T=47	T/C=2	C/C=0	T= 97.96	C=2.04	p=0.8841
ACAT2						
rs25683	A/A=3	A/C=20	C/C=26	A= 26.53	C=73.47	p=0.7421
APOA4						
rs5104	T/T=42	C/T=7	C/C=0	T= 92.86	C=7.14	p=0.5903
APOB						
rs676210	G/G=27	G/A=19	A/A=3	G= 74.49	A=25.51	p=0.8871
APOE						
variant	ε2/-=6	ε3/ε3=40	ε4/-=3	n/a	n/a	n/a
CETP						
rs5882	A/A=6	A/G=38	G/G=5	A= 51.02	G=48.98	p=0.0001
CYP7A1						
rs3808607	T/T=43	T/G=6	G/G=0	T= 93.88	G=6.12	p=0.648

Table 5.4 continued

Gene SNP	Genotypes			Allele frequency %		P values*
DHCR7						
rs760241	G/G=46	A/G=3	A/A=0	G= 96.94	A=3.06	p=0.8251
LDLR						
rs688	C/C=4	C/T=26	T/T=18	C= 35.42	T=64.58	p=0.2022
LIPA						
rs1051338	A/A=13	C/A=36	C/C=0	A= 63.27	C=36.73	p<0.0001
LIPC						
rs6083	A/A=12	A/G=25	G/G=12	A=50	G=50	p=0.8864
LIPF						
rs814628	A/A=42	A/G=7	G/G=0	A=92.86	G=7.14	p=0.5903
LIPG						
rs2000813	C/C=33	C/T=16	T/T=0	C=83.67	T=16.33	p=0.1720
LPL						
rs13702	T/T=29	T/C=18	C/C=2	T=77.55	C=22.45	p=0.7001
rs3200218	A/A=29	A/G=20	G/G=0	A=79.59	G=20.41	p=0.0727
LSS						
rs2839158	C/C=35	T/C=14	T/T=0	C=85.71	T=14.29	p=0.2433
rs34115287	T/T=35	C/T=14	C/C=0	T=85.71	C=14.29	p=0.2433
NPC1L1						
rs2073547	T/T=20	T/C=29	C/C=0	T=70.41	C=29.59	p=0.0033
PCSK9						
rs562556	A/A=28	A/G=18	G/G=3	A=75.51	G=24.49	p=0.9623
PPARA						
rs6008259	G/G=31	G/A=14	A/A=4	G=77.55	A=22.45	p=0.2091

Gene	Genotypes			Allele frequency %		P values*
SNP						
SCAP						
rs12487736	T/T=14	T/C=29	C/C=7	T=57	C=43	p=0.1952
SCD						
rs2234970	A/A=27	A/C=11	C/C=11	A=66.33	C=33.67	p=0.0005
SREBF1						
rs11868035	G/G=20	G/A=29	A/A=0	G=70.41	A=29.59	p=0.0033
rs2297508	C/C=9	C/G=28	G/G=12	C=46.94	G=53.06	p= 0.3032
SREBF2						
rs2228314	G/G=30	G/C=17	C/C=2	G=78.57	C=21.43	p=0.8320

*P values assessed using X^2 test for distribution for testing for Hardy-Weinberg equilibrium with two alleles. p<0.05 considered significant. Measured genotype frequencies significantly different from the expectations of Hardy-Weinberg equilibrium when p<0.05. *ABCA1*, ATP-binding cassette, sub-family A, member 1; *ABCG5*, ATP-binding cassette, sub-family G (WHITE), member 5; ABCG8, ATP-binding cassette, sub-family G (WHITE), member 8; *ACAT2*, acetyl-Coenzyme A acetyltransferase 2; *APOA4*, apolipoprotein A-IV; *APOB*, apolipoprotein B; *ApoE*, apolipoprotein E epsilon variations; *CETP*, cholesteryl ester transfer protein; *CYP7A1*, cholesterol 7 alpha-hydroxylase; *DHCR7*, 7-dehydrocholesterol reductase; *LDLR*, low density lipoprotein receptor; *LIPA*, lysosomal acid lipase gene; *LIPC*, hepatic lipase; *LIPF*, gastric lipase; *LIPG*, endothelial lipase; *LPL*, lipoprotein lipase; *LSS*, lanosterol synthase; *NPC1L1*, Niemann-pick C1-like 1 protein; *PCSK9*, proprotein convertase subtilisin/kexin type 9; *PPAR*, peroxisome proliferator-activated receptor alpha; *SCAP*, SREBF chaperone; *SCD*, stearoyl-CoA desaturase; *SREBF1*, sterol regulatory element binding transcription factor 1; *SREBF2*, sterol regulatory element binding transcription factor 2.

Table 5.5 Single nucleotide polymorphisms associated with changes in HDL-2 subclass in response to dietary cholesterol and plant sterol consumption

Gene SNP	ΔHDL-2 (mmol/L) after cholesterol supplementation*	ΔHDL-2 (mmol/L) after plant sterol supplementation*
ABCA1	G/A=0.107±0.04, p=0.0425	G/A=0.105±0.04, p=0.0376
rs2066714	A/A=0.018±0.01, p=0.8068	A/A=-0.005±0.01, p=0.9993
LIPF	A/A=0.017±0.01, p=0.8458	A/A=-0.001±0.01, p=0.9461
rs814628	A/G=0.112±0.04, p=0.0374	A/G=0.067±0.01, p=0.3391
SCD	A/A=-0.012±0.02, p=0.9980	A/A=-0.024±0.02, p=0.8798
rs2234970	A/C=0.047±0.03, p=0.7294	A/C=0.038±0.03, p=0.8698
	C/C=0.112±0.04, p=0.0022	C/C=0.060±0.04, p=0.3458
SREBF1	A/G=0.058±0.02, p=0.0174	A/G=0.026±0.02, p=0.6546
rs11868035	G/G=-0.012±0.02, p=0.9942	G/G=-0.014±0.02, p=0.9826

*mixed model effects by genotype assessed with SAS mixed procedure ANOVA with Tukey-Kramer adjustments when treatment × genotype interaction was significant (statistical significance level set at $p < 0.05$). All values are least square means ± SEM (n=49). ΔHDL-2: absolute change from control in mmol/L. *ABCA1*, ATP-binding cassette, sub-family A, member 1; *LIPF*, gastric lipase; *SCD*, stearoyl-CoA desaturase; *SREBF1*, sterol regulatory element binding transcription factor 1.

Table 5.6 Single nucleotide polymorphisms associated with changes in HDL-3 subclass in response to dietary cholesterol and plant sterol consumption

Gene SNP	ΔHDL-3 (mmol/L) after cholesterol supplementation*	ΔHDL-3 (mmol/L) after plant sterol supplementation*
ABCA1 *rs2066714*	G/A=0.069±0.01, p=0.0181 A/A=0.012±0.008, p=0.6680	G/A=0.057±0.02, p=0.0673 A/A=0.0005±0.008, p=0.9495
LIPC *rs6083*	A/A=-0.012±0.01, p=0.9976 A/G=0.039±0.01, p=0.0104 G/G=0.006±0.02, p=0.6998	A/A=0.013±0.01, p=0.9945 A/G=0.010±0.01, p=0.9877 G/G=-0.006±0.02, p=0.6843

*mixed model effects by genotype assessed by SAS mixed procedure ANOVA with Tukey-Kramer adjustments when treatment × genotype interaction was significant ($p<0.05$ considered significant). All values are least square means ± SEM (n=49). ΔHDL-3: absolute change from control in mmol/L. *ABCA1*, ATP-binding cassette, sub-family A, member 1; *LIPC*, hepatic lipase gene.

5.5 DISCUSSION

The major finding of this work is that dietary cholesterol consumption may modify large HDL particle subclass distribution in healthy individuals in a manner dependent on gender and specific gene polymorphisms, while plant sterol consumption does not affect lipoprotein subclass distribution. The findings of the current study imply that dietary cholesterol and plant sterols possibly follow different mechanisms through which they influence lipoprotein metabolism owing to the differences in their structure and biological functionality.

Our observations regarding incremental shifts in plasma HDL-C concentrations and large HDL-subclasses following cholesterol consumption concur with those of previous studies (9, 10, 11, 35, 36, 37, 38). Large HDL particles have been suggested to play a pivotal role in RCT and hence inversely been associated with CVD risk (3, 4, 5). Our results indicate that dietary cholesterol consumption may in fact prove to be beneficial for cardiovascular health and not contribute to CVD risk as is usually advocated. However, given the small increments in HDL-C and large HDL subclasses, the clinical significance in CVD risk reduction is only modest. As seen in our study, plant sterol intake has been known to have no effects on plasma HDL-C concentrations or HDL subclasses (39, 40, 18). Our observations with regards to such differential effects of dietary cholesterol versus plant sterol consumption on plasma HDL subclasses suggest that these sterols possibly follow different cholesterol trafficking patterns, exerting dissimilar biological functions upon intestinal absorption. Our study findings also suggest that women may benefit more from dietary cholesterol and plant sterol consumption than men by increasing large HDL subclasses. Clifton and Nestel (41) found that supplementing 650 mg/d of dietary cholesterol for 3 weeks accompanied by a low fat diet made women substantially increase HDL-

2 particle concentrations compared to men. In a later study, Clifton et al. (42) replicated their earlier findings, and assumed that differential fat distribution between men and women was responsible for the variations in plasma HDL-C and HDL subclass responses. Likewise, the gender related effects on HDL-2 concentrations due to plant sterol intake in our study could be ascribed to such body fat compositional differences. Overall, our results reinforce previous findings and call for the development of gender specific recommendations for dietary cholesterol and plant sterol consumption.

Using a candidate gene approach, we report emerging evidence that genotypes of *ABCA1 rs2066714*, *LIPC rs6083*, *LIPF rs814628*, *SCD rs2234970* & *SREBF1 rs2297508* contribute to shaping the HDL-subclass distribution to cholesterol and plant sterol supplementation. ABCA1 is a transmembrane protein which plays an important role in cellular cholesterol efflux mechanisms and is also involved in HDL metabolism (43). Common polymorphisms in *ABCA1* gene have been suggested to influence plasma HDL-C levels and HDL subclasses (44, 45). The study by Tsai et al. (45) remains the only attempt to our knowledge to have investigated the effect of *ABCA1 rs2066714* genotypes on plasma HDL subclasses, however, in response to fenofibrate treatment. The authors did not find any associations between *ABCA1 rs2066714* genotypes and changes in HDL subclass particle concentrations following fenofibrate therapy. We have shown that G allele carriers of *ABCA1 rs2066714* exhibit increased responsiveness of large HDL subclass (HDL-2) concentrations to both dietary cholesterol and plant sterol intake. Our results indicate that dietary cholesterol and plant sterols may independently work in concert with *rs2066714* genotypes and modify *ABCA1* expression, leading to altered HDL metabolism and plasma HDL subclass distribution.

Hepatic lipase encoded by *LIPC* gene is a key enzyme involved in LDL and HDL metabolism, HDL2 to HDL3 conversion and RCT (24, 25). For, instance, polymorphisms in *LIPC* gene have been shown to influence HDL-C levels, LDL buoyancy, hepatic lipase activity, HDL particle size and HDL-2c levels (46, 24, 25). However, in our study, the response pattern due to dietary cholesterol intake was unexpected and failed to show any allelic dose effect, implying the existence of potential confounding factors. Carr et al. (47) demonstrated that lipoprotein subclass distribution controlled by hepatic lipase activity was strongly interlinked with gender and *LIPC* genotype. Therefore, on this basis, we speculate that gender interferes with dietary cholesterol induced HDL subclass responsiveness related to *LIPC rs6083* genotypes. *LIPF* gene regulates gastric lipase activity, which is involved in the digestion of fats, and specifically, *rs814628* in *LIPF* has been linked to obesity (48, 49). It is known that BMI and fat distribution may affect HDL particle size distribution (42, 50). Hence we assume that the effect of *LIPF rs814628* genotypes on HDL-subclass responsiveness to cholesterol and plant sterols is dependent on inter-individual differences in adiposity and BMI in the study participants. Further, reaffirming our assumption, dietary cholesterol and plant sterols could be seen to behave differently, by affecting large and small subclasses respectively in concert with *rs814628* genotype specific to G allele. Therefore, possessing the G allele for *rs814628* in *LIPF* appears to confer cardiovascular benefits by favorably modifying specific HDL subclasses in response to both dietary cholesterol and plant sterol intake. Notwithstanding this, due to lack of research in this aspect, it is difficult to ascertain whether *LIPF rs814628* genotypes are directly related to HDL metabolism or rather indicate the effect of another gene polymorphism involved in HDL metabolism.

Stearoyl-coenzyme A desaturase (SCD1), apart from its primary role in monounsaturated fatty acid biosynthesis, is also involved in lipid homeostasis, lipoprotein metabolism, HDL subclass allocation and RCT (51, 52). Chu et al. (51) using a *SCD1*$^{-/-}$ mouse model showed that SCD1 deficiency increases plasma HDL-C levels by activation of liver X receptor. Our observations on SCD *rs2234970* genotype oriented responses reflect the findings of Chu et al. (51). We hypothesize that, in the presence of C allele for *SCD rs2234970*, cholesterol supplementation may reduce SCD activity and consequently increase plasma HDL-C concentrations, especially that of the HDL-2 subclass (51). As such, due to limited human studies, it remains difficult to arrive at a specific conclusion with regards to the specific role of *rs2234970* on HDL metabolism. Nevertheless, our results strongly suggest that common variants in *SCD* gene may serve as potential candidates in determining plasma HDL-C responses to dietary interventions.

Another important genetically dependent influence of dietary cholesterol on HDL metabolism was observed from the interaction of treatment with the *SREBF1* gene. *SREBF1* gene encodes the transcription factors, sterol regulatory element–binding protein (SREBP)-1a and -1c, involved in cholesterol and fatty acid synthesis respectively (53). Polymorphisms in *SREBF1* locus have been linked with variations in plasma HDL-C levels (54). However, the specific contribution of *SREBF1 rs11868035* genotypes on plasma HDL-C responses and HDL subclass distribution remains to be elucidated. One possibility relates to the decreased expression of *SREBF1* induced by dietary cholesterol consumption in A allele carriers of the SNP *rs11868035* in order to maintain cholesterol homeostasis (55, 56, 57). Decreased *SREBF1* expression could lead to elevated plasma HDL-C and HDL-2 subclass concentrations (57). However, this hypothesis needs to be tested by gene-expression studies.

The present research was the first to examine parallel associations between various candidate SNPs and lipoprotein subclass responsiveness to dietary cholesterol versus plant sterol consumption. Another major strength was the Hutterite based study population, which minimizes environmental and lifestyle related confounders. The present research also had some limitations. Minimal control over dietary intake was imposed due to the free-living arrangement. Nevertheless, the limitation was minimized owing to the fact that Hutterites have a community dining system with homogeneous dietary intakes, except for individual variations in portion sizes. Secondly, the sample size (n=49) may not be optimal for testing gene-diet interactions.

In summary, consumption of dietary cholesterol increased plasma HDL-C levels and altered large HDL subclasses in a manner dependent on gender and common genetic variants within lipoprotein metabolic pathways in healthy individuals, while plant sterol consumption did not impact lipoprotein subclass distribution. Our data suggest that dietary cholesterol and plant sterols follow different mechanisms through which they modify plasma lipoprotein concentrations and subclass distribution. Such knowledge regarding the differential functionality and biological effects of dietary cholesterol in contrast to plant sterols helps in understanding their relation to CVD risk in order to develop meaningful dietary guidelines and policies regarding cholesterol and plant sterol consumption.

5.6 ACKNOWLEDGMENTS

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BRIDGE TO CHAPTER VI

Results from Chapter IV showed a large inter-individual variability in plasma lipid responsiveness driven by variations in endogenous cholesterol synthesis and absorption. Common genetic polymorphisms involved in cholesterol metabolism have been associated with such differences in responsiveness across individuals. However, the manner in which these common variants affect plasma lipid and plant sterol responses to dietary cholesterol consumption versus plant sterol consumption in relation to cholesterol kinetics is not thoroughly understood. After having demonstrated a genetically-propelled impact on lipoprotein subclass distribution in Chapter V, data from Chapter VI delineate the influence of gene-diet interactions on the differential modulation of plasma lipid, plant sterol and cholesterol kinetic responses to dietary cholesterol and plant sterol intake. Findings from Chapter VI suggest that the non-responsive genoset combination of *CYP7A1 rs3808607* TT/*APOE ε3* isoform of the study population could have resulted in the lack of plasma LDL-C lowering responsiveness to plant sterols. Also, the following chapter expands on the findings pertaining to the roles of *ACAT2 rs25683* and *NPC1L1 rs2073547* genotypes influencing plasma campesterol and β-sitosterol responses to plant sterol intake respectively, and their effects on cholesterol absorption.

CHAPTER VI

MANUSCRIPT 4

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GENE-DIET INTERACTIONS AFFECT PLASMA LIPID, PLANT STEROL RESPONSIVENESS TO DIETARY CHOLESTEROL VERSUS PLANT STEROL CONSUMPTION BY MODULATING CHOLESTEROL KINETICS IN A HUTTERITE FOUNDER POPULATION

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6.1 ABSTRACT

Lowering total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) remains the primary target in CVD risk reduction strategies. Variations in the magnitude to which cholesterol synthesis reciprocally interacts with changes in absorption affects the efficacy of therapeutic and dietary interventions in cholesterol management. Genetic heterogeneity has been attributed to the variability in responsiveness to dietary cholesterol and plant sterols in a general population. However, there is a paucity of research on the effect of common variants linked to differential responses to dietary cholesterol and plant sterols in Hutterites. The objective of the study was to examine the effects of common polymorphisms in candidate genes involved in cholesterol metabolism on plasma lipid and plant sterol responses to dietary cholesterol versus plant sterol consumption, in relation to cholesterol trafficking, using stable isotope assessment in healthy Hutterite individuals. A randomized, double-blind, crossover, placebo-controlled clinical trial (n=49) with 3 treatment phases of 4-week duration each separated by a 4-week washout was conducted in a Manitoba Hutterite population. During each phase, participants consumed one of the three treatments containing 600 mg/d dietary cholesterol, 2 g/d plant sterol or a placebo after breakfast meal. Plasma lipid profile was determined and cholesterol absorption and synthesis were measured by stable isotope techniques. Participants were genotyped for 38 candidate SNPs across 25 genes. Plasma HDL-C concentration responses were influenced by genotypes of *ABCA1 rs2066714* (p=0.0010) to both cholesterol and plant sterol intake, whereas genotypes of SNPs *CYP7A1 rs3808607* (p=0.0192), *LIPA rs1051338* (p=0.0055), *LSS rs2839158* (p=0.0212) and *SCD rs2234970* (p=0.0184) modified HDL-C concentration responses to dietary cholesterol alone. No association was found between any candidate SNP and plasma LDL-C responsiveness to dietary cholesterol nor plant sterols. *ACAT2 rs25683* and *NPC1L1 rs2073547* genotypes

influenced plant sterol induced responses of circulating campesterol (p=0.0355) and β-sitosterol (p=0.0084), respectively, and correspondingly altered cholesterol absorption. In summary, our results demonstrate that common genetic variants involved in cholesterol metabolism modulate plasma HDL-C and plant sterol responsiveness differentially to dietary cholesterol versus plant sterol consumption through individualized cholesterol trafficking mechanisms in Hutterite individuals. Preponderance of the non-responsive genoset combination of *CYP7A1 rs3808607* TT/*APOE* ε3 isoform of the study population evidently contributes to the lack of plasma LDL-C lowering responsiveness to plant sterols. Plasma campesterol and β-sitosterol responses to plant sterol intake associate with *ACAT2 rs25683* and *NPC1L1 rs2073547* respectively, affecting cholesterol absorption, without altering plasma LDL-C levels. Overall, our results demonstrate the polygenic control of variability in plasma lipid and plant sterol responsiveness to cholesterol versus plant sterols.

6.2 INTRODUCTION

Cardiovascular disease (CVD) persists as the leading cause of mortality worldwide, and by 2030, the number of deaths resultant from CVD has been projected to reach 23.3 million annually (1). Lowering total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) is held as the primary prevention approach for CVD risk reduction (2). The reciprocal interaction between cholesterol absorption and synthesis to maintain cholesterol homeostasis affects cholesterol management strategies (3, 4, 5, 6). In addition, variations exist in the magnitude to which cholesterol synthesis reacts to changes in absorption and vice-versa, in response to therapeutic, dietary or environmental stimuli (4, 6). Dietary cholesterol consumption has been shown to raise plasma cholesterol levels in hyper-responders, where the maintenance of plasma cholesterol homeostasis falters due to deregulated compensatory mechanisms of cholesterol absorption relative to synthesis (7, 8, 9). Notably, the proportion of hyper-responders is about one fourth of the whole population, while the rest have a normal response to cholesterol intake (8, 9). Previous studies have shown that different levels of cholesterol intakes result in either no effect (10) or an decrease in cholesterol synthesis (11, 3, 12), reaffirming the existence of substantial inter-individual variability in cholesterol trafficking mechanisms.

The efficacy of plant sterol intake in lowering plasma LDL-C reduction has been well-established (13, 14). Consumption of 2 g/d of plant sterols results in plasma LDL-C reductions of 5-15% (13). Plant sterols are structurally similar to cholesterol and the North American diet provides about 300-400 mg of plant sterols per day (15). Small intestinal absorption of plant sterols is less (<2%) compared to that of dietary cholesterol, which is absorbed with ~50% efficiency (16). Plant sterols exert their plasma LDL-C lowering effect by inhibiting cholesterol

absorption (13). However, the precise mechanism of action of plant sterols has remained

unresolved (17). Large inter-individual variability of plasma TC and LDL-C responsiveness to

plant sterols is usually seen in plant sterol intervention studies (18, 19, 20).Various inherent

physiological factors have been suggested for such variability and lack of LDL-C lowering

efficacy, including baseline plasma LDL-C concentrations of individuals (21), basal cholesterol

synthesis status (19, 20) and genetic heterogeneity (19, 22). Noteworthy, plant sterol intake

results in a compensatory increase in cholesterol synthesis in response to a reduction in

cholesterol absorption, but not necessarily always to a corresponding degree of equivalent

magnitude (13, 23, 24). Rudkowska et al. (24) reported that non-responders to plant sterols

display suppressed reductions in cholesterol absorption compared to responders, but with similar

magnitudes of reciprocal upregulation of cholesterol synthesis. These findings lend support to

the notion of plant sterol induced hyper-reactive increase in cholesterol synthesis corresponding

to a minimal reduction in absorption, leading to non-responsiveness only in a proportion of the

population. Genetic heterogeneity to a large extent explains such variability in plasma lipid

responsiveness to dietary sterols (22, 25, 6). Polymorphisms in *ABCG5/8* have been reported to

affect plasma lipid responses to dietary cholesterol (26, 27). The effect of *APOE* polymorphisms

on plasma lipids and cholesterol synthesis in response to cholesterol feeding has been previously

studied (28, 29). *CYP7A1* promoter polymorphism (A-278C) has been shown to affect and

modify plasma HDL-C responses to cholesterol consumption (30, 31). Mackay et al. (22)

demonstrated that *CYP7A1 rs3808607* and APOE polymorphisms work in concert to determine

plasma LDL-C responses to plant sterol interventions. Variations in *ABCG5/8* and *NPC1L1*

polymorphisms are linked to plasma LDL-C responses to plant sterol intake (32, 24). These

findings underscore the potential role of gene polymorphisms in modulating cholesterol

homeostasis and the need to examine their associations with phenotypic variations in responsiveness to cholesterol lowering interventions.

In spite of having only minor structural variations, the functionality, absorption and metabolism of cholesterol differs largely from that of plant sterols (33). Hence, investigating whether dietary cholesterol and plant sterols follow similar mechanisms through which they modulate plasma lipids in the context of genetic heterogeneity would offer valuable insights on the functional relation of these dietary sterols in cardiovascular health. Genetic isolates and founder populations have always been of great interest for genetic mapping studies with respect to Mendelian diseases, but are now being increasingly recognized towards understanding common diseases, due to reduced genetic and environmental heterogeneity (34). Amongst founder populations, the Hutterites are a genetically isolated population of European descent practicing a unique community lifestyle (35). Only one study has been conducted previously which evaluated the effects of plant sterol intervention in healthy Hutterites (23). Therefore, it is important to also question whether associations of common polymorphisms and plasma lipid responses to dietary sterols known in the general population, are also consistently observed in the Hutterite population. Hence, we hypothesized that dietary cholesterol and plant sterols would differentially modulate plasma lipids in association with common polymorphisms in genes controlling cholesterol metabolism in a healthy Hutterite population. To support such notion, the current study aimed to explore the effects of common polymorphisms in candidate genes controlling cholesterol metabolism on plasma lipid and plant sterol responses in relation to cholesterol trafficking using stable isotope assessment in healthy Hutterite individuals.

6.3 STUDY PARTICIPANTS AND METHODS

6.3.1 Participants

The study participant characteristics are discussed in Chapter IV, section 4.3.1 and table 4.1

6.3.2 Study design and protocol

The study design and protocol are elaborated in Chapter IV, section 4.3.3.

6.3.3 Blood collection and plasma lipid profile

Plasma lipid profile was determined by automated enzymatic methods on a Vitros-350 chemistry analyzer (Ortho-Clinical Diagnostics, Markham, ON, Canada). Low density lipoprotein cholesterol (LDL-C) levels were calculated by using the Friedewald equation (36).

6.3.4 Quantification of plasma plant sterols and non-cholesterol sterols

Plasma non-cholesterol sterols (NCS) were determined by using a gas-chromatograph equipped with a flame ionization detector (GC-FID) (Varian 450; Varian Canada Inc., Mississauga, ON, Canada) as described with some modifications in the protocol (13). Plasma samples were saponified using methanolic KOH after addition of an internal standard, 5-α-cholestane. Following this, the mixture was extracted for sterols twice with petroleum ether and then evaporated to dryness under nitrogen gas and resuspended in hexane. To the extract, 100µl of HMDS+TMCS+Pyridine (3:1:9) (Supelco) was added for trimethylsilyl (TMS) derivatization. NCS were determined by GC-FID in duplicate (1µl) injections. The injector and detector temperatures were set to 280°C and 300°C respectively. The column temperature programming

was done by initially setting the temperature to 130°C for 2 min, followed by a ramp to 270°C at 30°C/min and holding for 10 min, then ramping to 290°C at 10°C /min and holding for 9 min, then finally ramping to 320°C at 40°C/min and holding for 5 min. The carrier gas (helium) flow rate was 1.0 ml/min with the inlet splitter set at 22.9:1. Desmosterol, lathosterol, campesterol, and sitosterol were separated and expressed as ratios in µmol/L. Authenticated standards (Sigma-Aldrich Canada Ltd., Oakville, ON) were used for the identification of individual sterols and stanols.

6.3.5 Determination of cholesterol absorption

Cholesterol absorption was determined using the stable-isotope single tracer method (37). Red blood cell (RBC) fraction of blood collected on days 24, 25, 26 & 28 were used for the free cholesterol extraction as previously discussed in Chapter IV under section 4.3.6 (38).

6.3.6 Determination of cholesterol synthesis

Cholesterol fractional synthetic rates (FSR) (% per day) were calculated as the rate of incorporation of deuterium within the body water pool into the RBC cholesterol pools by measuring the deuterium RBC enrichment over 24 h as described in Chapter IV under section 4.3.7 (39). Cholesterol FSR was calculated using the method adopted from Jones et al. (12) and Myrie et al. (23).

6.3.7 DNA extraction and SNP genotyping

Genotyping procedures and methods used are described in Chapter V under section 5.3.5.

Genotyping of a set of 38 candidate SNPs across 25 different genes previously known to be

associated with cholesterol metabolism was performed on the study participant samples

(Chapter V, Table 5.1).

6.3.8 Statistical analysis

Statistical software, SAS version 9.2 (SAS Inc) was used for statistical analysis. Endpoint

measurements of the treatment and control phases were compared. Values were expressed as

least square means and standard error of the mean (SEM), unless otherwise stated. Effects of

dietary treatments were examined using a mixed model ANOVA procedure with participant ID

as a random factor and treatment as an independent factor followed by Tukey-Kramer

adjustments for multiple comparisons. The effect of treatment, sequence, and gender were

included in the model as fixed factors. Genotype and treatment by genotype were included as

fixed factors. Mixed model ANOVA procedure was used to analyze gene-diet interactions were

assessed, where genotype and gender were used as fixed effects, and Tukey-Kramer adjustments

were applied for multiple comparisons. Data are expressed as absolute changes from control in

response to dietary cholesterol and plant sterol treatments (Δ values). Statistical significance for

all analyses was set at $p < 0.05$.

6.4 RESULTS

6.4.1 Effects of dietary cholesterol and plant sterol consumption on plasma lipids, plasma plant sterols and non-cholesterol sterols

The influence of dietary cholesterol and plant sterol consumption on plasma lipids has been discussed elaborately in Chapter IV under section 4.4.2. Briefly, dietary cholesterol consumption of 600 mg/day for 4 weeks increased plasma total cholesterol (0.16±0.06 mmol/L, p=0.0179) and HDL-C (0.08±0.03 mmol/L, p=0.0216) concentrations compared to the control in the study population. Plant sterol consumption of 2 g/day did not result in any reductions in total cholesterol (p=0.3327) and LDL-C (p=0.3525) concentrations compared to the control. No changes in TG were found due to dietary cholesterol (p=0.9523) or plant sterol consumption (p=0.6525) compared to the control. Effects of dietary cholesterol and plant sterol consumption on plasma plant sterol concentrations is presented in **Table 6.1**. An increase in plasma campesterol (2.72±0.96 µmol/L, p=0.0157) and β-sitosterol (1.75±0.60 µmol/L, p=0.0122) concentrations and lathosterol to cholesterol ratio (0.46±0.18, p=0.0379) was seen following plant sterol consumption. No changes in campesterol, β-sitosterol concentrations and lathosterol to cholesterol ratio were observed due to cholesterol supplementation.

Table 6.1 Plasma non-cholesterol sterol responses to dietary cholesterol and plants sterol treatment after 4 weeks

Plasma non-cholesterol sterols	Control	Cholesterol	Plant sterols
Campesterol (µmol/L)	5.24±0.83[a]	4.79±0.82[a]	7.96±0.85[b]
Absolute change from control		-0.45±0.94	2.72±0.96
β-sitosterol (µmol/L)	3.38±0.44[a]	2.98±0.44[a]	5.29±0.45[b]
Absolute change from control		-0.40±0.57	1.88±0.58
Lathosterol (µmol/L)	2.87±0.77	3.17±0.78	4.62±0.77
Absolute change from control		0.29±0.99	1.74±1.01
Desmosterol (µmol/L)	3.61±0.53	2.07±0.54	3.64±0.53
Absolute change from control		-1.53±0.77	0.03±0.77
Lathosterol/cholesterol	0.47±0.14 [a]	0.43±0.14[a]	0.92±0.13[b]
Absolute change from control		-0.04±0.19	0.46±0.18

All values are least square means ± SEM (n=49). Differences between treatments were assessed by SAS mixed procedure ANOVA with Tukey-Kramer adjustments; $p < 0.05$ is considered significant. Values with different superscript letters are significantly different from each other. LDL, low density lipoprotein; HDL, high density lipoprotein.

6.4.2 Influence of genotype on response of plasma lipids to dietary cholesterol and plant sterol consumption

No genotype specific changes in plasma LDL-C concentrations were observed for any of the SNPs in response to cholesterol or plant sterol consumption. Candidate SNPs *ABCA1 rs2066714*, *CYP7A1 rs3808607*, *LIPA rs1051338*, *LSS rs2839158* and *SCD rs2234970* were associated with changes in plasma HDL-C concentrations in response to cholesterol and plant sterol consumption (**Table 6.2**). In *ABCA1 rs2066714* G/A heterozygotes, plasma HDL-C concentrations increased after dietary cholesterol consumption, whereas A/A homozygotes did not respond (ΔHDL-C (mmol/L): 0.31±0.08 for G/A vs. 0.07±0.03 for A/A). An identical association was seen with plant sterol consumption (ΔHDL-C (mmol/L): 0.25±0.08 for G/A vs. -0.03±0.03 for A/A) (**Table 6.2**). *CYP7A1 rs3808607* G/T heterozygotes increased plasma HDL-C concentrations following dietary cholesterol consumption, while T/T homozygotes did not respond (ΔHDL-C (mmol/L):0.29±0.09 for G/T vs. 0.07±0.03 for T/T). No effects of *rs3808607* genotypes on plasma lipids were observed after plant sterol consumption (**Table 6.2**).

Similarly, when stratified by *LIPA rs1051338* genotypes, cholesterol or plant supplementation did not affect C/A heterozygotes, while A/A homozygotes alone displayed an increase in plasma HDL-C concentrations post cholesterol consumption (ΔHDL-C (mmol/L): 0.22±0.06 for A/A vs. 0.05±0.03 for C/A) (**Table 6.2**). When stratified by *LSS rs2839158*, C/C homozygotes increased plasma HDL-C compared to C/T heterozygotes in response to dietary cholesterol consumption (ΔHDL-C (mmol/L): 0.18±0.02 for C/C vs. 0.06±0.02 for C/T) (**Table 6.2**). No effects of *rs2839158* genotypes on plasma lipids were noticed after plant sterol intake.

In *SCD rs2234970* C/C homozygotes, plasma HDL-C concentrations increased after dietary cholesterol consumption, whereas A/A or A/C genotype individuals did not respond (ΔHDL-C (mmol/L): 0.25±0.06 for C/C vs. 0.03±0.04 for A/A and 0.11±0.06 for A/C) (**Table 6.2**).

Only *LIPF rs814628* genotypes were associated with changes in plasma TG concentrations in relation to plant sterol consumption. *LIPF rs814628* A/G heterozygotes increased plasma TG concentrations following plant sterol supplementation, while A/A homozygotes did not (ΔTG (mmol/L): 0.38±0.14 for A/G vs. 0.06±0.05 for A/A) (**Table 6.2**). No effects of *rs814628* genotypes were observed after cholesterol supplementation.

6.4.3 Influence of genotype on response of plasma plant sterols and non-cholesterol sterols to dietary cholesterol and plant sterol consumption

In *ACAT2 rs25683* A/A and A/G genotype individuals, plasma campesterol concentrations increased following plant sterol consumption, while G/G homozygotes did not (Δcampesterol (µmol/L): 4.22±1.33 for A/A and A/G vs. 1.36±1.20 for G/G) (**Table 6.3**). *NPC1L1 rs2073547* T/T homozygotes alone increased plasma β-sitosterol concentration after plant sterol consumption, while T/C heterozygotes did not (Δβ-sitosterol (µmol/L): 3.33±0.75 for T/T vs. -0.32±0.89 for T/C) (**Table 6.3**).

Table 6.2 Single nucleotide polymorphisms associated with changes in plasma HDL-cholesterol and triglyceride concentrations following dietary cholesterol and plant sterol supplementation

Lipid variable	Gene SNP	Δ (mmol/L) after cholesterol supplementation*	Δ (mmol/L) after plant sterol supplementation*
	ABCA1	G/A=0.31±0.08, p=0.0033	G/A=0.25±0.08, p=0.0071
	rs2066714	A/A=0.07±0.03, p=0.1570	A/A=-0.03±0.03, p=0.8269
	CYP7A1	T/T=0.07±0.03, p=0.1830	T/T=-0.02±0.01, p=0.9617
	rs3808607	G/T=0.29±0.09, p=0.0186	G/T=0.17±0.08, p=0.2150
	LIPA	A/A=0.22±0.06, p=0.0013	A/A=0.15±0.06, p=0.1274
HDL-C	*rs1051338*	C/A=0.05±0.03, p=0.6796	C/A=-0.04±0.03, p=0.6897
	LSS	C/C=0.18±0.02, p=0.0048	C/C=0.05±0.02, p=0.5491
	rs2839158	C/T=0.06±0.02, p=0.9889	C/T=-0.10±0.02, p=0.2082
	SCD	A/A=0.03±0.04, p=0.9993	A/A=-0.05±0.04, p=0.8158
	rs2234970	A/C=0.11±0.06, p=0.5041	A/C=0.03±0.06, p=0.9967
		C/C=0.25±0.06, p=0.0013	C/C=0.10±0.06, p=0.6595

Table 6.2 continued

Lipid variable	Gene SNP	Δ (mmol/L) after cholesterol supplementation*	Δ (mmol/L) after plant sterol supplementation*
TG	*LIPF*	A/A=-0.001±0.05, p=0.9993	A/A=0.06±0.05, p=0.9103
	rs814628	A/G=0.02±0.14, p=0.9889	A/G=0.38±0.14, p=0.0214

*mixed model effects by genotype assessed with SAS mixed procedure ANOVA with Tukey-Kramer adjustments when treatment × genotype interaction was significant (statistical significance level set at $p<0.05$). All values are least square means ± SEM (n=49). Δ, absolute change of HDL-C or triglycerides concentration from control in mmol/L. *ABCA1*, ATP-binding cassette, sub-family A, member 1; *CYP7A1*, cholesterol 7 alpha-hydroxylase; *LIPA*, lysosomal acid lipase; LIPF, gastric lipase gene; *LSS*, lanosterol synthase; *SCD*, stearoyl-CoA desaturase HDL-C, high density lipoprotein cholesterol; TG, triglycerides.

Table 6.3 Single nucleotide polymorphisms associated with changes in plasma campesterol and β-sitosterol concentrations in response to dietary cholesterol and plant sterol consumption

Plant sterol	Gene SNP	Δ (µmol/L) after cholesterol supplementation*	Δ (µmol/L) after plant sterol supplementation*
Campesterol	*ACAT2* *rs25683*	A/A+A/G=2.09±1.27, p=0.5717 G/G=-2.59±1.25, p=0.3275	A/A+A/G=4.22±1.33, p=0.0243 G/G=1.36±1.20, p=0.8661
β-sitosterol	*NPC1L1* *rs2073547*	T/T=0.66±0.76, p=0.9583 T/C=-1.56±0.90, p=0.8937	T/T=3.33±0.75, p=0.0004 T/C=-0.32±0.89, p=0.9992

*mixed model effects by genotype assessed with SAS mixed procedure ANOVA with Tukey-Kramer adjustments when treatment × genotype interaction was significant (statistical significance level set at $p<0.05$). All values are least square means ± SEM (n=49). Δ, absolute change from control. *ACAT2*, acetyl-Coenzyme A acetyltransferase 2; *NPC1L1*, Niemann-pick C1-like 1 protein.
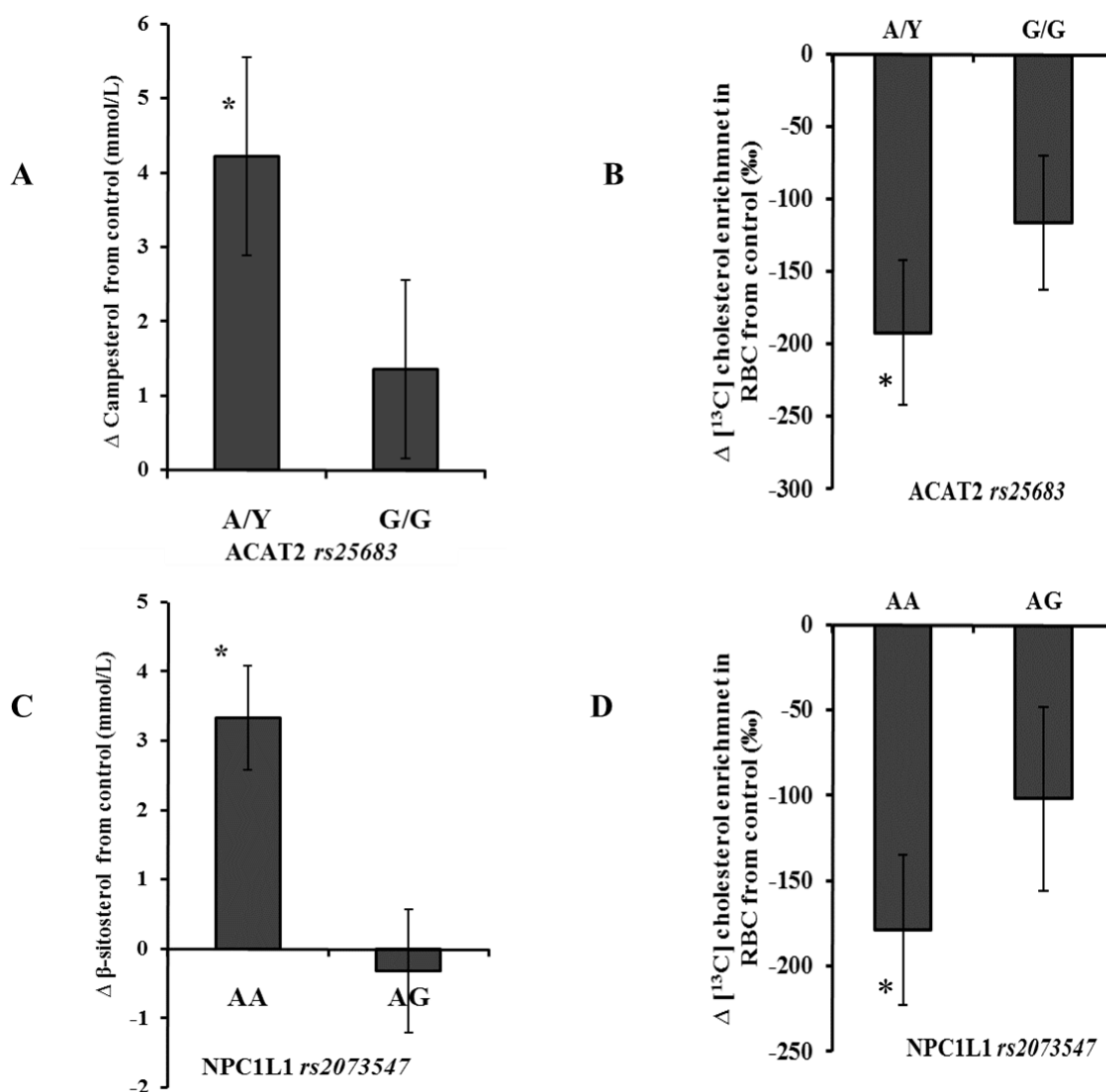
Figure 6.1 (A), Responses of plasma campesterol and (B), changes in cholesterol absorption to 4-week plant sterol consumption compared with 4-week control in participants stratified by *ACAT2 rs25683*. (C), Responses of plasma β-sitosterol and (D), changes in cholesterol absorption to 4-week plant sterol consumption compared with 4-week control in participants stratified by *NPC1L1 rs2073547*. Cholesterol absorption measured by area under the [3, 4]-^{13}C cholesterol RBC enrichment curve, 72 h post-^{13}C-cholesterol ingestion. *ACAT2*, acetyl-Coenzyme A acetyltransferase 2; *NPC1L1*, Niemann-pick C1-like 1 protein.

6.4.4 Impact of genotype on response of cholesterol absorption and synthesis to dietary cholesterol and plant sterol consumption

The effects of dietary cholesterol and plant sterol consumption on cholesterol absorption (area under the [3, 4]-^{13}C cholesterol RBC enrichment curve) and FSR (proportion of the rapidly turning over cholesterol pool synthesized per day) in our study participants have been discussed previously (Chapter IV). Briefly, cholesterol consumption of 600 mg/d for 4 weeks did not result in any changes in cholesterol absorption or FSR compared to the control. However, plant sterol consumption of 2 g/d for 4 weeks reduced cholesterol absorption (p=0.0004) and markedly increased cholesterol FSR (p=0.0026) compared to the control. No genotype and treatment interactions were found between any of the candidate SNPs and cholesterol absorption.

6.5 DISCUSSION

Our data suggest that common variants in genes involved in cholesterol metabolism modify plasma lipid and plant sterol responsiveness to dietary cholesterol and plant sterol supplementation by interacting with cholesterol trafficking mechanisms. Dietary cholesterol or plant sterol consumption did not result in any changes in plasma LDL-C concentrations in this group of Hutterites. The lack of plasma LDL-C lowering efficacy of plant sterols can be evidently linked to the predominant non-responsive genoset combination of *CYP7A1 rs3808607* TT allele and *APOE* ε3 isoform of the study population, as previously demonstrated by Mackay et al. (22). Genotypes of the SNPs *ABCA1 rs2066714*, *CYP7A1 rs3808607*, *LIPA rs1051338*, *LSS rs2839158* and *SCD rs2234970* influenced plasma HDL-C responsiveness to dietary cholesterol. Plasma campesterol and β-sitosterol responses to plant sterol intake were modulated

by *ACAT2 rs25683* and *NPC1L1 rs2073547* respectively, and correspondingly, altered cholesterol absorption, without affecting plasma LDL-C levels.

Various studies have previously shown that the majority of individuals do not exhibit any increase in plasma LDL-C concentrations after cholesterol consumption, suggesting variability in responsiveness to dietary cholesterol (40, 41, 42, 8, 9). However, we could not find any associations with candidate SNPs and plasma LDL-C changes in response to cholesterol consumption to explain the role of genetic variants in determining the responsiveness.

On the other hand, the absence of plasma LDL-C reductions due to plant sterol intake in our study participants was in contrast to the well-established plasma LDL-C lowering efficacy of plant sterols observed in various studies (13, 16, 23, 43, 44). Common variants in genes controlling cholesterol metabolism, especially *CYP7A1* and *APOE* polymorphisms have been increasingly linked to the responsiveness of plasma LDL-C to plant sterol consumption (19, 45, 22). However, we did not find any gene-diet interactions between plasma LDL-C concentrations and any of the candidate SNPs tested in our study population due to plant sterol consumption. Miettinen and Vanhanen (46) in their study involving patients with primary hypercholesterolemia reported an 8% LDL-C reduction in individuals with *APOE* ε4 allele following sitostanol supplementation. However, the LDL-C reductions were found to be insignificant in patients with *APOE* ε3 allele. On the other hand, De Castro-Oros et al. (45) demonstrated that G allele carriers of *CYP7A1 rs3808607* polymorphism exhibited enhanced LDL-C lowering responsiveness to plant sterol consumption compared to T/T homozygotes. *CYP7A1* gene encodes cholesterol 7α-hydroxylase, the rate limiting enzyme which plays a

crucial role in bile acid synthesis and cholesterol homeostasis (47, 22). G allele carriers have been shown to have increased *CYP7A1* gene expression compared to T/T homozygotes, which increases their bile acid synthesis and expands the bile acid pool and subsequently leads to increased cholesterol absorption (45). Mackay et al. (22) showed that *CYP7A1 rs3808607* G-allele and *APOE* ε4 were associated with increased plasma LDL-C lowering responsiveness to plant sterol consumption. The genotype distributions of common polymorphisms in the Hutterites have been reported to be different from that of the general population, especially *CYP7A1 rs3808607* and *APOE* variants (48, 49). Mackay et al. (22) demonstrated that individuals with the genoset combination of TT/*APOE* ε3 did not exhibit any plasma LDL-C lowering response after plant sterol consumption. Noteworthy, 71% (n=35) of our study participants possessed the combination of TT genotype for *CYP7A1 rs3808607* and *APOE* ε3 isoform. Hence, the lack of plasma LDL-C lowering due to plant sterol consumption can be attributed to the predominant non-responsive *CYP7A1 rs3808607* TT and *APOE* ε3 genoset in our study population. Another 10% (n=5) of the participants had the genoset combination of *CYP7A1 rs3808607* TT and *APOE* ε2, also a non-responsiveness genotype shown by Mackay et al (22). The other genoset combinations observed in the study population were *CYP7A1 rs3808607* G/T and *APOE* ε3 (10%; n=5), who showed moderate to low LDL-C lowering response to plant sterol consumption. There were no participants with *CYP7A1 rs3808607* G/- and *APOE* ε4 combination, and only 6% (n=3) had *CYP7A1 rs3808607* TT and *APOE* ε4 genotype. Only one participant presented with *CYP7A1 rs3808607* G/T and *APOE* ε2 genotype. Put together, our results reinforce the findings of Miettinen and Vanhanen (46), Mackay et al. (22) and De-Castros et al. (45) and corroborate the predictive role of *CYP7A1 rs3808607* and *APOE* polymorphisms in determining an individual's response to plant sterol intervention.

Unlike plant sterols, dietary cholesterol consumption increased plasma HDL-C concentrations in our study participants, whereas no changes in HDL-C were noticed due to plant sterol consumption. The plasma HDL-C raising effect of dietary cholesterol consumption has been demonstrated by other studies as well (50, 51, 52, 53). This clearly indicates that plant sterols and dietary cholesterol differentially modulate plasma lipids, possibly through different mechanisms. Plasma HDL-C responsiveness to dietary cholesterol consumption was associated with *ABCA1 rs2066714*, *CYP7A1 rs3808607*, *LIPA rs1051338*, *LSS rs2839158* and *SCD rs2234970* polymorphisms, and the SNP *ABCA1 rs2066714* alone showed an interactive effect with plasma HDL-C responsiveness and plant sterol consumption. Our results suggest that individuals carrying the C allele for *ABCA1 rs2066714* might benefit to a greater extent by consuming plant sterols and even dietary cholesterol consumption seems to be atheroprotective by raising HDL-C concentrations in these individuals. Such an effect has not been reported previously. ATP-binding cassette transporter A1 (ABCA1) is a transmembrane protein which plays a pivotal role in cellular cholesterol efflux and also orchestrates HDL metabolism and reverse cholesterol transport (54, 55). Common polymorphisms in *ABCA1* gene have been linked with altered plasma HDL-C concentrations (56, 57, 58). *ABCA1 rs2066714* is a nonsynonymous mutation associated with variability in plasma HDL-C concentrations (56, 59, 60). These studies and our results suggest that *ABCA1 rs2066714* polymorphism may potentially contribute to variability in plasma HDL-C responses due to dietary cholesterol and plant sterol supplementation.

Our observation of the increased plasma HDL-C response in G allele carriers of *CYP7A1 rs3808607* to cholesterol consumption concurs with the previous work of Hoffman et al. (31).

The authors reported that *CYP7A1 rs3808607* GG homozygotes displayed a larger increase (0.17±0.04 mmol/L) in plasma HDL-C compared to TT homozygotes (0.00±0.02 mmol/L) after consuming a cholesterol rich diet. Wang et al. (30) observed that men homozygous for the G allele display significantly higher plasma HDL-C concentrations. Machleder et al. (61) using mouse models suggested that a genetic link involving *CYP7A1* gene exists between bile acid metabolism and HDL metabolism. However, at present, the exact mechanism through which *CYP7A1 rs3808607* modulates dietary cholesterol induced plasma HDL-C responses remains unclear. Nevertheless, our results suggest that *CYP7A1* polymorphism may influence dietary cholesterol and plant sterol responsiveness via separate mechanisms.

Our findings report for the first time an association of *LIPA rs1051338* with dietary cholesterol induced HDL-C responsiveness. Lysosomal acid lipase (LIPA) plays a critical role in triglyceride and cholesterol ester hydrolysis and is also involved in the regulation of intracellular cholesterol processes (62, 63). Two rare genetic disorders related to cholesterol metabolism, Wolman disease and cholesterol ester storage disorder are known to be linked with LIPA activity (64). Common polymorphisms in *LIPA* gene have been previously associated with plasma HDL-C levels (63). Results from our study suggest that *LIPA rs1051338* may potentially contribute to the inter-individual variability in plasma HDL-C concentrations due to dietary cholesterol consumption.

Similarly, we also report the potential contribution of *LSS rs2839158* polymorphism in influencing plasma HDL-C responsiveness to dietary cholesterol intake. Lanosterol synthase (LSS) is a key enzyme involved in cholesterol biosynthesis pathway and changes in the activity

of the enzyme could modulate circulating cholesterol concentrations (65, 66). However, the precise role of the *LSS* polymorphism in modulating HDL-C levels is unclear due to a paucity of available research. Future studies directed towards scrutinizing this association will help validate our findings.

We observed a novel allelic dose effect of the SNP *rs2234970* in *SCD* gene with plasma HDL-C responsiveness to dietary cholesterol. Stearoyl-coenzyme A desaturase regulates monounsaturated fatty acid (MUFA) biosynthesis and is also involved in lipid homeostasis, lipoprotein metabolism, HDL subclass allocation and reverse cholesterol transport (67, 68). SCD deficiency has been linked to elevated plasma HDL-C levels (67). However, the polymorphism *rs2234970* in *SCD* gene has never been previously shown to modify the dietary influence of plasma HDL-C responses. Landau et al. (69) showed that cholesterol feeding in mice influenced hepatic SCD activity and suggested an indirect regulatory effect on HDL-C levels. More research is needed to evaluate the clinical relevance of *SCD rs2234970* mediated effects of dietary cholesterol on plasma HDL-C levels. Put together, results from our study and findings of others point to a possible link between *SCD rs2234970* and variability in plasma HDL-C responsiveness to dietary cholesterol.

ACAT2 rs25683 genotypes modulated the response of plasma campesterol concentrations to plant sterol intake in our study population. Acetyl-coenzyme A acetyltransferase 2 (ACAT2) serves to esterify cholesterol into cholesteryl esters for packaging into the neutral lipid core of nascent chylomicrons and controls the selective absorption of dietary sterols (70, 71). Carriers of *ACAT2 rs25683* A allele displayed increased plasma campesterol concentrations due to plant

sterol consumption in our study. Correspondingly, carriers of A allele alone displayed significant reductions in cholesterol absorption due to plant sterol consumption (**Figure 6.1B**). These findings indicate that *rs25683* polymorphism in *ACAT2* gene potentially modulates selective plant sterol absorption in the intestine and thereby affects cholesterol absorption.

A similar effect was observed with *NPC1L1 rs2073547* and β-sitosterol to plant sterol intake (**Figure 6.1D**). Niemann-Pick C1 Like 1 (NPC1L1) protein is a key intestinal cholesterol and plant sterol transporter and plays a crucial role in cholesterol homeostasis (72). Non-synonymous mutations in *NPC1L1* have been linked with NPC1L1 expression and function, influencing cholesterol absorption and plasma LDL-C levels (73, 74). Jesch et al. (75) demonstrated the repression of intestinal *NPC1L1* mRNA expression by β-sitosterol, suggesting a new mechanism through which plant sterol consumption could likely inhibit intestinal cholesterol absorption. These studies support our findings and evince the possible role of *NPC1L1 rs2073547* mediated plant sterol uptake and effect on cholesterol absorption responsiveness. However, in both the cases of *ACAT2 rs25683* and *NPC1L1 rs2073547*, no significant changes in plasma LDL-C responses between the genotypes were observed, which suggests that other overriding mechanisms exist which could have offset the effects of these SNPs on cholesterol absorption controlled via intestinal plant sterol uptake.

Examining the associations of 38 candidate SNPs with plasma lipid and plant sterol responsiveness in relation to cholesterol trafficking to dietary sterol consumption in healthy individuals was a major strength of the study. The double-blind randomized crossover placebo-controlled clinical trial design with 4 weeks of dietary intervention was another major strength of

our study. Noteworthy, our study was conducted in a founder Hutterite population, which enabled us to minimize environmental and lifestyle related variabilities. Nevertheless, our study did have certain limitations. The study was free-living and minimal control over dietary intake was exerted. However, as mentioned previously, Hutterites practice a community dining system with homogeneous dietary intake patterns, which potentially averts dietary interferences affecting the study's objectives. The sample size (n=49) may not be optimal when it comes to testing for gene-diet interactions. Most of the candidate SNPs tested lacked sufficient number of individuals (<5%) falling into all three genotypes for a given SNP. This was expected as the Hutterites are a genetically isolated founder population and the genotype distributions and frequency of common polymorphisms in Hutterites are quite different from that of the general population (48, 49). Hence a large sample size is needed in future studies to avert such shortcomings.

In summary, our results demonstrate that common genetic polymorphisms involved in cholesterol metabolism modulate plasma HDL-C and plant sterol responses differentially to cholesterol versus plant sterol consumption, by mediating cholesterol trafficking mechanisms in healthy Hutterite individuals. The lack of plasma LDL-C lowering efficacy of plant sterols in our study can be clearly evinced to the predominant non-responsive genoset combination of *CYP7A1 rs3808607* TT genotype and *APOE* ε3 isoform of the study population (22). Also, dietary cholesterol induced responsiveness of plasma HDL-C was modulated by *ABCA1 rs2066714*, *CYP7A1 rs3808607*, *LIPA rs1051338*, *LSS rs2839158* and *SCD rs2234970*. Plasma campesterol and β-sitosterol responses to plant sterol consumption were associated with *ACAT2 rs25683* and *NPC1L1 rs2073547* respectively, and correspondingly, altered cholesterol absorption, without

any impact on plasma LDL-C levels. Overall, our findings emphasize the importance of understanding genetic heterogeneity of responsiveness to dietary sterols. Such prognostic knowledge will enable the development of efficacious and personalized cholesterol management strategies.

6.6 ACKNOWLEDGMENTS

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GENERAL DISCUSSION

7. 1 SUMMARY AND IMPLICATIONS

The findings of the studies presented in this thesis bear important implications for the management of plasma cholesterol and cardiovascular disease (CVD). Plasma total cholesterol (TC) and LDL-cholesterol (LDL-C) are established risk factors of CVD (1), and lowering their concentrations represents the main target for the control and prevention of CVD risk and ailments. A reduction of 10% plasma TC concentrations has been associated with 20-50% lowered risk of coronary heart disease (CHD) and CHD-related morbidity and mortality over the long term (2). Likewise, a 10% reduction in LDL-C concentrations translates to a 20% reduction in cardiovascular events (2). Also, it is evident that plasma HDL-C concentrations are inversely linked to CVD risk (3, 4, 5). Analyzing LDL and HDL particle size and subclass distribution serves to reinforce the CVD risk estimating power of plasma LDL-C and HDL-C measurements and helps in monitoring the efficacy of therapeutic or dietary interventions in improving the lipid profile (6, 7, 8). Diet and dietary components play a major role in influencing plasma cholesterol concentrations. Dietary cholesterol and plant sterol intake affect plasma TC and LDL-C, although differentially, by modulating the interaction between cholesterol absorption and synthesis (5, 9, 10). Dietary recommendations pertaining to the intake of cholesterol in healthy individuals have been called into question due to the fact that dietary cholesterol consumption does not unfavorably alter plasma lipid profile in the majority of the population, and in hyper-responders, who constitute only about one fourth of the entire population, an elevation in plasma

TC is seen (11, 12). In most cases, the maintenance of LDL-C: HDL-C ratio is seen in these hyper-responders, with an increase in large LDL and HDL particles following higher intakes of dietary cholesterol (13, 14, 15). Data from the present research demonstrate that dietary cholesterol consumption modifies plasma cholesterol concentrations, but not unfavorably, and possibly does not confer atherogenic risk. Also, the impact dietary cholesterol consumption bears on the rates of cholesterol absorption and synthesis appear to be very modest.

Plant sterols are known to substantially reduce plasma LDL-C concentrations by interfering with cholesterol absorption, and resulting in a compensatory increase in cholesterol synthesis (16, 17, 18). However, such increase in synthesis does not always correspond in magnitude equivalent to the reduction in absorption (10, 19). This reciprocal interaction has important implications in the efficacy of plant sterols in cholesterol management, since non-compensatory increases in cholesterol synthesis in response to a reduction in absorption could obfuscate the beneficial effects of plant sterols in improving plasma lipid profile. The findings of the present research demonstrate a hyper-reactive increase in cholesterol synthesis corresponding to a modest reduction in absorption due to plant sterol consumption, leading to a lack of plasma LDL-C reduction in the study population. Given the fact that dietary cholesterol and plant sterols modulate plasma lipid responsiveness differentially in a manner dependent on the interaction between cholesterol synthesis and absorption, the observations in Chapter IV, using stable isotope techniques, address the gap in research on the reciprocal interaction and underscore the importance of the relationship in cholesterol management.

Inter-individual variability is commonly seen in the plasma LDL-C responsiveness to dietary cholesterol and plant sterols (20, 13, 21, 22). The observations from Chapter IV of this thesis demonstrate a range of changes in plasma LDL-C concentrations contributed largely by the inter-individual variability in endogenous cholesterol synthesis due to dietary cholesterol and plant sterol consumption. Apart from factors such as baseline plasma LDL-C levels (23), basal cholesterol synthesis status (21, 22) affecting responsiveness, genetic heterogeneity is considered to predominantly influence responsiveness and contribute to a large extent to the variability. Chapter VI presents data demonstrating associations of plasma lipid and plant sterol responsiveness with common variants in candidate genes involved in cholesterol metabolism in relation to changes in cholesterol synthesis and absorption. Novel associations between variants in genes such as *ABCA1*, *CYP7A1*, *LIPA*, *LSS* and *SCD* and changes in plasma HDL-C responses due to dietary cholesterol consumption underscore the role of these polymorphisms in determining dietary responses, and their implications in CVD risk management. The findings of the present research clearly show that the genoset combination involving *CYP7A1* and *APOE* polymorphisms strongly influence the responsiveness to LDL-C lowering efficacy of plant sterol intervention, in agreement with the observations from previous studies (24, 25). Data from Chapter VI therefore indicate a potential cross-talk between *CYP7A1* and *APOE* genoset and cholesterol synthesis pathway. Also, data from Chapter VII delineate the control of plasma plant sterol responses of two major plant sterols, campesterol and β-sitosterol by *ACAT2* and *NPC1L1* gene variants respectively, by interacting with cholesterol absorption. These research outcomes enhance our understanding on the function, absorption and metabolism of dietary cholesterol and plant sterols in alignment with the genetic heterogeneity. Put together with Chapter IV, data from Chapter VI show that specific polymorphisms control plasma LDL-C responsiveness of dietary

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sterols and can serve as predictive tools for administering optimal and personalized cholesterol management interventions based on an individual's genetic makeup.

Data from Chapter V demonstrate the effect of dietary cholesterol and plant sterols on LDL and HDL subclass distribution in association with common genetic variants in candidate genes in the lipoprotein and cholesterol metabolic pathways. The findings have important implications in terms of the differential effects of dietary cholesterol and plant sterols in modulating the lipoprotein distribution. Data from the present research evidently demonstrates that dietary cholesterol consumption increases plasma HDL-C and large HDL subclasses more prominently in women than in men. Large HDL subclasses are inversely linked to CVD risk (3, 4). Also, the LDL particle size increases following cholesterol consumption, specifically increasing large buoyant non-atherogenic LDL particles. The findings in Chapter V are in agreement with previous reports (3, 4, 26) and indicate that cholesterol consumption could be more beneficial to women, and not harmful for men in relation to CVD, by inducing elevation of only plasma large LDL subclass enrichment. On the other hand, findings of the present research also evince that plant sterols do not influence lipoprotein subclass distribution, regardless of their plasma LDL-C lowering effects, concurring with the observations of previous research studies (27, 28, 29, 9). Hence, plant sterols may act in modifying plasma lipids in a manner different to that of dietary cholesterol, irrespective of the similarity in chemical structure. Some studies advocate that plant sterols in fact may increase atherogenic risk (30, 31). The observations from Chapter V show that plant sterol consumption does not change lipoprotein subclass distribution, especially the small LDL and HDL subclasses, and therefore less likely to increase CVD risk. The observations in Chapter V pertaining to genetic variants and their associations with lipoprotein subclass

distribution address the research gap regarding the role of genetic variants affecting lipoprotein

subclass distribution in response to dietary sterols. Data from the Chapter V show novel

associations of changes in large HDL subclasses and their relative enrichment with common

polymorphisms in *ABCA1*, *SCD*, *SREBF1*, *LIPF* and *LIPC* genes. The findings of the study

broaden our understanding with regards to the role of genetics influencing inter-individual

variability in lipoprotein particle size distribution in response to dietary sterols. Such knowledge

will help in establishing population-wide recommendations for dietary cholesterol and plant

sterol consumption for cholesterol management and CVD risk. Overall, the findings herein

suggest that dietary cholesterol consumption has a very modest impact on cholesterol kinetics,

and may not unfavorably modify CVD risk biomarkers, while the LDL-C lowering efficacy of

plant sterol consumption depends on the reciprocal interaction between endogenous cholesterol

synthesis and absorption, with common genetic variants possibly influencing plasma lipid

responsiveness. The findings thus emphasize the importance of understanding genetic

heterogeneity of responsiveness to dietary sterols. Such prognostic knowledge will enable the

development of efficacious and personalized cholesterol management strategies.

7.2 LIMITATIONS AND FUTURE DIRECTIONS

Certain limitations are acknowledged to exist in the present research, and possibly could have

resulted in type I (false positive) and type II (false negative) errors. One of the limitations is that

the present research utilized a free-living design, to enable testing the hypotheses in a real-life

scenario. But often, the free-living design is not considered the "gold standard method" for

examining the health benefits of dietary interventions, as opposed to a fully-controlled design,

which ensures more precise conclusions (32). However, as an advantage, the Hutterites have a

community dining system with all individuals in the population consuming typically the same diet, except for individual variations in caloric intake. This in fact enabled the present research to conduct the dietary intervention to the level of a fully-controlled feeding design. Yet, it is still not possible to closely supervise dietary intakes ensuring consistency in intake and monitor deviation from the advised dietary intake patterns throughout the study period. Also, the dietary composition and patterns of intake in Hutterites may vary from that of the general population limiting the generalizability of the findings. Hence in future studies, fully-controlled dietary interventions, where all the meals and study treatment products are prepared and administered by the research personnel, and consumption of the study treatments are supervised closely, would furnish more accurate research conclusions.

Secondly, the sample size (n=49) may not be optimal when it comes to testing for gene-diet interactions. Although, the population size is similar to most similar dietary intervention studies (24, 18, 22), and many findings related to gene-diet interactions replicated previous findings, the sample size can be still regarded small when compared to genome-wide association studies (33, 34). Inter-related to this, the present research with Hutterite participants encountered a unique limitation, usually not observed in studies conducted in a general population with similar sample size. Most of the candidate single nucleotide polymorphisms (SNPs) tested lacked sufficient number of individuals (<5%) falling into all three genotypes for a given SNP, despite preselecting candidate SNPs with a MAF ≥0.06. This was expected as the Hutterites are a genetically isolated founder population and the genotype distributions and frequency of common polymorphisms in Hutterites are quite different from that of the general population (35). As a result, the findings pertaining to novel associations from the present research would require

replication in future full-feeding dietary intervention studies powered with large sample sizes, which will eliminate such shortcomings.

Thirdly, in context with the limitation above, the present research also poses another limitation, related to the challenges in extrapolating the findings from the Hutterite population to the general population. Examining the effects of dietary sterols and their implications in a founder population like the Hutterites using a gene-diet approach provides a more homogeneous testing environment with respect to lifestyle, genetics, environment and dietary patterns, facilitating robust research outcomes (36). Nevertheless, it has to be acknowledged that the community living, dietary pattern and the genetic makeup of the Hutterites are strikingly dissimilar from that of the general population, where large heterogeneity in life-style related factors could affect health outcomes. Notably, CVD is a complex condition with multifactorial etiology and knowledge of an individual's genetic makeup still cannot accurately predict the onset and disease progression (37). CVD develops as a result of an interaction between an individual's genotype and environment factors (37). Hence, generalizing the findings of the present research is limited by the study population. Also, comparing the effects of the dietary interventions in Hutterites to the general population is limited by the sample size of the present research. Future studies with large population sizes, comparing the effects of dietary sterols in Hutterites with that of the general population will lead to valuable information on the complex etiology of CVD.

Finally, taking into account that a candidate gene approach was adopted, only a limited number of systematically-chosen SNPs within a selected assortment of genes involved in cholesterol and lipoprotein metabolism were studied in the present research. However, failure to notice an

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association with these SNPs does not preclude the possibility that other SNPs in the candidate genes affect cholesterol and lipoprotein metabolic pathways upon consumption of dietary sterols. Future studies can use next-generation DNA sequencing (38), to analyze the entire genes of interest for associations (39), and machine learning approaches (40) to obviate such potential type II errors. These approaches would help unravelling and understanding the manner in which multiple SNPs in the same gene or SNPs of different non-related genes (epistasis) interact with each other in modulating responsiveness to dietary components.

Considering these limitations, future dietary intervention studies with fully-controlled design need to adopt a priori selection and recruitment of a large sample size of carriers of genotypes known to be associated with hypo and hyper-responsiveness to dietary sterols. Such studies would enable thorough understanding of genetic heterogeneity to plasma lipid responsiveness to dietary cholesterol and plant sterol consumption.

7.3 FINAL CONCLUSIONS

The burgeoning prevalence of CVD and its associated morbidity and mortality is a major concern worldwide. Improving the lipid profile through dietary and therapeutic interventions, specifically aimed at decreasing plasma TC and LDL-C, and increasing HDL-C concentrations offers substantial reduction of CVD risk (1, 2). Also, the prognostic values of these CVD risk biomarkers in monitoring the efficacy of therapeutic or dietary interventions are reinforced by incorporating LDL and HDL particle size measures and by analyzing subclass distribution (6, 7, 8). Noteworthy, the plasma levels of the biomarkers and their relative distribution heavily depends on the reciprocal interaction between endogenous cholesterol absorption and synthesis,

and the manner in which each mechanism drives one another (10). Dietary components play a vital role in modulating plasma cholesterol concentrations. Specifically, dietary sterols such as dietary cholesterol and plant sterol intake affect plasma TC and LDL-C, by inflecting the interaction between cholesterol absorption and synthesis (16, 10). Dietary recommendations related to the intake of these dietary sterols in a healthy population are profoundly influenced by the inter-individual variabilities in endogenous mechanisms. Hypo and hyper-responsiveness to dietary sterols with respect to plasma lipids are generally observed in most studies investigating the effect of the sterols in cholesterol management (13, 14, 21).

Genetics play a huge role in modulating the responses to the dietary sterols and underscores the importance of personalized nutrition. The research work encompassed within this thesis provides new evidence in regards to the impact of dietary cholesterol and plant sterol consumption on plasma lipid biomarkers of cardiovascular health in relation to gene-diet interactions. By using stable isotopic techniques and lipoprotein particle size distribution analyses and by examining gene-diet interactions, the studies comprising this thesis reveal the crucial roles of genetic heterogeneity and variations in endogenous cholesterol metabolism in determining the response of plasma lipid biomarkers to dietary sterols. Specifically, the present research discriminates the effects of dietary cholesterol and plant sterols on plasma lipid responsiveness by demonstrating how differently they affect the rates of cholesterol absorption and synthesis, and how different genetic variants control these mechanisms. The findings of the present research also reinforce the valuable prognostic information that could be obtained by estimating lipoprotein particle size and distribution. Therefore, the observations from this thesis serve to highlight the important implications of inter-individual variability in responsiveness to dietary sterols and its association

with the genomic architecture of an individual. Given the complex nature of CVD and the challenges that exist in cholesterol management and CVD risk reduction through dietary manipulation, the current thesis findings represent an important step in advancing knowledge towards personalized nutrition. In the longer term, measuring lipoprotein subclass particles together with the use of genetic markers for risk assessment in hypo and hyper-responders to dietary cholesterol and plant sterols will lead to an advanced understanding of the pathophysiological processes concerning CVD, and the effective management of health and economic burdens related to the pandemic.

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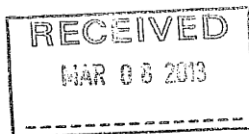40. Libbrecht MW, Noble WS (2015) Machine learning applications in genetics and genomics. Nat Rev Genet 16: 321-332.

APPENDICES

APPENDIX I

ETHICS APPROVAL FOR STUDIES CORRESPONDING TO CHAPTERS IV, V & VI

RECEIVED

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OF MANITOBA | Research Ethics Boards

BIOMEDICAL RESEARCH ETHICS BOARD (BREB)
CERTIFICATE OF FINAL APPROVAL FOR NEW STUDIES
Full Board Review

PRINCIPAL INVESTIGATOR:	INSTITUTION/DEPARTMENT:	ETHICS #:
Dr. P. Jones	UofM / RCFFN	B2013:019
BREB MEETING DATE:	APPROVAL DATE:	EXPIRY DATE:
February 25, 2013	March 6, 2013	February 25, 2014
STUDENT PRINCIPAL INVESTIGATOR SUPERVISOR (If applicable): ᴧᴧ		

PROTOCOL NUMBER:	PROJECT OR PROTOCOL TITLE:
NA	Effect of a Plant Sterol-Fortified Low-Fat Milk Product on Plasma Lipid Levels of Humans in Relation to Different Infant Feeding Practices and Later Life Cholesterol Metabolism
SPONSORING AGENCIES AND/OR COORDINATING GROUPS:	
Dairy Farmers of Canada	

Submission Date(s) of Investigator Documents:	REB Receipt Date(s) of Documents:
February 11 and March 6, 2013	February 11 and March 6, 2013

THE FOLLOWING ARE APPROVED FOR USE:

Document Name	Version(if applicable)	Date
Protocol:		
Protocol		March 6, 2013
Consent and Assent Form(s):		
Research,Participant Information and Consent Form		March 6, 2013
Other:		
Screening Form		02/11/2013
3 Day Food Diary received February 11, 2013		
Advertisement		02/11/2013

CERTIFICATION
The University of Manitoba (UM) Biomedical Research Board (BREB) has reviewed the research study/project named on this *Certificate of Final Approval* at the *full board meeting* date noted above and was found to be acceptable on ethical grounds for research involving human participants. The study/project and documents listed above was granted final approval by the Chair or Acting Chair, UM BREB.
BREB ATTESTATION
The University of Manitoba (UM) Biomedical Research Board (BREB) is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement 2, and the applicable laws and regulations of Manitoba. In respect to clinical trials, the BREB complies with the membership requirements for Research Ethics Boards defined in

- 1 -

Division 5 of the Food and Drug Regulations of Canada and carries out its functions in a manner consistent with Good Clinical Practices.

QUALITY ASSURANCE

The University of Manitoba Research Quality Management Office may request to review research documentation from this research study/project to demonstrate compliance with this approved protocol and the University of Manitoba Policy on the Ethics of Research Involving Humans.

CONDITIONS OF APPROVAL:

1. The study is acceptable on scientific and ethical grounds for the ethics of human use only. *For logistics of performing the study, approval must be sought from the relevant institution(s).*
2. This research study/project is to be conducted by the local principal investigator listed on this certificate of approval.
3. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to the research study/project, and for ensuring that the authorized research is carried out according to governing law.
4. **This approval is valid until the expiry date noted on this certificate of approval.** A Bannatyne Campus Annual **Study Status Report** must be submitted to the REB within 15-30 days of this expiry date.
5. Any changes of the protocol (including recruitment procedures, etc.), informed consent form(s) or documents must be reported to the BREB for consideration in advance of implementation of such changes on the **Bannatyne Campus Research Amendment Form.**
6. Adverse events and unanticipated problems must be reported to the REB as per Bannatyne Campus Research Boards Standard Operating procedures.
7. The UM BREB must be notified regarding discontinuation or study/project closure on the **Bannatyne Campus Final Study Status Report**.
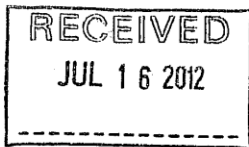

Sincerely,
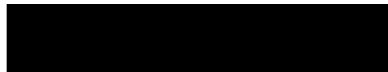
Lindsay Nicolle, MD, FRCPC
Chair, Biomedical Research Ethics Board
Bannatyne Campus

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BIOMEDICAL RESEARCH ETHICS BOARD (BREB)
CERTIFICATE OF FINAL APPROVAL FOR AMENDMENTS AND ADDENDUMS

PRINCIPAL INVESTIGATOR:	INSTITUTION/DEPARTMENT:	ETHICS #:	
Dr. P. Jones	UofM/RCFFN	B2013:019	
BREB MEETING DATE (If applicable):		APPROVAL DATE: July 8, 2013	
STUDENT PRINCIPAL INVESTIGATOR SUPERVISOR (If applicable):			

PROTOCOL NUMBER:	PROJECT OR PROTOCOL TITLE:
NA	Effect of a Plant Sterol-Fortified Low-Fat Milk Product on Plasma Lipid Levels of Humans in Relation to Different Infant Feeding Practices and Later Life Cholesterol Metabolism
SPONSORING AGENCIES AND/OR COORDINATING GROUPS:	
Dairy Farmers of Canada	

REMINDER: THE CURRENT BREB APPROVAL FOR THIS STUDY EXPIRES: February 24, 2014

REVIEW CATEGORY OF AMENDMENT:	Full Board Review ☐	Delegated Review ☒
Submission Date of Investigator Documents: June 26, 2013	BREB receipt date of Documents: June 27, 2013	

THE FOLLOWING AMENDMENT(S) and DOCUMENTS ARE APPROVED FOR USE:

Document Name	Version(if applicable)	Date

Protocol:
Protocol Amendment received June 26, 2013
Consent and Assent Form(s):
Research Participant Information and Consent Form - Genetic Analysis   June 26, 2013

Other:

CERTIFICATION
The University of Manitoba (UM) Biomedical Research Board (BREB) has reviewed the amendment to the research study/project named on this *Certificate of Approval* as per the category of review listed above and was found to be acceptable on ethical grounds for research involving human participants. The amendment and documents listed above were granted final approval by the Chair or Acting Chair, UM BREB.
BREB ATTESTATION
The University of Manitoba (UM) Biomedical Research Board (BREB) is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement 2, and the applicable laws and regulation of Manitoba. In respect to clinical trials, the BREB complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations of Canada and carries out its functions in a manner consistent with Good Clinical Practices.

QUALITY ASSURANCE

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APPENDIX II

FORMS CORRESPONDING TO STUDIES DESCRIBED IN CHAPTERS IV, V, & VI

Study Advertisement – Poster

Participant Consent form corresponding to studies described in Chapters IV, V & VII

UNIVERSITY OF MANITOBA

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

Title of Study: Effect of a Plant Sterol-Fortified Low-Fat Milk Product on Plasma Lipid levels of Humans in Relation to Different Infant Feeding Practices and Later Life Cholesterol Metabolism

Investigator: Peter Jones, PhD

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Montreal, QC H3A 2N4
Phone: (514) 284-1092

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family. This consent form may contain words that you do not understand. Please ask the study staff to explain any words or information that you do not clearly understand.

Purpose of study
The purpose of the study is to evaluate the lipid-lowering efficacy and safety of consumption of a plant sterol (PS) formulation compared to a placebo product and a cholesterol enriched diet. The study also attempts to identify the effects of breast feeding and its duration on later life lipid metabolism. It has been shown that the ingestion of plant sterols result in a favorable modification of lipid profiles. Therefore, it is anticipated that consumption of these plant sterol enriched dairy products will improve lipid profile, as well as other health-related markers.

Study procedures
The study requires you to provide us information on the type and duration of your early feeding during infancy. If you agree to take part in this study, as part of a pre-screening visit, you will be asked to have a fasting (nothing to eat or drink 12 hours before the test) blood sample of approximately two teaspoons taken to measure your blood fat levels. If you meet eligibility

Effect of a Plant Sterol-Fortified Low-Fat Milk Product on Plasma Lipid levels of Humans in Relation to Different Infant Feeding Practices and Later Life Cholesterol Metabolism

requirements, you will be invited back for further screening where a fasting blood sample of four teaspoons will be taken to do a complete blood count and biochemistry profile. All baseline values must be normal as verified by the study investigators prior to enrollment in the study and any abnormality in tests performed at screening will result in exclusion. Pregnancy tests will be performed for all pre-menopausal female participants at screening visits and at the beginning of each phase, if the test is positive at screening or during the study they will be asked to stop taking study treatment immediately and be withdrawn from the study. Any change in your health status at any point during the study needs to be reported to the study investigators.

The study will consist of 3 phases of 30 days during which you will consume your supper time meal along with your assigned treatment under supervision. You will follow your normal eating routine for the other 2 meals of the day. We ask that you limit your consumption of alcohol and caffeinated beverages throughout the phases. At the end of each phase, a washout period of 4 weeks will be followed during which you will consume your habitual diets. The 3 phases of treatments will include:

1) Study meal with placebo (240 ml of 2% milk shake; 50 mg cholesterol).
2) Study meal with cholesterol enrichment (240 ml of 2% milk shake; 600 mg cholesterol).
3) Study meal with plant sterols formulation (240 ml of 2% milk shake; 50 mg cholesterol). The study treatment will provide 2.0g/d of plant sterols

This study is with a double –blinded design which means neither you nor the clinical staff will know which variation of the treatments you will be receiving. You will receive all 3 treatments, however, it will be unknown the order you will be given and in what phase. In an emergency, this information will be made available.

During days 1, 2, 29 and 30 of each phase fasting blood samples approximately 2 tablespoons will be taken on each blood draw day. On days 26, 27 and 28 of each phase fasting blood samples approximately 1 tablespoon of blood will be taken on each blood draw day. These blood samples will be obtained for assessment of blood fat levels and fat metabolism. Each blood test will take approximately 5 minutes. The total amount of blood drawn during each phase of the study will be approximately 11 tablespoons.

On day 1 and 30 of each phase you will be asked to give a urine sample. In addition, you will be asked to fill out a 3-day food diary at the beginning and end of each phase.

Risks and discomforts
As with any clinical trial, there may be as yet unknown or unforeseen risks of taking part.
The plant sterols formulated with the proposed preparation procedures and at the proposed dose level, have been shown to have no known direct negative side effects on health in existing animal and human experiments. **However there may be slight gastro-intestinal discomforts as a result of plant sterol consumption.** Some known risks, although rare, are associated with placing a needle into a vein. These include the possibility of infection, perforation or penetration

Effect of a Plant Sterol-Fortified Low-Fat Milk Product on Plasma Lipid levels of Humans in Relation to Different Infant Feeding Practices and Later Life Cholesterol Metabolism

of the needle through the vein, and bleeding, pain, or bruising at the site. In case you feel any discomfort during the experimental trial a physician, Dr. Shahrokh Nejad Ghaffar, will be available to contact at any time. Dr. Shahrokh Nejad Ghaffar can be reached at (204) 805-1811.

Benefits
You may not benefit from participation in this research; however, the study should contribute to a better understanding of the effect of early nutrition and plant sterol formulations on health. It is also anticipated that oral intervention with these plant sterol products may provide positive effects on lipid lowering efficacy and other health-related markers. In addition to the above, you will also receive your results when they become available.

Costs
All clinic and professional fees, diagnostic and laboratory tests that will be performed as part of this study are provided at no cost to you. There will be no cost for the study treatment that you will receive. The study cost and honorariums will be covered by Dairy Farmers of Canada, the study sponsor.

Payment for participation
You will receive up to a maximum of $200 at completion of this study for your time and inconvenience of the study schedule. If you withdraw early from the study, you will receive an appropriate pro-rated fraction of this amount.

Alternatives
You do not have to participate in this study.   The study coordinators, physician and principal investigator will answer any questions you have about the experimental group of this study. You should be aware that medications exist as an alternative to treatment of lowering lipid and blood cholesterol levels.

Confidentiality
Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. The RCFFN staff involved with your care may review/copy medical information that may reveal your identity. The Biomedical Research Ethics Board at the University of Manitoba may also review your research-related records for quality assurance purposes. If the results of the trial are published, your identity will remain confidential. Personal information such as your name, address, telephone number and/or any other identifying information will not leave the RCFFN.

You will be assigned a participant code. The coding system of the study for participant identification will be the initials of each participant followed by a three-digit number. The three-digit number will be based on chronological order of participant selection. The identification codes corresponding to the study participants will be on the written documents which will only be available to the RCFFN staff. Study samples will be stored in the freezer at the RCFFN. Only the study coordinators and the principal investigator will have access to the samples. Your samples will not be used for any additional analyses, nor stored for any longer than 2 years, nor

Initials of participant: _____

Effect of a Plant Sterol-Fortified Low-Fat Milk Product on Plasma Lipid levels of Humans in Relation to Different Infant Feeding Practices and Later Life Cholesterol Metabolism

shared with any other group, other than is indicated in the protocol, without your specific consent. **ClinicalTrials.gov is a website that provides information about federally and privately supported clinical trials. A description of this clinical trial will be available on http://ClinicalTrials.gov. This website will not include information that can identify you. At most, the website will include a summary of the results. You can search this website at any time.**

Voluntary participation/withdrawal from the study
Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision to not participate or to withdraw from the study will not affect your other medical care.

Your participation in this study may be terminated without your consent by the study coordinators, physician or principal investigator. The study staff will withdraw you if he/she feels that participation is no longer in your best interest, or if you fail to follow the directions of the study staff.

If you decide to participate, you will agree to cooperate fully with the study visit schedule, and will follow the study staff's instructions.

We will tell you about new information that may affect your health, welfare, or willingness to stay in this study. Should you wish to withdraw your participation from the study, you must inform the study coordinators so that your file can be officially closed.

Medical care for injury related to the study
In the event of an injury that occurs to you as a direct result of participating in this study, or undergoing study procedures you should immediately notify the study physician, Dr. Shahrokh Nejad Ghaffar (204) 805-1811 or go to your nearest emergency room to receive necessary medical treatment. You are not waiving any of your legal rights by signing this consent form or releasing the investigator or the sponsor from their legal and professional responsibilities. If any health abnormalities are identified in the clinical tests conducted during this experiment, Dr. Shahrokh Nejad Ghaffar will be contacted, who will inform you of the results.

Questions
You are free to ask any questions that you may have about your treatment and your rights as a research participant. If any questions come up during or after the study or if you have a research-related injury, contact the study doctor and the study staff.

| Investigator: | Dr. Peter Jones | Tel No. | 204-474-9989 |
| Study Physician | Dr. Shahrokh Nejad Ghaffar | Tel No. | 204-805-1811 |

For questions about your rights as a research participant, you may contact:
The Biomedical Research Ethics Board, University of Manitoba at 789-3389

Initials of participant: _____

Effect of a Plant Sterol-Fortified Low-Fat Milk Product on Plasma Lipid levels of Humans in Relation to Different Infant Feeding Practices and Later Life Cholesterol Metabolism

Do not sign this consent form unless you have a chance to ask questions and have received satisfactory answers to all of your questions.

<u>Consent</u>
I agree to allow the study doctor to inform my family doctor that I am participating in this study or to obtain information regarding my medical history.

<div align="center">Yes ☐ No ☐</div>

1. I have read and understood this Information and Consent Form, and I freely and voluntarily agree to take part in the clinical trial (research study) described above.

2. I understand that I will be given a copy of the signed and dated Information and Consent Form. I have received an explanation of the purpose and duration of the trial, and the potential risks and benefits that I might expect. I was given sufficient time and opportunity to ask questions and to reflect back my understanding of the study to study personnel. My questions were answered to my satisfaction.

3. I agree to cooperate fully with the study doctor and will tell him if I experience any side effects, symptoms or changes in my health.

4. I am free to withdraw from the study at any time, for any reason, and without prejudice to my future medical treatment.

5. I have been assured that my name, address and telephone number will be kept confidential to the extent permitted by applicable laws and/or regulations.

6. By signing and dating this document, I am aware that none of my legal rights are being waived.

Signature:_____ Date/Time: _____

Printed name of above:_____

_____

For Clinical Study Coordinator: I confirm that I have explained the purpose, duration etc of this clinical trial, as well as any potential risks and benefits, to the participant whose name and signature appears above. I confirm that I believe that the participant has understood and has knowingly given their consent to participate by his/her personally dated signature.

Signature:_____ Date/Time: _____

Printed name of above:_____ Study role:_____

<div align="center">ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE</div>

Participant Consent form corresponding to Genetic studies described in Chapters V & VI

UNIVERSITY
OF MANITOBA

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

ADDITIONAL RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM
FOR GENETIC ANALYSIS

Title of Study: Effect of a Plant Sterol-Fortified Low-Fat Milk Product on Plasma Lipid levels of Humans in Relation to Different Infant Feeding Practices and Later Life Cholesterol Metabolism

Investigator: Peter Jones, PhD

        Richardson Centre for Functional Foods and Nutraceuticals
        University of Manitoba
        196 Innovation Drive, SmartPark
        Winnipeg, Manitoba R3T 2N2
        Phone: (204) 474-9989

Sponsor: Dairy Farmers of Canada
        1801 McGill College Avenue
        Suite # 700
        Montreal, QC H3A 2N4
        Phone: (514) 284-1092

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family. This consent form may contain words that you do not understand. Please ask the study staff to explain any words or information that you do not clearly understand.

Nature and Duration of Procedure
From the blood drawn during the clinical study entitled "Effect of a Plant Sterol-Fortified Low-Fat Milk Product on Plasma Lipid levels of Humans in Relation to Different Infant Feeding Practices and Later Life Cholesterol Metabolism", we would like to extract DNA and perform genetic analyses using a laboratory technique that augments and recognizes specific genes. This will allow us to determine why some people respond differently than others when consuming plant sterols and cholesterol as shown by their plasma lipid levels. DNA is a molecule found in the cells of your body that is organized into genes that contain all of the information needed to make the proteins, which perform specific biological functions in your body.

Initials of participant: _____

Appendix II

Effect of a Plant Sterol-Fortified Low-Fat Milk Product on Plasma Lipid levels of Humans in Relation to Different Infant Feeding Practices and Later Life Cholesterol Metabolism

Confidentiality and Safekeeping of DNA Samples

All of the information obtained about you and the results of the research will be treated confidentially. We will protect your confidentiality by assigning your DNA sample a specific code. This code will link you to your DNA sample and can only be decoded by the principal researcher or an individual authorized by the latter. Samples of your DNA will be kept at the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, under the supervision of Dr. Peter Jones for a 2-year period following the end of the research project. After this time, all samples will be destroyed. Your DNA samples will only be used for the purpose of this research project. Your samples will not be used for any additional analyses, nor stored for any longer than 2 years, nor shared with any other group, other than is indicated in the protocol, without your specific consent.

Your participation and the results of the research will not appear in your medical record. Although the results of this study may be published or communicated in other ways, it will be impossible to identify you. Unless you have provided specific authorization or where the law permits or a court order has been obtained, your personal results will not be made available to third parties such as employers, government organizations, insurance companies, or educational institutions. This also applies to your spouse, other members of your family and your physician. However, for the purposes of ensuring the proper management of research, it is possible that a member of an ethics committee, a Health Canada representative, or a representative from the Richardson Center for Functional Foods and Nutraceuticals may consult your research data and record. You can communicate with the research team to obtain information on the general progress or the results of the research project. Project updates will be mailed at the end of the project. However, we will not communicate any individual results to you.

Potential Risks and/or Benefits

As the DNA will be extracted from blood samples that have already been taken, there are no additional invasive procedures to undergo and no physical risks to you. While there may be no direct benefits to you for taking part in these additional analyses, we hope that the results will provide novel information on the influence of genetic characteristics of people and their response to plant sterols and cholesterol intake during the study time, particularly with regard to plasma lipid levels.

Signature of Participant

The content of the procedure and procedures associated with it have been fully explained to me, and all experimental procedures have been identified. I have had the opportunity to ask questions concerning any and all aspects of the project and procedures involved, and may continue in the future to ask further questions at any time, as it is my right to do so. I am aware that I may refuse to participate as well as withdraw my consent at any time. I acknowledge that no guarantee or assurance has been given by anyone as to the results to be obtained and that my participation in this study is completely voluntary. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. Samples will neither be utilized for any additional analyses, nor stored for any prolonged period, or shared with any other group other than is indicated in the protocol without my specific consent.

Page 2 of 3
June 26, 2013   Initials of participant: _____

Appendix II

Effect of a Plant Sterol-Fortified Low-Fat Milk Product on Plasma Lipid levels of Humans in Relation to Different Infant Feeding Practices and Later Life Cholesterol Metabolism

I, _____, have read the above description. I have been made aware of all the procedures, advantages and disadvantages of the study, which have been explained to me. By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

_____   _____
Signature of Participant   Date

For Clinical Study Coordinator: I confirm that I have explained the purpose, duration etc of this clinical trial, as well as any potential risks and benefits, to the participant whose name and signature appears above. I confirm that I believe that the participant has understood and has knowingly given their consent to participate by his/her personally dated signature.

Signature: _____   Date/Time: _____

Printed name of above:_____   Study role:_____

<div align="center">ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE</div>

Participant Screening Form

Richardson Centre for Functional Foods and Nutraceuticals

DAIRY CLINICAL STUDY   VISIT 1 (SCREENING)

Participant Code: _____

Participant code:_____   **Date of Visit:___/___/_____**

Surname: _____ Given Name(s): _____

(Day/Month/Year)
Date of Birth: _____/___/_____

Address: _____
(Street)

_____
(City)   (Province)

_____
(Postal code)

Phone Number: (Home)   ☐☐☐ ☐☐☐ ☐☐☐☐

(Work)   ☐☐☐ ☐☐☐ ☐☐☐☐

(Mobile)   ☐☐☐ ☐☐☐ ☐☐☐☐

Email Address:_____

INFORMED CONSENT

Please note: written informed consent must be given before any study specific procedures take place or any current therapy is discontinued for the purposes of participation in this study.

Has the participant freely given written informed consent?   Yes ☐   No ☐

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Richardson Centre for Functional Foods and Nutraceuticals

DAIRY CLINICAL STUDY   VISIT 1 (SCREENING)

Participant Code: _____

ANTHROPOMETRICS

Age (yrs): [ ][ ]   **Sex:**   **Male** [ ]   **Female** [ ]

Height (m):_____   **Weight (kg):**_____   **BMI (kg/m²):**_____

Waist girth 1: _____   Hip girth 1: _____
Waist girth 2: _____   Hip girth 2: _____
Waist girth 3: _____   Hip girth 3: _____
Ave. waist girth (cm):_____   **Ave. hip girth (cm):**_____
Waist/hip ratio: _____

Completed by: _____

BLOOD PRESSURE (after 10 minutes of rest)

ARM: **Left** [ ]   **Right** [ ]
TYPE OF DEVICE: **Automatic** [ ]   **Mercury** [ ]
Reading 1   systolic BP (mmHg): _____   diastolic BP (mmHg): _____
Reading 2   systolic BP (mmHg): _____   diastolic BP (mmHg): _____
Reading 3   systolic BP (mmHg): _____   diastolic BP (mmHg): _____
Avg. Systolic BP (mmHg): _____
Ave. diastolic BP (mmHg):_____
Heart rate (beats/min): _____

Completed by: _____

Richardson Centre for Functional Foods and Nutraceuticals

DAIRY CLINICAL STUDY   VISIT 1 (SCREENING)

Participant Code: _____

Infancy Breast feeding duration: _____

MEDICAL HISTORY		
Is there any relevant medical history in the following systems?		

System	*Yes	No	System	*Yes	No
Cardiovascular			Neoplasia		
Respiratory			Neurological/psychological		
Hepato-biliary			Lactose intolerance		
Gastro-intestinal			Immunological		
Genito-urinary			Dermatological		
Endocrine			Allergies		
Haematological			Eyes, ear, nose, throat		
Musculo-skeletal			Other		

COMMENTS:

MEDICATIONS   Yes ☐   No ☐

Name of drug	Dose	Freq.	Indication	Start	Stop	*
_____	____	____	_____	__/__/__	__/__/__	☐
_____	____	____	_____	__/__/__	__/__/__	☐
_____	____	____	_____	__/__/__	__/__/__	☐
_____	____	____	_____	__/__/__	__/__/__	☐
_____	____	____	_____	__/__/__	__/__/__	☐
_____	____	____	_____	__/__/__	__/__/__	☐

Richardson Centre for Functional Foods and Nutraceuticals

DAIRY CLINICAL STUDY   VISIT 1 (SCREENING)

Participant Code: _____

NATURAL HEALTH PRODUCTS

Do you take dietary supplements, vitamins and/or minerals, homeopathic remedies, or other natural products (probiotics, medicinal plants, omega-3, etc)?   **Yes** ☐   **No** ☐

Name of product	Dose	Freq.	Indication	Start	Stop	*
_____	_____	_____	_____	___/___/___	___/___/___	☐
_____	_____	_____	_____	___/___/___	___/___/___	☐
_____	_____	_____	_____	___/___/___	___/___/___	☐
_____	_____	_____	_____	___/___/___	___/___/___	☐
_____	_____	_____	_____	___/___/___	___/___/___	☐
_____	_____	_____	_____	___/___/___	___/___/___	☐
_____	_____	_____	_____	___/___/___	___/___/___	☐
_____	_____	_____	_____	___/___/___	___/___/___	☐

FOR WOMEN ONLY:

PLEASE CHECK ONE THE FOLLOWING:

   PREMENOPAUSAL ☐   **POST MENOPAUSAL** ☐

ARE YOU CURRENTLY OR PLANNING TO BE PREGNANT IN THE NEXT 7 MONTHS:

Yes ☐   No ☐

Are you currently a nursing mother: Yes ☐   No ☐

INCLUSION CRITERIA   Yes   No

		Yes	No
1	Is the participant a healthy individual aged between 18 and 50 years?	☐	☐
2	BMI<30 kg/m^2	☐	☐
3	Plasma triglycerides <3.0 mmol/L	☐	☐
4	If female not pregnant or nursing	☐	☐

Richardson Centre for Functional Foods and Nutraceuticals

DAIRY CLINICAL STUDY   VISIT 1 (SCREENING)

Participant Code: _____

EXCLUSION CRITERIA   Yes   No

1   Chronic alcohol consumption > 2 drinks/day or substance abuse or chronic smoking

2   Pregnant or planning for pregnancy during the next 5 months

3   Physical activity /exercise greater than walking or running 15 miles/wk or 4,000 kcal/wk

4   Sitosterolemia

5   History of recent (i.e. less than 1 mo) or chronic use of oral hypolipidemic therapy, including fish oils, or probucol within the last 3 months

6   History of myocardial infarction, coronary artery bypass, kidney disease, liver disease or other major surgical procedures within the last six months,

7   Recent onset of angina, congestive heart failure, inflammatory bowel disease, pancreatitis, diabetes, or significant current gastrointestinal, renal, pulmonary, hepatic or biliary disease or cancer

Is the Participant eligible for the study

                                                              Yes   No

3-day Participant Food Record

3-Day Food Diary_ DP 2013_Peter J.H Jones_ Phase__; Day : Date:_____;Participant code:_____

DAY 1	Breakfast	Lunch	Dinner	Snacks
Meats& Dairy				
Vegetables & Fruit				
Breads, Grains & Cereals				
Fats (butter, oil etc.)				
Candy & Sweets				
Water				
Other Drinks				
Additional comments				

DAY 2	Breakfast	Lunch	Dinner	Snacks
Meats& Dairy				
Vegetables & Fruit				
Breads, Grains & Cereals				
Fats (butter, oil etc.)				
Candy & Sweets				
Water				
Other Drinks				
Additional comments				

DAY 3	Breakfast	Lunch	Dinner	Snacks
Meats& Dairy				
Vegetables & Fruit				
Breads, Grains & Cereals				
Fats (butter, oil etc.)				
Candy & Sweets				
Water				
Other Drinks				
Additional comments				

Participant Baseline and Endpoint Data collection sheet

DAIRY STUDY–DP 2013

PARTICIPANT DATA-BASELINE AND END POINT

Participant Code: _____

Study Phase: _____   Treatment _____

Start Date: _____   End Date   _____

ANTHROPOMETRIC DATA

SECTION 1: START AND END WEIGHT

Day 1: Weight (Kg) _____   Staff Initials _____

Day 2: Weight (Kg) _____   Staff Initials _____

Day 29: Weight (Kg) _____   Staff Initials _____

Day 30: Weight (Kg) _____   Staff Initials _____

SECTION 2: WAIST & HIP CIRCUMFERENCE

Day 1: First measurement (cm) _____   Second measurement (cm) _____

Day 30: First measurement (cm) _____   Second measurement (cm) _____

Day 1 Hip: First measurement (cm) _____   Second measurement (cm) _____

Day 30 Hip: First measurement (cm) _____   Second measurement (cm) _____

SECTION 3: SEATED BLOOD PRESSURE (after 10 minutes of rest)

Left Arm ☐   Right Arm ☐

Day 1 or 2:

a. First blood pressure measurement: _____ / _____ (SBP / DBP)

b. Second blood pressure measurement: _____ / _____

c. Third blood pressure measurement: _____ / _____

d. Average (2nd and 3rd) blood pressure measurement: _____ / _____

Day 29 or 30:

a. First blood pressure measurement: _____ / _____ (SBP/ DBP)

b. Second blood pressure measurement: _____ / _____

c. Third blood pressure measurement: _____ / _____

d. Average (2nd and 3rd) blood pressure measurement: _____ / _____

Study Report, Sample

UNIVERSITY
OF MANITOBA

Date: Feb 3, 2014

PARTICIPANT STUDY REPORT

Richardson Centre for Functional Foods and Nutraceuticals	**Participant Name:**
	Principal Investigator: Dr. Peter Jones

Study title:

Effect of a Plant Sterol-Fortified Low-fat Milk Product on Plasma Lipid Levels of Humans

Study overview:

Plant sterols/stanols (PS) occur naturally in plants and are found at low levels in everyday foods such as vegetable oils, nuts, seeds, grain products, and fruits. Typical consumption of plant sterols in Western diet is less than 400 mg/day. Consumption of PS at level of 2 g/day has been recommended for lowering blood lipid concentrations; particularly bad cholesterol levels (low density lipoprotein-cholesterol/LDL-C) levels. The purpose of the study you participated in was to test the effect of a plant sterol ingredient versus added dietary cholesterol in dairy products on your blood lipid levels and response. We tested this product by adding it to milkshakes and comparing it against a placebo or milkshakes with no added plant sterols or cholesterol. The tables below describe your blood lipids at the beginning and end of each phase of the experiment.

Results:

The results of the study indicated in the following report outline the blood total cholesterol, triglyceride, LDL-C and HDL-C at the start and end of each 4-week phase.

What do the numbers in the test results mean?

Table 1 will help you to understand your results. The table provides common guideline for the major classes of blood lipids. However, cholesterol test results should be considered together with other risk factors, medical history and present health. Your doctor is the best person to interpret your test results and can advise you if you need to take action.

Richardson Centre
for Functional Foods
and Nutraceuticals

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Table1. Target Cholesterol Ranges

Type of lipid	Total cholesterol	LDL-cholesterol	HDL-cholesterol	Triglycerides
Target Level	Less than 5.22 mmol/L	Less than 3.5 mmol/L	Men: Higher than 1.0 mmol Women: 1.3 mmol	Less than 1.7 mmol/L

Did plant sterols work for you?

Table 2 reveals your blood lipid results at the beginning and end of each phase. The percent (%) change is the change in your results from start of the phase to the end of the phase. In the % change column, a negative number (i.e. -10%) means that there was a decrease in your cholesterol after 4 weeks of the treatment (plant sterol worked for you). Whereas, a positive number (i.e. +8%) means that there was an increase in your cholesterol after 4 weeks of the treatment (plant sterol didn't work for you).

Table2: Your Lipid Results

Phase / Treatment	Milkshake without added plant sterol/cholesterol (A)			Milkshake with added cholesterol (B)			Milkshake with added plant sterol (C)		
	Start	End	% Change	Start	End	% Change	Start	End	% Change
Plasma Lipids									
Total cholesterol (mmol/l)									
LDL cholesterol (mmol/l)									
HDL cholesterol (mmol/l)									
Triglycerides (mmol/l)									

Note: These results are for research purposes only.

We would like to thank you for your participation and commitment!

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and Nutraceuticals