Genetic basis for heterogeneity of response of LDL cholesterol to plant sterols

By

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Abstract

Plant sterols and their saturated counterparts, plant stanols share a similar chemical structure to cholesterol, differing only in side chains and double bond placement. Plant sterols are naturally found in plants and are typically ingested in the 400mg/day range. Consumption of 1-3 g of plant sterols a day has been repeatedly shown to lower total and LDL cholesterol. However, data from nutritional trials involving plant sterols demonstrate considerable inter-individual variations in response to plant sterol consumption. The objective of this research was to investigate the metabolic and genetic factors that underlie this heterogeneity of responsiveness of LDL cholesterol to plant sterol consumption. A study was conducted to test the effectiveness of lathosterol to cholesterol ratio (L/C), a surrogate marker of cholesterol synthesis, as a predictor of LDL cholesterol lowering in response to plant sterol consumption. 63 mildly hypercholesterolemic adults, with high (n=24, L/C = 2.03 ± 0.39umol/mmol) or low (n=39, L/C =0.99±0.28 umol/mmol) L/C ratio at baseline, consumed either 0 or 2g/d of PS for 28 days in a dual-center, single-blind, randomized, crossover design. Plasma lipid and non-cholesterol sterol concentrations were measured at the end of each phase. Single nucleotide polymorphisms (SNPs) in candidate genes involved in cholesterol metabolism were investigated for potential gene by nutrient interactions. Plant sterol consumption lowered total and LDL cholesterol concentrations overall, but only individuals with low L/C ratio responded to plant sterol treatment by lowering TC and LDL-C, while individuals with high L/C ratio showed no marked improvement. The rs3808607 T-allele in the promoter of the CYP7A1 gene was associated with decreased LDL-C responsiveness to plant sterol consumption. The rs3808607 G-allele and ApoE ε4 were

associated with increased LDL-C responsiveness to plant sterol consumption. PS consumption did not lower TG overall (p=0.0506), but had an interaction with *rs5882* in CETP (p=0.0080**)**. Homozygous minor allele (G/G=-0.47mmol/L, p=0.0002, n=10) carriers of *rs5882* had reductions in TG concentrations with PS consumption, while A/A (+0.01mmol/L, p =0.6634, n=25) and A/G (-0.04mmol/L, p= 0.3399, n=28) carriers did not. Baseline L/C predicts LDL-C lowering due to PS consumption, which is associated with rs3808607 genotype in the promoter of the CYP7A1 gene. *rs5882* in CETP is associated with TG lowering due to PS consumption. rs3808607, *rs5882* and ApoE variant are potential genetic markers which could identify individuals who would derive maximum benefit from plant sterol consumption.

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Dedication

I dedicate this thesis to my family.

To my wife Kim, and my children Chloe and Isaac.

To my parents Dennis and Annette.

I would not have been able to do this without you.

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Abbreviations

7αHC	7-alpha-hydroxycholesterol
ABCG5	ATP-binding cassette, sub-family G (WHITE), member 5
ABCG8	ATP-binding cassette, sub-family G (WHITE), member 8
ADH	Autosomal dominant hypercholesterolemics
AFM	Advanced Foods and Materials Canada
APOA4	Apolipoprotein A-IV
ApoE	Apolipoprotein E
BMI	Body mass index
CETP	Cholesteryl ester transfer protein
CHD	coronary heart disease
CIHR	Canadian Institute of Health Reseasch
CVD	Cardiovascular disease
CYP7A1	Cholesterol 7 alpha-hydroxylase
DNA	deoxyribonucleic acid
FCH	Familial combined hypercholesterolemia
FCHL	Food Components and Health Laboratory
FH	Familial hypercholesterolemia
FHADH	Non-familial hypercholesterolemia autosomal dominant hypercholesterolemia
FSR	Fractional synthesis rate
GC-C	Gas chromatography- combustion
GCFID	Gas chromatography- flame ionization detector
GC-MS	Gas chromatography- mass spectrometry
GC-P	Gas chromatography- pyrolysis
GWAS	Genome-Wide Association Study
HDL	high density lipoprotein
HMG-CoAR	3-hydroxy-3-methyl-glutaryl-CoA reductase
HPLC	High performance liquid chromatography
HS	High synthesizer
IRMS	Isotope ratio mass spectrometry
LDL	Low density lipoprotein
LS	Low synthesizer

LXR	Liver X receptor
MetS	Metabolic syndrome
MIDA	Mass isotopomer distribution analysis
MRI	Magnetic resonance imaging
MS/MS	Tandem mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	non-alcoholic fatty liver disease
NCBI	National Center for Biotechnology Information
NCEP ATP III	National Cholesterol Education Program Adult Treatment Program III
NCS	Non-cholesterol sterols
NPC1L1	Niemann-Pick C1-Like 1
NRU	Nutritional Research Unit
PDB	Pee Dee belemnite
PH	Polygenic hypercholesterolemics
pHBL	primary hypobetalipoproteinemia
PS	Plant sterol
RBC	Red blood cells
RCFFN	Richardson Center for Functional Foods and Nutraceuticals
RNA	Ribonucleic acid
SIM	Single ion monitoring
SMOW	Standard mean ocean water
SNP	Single nucleotide polymorphism
SR-BI	Scavenger receptor class B member 1
SREBF2	Sterol regulatory element-binding protein 2
TC	Total cholesterol
TG	Triglyceride
TICE	Trans-intestinal cholesterol exchange
UPLC	Ultra-performance liquid chromatography
VFA	Visceral fat area

Chapter 1

OVERALL INTRODUCTION

1.1 INTRODRUCTION

Plant sterols, and their saturated plant stanol counterparts, are steroid compounds similar to cholesterol which occur naturally in plants. Although almost structurally identical to cholesterol, plant sterols (PS) possess extra carbon groups at the C24 position. Dietary plant sterols are found principally in foods such as nuts, legumes and vegetable fats and are typically ingested at levels of less than 400 mg/day (1,2). The most common plant sterols in the diet are sitosterol, campesterol and stigmasterol, which account for about 65%, 30% and 3% of diet contents, respectively (3).

Consumption of supplemental PS, typically over 1 g/day has been demonstrated to reduce circulating cholesterol concentrations, particularly low density lipoprotein-cholesterol (LDL-C) concentrations, in humans (4,5). Interest in the use of PS to control circulating lipid concentrations has existed since the 1950s (6) and a mix of free PS, primarily sitosterol suspended in oil or methylcellulose was marketed under the name Cytellin for the treatment of elevated cholesterol from 1954-1982 (7,8). The PS dose provided by Cytellin to lower serum cholesterol concentrations was in the 15-20 g/day range, and the product fell out of favor with the discovery of statin drugs.

The development of PS fatty acid esters led to a resurgence in use of PS as dietary agents capable of modulating circulating lipid concentrations in the 1990s (4,7). Functional foods in numerous matrices both high and low in fat, as well as nutraceuticals such as

capsules and tablets containing free and esterified PS, have since been introduced into the global food marketplace. Numerous randomized clinical trials have been conducted demonstrating LDL-C lowering efficacy of PS in the 10% range with PS intakes of 1-2 g per day (4,5,9).

In May 2010, Health Canada's Food Directorate approved the addition of PS into spreads, mayonnaise, margarine, calorie-reduced margarine, salad dressing, yogurt and yogurt drinks, as well as vegetable and fruit juices (10). Accordingly, several major food companies have since started marketing food products enriched with supplemental PS in Canada.

Despite repeated demonstration of the efficacy of plant sterols in lowering mean circulating cholesterol concentrations of humans in clinical trials, significant inter-individual variation in cholesterol lowering has been seen (11,12)(Figure 1.1). While food matrixing and dosage could contribute to variability between different clinical trials, it is now increasingly clear that independent of such factors, some individuals respond immediately and with a more major shift in lipid profiles, compared with other individuals who are much more resistant or completely insensitive to plant sterol intervention (12-14).

The change in an individual's cholesterol concentrations in response to plant sterol consumption can be classified as a phenotype as it has been shown to be repeatable within individuals upon repeated plant sterol interventions (13). The metabolic or genetic factors which modify an individual's plant sterol response phenotype have yet to be fully established.

Cholesterol metabolism can be estimated through the use of non-cholesterol sterols, with cholesterol precursors, such as desmosterol or lathosterol, used as markers for endogenous cholesterol synthesis and plant sterols, such as campesterol and sitosterol, used as markers of cholesterol absorption (15). These non-cholesterol sterols are typically expressed as ratios to cholesterol, as these more tightly correlate with direct measures of cholesterol metabolism than the absolute values (16). Overall cholesterol metabolism has been estimated through the use of ratios of cholesterol synthesis to absorption markers (17).

Characteristics of an individual's cholesterol metabolism have been related to their response to PS consumption. Using non-cholesterol sterols as surrogate markers of cholesterol metabolism (15,18), it has been shown, retrospectively, that individuals with high cholesterol absorption and/or low cholesterol synthesis have enhanced LDL lowering compared to those with low cholesterol absorption and/or high cholesterol synthesis when given dietary PS (19-22).

To investigate the impact of cholesterol metabolism on response in a prospective fashion Thuluva et al.(23) recruited individuals with the highest (n=8) and lowest (n=8) lathosterol to campesterol ratios, where a high value would represent greater cholesterol synthesis and a lower value greater cholesterol absorption. A population of 137 male hypercholesterolemics was examined. In this group only individuals with the lowest lathosterol to campesterol ratio had significant reductions in total and LDL cholesterol after 4 weeks of plant stanols at a dose of 1g/d.

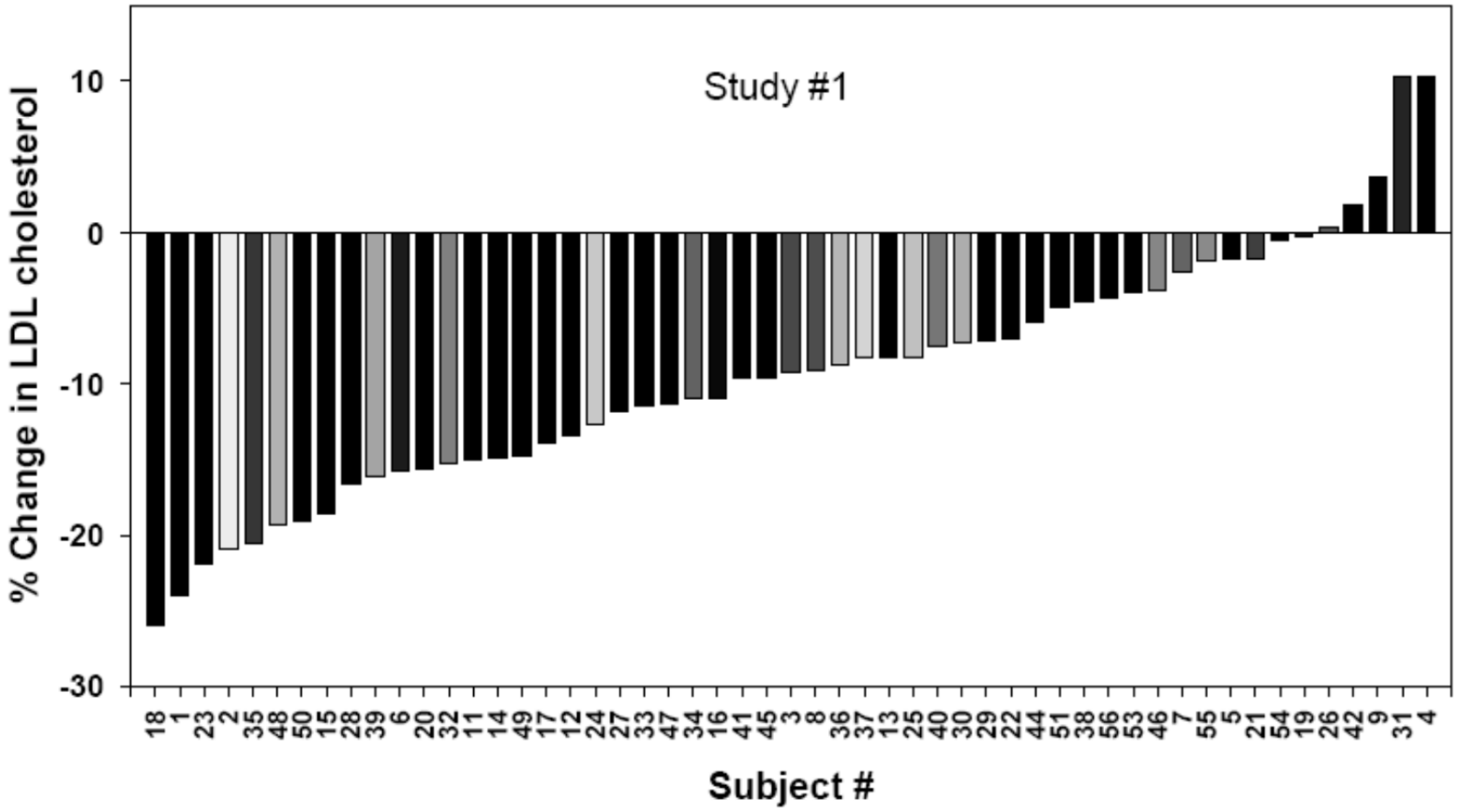

Figure 1.1 Range of responsiveness in plasma LDL-C to a plant sterol intervention at the Beltsville Human Nutritional Research Center

Houweling et al. (24) conducted a trial to assess the impact of baseline sitosterol and campesterol concentrations, both surrogate markers for cholesterol absorption, on response of plasma lipids to PS in 82 mildly hypercholesterolemic men in a crossover format. Individuals with high plant sterol concentrations at baseline did not have significantly different responses in LDL-C to 4 weeks of PS consumption compared to individuals with low baseline plant sterol concentrations. Baseline plant sterol concentrations, used by Houweling et al. (24), reflected only cholesterol absorption, whereas Thuluva et al. (23) used the ratio of lathosterol to campesterol which also reflected cholesterol synthesis.

These findings suggest that the amount of cholesterol produced endogenously, through cholesterol synthesis, may be important in determining responsiveness to plant sterols. The association between cholesterol synthesis and responsiveness to plant sterols is further reinforced by a retrospective clinical analysis showing significantly higher cholesterol synthesis in non-responders to plant sterols than responders (Rideout et al. Manuscript 4 in thesis).

Genetic variations in genes related to cholesterol metabolism have been investigated for associations with responsiveness to PS consumption. Variations in genes such as ABCG5 and G8, NPC1L1, APOA4, SR-BI, HMG-CoAR, CETP, ApoE and CYP7A1 have been studied in relation to cholesterol lowering response to plant sterols and stanols (13,14,25-29). As yet very few trials have demonstrated associations between a single nucleotide polymorphism (SNP) in a gene and serum LDL-C lowering response to plant sterol or stanol consumption.

Zhao et al. showed that the ABCG8 rs4148217 polymorphism associated with PS response in individuals with high basal plant sterol concentrations in a 4 week randomized crossover trial with 82 participants (14). However, the same rs4148217 polymorphism in ABCG8 was found not associated with plant sterol or stanol response in studies by Plat et al. (25) in an 8 week parallel arm trial with 112 participants, by Rudkowska et al. (13) in a 4 week randomized crossover with 26 participants, and by Gylling et al. (27) in a 1 year parallel arm trial with 282 participants.

Lottenberg et al. (30) investigated the impact of CETP I405V (*rs5882*) genotype on cholesterol lowering after PS consumption in a double blinded crossover study. Only the II and IV carriers had significant reductions in total cholesterol and only the II carriers had significant reductions in LDL-C with PS consumption. CETP concentrations were only reduced in the II carriers in response to PS consumption. Plat et al.(29) looked at response to plant stanol consumption in relation to the CETP taqIB (*rs708272*) variant and saw no association between genotype and cholesterol lowering. The CETP taqIB (*rs708272*) variation, unlike CETP I405V (*rs5882*) is in an intron and does not cause a change in the amino acid sequence of the CETP protein.

De Castro-Oros et al. (26) showed that *rs3808607* (-204A>C) in the promoter region of the CYP7A1 gene, which encodes for the rate limiting enzyme in the synthesis of bile acid from cholesterol, was associated with response to plant sterol consumption. Compared to AA carriers of the variant, C-carriers showed significantly higher reductions in LDL-C concentrations (0.13 vs. 0.43 mmol/L) in response to PS consumption. Due to this previous association CYP7A1 *rs3808607* (-204A>C) exists as an excellent candidate

polymorphism in which to investigate cholesterol lowering in response to PS consumption.

The ApoE gene codes for the apolipoprotein E, a glycoprotein present in triglyceride-rich lipoproteins, such as chylomicrons and VLDLs, as well as HDL. ApoE is a gene that is highly polymorphic, with 3 major variations, named epsilon 2, 3 or 4. These variants are caused by a combination of 2 SNPs in the ApoE gene. Sanchez-Muniz et al. (28) investigated the effect of ApoE variants on PS supplementation and showed ApoE ε4 variant carriers may not respond as well to PS consumption as ε3 and ε2 carriers. These findings were contrary to those of Vanhanen et al. (31) who associated ApoE ε4 variant with a more consistent response to plant stanols, and Geelen et al. (32) who did not see any association between magnitude of cholesterol response to PS consumption and ApoE variants. The impact of ApoE variants on cholesterol lowering in response to PS consumption requires further investigation.

Overall, genetic variants that have been associated with plant sterol response have been inconsistent or have not been replicated, something which is critical since the populations examined in plant sterol clinical trials have been relatively small. Most of the plant sterol clinical trials that have looked at genetic polymorphisms have been parallel arm, which is not ideal for the investigation of genetic polymorphism because the genetic diversity between participants receiving the control or placebo treatment is never the same as the participants receiving the PS intervention. Since the trial populations in the PS trials were typically small ($n<100$) (5), the added statistical strength of a crossover format is also a viewed as a large asset. Therefore, a crossover trial format should be selected for the investigation of response to PS consumption in relation to genetic polymorphisms.

1.2 RATIONALE

The substantial range of individual responsiveness to plant sterols has important ramifications. Marked differences across individuals in particular aspects of the cholesterol metabolic pathway must alter the impact of plant sterol consumption. Discerning which factors are responsible for inter-individual variations in response will permit tests to be developed to distinguish individuals for whom plant sterols would exist as useful dietary adjuncts, compared with those who may require either alternative dietary approaches, or a pharmaceutical regimen, to effectively manage circulating lipid concentrations. As such, a pronounced need exists to understand the genetic and metabolic factors that explain the substantial degree of heterogeneity in response of lipid concentrations to plant sterols across individuals.

Based on the association between elevated cholesterol synthesis and non-response to plant sterol consumption (Manuscript 4), and using a human intervention trial (Manuscript 5), the primary focus of this research is to delineate the impact of differing cholesterol synthesis levels on response of LDL-C and other plasma lipids to plant sterol consumption. Participants pre-identified as high or low endogenous cholesterol synthesizers, according to their screening level of lathosterol to cholesterol ratios, were given PS or a placebo containing margarine to consume under supervision for 4 weeks in a crossover design. The trial characterized the responsiveness of the participants' total, LDL, and HDL cholesterol, as well as triacylglycerol (TG) concentrations, to PS consumption. This research will determine if cholesterol synthesis phenotype predicts the responsiveness of lipids to plant sterol consumption. Variations in candidate genes involved in cholesterol metabolism will also be investigated in order to find associations

with both cholesterol metabolism phenotypes and responsiveness of lipids to plant sterols. The output of this research will be to advance the knowledge of which genetic factors influence the degree of cardiovascular benefit derived from PS through lipid lowering.

1.3 OBJECTIVES

The overarching objective of the present research program is to identify a genetic basis for heterogeneity in responsiveness of lipids to plant sterol consumption, and to identify which regulators of cholesterol metabolism associate with the genetic factors identified. The long-term goal is to predetermine who will, and will not, respond to plant sterols as a lipid lowering nutritional therapy. Specific objectives include:

1) Characterize a continuum of responsiveness of plasma LDL-C concentrations to PS consumption in a cohort of individuals with mild hypercholesterolemia who are high or low cholesterol synthesizers, as estimated by plasma lathosterol concentrations.

2) Evaluate the impact of PS consumption on cholesterol metabolism, as characterized by non-cholesterol sterol concentrations, in a cohort of individuals with mild hypercholesterolemia who are high or low cholesterol synthesizers, as estimated by plasma lathosterol concentrations.

3) Associate SNPs within candidate genes involved in cholesterol metabolism with the degree of plasma total, LDL, and HDL cholesterol as well as TG concentrations responsiveness to PS consumption.

4) Associate SNPs within candidate genes involved in cholesterol metabolism with changes in cholesterol metabolism as characterized by non-cholesterol sterol concentrations due to PS consumption.

1.4 HYPOTHESES

The hypotheses to be tested include:

1) Responsiveness of lipid lowering efficacy to plant sterol consumption will differ between low and high cholesterol synthesizing individuals. Low endogenous cholesterol synthesizers will have greater cholesterol lowering following PS consumption compared to high cholesterol synthesizers.

2) Variations in candidate genes involved in cholesterol metabolism will associate with the degree of cholesterol lowering efficacy, and with changes in cholesterol metabolism following PS consumption in low and high cholesterol synthesizing individuals.

The following manuscripts will give an overview of the plant sterol consumption in human nutrition (Manuscript 1), methods available for the assessment of cholesterol metabolism (Manuscript 2), the use of non-cholesterol sterols as surrogates of cholesterol metabolism (Manuscript 3), and the relationship between cholesterol synthesis and non-response to plant sterols (Manuscript 4). Thereafter, the manuscripts will address the ability of lathosterol to cholesterol ratio to predict cholesterol lowering response to PS consumption (Manuscript 6), and the genetic basis for heterogeneity of response of total and LDL cholesterol (Manuscript 7) and TG (Manuscript 8) to plant sterols.

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Bridge to chapter II

The following chapter comprises a manuscript which provides a broad overview of plant sterols in the context of human nutrition. Special attention is focused on plant sterols as food ingredients for cholesterol lowering. How plant sterols are incorporated in foods and there physiological functions when consumed are also reviewed. This chapter is an introduction to the field of plant sterols as cholesterol lowering food ingredients in general. Dylan S. MacKay was the principal manuscript author, and Peter J. H. Jones contributed to the preparation of the manuscript.

Chapter II

Manuscript 1: Literature review

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Phytosterols in human nutrition: Type, formulation, delivery and physiological function

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2.1 Abstract

Phytosterols are a family of compounds similar to cholesterol which have been shown to lower cholesterol levels when supplemented in the diet. A daily dose of 2-3 grams of phytosterols has been shown to reduce LDL-cholesterol levels by 5-15%. Phytosterol supplementation can be undertaken using phytosterol enriched functional foods or nutraceutical preparations. The type of phytosterol supplemented, such as plant sterol or saturated plant stanol appear to be equally effective in lowering cholesterol levels. Phytosterols, whether in esterified or free form have both been shown to lower cholesterol level, with esterified phytosterol formulations having a greater number of clinical trials demonstrating efficacy. The functional food or nutraceutical matrix which is used to deliver supplemental phytosterols can significantly affect cholesterol lowering efficacy. Effective cholesterol lowering by phytosterols depends on delivery of phytosterols to the intestine in a form which can compete with cholesterol for absorption. New phytosterol functional food and nutraceuticals products should always be tested to demonstrate adequate delivery of phytosterol dose and effective total and LDL-cholesterol lowering. Phytosterol products which do not effectively lower cholesterol will negatively impact the perception and use of phytosterols, and must not be allowed on the marketplace.

2.2 Article

Phytosterols are a group of lipophilic steroid alcohols found in plants which are chemically similar to cholesterol, but differ in side chains and ring structure saturation. Phytosterols can be sub-divided into two classes, plant sterols or "Δ5-sterols" with a double bond at the 5 position in the sterol ring, and plant stanols or 5α-sterols with a saturated sterol ring structure. The human diet naturally contains both plant sterol and to a much lesser extent stanol forms of phytosterols found in fruits, vegetables, grains and nuts. Phytosterol intakes range from 150-450 mg/day, depending dietary choices, especially of plant based fats and oils which are rich sources of dietary phytosterols [10,36]. The cholesterol lowering ability of phytosterols was first demonstrated in humans in 1953 [40], and a mix of free phytosterols, primary β-sitosterol suspended in oil or methylcellulose was subsequently marketed under the name Cytellin for the treatment of elevated cholesterol from 1954-1982 [21]. The cholesterol lowering ability of phytosterols was re-discovered through the development of functional foods containing phytosterols, specifically plant stanol esters which were used to make functional margarines introduced into the Finnish market in 1995. Functional foods in numerous matrices both high and low in fat, as well as nutraceuticals such as capsules and tablets containing free and esterified phytosterols, have since been introduced into the global marketplace [1]. The objective of this article is to discuss how the magnitude of cholesterol lowering and, therefore, the physiological effects of the phytosterol supplementation vary depending on the type, formulation and delivery of phytosterol.

The primary purpose of phytosterol supplementation is for improvement of plasma cholesterol levels. Cholesterol absorption requires solubilisation of cholesterol

into micelles within the intestinal chyme, these micelles allow hydrophobic compounds like cholesterol, fatty acids and fat soluble vitamins to make contact with the apical membrane of the enterocytes lining the intestinal tract [34]. Cholesterol is transported across the apical membrane into the enterocytes by an NPC1L1 dependant process [3]. Cholesterol that enters the enterocytes is then packaged into chylomicrons for secretion into lymph and blood via the enterocytes' basolateral membrane. Due to the chemical similarity of phytosterols to cholesterol, it is via a competitive inhibition of cholesterol absorption that phytosterols bring about the total and LDL cholesterol lowering seen when phytosterols are supplemented in the diet [22]. Phytosterols' main site of competitive inhibition with cholesterol is for incorporation into the mixed micelles in the intestinal chyme, causing a greater amount of cholesterol to remain unsolubilized reducing absorption [37]. Phytosterols may also compete with cholesterol during NPC1L1 dependant transportation into the enterocytes, as well as packaging into chylomicrons in the enterocytes. However, once in the enterocytes phytosterols are preferentially transported back out into the lumen by ABCG5 and G8 heterodimer complex. Phytosterols which do get packaged into chylomicrons and enter into the circulatory system are also preferential excreted from the liver into the bile by the same ABCG5/G8 system [22]. This process of preferential phytosterol excretion maintains total phytosterols concentrations in the 12-20 μmol/L range [26], that are 0.01-0.14% of cholesterol levels for sitosterol and campesterol [32] and 0.003-0.004% for stanol levels [17], even though both cholesterol and phytosterols are found in similar concentrations in the diet.

These two classes of phytosterols are absorbed at different level by the human body. Plant sterols are absorbed in the 0.5 -1.9% range, whereas stanols are absorbed in the < 0.1% range [37].The differential absorption of phytosterols is reflected in their circulating levels, with plant sterols having higher concentrations than plant stanols in the plasma.

The ability of phytosterols, both sterols and stanols, to lower total and LDL-cholesterol levels has been repeatedly demonstrated in many clinical trials [24,27]. A recent meta-analyses of randomized, double blinded, single intervention, clinical trials looking at phytosterol supplementation lasting longer than 2 weeks identified 81 trials demonstrating that phytosterols lower LDL-cholesterol in a dose dependent manner [11]. The meta-analysis concluded that a mean dose of 2.15 g/day of phytosterols yielded a pooled LDL-cholesterol lowering of 0.34mmol/l or 8.8%. Other meta-analyses of phytosterol trials have made similar conclusions [1,24,33,48]. The addition of phytosterol supplementation in individuals with on-going statin therapy also lowers cholesterol levels beyond that seen with statins alone [9,44].

Interestingly the meta-analyses conducted on trials of phytosterol supplementation show large heterogeneity in mean cholesterol lowering across different trial populations in response to the same dose of phytosterols [1,11,24,27,33]. This heterogeneity of response suggests that differences in trial design affect the cholesterol lowering efficacy of the phytosterols. The different sources of phytosterols in these trials are not likely to have contributed to the heterogeneity as phytosterols from varying sources have been shown to be equally effective in cholesterol lowering in direct comparisons [5,18].The effect of genetic variations, which modulate phytosterol response [8,43], can be diluted in

the mean response of a trial population if the genetic variations is not shared across the whole population, however genetic variations often have geographic or ethnic distributions and can therefore contribute to some inter-trial heterogeneity. The type, formulation and delivery of phytosterols are also likely contributing factors to the variability seen between trials.

The type of phytosterol, whether plant sterol or stanol, and their respective ability to lower cholesterol continues to be debated. Plant sterols and plant stanols when compared head to head in clinical trials have been shown to equally reduce cholesterol levels [17,35,51] and large meta-analyses have also concluded that sterols and stanols work equivalently [1,11,24]. A recent meta-analysis of 14 randomized controlled trials comparing the effect of plant sterols to plant stanols directly at doses of 0.6 to 2.5 g/day in healthy and hypercholesterolemic patients showed no significantly different effects between the two on total cholesterol, LDL cholesterol, HDL cholesterol, or triglyceride levels [48]. However, a recent meta-analysis by Musa-Veloso et al. [33] which re-analyzed the trials in the Demonty et al. [11] concluded that plant stanols had a significantly greater maximal dose response curve than plant sterols, but only at intakes much higher than the currently recommended 2-3 g per day range. Trials looking at high doses (> 4g/day) of plant sterols or stanols are very limited [7,16,31], and none have yet to be completed comparing the same high dose of plant sterol to plant stanol. Until a direct comparison between high dose plant sterol to plant stanol supplementation is completed it cannot be said that either is more efficacious at lowering cholesterol.

The debate regarding sterol versus stanol safety is centered on their differing absorption plasma levels and plasma concentrations [52]. Due to the extremely elevated

levels of phytosterols seen in the rare genetic disorder phytosterolemia, which is associated with rapidly progressing CVD, it was hypothesized that plant sterols themselves may be atherogenic. Glueck et al. [15] suggested that elevated plant sterol levels may be associated with increased CVD risk. This conclusion was based on campesterol and total plant sterol levels correlating positively with cholesterol levels and high campesterol being associated with increased personal or familial coronary heart disease in the top quintile of the 3472 hypercholesterolemic patients accessed. Several other studies have since suggested that elevated plant sterol levels may be a risk factor for CVD [2,41,47]. However, it must be noted that in these studies the circulating levels of plant sterols were not as high as levels seen in phytosterolemia which can range from 500-2250 μmol/L which are 20-100 times higher than those seen in the normal population [20,26,36]. In phytosterolemia the rapid development of CVD may still be due to the improper handling of cholesterol, which is elevated in most cases and accounts for the vast majority of sterols in phytosterolemics [36,42]. Studies showing increased plant sterol levels are a risk for CVD have been contrast by similar studies showing a decreased risk of CVD with increasing plant sterol concentrations [14,39,45]. The safety debate regarding plant sterol levels and CVD is further complicated by the fact that circulating plant sterol levels reflect the level of cholesterol absorption [32,49]. This relationship has led some to conclude that elevated cholesterol absorption, rather than plant sterol levels themselves, are atherogenic or otherwise associated with increased CVD risk [46].

Incorporation of phytosterols into the mixed micelles of the intestinal chyme is critical to their cholesterol lowering effect; therefore, the formulation of the phytosterol

and its delivery vehicle is important in predicting optimum cholesterol lowering. If supplemented phytosterols remain crystallized or are trapped in their delivery matrix when in the chyme and cannot enter into the mixed micelles, the effective dose of phytosterols is reduced as is the subsequent cholesterol lowering capacity. Therefore, the appropriate delivery vehicle for supplemental phytosterols is critical for successful cholesterol lowering. Currently phytosterol supplementation is available in functional food or nutraceutical forms and may not always be optimally delivered.

Cytellin, the pharmaceutical preparation of phytosterols was discontinued in 1985 due to lack of palatability, which would have affected compliance and effectiveness. The lack of palatability was likely due to the difficulty in dissolving high doses of free plant sterols in an aqueous mixture, yielding an unpleasant gritty texture and taste [28]. The first functional food introduced containing phytosterols was a margarine containing phytosterol fatty acid esters introduced in Finland in 1995 [21]. Free phytosterols exist as a crystalline powder when extracted from vegetable oils, insoluble in water and poorly soluble in oil [13]. Production of phytosterol fatty acid esters was critical to the development of the first functional foods containing phytosterols. Esterification of phytosterols increases the solubility in oil by ten-fold and allows for greater incorporation of phytosterol into a functional margarine without significantly impacting taste or texture.

The use of phytosterol esters may not optimal in all food matrices. Phytosterol esters are larger, heavier molecules than free phytosterols and deliver less phytosterol and more calories from fat on a per gram basis [29]. Since phytosterol food products are marketed to lower cholesterol having phytosterol enriched higher fat foods like margarine may not ideal individuals following low fat diets. Functional foods have since been

developed in which free phytosterols have been incorporated directly into foods, through homogenization and emulsion techniques often using stabilizing agents such a lecithin. These different methods have and are also being used to prepare lower fat or fat free functional foods.

Numerous clinical trials have been conducted using phytosterol functional foods in the free or esterified forms across many different types of matrices. Meta-analyses of these trials have found no significant difference between LDL cholesterol lowering between esterified and free phytosterol [1,11,33]. However, it must be noted that in these meta-analyses "free" phytosterols refer to all the various methods free phytosterols can be incorporated into foods. No meta-analyses have yet to access the cholesterol lowering efficacy between functional foods made with different free phytosterols incorporation methods, likely due to the lower number of published trials using free phytosterols and the proprietary and often vague descriptions of the incorporation methods. Some of the incorporation methods for free phytosterols include emulsion with lecithin [38], supersaturation using crystallization inhibitors [13], sub-particularization using aerosol solvent extraction [53], and microemulsion by solvent displacement [28,29] or rapid expansion of supercritical solution into aqueous solution [50]. Each of these methods have been developed to incorporate free phytosterols into functional foods and center around reducing crystallization and or particle size while maximizing the free phytosterols solubility. These newer nanotechnology methods for phytosterol incorporation into functional foods offer great opportunity, however due to the overwhelming risk of introducing a food product into the marketplace that is harmful,

foods created with these new technologies must be tested, first in animals and then in humans before introduction into the broader marketplace.

Phytosterols with smaller particle sizes are thought to improve the water solubility of the phytosterols and potentially enhance the cholesterol lowering dose response [29]. A trial in hamsters comparing nanoscale (particle size: 200–300 nm) versus customary (particle size: 10000–90000 nm) free sitosterol found that both preparations increased sitosterol concentrations in the hamsters compared to control, but no differences in tissue concentrations between preparations were seen [25]. The cholesterol levels of the treated hamsters were slightly but not significantly reduced by both sitosterol preparations. This lack of significant cholesterol lowering was attributed to the use of a cholesterol free diet. This lone animal trial does not disprove the theory of enhanced efficacy of phytosterols with smaller particle sizes; however, further animal trials testing safety and then human clinical trials evaluating the effect of varying particle size must still be conducted.

The variety of food matrices used in phytosterol functional foods has expanded beyond margarine spreads as incorporation techniques for esterified and free phytosterols have improved. Mayonnaise, salad dressings, dairy and non-dairy beverages, chocolates , meat, cheese, and baked goods have all been used to deliver phytosterols in clinical trials [1]. Clifton et al. [6] compared the LDL cholesterol lowering effects of 1.6 g/day of phytosterols delivered by milk, yogurt, bread or cereal. The LDL cholesterol lowering action of the phytosterol enriched milk was 15.9%, almost twice that of the yogurt (8.6%) and three times that of bread and cereal (6.5% and 5.4% respectively). This was the first study to show conclusively using a head to head comparison that food matrix can significantly affect LDL cholesterol lowering of phytosterols. The effect of food matrices

on LDL cholesterol lowering of phytosterol supplementation has since been investigated in a meta-analysis by Abumweis et al. [1] who concluded that phytosterols incorporated into fat spreads, mayonnaise, salad dressing or dairy reduced LDL levels to a greater extent than incorporated into other products such as baked goods and juices. Demonty et al. [11] also reviewed the effect of phytosterol food matrix on cholesterol lowering, high fat to low fat foods and dairy to non-dairy foods were compared and no differences in the absolute or relative dose response curves of the different food formats was seen. These findings of the Demonty et al.[11] and Abumweis et al.[1] meta-analyses are not contradictory because classification of food formats compared differed between analyses.

Phytosterol nutraceuticals are available in various tablets and capsule forms. Clinical trials into phytosterol nutraceuticals are limited, with only 7 trials having published results (Table 2.1). The dosages used in these trials ranged from 0.8-3.0 g/day of varying phytosterols forms, including plant sterol and stanol esters as well as free stanols. Of the 7 studies, 5 studies and one study arm reported significant reductions in LDL cholesterol ranging from -0.22 to -0.65 mmol/L (4-11%), only 1 study and a study arm reported non-significant reductions in LDL cholesterol levels in response to the phytosterol. Denke et al. [12] concluded that the lack of LDL cholesterol lowering seen in response to sitostanol capsules may have been due to the experimental diet being low cholesterol (<200 mg/day) or due to the formulation of the capsule which may not have mixed with the intestinal contents. It has previously been shown that phytosterol supplementation can reduce LDL-cholesterol when supplemented while consuming a low cholesterol diet [4,19,23], therefore, the formulation of the capsule is more likely to have contributed to the lack of effect seen by Denke et al. Mcpherson et al. [30] also reported

Table 2.1 Cholesterol lowering action in phytosterol trials using capsules or tablets

Authors	Study size	Study design	Matrix	Type of phytosterol	Dose free phytosterol	Duration	ΔLDL (mmol/L)[a]
Acuff et al. [54]	$n = 16$	Double-blind, placebo-controlled, sequential study	Capsule	Plant sterol ester	0.8 g/day in 2 capsules	4 wk	−0.34
Carr et al. [55]	$n = 32$	Randomized, double-blind, two-group parallel, placebo-controlled	Capsule	Plant sterol ester of stearate	1.8 g/day in 3 capsules	4 wk	−0.35
Denke et al. 1994	$n = 33$	Sequential study, low cholesterol diet	Capsule	Free Sitostanol in oil	3.0g/day in 12 capsules	1 month	−0.15[b]
Earnest et al. [56]	$n = 54$ (25 active, 29 placebo)	Randomized, double-blinded, parallel, placebo-controlled	Capsule	Plant sterol ester	1.56 g/day of ester in 4 capsules	12 wk	−0.38
Goldberg et al. [57]	$n = 26$ (13 per arm)	Double-blind, placebo-controlled, parallel clinical trial	Tablet	Plant stanol lecithin complex	1.8 g/day in 8 tablets	6 wk	−0.68
McPherson et al. [53]	$n = 27$ per	Placebo-controlled, double-blind	Capsule time release >60 min	Aqueous stanol lecithin complex	1.01 g/day in 8 capsules	6 wk	−0.28
McPherson et al. [53]	$n = 25$	Placebo-controlled, double-blind	Tablet fast dissolve <10 min	Spray dried stanol lecithin complex	1.26 g/day in 6 tablets	6 wk	−0.68
Woodgate et al. [58]	$n = 29$ (14 active, 15 control)	Randomized, double-blind, placebo-controlled	Softgel capsule	Stanol ester	1.6 g/day in 3 capsules	4 wk	−0.39

[a] In cross-over trials ΔLDL = mean LDL end of treatment − mean LDL end of control. In parallel trials ΔLDL = ΔLDL treatment − ΔLDL control.
[b] In Denke et al. mean LDL of preceding and subsequent step one diet phases used as LDL control.

non –significant reductions in LDL-cholesterol in one arm of their study. The time release capsule, which took longer than 60 minutes to dissolve, failed to lower cholesterol while the quick dissolving tablet, which dissolved in less than 10 minutes, lowered LDL cholesterol by 10.4%. The type of phytosterols used in both negative trials was free plant stanols, which has shown LDL lowering efficacy in other vehicles [33], suggesting that failure to interact sufficiently with the mixed micelles in the intestine was due to the delivery matrix contributing to the lack of effect.

Phytosterols have a long demonstrated history of lowering total and LDL cholesterol and are being more widely accepted as an alternative or adjunct to pharmaceuticals in hypocholesterolemic regimes. The range of methods available to incorporate free and esterified phytosterols into new functional foods and nutraceuticals is offering consumers a wider choice in the delivery of phytosterol supplementation and will lead to a increase in use provided that the new products continue to demonstrate cholesterol lowering. When developing new phytosterol enriched functional foods or nutraceuticals it is critical that, regardless of the type and formulation of phytosterol or matrices used, the product is clinically tested to insure that phytosterols are delivered to the gut in such a way that they can effectively compete with cholesterol for absorption. If some or all of the phytosterols delivered to the gut are in a form which cannot interact with mixed micelles and compete with cholesterol for incorporation then the effective dose of phytosterol is lower and the corresponding LDL-lowering efficacy is reduced. If new phytosterol products which ineffectively lower cholesterol are allowed to enter the marketplace with the same health claims as effective products than the perceived health

benefit of phytosterols will be diluted and consumers will resist trying them or discontinue their use.

2.3 Conflict of Interest

DSM has declared no conflict of interest. PJHJ is a consultant for Danone, Unilever, Forbes Meditech, Whitewave and Enzymotec Inc.

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Bridge to chapter III

The following chapter comprises a manuscript which provides an overview of methods available to researchers to estimate or measure cholesterol absorption or synthesis in humans. This chapter looks at the benefits and drawbacks of the various methods available for assessing cholesterol metabolism. These methods could be used to assess the impact of plant sterol consumption on cholesterol metabolism. Dylan S. MacKay was the principal manuscript author and Peter J. H. Jones contributed to the preparation of the manuscript.

Chapter III

Manuscript 2: Literature review

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Evaluation of Methods for the Determination of Cholesterol Absorption and Synthesis in Humans

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3.1 Abstract

Cardiovascular disease risk and its associated complications correlate positively with circulating cholesterol levels. Plasma cholesterol levels are maintained by reciprocally related endogenous cholesterol synthesis and cholesterol absorption from dietary and biliary sources. Numerous *in vivo* clinical methods exist to quantify the absorption and synthesis of cholesterol in humans. This review summarizes these different methods available to study cholesterol absorption and synthesis, highlighting each method's strengths and weaknesses, as well as their applicability in different types of trials.

3.2 Introduction

Elevated cholesterol, particularly low density lipoprotein (LDL) cholesterol, is a well defined risk factor for the development of atherosclerosis (1) through the formation of atherosclerotic lesions (2). Atherosclerosis is associated with cardiovascular disease (CVD), the leading cause of mortality and disability in developed countries. Cholesterol levels are maintained and regulated by cholesterol absorption and synthesis, which have a reciprocal relationship, and by cholesterol elimination into the bile. Therefore, the proper evaluation of absorption, synthesis and trafficking of cholesterol throughout the body is critical to health research.

Plasma cholesterol can be synthesized hepatically or extra-hepatically, or absorbed from the intestine, derived from dietary or biliary sources. Statins, a family of HMG-COA reductase inhibitors, have been shown to effectively reduce cholesterol synthesis, achieving plasma LDL cholesterol lowering of up to 60% (3) and a CVD risk reduction of one-third (4). Although efficacious in lowering LDL-C levels, statin use is associated with adverse events including muscle cramping and rhabdomyolysis (5). Therapies which reduce cholesterol by inhibiting intestinal cholesterol or bile acid absorption are also available. Plant sterols/stanols, dietary fibre, and bile acid sequestrants have been shown to be effective in treating hyperlipidemia, with LDL cholesterol reductions of 10-15%, 8.5-13% and 5-30%, respectively (6). Use of Ezetimibe, a potent cholesterol absorption inhibitor, led to the discovery of the Niemann-Pick C1 Like 1 (NPC1L1) protein and its role in cholesterol absorption (7). NPC1L1, a novel sterol transporter highly expressed in the jejunum, is essential for the absorption of cholesterol. NPC1L1 null mice display reduced cholesterol absorption by upwards of 90%(8). Ezetimibe has

been demonstrated to lower LDL cholesterol levels by 16-19% (9). The wide range of cholesterol lowering response seen after each treatment is likely the product of genetic factors which modify cholesterol synthesis and absorption, as well as modulate the effectiveness of each intervention (10-12). Given this considerable genetic heterogeneity, a need exists for precise measurement of cholesterol synthesis and absorption as well as their response to different dietary, pharmaceutical and lifestyle interventions.

Methods used to elucidate the different facets of cholesterol trafficking have undergone significant evolution since their inception in the late 1950's. As new technologies and instrumentation have been adopted into the older techniques, new methods have evolved which are now available to more safely and thoroughly evaluate cholesterol absorption and synthesis. To comprehend many of the methods used today it is important to understand how older approaches evolved and were validated. The purpose of this review is to describe and systematically compare the various methods previously and currently used to monitor cholesterol synthesis and absorption in humans. These comparisons will aid investigators in the selection of methods most appropriate for a particular application with respect to their advantages, drawbacks and assumptions.

3.3 Methods to assess cholesterol absorption

Direct methods

Direct methods of measuring cholesterol absorption measure cholesterol flux from gut to lymph. Such methods are so termed as the amount of cholesterol absorbed across a section of intestine is assessed directly, without using faecal or plasma levels of cholesterol to infer absorption. The only direct method available for humans is the

intestinal perfusion technique introduced by Grundy and Mok (13). This perfusion method required intubation with a triple-lumen tube. Liquid formula containing a known amount of cholesterol and ß-sitosterol is infused into the duodenum at the Ampulla of Vater through the first tube, while intestinal contents are aspirated from the 2nd and 3rd tube, positioned 10 and 100 cm distal to the first, respectively. Analysis of the aspirate allows for calculation of net hourly cholesterol absorption across a 100 cm gut segment. Discriminating between exogenous and endogenous sources of cholesterol is performed by infusing labelled cholesterol. A drawback is that cholesterol undergoes transformation into other sterol products by gut bacteria, this loss of cholesterol must be accounted for in this method. ß-sitosterol was used as a marker for cholesterol loss in the gut. ß-sitosterol was originally thought to be non-absorbable, unaffected by gut bacteria, and unable to affect cholesterol absorption. However, Grundy and Mok (13) noticed that even small increases in ß-sitosterol concentration used in this method were associated with noticeable reductions in measured cholesterol absorption. It has since been shown that ß-sitosterol and other plant sterols are absorbed in the range of 5 -15% and inhibit cholesterol absorption, even at the concentrations used in this method (13-15). Plant sterols compete with cholesterol for absorption into gut enterocytes, then are largely exported back into gut and liver by ABCG5 and ABCG8 transporters (16). The use of an inhibitor of cholesterol absorption such as ß-sitosterol in a method used to assess cholesterol absorption therefore leads to an underestimation of actual cholesterol absorption capacity. The largest limitation to use of direct measurement techniques is the substantial level of invasiveness involved. Notwithstanding, only direct methods yield

objective measures of total, exogenous and endogenous cholesterol absorption across the intestine (17).

Indirect methods

The following methods infer cholesterol absorption from plasma or faecal values, rather than directly measuring the flux of cholesterol from gut to lymph.

Cholesterol balance methods

Cholesterol balance methods assess absorption as the difference between dietary sterol intake and faecal cholesterol elimination, excluding cholesterol of endogenous origins (18). The technique has typically relied on labelled cholesterol to distinguish endogenous from exogenous sourced cholesterol within the faecal and plasma cholesterol pools measured. Four main balance methods exist, namely Method I, II, III and V , following the nomenclatures from Grundy and Arhens (19).

Method I consists of a single dose of [14C] or [3H] radioactively labelled cholesterol administered intravenously (19,20). This radio-isotope labels endogenous cholesterol and its subsequent steroid products using the formulas:

Formula 1:

$$Daily\ exogenous\ absorbed\ cholesterol\ \left(\frac{mg}{day}\right) =$$

$$daily\ cholesterol\ intake\ \left(\frac{mg}{day}\right) -\ daily\ unabsorbed\ dietary\ cholesterol\ \left(\frac{mg}{day}\right)$$

Formula 2:

$$Daily\ unabsorbed\ cholesterol\ \left(\frac{mg}{day}\right) =$$

$$faecal\ neutral\ steroids\ per\ day\ \left(\frac{mg}{day}\right) -\ endogenous\ faecal\ neutral\ steroids\ per\ day\ \left(\frac{mg}{day}\right)$$

Endogenous faecal neutral steroids are calculated by dividing the total radioactivity (dpm/day) in total faecal neutral steroids by the specific activity (dpm/mg) of plasma cholesterol 1-2 days prior, depending on gastrointestinal transit time, accessed by chromic oxide administration (19,20).

Method II requires continuous oral labelling with radioactive cholesterol (19,20). The radioactively labelled cholesterol is generally incorporated into a liquid diet to ensure a precise daily intake for many weeks. Cholesterol absorption is calculated using the formula:

Formula 3: $Z = X + Y$

$$Z = total\ faecal\ neutral\ steroids\ \left(\frac{mg}{day}\right)$$

$$X = faecal\ neutral\ steroids\ of\ endogenous\ origin\ \left(\frac{mg}{day}\right)$$

$$Y = daily\ unabsorbed\ dietary\ cholesterol\ \left(\frac{mg}{day}\right)$$

Formula 4: $Z \times SA(Z) = X \times SA(X) + Y \times SA(Y)$

$$SA(Z) = \text{specific activity of total faecal neutral steroid } \left(\frac{dpm}{mg}\right)$$

$$SA(X) = \text{specific activity of plasma cholesterol } \left(\frac{dpm}{mg}\right)$$

$$SA(Y) = \text{specific activity of dietary cholesterol } \left(\frac{dpm}{mg}\right)$$

The equations are solved for Y, with absorption measurements assessed at any time after four days of continuous radioisotope feeding. Isotopic steady state is not required. Results for cholesterol absorption measured by Methods I and II yield similar results (19). However, it has been shown that Method II provides more consistent estimates of cholesterol absorption than Method I.

Method III, introduced by Wilson and Lindsey (21), required attaining of isotopic steady state. Continuous feeding with radioisotope labelled cholesterol for greater than 100 days is often required to reach an isotopic steady state. When an isotopic steady state is reached, daily cholesterol turnover is calculated, and daily absorbed dietary cholesterol is arrived at by the formula:

Formula 5:

$$\text{Dietary cholesterol absorption } \left(\frac{mg}{day}\right) =$$

$$\text{daily cholesterol turnover } \left(\frac{mg}{day}\right) \times$$

$$\left(\frac{\text{specific activity of plasma cholesterol}}{\text{specific activity of dietary cholesterol}}\right).$$

Failure to reach isotopic steady state will yield an underestimate of actual dietary cholesterol absorbed (17). The lengthy period required reaching isotopic steady state and

the difficulty determining when this state has been attained renders Method III difficult and unreliable. Method III was shown to give an approximately 20 % lower value of cholesterol absorption than Method II, when compared directly (20).

Method V is a combination of methods I and II (22). Subjects are continuously fed radioactively labelled cholesterol as in Method I, then given a single bolus of different radioactively labelled cholesterol as required in Method II. The equations for Method II are used, with only the modification of the X *$SA_{(X)}$ term which is replaced by

[(R)($SA_{(X\ oral)}$) / ($SA_{(X\ intravenous)}$)], where R= daily faecal neutral steroid excretion of intravenous cholesterol (mg/day), $SA_{(X\ oral)}$ = plasma specific activity of oral isotope taken 1 day before R (dpm/mg), and $SA_{(X\ intravenous)}$ = plasma specific activity of intravenous isotope taken 1 day before R. Method V was designed for unusually high rates of cholesterol synthesis as a result of surgical or pharmacological interference with the enterohepatic circulation, which can lead to endogenous cholesterol being secreted into the gut prior to isotopic equilibration (17).

Methods I, II and V all require use of non-absorbable markers to track gastrointestinal transit time, and for cholesterol loss in the gut. Chromic oxide is often used as such a marker of gastrointestinal transit time. Transit time must be accurately calculated in methods that measure faecal steroid activity to ensure that faeces sampled correspond with the timing of isotope administration. ß-sitosterol was commonly used to control for cholesterol loss in the gut. Loss of cholesterol due to bacterial degradation can be upwards of 25% (23). Therefore if the cholesterol loss in the gut is not accounted for by

these methods, the calculated cholesterol absorption will be higher than its actual value (20).

Isotope ratio methods

Isotope ratio methods are capable of measuring percent, but not net, cholesterol absorption rates (24). Similar to balance methods, isotope ratio methods require accurate records of dietary cholesterol intake to estimate the mass of exogenous cholesterol consumed, then derive the proportion absorbed from the fractional data obtained (18).

Method IV, also termed faecal isotope ratio method, first introduced by Borgstrom (25), calculates cholesterol absorption as the percentage of a single oral dose of radioactively labelled cholesterol not recovered in the faeces. Single doses of both radioactively labelled cholesterol and ß-sitosterol are administered orally, after which faeces are collected for seven to eight days. The ratio of labelled cholesterol to labelled ß-sitosterol in a sample of the pooled faeces samples is compared to the ratio given orally. Cholesterol absorption is calculated using the formula:

Formula 6:

$$Cholesterol\ Absorption\ (\%) =$$

$$1 - \left(\left(\frac{Faecal\ Cholesterol\ (dpm)}{Faecal\ ß{-}sitosterol\ (dpm)}\right) \times \left(\frac{Oral\ Cholesterol\ (dpm)}{Oral\ ß{-}sitosterol\ (dpm)}\right)\right) \times 100$$

Method IV, despite requiring a prolonged period of faecal collection, uses a far smaller dose of radioactivity than do balance methods, and is relatively straightforward and simplistic to execute (17). Method IV does still require a marker of cholesterol loss in the gut, so it can share certain of the drawbacks previously discussed.

The plasma isotope ratio method (Method VI) was first introduced by Zilversmit (26) in rats and subsequently applied to humans (27). Method VI involves simultaneous oral and intravenous administration of [14C] and [3H] radio-labelled cholesterol and requires only a single blood sample 3-4 days afterward afterwards. The methodological principles are based on the measurement of drug absorption used in pharmacology (17). If the absorption of cholesterol was 100% then the specific activity curve of both radiolabelled cholesterol tags would converge, assuming equal doses of the different labels. If absorption is zero, none of the orally administered cholesterol, and therefore zero radioactivity from the oral radioisotope, would appear in the plasma. Since cholesterol absorption falls between zero and 100%, the ratio of the two plasma specific activities, after normalization for dose administered, is used to calculate absorption using the formula:

Formula 7:

$$Cholesterol\ Absorption\ (\%) = 100 \times \left(\frac{\%\ oral\ dose\ in\ plasma}{\%\ IV\ dose\ in\ plasma} \right)$$

where % oral dose and % I.V. dose in plasma are the percentage of IV and oral tracer in the plasma sample, respectively (28) . The ratio is calculated 3-4 days after radioisotope administration to enable equilibration of each tracer, as the isotopes distribute across body sterol compartments and commence being lost at equivalent rates. Curiously, a delay of up to 48 h occurs in the peak appearance of oral cholesterol isotope in the blood of humans, likely related to the multiple sub-pools associated with the process of cholesterol absorption (17). The advantages of Method VI over alternative approaches include that: i) only a single blood sample is required, ii) a low dose of radioisotope is

required, iii) faecal collections are avoided, and iv) the method does not depend on markers such as ß-sitosterol to correct for faecal losses. Method VI lends itself to repeated use because of its short duration and low level of labelling. Method VI allows for investigation of cholesterol absorption under different experimental parameters in the same individual within a comparatively short time frame. Method VI has been validated in humans numerous times against Method IV under different conditions yielding similar results. Samuel *et al.*(27) comparing Method VI to Method IV in 12 hospitalized individuals showed that results from Method VI correlated with Method IV (r=0.83) and that results from both methods agreed within 5%. Samuel *et al.* (28) further validated Method VI against Method IV in an additional 8 individuals, demonstrating a level of accuracy of 3.5% at a 95% confidence level.

A third isotope ratio method, the continuous isotope feeding method (Method VIII) introduced by Crouse and Grundy (29) is similar to Method IV since average cholesterol absorption is calculated using the ratio administered cholesterol to ß-sitosterol measured in the faeces, but differs in method of isotope administration. [14C] Cholesterol and [3H] ß-sitosterol are administered orally three times daily over 10 days, with faeces collected from days 4-10 (17,18). Following day 3 of isotope administration the ratio of isotopes in the faeces becomes essentially constant and cholesterol absorption becomes calculable by the formula:

Formula 8:

$$Absorption\ (\%) = 100 \times \left(\frac{Faecal\ cholesterol\ (dpm)}{Faecal\ sitosterol\ (dpm)} \Big/ 1 - \frac{dietary\ cholesterol\ (dpm)}{dietary\ sitosterol\ (dpm)}\right)$$

Method VIII requires knowledge only of the ratio of radioactivity within a single faecal sample; faecal mass need not be calculated (29). Since ß-sitosterol is also administered with the labelled cholesterol, Method VIII may underestimate actual cholesterol absorption.

Plasma cholesterol specific radioactivity following the consumption of a test meal containing radioactively labelled cholesterol (hereafter called Method VIIII) has also been investigated as a novel measure of cholesterol absorption. Lin *et al.* (30) assessed cholesterol absorption in 11 individuals with Smith-Lemli-Opitz syndrome (SLOS), a cholesterol synthesis disorder, and compared Method VIIII with cholesterol absorption measured by Method IV. These investigators sampled blood at 24 and 48 hours following radioisotope enriched tests meals and calculated the specific radioactivity of cholesterol in plasma. Values obtained correlated well with those calculated by Method IV ($r=0.594$, $p=0.009$, and $r=0.474$, $p=0.047$ over 24 and 48 hours, respectively). While Method VIIII fails to calculate cholesterol mass or percent absorption, it does allow for investigation of relative changes in cholesterol absorption within an individual following different interventions. Method VIIII requires only a single blood sample, and no stool collection. Method VIIII is very similar to the single isotope tracer method that will be discussed in the stable isotope methods section below (31). The robustness of the relationship between plasma radioactivity at 24 hours and cholesterol absorption, however, requires further validation, especially in healthy individuals.

While use of radio-isotopes has been invaluable in the investigation of cholesterol absorption, the advent of safer stable isotope laboratory techniques, and developments in isotope ratio mass spectrometry, has propelled the field much further (24). The switch to

stable isotopes has reduced logistic difficulties related to containment, handling, disposal and overall safety associated with radioisotopes, allowing for investigation of cholesterol absorption in certain populations including children, pregnant and lactating mothers, which had previously been excluded due to ethical considerations around radio-isotope administration.

Stable isotope methods for cholesterol absorption

Cholesterol labelled with stable isotopes has been shown to possess identical kinetics as radio-labelled cholesterol (24), leading to the development of stable isotope techniques to investigate cholesterol absorption. Lutjohann *et al*. (32) introduced a stable isotope version of Crouse and Grundy 's (29) Method VIII (continuous isotope feeding) discussed above. [2H6] cholesterol and [2H4] sitostanol were used in place of [14C] cholesterol and [3H] ß-sitosterol, quantified using gas-liquid chromatography combined with selected ion monitoring mass spectrometry. Cholesterol absorption was calculated as in Method VIII. The stable isotope method was twice compared to the radio isotope Method VIII, in six monkeys, yielding similar results. The stable isotope method produced an absorption range of 49-73% (mean of 60%), with coefficients of variation ranging from 3.9%-15.1% (mean 7.1%). The radioisotope produced a range of 51-69% (mean 61%) with coefficients of variation ranging from 1.9-13.6% (mean 5.1%) (32). The stable isotope Method VIII also demonstrated good reproducibility, with the measured mean cholesterol absorption across 7 individuals being identical two weeks apart (32). These similar results demonstrated that the stable isotope Method VIII was as effective as the radio-isotope Method VIII, without the risk to radioactive exposure of subjects and research staff. This method uses sitostanol, rather than β-sitosterol, as a marker for faecal losses of

cholesterol. The use of sitostanol rather than β-sitosterol is an improvement as sitostanol is absorbed to a much lower extent than β-sitosterol, and the sitostanol is given at a dosage that is unlikely to affect cholesterol absorption (14,15,32,33).

The plasma isotope ratio method (Method VI) (26,28) was also adapted to use stable isotopes. Bosner *et al*. (34) used [2H] labelled oral and [13C] labelled IV cholesterol to calculate the plasma stable isotope ratio and cholesterol absorption percentage. The Bosner *et al*. method uses gas-chromatography – mass spectrometry with selected ion monitoring (GC/MS-SIM) or GS/MS- chemical ionization mode (CI) to determine isotopic enrichment. This stable isotope adaptation of Method VI demonstrated good reproducibility, repeat measures of cholesterol absorption were performed 4-6 weeks apart in 5 individuals, yielding a standard deviation between tests of 2.8% (34). Bosner *et al.* (35) further modified Method VI to a single isotope dual tracer method, using oral [2H]5 and IV [2H]6 cholesterol. Isotope detection in plasma cholesterol was done by GC/MS- selected mass monitoring. Jones *et al.* (36) were the first to use isotope ratio mass spectrometry to determine isotopic enrichment using the plasma isotope ratio method. Oral [13C] and IV [3H] cholesterol were administered to 15 hypercholesterolemic men, followed by blood sampling 2-3 days post administration. Free cholesterol from red blood cells was purified by thin layer chromatography and subsequently combusted to yield carbon dioxide and water. The CO_2 was then measured for [13C] enrichment against the international standard Pee Dee Belemite (PDB) utilizing an isotope ratio mass spectrometer (IRMS). Water samples were reduced to hydrogen gas via zinc reduction and 3H enrichment was measured against Standard Mean Ocean Water (SMOW) international standard by IRMS. The ratio of plasma enrichment of [13C] to

[2H] cholesterol on 72 hours following tracer administration was used to calculate cholesterol absorption. The plasma ratio is measured 72 hours after isotope administration, similarly to the radio-isotope Method VI, to allow equilibration of oral isotope in the plasma (36). Recently, continuous flow gas chromatograph combustion IRMS (GC/C/IRMS) systems for [13C] cholesterol and gas chromatograph pyrolysis IRMS (GC/P/IRMS) systems for [18O] or [2H] cholesterol have been used in calculating cholesterol absorption(37,38). The use of IRMS vs GC/MS-SIM has vastly increased the precision of this method. These plasma stable isotope ratio techniques, and the Zilversmit method using radioisotopes (26,27), also estimate cholesterol turnover in the main M1 free cholesterol pool (39,40) from the decay curve of the intravenously injected radio or stable isotope (36). The additional information regarding cholesterol turnover yielded by these methods make them desirable choices in situations when shifts in cholesterol turnover as well as absorption may be of interest.

Stable isotope versions of Method VIII and Method IV have not been compared against each other; both have been validated against their corresponding methods using radio-isotopes. These methods are two of the most widely used methods for accessing cholesterol absorption and merit direct comparison.

Two single stable isotope methods for assessing cholesterol absorption have been introduced by Ostlund *et al.* (41) and Wang *et al.* (31). Ostlund *et al.* (41) administered [2H] cholesterol to volunteers and measured the average oral cholesterol tracer in plasma ((mmol [2H] cholesterol/mol natural cholesterol) in blood samples taken 4 and 5 days post tracer administration using GC/MS. Wang *et al.* (31) administered [13C] cholesterol orally followed by blood sampling at 24, 48, 72 and 96 hours. 13C

enrichment in plasma free cholesterol was measured using GC/C/IRMS. Average [13C] enrichment from 24-96 hours and area under the curve (24-96 hours) of [13C] enrichment were compared to cholesterol absorption percentage assessed using the stable isotope Method VI (34) in 2 studies. Both the average and area under the curve of [13C] enrichment in plasma free cholesterol correlated with cholesterol absorption percentage measured by stable isotope method VI (r values ranging from r=0.81, p=0.0001 to r=0.88, p=0.0001)(31). Both these single isotope methods can be employed to compare treatment effects, such as pharmaceutical or dietary interventions, relative to controls, on cholesterol absorption.

Indirect methods of assessing cholesterol absorption all assume that endogenous cholesterol is excreted via bile. However, it has been demonstrated by Simmonds *et al. (42)* in a study using the intestinal perfusion Method VII that direct trans-intestinal cholesterol excretion (TICE) occurs in the proximal jejunum. The net contribution of TICE to faecal neutral sterol loss has not been quantified in humans (43). In mice, TICE has been shown to decrease along the length of the small intestine (proximal > medial >distal) and depends on the presence of a cholesterol acceptor in the lumen (44). The impact of TICE on the indirect methods for measuring cholesterol absorption has not been investigated, however, TICE would be expected to parallel biliary cholesterol secretion. Method VI (plasma dual isotope ratio) would be able to adjust for TICE, as the isotope administered via intravenously would be lost via biliary and TICE routes. Since Method VI has demonstrated excellent agreement with method IV (27,28), which has itself been validated against other methods (20), the impact of TICE on the indirect methods of cholesterol absorption is likely small, provided the cholesterol lost via TICE

originates from the main (M1) plasma pool of cholesterol. The impact of re-secretion of isotopically labelled cholesterol via TICE in all these indirect methods is also unknown. However, this re-secreted isotopically labelled cholesterol would hypothetically be available for immediate re-absorption, in which case the re-secretion of isotopically labelled cholesterol would have a minimal impact on the overall measure of cholesterol absorption between the gut lumen and the main (M1) plasma cholesterol pools.

Absorption surrogate method

The use of serum plant sterol levels to predict cholesterol absorption was first proposed by Tilvis and Miettinen (45). These researchers showed that serum levels of campesterol, when normalized for total serum cholesterol, correlated positively with cholesterol absorption as measured by Method VIII. This approach involves lipid extraction from a single blood sample followed by evaluation of serum plant sterol and cholesterol levels using GCFID, HPLC or GCMS methods. The use of the ratios of campesterol, cholestanol, or ß-sitosterol relative to cholesterol has been subsequently employed numerous times to measure cholesterol absorption (33,46-51). Miettinen *et al.* (51) demonstrated that baseline cholestanol levels, as a measure of cholesterol absorption, were predictive of an individual's response to statin treatment. Nissinen *et al.* (47) showed that ß-sitosterol to cholesterol ratios were more strongly correlated with cholesterol absorption than were the ratios of campesterol or cholestanol relative to cholesterol, as measured by Method VIII, across three diets varying in both cholesterol and lipid levels in 29 healthy male volunteers. When using serum plant sterols as surrogates for cholesterol absorption it is imperative that factors which are known to change serum plant sterol levels be controlled for, so as not to identify changes in

cholesterol absorption which may not exist (52). Serum plant sterol levels vary substantially within and across different populations, with the greatest variability being attributed to the method used to measure them, however, genetics, gender, diet and disease also play a factor (53). Recently it has also been shown that serum plant sterol concentrations fail to accurately reflect cholesterol absorption in individuals with Smith-Lemli-Opitz syndrome (54). Therefore, the use of serum plant sterols as surrogates for cholesterol absorption should be carefully verified and validated prior to its use within a particular population. Use of plant sterol surrogates does benefit from relative speed and simplicity compared to other previous discussed methods (18,24,33). Furthermore this approach represents the only estimate of cholesterol absorption which can be done in large scale studies.

3.4 Methods of assessment of cholesterol synthesis

Cholesterol synthesis contributes substantially more to circulating cholesterol pools than cholesterol absorption, yielding approximately 700-900 mg a day (24,55). This synthesis has been shown to undergo diurnal periodicity, maintaining cholesterol levels during fasting (56). Accurate assessment of cholesterol synthesis is essential to the field of cholesterol research.

Balance method

Cholesterol synthesis can be estimated when intake of dietary cholesterol and excretion of total cholesterol are known during metabolic steady state (19). The criteria needed for this metabolic steady state are constant plasma cholesterol and faecal cholesterol excretion levels during a period of constant weight. In such steady state conditions,

cholesterol synthesis can be taken as the difference between cholesterol excretion, both faecal neutral sterols and bile acids, and assessed dietary intake. Dietary cholesterol intake must be accurately measured and faeces reliably collected over the experimental period for the method to yield accurate data. Faecal flow must also be monitored with oral administration of a marker such as $Cr_2O_{3,}$ to assure faeces collected stems from the experimental periods. Faecal neutral sterols and bile acids are measured in faeces, typically by GC-FID or GC-MS, and cholesterol synthesis for a given period of time is calculated (33,57,58). Although this method represents the gold standard for calculating cholesterol synthesis, it is vulnerable to errors in the estimation of both dietary cholesterol intake and faecal excretion, both of which can potentially cause significant errors. The balance method determines the actual mass of cholesterol synthesized during a given period. However, the technique does require metabolic steady state, as well as accurate measurement of cholesterol intake and a need for comprehensive faecal collection across the period of interest. Therefore, this method is not optimally suited for larger trials.

Fractional conversion of squalene

Daily cholesterol synthesis rate can be also estimated as the fraction of infused radiolabelled mevalonic acid converted to cholesterol (59,60). Here, dosages of both [14C] mevalonic acid and [3H] cholesterol are administered intravenously. It is then assumed that the infusion of labelled mevalonic acid rapidly labels the plasma squalene pool, reaching a maximum enrichment within approximately 100 minutes. Cholesterol synthesis is subsequently estimated through measurement of squalene synthesis. Squalene synthesis is calculated by the percentage of mevalonic acid dose converted to cholesterol,

divided by the area under the curve of plasma squalene specific activity. This method assumes that plasma squalene synthesis is equivalent to cholesterol synthesis, which may not always be the case. Cholesterol synthesis rates estimated using this method have been shown to agree with cholesterol synthesis calculated by the balance method within 8% (60). Benefits of this method are that the protocol requires only 1 hour of a participant's time, and can be repeated every 3 weeks (59). The method does, however, require the administration of intravenous radio-isotopes.

Mass isotopomer distribution analysis (MIDA)

Mass isotopomer distribution analysis (MIDA) measures the synthesis of several biological polymers *in vivo* (61) . The technique uses the relative abundance pattern or distribution of polymer species which differ only in mass of isotopomers produced during the administration of stable isotope labelled precursors. The distribution of the polymer species produced is compared to theoretical distribution patterns predicted by binomial and polynomial expansion. Using these theoretical distributions, parameters such as fractional synthesis rate can be calculated by combinatorial probability modelling. Since cholesterol is synthesized from subunits of acetyl-CoA, fractional synthesis of cholesterol can be calculated during the infusion of 13C labelled acetate (62). This method is invasive, requiring a 24-hr intravenous infusion and serial blood sampling from an indwelling catheter (63). Cholesterol in the blood samples is measured by GC-MS to determine the distribution pattern of isotopomers from which the rate of synthesis is obtained. The data analysis relies on more complex mathematical modelling constructs than do other methods for estimating cholesterol synthesis. The FSR measured by MIDA

agrees well with FSR measured by deuterium incorporation, showing a significant correlation (r=0.84, p=0.0007) across both techniques in 12 subjects (63).

Deuterium incorporation

This method is based on the tritiated water uptake method by Dietchy and Spady (64) used originally in animals. Deuterium incorporation methodology uses water as a tracer to determine the synthesis of free cholesterol (FC). The fractional synthesis rate (in pools/day) of free cholesterol is calculated from the rate of incorporation of deuterated water into *de novo* synthesized plasma or erythrocyte cholesterol. Orally administered deuterated water equilibrates across the body water pool as well as with intracellular NADPH pools. Body water and NADPH exist as the precursors for 22 of the 46 hydrogens in synthesized cholesterol (65). Deuterium enrichment of the precursor pool, plasma water, and in erythrocyte or plasma cholesterol is measured by IRMS. Fractional synthesis rate of free cholesterol (FSR-FC) is calculated using the following formula:

Formula 9:

$$FSR - FC\left(\frac{pools}{day}\right) = \left(\frac{\delta\ cholesterol\ ‰}{\delta\ plasma\ water\ ‰ \times 0.478}\right)$$

Where δ refers to the change in deuterium enrichment over 24 hours and 0.478 is the ratio of cholesterol from body water and NADPH to total hydrogen in a cholesterol molecule, or the ratio of hydrogen which could be enriched by oral D2O administration (66). From the FSR-FC the ASR -FC (g/day) can be calculated by multiplying the FSR-FC by an estimate of the M1 pool size (40) and 0.33 the proportion of FC in total

cholesterol. The ASR-FC approximates the daily production of newly synthesized cholesterol.

Three main assumptions required for deuterium incorporation include: i) that the fraction of hydrogen derived from plasma water (22/46, or 0.478) is constant in *denovo* synthesized cholesterol; ii) that *de novo* synthesized free cholesterol rapidly exchanges between the site of synthesis and the major free cholesterol (M1, plasma compartment) pool, and within this pool cholesterol migrates rapidly between cellular membranes and lipoproteins as well as between different classes of lipoproteins; iii) that deuterium uptake into free cholesterol of the major pool of cholesterol reflecting synthesis and iv) that synthesis of cholesterol in the major pool provides a reasonable measurement of total cholesterol synthesis as most sterol synthesis occurs in the gut and liver which contribute to the plasma cholesterol pool. It must be acknowledged that the major plasma pool of cholesterol is at equilibrium with two other slow turnover pools outside the plasma, and that the slow inter-pool cholesterol exchange could cause insignificant entry of labelled free cholesterol into the central pool within a 24 hour time period (64,65). Although these assumptions are not without imperfection, the cholesterol synthesis estimates yielded by deuterium incorporation have been sensitive enough to show differences in cholesterol synthesis due to genetic factors, as well as dietary and pharmaceutical interventions. Deuterium incorporation has also been shown to correlate well with cholesterol synthesis measured by the balance method (57), MIDA (63) and cholesterol synthesis surrogate levels (67).

Cholesterol synthesis precursors

Concentrations of plasma intermediates along the synthesis pathway of cholesterol represent another approach to indirectly measuring cholesterol synthesis. Squalene (68), mevalonic acid (69), lanosterol, desmosterol and lathosterol have all been variously used as surrogates for cholesterol synthesis (67,70). Such precursors have been shown to fluctuate with diurnal cholesterol synthesis patterns, as well as in conditions in which cholesterol synthesis is elevated or reduced (68). Levels of these precursors correlate closely with measured cholesterol synthesis when they are normalized for plasma cholesterol level, then when taken the absolute amount of precursor and are normally expressed in mmol/mol cholesterol (33,47,70). When using cholesterol precursors as surrogates for cholesterol synthesis, dietary intakes of each precursor should be controlled; this is particularly important for squalene, which is abundant in olive oil and is at least partially absorbed into the blood (14). The most apparently reliable surrogate for cholesterol synthesis has been lathosterol (47,58). Since cholesterol synthesis surrogates require only one blood sample they are ideal for estimating cholesterol synthesis in large cross sectional studies or in epidemiological trials.

Combined cholesterol absorption and synthesis technique

Advancements in quantification of cholesterol homeostasis must still be continued with the goal of finding more accurate methods to measure cholesterol synthesis and absorption simultaneously. The ability to simultaneously measure changes in cholesterol absorption and synthesis is a valuable asset, particularly in investigating the reciprocal relationship between absorption and synthesis. At present, the plasma dual isotope method described by Bosner *et al.* (34) can be applied in concert with the deuterium incorporation approach (65) to measure cholesterol absorption, turnover and synthesis in

a relatively short period of time (5 days)(37). However, in this method as demonstrated by Jones *et al.* (36), cholesterol synthesis is measured directly following assessment of absorption and turnover, not at the same time. It is possible to measure synthesis and absorption of cholesterol simultaneously using a triple stable isotope technique which combines the plasma dual isotope method and deuterium incorporation techniques(37). In this combined technique 18O-cholesterol or 13C cholesterol is administered intravenously, while the other isotopically labelled cholesterol and D2O is administered orally (37,71). This method yields absorption and synthesis measures from the same time frame and with some modifications could also measure cholesterol turnover simultaneously using the depletion of the I.V administered isotope (36).

3.5 Conclusion

Methods used to quantify cholesterol absorption and synthesis reviewed in this article yield invaluable information, as well as provide effective means of measuring the experimental effects of various dietary, physiological and pharmacological interventions on whole body cholesterol trafficking and homeostasis. Methods have evolved substantially over the years, from radio-isotopes to stable isotopes, and from highly invasive to less invasive procedures. Advantages and drawbacks, as well as the type of information yielded by each approach, should be weighed carefully when selecting an appropriate method. The cost and available technical expertise and facilities will also limit which methods are available to each investigator. In sum, a veritable tool box of techniques that possess relative strengths and weaknesses are available to investigators, and from this array we must apply the most appropriate methods for each particular experimental question.

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Bridge to chapter IV

The following chapter comprises a manuscript which provides a review of the current use of non-cholesterol sterols, introduced in chapter III, as surrogate markers for the estimation of cholesterol absorption and synthesis. These surrogate markers were used in the design of the nutritional trial which is presented in chapter VI, VII and VIII. Dylan S. MacKay was the principal manuscript author, and Peter J. H. Jones contributed to the preparation of the manuscript.

Chapter IV

Manuscript 3: Literature review

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Plasma non-cholesterol sterols: Current uses, potential and need for standardization

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4.1 Abstract

Purpose of review: Non-cholesterol sterols (NCSs) in plasma encompass endogenous cholesterol precursors and exogenous phytosterols and cholesterol metabolites and are used as surrogate measures of cholesterol synthesis and cholesterol absorption, respectively. The ratios of cholesterol synthesis to cholesterol absorption surrogates are also utilized to assess the overall balance of cholesterol metabolism, with higher values representing more synthesis and lower more absorption. The objective of this review is to focus on recent findings using plasma NCSs and their potential in customizing dietary and pharmacological hypolipidemic therapies.

Recent findings: NCSs are often used to assess the impact of pharmacological and dietary interventions on cholesterol synthesis and absorption. Various forms of dyslipidemia have been characterized using NCSs, and NCSs may be a valuable tool in selecting appropriate treatment therapies. NCSs levels are affected by genetic, dietary and physiological factors and have been related to cardiovascular disease risk.

Summary: The expanded use of plasma NCSs is currently limited by the lack of standardized methodology. However, non-cholesterol sterols are still a valuable research tool for the overall assessment of cholesterol metabolism and may have clinical potential in the personalization of diet and medicine.

Keywords: lathosterol, desmosterol, sitosterol, campesterol, cholesterol, non-cholesterol sterols

4.2 Introduction

The relationship between cardiovascular disease (CVD) and elevated cholesterol, although often debated, is still widely viewed as important and lowering LDL cholesterol levels is the basis for most CVD primary treatment and prevention strategies (1). Cholesterol in the body has two potential origins, via endogenous synthesis or through absorption of biliary and dietary cholesterol (2). Non-cholesterol sterols (NCSs) in plasma could refer to any sterol other that cholesterol; however, generally NCSs refer to the sterol precursors on the cholesterol synthesis pathway, phytosterols of plant origin and certain cholesterol derivatives (Figure 4.1). Plasma NCSs are almost always reported as a ratio to cholesterol to normalize differences caused by variable levels of lipoproteins which transport cholesterol and non-cholesterol sterols (3). NCSs are used as surrogate markers of cholesterol synthesis and cholesterol absorption as their levels have been shown to correlate with cholesterol synthesis and absorption measured using the gold standard radio and stable isotopic methods (4, 5). However, in a recent review Miettinen et al. (6) cautioned against the assumption that NCS surrogate markers of cholesterol metabolism are valid in all circumstances and recommended measuring numerous NCS to use as surrogates and that absolute measures of cholesterol metabolism be measured whenever possible. The use of surrogate markers is qualitative in nature and cannot replace or yield the quantitative data derived by isotopic methods. However, compared to the more complicated and exact isotopic measures of cholesterol synthesis and absorption, the assessment of NCSs is affordable and can be performed on large populations (7). This has led to the wide use of NCSs to measure and characterize cholesterol metabolism across numerous genetic, dietary and physiological variables.

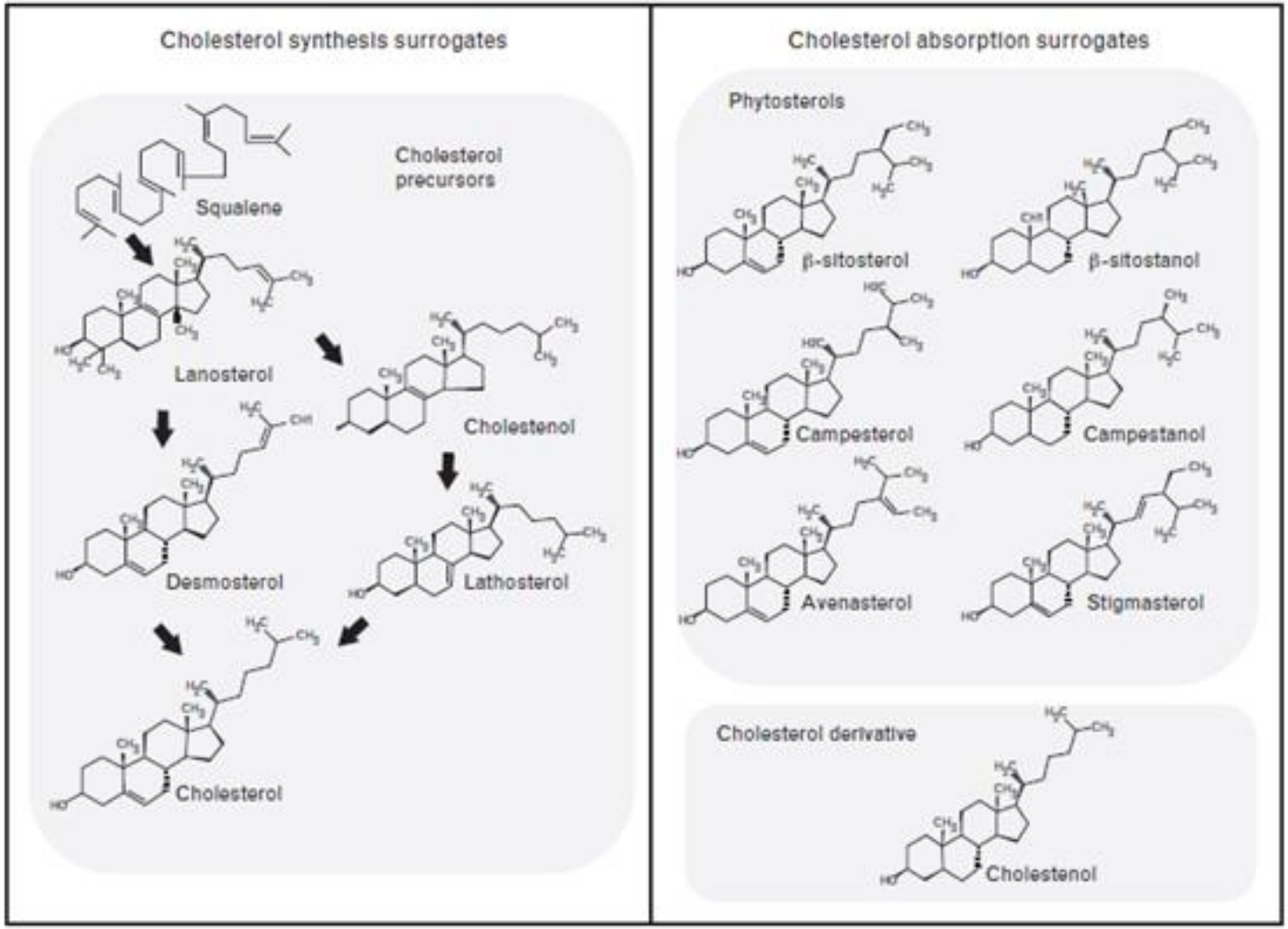

Figure 4.1 Common noncholesterol sterols used as cholesterol synthesis or absorption surrogates.

This paper will review recent findings made using NCSs and discuss the need for standardization in the measurement of NCSs.

4.3 Surrogates of cholesterol absorption and synthesis in clinical trials

Serum or plasma levels of cholesterol precursors, phytosterols and cholestanol are often measured in clinical trials to assess the impact of various treatments on cholesterol synthesis and absorption (7). The effect of statin and ezetimibe on cholesterol synthesis and absorption measured by NCSs has been extensively reviewed by Descamps et al. (8). Statins have demonstrated a consistent ability to increase cholesterol absorption, which correlates with a reduction in cholesterol synthesis, in response to supplementation, while ezetimibe supplementation consistently increases cholesterol synthesis surrogates.

Effects of dietary interventions on cholesterol metabolism can also be monitored using NCSs. Athyros et al (9) conducted a randomized placebo controlled clinical trial comparing the effects of Mediterranean or plant stanol supplemented diets on estimated CVD risk. Serum lathosterol was measured as a surrogate for cholesterol synthesis and campesterol and sitosterol as surrogates of cholesterol absorption. Since this trial included a plant stanol treatment arm, sitostanol and campestanol were also measured to monitor increases in these phytosterols in response to supplementation. The Mediterranean diet resulted in a similar estimated CVD risk reduction as plant stanol supplementation after 4 months, but had no significant impact on lathosterol, campesterol or sitosterol, whereas the plant stanol supplementation significantly increased lathosterol (+21%) and decreased campesterol and sitosterol (-32 and -52 %, respectively), suggesting a decrease in cholesterol absorption and an increase in synthesis. Campestanol

and sitostanol levels also increased (42 and 46%, respectively) in response to the plant stanol supplementation. Ramos et al. (10) investigated the effects of fiber supplementation on cholesterol synthesis and absorption during effective lipid lowering therapy of statins and/or ezetimibe. Changes in cholesterol synthesis were measured using desmosterol and in cholesterol absorption using campesterol and sitosterol. NCSs were reported as absolute values, not in ratios to cholesterol. Fiber supplementation on top of effective pharmacological lipid lowering decreased campesterol and sitosterol. The authors concluded that fiber is an effective and safe adjunct therapy to reduce the absorption of phytosterols which increase with statins. Miettinen et al. (11) used NCSs in serum and in arterial tissue to measure the impact of plant stanol compared to plant sterol supplementation on cholesterol metabolism in statin treated patients undergoing carotid endartectomy. Cholesterol metabolism was assessed using the ratio of synthesis surrogates (lathosterol) to absorption surrogates (campesterol, sitosterol and cholestanol). Plant stanol and sterol supplementation both lowered LDL cholesterol (-17.2 % and 13.8%, respectively) but had opposite effects on campesterol and sitosterol concentrations, with stanols lowering and sterols raising serum levels. Arterial tissue levels of NCSs were not significantly different between stanol and sterol groups. The ratios of lathosterol/campesterol and lathosterol/sitosterol were higher for plant stanol than sterol groups for both serum and arterial tissues. This difference was due to the intake of campesterol and sitosterol in the plant sterol margarine. Cholestanol levels were not significantly different between plant stanol or sterol groups, suggesting that they had similar impacts on cholesterol absorption.

Considerable interest has been directed at use of NCS in situations where plant sterol and stanol levels vary. The use of phytosterols such as campesterol and sitosterol as absorption surrogates during increased intake of phytosterols either through supplementation or diet should be avoided as the increase in campesterol and sitosterol will be in response to the supplementation itself, and not to an increase in cholesterol absorption (12, 13). Cholestanol is a better surrogate in these circumstances as it is not a phytosterol.

Serum phytosterols are often measured during phytosterol supplementation to monitor compliance. In a randomized controlled trial looking at the effects of combining foods with recognized cholesterol lowering properties, a dietary portfolio, which included plant sterol margarine total, phytosterols were measured at the end of the dietary intervention. Both intensities of dietary portfolio lowered LDL cholesterol (-13.8 and -13.1 % for intensive and routine portfolio, respectively) and increased phytosterol levels compared to the control (14).

Although in smaller clinical trial populations it may be possible to measure cholesterol absorption or synthesis directly using a balance or stable isotope technique, the use of NCSs is still the best option for the majority of trials (7). However, it is important to establish that the NCSs used as surrogate measures of cholesterol metabolism are appropriate for each intervention. Restraint must also be maintained when interpreting changes in surrogate measures in response to a clinical intervention, especially changes in ratios of one surrogate to another.

4.4 Cholesterol metabolism in dyslipidemias

Non-cholesterol sterols have been used to characterize the cholesterol metabolism phenotype of various diseases and conditions. Different forms of dyslipidemia have been assessed to see if they differ in cholesterol synthesis or absorption using NCSs as surrogates (2, 15, 16). Familial combined hypercholesterolemia (FCH), which is seen in 10-30% of premature coronary heart disease (CHD) cases, presents with increased circulatory cholesterol, triglycerides and apoB levels, believed to be due to an overproduction of apo B-100 rich lipoproteins in the liver (2). Cholesterol metabolism in FCH has been characterised using NCSs as represented by elevated synthesis and decreased absorption of cholesterol than in controls (2). Polygenic hypercholesterolemics (PH), who present with elevated LDL levels and normal triglyceride and HDL levels have been characterised as having higher absorption of cholesterol, compared to control and FCH, by NCSs. Congruently, PH respond well to dietary hypercholesterolemic interventions (2). Non-familial hypercholesterolemia autosomal dominant hypercholesterolemia (non-FHADH) also shows a lowered synthesis (lathosterol) and elevated absorption (campesterol, sitosterol and stigmasterol) of cholesterol, suggesting non-FHADH may be due to potential defect in cholesterol absorption (17). The NCS phenotypes have been proposed as a means to differentiate different hypercholesterolemias, particularly FCH from FH (17). NCSs may also have potential in the diagnosis of paediatric hypercholesterolemia (18). The early diagnosis of genetic hypercholesterolemia is strongly recommended so that early lifestyle, dietary and pharmacological treatments can be started to prevent cardiovascular events. Noto et al. (2010) investigated the use of NCSs in the diagnosis of hypercholesterolemia in children

(18). Autosomal dominant hypercholesterolemics (ADH), FCH and PH were assessed for their NCSs phenotype. The NCS phenotypes were then used along with lipid values in a post-hoc discriminant analysis to separate individual types of hypercholesterolemias and were able to correctly classify all ADH patients, whereas the discriminant analysis using only lipid values could only classify 73% of ADH patients correctly.

The ability of NCSs levels to predict primary hypobetalipoproteinemia (pHBL), characterised by total cholesterol levels $<5^{th}$ percentile, has also been investigated (19). Patients with pHBL were compared to non-pHBL low cholesterol patients and normal cholesterol controls, pHBL patients showed elevated cholesterol absorption (campesterol/cholesterol) compared to low cholesterol patients. The use of NCSs in a discriminant analysis, however, was not able to distinguish pHBLs patients from low cholesterol controls. Therefore NCSs may have benefit in distinguishing between some forms of dyslipidemia, but in pHBL NCSs do not provide any additional diagnostic benefit.

4.5 Cholesterol metabolism in metabolic syndrome

Metabolic syndrome (MS) is a clustering of visceral obesity, hypertension, low-HDL, high triglycerides and impaired fasting glucose, symptoms which are strongly associated with insulin resistance and is diagnosed according to NCEP:ATPIII by the presence of 3 or more of the 5 characteristics (20). MS greatly increases the risk of diabetes and CVD. Metabolic syndrome and its components, insulin resistance and type 2 diabetes have all been associated with increased synthesis and reduced absorption of cholesterol as detected using NCSs (2). Patients with MS have been shown to have higher lathosterol

and lower campesterol levels compared with healthy controls (21), and in a cross-sectional study high lathosterol levels correlated with features of MS (22). Visceral fat area (VFA), measured by MRI, is a measure of visceral obesity and has been shown to correlate positively with desmosterol and lathosterol and negatively with cholestanol in high risk vascular (23) and dyslipidemic patients (24) . VFA, which is an independent predictor of CVD, correlated negatively with HDL and positively with triglycerides and VLDL cholesterol, but unexpectedly showed a negative correlation with LDL cholesterol, suggesting a possible loss of association between LDL and CVD risk in obesity (23). Healthy participants with low HDL, a component of MS, were shown to have higher lathosterol and lower campesterol and sitosterol than age and gender paired participants with high-HDL (25). The activities of LCAT and CETP which are involved in the reversed cholesterol transport did not correlate with any of the plasma sterol markers measured. HDL levels in the study population were inversely related to measures of insulin resistance in spite of the fact that participants with metabolic syndrome were excluded. Insulin resistance has been associated with increased desmosterol and lathosterol, and type 2 diabetics have been shown to have significantly higher synthesis and lower absorption markers of cholesterol than non-diabetic controls (2). This increase in synthesis in relation to insulin resistance is likely due to elevated insulin levels which drive cholesterol synthesis via LXR (26)and SREBP-2 (27). Insulin resistance is a hallmark non-alcoholic fatty liver disease (NAFLD). The cholesterol metabolism in NAFLD has been assayed using NCSs, showing an increase in cholesterol synthesis (desmosterol and lathosterol) and a decrease in cholesterol absorption (cholestanol, campesterol and sitosterol) compared to controls, independent of BMI (28).

4.6 Non-cholesterol sterols to guide hypolipidemic treatment

The ability to use NCSs to assess cholesterol metabolism in different populations may be beneficial in selecting the appropriate hypolipidemic treatment. Populations with high synthesis and low absorption of cholesterol may be best served with statin therapy, whereas populations with low synthesis and high absorption may benefit more from ezetimibe, bile acid binding resin or phytosterols (29). Populations with a balance of both synthesis and absorption of cholesterol could require a combination of statins and cholesterol absorption lowering therapy. Poor responders to statins have been shown to have lower lathosterol/cholesterol, lathosterol/cholestanol, desmosterol/cholesterol and desmosterol/cholestanol baseline levels than good responders (30). This lower synthesis/higher absorption phenotype at baseline was also shown to be associated with recurrence of cardiovascular events in a subgroup of the 4S trial (31). In this subgroup serum cholesterol was not lowered as much as in individuals with a better prognosis. However, the association between poor response to statins and low synthesis/ high absorption of cholesterol has not been seen in all trials, and the association between low synthesis/high absorption and recurrence of CVD events was not reproduced in the PROSPER trial (8).

An association between low cholesterol synthesis and high absorption phenotype with CVD was seen by Weingartner et al. (32) who investigated NCSs and CVD risk in patients referred for coronary angiography. Patients with CVD (n=66) had lower lathosterol/cholesterol and higher campesterol/cholesterol ratios than patients without CVD (n=111). Logistic regression analysis including the established CVD risk factors age, gender, total cholesterol, arterial hypertension, body mass index and smoking,

revealed that total campesterol and campesterol/cholesterol ratios were significant predictors of CVD risk. The association between low synthesis and high absorption of cholesterol with CVD has been seen in epidemiological trials (33-35) and genome wide association (36), but is at odds with the high synthesis and low absorption phenotype seen in MS and type 2 diabetes, both of which are associated with elevated CVD risk (20). It is likely that the boundaries of both cholesterol absorption or synthesis spectrums are associated with dyslipidemia and the development of CVD.

NCSs at baseline have also been shown to predict response to phytosterol therapy. Numerous studies have shown that basal levels of cholesterol absorption markers predict lowering to phytosterols, with individuals with high absorption indices having greater LDL cholesterol lowering (37-39). High basal levels of cholesterol synthesis markers have also been shown to predict poor response to phytosterol therapy (40, 41). This is congruent with the finding that fractional cholesterol synthesis, measured directly using a stable isotope tracer, in poor responders to phytosterol intervention was higher than in responders whose LDL cholesterol levels were lowered more substantially (42).

The ability of NCSs to predict hypolipidemic therapies response post-hoc deserves further research, but studies selecting and comparing individuals with specific NCSs phenotypes in a prospective manner must be undertaken to fully evaluate the potential of NCSs to personalize treatments.

4.7 The need for standardized measurement of NCSs

The ease with which NCSs can be measured is their greatest asset, with a single fasting blood sample yielding a picture of an individual's cholesterol metabolism. This ease,

however, has resulted in a wide and heterogeneous selection of methods to assess NCSs. Extraction and derivitization of NCSs differ between research groups, as do separation techniques, which include GC, LC and UPLC systems (10, 11, 14, 18, 23, 32, 33). The quantification of NCSs is divided between flame ionization (11) and mass spectrometry(32) detection, unfortunately, considerable variability exists between measurement techniques. When factors affecting plasma concentrations of plant sterols were investigated by Chan et al (2006), measurement method had the largest impact (22%) on circulating phytosterol levels ahead of gender (18%), diabetes or metabolic syndrome (17%), and diet (3.6%) (43). Keller et al. (2011) reported on a case study of a proband with elevated plant sterol levels (44). Plant sterols were measured by GC-FID and LC/MS-MS with the coefficient of variation between analysis methods being 13 and 15 % for sitosterol and campesterol, respectively. The potential uncertainty in NCSs measured across different methods can make cross-comparison between studies difficult. Meta-analyses using NCSs measured using different methods requires normalization which may lower the ability of an analysis to detect differences or failure to normalize may detect differences in populations that are simply due to different methodologies. The ability to reliably cross-compare NCS data is critical because NCSs are already relative surrogates, not actual measurements of cholesterol synthesis and absorption levels and therefore subject to more variability (6).

The reporting of NCSs is also highly variable, with some groups reporting exact values and others values normalized to cholesterol (10, 11). Even the cholesterol level used to normalize can be variable with some groups normalizing cholesterol measured in the same sample and method as the NCSs (3, 11), or others to cholesterol measured using

enzymatic methods (35). The number of sterols reported by different research groups also varies from just total phytosterols alone, (14) up to 9 individual sterols (11).

The heterogeneity in measurement and reporting of NCSs limits their potential in research which increasingly demands multi-center trials and in clinical settings where widespread access to standardized measurement is critical. Similar to basic lipid measurements in the past the measurement of NCSs is in need of standardization.

4.8 Conclusions

Non-cholesterol sterols represent a valuable research tool offering the potential to assess key elements of cholesterol trafficking using only a single blood sample and relatively unsophisticated instrumentation. The ability of NCSs to predict response to dietary or drug therapies offers the potential to personalize medicine and improve the overall health for individuals. Unfortunately the variability in, and lack of access to, methods used to assess NCSs negatively impacts their ability to move beyond use as a primary research tool. The ability to verify and cross-compare NCSs between different methods would allow for greater meta-analyses using NCSs data, thereby offering the potential for new findings and expanding the use of NCSs into clinical practice.

4.9 Key points

Non-cholesterol sterols are an easy and affordable means of estimating cholesterol metabolism which can be carried out in even the largest of trials.

Cholesterol metabolism of different disease states can be phenotyped with NCSs and this may help in identifying appropriate hypolipidemic treatments.

The measurement and reporting of NCSs is very heterogeneous and standardization would allow better comparison or meta-analyses of results across different assessment methods and improve the use of NCSs in both research and clinical settings.

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Bridge to chapter V

The following chapter comprises a manuscript which presents a retrospective analysis of previously completed nutritional intervention trials involving plant sterols with supporting data from an animal trial. The data in this chapter show that endogenous cholesterol synthesis levels are associated with response of LDL-C to plant sterol consumption. These data helped design the nutritional trial which is presented in chapters VI, VII and VIII. Dylan S. MacKay was responsible for the human data analysis in the retrospective clinical analysis and contributed to the preparation of the manuscript. Todd. C. Rideout was the principal manuscript author and project lead on the animal model experiments and one of the clinical studies included in the retrospective analyses. Scott V. Harding conducted the stable isotope work on the animal model experiments and contributed to the preparation of the manuscript. Suhad S. Abumweis organized the data from the previously run clinical studies and revised the final manuscript. Peter J. H. Jones was principal investigator on the human clinical studies and contributed to the preparation of the manuscript.

Chapter V

Manuscript 4

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High basal fractional cholesterol synthesis is associated with non-response of plasma LDL-cholesterol to plant sterol therapy

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Running title: Cholesterol synthesis and response to plant sterols

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5.1 Abstract

Background: The cholesterol-lowering effectiveness of plant sterol (PS) therapy is hindered by a wide-ranging variability in LDL-cholesterol (LDL-C) responsiveness across individuals. To capitalize on the LDL-C lowering potential of PS in the clinical setting it is paramount to characterize the metabolic factors that underlie this heterogeneity of responsiveness.

Objectives: To investigate the relationship between cholesterol synthesis and plasma LDL-C reductions in response to PS consumption.

Design: We evaluated previously conducted clinical PS interventions incorporating stable isotope measures of cholesterol synthesis and conducted feeding studies in animal models of response (Syrian Golden hamsters) and non-response (C57BL/6J mice) to PS consumption.

Results: From our clinical study population (n=113), we identified 47 non-responders (3.73 ± 1.10 % change in LDL-C) and 66 responders (-15.16 ± 1.04 % change in LDL-C) to PS therapy. Basal cholesterol fractional synthesis rate (FSR) as measured by direct deuterium incorporation was 23% higher (P = 0.003) in the non-responder subgroup compared with responders to PS therapy. Basal cholesterol FSR correlated (r = 0.22, P = 0.02) with % change in LDL-C following PS intervention. Supporting our clinical observations, non-responding mice demonstrated a 77% higher (P = 0.001) basal cholesterol-FSR compared with responding hamsters. Compared with control mice, PS-fed mice demonstrated an increase in hepatic nuclear SREPB2 protein abundance (1.3 fold of control, P = 0.04) and HMG-CoA reductase mRNA expression (2.4 fold of control, P=0.00).

Conclusions: Results suggest that subjects with high basal cholesterol synthesis are less responsive to PS treatment compared with subjects with low basal cholesterol synthesis.

Key words: Plant sterols; cholesterol synthesis; humans; mice; hamsters

5.2 Introduction

Plant sterols (PS) have a long-standing history as effective dietary cholesterol-lowering agents by interfering with intestinal cholesterol absorption. Recent analyses report a 5-15% reduction in plasma LDL-cholesterol (LDL-C) in response to PS therapy (1-3). However, clinical interventions demonstrate significant inter-individual variability in the extent of cholesterol reductions and in many cases a complete non-response of plasma cholesterol to PS therapy is evident (4, 5). Although compliance, food matrixing, dosage, and timing issues are thought to play a role in the lack of consistent response to PS across individuals within a population, it is clear that independent of such factors, some individuals respond with substantial reductions in circulating cholesterol, while other individuals are much more resistant or completely insensitive to PS challenges. Understanding the genetic and metabolic factors that underlie this heterogeneity of responsiveness is integral in establishing PS as effective cholesterol-reducing agents in clinical practice.

Hepatic cholesterol synthesis is integral to the maintenance of whole-body cholesterol homeostasis and is regulated by multiple dietary factors (6-8). It has been suggested that high basal hepatic cholesterol synthesis confers protection against diet-induced hypercholesterolemia by creating a cholesterol buffering capacity in which dietary cholesterol can maximally reduce cholesterol biosynthesis through negative feedback inhibition (9, 10). However, high basal cholesterol synthesis may also render pharmacological inhibition of cholesterol absorption with ezetimibe less effective than under conditions of low cholesterol synthesis (11). Therefore, we hypothesized that basal cholesterol synthesis capacity may underlie much of the variable cholesterol-lowering

response to PS therapy. To test this hypothesis, we conducted a retrospective evaluation of clinical data from several of our human studies to determine if basal cholesterol synthesis as measured by direct stable isotope methodology was different between hyper- and hypo-responders to PS intervention. Additionally, to further investigate the impact of hepatic cholesterol biosynthesis and sterol trafficking on the hypocholesterolemic response to PS, we conducted studies in animal models of response (Syrian Golden hamsters) and non-response (C57BL/6J mouse) to PS consumption.

5.3 Materials and Methods

Human clinical studies

Three previously published PS clinical studies from our group were chosen based on similarity in study design and availability of data concerning cholesterol fractional synthesis rates directly measured by deuterium incorporation (5, 12, 13). Study 1 was conducted at the Clinical Nutrition Research Unit at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN), University of Manitoba while studies 2 and 3 were conducted at the Mary Emily Clinical Nutrition Research Unit of McGill University. Clinical studies were compliance-controlled feeding, randomized cross-over investigations consisting of 4-week control and PS-intervention phases separated by a washout period of at least 4 weeks. The pooled study population included 135 hypercholesterolemic subjects (LDL-C >3.5 mmol/L) with a BMI between 22 and 32 kg/m^2 and an age range of 20-80 years (**Table 5.1**). All studies excluded subjects who were smokers, had a history of diabetes, hypertension or hypothyroidism, or were taking cholesterol-lowering medication in the six months prior to the start of the study.

Table 5.1

Study and subject characteristics from clinical plant sterol interventions[1]

	Clinical studies (reference)		
	Study 1 (5)	Study 2 (12)	Study 3 (13)
Study characteristics			
Design	Crossover	Crossover	Crossover
Diet	Controlled	Controlled	Controlled
Plant sterol intake (g/d)	1.95	2	1.6
No. of subjects	23	82	30
Male/female	10/13	82/0	17/13
Duration (d)	28	30	30
Subject characteristics (baseline)			
Mean age (y)	43	51	60
BMI (kg/m^2)	29–30	28–29	27–30
Total cholesterol (mmol/L)	6.46 ± 0.30^d	5.82 ± 0.10^e	5.74 ± 0.17^e
LDL cholesterol (mmol/L)	4.23 ± 0.21^d	3.71 ± 0.10^e	3.61 ± 0.15^e
HDL cholesterol (mmol/L)	1.27 ± 0.07	1.21 ± 0.03	1.37 ± 0.09
Triglycerides (mmol/L)	2.33 ± 0.39	2.03 ± 0.10	1.87 ± 0.21

[1] Values with a different superscript letter are significantly different from each other ($P < 0.05$).

Study diets were designed to contain 35% of energy as fat, 50% as carbohydrate and 15% as protein and meet the energy requirements of each subject using the Mifflin equation (14). Subjects were instructed to consume only the foods and beverages provided by the clinical research staff and to refrain from coffee and alcohol consumption. PS were supplied in soy beverage, yogurt, or margarine food vehicles at approximately 2 g/d. To monitor compliance, subjects consumed one meal per day with PS treatment or placebo under direct supervision. The remaining meals and treatments were packed for take-out. Each study protocol was approved by the Bioethical Research Ethics Board at the University of Manitoba or the Faculty of Medicine Institutional Review Board of McGill University. All subjects signed informed consent to participate in the studies.

Fasting blood samples were taken at the beginning and at the end of each experimental period for plasma lipid analyses. Twenty-four hours before the end of each experimental phase, subjects were given an oral dose of deuterium oxide (0.7 g/kg estimated body water) prior to breakfast as a tracer for measuring cholesterol fractional synthesis (FSR) according to previously established procedures (15). Cholesterol FSR measured at the end of the control period was used as an estimate of basal cholesterol synthesis. Specific details on experimental design and sample collection and analyses for the three studies have been published previously (5, 12, 13).

Animal experiments

In two separate studies, sixteen male C57BL/6J mice and Syrian Golden hamsters were acquired from Charles Rivers and brought to the Animal Model Research Facility at the RCFFN. Mice were pair-housed in plastic cages with shavings in a temperature-controlled room (20°C) in a 12h light/dark cycle. Hamsters were housed individually

under similar conditions. Animals had free access to water and were acclimatized to the facility and research staff for 1 week on a standard rodent non-purified diet (Prolab RMH 3000, LabDiet) prior to commencement of the experiment. At the initiation of the experiment, animals were randomized to a hypercholesterolemic (0.25%) rodent diet with and without 2% PS (Reducol, Forbes Meditech) for 4-6 weeks. Each study was designed as a randomized complete block. All procedures were approved by the Animal Care Committee at the University of Manitoba and the animals were cared for in accordance with the guidelines established by Canadian Council of Animal Care.

Sample collection and processing. At the end of each study, animals were anesthetized with isoflurane for blood and tissue collection. Fasting blood was collected by cardiac puncture into EDTA tubes. Plasma was separated from whole blood by centrifugation at 1,000 $\times g$ for 10 min and stored in aliquots at -20°C. Livers from mice were collected and processed according to previously reported procedures (16).

Plasma lipid analyses. Plasma total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), non-HDL cholesterol, and triglycerides (TG) were determined by automated enzymatic methods on a Vitros 350 chemistry analyzer (Ortho-Clinical Diagnostics, Markham, Ontario, Canada).

RNA preparation and real-time RT-PCR. Total RNA was isolated from mouse whole liver tissue using TRIzol reagent (Invitrogen Canada Inc., Burlington, ON). RNA concentration and integrity was determined with spectrophotometry (260 nm) and agarose gel electrophoresis, respectively. RNA preparation and real-time RT-PCR was conducted using a one-step Quantitect SYBR Green RT-PCR kit (Qiagen Inc.,

Mississauga, ON, Canada) on an Applied Biosystems 7500 system according to previously established protocols (16). Sense and antisense primers were obtained from previously published sequences for HMG-CoAr, SREBP2, and β-actin (17) and ABCG5 and G8 (18).

Immunoblot Analyses. Immunoblots were prepared as previously described (16). Nuclear and cytoplasmic extracts for immunoblot analyses of SREBP2 (SC-5603, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were separated using CelLytic™ NuCLEAR™ extraction kit (Sigma, Saint Lois, Missouri, USA). Microsomal extracts for the immunoblot analyses of HMG-CoA reductase (SC-33827, Santa Cruz Biotechnology) and β-actin (Ab8229, Abcam, Cambridge, MA, USA), were prepared based on the previously published procedures (19). β-actin was used as the housekeeping protein for all target proteins (Ab8229, Abcam, Cambridge, MA, USA).

Cholesterol synthesis and absorption. Cholesterol FSR (FSR; % pool/day) was quantified using the uptake rate of deuterium from body water into the newly synthesized RBC free cholesterol pool extracts over 2 h at the end of the feeding experiment (20, 21). Cholesterol absorption was measured using the single stable isotope tracer approach with 48-hour [3,4]-^{13}C cholesterol RBC enrichment reflecting cholesterol absorption efficiency (22).

Statistical analyses

Subjects on the control phase averaged a reduction in LDL-C of 4% compared with baseline measurements, presumably due to the strict diet provided to the subjects. Therefore, subjects who did not demonstrate a reduction in LDL-C beyond that observed on the control phase in response to PS intervention (<5%) were identified as 'non-

responders'. Accordingly, clinical study subjects with an LDL-C reduction >5% in response to PS intervention were identified as 'responders'. LDL-C responses were analyzed as the % difference between end of treatment versus control phases.

Basal cholesterol synthesis rates (control phase FSR, % pool/d) between responder and non-responder sub-groups were analyzed using general linear model (GLM) with trial as a fixed factor. Association between LDL-C response and basal cholesterol synthesis rates was assessed using Pearson correlation coefficients. Plasma LDL-C response stratified by low, medium, and high basal cholesterol synthesis rates was compared using Tukey's post hoc test.

Endpoint parameters within and between animal studies were compared using the GLM univariate analyses with block and study included as a fixed factor. All data were analyzed using SPSS 16 for Mac. Differences for clinical and animal studies were considered significant at $P < 0.05$.

5.4 Results

Human studies

From a study population of 135 subjects, 113 subjects had complete data sets for plasma lipid and cholesterol synthesis. Out of the 113 subjects included in our analyses, 47 non-responders and 66 responders to PS therapy were identified using the threshold of 5% reduction in plasma LDL-C. The pooled LDL-C reduction (% change from control phase) for all study subjects in response to PS therapy was -7.3 ± 1.2 %. Plasma LDL-C was increased ($P = 0.01$) in the non-responder subgroup (3.7 ± 1.1 %) and decreased ($P = 0.01$) in the responder subgroup (-15.2 ± 1.0 %) following PS intervention compared with control phase.

Basal cholesterol FSR was 23% higher (P = 0.003) in the non-responder subgroup compared with responders to PS (**Figure 5.1**). Following the 4 week PS intervention, non-responders had a 14% higher (P = 0.04) FSR compared with the responder subgroup (**Figure 5.1**). Basal FSR was correlated (r = 0.22, P = 0.02) with % change in LDL-C. Subjects with the lowest basal FSR demonstrated greater reductions in plasma LDL-C compared with subjects with higher FSR (**Figure 5.2**). Additionally, when subjects were stratified by FSR (top and bottom 25%), those with the lowest basal FSR responded with a higher (P = 0.03) % reduction in LDL-C (-12.31 ± 2.22 vs. -3.17 ± 0.66 %) compared with subjects with highest FSR (**Figure 5.3**).

Animal studies

Compared with hamsters, mice demonstrated a non-response to PS consumption with little reduction (P<0.05) of plasma total-cholesterol (-4.5 ± 4.6 vs -55.3 ± 4.8%), non-HDL cholesterol (-1.9 ± 11.0 vs -85.5 ± 9.6%), and HDL-cholesterol (-1.3 ± 1.4 vs -24.0 ± 6.4%) following PS consumption. Furthermore, compared with hamsters, non-responding mice demonstrated a higher (P < 0.01) cholesterol-FSR in the control (4.35 ± 0.35 vs 1.92 ± 0.42 % pool/d) and PS-supplemented phase (6.96 ± 0.78 vs 3.92 ± 0.33 % pool/d). Compared with control mice, PS-fed mice had lower (P<0.05) intestinal cholesterol absorption (4.61 ± 0.42 vs. 6.82 ± 0.83 %/d) and hepatic total cholesterol concentration (1.81 ± 0.37 vs. 8.62 ± 0.46 μmol/g tissue).

mRNA and protein expression patterns of hepatic sterol regulatory targets are presented in Table 5.2. PS-fed mice had higher hepatic HMG-CoAr mRNA expression

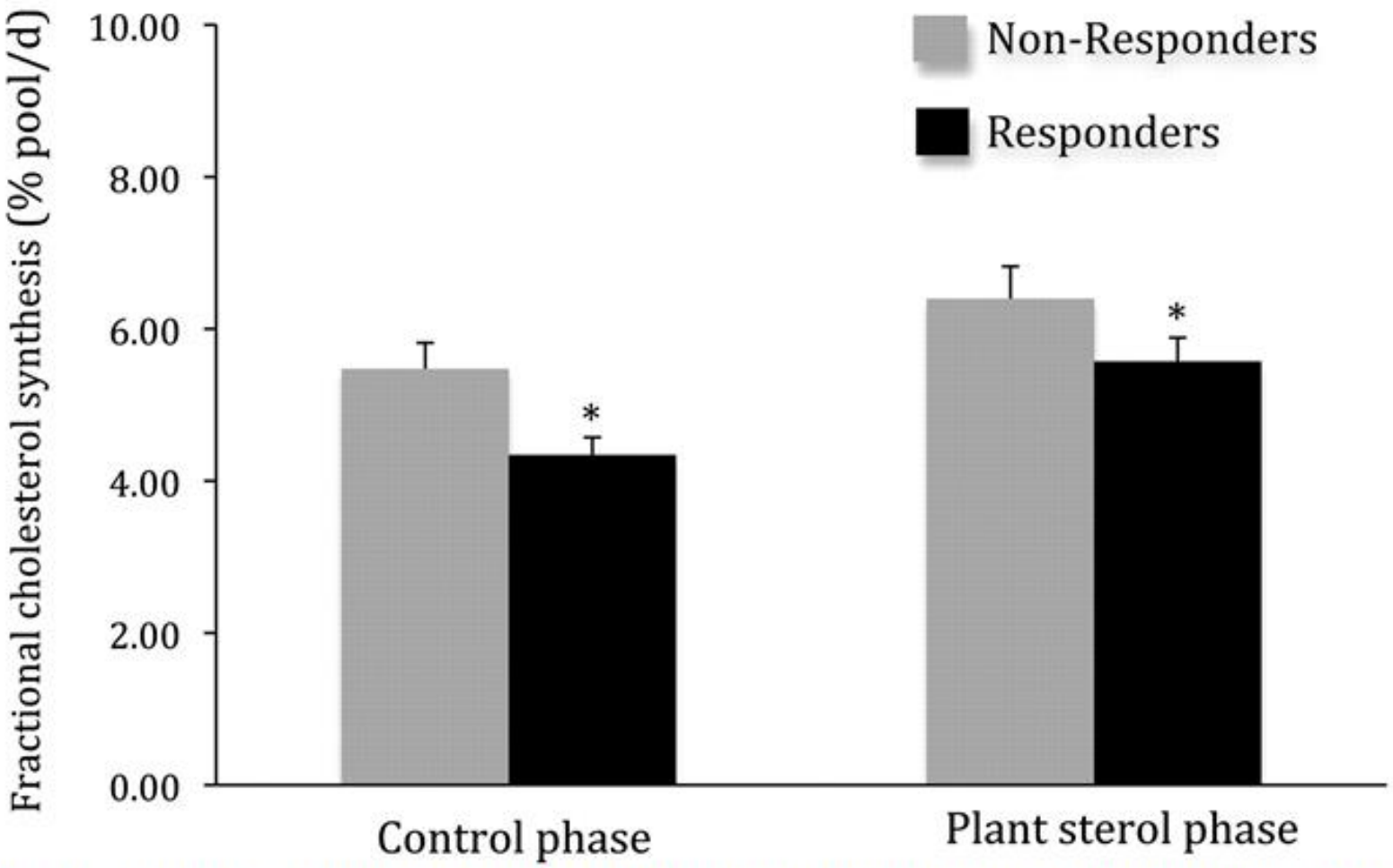

Figure 5.1 Mean (±SE) cholesterol fractional synthesis rates (% pool/d) in responder and nonresponder subgroups during control and plant sterol supplemented phases. *Significantly different from nonresponder subgroups, P < 0.05

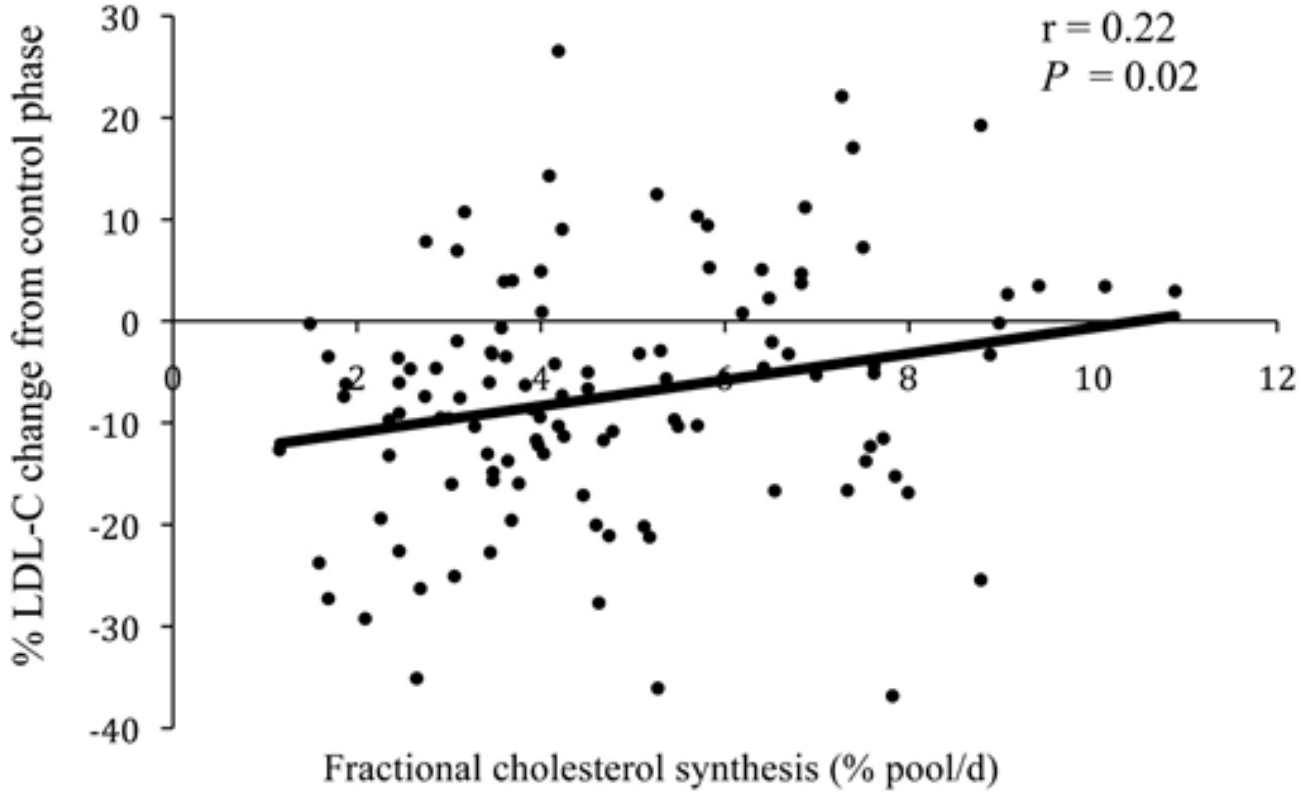

Figure 5.2 Pearson's product-moment correlation (r) between basal cholesterol fractional synthesis rate (% pool/d) and percentage of LDL cholesterol (LDL-C) change from control phase in response to plant sterol consumption

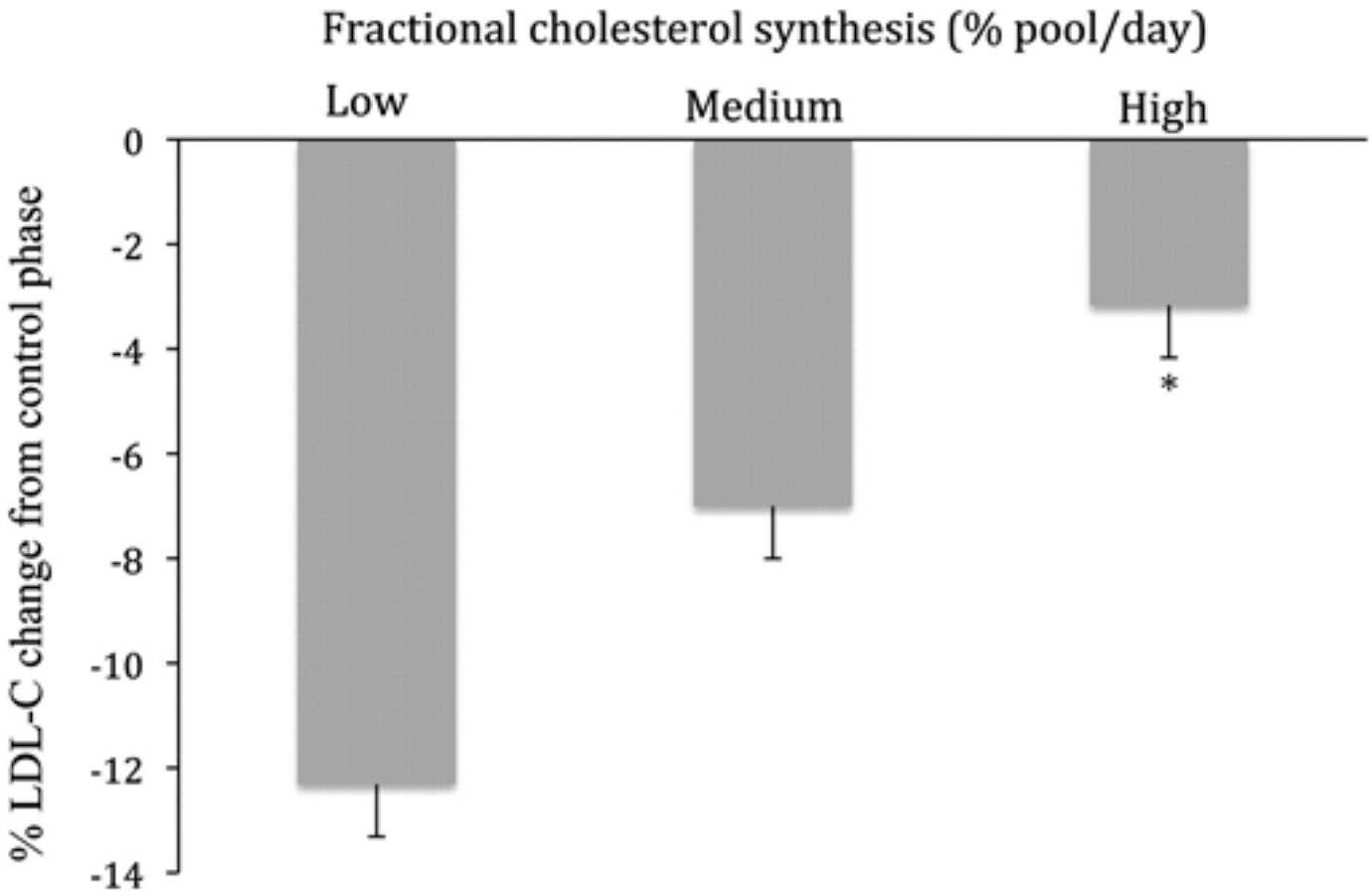

Figure 5.3 Mean ({+/-}SE) percentage change in LDL cholesterol (LDL-C) in the top 25% and bottom 25% of subjects stratified by low (n = 28), medium (n = 57), and high (n = 28) basal cholesterol fractional synthesis rate (% pool/d)

Table 5.2

mRNA and protein expression patterns (in arbitrary units) of hepatic regulatory targets in C57BL/6J control and plant sterol–supplemented mice[1]

	mRNA		Protein	
Target gene/protein	Control	Plant sterol	Control	Plant sterol
HMG-CoAr	1.0 ± 0.1	2.4 ± 0.2[2]	1.0 ± 0.1	0.9 ± 0.1
ABCG5	1.0 ± 0.1	0.4 ± 0.03[2]	—	—
ABCG8	1.0 ± 0.1	0.5 ± 0.1[2]	—	—
SREBP2 (cytoplasmic)	—	—	1.0 ± 0.1	0.9 ± 0.1
SREBP2 (nuclear)	—	—	1.0 ± 0.1	1.4 ± 0.1[2]

[1] Values are means ± SEs; $n = 8$. All data were analyzed by general linear model univariate analyses with block as a fixed factor. *HMG-CoAr*, β-hydroxy-β-methylglutaryl coenzyme A reductase; *ABCG5* and *ABCG8*, ATP binding cassette transporters G5 and G8, respectively; *SREBP2*, sterol regulatory element binding protein 2.

[2] Significantly different from control, $P < 0.05$.

(2.4 fold of control, $P<0.01$), however no difference ($P=0.29$) in HMG-CoAr protein abundance was observed between the control and PS-fed mice. Although PS consumption did not affect ($P = 0.41$) hepatic protein abundance of cytoplasmic precursor SREPB2, the nuclear active form was higher (1.3 fold of control, $P = 0.04$) in the PS-fed mice compared with control mice. PS-fed mice had lower mRNA expression of hepatic ABCG5 (2.6 fold of control, $P =0.01$) and ABCG8 (1.9 fold of control, $P =0.01$) compared with control mice.

5.5 Discussion

The present study demonstrates in multiple species that the degree of plasma LDL-C reduction in response dietary PS therapy is influenced by basal cholesterol synthesis rate. In the human work presented, individuals with low basal cholesterol synthesis respond to PS therapy with clinically significant reductions in LDL-C (-15.2 ± 1.0 %) while those with relatively high basal cholesterol synthesis are unresponsive to PS therapy, displaying no reduction in LDL-C following PS consumption (3.7 ± 1.1 %). Results from our studies in animal models of response (hamster) and non-response (mouse) to PS consumption support the association between basal cholesterol synthesis and LDL-C lowering response to PS therapy in humans.

LDL-C reductions in subjects from the current retrospective analysis ranged from -40 to -0.19%, confirming recent reports on the variable cholesterol-lowering response to PS consumption (4, 23). As the hypocholesterolemic effects of PS are achieved through direct interference with intestinal cholesterol absorption, the substantial range of responsiveness is most often examined in the context of genetic factors that underscore the wide variability in intestinal cholesterol absorption across populations (13, 23-25).

Basal serum ratios of campesterol to cholesterol, an indirect measure of intestinal cholesterol absorption, have been reported to predict the LDL-C lowering response to PS (26, 27). Overall, results from these studies suggest that subjects with high intestinal cholesterol absorptive efficiency respond with greater reductions in plasma LDL-C to PS therapy than individuals with low absorption efficiency. Similarly, several studies have observed a lower LDL-C lowering response to PS therapy in subjects with high basal sterol synthesis marker concentrations that provides an indirect reflection of whole-body cholesterol synthesis (28-30). After pre-screening for high and low plasma lathosterol/campesterol ratios, greater LDL-C reductions have been observed in a small group of subjects (n=8) with plasma marker ratios indicative of low basal cholesterol synthesis compared with subjects with marker ratios reflecting high basal synthesis in response to plant stanol consumption (-13.8 vs +4.2%) (31). Our study confirms these results and is the first to demonstrate non-response to PS therapy in subjects with high basal cholesterol synthesis evaluated using direct kinetic stable-isotope measures of endogenous cholesterol synthesis (%/d).

Through an elegant feedback mechanism and precise molecular regulation of enterohepatic sterol homeostasis, cholesterol absorption and synthesis maintain a reciprocal relationship to sustain body pools (32-36). As PS therapy has been shown to work most effectively in subjects with high intestinal cholesterol absorption efficiency (13), it is reasonable that PS responsive subjects would possess low cholesterol synthetic capacities. Alternatively, non-responders to PS therapy may compensate for PS-induced reductions in intestinal cholesterol absorption by increasing whole-body cholesterol synthesis, thereby maintaining plasma cholesterol concentrations. Although measures of

intestinal cholesterol absorption were not available for all study subjects included in this analyses, results from our animal studies suggest that mice demonstrate a non-response to PS consumption even in the face of significant reductions (-39%) in intestinal cholesterol absorption.

Although this is the first study to specifically utilize wild-type C57BL/6J mice as a model of non-response to PS consumption, three previous reports have observed similar findings (37-39). Alternatively, hamsters demonstrate consistent reductions in intestinal cholesterol absorption and plasma cholesterol in response to dietary PS supplementation (40-42). In support of our clinical findings, basal cholesterol synthesis in non-responsive mice was considerably higher (77%) than in responsive hamsters. A previous analysis confirms that mice possess a higher cholesterol synthetic capacity than hamsters (160 vs. 40 mg/kg/d) (43).

The inhibition of intestinal cholesterol absorption and associated reduction in hepatic cholesterol stores observed in the PS-fed mice were associated with modulation in the expression of hepatic sterol regulatory and trafficking machinery. As far as we are aware, this is the first report of enhanced SREBP2 nuclear protein abundance in response to PS consumption. SREBP2 is a master transcriptional regulator of genes involved in hepatic cholesterol synthesis (44) and was likely responsible for the compensatory increase in HMG-CoAr mRNA and cholesterol synthesis in PS-fed mice displaying reduced hepatic cholesterol concentrations. Alternatively, the observed reduction in hepatic ABCG5/G8 mRNA expression in the current study has been reported previously in the same mouse model following PS consumption (37) and may be a mechanism to reduce biliary sterol loss under conditions of cholesterol depletion.

In summary, with the wide degree of LDL-C lowering responsiveness to PS therapy it is important to characterize metabolic factors to explain this variability and assist in identifying patients for whom PS therapy would be an appropriate therapeutic strategy. Our results in human clinical studies and animal models of non-response (mouse) and response (hamster) to PS therapy suggest that patients with high basal cholesterol synthesis are unresponsive to PS treatment.

5.6 Acknowledgments

TCR was principle manuscript author and project lead on the animal model experiments and one of the clinical studies included in the retrospective analyses; SVH conducted the stable isotope work on the animal model experiments and contributed to the preparation of the manuscript; DM assisted in analyzing the human clinical data and revised the final manuscript; SSA organized the data from the previously run clinical studies and revised the final manuscript; PJH was principle investigator on the human clinical studies and contributed to the preparation of the manuscript. The authors have no conflicts to declare. The authors gratefully acknowledge Maggie Wilson and Derek Davlut for their assistance with mRNA and protein expression analyses and Peter Frohlich for his help with the animal experiments.

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Bridge to Chapter VI

Manuscript 4 has identified endogenous cholesterol synthesis level as a potential predictor of cholesterol lowering in response to plant sterol consumption. Using high or low endogenous cholesterol synthesis, estimated by L/C ratio, as recruitment criteria a nutritional intervention trial was set up to investigate endogenous cholesterol synthesis on LDL-C lowering in response to PS consumption. Chapter VI comprises a manuscript which demonstrates that L/C ratio can predict cholesterol lowering in response to PS consumption. Dylan S. MacKay was the principal manuscript author and project lead on the human trial and human data analysis. Sarah K. Gebauer was involved in conducting the human clinical trial and contributed to the preparation of the manuscript. Peter K. Eck contributed to the preparation of the manuscript. David J. Baer was principal investigator at the Beltsville human clinical trial site and contributed to the preparation of the manuscript. Peter J. H. Jones was principal investigator at the Winnipeg human clinical trial site and contributed to the preparation of the manuscript.

Chapter VI

Manuscript V

This manuscript will be submitted for publication in 2013

Lathosterol to cholesterol ratio in serum predicts cholesterol lowering response to plant sterol consumption in a dual center, randomized, single-blind placebo controlled trial

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Running title: Lathosterol to cholesterol ratio and plant sterol response

6.1 Abstract

Background: Plant sterol (PS) consumption for cholesterol lowering is hampered by large variability in efficacy across individuals. We have previously shown that high fractional cholesterol synthesis measured by deuterium incorporation was associated with non-response to PS consumption; however this association was done retrospectively.

Objective: The goal was to test the effectiveness of lathosterol to cholesterol ratio (L/C), a surrogate marker of cholesterol synthesis, as an *a priori* predictor of cholesterol lowering in response to PS consumption.

Design: 63 mildly hypercholesterolemic adults with high (n=24, L/C = 2.03 ± 0.39umol/mmol) or low (n=39, L/C =0.99±0.28 umol/mmol) L/C ratio at baseline, consumed either 0 or 2g/d of PS for 28 days in a dual-center, single-blind, randomized, crossover design. Plasma lipid and non-cholesterol sterol levels were measured at the end of each phase.

Results: PS consumption lowered total cholesterol (TC,-0.25 ±0.05 mmol/L, $p < 0.0001$) and LDL cholesterol (LDL-C, -0.16 ± 0.04 mmol/L, $p<0.0001$) overall. Specifically, individuals with low L/C ratio responded to PS treatment with a reduction in TC (-0.40 ± 0.07 mmol/L, $p<0.0001$) and LDL-C (-0.29 ±0.05 mmol/L, $p=0.0002$) while individuals with high L/C ratio failed to showed a lowering in those parameters.

Conclusions: The L/C ratio predicts the extent of reduction in TC and LDL-C in response to PS consumption. Cholesterol synthesis assessment may thus have a clinical use in identifying responders and non-responders to PS therapy.

6.2 Introduction

Higher LDL cholesterol (LDL-C) levels are associated with elevated cardiovascular disease (CVD) risk, and this relationship between LDL-C and CVD is believed to be continuous over a wide range of LDL-C levels (1). Therefore, LDL-C lowering therapy remains the primary prevention strategy for CVD (2). Plant sterols are steroid compounds which closely resemble cholesterol but have differing C24 carbon side chain lengths and are found in plants (3). Plant sterols (PS), or their saturated equivalents plant stanols, have well established cholesterol lowering properties, with a significant database of clinical trials that typically demonstrate mean LDL-C lowering in the 5-15% range (4-6). PS have been shown to reduce cholesterol absorption, and it is primarily through lowered cholesterol absorption that the LDL-C reductions are achieved (7). Additional mechanisms with which PS may bring about cholesterol lowering have been reviewed in detail by Smet et al. (8).

Within clinical trials significant inter-individual variability in LDL-C lowering in response to PS consumption exists (9-11), with individuals showing better than average, non- or even adverse response to PS consumption (12, 13). Distinct inter-individual responses to PS consumption have been shown to be reproducible in individuals across repeated PS interventions (14), where some subjects had consistently lowered LDL-C when ingesting PS, while others did not. The strong correlations between participants' multiple responses to PS indicate an endogenous determinant, such as cholesterol metabolism.

Cholesterol metabolism can be estimated through the use of non-cholesterol sterols, with cholesterol precursors such as desmosterol or lathosterol used as markers for endogenous

cholesterol synthesis and plant sterols, such as campesterol and sitosterol, used as markers of cholesterol absorption (15). Endogenous synthesis of cholesterol can occur from lanosterol via the Bloch and Kandutsch-Russell pathways, depending on when the double bond at C24 is reduced (16). In the Bloch pathway the C24 double bond is reduced in the last reaction step, in the Kandutsch-Russell pathway the C24 double bond is reduced earlier. Desmosterol and lathosterol are intermediates which occur in the Bloch and Kandutsch-Russell pathways, respectively.

Non-cholesterol sterol levels are typically expressed as ratios to cholesterol, as these more tightly correlate with direct measures of cholesterol metabolism than do the absolute values (17). Overall cholesterol metabolism has been estimated through the use of ratios of cholesterol synthesis to absorption markers (18), higher ratios indicating more cholesterol synthesis and lower ratios indicating more cholesterol absorption.

Few studies have been undertaken which directly aim to determine the influence of endogenous characteristics of cholesterol metabolism on the ability to positively respond to PS consumption. In a small trial Thuluva et al. (19) determined that only individuals with low endogenous cholesterol synthesis, estimated by lathosterol to campesterol ratio, reduced their LDL-C cholesterol after 4 weeks of plant stanols at a dose of 1gram/day. This study was small, limited to only eight individuals in each group.

Surrogate markers of cholesterol metabolism (15, 20), have also been used retrospectively to show that high cholesterol absorption and/or low cholesterol synthesis phenotypes have enhanced LDL-C lowering compared to low cholesterol absorption and/or high cholesterol synthesis in response to PS (11, 21-23). Similarly, Rideout et al.

(24) determined in a retrospective analysis of three clinical trials that individuals who do not lower LDL-C in response to PS had higher basal cholesterol synthesis.

Houweling et al. (25) reported that mildly hypercholesterolemic individuals with a low capacity to absorb cholesterol, as assessed by baseline serum plant sterol levels, responded to PS consumption just as well as individuals with a higher cholesterol absorption in a single blinded, randomized, diet controlled crossover trial. This finding contradicted those of Mussner et al.(23) and Fuentes et al. (21) , but may suggest that cholesterol absorption, estimated by non-cholesterol sterols, may not be a good predictor of the extent of PS derived cholesterol lowering.

Although the relationship between cholesterol absorption and synthesis is often described as reciprocal (26) and balanced to maintain cholesterol levels, cholesterol synthesis is responsible for a much larger contribution to circulating cholesterol levels than cholesterol absorption (27). The majority of cholesterol available for absorption in the intestinal lumen is produced by cholesterol synthesis (via biliary cholesterol excretion) which is in the ~1000mg/day range (28), and not of dietary origin which is typically in the ~300 mg/day range (29) .

Taken together, substantial evidence supports the theory that endogenous cholesterol synthesis, rather than the capacity to absorb cholesterol, serves as a key determinant of an individual's ability to respond to plant sterol consumption (19, 24). Therefore, we hypothesize that basal cholesterol synthesis may modulate an individual's cholesterol response to PS consumption.

Previous trials investigating the impact of cholesterol synthesis on responsiveness to PS consumption were either limited by low subject numbers, use of a parallel-arm design or

were retrospective in their analyses (19, 22, 30). Therefore, the present objective was to perform clinical trial involving prospective recruitment of individuals with high or low lathosterol to cholesterol (L/C) ratio, a surrogate marker of cholesterol synthesis. This requirement criterion was selected to increase the likelihood of having individuals with low endogenous cholesterol synthesizers who we hypothesized would respond well, and high endogenous cholesterol synthesizers who we hypothesized would respond poorly, to PS consumption.

6.3 Methods

Recruitment

Mildly hypercholesterolemic, but otherwise healthy, individuals (42 female, 29 male) aged 30-75 years were recruited from Winnipeg, MB and Beltsville, MD areas using media and direct mail advertisements. Fasting blood samples were collected and screened for biochemical and hematologic variables. Otherwise healthy was defined as the absence of a known chronic or infectious disease and supported by the fasting blood sampling. Inclusion criteria were fasting LDL cholesterol ≥3.0 mmol/L, age 30–75 y, and fasting glucose <6.1 mmol/L. Exclusion criteria included history of cardiovascular disease, diabetes, uncontrolled hypertension, kidney disease, cancer, smoking, use of medications or natural health products known to affect lipid metabolism including cholestyramine, colestipol, niacin, clofibrate, gemfibrozil, probucol, HMG CoA R inhibitors, methotrexate, high dose dietary supplements, and plant sterols for at least the previous 2 months, chronic alcohol consumption (>2 servings/d), or excessive exercise expenditure (>4000 kcal/wk). Individuals meeting these inclusion and exclusion criteria had their serum ratio of lathosterol to cholesterol (L/C) assessed by GC-MS. Individuals above the

25th and below the 75th percentiles of L/C ratio, based on the first 40 individuals screened, were enrolled into the trial. A final sample size of 71 participants were recruited into the study (n=27 high L/C ratio or high synthesis (HS) participants and n=44 L/C ratio of low synthesis (LS) participants). The study was conducted according to the principles expressed in the Declaration of Helsinki. The study procedures were approved by the University of Manitoba's Biomedical Research Ethics Board (protocol no. B2007:073). All participants provided written informed consent. Registered at clinicaltrials.gov as NCT01131832.

Study Design

A dual center, randomized, single blind, crossover, placebo controlled clinical trial was conducted at the Nutritional Research Unit (NRU) of the Richardson Center for Functional Foods and Nutraceuticals, University of Manitoba, and the Food Components and Health Laboratory (FCHL), at the USDA Beltsville Human Nutrition Research Center. The study was designed comprising two phases with two 28 day phases separated by a minimum of 4 weeks washout period during which participants consumed their habitual diets. Participants were randomized to the two experimental diets using *simple* randomization. During the two 4-week phases participants attended the research site (NRU or FCHL) and consumed one meal a day under supervision for a minimum of 4-5 days a week, and without supervision at home 2-3 days a week. The treatment meal contained either 25 g of margarine with 2 g/d free plant sterols, as esters, (treatment) or without (placebo) plant sterols depending on the phase. Participants were asked to refrain from drinking >2 alcoholic or caffeinated beverages a day. Participants were also advised to maintain their typical diet and physical activity levels and were asked to report any

changes in diet or physical activity and symptoms or changes in health and medications throughout the study. Post-treatment plant sterol concentrations served as an indicator of compliance. Body weight was measured under supervision on a weekly basis during each phase.

Blood sampling and serum lipid analysis

On days 1, 2, 24, 25, 26, 27 and 28 of each phase, 12 h fasted serum and plasma samples were collected. Within 1 h of blood collection, serum, plasma, buffy coat and erythrocyte fractions were separated by centrifugation at 3000 rpm for 20 min at 4°C, aliquoted and immediately stored at -80°C until further analysis.

Serum total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), triglycerides (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 the Friedewald equation(31). Non-cholesterol sterols (NCS) and cholesterol were determined by using a gas-chromatograph equipped with a flame ionization detector (GC-FID)(Agilent Technologies) as described with some modifications (32). An internal standard, 5-α-cholestane, was added to 500 ul of serum samples that were then saponified with methanolic KOH. Sterols were extracted twice from the mixture with petroleum ether, evaporated to dryness under nitrogen gas and resuspended in 400ul hexane and trimethylsilyl (TMS) derivatized with the addition of 100ul HMDS+TMCS+Pyridine (3:1:9)(Supelco). NCS and cholesterol were determined by GC-FID in duplicate (1µl) injections. The injector temperature was set to 280°C and detector temperature was 300°C. The column temperature was initially set to 130°C for 2 minutes, followed by a

ramp to 270°C at 30°C/min and held for 10 minutes, then ramped to 290°C at 10°C /minutes and held for 9 minutes, then finally ramped to 320°C at 40°C/minutes and held for 5 minutes. The carrier gas (helium) flow rate was 1.0 ml/min with the inlet splitter set at 22.9:1. Cholestanol, desmosterol, lathosterol, campesterol, and sitosterol were separated and expressed as ratios in umol/mmol of cholesterol, which was measured in each sample by GC-FID. Individual sterols and stanols were identified using authenticated standards (Sigma-Aldrich Canada Ltd., Oakville, ON).

Statistical analysis

Using a crossover design, endpoints of the treatment and control phases were compared. The statistical analyses were performed by using SAS 9.2 (SAS Inc). Dependent variables normality was determined using the Shapiro-Wilk test. Non-normal variables (HDL-C, TG) were log transformed before analysis. Results are expressed as estimated least square means ± SEM for all untransformed values, actual means ± SEM reported for transformed variables. Effects of treatment were analyzed by the SAS MIXED procedure with participant ID and site as random factors and treatment as an independent factor. Effect of treatment, sequence, and gender were included in the model as fixed factors. Synthesis and treatment by synthesis were also included as fixed factors when investigating the impact of high or low starting L/C ratios. Significant treatment by synthesis effects were examined by the SAS SLICE function, with Bonferroni correction for the number of slices. Treatment effect sizes by synthesis, from significant one way treatment by synthesis interactions, were compared by T-test or ANOVA using difference in mixed model least square means summary statistics for the treatment effect slices, with

Tukey-Kramer adjustment for multiple comparisons. Statistical significance was set at $p < 0.05$ for all analyses, with adjustments as above.

6.4 Results

Baseline characteristics

63 individuals (n=24 HS, n=39 LS) completed the 2-phase study. One participant withdrew without explanation, 3 participants were asked to leave the trial due to inability to make minimum visits to the research center for meals, and 4 participants were excluded prior to sample analysis, one due to admitted consumption of plant sterol products throughout the trial, one due to illness and subsequent medication, and two due to large changes in physical activity during the trial. Baseline characteristics of the participants who completed the study are presented in Table 6.1. Participants in the HS group by design possessed higher serum L/C ratios ($p<0.0001$) than LS participants. HS participants also had higher body weight ($p=0.007$), BMI ($p=0.0032$) and TG concentrations ($p=0.0017$), and had lower HDL-C ($p=0.0001$) and sitosterol to cholesterol ratio ($p=0.0164$) and cholestanol to cholesterol ratio ($p=0.0034$) than the LS participants. No side effects were associated with the experimental diets. The participants in final population reported no change in physical activity, and no significant differences were observed in body weight during the trial.

Table 6.1 Baseline participant characteristics (Mean ± STD)

	All Participants (n=63)	High Synthesis (HS, n=24)	Low Synthesis (LS, n=39)	P-values (HS vs. LS)
Lathosterol[a]	1.39 ± 0.60	2.03 ± 0.39	0.99 ± 0.28	**p<0.0001**
Gender (male/female)	24/39	12/12	12/27	p=0.1821[b]
Age (years)	55.2 ± 8.98	54.9 ± 8.6	55.8 ± 8.5	p=0.6804
Body weight (kg)	81.6 ± 19.0	96.0 ± 24.9	75.1 ± 14.4	**p=0.0007**
BMI (kg/m2)	28.8 ± 6.0	32.1 ± 7.0	27.0 ± 4.2	**p=0.0032**
TC (mmol/L)	6.01 ± 0.89	5.77 ± 0.84	6.08 ± 0.89	p=0.1841
LDL-C(mmol/L)	3.79 ± 0.77	3.65 ± 0.71	3.84 ± 0.76	p=0.3217
HDL-C (mmol/L)*	1.48 ± 0.40	1.24 ± 0.24	1.60 ± 0.43	**p<0.0001**
TG (mmol/L) *	1.64 ± 0.68	1.99 ± 0.72	1.44 ± 0.60	**p=0.0017**
Glucose (mmol/L)	4.89 ± 0.46	5.06 ± 0.40	4.86 ± 0.52	p=0.1219
Campesterol[a]	2.23 ± 0.90	2.01 ± 0.87	2.36 ± 0.91	p=0.1297
Sitosterol[a]	1.16 ± 0.49	0.98 ± 0.41	1.28 ± 0.51	**p=0.0164**
Cholestanol[a]	1.65 ± 0.31	1.51 ± 0.26	1.74 ± 0.30	**p=0.0034**

p-values for HS vs LS by unpaired t-test
a= non-cholesterol sterols reported as µmol/mmol cholesterol
b=calculated by two-tailed fisher's exact test
*=values were log transformed prior to analysis, actual means and standard deviation in the mean reported

Effects of plant sterol consumption on serum lipids and NCS

Consumption of 2.0 grams per day of PS for 28 days reduced total cholesterol (TC) in all participants (-0.25 ±0.05 mmol/L, $p < 0.0001$) compared to the placebo (Table 6.2). LDL-C concentration was reduced by PS consumption across all participants (-0.16 ± 0.04 mmol/L, $p<0.0001$) compared to the placebo. No reduction in HDL-C ($p=0.0639$) or TG ($p=0.0506$) concentrations were seen following plant sterol consumption. However, significant heterogeneity in both total and LDL cholesterol can be seen in the study population (Figure 6.1, Figure 6.2, respectively. Serum campesterol ($p<0.0001$) and sitosterol ($p<0.0001$) to cholesterol ratios were significantly increased across all the participants following PS consumption compared to the placebo, indicating compliance to the diets. Cholestanol to cholesterol ratio was not significantly reduced by PS consumption ($p=0.0651$) compared to placebo. Across all study participants, lathosterol ($p=0.5384$) and desmosterol ($p=0.9195$) to cholesterol ratios were not significantly changed following PS consumption compared to placebo.

Table 6.2 Absolute changes in lipids and non-cholesterol sterols following plant sterol consumption.

Δ LSmean (treatment-placebo)	All participants (n=63)	HS participants (n=24)	LS participants (n=39)	P-values		
				Treatment	Treatment by synthesis	Simple effects[c]
TC(mmol/L)	-0.25±0.05	-0.09±0.09	-0.40±0.07	**p<0.0001**	**p=0.0054**	HS=0.2821[‡] **LS<0.0001**[†]
LDL-C (mmol/L)	-0.16±0.04	-0.04±0.07	-0.29±0.05	**p=0.0002**	**p=0.0055**	HS=0.4904[‡] **LS<0.0001**[†]
HDL-C (mmol/L)[a]	-0.04±0.07	-0.04±0.07	-0.04±0.09	p=0.0629	n/a	n/a
TG (mmol/L)[a]	-0.11±0.11	-0.02±018	-0.17±0.12	p=0.0506	n/a	n/a
Desmosterol[b]	-0.001±0.01	-0.01±0.03	+0.01±0.02	p=0.9195	n/a	n/a
Lathosterol[b]	+0.03±0.04	-0.01±0.06	+0.06±0.05	p=0.5384	n/a	n/a
Campesterol[b]	+1.23±0.07	+0.97±0.11	+1.48±0.09	**p<0.0001**	**p=0.0007**	**HS<0.0001**[‡] **LS<0.0001**[†]
Sitosterol[b]	+0.39±0.03	+0.33±0.05	+0.44±0.04	**p<0.0001**	p=0.0877	n/a
Cholestanol[b]	-0.03±0.02	-0.04±0.02	-0.02±0.02	p=0.0651	n/a	n/a

All values reported as estimated least square means ± SEM unless otherwise labeled. a= values log transformed prior to analysis, actual means ± SEM reported. b=Non-cholesterol sterols reported as umol/mmol cholesterol. c= values log transformed prior to analysis, actual means ± SEM in nmol/mmol cholesterol reported. d=simple effects of treatment tested for synthesis groups using SAS SLICE function when treatment*synthesis interaction was significant. Different superscripts had significantly different treatment effect size, tested by T-test.

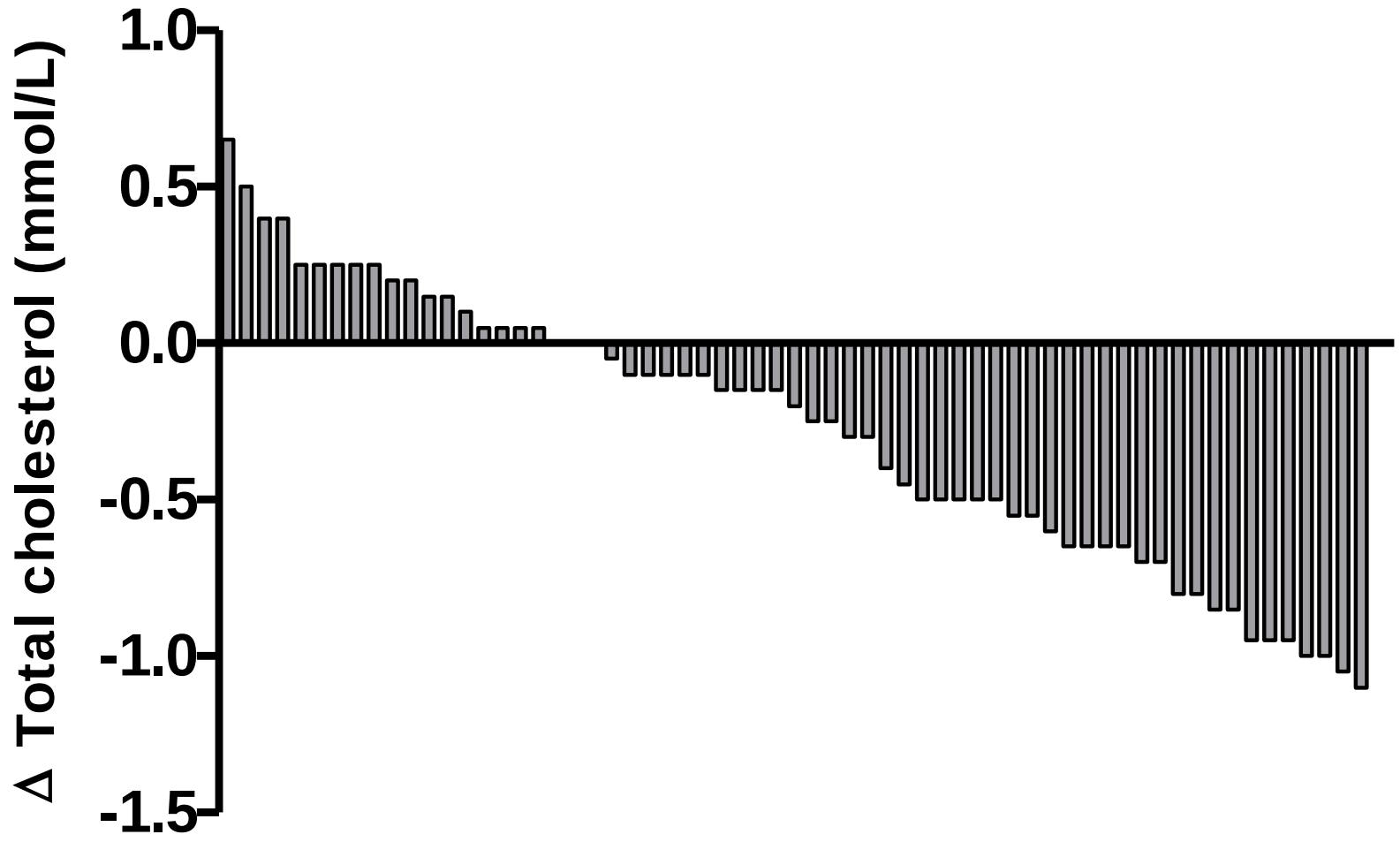

Figure 6.1 Individual changes in total cholesterol in response to plant sterol consumption compared to control.

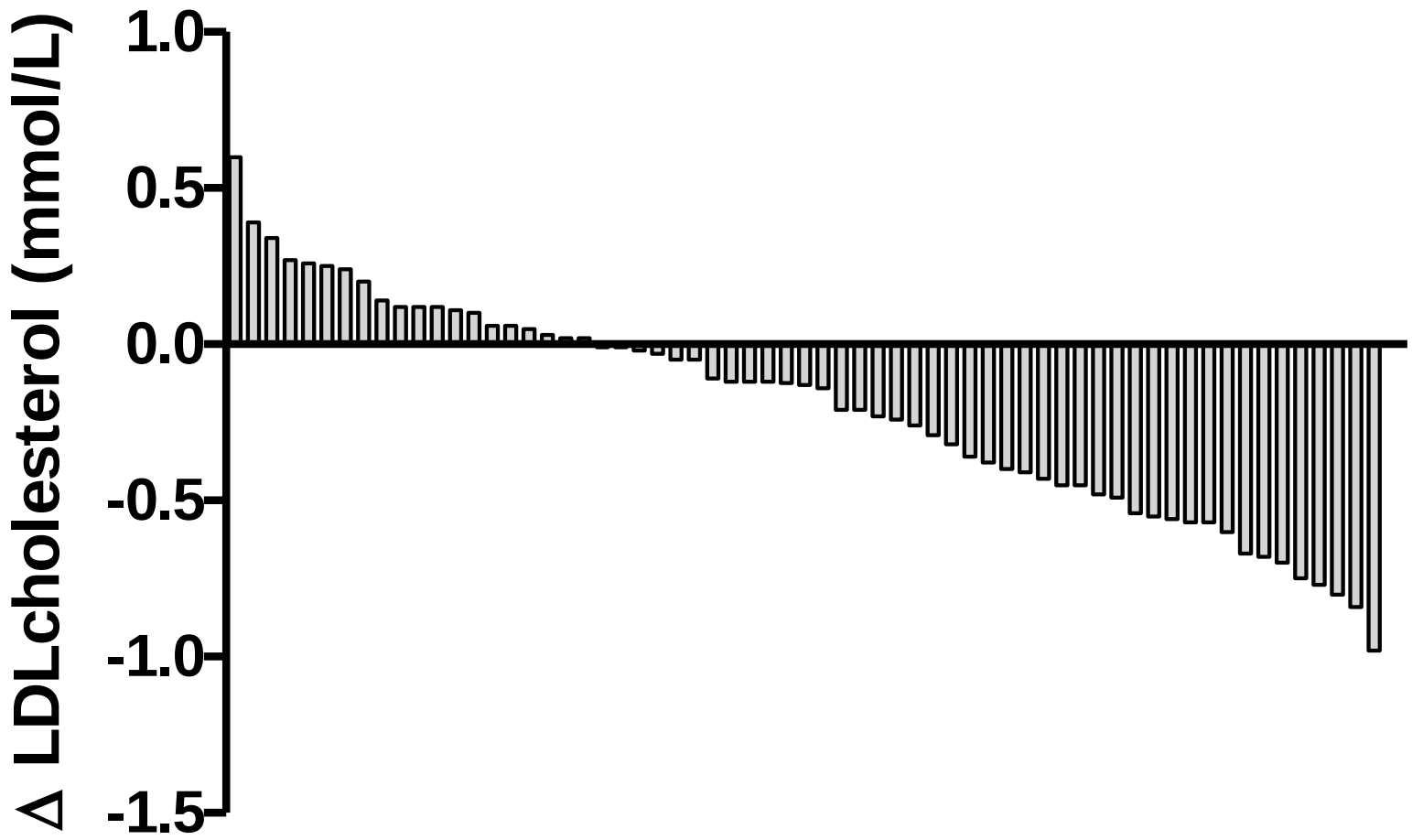

Figure 6.2 Individual changes in LDL cholesterol in response to plant sterol consumption compared to control.

Influence of endogenous cholesterol synthesis on the response of lipids and NCS to plant sterol consumption

Above we reported a reduction of TC and LDL-C for the participants as a whole. However, when stratified by endogenous cholesterol synthesis phenotype, PS consumption reduced TC and LDL-C for LS participants (-0.40±0.07mmol/L, $p<0.0001$ and -0.29±0.05 mmol/L, $p<0.0001$ for TC and LDL-C, respectively), not for HS participants (-0.09±0.09 mmol/L, $p=0.2821$ and -0.04±0.07 mmol/L, $p=0.4904$ for TC and LDL-c, respectively), compared to placebo (Table 6.2; Figure 6.3 and 6.4). Endogenous cholesterol synthesis phenotype had an interactive effect with PS consumption in relation to campesterol to cholesterol ratio. Campesterol to cholesterol ratio was raised in both HS and LS participants (+0.97±0.11, $p<0.0001$ and +1.48±0.09, $p=<0.0001$, for HS and LS respectively), but LS participants had higher campesterol to cholesterol ratio following PS consumption compared to HS participants.

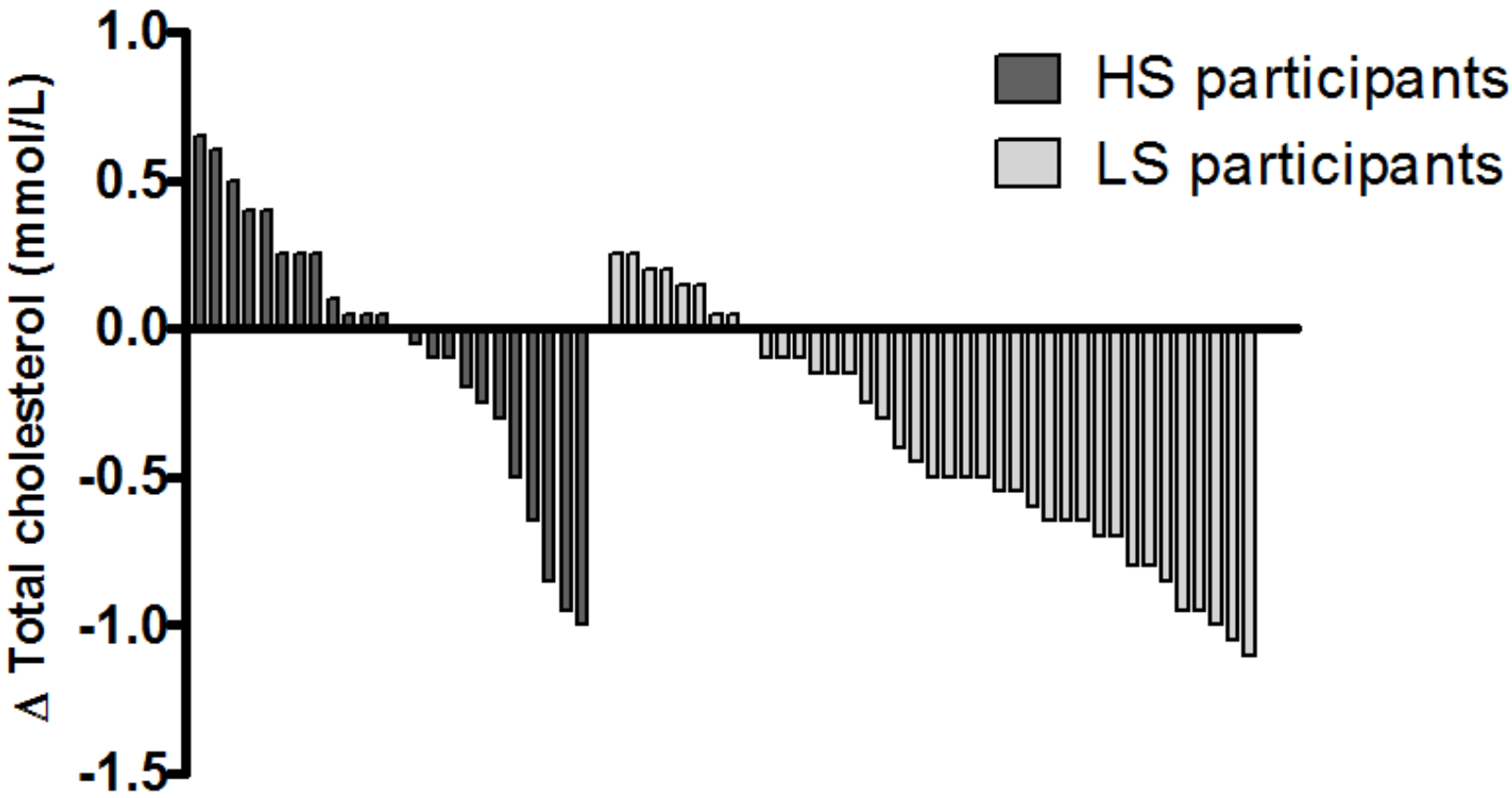

Figure 6.3 Individual changes in total cholesterol in response to plant sterol consumption compared to control stratified by endogenous cholesterol synthesis phenotype.

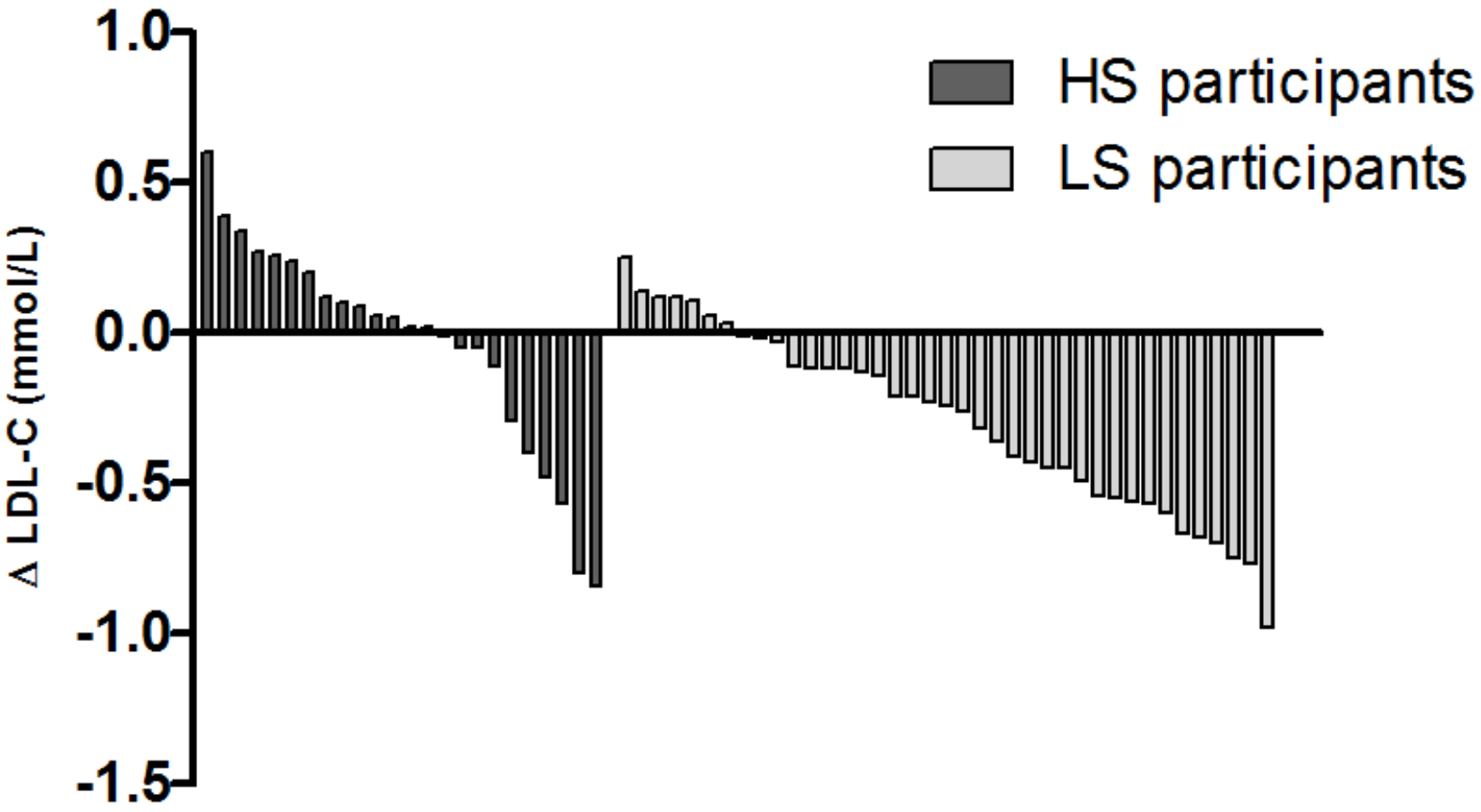

Figure 6.4 Individual changes in LDL cholesterol in response to plant sterol consumption compared to control stratified by endogenous cholesterol synthesis phenotype.

6.5 Discussion

The presented data show for the first time that L/C ratio can be used to predict an individual's response to PS consumption *a priori.* This supports the hypothesis that an individual's response to plant sterol intervention is determined by the degree of endogenous cholesterol synthesis.

Our findings expand on those of Rideout et al.(24) who showed that non-response of LDL-C to PS consumption was associated with high basal fractional cholesterol synthesis, and Carr et al.(22) who demonstrated a strong negative correlation between baseline lathosterol levels and percentage reductions in LDL-C in adults consuming PS. However, our data also indicate that additional modifying factors remain to be determined, as seen when we scrutinize each individual's LDL-C lowering as a response to PS intervention. Although the overwhelming majority of LS strongly respond, a few LS individuals do not (Figure 6.3 and Figure 6.4). Moreover, some of the HS show a strong response, while others counteract this effect by elevating their LDL-C concentrations (Figure 6.3 and Figure 6.4). Therefore, while LS seems to be a good predictor of the degree of LDL-C lowering subsequent to PS intervention, HS does not rule out PS effectiveness. This is evidenced by the substantial proportion of HS individuals who did have lowered LDL-C after PS consumption, but this was totally masked by the fact that more that 50% of HS individuals had elevated LDL-C levels (Figure 6.4).

PS consumption caused an elevation in campesterol to cholesterol ratio in both HS and LS groups. However, a greater elevation of campesterol to cholesterol ratio was seen in the LS group compared to the HS group. This interactive effect between endogenous

cholesterol synthesis and PS consumption was not seen in sitosterol to cholesterol ratio as LS participants already had higher sitosterol to cholesterol ratios than HS participants at baseline. Typically campesterol and sitosterol are only used as markers of compliance and not cholesterol absorption surrogates in PS studies because PS formulations always contain high levels of both campesterol and sitosterol (33, 34). When campesterol and sitosterol intakes are increased circulating campesterol and sitosterol levels also increase, which reflects the increased consumption and not an increase in cholesterol absorption. However, the higher sitosterol to cholesterol levels in the LS participants at baseline and larger increase in campesterol to cholesterol ratio compared after PS consumption may indicate elevated sterol absorption in the LS participants compared to HS participants. Elevated sterol absorption in the LS participants would be expected as endogenous cholesterol synthesis does tend to show a reciprocal relationship in cholesterol absorption (26).

While the relationship between endogenous cholesterol synthesis and cholesterol absorption may be reciprocal, but they are not equal contributors to the cholesterol pool, with synthesis contributing more cholesterol than absorption (28). This greater contribution of endogenous cholesterol synthesis to the cholesterol pool than cholesterol absorption may explain why our trial showed differing responses between LS and HS participants to PS consumption, but Houweling et al.(25) failed to show any different response in groups based on baseline circulating PS concentrations. These findings suggest that surrogate markers of endogenous synthesis are better predictors of response to PS than surrogate markers of cholesterol absorption.

At baseline, HS participants had higher body weight, BMI, and TG and lower HDL than LS participants. This constellation of traits is reminiscent of a metabolic syndrome phenotype (35). Metabolic syndrome itself (36, 37), and individual traits of metabolic syndrome such as elevated visceral fat (38), low HDL-C (39) and insulin resistance (40) have all been linked with elevated cholesterol synthesis and depressed cholesterol absorption phenotypes. A proposed mechanism for the like between metabolic syndrome with elevated cholesterol synthesis is insulin resistance, where elevated insulin concentrations drive cholesterol synthesis via activation of SREBF2 (41). Our data would suggest that individuals with high synthesis, such as those with metabolic syndrome may not receive the greatest health benefit from PS consumption.

The impact of PS consumption for individuals with metabolic syndrome has not been consistently demonstrated (13), with some trials showing cholesterol lowering (42, 43) and other failing to show benefits (44, 45). This inconsistency in results of trials involving individuals with metabolic syndrome may be due to the wide-ranging criteria that define the metabolic syndrome (35). These criteria may lead to populations of individuals, diagnosed with metabolic syndrome, who may not necessarily share the same metabolic characteristics that are effecting response to PS consumption (13). Our data would suggest that the metabolic characteristic most likely to be modulating response to PS consumption is endogenous cholesterol synthesis. The relationship between insulin resistance and elevated endogenous cholesterol synthesis and its impact on responsiveness to PS consumption needs further investigation. Particularly since many individuals with insulin resistance will have hypercholesterolemia and may be more likely to try PS products to help control their cholesterol.

The recruitment of individuals with high or low L/C ratio could have obscured the ability of this trial to adequately measure the impact of cholesterol synthesis on response to PS consumption across its full spectrum. However, participants with mean endogenous cholesterol synthesis would be expected to show a range of responsiveness somewhere between the HS and LS groups seen in our trial. Within the LS and even more so in the HS group there was a range of responsiveness seen for total and LDL cholesterol in response to PS consumption. This range of responsiveness suggests that additional factors which modify response to PS consumption, beyond endogenous cholesterol synthesis, remain to be determined. These factors likely have a genetic underpinning. Therefore, investigation of genetic polymorphism in genes in cholesterol metabolism pathways should be undertaken to see if any polymorphisms are associated with the degree of lipid responsiveness to PS consumption.

In summary, our data represent a first step in evaluating the utility of measuring endogenous cholesterol synthesis to predict an individual's response to PS intervention. Our results demonstrate that the response of plasma lipids to PS consumption has high inter-individual variability which is strongly influenced by endogenous cholesterol synthesis phenotype. Our data shed light on the controversial results reported in previous trials, where large inter-individual variation has hampered the ability to decisively determine if and to what degree PS consumption contributes to decreased total cholesterol and LDL-C and therefore reduced CVD risk. Only one previous dietary intervention trial, which used plant stanols, has considered an individual's endogenous cholesterol synthesis capacity *a prior* (19), which we have shown could certainly disturb

PS cholesterol lowering results should individuals with high or low endogenous cholesterol synthesis capacity be overrepresented in a study's cohort.

Our findings could be used in the context of personalized nutritional recommendations to help predicted response to PS consumption and thereby maximize efficacy in reducing CVD risk factors.

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Bridge to chapter VII

Manuscript 5 demonstrated that endogenous cholesterol synthesis level was a predictor of cholesterol lowering response to PS consumption. However, many factors are involved in setting endogenous cholesterol synthesis levels, many of which may be due to genetic variations. The following chapter comprises a manuscript which presents the associations between candidate single nucleotide polymorphisms in genes related to cholesterol metabolism and changes in cholesterol concentrations in the nutritional trial described in Manuscript 5. Dylan S. MacKay was the principal manuscript author and project lead on the human trial and human data analysis. Sarah K. Gebauer was involved in conducting the human clinical trial and contributed to the preparation of the manuscript. Peter K. Eck was involved in the investigation of nutrient by gene interactions and contributed to the preparation of the manuscript. David J. Baer was principal investigator at the Beltsville human clinical trial site and contributed to the preparation of the manuscript. Peter J. H. Jones was principal investigator at the Winnipeg human clinical trial site and contributed to the preparation of the manuscript.

Chapter VII

Manuscript VI

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The genetic basis for heterogeneity of response of total and LDL cholesterol to plant sterols

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Running title: genetic basis for heterogeneity of cholesterol response to plant sterols

7.1 Abstract

Cholesterol lowering in response to plant sterol (PS) consumption has large and reproducible variability in efficacy across individuals. Genetic polymorphisms are thought to contribute to this heterogeneity in cholesterol lowering in response to PS consumption. We associated candidate SNPs, in genes related to cholesterol metabolism, with change in cholesterol concentrations following PS consumption in a trial that recruited individuals with high or low endogenous cholesterol synthesis, estimated by lathosterol to cholesterol (L/C) ratio. 63 mildly hypercholesterolemic adults, pre-assessed as possessing high (n=24, L/C = 2.03 ± 0.39 umol/mmol) or low (n=39, L/C =0.99±0.28 umol/mmol) L/C, consumed either 2g/day (free sterol) of PS ester enriched or PS free margarine for 28 days in a dual-center, single-blind, randomized, 2-phase crossover design. Plasma lipids and non-cholesterol sterol concentrations were measured at the end of each phase. Candidate SNPs and ApoE variant were accessed by TaqMan genotyping assay. PS consumption lowered LDL-C overall, with an interaction with L/C. The rs3808607 T-allele in CYP7A1 was associated with decreased LDL-C responsiveness to PS consumption. The rs3808607 G-allele and ApoE ε4 were associated with increased LDL-C responsiveness to PS consumption. The SNP rs3808607 in the promoter region of the CYP7A1 had a different distribution across high and low L/C groups (p=0.0126), ApoE alleles were not differently distributed between high and low L/C groups (p=0.7000). Results show that rs3808607 and ApoE variant are associated with cholesterol lowering due to PS consumption, and could serve as potential predictive geneticmarkers to identify individuals who would derive maximum LDL-C lowering with PS consumption.

7.2 Introduction

The relationship between low density lipoprotein cholesterol (LDL-C) concentrations and cardiovascular disease (CVD) risk is believed to be linear over a wide range of LDL-C (1). Therefore, LDL-C lowering therapy is the primary prevention strategy for CVD (2). Plant sterols (PS) have well established cholesterol lowering properties, likely achieved through reduced intestinal cholesterol absorption (3). Clinical trials typically demonstrate mean LDL-C lowering in the 5-15% range in response to PS consumption (4-6). Additional cholesterol lowering mechanisms have been proposed for PS consumption and have been reviewed in detail by Smet et al. (7).

Within clinical trials inter-individual variability in LDL-C lowering in response to PS consumption exists (8-10), with certain individuals showing better than average, non- or even adverse response to PS consumption (11, 12). Distinct inter-individual responses to PS consumption have been shown to be reproducible in individuals across repeated PS interventions (13), where some participants consistently lowered their LDL-C when ingesting PS, while others did not. These strong correlations between participants' repeated responses to PS indicate a potentially genetic determinant of responsiveness.

Using lathosterol to cholesterol ratio to estimate endogenous cholesterol synthesis, we have previously shown that individuals with low endogenous cholesterol synthesis respond better to PS consumption, than those with high endogenous cholesterol synthesis (Chapter 6). Since it has also been hypothesized that much of the variability in response to PS consumption may have a genetic basis (11, 14, 15), we investigated the impact of genetic variations in candidate genes related to cholesterol metabolism (Table 7.1).

One specific variation of interest *rs3808607* in the promoter region of the CYP7A1 gene has previously been shown to affect total cholesterol lowering response to PS by De Castro-Oros et al.(16). The CYP7A1 gene codes for cholesterol 7 alpha-hydroxylase, the enzyme responsible for the rate limiting first step in bile acid synthesis (17). The product of this rate limiting step in bile acid synthesis, 7-alpha-hydroxycholesterol (7αHC), was also measured as a surrogate marker of bile acid synthesis (18).

Apolipoprotein E (ApoE), involved in the transport and metabolism of cholesterol and triacylglycerols (TG), is a lipoprotein found in TG-rich chylomicrons, VLDL and their remnant particles, and in HDL particles. ApoE is a gene that is highly polymorphic, with 3 major variation, named epsilon 2, 3 or 4. These ApoE variants are created by different combinations of the *rs7412* and *rs429358* genetic polymorphisms. ApoE variants have been associated with the magnitude of lipid lowering in response to plant sterol consumption. Sanchez-Muniz et al. (19) have suggested that ApoE ε4 variant carriers may not respond as well to plant sterol consumption as ε3 and ε2 carriers. Conversely, Miettinen et al.(20) and Vanhanen et al. (21) suggested cholesterol lowering response would be better in ApoE ε4 variant carriers. Geelen et al. (22) showed no effect of ApoE variant on cholesterol lowering in response to PS consumption. The impact of ApoE variant on cholesterol lowering response to PS consumption has been variable between trials and requires further research and replication before any strong conclusions can be drawn.

Previous trials investigating the impact of cholesterol synthesis on responsiveness to PS consumption were either limited by low subject numbers, parallel-arm design or were retrospective analyses (23-25). We performed a nutritional trial involving prospective

recruitment of individuals with high or low lathosterol to cholesterol (L/C) ratio, a surrogate marker of cholesterol synthesis (Chapter 6). This trial yielded a wide range of responsiveness and possessed a crossover design, which balances the genetic diversity in the placebo and PS treatments, making it ideal for investigating the impact of genetic polymorphisms on cholesterol lowering in response to PS consumption.

7.3 Methods

Recruitment

Mildly hypercholesterolemic individuals (42 female, 29 male) aged 30-75 years were recruited from Winnipeg, MB and Beltsville, MD as has been reported previously (Chapter 6). The study was conducted according to the principles expressed in the Declaration of Helsinki. Study procedures were approved by the University of Manitoba's Biomedical Research Ethics Board (protocol no. B2007:073). All participants provided written informed consent.

Study Design

A dual center, randomized, single blind, crossover, placebo controlled nutritional trial was designed with two 28 day phases a minimum of 4 week washout period between phases during which the subjects consumed their habitual diets. During the PS phase participants consumed 2 g/day PS in margarine under supervision of a trial coordinator. This trial was conducted at the Nutrition Research Unit (NRU) of the Richardson Center for Functional Foods and Nutraceuticals, University of Manitoba, and the Food Components and Health Laboratory (FCHL), at the USDA Beltsville Human Nutrition Research Center. Full study design details have been reported previously (Chapter 6).

Individuals with high (HS) or low (LS) endogenous cholesterol synthesis were selectively recruited into this trial using lathosterol to cholesterol ratio as a surrogate marker of cholesterol synthesis.

Blood sampling, serum lipid, non-cholesterol sterol and 7-alpha-hydroxycholesterol analysis

Blood sample collection, lipid analysis and non-cholesterol sterol analysis protocols have been reported previously (Chapter 6).

7-alpha-hydroxycholesterol (7αHC) was measure by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) adapted from Steiner et al. (26). Briefly, plasma sample preparation was performed following addition of a deuterated 7αHC- d7 standard (Avanti Polar Lipids Inc., Alabaster, AL, US) using C18 solid phase extraction cartridges (HF BOND ELUT-C18, 200 mg, 3ml, Agilent Technologies, Mississauga, ON). Cartridges were conditioned with 2 ml methanol followed by 2 ml ddH_2O, samples (~850 ul) were then loaded onto the cartridges with light vacuum, washed twice with 2 ml ddH_2O and eluted in 2 ml methanol. The elution was then passed through a 4 mm syringe filter (Phenex 0.2 µm PTFE membrane; Phenomenex, Torrance, CA, US) before 10µl was injected into the Waters Acquity UPLC system, including an Acquity UPLC binary pump and a sample manager (Waters Corporation, Milford, MA). The column used for the separation was a Kinetix XB-C18 column (2.1 x 100mm, particle size 1.7 µm; Phenomenex, Torrance, CA, US) with an in line filter (KrudKatcher ULTRA HPLC In-Line Filter, 0.5µm Depth Filter x 0.004in; Phenomenex, Torrance, CA, US). The column temperature was maintained at 35°C, and the gradient system was used with a mobile phase A (0.1% formic acid in ddH_20) and a

mobile phase B (0.1% formic acid in acetonitrile), at a total flow rate of 0.20 mL/min. The gradient program was started at 10% phase A and 90% phase B for 6 min, increased linearly to 100% phase B for 4 min, held at 100% phase B for 4 min, then returned to initial conditions and re-equilibrated for 4 min. The total run time for each sample analysis was 16 min. The samples for analysis were maintained at 4°C.

The MS-MS detection was performed in positive ionization mode on a tandem quadrupole mass spectrometer (Waters Micromass Quattro Micro API, Waters Corporation, Milford, MA) equipped with an electrospray ionization interface. Quantification of 7αHC was performed using multiple reaction monitoring (MRM) mode using peak areas. The MS parameters were as follows: capillary voltage 3.50 kV; source temperature 100°C; desolvation temperature 400°C; nitrogen gas with flow rates of desolvation and cone gas of 400 and 50 L/hr, respectively; argon was used as the collision gas; cone voltage was 20V; collision energy was 20 eV. The MRM transitions for 7αHC were 401.4 > 383.4 *m/z* and for 7αHC-d7 were 408.4 > 390.4 *m/z*.

Genotyping

To examine the influence of genetics in cholesterol lowering in response to PS consumption candidate SNPs were selected in genes directly related to cholesterol absorption, synthesis and trafficking (Table 7.1). The selection of candidate SNPS first included SNPs previously associated with response to plant sterols (*rs3808607*, *rs5882*, *rs4148217and* APOE variant), additional SNPs were selected because they were either missense mutations, or previously associated with changes in cholesterol metabolism, with minor allele frequencies of at least 0.06 according to NCBI's dbSNP. Candidate SNPs (19 SNPS across 11 genes) were primarily non-synonymous missense mutations

(15 SNPs), with some in the near gene 5´ region (2 SNPS), and with one in the 3´untranslated region and one synonymous mutation (found in the LDL-receptor). ApoE variant (ε2,ε3,ε4) was also assessed by genotyping SNPs *rs7412* and *rs429358* in the ApoE gene. Participants were genotyped for each candidate SNP. Genomic DNA was extracted from white blood cells by using a column based DNA extraction kit (DNeasy Blood and Tissue Kit, QIAGEN Sciences) according to the manufacturer's instructions.

The concentration and integrity of the genomic DNA were assessed by micro-volume spectrophotometer (NanoDrop 2000,Thermo Fisher Scientific). DNA samples were genotyped by TaqMan SNP genotyping assay (Table 7.2) (Life Technologies, Burlington, ON) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Life Technologies, Burlington, ON).

Statistical analysis

Endpoint to endpoint measurements of the treatment and control phases were compared. The statistical analysis was performed by using SAS 9.2 (SAS Inc). Dependent variables normality was determined using the Shapiro-Wilk test. Non-normal variables (HDL-C and 7αHC) were log transformed before analysis. The results are expressed as estimated least square means ± SEM for all untransformed values, actual means ± SEM reported for transformed variables.

The effects of treatment were analyzed by the SAS MIXED procedure with participant ID and site as random factors and treatment as an independent factor. 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 investigating the impact

Table 7.1 Candidate SNPs in genes directly related to cholesterol absorption, synthesis and trafficking

Gene *SNP*	**Type of SNP**	**Variation**	**Minor allelic frequency**[†]	**Function of gene**
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1			Cholesterol efflux pump in the cellular lipid removal pathway.
rs2230808	NS - missense	C to T	T=0.410	
rs2066714	NS - missense	T to C	C=0.366	
ABCG5	ATP-binding cassette, sub-family G (WHITE), member 5			Half-transporter (with ABCG8) that promotes intestinal and biliary excretion of sterols.
rs6720173	NS - missense	G to C	C=0.211	
rs6756629	NS - missense	G to A	A=0.074	
rs2278356	3'-UTR	A to C	C=0.403	
rs11887534	Near gene-5'	G to C	C=0.065	
ABCG8	ATP-binding cassette, sub-family G (WHITE), member 8			Half-transporter (with ABCG5) that promotes intestinal and biliary excretion of sterols.
rs4148211	NS - missense	A to G	G=0.441	
rs4148217	NS - missense	C to A	A=0.218	
rs6544718	NS - missense	C to T	T=0.106	
CETP	Cholesteryl ester transfer protein			Facilitates the transport of cholesteryl esters and triglycerides between the lipoproteins
rs5882	NS - missense	A to G	G=0.448	
CYP7A1	Cholesterol 7 alpha-hydroxylase			The rate-limiting enzyme in the synthesis of bile acid in the classic pathway.
rs3808607	Near gene-5'	T to G	G=0.450	
DHCR7	7-dehydrocholesterol reductase			Enzyme which catalyzes the conversion of 7-dehydrocholesterol to cholesterol.
rs760241	NS - missense	G to A	A=0.179	
LDLR	low density lipoprotein receptor			A cell surface protein involved in receptor-mediated endocytosis.
rs688	Synonymous	A to G	G=0.280	
LSS	Lanosterol synthase			The protein encoded by this gene catalyzes the conversion of (S)-2,3 oxidosqualene to lanosterol.
rs2839158	NS - missense	C to T	T=0.137	
rs34115287	NS - missense	T to C	C=0.136	
PCSK9	Proprotein convertase subtilisin/kexin type 9			A convertase belonging to the proteinase K subfamily which induces LDLR degradation
rs562556	NS - missense	A to G	G=0.148	

SCAP	SREBF chaperone			a protein with a sterol sensing domain which is involved in SREBFs regulation
rs12487736	NS - missense	C to T	T=0.476	
SREBF2	Sterol regulatory element binding transcription factor 2			Transcription factor that controls cholesterol homeostasis by stimulating transcription of sterol-regulated genes
rs2228314	NS - missense	G to C	C=0.366	
rs2228313	NS - missense	G to C	C=0.066	
ApoE variant	E2	E3	E4	Apolipoprotein E is a glycoprotein present in human plasma; ApoE is associated with triglyceride-rich lipoproteins (chylomicrons and VLDLs) and HDL.
Typical frequency*	7.9%	78.6%	13.5%	

†Frequency according dbSNP in July 2013 *frequency from (n=1209) Ordovas et al. 1987 (27)

Table 7.2 TaqMan genotyping assay identification numbers*

Gene *SNP*	**TaqMan** Assay ID#
ABCA1	
rs2230808	*C_2741104_1_*
rs2066714	*C_2741083_1_*
ABCG5	
rs6720173	*C_29001998_10*
rs6756629	*C_29001991_10*
rs2278356	*C_15965282_10*
rs11887534	*C_26135643_10*
ABCG8	
rs4148211	*C_29535502_10*
rs4148217	*C_375061_10*
rs6544718	*C_25642779_10*
CETP	
rs5882	C_790057_10
CYP7A1	
rs3808607	C_27492121_20
DHCR7	
rs760241	C_182603_20
LDLR	
rs688	C_2804264_20
LSS	
rs2839158	C_15832495_10
rs34115287	C_25622398_20
PCSK9	
rs562556	C_998751_10
SCAP	
rs12487736	C_33912364_10
SREBF2	
rs2228314	C_2189943_10
rs2228313	C_16170982_10
ApoE variant	
rs7412	C__27492121_20
rs429358	C___3084793_20

*www.lifetechnologies.com

of genotype. Significant treatment by synthesis or treatment by genotype effects, were examined by the SAS SLICE function, with Bonferroni correction for the number of slices. Treatment effect sizes by genotype or synthesis, from significant one way treatment by synthesis or treatment by genotype interactions, were compared by T-test or ANOVA using difference in mixed model least squared means summary statistics for the treatment effect slices, with Tukey-Kramer adjustment to correct for multiple comparisons. Combinations of genotypes and phenotype which were significant by themselves were analyzed together with appropriate 3- way and 4-way interactive terms. Statistical significance was set at $p < 0.05$ for all analyses, with adjustments as above.

Due to the strong candidate nature of the SNPs selected no corrections for family-wise multiple comparisons in association with genotypes were used because risk of type 1 error was minimal. Pearson correlation analyses were conducted to test associations. Pearson Chi-Squared tests were used to test the distribution of candidate SNPs between HS and LS groups. Linkage of candidate SNPs was accessed by the LD TG SNP Selection tool on the National Institute of Environmental Health Sciences website (http://snpinfo.niehs.nih.gov/snpinfo/snptag.htm) and pairwise r^2 for tightly linked SNPs using CubeX software (28).

7.4 Results

Baseline characteristics

63 individuals (n=24 HS, n=39 LS) completed the 2-phase study design and were genotyped for candidate SNPs. One participant withdrew without explanation, 3

participants were asked to leave the trial due to inability to make minimum visits to the research center for meals, and 4 participants were excluded prior to sample analysis, one due to admitted consumption of plant sterol products throughout the trial, one due to illness and subsequent medication, and two due to large changes in physical activity during the trial. Baseline characteristics of the study population (n=63) who completed the study have previously been published (Chapter 6). All participants (n=63) were successfully genotyped for each SNP of interest. The participants in final population reported no change in physical activity, and no differences were observed in body weight during the trial.

Distribution of candidate single nucleotide polymorphism genotypes in the study population

The distribution of each of the candidate SNPs overall and between HS and LS participants is shown in Table 7.3. Two pairs of candidate SNPs (*rs6756629* and *rs11887534)* in ABCG5 and (*rs2839158* and *rs34115287*) LSS were found to be in tight linkage (r^2=0.9151 and r^2=0.9434). Four SNPs, *rs6756629*, (p=0.003) and *rs11887534* (p=0.001) in ABCG5, *rs3808607* in (p=0.011) CYP7A1, and *rs688* (p=0.050) in LDLR were found to be unequally distributed between HS and LS participants.

Table 7.3 Frequency of candidate SNPs in study population

Gene *SNP*	**High synthesizers (HS)**	**Low synthesizers (LS)**	**All**	**X^2 test for distribution between HS vs LS**
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1			
rs2230808				Pearson Chi-Square = 3.093
C/C	9	20	29	DF = 2
C/T	12	11	23	P-Value = 0.213
T/T	3	8	11	
rs2066714				Pearson Chi-Square = 0.724
T/T	12	21	33	DF = 2
T/C	7	13	20	P-Value = 0.696
C/C	5	5	10	
ABCG5	ATP-binding cassette, sub-family G (WHITE), member 5			
rs6720173				Pearson Chi-Square = 0.185
G/G	16	28	44	DF = 1
C/S	8	11	19	P-Value = 0.667
rs6756629				**Pearson Chi-Square = 8.851**
G/G	16	37	53	**DF = 1**
A/R	8	2	10	**P-Value = 0.003**
rs2278356				Pearson Chi-Square = 0.140
A/A	8	14	22	DF = 2
A/C	14	21	35	P-Value = 0.933
C/C	2	4	6	
rs11887534				**Pearson Chi-Square = 11.487**
G/G	16	38	54	**DF = 1**
C/S	8	1	9	**P-Value = 0.001**
ABCG8	ATP-binding cassette, sub-family G (WHITE), member 8			
rs4148211				Pearson Chi-Square = 0.006
A/A	10	16	26	DF = 2
A/G	9	15	24	P-Value = 0.997
G/G	5	8	13	
rs4148217				Pearson Chi-Square = 0.445
C/C	14	26	40	DF = 1
A/M	10	13	23	P-Value = 0.505
rs6544718				Pearson Chi-Square = 2.726
C/C	19	23	42	DF = 1
T/Y	5	16	21	P-Value = 0.099
CETP	Cholesteryl ester transfer protein			
rs5882				Pearson Chi-Square = 0.806
A/A	11	14	25	DF = 2
A/G	9	19	28	P-Value = 0.668
G/G	4	6	10	

(Table 7.3, continued)

CYP7A1	Cholesterol 7 alpha-hydroxylase			
rs3808607				**Pearson Chi-Square=8.996**
T/T	13	7	20	**DF=2**
T/G	9	26	35	**P-value=0.011**
G/G	2	6	8	
DHCR7	7-dehydrocholesterol reductase			
rs760241				Pearson Chi-Square = 2.734
G/G	21	27	48	DF = 1
A/R	3	12	15	P-Value = 0.098
LDLR	low density lipoprotein receptor			
rs688				**Pearson Chi-Square = 5.990**
C/C	12	13	25	**DF = 2**
C/T	10	12	22	**P-Value = 0.050**
T/T	2	14	16	
LSS	Lanosterol synthase			
rs2839158				Pearson Chi-Square = 0.242
C/C	18	27	45	DF = 1
T/Y	6	12	18	P-Value = 0.623
rs34115287				Pearson Chi-Square = 0.490
T/T	18	26	44	DF = 1
C/Y	6	13	19	P-Value = 0.484
PCSK9	Proprotein convertase subtilisin/kexin type 9			
rs562556				Pearson Chi-Square = 0.000
A/A	16	26	42	DF = 1
G/R	8	13	21	P-Value = 1.000
SCAP	SREBF chaperone			
rs12487736				Pearson Chi-Square = 0.293
T/T	5	9	14	DF = 2
T/C	12	21	33	P-Value = 0.864
C/C	7	9	16	
SREBF2	Sterol regulatory element binding transcription factor 2			
rs2228314				Pearson Chi-Square = 1.111
G/G	10	18	28	DF = 2
G/C	11	19	30	P-Value = 0.574
C/C	3	2	5	
rs2228313				Pearson Chi-Square = 0.399
G/G	21	36	57	DF = 1
C/S	3	3	6	P-Value = 0.528
ApoE variant	Apolipoprotein E epsilon variations			
ε2/ -	2	2	4	Pearson Chi-Square = 0.357
ε3/ ε3	14	22	36	DF = 2
ε4/ -	8 (2 ε4/ ε4)	15 (3 ε4/ ε4)	23	P-Value = 0.837

Effects of plant sterol consumption on of serum total, LDL and HDL cholesterol, NCS and 7α-hydroxy-cholesterol

The impacts of 2.0 g per day of PS for 28 days on serum lipids and NCS have been previously reported (Chapter 6). Briefly, across all participants total and LDL cholesterol was reduced following PS consumption compared to placebo; HDL-C was not changed. The interactive effect of endogenous cholesterol synthesis phenotype with PS consumption with relation to lipid and NCS alterations have been reported previously (Chapter 6). Briefly, individuals with low L/C ratio responded to PS treatment by lowering TC and LDL-C, while individuals with high L/C ratio showed no marked improvement.

HS participants have higher 7αHC to cholesterol ratio at the end of the control phase compared to the LS participants (Table 7.4). Across all participants 7αHC was reduced following plant sterol consumption compared to placebo ($p=0.0433$) (Table 7.5). Endogenous synthesis phenotype did not show an interaction with PS consumption and 7αHC to cholesterol ratio. This finding suggests that endogenous bile acid synthesis is higher in HS participants.

Table 7.4 End of control phase 7α-hydroxy-cholesterol to cholesterol ratios (Mean ± STD)

	All participants (n=63)	High Synthesis (HS, n=24)	Low Synthesis (LS, n=39)	P-values (HS vs. LS)
7αHC[c*]	6.88 ± 6.07	9.19 ± 7.29	5.46 ± 4.76	**p=0.0008**

p-values for HS vs LS by unpaired t-test
c= values from end of placebo phase, not baseline of trial, reported as $x10^3$ nmol/mmol cholesterol
*=values were log transformed prior to analysis, actual means and standard deviation in the mean reported

Table 7.5 Absolute change in 7α-hydroxy-cholesterol to cholesterol ratios following plant sterol consumption.

Δ LSmean (treatment-placebo)	All participants (n=63)	HS participants (n=24)	LS participants (n=39)	P-values		
				Treatment	Treatment by synthesis	Simple effects[c]
7α-HC [c]	-1.39±0.72	-1.96±1.66	-1.04±0.56	**p=0.0433**	p=0.9265	n/a

All values reported as estimated least square means ± SEM unless otherwise labeled. a= values log transformed prior to analysis, actual means ± SEM reported. c= values log transformed prior to analysis, actual means ± SEM in nmol/mmol cholesterol reported.

Influence of genotype on response of total cholesterol to plant sterol consumption

Candidate SNP associations with total, LDL and HDL cholesterol in response to plant sterol consumption are shown in Table 7.6. Two candidate SNPs, *rs3808607* (p=0.0204 for interaction) in CYP7A1 and *rs34115287* (p=0.0209 for interaction) in LSS, as well as ApoE variant (p=0.0226 for interaction) associated with TC lowering in response to PS consumption (Table 7.7). *rs3808607* in CYP7A1 showed an allelic dose effect on response to PS consumption. T/T carriers (n=20) did not have a reduction in TC after PS consumption. G/T and G/G allele carriers had reductions in TC after PS consumption. The size of TC lowering following PS consumption was greater in G/G individuals compared to T/T individuals, with G/T falling in-between (Table 7.7). Both T and C carriers of *rs34115287* showed reductions in total cholesterol (p=0.0023 and p<0.0001, for T/T and T/C+C/C, respectively) following PS consumption, while T/C and C/C carriers had a greater cholesterol lowering than T/T carriers. ε3 and ε4 variants for ApoE had reductions in total cholesterol (p=0.0011 and p=0.0001, respectively) after PS consumption, while ε2 variants did not (p=0.4399). The TC lowering effect size of PS consumption was greater in ε4 variants than ε2 variants with the effect size in ε3 variants falling in between.

The SNPs and APOE variants which had interactive effects with PS consumption in relation to TC lowering were combined with synthesis phenotype to determine if the genotypes and synthesis phenotype may be related to one another in their association with TC lowering (Table 7.8).

rs3808607 in CYP7A1 continued to have an interactive effect with PS consumption in relation to TC lowering when combined with the synthesis phenotype, while the

phenotype did not, with no 3-way interactive effects. This finding suggests that phenotype and genotype are related in their association with PS induced TC lowering, but the genotype is more strongly associated than phenotype. This notion is further reinforced by the unequal distribution of the *rs3808607* SNP between the HS and LS participants. The T/T carriers were more prevalent in the HS group, while G/T and G/G carriers made up more of the LS group.

Table 7.6 Candidate SNP associations with total, LDL and HDL cholesterol in response to plant sterol consumption

Gene *SNP*	**Total cholesterol**		**LDL-C**		**HDL-C**	
ABCA1	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs2230808	p=0.1057	p=0.1229	p=0.0924	**p=0.0379**	p=0.3374	p=0.1925
rs2066714	p=0.6911	p=0.8158	p=0.9312	p=0.6220	p=0.0560	p=0.9160
ABCG5	**Overall**	TX by SNP	**Overall**	TX by SNP	Overall	TX by SNP
rs6720173	p=0.1192	p=0.3086	p=0.0727	p=0.3227	p=0.6065	p=0.9372
rs6756629	**p=0.0308**	p=0.6349	**p=0.0399**	p=0.8250	p=0.1925	p=0.6921
rs2278356	**p=0.0037**	p=0.3774	**p=0.0003**	p=0.0623	p=0.3562	p=0.7498
rs11887534	**p=0.0383**	p=0.4490	**p=0.0413**	p=0.8896	p=0.1140	p=0.7001
ABCG8	**Overall**	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs4148211	p=0.1926	p=0.8786	p=0.1918	p=0.5709	p=0.7602	p=0.1818
rs4148217	p=0.9534	p=0.5492	p=0.4199	p=0.1705	p=0.1270	p=0.5211
rs6544718	p=0.1927	p=0.6725	p=0.2111	p=0.9430	p=0.4926	p=0.3203
CETP	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs5882	p=0.4125	p=0.4381	p=0.6377	p=0.5994	p=0.8758	p=0.3460
CYP7A1	Overall	**TX by SNP**	Overall	TX by SNP	Overall	TX by SNP
rs3808607	p=0.1639	**p=0.0204**	p=0.1708	**p=0.0126**	p=0.4866	p=0.3852
DHCR7	**Overall**	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs760241	**p=0.0497**	p=0.2713	**p=0.0291**	p=0.6150	p=0.7269	p=0.9266
LDLR	Overall	TX by SNP	**Overall**	TX by SNP	**Overall**	TX by SNP
rs688	p=0.0805	p=0.5840	**p=0.0422**	p=0.9222	**p=0.0106**	p=0.0544
LSS	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs2839158	p=0.0973	p=0.0584	p=0.2369	p=0.1681	p=0.4783	p=0.8805
rs34115287	p=0.0804	**p=0.0209**	p=0.2467	p=0.0672	p=0.6028	p=0.6326
PCSK9	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs562556	p=0.5922	p=0.7172	p=0.8791	p=0.4800	p=0.3669	p=0.6021
SCAP	**Overall**	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs12487736	**p=0.0495**	p=0.5312	p=0.0703	p=0.2427	p=0.6822	p=0.5142
SREBF2	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs2228314	p=0.2511	p=0.1529	p=0.1133	p=0.3423	p=0.7879	p=0.7381
rs2228313	p=0.7902	p=0.3623	p=0.4010	p=0.6402	p=0.4187	p=0.1356
ApoE	Overall	**TX by SNP**	Overall	**TX by SNP**	Overall	TX by SNP
Variant	p=0.1811	**p=0.0226**	p=0.2913	**p=0.0466**	p=0.1174	p=0.2518

Mixed model effects of genotype overall and treatment*genotype interaction

Table 7.7 SNPs associated with total cholesterol lowering in response to plant sterol consumption

Gene *SNP*	**Treatment** *p-value*	**Treatment x genotype** *p-value*	**Simple effects by genotype*** ΔTC (mmol/L), p-value, n
CYP7A1 *rs3808607*	**p=<0.0001**	**p=0.0204**	T/T= -0.09, p=0.3252 (n=20)[‡] **G/T=-0.32, p=0.0001 (n=35)**[†‡] **G/G=-0.56, p=0.0002 (n=8)**[†]
LSS *rs34115287*	**p=<0.0001**	**p=0.0209**	**T/T=-0.20, p=0.0023**[†] **T/C and C/C= - 0.47, p<0.0001**[‡]
ApoE Variant	**p=0.0366**	**p=0.0226**	ε2= 0.16, p=0.4399 (n=4) [‡] ε3**=-0.23, p=0.0011(n=35)**[†‡] ε4**=-0.42, p=0.0001 (n=24)**[†]

*mixed model simple effects of treatment sliced by genotype using SAS SLICE function when treatment and treatment*genotype was significant (statistical significance level set to p=0.0167, Bonferroni correction for 3 slices, p=0.0250, Bonferroni correction for 2 slices).

Genotypes within an SNP with different superscripts had significantly different treatment effect size, tested by T-Test or ANOVA with Tukey –Kramer adjustment

7.8 Association between phenotype, genotype and total cholesterol lowering in response to plant sterol consumption

	Synthesis phenotype (HS vs LS)	**Interpretation**
CYP7A1 *rs3808607**	Treatment (Tx) by Synthesis: not significant **Tx by Genotype: p= 0.0328** Genotype by Synthesis: not significant Tx by Genotype by Synthesis: not significant	*rs3808607* in CYP7A1 overrides the synthesis phenotype association with PS induced total cholesterol lowering.
LSS *rs34115287*	**Treatment (Tx) by Synthesis: p= 0.0074** Tx by Genotype: not significant Genotype by Synthesis: not significant Tx by Genotype by Synthesis: not significant	The synthesis phenotype overrides the association between PS induced total cholesterol lowering and *rs34115287* in LSS.
ApoE Variant	**Treatment (Tx) by Synthesis: p= 0.0070** Tx by Genotype: **p= 0.0268** Genotype by Synthesis: not significant Tx by Genotype by Synthesis: not significant	Phenotype and genotype independently associate with PS induced total cholesterol lowering

*as seen in table 5.5 this SNP is unequally distributed across synthesis phenotypes

rs34115287 in LSS no longer had an interactive effect with PS consumption in relation to TC lowering when combined with the synthesis phenotype. The synthesis phenotype continued to have an interactive effect with PS consumption in relation to TC lowering, with no 3-way interactive effects. This finding suggests the genotype is associated with the phenotype, but that phenotype is more strongly associated with response than the genotype. *rs34115287* was not unequally distributed between HS and LS participants.

ApoE variant continued to have an interactive effect with PS consumption in relation to TC lowering when combined with the synthesis phenotype. The synthesis phenotype also continued to have an interactive effect with PS consumption in relation to TC lowering, with no 3-way interactive effects. This finding suggests that ApoE variants association with TC lowering in response to PS consumption is independent from the association with synthesis phenotype.

The unequal distribution of *rs3808607* in CYP7A1 across the synthesis phenotypes, and the stronger association of *rs3808607* with TC lowering in response to PS consumption suggest that *rs3808607* in CYP7A1 is driving the association between TC lowering in response to PS consumption and synthesis phenotype. Therefore synthesis phenotype was not included when SNPs which had interactive effects with PS consumption in relation to TC lowering and ApoE variant were combined to determine if they may be related with one another in their association with TC lowering (Table 7.9).

Table 7.9 SNP combinations and total cholesterol lowering in response to plant sterol consumption

	LSS *rs34115287*	**ApoE** Variant
CYP7A1 *rs3808607*	**Tx by CYP7A1: p=0.0029** Tx by LSS: p=0.2248 CYP7A1 by LSS: not significant Tx by CYP7A1 by LSS: not significant	Tx by CYP7A1: p=0.1143 **Tx by ApoE: p= 0.0352** CYP7A1 by ApoE: not significant Tx by CYP7A1by ApoE: not significant
ApoE Variant	**Tx by LSS: p= 0.0398** **Tx by ApoE: p= 0.0397** LSS by ApoE: not significant Tx by LSSby ApoE: not significant	**CYP7A1xApoExLSS** **Tx by CYP7A1: p= 0.0409** Tx by ApoE: p= 0.1008 Tx by LSS: p=0.2100 All 3-way: not significant 4-way: not significant

rs3808607 in CYP7A1 continued to have an interactive effect with PS consumption in relation to TC lowering when combined with *rs34115287* in LSS, while LSS did not, with no 3-way interactive effects. This finding suggests that *rs3808607* is more strongly associated with TC lowering due to PS consumption than *rs34115287*. This suggestion is supported by the fact that both T/T and T/C+C/C carriers of *rs34115287* had reductions in TC concentrations with PS consumption, just different TC lowering magnitudes. T/C+C/C carriers having twice the TC reduction following PS consumption compared to T/T carriers. In *rs3808607* there was an allelic dose effect of the G allele and no reduction in the T/T carriers in relation to TC lowering after PS consumption.

rs34115287 in LSS continued to have an interactive effect with PS consumption in relation to TC lowering when combined with ApoE variant. The ApoE variant also remaining associated with TC lowering in response to PS consumption, with no 3-way interactive effects. This suggests that the ApoE variant and *rs34115287* associations with PS induced TC lowering were independent of each other.

ApoE variant continued to have an interactive effect with PS consumption in relation to TC lowering when combined with *rs3808607* in CYP7A1. *rs3808607* in CYP7A1 no longer had an interactive effect with PS consumption in relation to TC lowering. This observation suggests that ApoE variant is more strongly associated with TC lowering than *rs3808607*. However, when *rs34115287* in LSS, is also combined with ApoE variant and *rs3808607* in CYP7A1, only *rs3808607* in CYP7A1 remains had an interactive effect with PS consumption in relation to TC lowering, with no other 3-way or 4-way interactive effects.

The above results suggest that *rs3808607* in CYP7A1 most strongly influences the TC lowering in response to PS consumption. However, as seen in Table 7.10, all three traits do seem to modify total cholesterol response showing a trend from ε2, T/T, T/T to ε4, G/G, C/Y of increasing TC lowering, but due to the limited numbers of participants at each combination, and correction for multiple comparisons, only 2 combinations reach statistically significant reductions in total cholesterol following PS consumption.

rs6756629 (p=0.0308), *rs2278356* (p=0.0037) and *rs11887534* (p=0.0383) in ABGC5, *rs760241* (p=0.0497) in DHCR7 and rs12487736 (p=0.0475) in SCAP all showed associations with overall TC concentration, independent of PS consumption (Table 7.6). *rs6756629* and *rs11887534* in ABGC5 were in tight linkage (r^2=0.9151) in our study population, and *rs6756629* has been previously associated with TC concentrations by GWAS by Aulchenko et al.(29). As in the GWAS, the A/R carriers for *rs6756629* and C/S for *rs11887534*, possessed lower TC concentrations than did G/G carriers in this study population. C/C carriers in *rs227835* in ABCG5 had lower TC than C/A (p=0.0028) and A/A (p=0.0010), independent of PS consumption. A-allele carriers for *rs760241* in DHCR7 had higher total cholesterol than G/G carriers, independent of PS consumption. While *rs12487736* in SCAP was associated with TC concentration overall (p=0.0495), T/T carriers were not different from T/C (p=0.0593) or C/C (p=1.000) after correction for multiple comparisons. rs227835 in ABCG5, rs760241 in DHCR7 and rs12487736 in SCAP have not previously been associated with TC concentrations.

Table 7.10 Best SNPs associated with total cholesterol lowering in response to plant sterol consumption

SNP combination	**Treatment** *p-value*	**Treatment x genotype** *p-value*	**Simple effects by SNP combination ***					
			ApoE	**CYP7A1**	**LSS**	**ΔTC (mmol/L)**	**p-value**	**n**
ApoE variant by **CYP7A1** *rs3808607* by **LSS** *rs34115287*	**p=<0.0064**	**TxbyCYP7A1:p=0.0409** Tx by ApoE: p= 0.1008 Tx by LSS: p=0.2100 All 3-way: not significant 4-way: not significant	ε2	T/T	T/T	+0.08	0.7822	2
			ε2	T/G	T/T	+0.25	0.3578	2
			ε3	T/T	T/T	-0.05	0.7125	8
			ε3	T/T	C/Y	+0.10	0.6024	10
			ε3	T/G	T/T	-0.06	0.6504	4
			ε3	**T/G**	**C/Y**	**-0.62**	**0.0001**	**8**
			ε3	G/G	T/T	-0.36	0.0611	4
			ε3	G/G	C/Y	-0.75	0.0067	2
			ε4	T/T	T/T	-0.33	0.0354	6
			ε4	**T/G**	**T/T**	**-0.40**	**0.0008**	**11**
			ε4	T/G	C/Y	-0.49	0.0124	1
			ε4	G/G	T/T	-0.60	0.1201	4
			ε4	G/G	C/Y	-0.95	0.0148	1

*mixed model simple effects of treatment sliced by SNP combination using SAS SLICE function when treatment and treatment*SNP combination was significant (statistical significance level set to p=0.0038, Bonferroni correction for 13 slices)

Influence of genotype on response of LDL cholesterol to plant sterol consumption

Two candidate SNPs, *rs2230808* (p=0.0379 for interaction) in ABCA1 and *rs3808607* (p=0.0126 for interaction) in CYP7A1, as well as ApoE variant (p=0.0466 for interaction) associated with LDL-C lowering in response to PS consumption (Table 7.11).

rs2230808 in ABCA1 did not show an allelic dose effect relationship with LDL-C lowering following PS consumption. C/C carriers and T/T carriers showed reductions in LDL-C, with no reduction in LDL-C for the C/T carriers (Table 7.11). This response pattern was unexpected and does not show any allelic dose effect; therefore a literature search for *rs2230808* was performed to look for potential confounding factors. This literature search indicated that *rs2230808* in ABCA1 had been previously shown to have a gender specific association with LDL-C concentrations in a study of Greek nurses by Kolovou et al. (30). Our analysis was repeated with gender by genotype interactions. In repeated analyses with the gender by genotype interaction, *rs2230808* was not associated with LDL-C lowering in response to PS consumption. The interaction between *rs2230808* in ABCA1 and gender was associated with overall LDL-C concentrations, independent of treatment, replicating the association seen by Kolovou et al. (30) with female homozygous minor allele carriers having lower LDL-C. Therefore, *rs2230808* in ABCA1 was not carried forward into associations with phenotype or other genotypes associated with LDL-C lowering by PS consumption.

rs3808607 in CYP7A1 showed an allelic dose effect on response to PS consumption. T/T carriers (n=20) failed to show a reduction in TC concentrations after PS consumption, whereas G/T and G/G allele carriers had reductions in LDL-C after PS

Table 7.11 SNPs associated with LDL-C lowering in response to plant sterol consumption

Gene *SNP*	**Treatment** *p-value*	**Treatment x genotype** *p-value*	**Simple effects by genotype*** ΔTC (mmol/L), p-value, n
ABCA1 *rs2230808*	**p <.00001**	**p= 0.0379**	**C/C=-0.28, p <.0001 (n=29)** ‡ C/T=-0.05, p= 0.4593 (n=23) † **T/T=-0.29, p=0.0052 (n=11)** †‡
CYP7A1 *rs3808607*	**p <0.0001**	**p=0.0126**	T/T=-0.05, p=0.5124 (n=20)‡ **G/T=-0.22, p=0.0001 (n=35)**†‡ **G/G=-0.46, p=0.0002 (n=8)**†
ApoE Variant	p=0.0547	**p=0.0466**	ε2=+0.10, p=0.5652 (n=4) ‡ ε3=**-0.16, p=0.0066(n=36)**‡ ε4=**-0.31, p<0.0001 (n=23)** ‡

*mixed model simple effects of treatment sliced by genotype using SAS SLICE function when treatment and treatment*genotype was significant (statistical significance level set to p=0.0167,Bonferroni correction for 3 slices) Genotypes within an SNP with different superscripts had significantly different treatment effect size, tested by T-Test or ANOVA with Tukey–Kramer adjustment.

consumption. The extent of LDL-C lowering following PS consumption was greater in G/G individuals compared to T/T individuals, with G/T falling in-between (Figure 7.1).

ε3 and ε4 variants for ApoE had reductions in LDL-C after PS consumption, while ε2 variants did not. The LDL-C lowering effect size of PS consumption was greater in ε4 variants than ε2 variants with the effect size in ε3 variants falling in between (Figure 7.2).

rs3808607 in CYP7A1 and ApoE variants were independently combined with synthesis phenotype to determine if the genotypes and phenotypes may be related to one another in their association with LDL-C lowering due to PS consumption (Table 7.12).

rs3808607 in CYP7A1 continued to have an interactive effect with PS consumption in relation to LDL-C lowering when combined with the synthesis phenotype, while phenotype did not, with no 3-way interactive effects. This finding suggests that the phenotype and genotype are related in their association with PS induced LDL-C lowering, but the association with genotype is stronger. This is further reinforced by the unequal distribution of the rs3808607 SNP between the HS and LS participants. The T/T carriers were more prevalent in the HS group, while G/T and G/G carriers make up more of the LS group.

ApoE variant did not have an interactive effect with PS consumption in relation to LDL-C lowering ($p=0.0559$) when combined with the synthesis phenotype, and phenotype remained significant, with no 3-way interactive effects. This suggests that the association of LDL-C lowering in response to PS consumption with ApoE variant is independent of the association with synthesis phenotype.

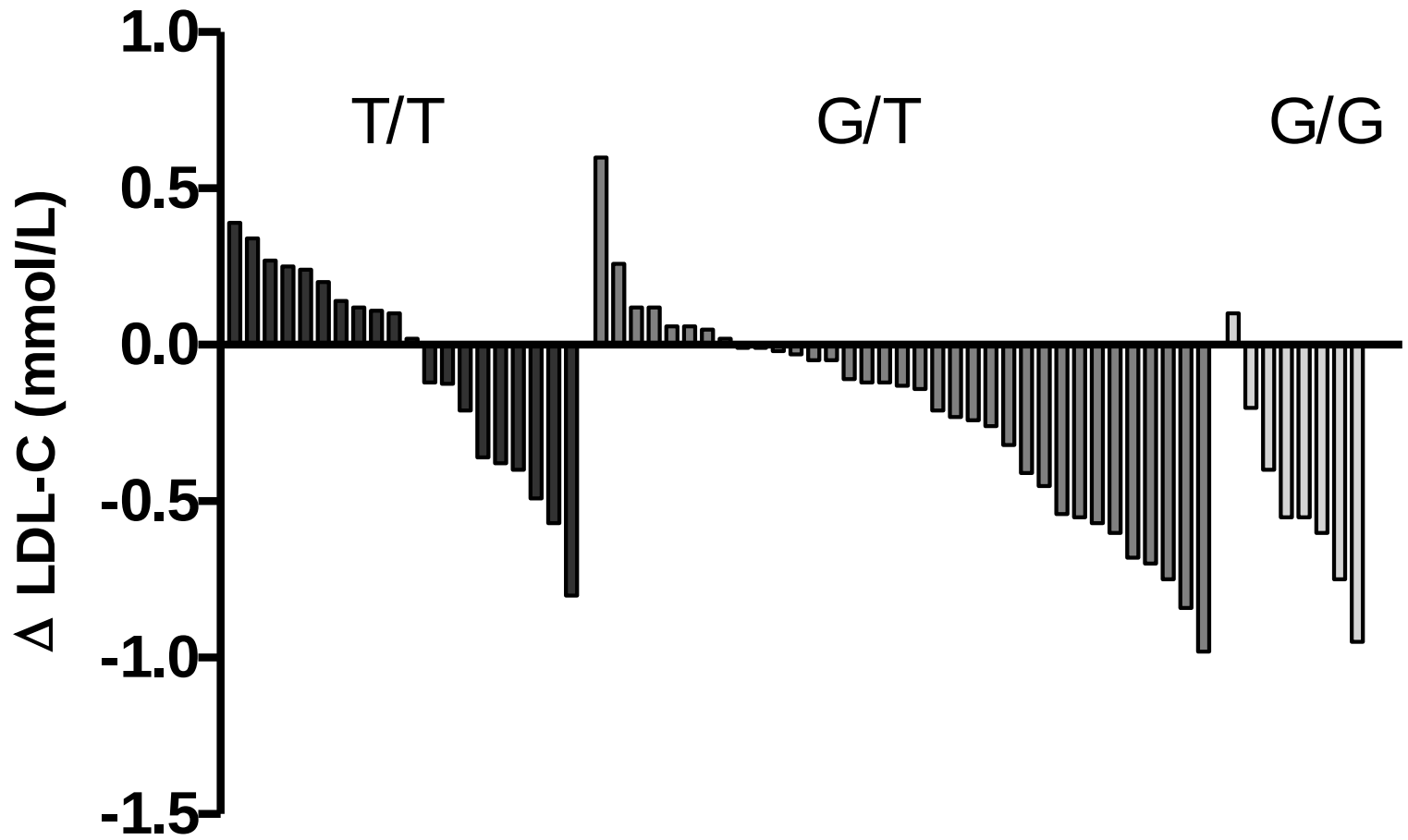

Figure 7.1 Individual changes in LDL cholesterol in response to plant sterol consumption compared to control stratified by *rs3803607* genotype in CYP7A1 promoter region.

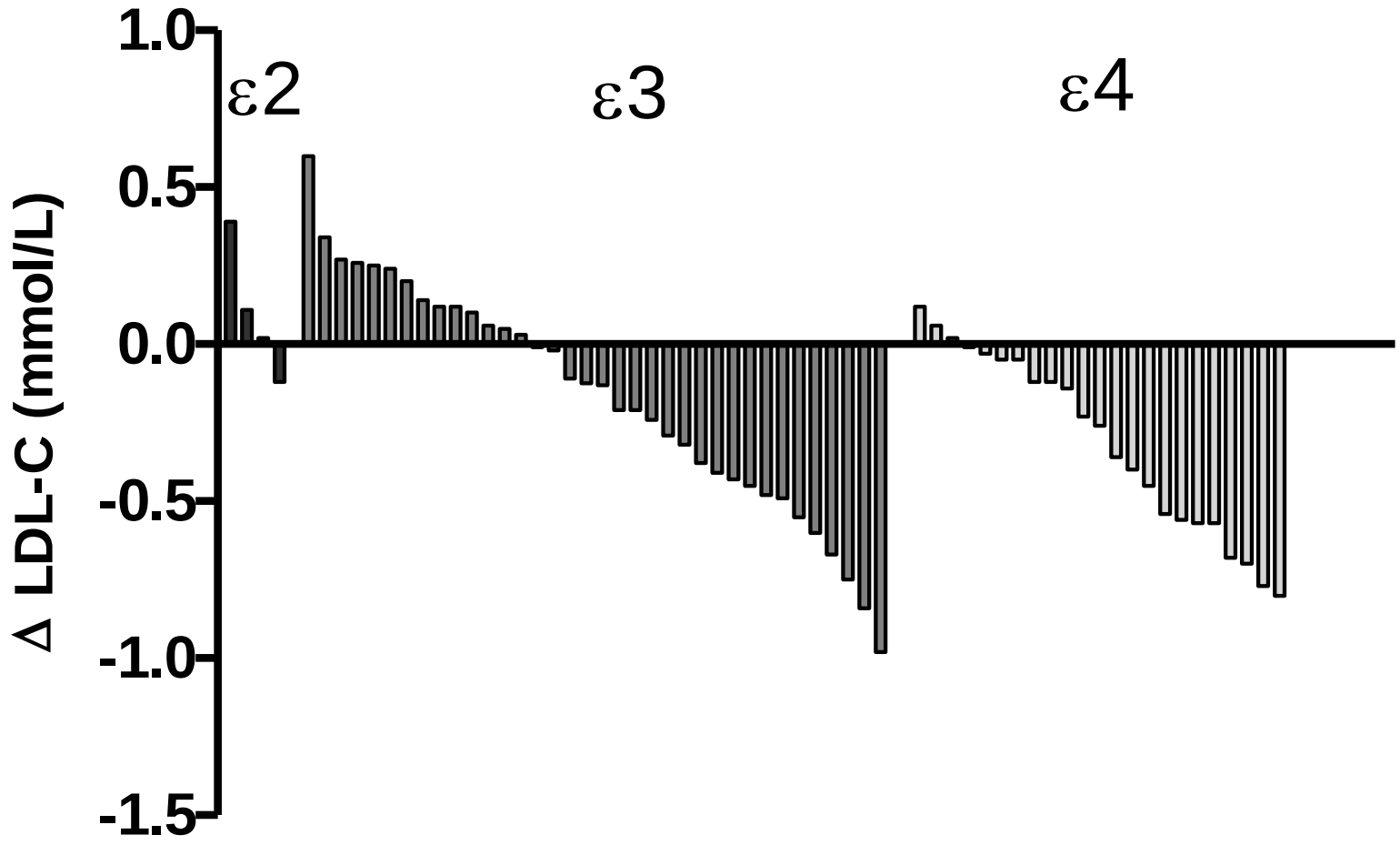

Figure 7.2 Individual changes in LDL cholesterol in response to plant sterol consumption compared to control stratified by ApoE variant.

Table 7.12 Association between synthesis phenotype, genotype and LDL-C lowering in response to plant sterol consumption

	Synthesis phenotype (HS vs LS)	**Interpretation**
CYP7A1 *rs3808607**	Treatment (Tx) by Synthesis: not significant **Tx by Genotype: p= 0.0491** Genotype by Synthesis: not significant Tx by Genotype by Synthesis: not significant	*rs3808607* in CYP7A1 overrides the synthesis phenotype association with PS induced total cholesterol lowering.
ApoE Variant	**Treatment (Tx) by Synthesis: p= 0.0073** **Tx by Genotype: p= 0.0559** Genotype by Synthesis: not significant Tx by Genotype by Synthesis: not significant	The synthesis phenotype barely overrides the association between PS induced total cholesterol lowering and ApoE variant

*as seen in Table 5.5, this SNP is unequally distributed across synthesis phenotype

The unequal distribution of *rs3808607* in CYP7A1 across the synthesis phenotypes, and the stronger association of *rs3808607* with LDL-C lowering in response to PS consumption suggest that *rs3808607* in CYP7A1 is driving the association between LDL-C lowering in response to PS consumption and synthesis phenotype. Therefore, synthesis phenotype was not included when *rs3808607* in CYP7A1 and ApoE variant were combined to determine if they may be related with one another in their association with LDL-C lowering. *rs3808607* in CYP7A1 and ApoE variant were both independently associated with LDL-C lowering by PS (Table 7.13). None of the ε2 variant carriers displayed LDL-C lowering after PS consumption, nor did T/T carriers of *rs3808607* unless they possessed the ε4 variant (Figure 7.3). However, this three way interactive effect between T/T and ε4 did not reach statistical significance (p=0.1603). Baseline participant characteristics stratified by *rs3803607* in CYP7A1 and ApoE variant can be seen in Tables 7.14 and 7.15, respectively.

rs6756629 (p=0.0399), *rs2278356* (p=0.0003) and *rs11887534* (p=0.0413) in ABGC5, *rs760241* (p=0.0291) in DHCR7 and *rs688* (p=0.0422) in LDLR all showed associations with overall LDL-C concentration, independent of PS consumption (Table 7.6). *rs6756629* and *rs11887534* in ABGC5 were in tight linkage (r^2=0.9151) in our study population and *rs6756629* has been previously associated with LDL-C concentrations through Genome wide association (GWAS) by Aulchenko et al.(29). As in the GWAS the A/R carriers, for *rs6756629* and C/S, for *rs11887534*, had lower LDL-C concentrations than G/G carriers in this study population.

Table 7.13 SNP combination associated with LDL-C lowering in response to plant sterol consumption

	Treatment *p-value*	**Treatment x genotype** *p-value*	**Simple effects by SNP combination*** ΔTC (mmol/L), p-value, n
CYP7A1 *rs3808607* *By* ***ApoE*** variant	**p=<0.0016**	**Tx x CYP7A1 p=0.0327** **Tx x ApoE p=0.0201** **All other 2-way or 3-way interactive effects were not significant**	ε2+T/T= +0.25, p=0.2732 (n=2) ε2+T/G=-0.05,p=0.8226 (n=2) ε3+T/T=+0.06, p=0.4832 (n=12) **ε3+T/G=-0.23,p=0.0028(n=18)** **ε3+G/G=-0.38, p=0.0035 (n=6)** **ε4+T/T= -0.37, p=0.0046 (n=6)** **ε4+T/G=-0.24,p=0.0035(n=15)** **ε4+G/G=-0.67, p=0.0032 (n=2)**

*mixed model simple effects of treatment sliced by haplotype using SAS SLICE function when treatment and treatment*haplotype was significant (statistical significance level set to p=0.0063, Bonferroni correction for 8 slices)

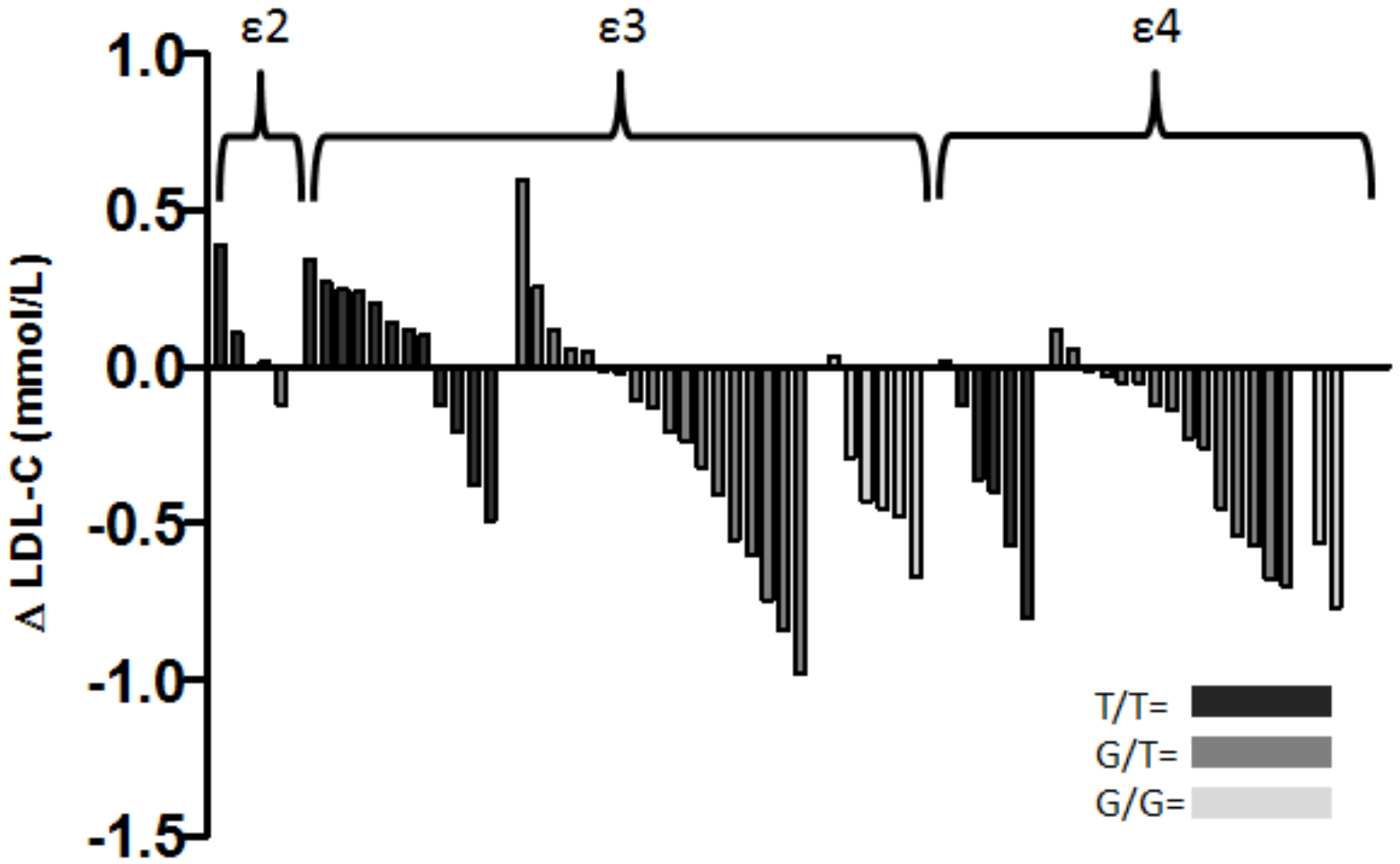

Figure 7.3 Individual changes in LDL cholesterol in response to plant sterol consumption compared to control stratified by *rs3808607* genotype and ApoE variant

Table 7.14 Baseline participant characteristics stratified by *rs3808607* genotype in CYP7A1 (Mean ± STD)

	T/T participants (n=20)	G/T participants (HS, n=35)	G/G participants (n=8)	P-values
Lathosterol[a]	1.72 ± 0.66[†]	1.22 ± 0.54[‡]	1.30 ± 0.41[†‡]	**p=0.0102**
Gender (male/female)	8/12	12/23	4/4	p=0.7270[b]
Age (years)	55.00 ± 8.79	56.08 ±8.70	53.75 ±7.63	P=0.7577
Body weight (kg)	93.29 ± 20.14[†]	78.46 ± 21.44[‡]	77.70 ± 17.49[†‡]	**p=0.0332**
BMI (kg/m2)	31.68 ± 7.76[†]	27.65 ±4.76[‡]	26.74 ± 4.32[†‡]	**p=0.0321**
TC (mmol/L)	5.81 ± 0.95	6.10 ±0.88	5.74 ±0.58	p=0.3636
LDL (mmol/L)	3.78 ± 0.77	3.82 ±0.76	3.47 ±0.66	p=0.4905
HDL * (mmol/L)	1.33 ± 0.31	1.51 ± 0.41	1.57 ± 0.54	p=0.1940
TG * (mmol/L)	1.57 ± 0.62	1.72 ±0.67	1.56 ± 1.03	P=0.7157
Glucose (mmol/L)	5.00 ± 0.54	4.93 ± 0.49	4.84 ± 0.35	p=0.7465
Cholestanol[a]	1.59 ± 0.28	1.69 ± 0.32	1.64 ± 0.31	p=0.5502
Campesterol[a]	2.12 ± 0.70	2.25 ±1.06	2.40 ± 0.62	p=0.7648
B-sitosterol[a]	1.14 ± 0.41	1.16 ± 0.56	1.23 ± 0.38	p=0.9028
7αHC[c*]	6.91 ± 4.84	7.27 ± 7.17	5.13 ± 2.95	p=0.1952

p-values from 1-way analysis of variance. Different superscripts indicate significant differences at p=0.05 by Tukey's HSD test.
a= non-cholesterol sterols reported as µmol/mmol cholesterol
b=calculated by Pearson chi-square test
c= values from end of placebo phase, not baseline of trial, values from end of placebo phase, not baseline of trial, reported as nmol/mmol cholesterol
*=values were log transformed prior to analysis, actual means and standard deviation in the mean reported

Table 7.15 Baseline participant characteristics stratified by ApoE variant (Mean ±STD)

	ApoE ε2 (n=4)	ApoE ε3 (n=36)	ApoE ε4 (n=23)	P-values
Lathosterol[a]	1.45 ± 0.83	1.38 ± 0.53	1.40 ± 0.70	p=0.9702
Gender (male/female)	2/2	16/20	6/17	p=0.3230
Age (years)	58.25 ± 11.30	55.83 ± 7.60	54.35 ±9.58	P=0.6473
Body weight (kg)	98.11 ± 15.88	85.22 ± 24.83	77.09 ± 14.01	p=0.1272
BMI (kg/m2)	32.02 ± 2.56	28.78 ±6.87	28.32 ± 4.98	p=0.5333
TC (mmol/L)	6.88 ± 1.10	5.87 ±0.70	5.95 ±1.03	p=0.0937
LDL (mmol/L)	4.64 ± 0.80†	3.69 ±0.69‡	3.74 ±0.76†‡	**p=0.0470**
HDL (mmol/L)	1.63 ± 0.29	1.43 ± 0.43	1.47 ± 0.39	p=0.6539
TG.(mmol/L)	1.36 ± 0.87	1.69 ±0.74	1.65 ± 0.63	P=0.6757
Glucose (mmol/L)	4.66 ± 0.35	4.96 ± 0.51	4.95 ± 0.45	p=0.5011
Cholestanol[a]	1.48 ± 0.19	1.65 ± 0.32	1.69 ±0.30	p=0.4606
Campesterol[a]	1.74 ± 0.31	2.40 ±1.02	2.05 ± 0.72	p=0.1958
B-sitosterol[a]	1.02 ± 0.32	1.23 ± 0.54	1.09 ± 0.43	p=0.4889
7αHC[c]	4.10 ± 4.89	6.61 ± 5.27	7.80 ± 7.36	p=0.4950

p-values from 1-way analysis of variance. Different superscripts indicate significant differences at p=0.05 by Tukey's HSD test.

a= non-cholesterol sterols reported as µmol/mmol cholesterol

b=calculated by Pearson chi-square test

c= values from end of placebo phase, not baseline of trial. Reported as nmol/mmol cholesterol

rs2278356 in ABCG5 has not previously been associated with LDL-C concentrations, in our study population homozygous minor allele carriers (C/C) had lower LDL-C concentrations than heterozygotes (C/A, p=0.0002) or homozygotes major allele carriers (A/A, p<0.0001). Allele carriers for *rs760241* in DHCR7 had higher LDL-C concentrations than G/G carriers (p=0.0291), independent of PS consumption, this SNP has not previously been associated with LDL-C concentrations.

rs688 in LDLR has previously been associated with LDL-C concentrations (31-33). While *rs688* in LDLR was associated overall with LDL-C (p=0.0422), there was no difference between groups when adjusted for multiple comparisons. The homozygous minor allele carriers (T/T) in our study did not have higher overall LDL-C concentrations than heterozygotes (C/T, p=0.0586) or homozygous major allele carriers (C/C, p=0.0967). While not reaching significance after adjusting for multiple comparisons, the pattern resembled what is typically seen in other trials which show an increase in LDL-C in T/T carriers.

Influence of genotype on response of HDL-C in response to PS consumption

No SNPs were associated with changes in HDL-C in response to PS consumption. *rs688* in LDLR was associated (p=0.0106) with overall HDL concentrations, independent of PS consumption (Table 7.6). Minor allele homozygous (T/T) carriers had higher HDL concentrations than heterozygous (C/T, p=0.0077), but not homozygous, major allele (C/C p=0.1017) carriers.

Influence of genotype on changes in cholesterol absorption surrogates in response to plant sterol consumption

One SNP *rs2066714* in ABCA1 (p=0.0176 for interaction) was associated with a change in cholestanol to cholesterol ratio after PS consumption (Table 7.16). C/C (n=10) carriers of *rs2066714* in ABCA1 had a reduction in cholestanol to cholesterol ratio following PS consumption compared to placebo, while T/T and T/C carriers did not (Table 7.17). No SNPs were related to overall cholestanol to cholesterol ratio, independent of PS consumption.

rs3808607 in CYP7A1 (p=0.0228 for interaction) was associated with a change in campesterol to cholesterol ratio after PS consumption (Table 7.18). All genotypes, T/T, T/G and G/G had increases in campesterol to cholesterol ratio following PS consumption compared to placebo. The magnitude of increase in this ratio in *rs3808607* G/G carriers was larger than T/T individuals with T/G individuals falling in between. These changes in campesterol to cholesterol ratio could reflect: 1) differences in the amount of campesterol that was absorbed, perhaps indicating different absorption levels; 2) differences in the amount of cholesterol lowering difference already associated with rs3808607 in CYP7A1; 3) differences in the amounts of campesterol consumed (campesterol was one of the sterols in the PS treatment).

Table 7.16 Candidate SNP associations with cholesterol absorption surrogates in response to plant sterol consumption

Gene *SNP*	**Cholestanol**		**Campesterol**		**Sitosterol**	
ABCA1	Overall	**TX by SNP**	Overall	TX by SNP	Overall	TX by SNP
rs2230808	p=0.6001	p=0.3832	p=0.7729	p=0.6383	p=0.6084	p=0.0862
rs2066714	p=0.6107	**P=0.0176**	p=0.5577	p=0.4499	p=0.4323	p=0.4870
ABCG5	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs6720173	p=0.5135	p=0.6327	p=0.6007	p=0.6279	p=0.7546	p=0.7546
rs6756629	p=0.9400	p=0.1246	p=0.7678	p=0.8325	p=0.8552	p=0.9717
rs2278356	p=0.4897	p=0.1217	p=0.9940	p=0.9178	p=0.9567	p=0.6365
rs11887534	p=0.8370	p=0.0570	p=0.8281	p=0.3711	p=0.6522	p=0.5368
ABCG8	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs4148211	p=0.3908	p=0.8888	p=0.5441	p=0.7180	p=0.4535	p=0.5276
rs4148217	p=0.4367	p=0.4943	p=0.2997	p=0.5513	p=0.4500	p=0.9720
rs6544718	p=0.9486	p=0.2987	p=0.6660	p=0.9962	p=0.7308	p=0.2941
CETP	Overall	TX by SNP	Overall	TX by SNP	Overall	**TX by SNP**
rs5882	p=0.5561	p=0.8419	p=0.4070	p=0.2532	p=0.9187	**p=0.0071**
CYP7A1	Overall	TX by SNP	Overall	**TX by SNP**	Overall	**TX by SNP**
rs3808607	p=0.1277	p=0.5710	p=0.2123	**p=0.0228**	p=0.3360	**p=0.0338**
DHCR7	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs760241	p=0.4059	p=0.9572	p=0.5724	p=0.5815	p=0.6439	p=0.8664
LDLR	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs688	P=0.8198	p=0.3412	p=0.6916	p=0.2925	p=0.4265	p=0.5076
LSS	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs2839158	p=0.1716	p=0.0732	p=0.7583	p=0.4591	p=0.8086	p=0.4651
rs34115287	p=0.3365	p=0.0866	p=0.9258	p=0.9386	p=0.8904	p=0.6696
PCSK9	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs562556	p=0.7340	p=0.7964	p=0.2355	p=0.4712	p=0.2394	p=0.9269
SCAP	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs12487736	p=0.4104	p=0.5424	p=0.9950	p=0.9238	p=0.8888	p=0.9328
SREBF2	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs2228314	p=0.3631	p=0.4244	p=0.8015	p=0.7419	p=0.7502	p=0.7317
rs2228313	p=0.8665	p=0.6857	p=0.8534	p=0.5424	p=0.8932	p=0.9660
ApoE	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
Isoform	p=0.2808	p=0.6833	p=0.4716	p=0.3660	p=0.5081	P=0.1106

Table 7.17 *rs2066714* in ABCA1 associates with cholestanol to cholesterol ratio lowering in response to plant sterol consumption

Gene *SNP*	**Treatment** *p-value*	**Treatment x genotype** *p-value*	**Simple effects by genotype*** Δ, p-value, n
ABCA1 *rs2066714*	**p <.0057**	**p= 0.0176**	A/A=-0.02, p =0.4908(n=25) † A/G=-0.001, p= 0.9690 (n=28) † **G/G=-0.14, p=0.0010 (n=10)** ‡

*mixed model simple effects of treatment sliced by genotype using SAS SLICE function when treatment and treatment*genotype was significant (statistical significance level set to p=0.0167, Bonferroni correction for 3 slices)
Genotypes within an SNP with different superscripts had significantly different treatment effect size, tested by ANOVA with Tukey –Kramer adjustment.

Table 7.18 *rs3808607* in CYP7A1 associates with campesterol to cholesterol ratio changes in response to plant sterol consumption

Gene *SNP*	**Treatment** *p-value*	**Treatment x genotype** *p-value*	**Simple effects by genotype*** Δ, p-value, n
CYP7A1 *rs3808607*	**p <.0001**	**p=0.0228**	T/T=+1.09, p<0.0001(n=20) † T/G=+1.30, p<0.0001 (n=35) †‡ G/G=+1.77, p<0.0001 (n=8) ‡

*mixed model simple effects of treatment sliced by genotype using SAS SLICE function when treatment and treatment*genotype was significant (statistical significance level set to p=0.0167, Bonferroni correction for 3 slices)
Genotypes within an SNP with different superscripts had significantly different treatment effect size, tested by ANOVA with Tukey –Kramer adjustment.

However, differences in PS consumption based on genotype are very unlikely. When changes in absolute campesterol concentrations (not ratios to cholesterol) after PS consumption were associated with *rs3808607* the interaction did not reach significance (p=0.0860). Therefore, it is more likely that the changes in cholesterol associated with *rs3808607* may be driving this association with changes in campesterol to cholesterol ratio.

Two SNPs, *rs3808607* in CYP7A1 (p=0.0338 for interaction) and *rs5882* in CETP (p=0.0071 for interaction) were associated with changes in sitosterol to cholesterol ratio after PS consumption (Table 7.19).

All genotypes, T/T, T/G and G/G in *rs3808607* had increases in sitosterol to cholesterol ratio following PS consumption compared to placebo. The magnitude of the increase in *rs3808607* G/G carriers was larger than in T/G carriers with T/T carriers falling in between. These results were slightly different from the pattern seen with campesterol.

All genotypes, A/A, A/G and G/G in *rs5882* had increases in sitosterol to cholesterol ratio following PS consumption compared to placebo. The magnitude of increase in A/G and G/G carriers was larger than A/A individuals.

No SNPs were associated with cholesterol absorption surrogates overall, independent of PS consumption (Table 7.16).

Table 7.19 SNPs associating with changes in sitosterol to cholesterol ratio in response to plant sterol consumption

Gene *SNP*	**Treatment** *p-value*	**Treatment x genotype** *p-value*	**Simple effects by genotype*** Δ, p-value, n
CYP7A1 *rs3808607*	**p <.0001**	**p=0.0338**	T/T=+0.42, p<0.0001(n=20) †‡ T/G=+0.35, p<0.0001 (n=35) † G/G=+0.59, p<0.0001 (n=8) ‡
CETP *rs5882*	**p <.0001**	**p=0.0228**	A/A=+0.29, p <0.0001 (n=25) † A/G=-+0.46, p<0.0001 (n=28) ‡ G/G=+0.52, p<0.0001 (n=10) ‡

*mixed model simple effects of treatment sliced by genotype using SAS SLICE function when treatment and treatment*genotype was significant (statistical significance level set to p=0.0167, Bonferroni correction for 3 slices).
Genotypes within an SNP with different superscripts had significantly different treatment effect size, tested by ANOVA with Tukey –Kramer adjustment.

Influence of genotype on response of cholesterol and bile acid synthesis surrogates to plant sterol consumption

rs4148211 in ABCG5 (p=0.0016) was associated with change in desmosterol to cholesterol ratio due to PS consumption (Table 7.20). The A/A genotype for *rs4148211* failed to manifest a change in desmosterol to cholesterol ratio due to PS consumption, while the A/G genotype had a decrease while the G/G genotype showed a increase. The effect size (and direction) of PS consumption on desmosterol to cholesterol ratio was different between A/G and G/G genotypes (Table 7.21). Three SNPs, *rs11887534* (p=0.0171) in ABCG5 and *rs2839158* (p=0.0443) and *rs34115287* (p=0.0233) in LSS were associated with overall desmosterol to cholesterol ratio, independent of PS consumption (Table 7.20).

rs11887534 in ABCG5 showed an overall association (p=0.0171) with total cholesterol concentration, and was unequally distributed between the synthesis phenotype groups, with C/S carriers having lower total cholesterol concentrations and a greater propensity to be in the HS group (8 HS vs 1 LS). The association with desmosterol to cholesterol ratio could be due to the previous association with cholesterol concentrations, however, when only absolute desmosterol (not ratio to cholesterol) was associated with *rs11887534* in ABCG5, an overall effect of gender and *rs11887534* was seen (p=0.0348), with male C/S carriers having higher desmosterol than male G/G carrier and all females. This suggests that C/S carriers, particularly males could have higher endogenous cholesterol synthesis. *rs11887534* in ABCG5 has been linked to gallstones by genome wide association studies, with the proposed mechanism being higher output of cholesterol into the bile in the C-allele carriers (34).

Table 7.20 Candidate SNP associations with cholesterol synthesis surrogates in response to plant sterol consumption

Gene *SNP*	**Desmosterol**		**Lathosterol**		**7α-hydroxycholesterol**	
ABCA1	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs2230808	p=0.7623	p=0.6194	p=0.8292	p=0.7676	p=0.8865	p=0.7294
rs2066714	p=0.2968	p=0.5320	p=0.5729	p=0.3929	p=0.5156	p=0.6829
ABCG5	**Overall**	TX by SNP	**Overall**	TX by SNP	**Overall**	TX by SNP
rs6720173	p=0.2697	p=0.0798	p=0.5626	p=0.4718	p=0.6201	p=0.4917
rs6756629	p=0.0609	p=0.3230	**p=0.0195**	p=0.2891	**p=0.0227**	p=0.9239
rs2278356	p=0.8299	p=0.0891	p=0.5507	p=0.7895	p=0.0818	p=0.3267
rs11887534	**p=0.0171**	p=0.2043	**p=0.0060**	p=0.1880	**p=0.0113**	p=0.7163
ABCG8	Overall	**TX by SNP**	Overall	TX by SNP	Overall	TX by SNP
rs4148211	p=0.7939	**p=0.0016**	p=0.9474	p=0.0881	p=0.7935	p=0.0555
rs4148217	p=0.3462	p=0.0806	p=0.3316	p=0.6338	p=0.2553	p=0.6549
rs6544718	p=0.1936	p=0.6621	p=0.2305	p=0.6737	p=0.3636	p=0.5836
CETP	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs5882	p=0.9187	p=0.6183	p=0.9449	p=0.5402	p=0.3817	p=0.2898
CYP7A1	Overall	TX by SNP	**Overall**	TX by SNP	Overall	TX by SNP
rs3808607	p=0.1939	p=0.7607	**p=0.0043**	p=0.5817	**p=0.0481**	**p=0.0427**
DHCR7	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs760241	p=0.4028	p=0.5254	p=0.1099	p=0.1465	p=0.6505	p=0.5789
LDLR	Overall	TX by SNP	**Overall**	TX by SNP	**Overall**	TX by SNP
rs688	p=0.1276	p=0.8967	**p=0.0259**	p=0.1811	**p=0.0402**	p=0.2000
LSS	**Overall**	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs2839158	**p=0.0443**	p=0.5033	p=0.3490	p=0.2925	p=0.8110	p=0.8751
rs34115287	**p=0.0233**	p=0.3677	p=0.2313	P=0.2580	p=0.6205	p=0.9191
PCSK9	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs562556	p=0.1860	p=0.5753	p=0.3328	p=0.6423	p=0.4721	p=0.9835
SCAP	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs12487736	p=0.8905	p=0.7063	p=0.4212	p=0.4269	p=0.9662	p=0.3419
SREBF2	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
rs2228314	p=0.2467	p=0.7695	p=0.7640	p=0.2279	**p=0.0283**	p=0.9476
rs2228313	p=0.2323	p=0.3285	p=0.9574	p=0.7708	p=0.8991	p=0.6330
ApoE	Overall	TX by SNP	Overall	TX by SNP	Overall	TX by SNP
Isoform	p=0.8427	p=0.6501	p=0.9308	p=0.4719	p=0.5693	p=0.1543

Table 7.21 ***rs4148211*** in **ABCG8 associates with changes in desmosterol to cholesterol ratio in response to plant sterol consumption**

Gene *SNP*	**Treatment** *p-value*	**Treatment x genotype** *p-value*	**Simple effects by genotype*** Δ, p-value, n
ABCG8 *rs4148211*	p=0.4136	**p=0.0016**	A/A=+0.01, p =0.5139 (n=26) †‡ **A/G=-0.05, p= 0.0120 (n=24)** † **G/G=+0.08, p=0.0109 (n=13)** ‡

*mixed model simple effects of treatment sliced by genotype using SAS SLICE function when treatment and treatment*genotype was significant (statistical significance level set to p=0.0167, Bonferroni correction for 3 slices)
Genotypes within an SNP with different superscripts had significantly different treatment effect size,
tested by ANOVA with Tukey –Kramer adjustment.

Higher cholesterol export into the bile would likely result in a compensatory increase in cholesterol synthesis explaining the association of *rs11887534* and desmosterol, as well as the unequal distribution of *rs11887534* between HS and LS groups. *rs11887534* and *rs6756629* in ABCG8 were in tight linkage in our trial population, however, the association between *rs6756629* and desmosterol to cholesterol ratio failed to reach significance (p=0.0609).

rs2839158 (p=0.0443) and *rs34115287* (p=0.0233) in LSS are in tight linkage and both showed an association with overall desmosterol to cholesterol ratio, independent of PS consumption. C/C for *rs2839158* and T/T for *rs34115287* had higher desmosterol to cholesterol than T/Y and C/Y carriers, for *rs2839158* and *rs3411528* respectively. This reduced desmosterol to cholesterol ratio in C/Y individuals could suggest decrease endogenous cholesterol synthesis rates. This reduction in endogenous cholesterol synthesis likely resulted in the increase cholesterol lowering in response to PS consumption, demonstrated by the interactive effect *rs3411528* had with total cholesterol lowering (p=0.0209).

Four SNPs, *rs11887534* (p=0.0060) and *rs6756629* (p=0.0195) in ABCG5, *rs3808607* (p=0.0043) in CYP7A1 and *rs688* (p=0.0259) in LDLR were associated with overall lathosterol to cholesterol ratio, independent of PS consumption. Intuitively, these four SNPs were also shown to be unequally distributed between HS and LS groups, since those groups were based on *a priori* lathosterol to cholesterol ratio.

rs11887534 and *rs6756629* in ABCG5 are in tight linkage, with C/S and A/R carriers having higher lathosterol to cholesterol ratios than T/T and G/G carriers, independent of PS consumption, for *rs11887534* (p=0.0060) and *rs6756629* (p=0.0195), respectively.

In *rs3808607* in CYP7A1 T/T carriers had higher lathosterol to cholesterol ratio than T/G carriers, but not G/G (p=0.1993). These findings suggest that T/T individuals have increased cholesterol synthesis, as is reflected in the synthesis groups, where 13/24 individuals are T/T carriers in the HS group, with only 7/39 are T/T in the LS group.

In *rs688* in LDLR G/G carriers had lower lathosterol to cholesterol ratios compared to A/A (p=0.0352) and A/G (p=0.0492) carriers. This finding suggests that G/G individuals have lower cholesterol synthesis.

rs3808607 in CYP7A1 (p=0.0427) was associated with change in 7αHC to cholesterol ratio in response to PS consumption (Table 7.27), and overall 7αHC to cholesterol ratio (Table 7.20). Gender also had interaction with treatment in 7αHC to cholesterol ratio as well. In response to PS consumption, both T/T and T/G female carriers had reductions in 7αHC to cholesterol, while G/G carriers, male or female did not. The change in 7αHC to cholesterol ratio due to PS consumption compared to control was different between T/G and G/G carriers and in opposite directions, with T/T carriers falling in between (Table 7.22). Independent of PS consumption, *rs3808607* was associated with 7αHC to cholesterol ratio, however when corrected for multiple comparisons, T/G carriers were not lower than T/T (p=0.0643) or G/G (p=0.1185) carriers.

rs6756629 (p=0.0227) and *rs11887534* (p=0.0113) in ABCG5, as well as *rs688* (p=0.0402) in LDLR and *rs2228314* (p=0.0283) in SREBF2 were also associated overall with 7αHC to cholesterol ratio, independent of PS consumption. *rs6756629* and *rs11887534* in ABCG5 are in tight linkage, C/S and A/R carriers had higher 7αHC to cholesterol ratios than T/T and G/G carriers, independent of PS consumption, for *rs11887534* and *rs6756629*, respectively. In *rs688* in LDLR, G/G carriers had lower

7αHC to cholesterol ratios than A/G carriers (p=0.0306), but not A/A carriers (p=0.3265). In *rs2228314* in SREBF2, C/C carriers had higher 7αHC to cholesterol ratios than G/C (p=0.0335) and G/G (p=0.0240) carriers.

Table 7.22 *rs38038607* in CYP7A1 associates with changes in 7αHC to cholesterol ratio in response to plant sterol consumption

Gene *SNP*	**Treatment** *p-value*	**Treatment x genotype** *p-value*	**Simple effects by genotype*** ΔTC (mmol/L), p-value, n
CYP7A1 *rs4148211*	p=0.6049	**p=0.0427**	**T/T=-0.26, p=0.0023, n=20**†‡ **T/G=-0.28,p=0.0043, n=35**† G/G=+0.38,p=0.4455, n=8‡

*mixed model simple effects of treatment sliced by genotype using SAS SLICE function when treatment and treatment*genotype was significant (statistical significance level set to p=0.0167, Bonferroni correction for 3 slices)
Genotypes within an SNP with different superscripts had significantly different treatment effect size, tested by ANOVA with Tukey –Kramer adjustment.

7.5 Discussion

Through the investigation of candidate genetic polymorphisms in genes directly related to cholesterol metabolism a significant genetic heterogeneity in response of total and LDL cholesterol to PS consumption was demonstrated within our study population. An impact of SNP *rs3808607* in CYP7A1, *rs34115287* in LSS and ApoE variant was shown on the degree of PS induced cholesterol lowering in our study population. These findings build on our previous results which have shown that response of total and LDL cholesterol to PS consumption is influenced by endogenous cholesterol synthesis (Chapter 6).

rs3808607 in CYP7A1 had the strongest and most consistent association with response to PS consumption, associating with both total and LDL cholesterol lowering. The association with total and LDL cholesterol lowering and PS consumption demonstrated an allelic dose effect, from non-response in the T/T carriers to an increasing response to PS consumption with each G-allele (Figure 7.1). *rs3808607* was also a much better predictor of response than the high and low endogenous synthesis phenotypes (Chapter 6). This observation was also reflected in the unequal distribution of *rs3808607* across the synthesis phenotypes, with more T-variant in the high and more G-variant in the low endogenous synthesis phenotype group.

De Castro-Oros et al. (16) has previously associated this SNP with response of TC to plant sterol consumption when the results of two previously completed plant sterol intervention trials were combined. Carriers of the G variant showed higher adjusted mean TC reductions compared to T variant following PS consumption. Our results have expanded on these findings by De Castro-Oros et al. replicating the association of *rs3808607* on total cholesterol and showing for the first time an association with LDL-C

lowering. The association of *rs3803607* with total and LDL cholesterol both showed an allelic dose effect, whereas in the De Castro-Oros et al. study all minor allele carriers were compared to homozygous major allele carriers.

rs3808607 is in the promoter region of CYP7A1 gene, which codes for cholesterol 7-alpha hydroxylase, the enzyme involved in the rate limiting step of the classic bile acid synthesis pathway. Bile acid synthesis is the major metabolic fate of cholesterol in the body and has a large influence on cholesterol homeostasis (35). De Castro-Oros et al. showed that HepG2 cells transfected with the G variant showed 78% higher transcriptional activity of CYP7A1 compared to cells transfected with the T variant (16). De Castro-Oros et al. (16) concluded that compared to the T variant, the G variant at SNP *rs3808607* is associated with enhanced gene expression and subsequently increased bile acid synthesis. This increased synthesis expands the bile acid pool improving cholesterol absorption, which would enhance the cholesterol lowering properties of PS consumption in the G variant carriers.

Our results reinforce and expand this conclusion, particularly through the association of *rs38038607* with changes in 7αHC to cholesterol ratio in response to PS consumption, and through the unequal distribution of *rs38038607* between high and low endogenous synthesis groups. In response to PS consumption, female T-allele carriers showed reduced bile acid synthesis, while homozygous female G/G carriers did not. No effects of genotype or PS consumption were seen in male participants. Our data are the first to show that in female G/G carriers, bile acid synthesis does not decrease in response to PS consumption, which could enhance cholesterol absorption, and increase cholesterol lowering due to PS consumption.

Gender differences have been previously reported in individual bile acid concentrations (36), however, to our knowledge gender and genotype interactions have not previously been associated with changes in bile acid synthesis in response to PS consumption. It must be noted that while we did see changes in 7αHC to cholesterol ratios, the 7αHC concentrations were measured at fasting and not in post-prandial conditions where even greater potential differences by genotype might have occurred. Similarly, Xiang et al. (36) also measured fasting conditions and reported large gender differences in bile acid concentrations between men and women, but did not see effects of the *rs3808607* or other genotypes.

A previous study investigating the impact of oat bran consumption on 7αHC concentrations found differences in 7αHC concentrations following oat bran versus wheat bran consumption, but only within the 8 h period following meals (33). Measurement during the post-prandial period where bile synthesis is being induced, in an interactive fashion with the dietary composition of the meal, is likely critical to understanding the impact of certain genotypes on bile acid metabolism. The unequal distribution of *rs3808607* between high and low endogenous synthesis groups further suggests that the G-variant may shift carriers towards a higher cholesterol absorption/low cholesterol synthesis phenotype, demonstrated by the strong distribution of G-variant carriers towards the low synthesis group (Table 7.3). This association between *rs3808607*and cholesterol metabolism could also have an application in the selection of pharmaceutical based cholesterol lowering pharmaceutical therapies (37). Individuals with high cholesterol absorption and low synthesis may not respond as well to statin therapy and may need additional pharmaceutical therapies to reach target cholesterol lowering.

Results of De Castro-Oros et al. (16) showed that *rs3808607* G-variant transfected cells had much lower transcription factor binding than T-variant cells, suggesting that the G variant may be interfering with transcription factor binding in the promoter region. This lowered transcription factor affinity of the G-variant site was not specific for a particular transcription factor, as numerous bands in the electrophoretic mobility shift assay, relating numerous unknown transcription factors, were all much lighter in the G-variant cells. Building on the results of De Castro-Oros et al. (16) we hypothesize that the *rs3808607* G-variant promoter region has reduced affinity for an as yet unknown transcription factor which is responsible for down-regulating CYP7A1 expression in response to lowered hepatic cholesterol. This dysregulation of CYP7A1, due to the G-variant of *rs3808607,* decreases the effectiveness of endogenous hepatic cholesterol synthesis to raise hepatic cholesterol concentrations, while simultaneously enhancing cholesterol absorption. Conversely, the T-variant of *rs3808607* is appropriately regulated, and therefore predisposes carriers to a high synthesis phenotype.

PS consumption lowers cholesterol absorption, which will have a greater impact on G-variant carriers due to their enhanced cholesterol absorption. This reduced cholesterol absorption will lower delivery of cholesterol to the liver, which in turn drops hepatic cholesterol concentrations triggering activation of SREBP2. SREBP2 turns down cholesterol efflux and increases hepatic cholesterol uptake via LDLR, while stimulating cholesterol synthesis. If bile acid synthesis is not down-regulated during PS consumption, as is suggested with the G-allele in *rs3808607*, LDL-C lowering would be further enhanced by the continued use of hepatic cholesterol as a substrate for bile synthesis and

subsequent increased LDL-C uptake via hepatic LDLR. This enhanced LDL-C lowering with each G-allele in *rs3808607* was clearly seen in our results.

However, in T-allele carriers, when hepatic cholesterol concentrations are being lowered, as in PS consumption or dietary cholesterol restriction, an unknown transcription factor is activated suppressing bile acid synthesis, reducing the impact of lowered cholesterol absorption on hepatic cholesterol concentrations, limiting LDL-C lowering.

The proposed hypothesis of deregulated bile acid synthesis in the *rs3808607* G-allele fits the association seen between *rs3808607* and lipid changes in response to a large dietary composition changes in a 8-year follow up study of 131 unrelated men in the Czech MONICA study (38). A large reduction in meat, eggs, butter, and animal fat consumption, combined with increased fruits and vegetable consumption, had a significant impact on dietary composition across the Czech Republic during the 8 year follow up period. While the exact impact of this dietary pattern shift on the individual diets of the 135 men could not be measured, it was suggested that the changes in dietary composition led to a decrease in cholesterol consumption. *rs3808607* in CYP7A1 had an interactive effect with dietary composition on changes in total and LDL cholesterol, with T/T carriers having smaller reductions in cholesterol than G/G carriers, with T/G carriers' reductions falling in between the homozygotes.

The proposed hypothesis of deregulated bile acid synthesis could also explain previous associations made between *rs3808607* and LDL-C concentrations (39, 40) because as proposed, T-variant carriers (associated with higher endogenous synthesis) respond less to dietary cholesterol, whereas than G-variant carriers (associated with more cholesterol absorption and lowered endogenous synthesis) possess circulating cholesterol

concentrations more reflective of dietary cholesterol. This would predispose G-variant individuals to higher cholesterol concentrations than T-variant carriers on the same western style diet, which contains significant dietary cholesterol sources. The G-variant in *rs3808607* has also been associated with reduced response to statin treatment (41, 42), which fits the proposed impaired down regulation of bile synthesis hypothesis, as it would be assumed that G-variants would be less impacted by the reduction in cholesterol synthesis due to statins if these individuals present with a higher cholesterol absorption/low cholesterol synthesis phenotype.

Interestingly the high endogenous synthesis group had higher 7αHC to cholesterol ratios than did the low endogenous synthesis group, independent of PS consumption, even though the G-variant for *rs3808607* was more strongly represented in the low synthesis group. The overall effect of *rs3808607* on 7αHC to cholesterol ratio suggested that T/T allele carriers had higher 7αHC to cholesterol ratios than T/G allele carriers, however, this association was not significant when corrected for multiple comparisons. These findings further support the hypothesis that the G-variant promoter region for *rs3808607* has less affinity for a down–regulating transcription factor, which is activated in response to PS consumption, rather than higher affinity for an up-regulating transcription factor that would cause G-allele carriers to constantly have higher bile acid synthesis than T-allele carriers.

In individuals with a distal ileum resection, which causes a decrease in bile reabsorption and therefore a subsequent up-regulation of CYP7A1, *rs3808607* T/T individuals had higher 7αHC to cholesterol ratio compared to G/G individuals (43). In the control group, without distal ileum resection, no differences were seen in 7αHC to cholesterol ratio. This

finding by Lenicek et al. (43) supports the theory that T-variants can be properly regulate CYP7A1, at least in circumstances of bile loss, and perhaps suggests that the G-variant of rs3808607 may have lower affinity for both an up-regulating transcription factor as well. The multiple bands on the De Castro-Oros et al. (16) electrophoretic mobility shift assay which were all lighter for the G-variant versus the T-variant allele for *rs3808607* suggest that the binding of numerous transcription factors could be impacted by this polymorphism. Ideally more research using human hepatic cell cultures should be done to investigate and identify which specific transcription factors are impacted by the *rs3808607* polymorphism, especially under high and low cholesterol conditions.

The influence of ApoE variant on response to PS consumption was evident in responses of both total and LDL cholesterol concentrations (Figure 7.2), and this impact was independent of the synthesis phenotype when both were associated with response (Table 7.12). ApoE ε2 variant individuals did not show a reduction in cholesterol in response to PS consumption, however, the number of individuals with the ε2 variant was very low (n=4) in our trial, which limits the interpretation of these results. More interestingly was the excellent response of the ApoE ε4 variant individuals (n=24), and the apparent ability of the ε4 variant to override the non-responsive T/T genotype in *rs3808607.* Within the ε3 variant (n=35) individuals the impact of *rs3808607* was clearly seen, however, as can be seen all ε4 variants showed good response to PS consumption (Figure 7.3). Unlike what could be expected from the previous Figure, no three way interaction of ApoE variant and *rs3808607* and PS consumption was seen with LDL-C reduction. The trial size (n=63) could have limited the ability to detect such higher order interactive effects.

This is the first time this dual association of ApoE variant and CYP7A1 has been shown with response of total or LDL cholesterol following PS consumption. Indeed, the association between ApoE ε4 variant with enhanced PS induced cholesterol lowering has previously been proposed by Miettinen et al. (20) and Vanhanen et al. (21) who both demonstrated enhanced cholesterol lowering in ε4 individuals following different plant stanol consumption regimens. Geelen et al.(22) did not see a difference between ApoE ε4 variant and ε3 variant individuals in a clinical trial that prospectively recruited to compare the ε4 and ε3 variants. Both ApoE ε4 variant and ε3 variant individuals showed similar response to 3 weeks of PS consumption. Sanchez Muniz et al. (19) on the other hand suggested that ApoE ε4 variant carriers were not responders to PS consumption. In this study LDL-C lowering was only seen in the ApoE ε2 and ApoE ε3 individuals. While this study was large (n=207), it was a parallel arm design (n=87 control, n=120 treatment) and had two treatment doses (1.1g/d or 2.2g/d of PS).

The Sanchez-Muniz et al. (19) trial, which did not show reductions in the ApoE ε4 variant, used a parallel arm design, in which the control group could potentially have a very different genetic makeup. Geelen et al.(22) used a crossover design, similar to the present trial, where individuals acted as their own control, while Vanhanen et al.(21) and Miettinen et al.(20) employed single arm interventions where individuals' endpoint values were compared to their own baseline values. A crossover design is the most advantageous for the investigation of nutrient-gene interactions because the genetic makeup of the treatment and control groups are identical, allowing for better isolation of single, or gene/gene interactive effects with a treatment.

Clearly, our data support the assertion that ApoE ε4 variant individuals respond well to PS consumption, similar to the ε3 variant carriers, except for those ε3 carriers who were also T/T carriers for *rs3808607*. The non-responsive effect of the T/T variant in *rs3808607* was somehow overcome in the ApoE ε4 variant carriers.

It has been suggested by some that ApoE ε4 carriers have higher cholesterol absorption, at least compared to ε2 variant carriers (44), and compared to ε2 variant carriers in a population of individuals with familial xanthomatous hypercholesterolemics (45). ApoE ε4 and ε3 proteins have upwards of 100 times higher affinity for LDLR than the ε2 variant (46, 47), and ApoE ε4 variant proteins have a preference for larger lipoproteins like chylomicrons and VLDLs, whereas ε3 and ε2 variants have higher affinity for smaller lipoproteins like HDL (47, 48). Therefore, a higher proportion of ApoE with the ε4 variant should end up on chylomicrons and VLDLs than with the ε3 and ε2 variant. Since the clearance of chylomicron remnants, which contain dietary or biliary derived cholesterol is ApoE dependent and primarily via LDLR, we propose that the hepatic delivery of cholesterol from the cholesterol absorption is faster for ApoE ε4> ε3> ε2 variants. This observation is supported by the fact that chylomicron remnant clearance is faster for ApoE ε4> ε3> ε2 variants (49) and chylomicron remnants are the primary hepatic delivery vehicle of absorbed cholesterol.

This tighter feedback between absorbed and hepatic cholesterol could explain many of the associations between ApoE variant and cholesterol concentrations, including the effect seen in our study. If the hepatic cholesterol concentrations are more closely linked to absorbed cholesterol in ApoE ε4 variant individuals, then the effect of lowered cholesterol absorption, due to PS consumption would be greatest in ApoE ε4 variant

individuals. The drop in absorbed cholesterol would have a greater impact on hepatic cholesterol concentrations in ApoE ε4 variants, triggering a cascade that leads to decrease hepatic cholesterol efflux, increased cholesterol synthesis and increased hepatic cholesterol uptake. This increased hepatic cholesterol uptake would result in total and LDL cholesterol lowering seen in ApoE ε4 variant individuals in our study. It is possible that this greater feedback of lower absorbed cholesterol due to PS consumption in ApoE ε4 variant individuals was able to overcome the tight regulation of bile acid synthesis seen in *rs3803807* T/T variant carriers, which we suggested as contributing to non-response of cholesterol concentrations to PS-consumption.

Another interesting aspect of the dual interaction between ApoE variant and *rs3808607* is the number of individuals who were ApoE ε2 or ε3 with rs3808607 T/T alleles, yet seem to have LDL-C concentrations which increased during PS consumption (Figure7.3). Six of the 14 individuals with ApoE ε2 or ε3 with rs3808607 T/T alleles had increases in LDL-C of 0.2 mmol/L or greater, compared to two of the other 49 individuals. It may be that in some individuals PS consumption causes some type of compensatory change in cholesterol metabolism which overcompensates for the lowering of absorbed cholesterol by PS consumption and leads to elevation in circulating cholesterol concentrations. The exact mechanism underlying this effect cannot be determined from this study, but may be related to slower clearance of chylomicron remnants and tight regulatory control of bile acid synthesis. Continued investigation of this adverse-response to PS consumption phenotype is required to allow identification of these individuals and to ascertain the exact mechanisms underlying this phenomenon.

In Summary, our data have shown for the first time an association between *rs34115287* in LSS with response of total cholesterol, but not for LDL-C to PS consumption. Both T/T carriers and C/Y carriers for *rs34115287* responded to PS consumption with a less pronounced total cholesterol lowering, however, C/Y carriers had a larger reduction in total cholesterol compared to T/T carriers. The LSS gene encodes for lanosterol synthase (also known as 2,3-oxidosqualene:lanosterol cyclase), a oxidosqualene cyclase involved in lanosterol synthesis from 2,3-oxidosqualene (50). This enzyme in cholesterol synthesis is rate-limiting and regulated, occurring downstream of the HMG-CoA reductase enzyme. *rs34115287* was not unequally distributed between high and low endogenous synthesis groups (based on L/C ratio), however, an overall effect of *rs34115287* was seen in desmosterol to cholesterol ratio, another marker of cholesterol synthesis. T/T carriers for *rs34115287* had higher desmosterol to cholesterol ratios than C/Y carriers, independent of PS consumption. This association between *rs34115287* and desmosterol concentrations has also not previously been reported.

Desmosterol and lathosterol occur in two intersecting pathways (Bloch and Kandutsch-Russell, respectively) of cholesterol synthesis (51) and are both surrogates of cholesterol synthesis, especially when reported as ratios to cholesterol (52). Carriers of one or more of the minor allele for *rs34115287* may have reduced cholesterol synthesis, at least via the Bloch pathway, and subsequently improved lowering of total cholesterol following PS consumption. The parallel nature of the Bloch and Kandutsch-Russell pathways for cholesterol synthesis reinforce the idea that more than just one surrogate marker of cholesterol synthesis may be valuable in evaluating overall cholesterol synthesis (52). This point is clearly demonstrated in the association of *rs34115287* with desmosterol

concentrations and response to PS consumption, even within our population of individuals selected based on lathosterol to cholesterol concentrations. This association between *rs34115287* and cholesterol synthesis is intriguing and should be investigated in a larger population in which non-cholesterol sterol data, or direct cholesterol synthesis measures are available.

Our study population (n=63) can be considered relatively small for probing of genetic associations. However, the specific goal of our study was to look for nutrient by gene interactive effects, which can only be seen in trials which carefully phenotype responsiveness to a given nutrient. We were able to replicate an association for *rs3803607* in CYP7A1 with total cholesterol lowering in response to PS consumption (16). This association was first reported in an equally small population, and replication is critical in this type of nutrigenetic research to strengthen the credibility of other reports. We were also able to replicate overall associations between *rs6756629* (and *rs11887534*) in ABGC5 with total and LDL cholesterol concentrations first reported through GWAS (29), as well as an association between *rs688* in LDLR with LDL-C concentrations (31-33). This replication of previous associations adds to the applicability of the findings in our study to other populations. However, some of the associations reported in our study for the first time must be interpreted in the context of our study's limited size (n=63) and our specially selected high and low endogenous synthesis population. Replication of these findings in future studies will be critical to confirming the associations we have reported.

At the same time our data shed light on the controversial results reported in previous trials, where large inter-individual variation hampered the ability to decisively determine

if and to what degree PS consumption contributes to decreased total and LDL cholesterol and, therefore, reduced CVD risk. Only one previous dietary intervention trial, which used plant stanols, considered endogenous cholesterol synthesis capacity *a priori* across individuals (23), which we have shown could certainly disturb PS cholesterol lowering results should individuals with high or low endogenous cholesterol synthesis capacity be overrepresented in the studies cohorts. Furthermore, the genetic polymorphisms, which we have associated with the response to PS consumption in our study, could modify reported effects of PS consumption in other populations which may be over or under representative of a particular polymorphism.

In summary, our data represent a first step in evaluating the use of endogenous cholesterol synthesis to predict an individual's response to PS intervention. Our results demonstrate that the response of plasma lipids to PS consumption has high inter-individual variability which is influenced by both endogenous cholesterol synthesis phenotype and common genetic variants. These genetic variants could be used in the future to identify individuals who will benefit the most from PS intervention and thereby positively modify their risk profiles in both primary and secondary prevention of CVD. The use of PS consumption, in the context of personalized nutritional recommendations based on predicted response, would greatly increase its efficacy in reducing CVD risk factors.

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Bridge to Chapter VIII

Manuscript 6 showed associations between numerous SNPs and cholesterol lowering in response to PS consumption. Evidence supporting the ability of PS consumption to lower TG concentrations is mixed, with only some trials reporting reductions. Changes in TG concentrations due to PS consumption, as seen in Manuscript 5, exhibit inter-individual variability, which may have a genetic underpinning. Therefore the following chapter comprises a manuscript which presents the associations between candidate single nucleotide polymorphisms in genes related to triglyceride metabolism and changes in triglyceride concentrations in the nutritional trial described in Manuscript 5. Dylan S. MacKay was the principal manuscript author and project lead on the human trial and human data analysis. Peter K. Eck was involved in the investigation of nutrient by gene interactions and contributed to the preparation of the manuscript. Todd C. Rideout contributed to the preparation of the manuscript. David J. Baer was principal investigator at the Beltsville human clinical trial site and contributed to the preparation of the manuscript. Peter J. H. Jones was principal investigator at the Winnipeg human clinical trial site and contributed to the preparation of the manuscript.

Chapter VIII

Manuscript VII

This manuscript will be submitted for publication in 2013

Cholesterol ester transfer protein polymorphism *rs5882* is associated with triglyceride lowering in response to plant sterol consumption

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Running title: genetic basis for heterogeneity of TG response to plant sterols

8.1 Abstract

Emerging evidence has suggested that plant sterol (PS) consumption may have the ability to lower triglyceride (TG) concentrations, on top of their well established LDL cholesterol lowering properties. However, the human clinical trial evidence is still inconsistent, with some trials showing TG reductions, and others no effect, of PS consumption. The potential exists that heterogeneity in responsiveness of TG concentrations to PS consumption has a genetic basis. Therefore we associated SNPs in genes related to TG metabolism with changes in TG concentrations following PS consumption in a trial that recruited individuals with high or low endogenous cholesterol synthesis, estimated by lathosterol to cholesterol (L/C) ratio. 63 mildly hypercholesterolemic adults, pre-assessed as possessing high (n=24, L/C = 2.03 ± 0.39 umol/mmol) or low (n=39, L/C =0.99±0.28 umol/mmol) L/C, consumed either 2g/day (free sterol) of PS ester enriched or PS free margarine for 28 days in a dual-center, single-blind, randomized, 2-phase crossover design. Plasma TG concentrations were measured at the end of each phase. Candidate SNPs and ApoE variant were accessed by TaqMan genotyping assay.

As a result of PS consumption, plasma TG levels were lowered in homozygotes for the minor allele of SNP *rs5882*-G (G/G=-0.47mmol/L, p=0.0002, n=10) in the CETP gene, encoding the Cholesteryl Ester Transfer Protein. However, individuals carrying the SNP *rs5882*-A allele did not show this TG lowering (*rs5882*-A/A +0.01mmol/L, p =0.6634, n=25; *rs5882*-A/G -0.04mmol/L, p= 0.3399, n=28). As a result, no TG lowering would have been observed over the experimental group when we would not have stratified by the genotype (p=0.0506).

We conclude that the SNP *rs5882*-G/G genotype could serve as a biomarker predicting the beneficial TG lowering effect of dietary PS intervention.

8.2 Introduction

Elevated circulating triglycerides (TG) are increasingly being recognized as an important independent risk factor for cardiovascular disease (CVD), that should be aggressively targeted with therapeutic lifestyle modifications (1). Plant sterol (PS) and stanol consumption has a well-recognized cholesterol lowering effectiveness, reducing LDL-C in the range of ~10% at an intake of 2g/day (2, 3). While not typically viewed as a TG lowering dietary agent, PS consumption has in some instances been shown to reduce TG concentrations in previous animal and human studies (4-13). A retrospective analysis of 5 plant stanol studies conducted at Maastricht University was able to show that plant stanol consumption lowered TG concentrations (14). This TG lowering effect was greatest in individuals with elevated baseline TG concentrations. This relationship between baseline TG concentrations and TG lowering through PS consumption was also replicated in a pooled analysis of 12 PS trials conducted by Demonty et al.(15). It has been hypothesized that the reduction in TG concentrations is due to a reduction in hepatic TG rich VLDL-1 particles (16).

Prevalence of hypertriglyceridemia is high, with 33.1% of Americans having "borderline high" TG concentrations and 19.5% falling into the "high "or "very high" categories. Elevated TG's are typically treated through body weight reduction strategies, but statins, fibrates, niacin and omega-3 fatty acids are available drug therapies to lower TG concentrations (17). The potential of PS consumption to reduce TG concentrations, on top of its demonstrated ability to lower LDL cholesterol, would make PS consumption a

viable recommendation for individuals looking to lower both cholesterol and TG concentrations. Unfortunately, the as yet unexplained wide range of responsiveness of TG in response to PS consumption hampers recommendations for PS and TG lowering. If individuals who benefit from TG lowering while consuming PS could be identified then PS consumption could be recommended to them.

In a previous trial (Chapter 6) TG lowering was not associated with endogenous cholesterol synthesis, however, overall PS consumption showed a trend towards TG lowering ($p=0.0506$). There was a wide range of TG responsiveness (Figure 8.1) to PS consumption in this trial and it had a crossover design, which balances the genetic diversity in the placebo and PS treatments. Therefore we investigated the impact of some common genetic polymorphisms in genes directly related to TG metabolism (Table 8.1) to investigate a potential genetic basis for the heterogeneity in TG response to PS consumption.

Table 8.1 Candidate SNPs in genes related to triglyceride metabolism

Gene *SNP*	**Type of SNP**	**Variation**	**Minor allelic frequency**	**Function of gene**
CETP	Cholesteryl ester transfer protein			Facilitates the transport of cholesteryl esters and triglycerides between the lipoproteins
rs5882	NS - missense	A to G	G=0.448	
LDLR	low density lipoprotein receptor			A cell surface protein involved in receptor-mediated endocytosis.
rs688	Synonymous	A to G	G=0.280	
PCSK9	Proprotein convertase subtilisin/kexin type 9			A convertase belonging to the proteinase K subfamily which induces LDLR degradation
rs562556	NS - missense	A to G	G=0.148	
ApoE variant	E2	E3	E4	A glycoprotein present in human plasma; ApoE is associated with triglyceride-rich lipoproteins (chylomicrons and VLDLs) and HDL.
Typical frequency*	7.9%	78.6%	13.5%	

*frequency from (n=1209) Ordovas et al. 1987 (18)

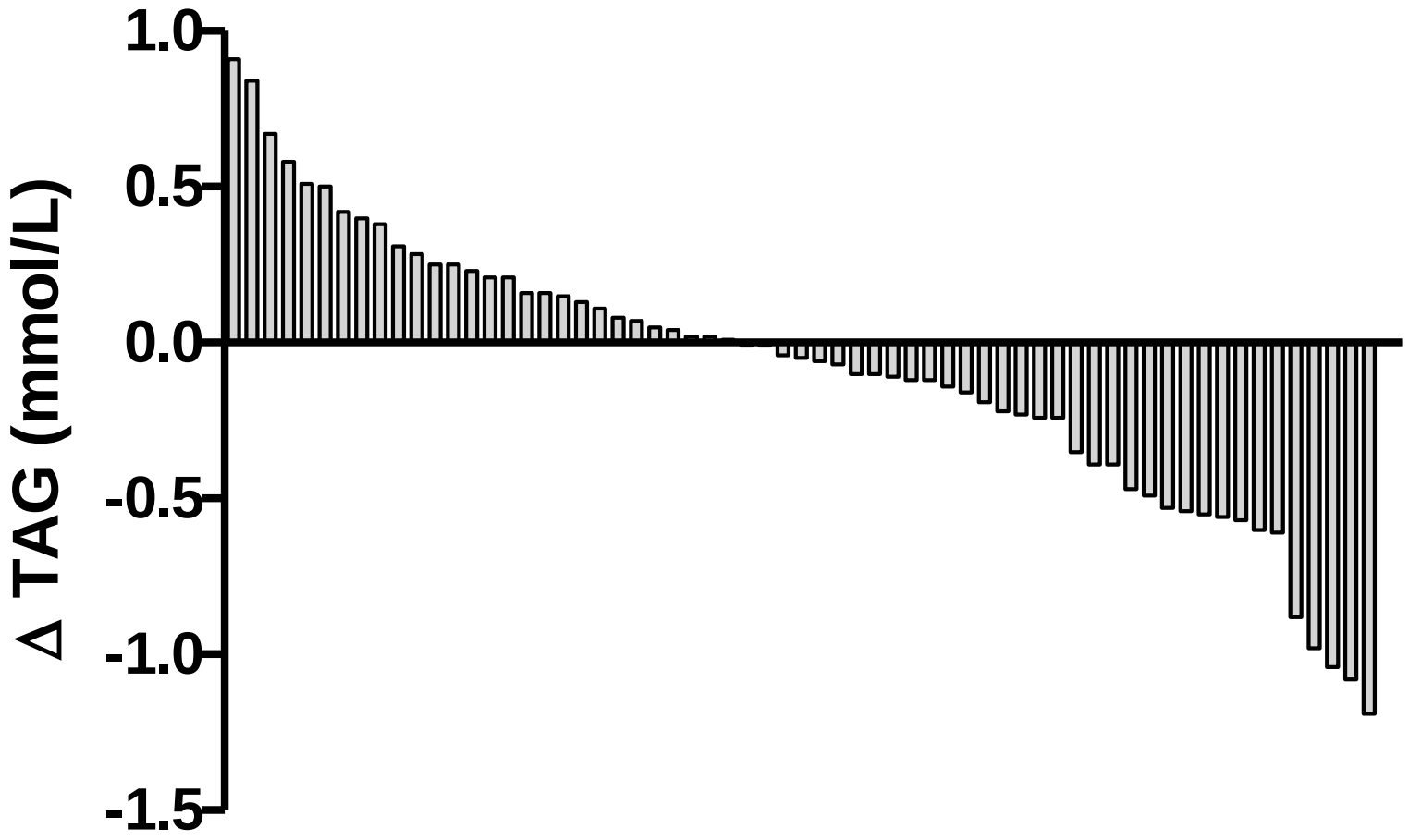

Figure 8.1 Individual changes in TG in response to plant sterol consumption compared to control.

8.3 Methods

Recruitment

Mildly hypercholesterolemic individuals (42 female, 29 male) aged 30-75 years were recruited from Winnipeg, MB and Beltsville, MD as has been reported previously (Chapter 6). The study was conducted according to the principles expressed in the Declaration of Helsinki. The study procedures were approved by the University of Manitoba's Biomedical Research Ethics Board (protocol no. B2007:073). All participants provided written informed consent. Registered at clinicaltrials.gov as NCT01131832.

Study Design

A dual center, randomized, single blind, crossover, placebo controlled clinical trial was conducted at the Nutrition Research Unit (NRU) of the Richardson Center for Functional Foods and Nutraceuticals, University of Manitoba, and the Food Components and Health Laboratory (FCHL), at the USDA Beltsville Human Nutrition Research Center as previously reported previously (Chapter 6). This trial selectively recruited for individuals with high (HS) or low (LS) endogenous cholesterol synthesis using lathosterol to cholesterol ratio as a surrogate marker of cholesterol synthesis.

Blood sampling and serum triglyceride analysis

Serum samples were collected on day 1, 2, and 27, 28 of each phase, following a 12 h fast. Serum, plasma, buffy coat and erythrocyte fractions were separated by centrifugation at 3000 rpm for 20 min at 4°C, aliquoted and immediately stored at -80°C until further analysis.

Serum triglycerides (TG) were determined by automated enzymatic methods on a Vitros-350 chemistry analyzer (Ortho-Clinical Diagnostics, Markham, ON, Canada).

Genotyping

To examine the influence of genetics in TG lowering in response to PS consumption candidate SNPs were selected,), in genes directly related to TG metabolism (Table 5.1). *rs5882* in CETP (TaqMan Assay ID# C_790057_10) and *rs562556* in PCSK9 (TaqMan Assay ID# C_998751_10) are non-synonymous missense mutations. *rs688* in LDLR (TaqMan Assay ID# C_2804264_20) is a synonymous mutation. All candidate genes had a minor allelic frequency of at least 0.06 according to NCBI's dbSNP. ApoE variant (ε2,ε3,ε4) was also assessed by genotyping SNPs *rs7412* (TaqMan Assay ID# C__27492121_20) and *rs429358 (*TaqMan Assay ID# C__3084793_20) in the ApoE gene. Participants were genotyped for each candidate SNP. Genomic DNA was extracted from white blood cells by using a column based DNA extraction kit (DNeasy Blood and Tissue Kit, QIAGEN Sciences) according to the manufacturer's instructions. The concentration and integrity of the genomic DNA were assessed by micro-volume spectrophotometer (NanoDrop 2000,Thermo Fisher Scientific). DNA samples were genotyped by TaqMan SNP genotyping assay (Table 5.2) (Life Technologies, Burlington, ON) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Life Technologies, Burlington, ON)

Statistical analysis

Endpoint measurements of the treatment and placebo phases were compared. The statistical analysis was performed by using SAS 9.2 (SAS Inc). Dependent variable normality was determined using the Shapiro-Wilk test. TG concentrations were found to be non-normal and were log transformed before analysis. The results are as actual means ± SEM and not estimated least squared means because of the log transformation. The effects of treatment were analyzed by the SAS MIXED procedure with participant ID and site as random factors and treatment as an independent factor. 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. Significant treatment by genotype effects, were examined by the SAS SLICE function, with Bonferroni correction for the number of slices. Treatment effect sizes by genotype from significant one way treatment by genotype interactions were compared by ANOVA using difference in mixed model least squared mean summary statistics for the treatment effect slices, with Tukey-Kramer adjustment for multiple comparisons. Statistical significance was set at $p < 0.05$ in all analyses, with adjustments as above. Due to the strong candidate nature of the SNPs selected no correction for family-wise multiple comparisons in association with genotypes were used because risk of type 1 error was minimal.

8.4 Results

Baseline characteristics

63 individuals (n=24 HS, n=39 LS) completed the 2-phase study design and were genotyped. Baseline characteristics of the study population (n=63) who completed the study have previously been published (Chapter 6). HS participants by design had higher

lathosterol to cholesterol ratio ($p<0.0001$) than LS participants. HS participants had higher body weight ($p=0.007$), BMI ($p=0.0032$), TG concentrations ($p=0.0017$), and lower HDL-C ($p=0.0001$) and sitosterol to cholesterol ratio ($p=0.0164$) and cholestanol to cholesterol ratio ($p=0.0034$) than the LS participants as previously reported (Chapter 6).

Effects of plant sterol consumption on serum TG concentrations

Consumption of 2.0 grams per day of PS for 28 days did not reduce TG concentrations ($p=0.0506$) across all participants. However, significant heterogeneity in TG lowering can be seen in our study population (Figure 8.1).

Influence of genotype on response of TG to plant sterol consumption

One SNP, *rs5882* in CETP ($p=0.0080$ for interaction) was associated with TG lowering after PS consumption (Table 8.3 and Figure 8.2). After PS consumption, plasma TG levels were lowered in homozygotes for the minor allele of the CETP SNP *rs5882*-G (G/G=-0.47mmol/L, $p=0.0002$, n=10). However, individuals carrying the SNP *rs5882*-A allele did not decrease plasma TG (*rs5882*-A/A +0.01mmol/L, $p =0.6634$, n=25; *rs5882*-A/G -0.04mmol/L, $p= 0.3399$, n=28) in response to PS intervention (Table 8.2). Baseline chracteristics of participants separated by *rs5882* in CETP are shown in Table 8.4.

rs688 in LDLR was associated with overall TG concentration, independent of PS consumption. Minor allele homozygous (T/T) carrier for *rs688* in LDLR had lower TG concentrations than heterozygous (C/T, $p=0.0024$), but not homozygous major allele (C/C, $p=0.0779$) carriers.

Table 8.2 Candidate SNP associations with triglyceride response to plant sterol consumption

Gene *SNP*	**TG**	
CETP **rs5882**	Overall p=0.9802	**TX by SNP** **p=0.0080**
LDLR ***rs688***	**Overall** **p=0.0036**	TX by SNP p=0.1143
PCSK9 *rs562556*	Overall p=0.5376	TX by SNP p=0.7391
ApoE Variant	Overall p=0.4228	TX by SNP p=0.8021

Table 8.3 *rs5882* in CETP associates with TG lowering in response to plant sterol consumption

Gene *SNP*	**Treatment** *p-value*	**Treatment x genotype** *p-value*	**Simple effects by genotype*** ΔTC (mmol/L), p-value, n
CETP ***rs5882***[a]	**p <.0005**	**p= 0.0080**	A/A=+0.01, p =0.6634(n=25) † A/G=--0.04, p= 0.3399 (n=28) † **G/G=-0.47, p=0.0002 (n=10)** ‡

a= values log transformed prior to analysis, actual means reported.*mixed model simple effects of treatment sliced by genotype using SAS SLICE function when treatment and treatment*genotype was significant (statistical significance level set to p=0.0167, Bonferroni correction for 3 slices)
Genotypes within an SNP with different superscripts had significantly different treatment effect size, tested by ANOVA with Tukey –Kramer adjustment.

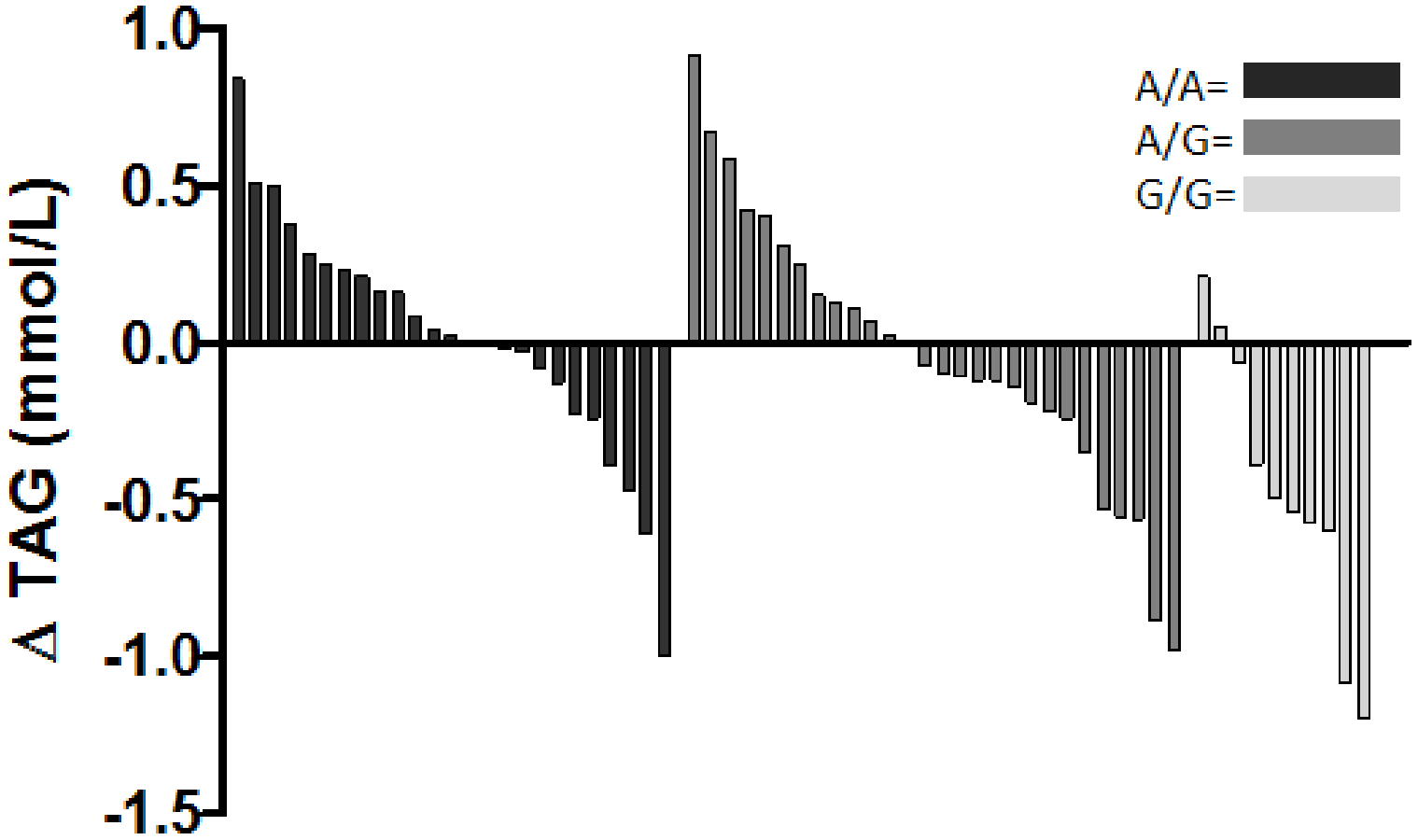

Figure 8.2 Individual changes in triglycerides in response to plant sterol consumption compared to control stratified by *rs5882* genotype in CETP.

Table 8.4 Baseline participant characteristics stratified by *rs5882* genotype in CETP (Mean ± STD)

	A/A participants (n=25)	A/G participants (HS, n=28)	G/G participants (n=10)	P-values
Lathosterol[a]	1.39 ± 0.59[†]	1.41 ± 0.65[‡]	1.34 ± 0.51[†‡]	p=0.9370
Gender (male/female)	11/14	9/19	4/6	p=0.4470[b]
Age (years)	56.44 ± 6.80	55.29 ±9.78	53.40 ±8.32	p=0.6060
Body weight (kg)	88.76 ± 25.88[†]	81.37 ± 17.92[‡]	73.64 ± 12.65[†‡]	p=0.1550
BMI (kg/m2)	29.44 ± 5.98[†]	29.44 ±6.39[‡]	25.49 ± 3.76[†‡]	p=0.1660
TC (mmol/L)	6.19 ± 0.88	5.78 ±0.79	6.44 ±1.01	p=0.2090
LDL (mmol/L)	3.95 ± 0.81	3.60 ±0.75	4.09 ±0.84	p=0.3650
HDL * (mmol/L)	1.47 ± 0.40	1.49 ± 0.36	1.56 ± 0.48	p=0.6520
TG * (mmol/L)	1.70 ± 0.67	1.53 ±0.63	1.75 ± 0.82	p=0.6170
Glucose (mmol/L)	4.90 ± 0.38	4.78 ± 0.37	4.87 ± 0.65	p=0.3240

p-values from 1-way analysis of variance. Different superscripts indicate significant differences at p=0.05 by Tukey's HSD test.
a= non-cholesterol sterols reported as µmol/mmol cholesterol
b=calculated by Pearson chi-square test
*=values were log transformed prior to analysis, actual means and standard deviation in the mean reported

8.5 Discussion

Through the investigation of candidate SNPs in genes related to TG metabolism we have shown a genetic basis for some of the heterogeneity in response of TG concentrations to PS consumption. One of the candidate genetic polymorphisms, *rs5882* in CETP was associated with TG lowering in response to PS consumption in an autosomal recessive pattern (Figure 8.2). Only homozygous G/G minor allele carriers had reductions in TG concentrations due to PS consumption.

This is the first study to demonstrate a genetic influence on TG lowering in response to PS consumption, although *rs5882* in CETP was previously shown to associate with the magnitude of total cholesterol lowering in response to PS consumption (19). In this trial by Lottenberg et al. only A/A and A/G, and not G/G carriers for *rs5882* showed cholesterol lowering in response to PS consumption, where no effect on TG concentrations was reported. *rs5882* G/G carriers have reduced CETP mass and activity, as well as lower CHD risk (20). CETP facilitates transfer of cholesteryl esters and TGs between lipoproteins in the plasma. Typically CETP facilitates the exchange of TG from TG-rich VLDL-1 particles for cholesteryl esters from HDL particles (21).

The effect of PS consumption on TG concentrations remains to be debated, with some trials showing reductions (6, 7, 9, 22), but the majority showing no effects individually (23). A pooled analysis of 12 randomized controlled trials showed that the TG lowering effect of PS consumption was dependent on baseline TG concentrations (23), with larger reductions for individuals with higher baseline TG concentrations. This same association between PS induced TG lowering and baseline TG concentration was also reported by Naumann et al.(14). Plat et al. (16) hypothesized that reductions in TG concentrations

following PS consumption were due to lower hepatic production of large TG rich VLDL-1 particles. If PS consumption leads to a decreased hepatic production of VLDL-1 particles as suggested by Plat et al.(16), the largest decrease in TG concentrations would be expected in individuals with the lowest CETP activity due to increased hepatic clearance.

In our study only individuals with the G/G variant for *rs5882* had reductions in TG concentrations due to PS consumption, the same genotype previously associated with reduced CETP mass and activity (19, 20). Evidence from work with CETP inhibitors suggests that lower CETP activity leads to increased VLDL-1 clearance from the circulation (24). This is due to a decreased exchange of triglycerides from the VLDL-1 particles for cholesteryl esters from HDL leading to more triglyceride rich VLDL-1. Higher triglyceride and lower cholesterol ester content has been hypothesized to make VLDL-1 particles a better substrate for lipoprotein lipase, leading to increased clearance and decreased transformation in LDL particles (24).

We did not measure CETP activity in our study, but individuals with the G/G variant for *rs5882* have been previously shown to have low CETP activity (19, 20), and these were the individuals who similarly had the greatest reduction in TG concentrations due to PS consumption in our study. The rs5882 G/G genotype has also been associated with increased longevity (25). It has been hypothesized that lowered CETP activity leads to a phenotype with less small dense LDL and HDL particles which is associated with a lower prevalence of hypertension and CVD.

Our study population (n=63) is relatively small for genetic associations. However, the specific goal of our study was to look for nutrient by gene interactive effects, which can

only be seen in trials which carefully phenotype response to a nutrient. Notwithstanding, some of the associations reported in our study for the first time must be in interpreted in the context of our study's limited size (n=63) and our specially selected high and low endogenous synthesis population. Replication of these findings in future studies will be critical to validating the associations reported presently. At the same time our data shed light on the controversial results reported in previous trials, where large inter-individual variation hampered the ability to decisively determine if and to what degree PS consumption contributes to decreased TG, and therefore reduced cardiovascular disease risk.

In summary, our data are the first to assert a genetic basis for the inter-individual variability in TG responses to PS consumption. Our data demonstrate that the response of TG concentrations to PS consumption may be influenced by a common genetic variant in CETP.

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Chapter IX

Overall conclusions

9.1 Summary and implications

The results of the present research have implications for the use of plant sterol containing products for the management of cardiovascular disease risk. Current recommendations for cardiovascular risk reduction focus primarily on lipid lowering (1), targeting optimal LDL-C concentrations of <2.60 mmol/L. This recommendation is based on a wealth of evidence showing a relationship between cholesterol concentrations and CVD risk (2-4). Plant sterol consumption is a recommended dietary therapeutic option to enhance LDL-C lowering by the National Cholesterol Education Program Adult Treatment Program III (NCEP ATP III)(1). According to the NCEP ATP III plant sterol consumption in the range of 2-3 grams per day will bring about reductions in LDL cholesterol in the 6-12% range. Data from the present research demonstrate that while mean reductions in LDL-C for a population may fall into this range, substantial inter-individual heterogeneity exists.

Our data exhibit a range of changes in LDL-C from substantial LDL-C lowering exceeding 12% to individuals who could be classified as adverse responder showing a increases in LDL-C following PS consumption of greater that 5%. This heterogeneity in responsiveness could be predicted by endogenous cholesterol synthesis level, which was measured in surrogate by lathosterol to cholesterol ratio. Low endogenous cholesterol synthesis was a good predictor of LDL-C lowering in response to PS consumption, while high endogenous cholesterol synthesis was associated with non-response. Furthermore, present data associated the magnitude of cholesterol lowering in response to PS consumption to genetic variations in the CYP7A1, ApoE and LSS genes.

These data also found a strong association between a genetic variation in the CETP and TG lowering in response to PS consumption, with only some participants benefitting from TG lowering after PS consumption. The significant genetic heterogeneity in cholesterol and TG lowering shown presently have important implications in regards to population wide recommendations for plant sterol product use. For individuals with a responsive phenotype and/or genotype in regard to PS consumption, PS consumption should be prioritized as they might receive a greater cholesterol lowering benefit than is typically reported by mean values of lipid lowering from clinical trials. Individuals with a non-responsive phenotype and/or genotype to PS consumption should be guided to other cholesterol lowering options as PS consumption does not provide them any benefit.

Clearly, one size fits all health recommendations are no longer sufficient in an era when genetic information is now easily accessible. Our data strongly support the concept of personalized nutrition, where an individuals' lifestyle and genetic makeup are used to optimize their own diet to maximize health benefits. Health and nutrition recommendations, just as pharmaceutical and nutritional products, must be implemented, prescribed or consumed in a context which realizes the unique nature of an individual's response.

9.2 Limitations and future directions

A limitation of this trial was the population size, with only 63 individuals studied. While this population was large enough to see significant genetic associations with PS consumption, it is small when compared to the size of populations used in genome wide association studies (5-7). Interestingly, the trial was able to replicate a previously reported association between a genetic variation in CYP7A1 and cholesterol lowering

observed in a population of 67 individuals in Spain (8). Replication of genetic associations is critical, especially when gene by nutrient interactions are being investigated and study populations are small. The novel associations reported in this thesis will require replication in subsequent clinical trials.

Lack of dietary control was also a limitation in our trial. Dietary intake was not assured phase to phase by full feeding, instead participants were asked to maintain their normal diet throughout the study and the intake of meals which contained the plant sterols of placebo was monitored. The crossover design of the trial, where individuals act as their own control, was also likely very important in minimizing the impact of this lack of dietary control on the trial's findings. Selection of a full feeding design may have removed variability from our results allowing for more or stronger associations between the genetic variations we investigated and cholesterol lowering in response to PS consumption.

Only a limited number of genetic variations, which were selected as having a minor allele frequency above 7%, were investigated for associations with response to plant sterol consumption. The small number of variations investigated is a potential limitation of this trial. Many variations that could have impacted response of LDL-C lowering to PS consumption were not evaluated. Ideally this type of limitation will be overcome through the use of whole genome sequencing in the future, where all possible variations in all possible genes will be evaluated.

Investigation of lipoprotein particle sizes through use of NMR (9) or a gel system (10) could have allowed us to see shifts in lipoprotein sub-particles which are not visible using just LDL-C, HDL-C and TG measures. This would have been very helpful in explaining

some of the TG lowering effects of plant sterol consumption that were associated with a variation in CETP. CETP mass or activity would have been an excellent additional analysis to help elucidate the association seen between TG lowering from PS and *rs5882* in CETP.

Future trials investigating the genetic heterogeneity of responsiveness to plant sterol consumption should seek to recruit for individuals with particular genotypes that have already have been associated with response to plant sterols. Unlike our trial, which recruited for a phenotype associated with response, trials with prospective recruitment based on genotype will be able to go beyond associating a genotype with response to plant sterols.

The use of whole genome sequencing, instead of candidate SNP selection, is a potential in the future as the costs of sequencing come down. Selection of candidate SNPs inherently causes a bias due to the restriction of all possible variations not selected as a candidate. Whole genome sequencing eliminates this potential bias, but does come with the disadvantage of increased type 1 error due to its hypothesis free multiple associations.

Another interesting future direction to the findings of this trial are the implications of changing endogenous cholesterol synthesis concentrations, perhaps through the use of statins, on response of cholesterol to plant sterol consumption. The impact of taking a low dose statin, which would lower endogenous cholesterol synthesis, on an individual's cholesterol lowering response to PS consumption warrants further investigation.

The findings of this thesis may be used to shape future research into other cholesterol lowering therapies. Ezetimibe is a pharmaceutical which lowers cholesterol absorption via interaction with the NPC1L1 transporter in the intestines (11). Dietary fibers are

thought to also lower cholesterol by lowering cholesterol absorption and the reabsorption of bile salts, and through down regulation of cholesterol synthesis via short chain fatty acid production (12). The associated genetic variations shown in our trial could also potentially be modifying factors in response to ezetimibe and dietary fibers.

9.3 Final conclusions

CVD remains a leading cause of death in Canada, accounting for 29% of all deaths in 2008 (13). Cholesterol lowering remains the primary prevention target for CVD prevention, with health behavior modification being the number one strategy to lower CVD risk (1). These recommended health behaviors include smoking cessation, diet modification, exercise, stress reduction and control of alcohol consumption. Plant sterol consumption in the 2-3 grams per day range has been repeatedly shown to be a dietary measure that can bring about significant reductions in total and LDL-C cholesterol concentrations (14-16). Based on these numerous clinical trials showing cholesterol lowering efficacy plant sterol containing products have been deemed by Health Canada as eligible for a health claim in Canada.

The totality of the present research demonstrates that significant inter-individual variability of response exists to plant sterol consumption. This inter-individual variability results in mean reported benefits of plant sterol consumption which does not adequately reflect the heterogeneity of their benefit to individuals looking to lower their cholesterol concentrations. Specific genetic variations, some of which have been identified in this research, are the contributors to this variability. The use of genetic technologies to identify responders, non-responders and even adverse responders to different dietary interventions, such as plant sterol consumption, will be critical in optimizing dietary

disease prevention strategies. Broad population based dietary recommendations will eventually need to be redefined and modified to specific sub-population recommendations to maximize disease risk reduction potential.

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Appendices

Appendix 1: Copyright licenses for previously published materials

Chapter II: Manuscript 1

Phytosterols in human nutrition: Type, formulation, delivery and physiological function

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Chapter III: Manuscript 2

Evaluation of methods for the determination of cholesterol absorption and synthesis in humans

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Chapter IV: Manuscript 3

Plasma non-cholesterol sterols: current uses, potential and need for standardization

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Chapter V: Manuscript 4

High basal fractional cholesterol synthesis is associated with non-response of plasma LDL-cholesterol to plant sterol therapy

Appendix 2: Ethics approval for study in chapter VI, VII and VIII

UNIVERSITY OF MANITOBA

BANNATYNE CAMPUS
Research Ethics Boards

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Tel: (204) 789-3255
Fax: (204) 789-3414

APPROVAL FORM

Principal Investigator: Dr. P. Jones
Sponsor: Minister of Science, Technology, Energy and Mines

Ethics Reference Number: B2007:073
Date of Approval: January 4, 2011
Date of Expiry: April 30, 2011**
****Expiry Date Anniversary**

Protocol Title: Genetic Basis for Heterogeneity in Response of Plasma Lipids to Plant Sterols

The following is/are approved for use:

- **Annual Approval**
- **Research Subject Information and Consent Form – 1, Version 4 dated 8/4/2010**
- **Research Subject Information and Consent Form – 2, Version 3 dated 8/4/2010**

The above was approved by Dr. Nicholas Anthonisen, Chair, Biomedical Research Board, Bannatyne Campus, and University of Manitoba on behalf of the committee per your submission dated December 8, 2010. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations of Canada*.

This approval is valid until the expiry date only. A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval should be sought from the relevant institution, if required.

Sincerely yours,

[redacted]

Nicholas Anthonisen, MD, Ph.D
Chair,
Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above Ethics Reference Number on all correspondence.
Inquiries should be directed to the REB Secretary **Telephone**: (204) 789-3255/ **Fax:** (204) 789-3414

www.umanitoba.ca/medicine/ethics

UNIVERSITY OF MANITOBA

BANNATYNE CAMPUS
Research Ethics Boards

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Tel: (204) 789-3255
Fax: (204) 789-3414

APPROVAL FORM

Principal Investigator: Dr. P. Jones
Sponsor: Minister of Science, Technology, Energy and Mines

Ethics Reference Number: B2007:073
Date of Approval: May 16, 2011
Date of Expiry: April 30, 2012

Protocol Title: Genetic Basis for Heterogeneity in Response of Plasma Lipids to Plant Sterols

The following is/are approved for use:

- **Annual Approval**
- **Research Subject Information and Consent Form – 1, Version 5 dated 24/1/2011**

The above was approved by Dr. Nicholas Anthonisen, Chair, Biomedical Research Board, Bannatyne Campus, and University of Manitoba on behalf of the committee per your submission dated May 5, 2011. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations of Canada*.

This approval is valid until the expiry date only. A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval should be sought from the relevant institution, if required.

Sincerely yours,

Nicholas Anthonisen, MD, Ph.D
Chair,
Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above Ethics Reference Number on all correspondence.
Inquiries should be directed to the REB Secretary **Telephone:** (204) 789-3255/ **Fax:** (204) 789-3414

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BIOMEDICAL RESEARCH ETHICS BOARD (BREB)

CERTIFICATE OF ANNUAL APPROVAL

PRINCIPAL INVESTIGATOR:	INSTITUTION/DEPARTMENT:	ETHICS #:
Dr. P. Jones	UofM / Richardson Centre for Functional Foods and Nutraceuticals	B2007:073
BREB MEETING DATE (If applicable):	**APPROVAL DATE:** November 22, 2012	**EXPIRY DATE:** April 30, 2013
STUDENT PRINICIPAL INVESTIGATOR SUPERVISOR (If applicable):		

PROTOCOL NUMBER:	PROJECT OR PROTOCOL TITLE:
NA	Genetic Basis for Heterogeneity in Response of Plasma Lipids to Plant Sterols
SPONSORING AGENCIES AND/OR COORDINATING GROUPS: Minister of Science, Technology, Energy and Mines	

Submission Date of Investigator Documents:	BREB Receipt Date of Documents:
November 19, 2012	November 22, 2012

REVIEW CATEGORY OF ANNUAL REVIEW: **Full Board Review** ☐ **Delegated Review** ☒

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

Document Name(if applicable)	Version(if applicable)	Date

Annual approval

*Annual approval implies that the most recent **BREB approved** versions of the protocol, Investigator Brochures, advertisements, letters of initial contact or questionnaires, and recruitment methods, etc. are approved.*

Consent and Assent Form(s):

CERTIFICATION

The University of Manitoba (UM) Biomedical Research Board (BREB) has reviewed the annual study status report for the research study/project named on this ***Certificate of Annual Approval*** as per the category of review listed above and was found to be acceptable on ethical grounds for research involving human participants. Annual approval was granted by the Chair or Acting Chair, UM BREB, per the response to the conditions of approval outlined during the initial review (full board or delegated) of the annual study status report.

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 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 annual 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,

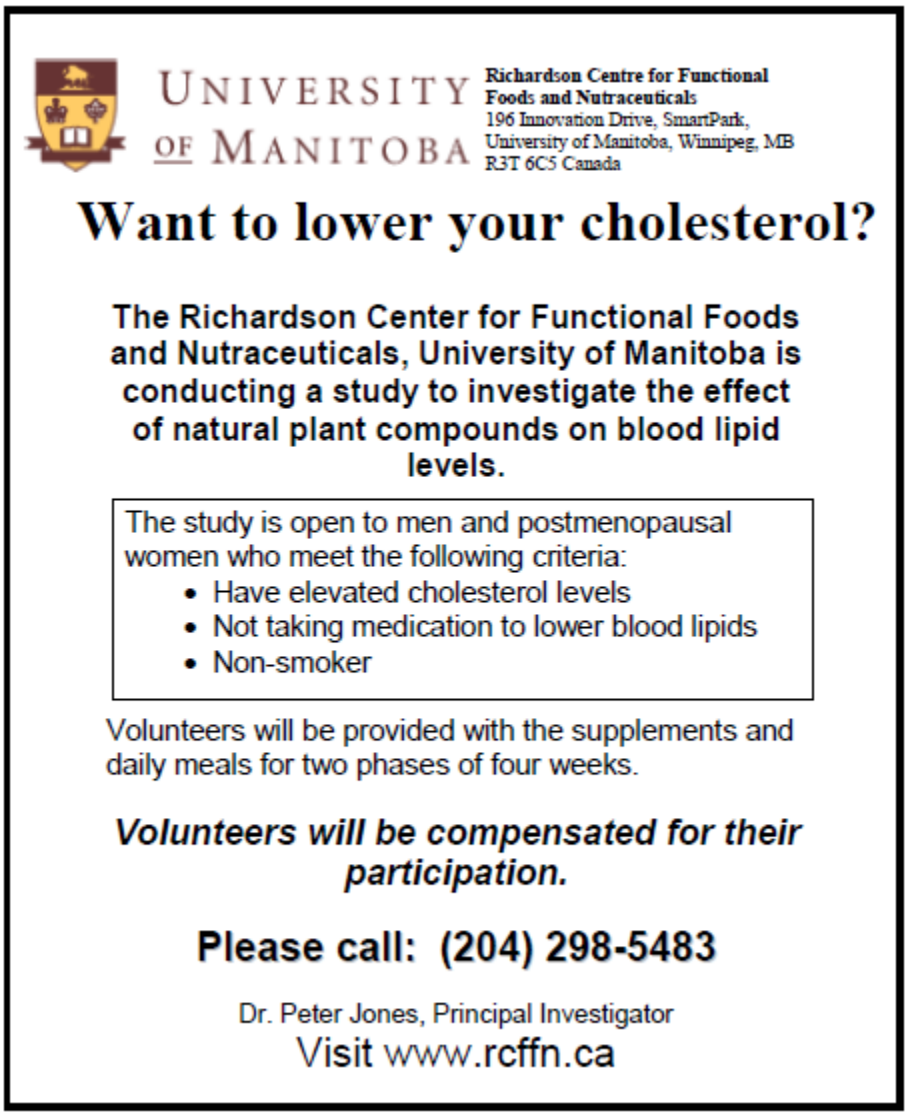
[redacted]

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

Please quote the above Human Ethics Number on all correspondence.
Inquiries should be directed to the REB Secretary **Telephone:** (204) 789-3255/ **Fax:** (204) 789-3414

Appendix 3: Study forms

Advertisements



UNIVERSITY OF MANITOBA

Richardson Centre for Functional Foods and Nutraceuticals
196 Innovation Drive, SmartPark,
University of Manitoba, Winnipeg, MB
R3T 6C5 Canada

Want to lower your cholesterol?

The Richardson Center for Functional Foods and Nutraceuticals, University of Manitoba is conducting a study to investigate the effect of natural plant compounds on blood lipid levels.

The study is open to men and postmenopausal women who meet the following criteria:

- Have elevated cholesterol levels
- Not taking medication to lower blood lipids
- Non-smoker

Volunteers will be provided with the supplements and daily meals for two phases of four weeks.

Volunteers will be compensated for their participation.

Please call: (204) 298-5483

Dr. Peter Jones, Principal Investigator

Visit www.rcffn.ca

2/23/2010

UNIVERSITY OF MANITOBA

Do you have high cholesterol?

The Richardson Center for Functional Foods and Nutraceuticals, University of Manitoba is conducting a study to investigate the effect of natural plant compounds on blood lipid levels.

The study is open to men and women who meet the following criteria:

-Have elevated cholesterol levels
-Not taking medication to lower blood lipids
-Non-smoker

Volunteers will be compensated for their participation!!!

Please call: (204) 298-5483

Dr. Peter Jones,

Principal Investigator

Visit www.rcffn.ca

196 Innovation Drive
Winnipeg, MB

7/30/2010

Participant consent forms

General consent form

UNIVERSITY OF MANITOBA

RESEARCH SUBJECT INFORMATION AND CONSENT FORM – 1
Version 5

Title of Study: **Genetic Basis for Heterogeneity in Response of Plasma Lipids to Plant Sterols**

Investigator: Peter Jones, PhD
Richardson Centre Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive, Smartpark
Winnipeg, Manitoba R3T 6C5
Phone: (204) 474-9787

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 doctor or study staff to explain any words or information that you do not clearly understand.

Purpose of study
The purpose of the study is to examine how a natural plant compound, plant sterols, will respond to blood fat levels and blood fat metabolism, as a result of individual gene profile, which is involved in blood fat metabolism. The plant sterols will be supplemented in margarine and will be provided with a daily meal by the metabolic kitchen at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) at the University of Manitoba.

Study procedures
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 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 physician prior to enrollment in the study and any abnormality in tests performed at screening will result in exclusion. An electrocardiogram (EKG) may be performed at the discretion of the physician in charge. Prior to beginning the study, you will undergo a physical examination by a physician to ensure that you are in good health. During the physical examination, the physician will measure your vital signs examine the normality of body systems and ask you some questions regarding your medical history.

Any change in your health status at any point during the study needs to be reported to the study investigators.

Initials of Subject: _____

You will need to ensure that you have not consumed any medication and/or natural health supplements affecting fat metabolism (such as, cholestyramine, colestipol, niacin, clofibrate, gemfibrozil, probucol, HMG CoA reductase inhibitors, high dose dietary supplements, fish oil capsules or plant sterol) for at least the last 3 months. You will also need to ensure that you will not be consuming any of these medications/supplements during the study. You will be required to report the use of any new medications and/or natural health products. The use of medications including natural health products known to affect lipid metabolism during the trial will be asked to withdraw from the trial.

In addition, you have to ensure that you do not smoke or consume large amounts of alcohol (>2drinks/day) or coffee (>2drinks/day); you do not have any major food allergies or are not vegetarian.

You will also need to ensure that you do not currently have diabetes, thyroid, kidney, heart or liver disease or have had any of these diseases at any time during the past three months, in addition any development of diabetes mellitus, thyroid, kidney, heart or liver disease during the trial will lead to exclude from the trial.

The study will consist of 2 phases of 28 days each during which you will consume a daily meal at the Richardson centre and follow a weight-maintaining diet. At the end of each phase, a washout period of 4 weeks will be followed during which you will consume your habitual diets. The 2 phases of treatments will include:

1) Control phase where no plant sterols are supplemented
2) Plant sterol phase where plant sterols are given

This study is with double-blind design which means that neither you nor the study staff will know which variation of the treatments that you will be receiving. In an emergency, this information will be made available.

One daily meal will be prepared in the metabolic kitchen of the RCFFN. You will consume at this meal at the RCFFN or the Deerlodge Center under supervision.

During days 1, 2, 25, 26, 27, 28, of each four-week test diet phase, fasting blood samples (approximately 6 teaspoons) will be obtained for assessment of blood fat levels and blood fat metabolism. On day 26 of the trial, you will be required to consume a quantity of carbon-labeled cholesterol. This labeled cholesterol is nearly identical to regular cholesterol but can be distinguished from normal cholesterol as they contains a heavier form of carbon. In addition, you will be required to consume approximately three tablespoons of water that has been tagged with a heavy form of hydrogen (known as deuterium). The movement of these tagged materials will permit assessment of how much cholesterol your body absorbs from your diets and how much cholesterol is produced inside your body. These tagged materials are non-radioactive, non-toxic, and do not pose any health risk to you.

Initials of Subject: _____

Each blood test will take approximately 5 minutes. The total amount of blood drawn during each phase of the study will be approximately 12 tablespoons. The total blood volume required for this trial will be approximately 1.5 cups.

At the end of each phase, a fecal sample will be collected using a take home stool collection kit to assess changes in intestinal bacterial populations in response to plant sterol supplementation.

Risks and discomforts
As with any clinical trial, there may be as yet unknown or unforeseen risks of taking part. The plant sterols supplementation at the proposed level has been shown to have no known direct negative side effects on health in several dozen existing animal and human experiments. Some known risks, although rare, are associated with placing a needle into a vein. These include the possibility of infection, perforation or penetration 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. Kesselman, will be available to contact at any time. Dr. Kesselman can be reached at (204) 954-4486.

Benefits
You may not benefit from participation in this research; however, the study should contribute to a better understanding of the response of plant sterols to blood fat levels and blood fat metabolism, as a result of individual gene profile, which is involved in blood fat metabolism. You will also receive access to your test 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.

Payment for participation
You will receive up to a maximum of **$280** at completion of this study for your time and inconvenience of the study schedule. This amount will be divided into 2 equal portions and 1 portion given after each phase. 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 lipid lowering medications exist as an alternative to lowering 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. With your permission, the study doctor will also write to your Family Doctor to tell him/her that you are taking part in a study or

Initials of Subject: _____

to obtain further medical information. 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 Richardson Centre for Functional Foods and Nutraceuticals.

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 shared with any other group, other than is indicated in the protocol, without your specific consent.

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 close.

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. Kesselman at (204) 954-4486 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 nor 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. Kesselman 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 subject. 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. 204-474-9787

Initials of Subject: _____

Genetic Basis for Heterogeneity in Response of Plasma Lipids to Plant Sterols

Study Physician Dr. Edward Kesselman Tel No. 204-954-4486

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

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

Consent

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.

Yes ☐ No ☐

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:_____

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

Signature:_____ Date/Time: _____

Genetic analysis consent form

UNIVERSITY OF MANITOBA

RESEARCH SUBJECT INFORMATION AND CONSENT FORM – 2
(Version 3)

ADDITIONAL RESEARCH SUBJECT INFORMATION AND CONSENT FORM FOR GENETICS ANALYSIS

Title of Study: **Genetic Basis for Heterogeneity in Response of Plasma Lipids to Plant Sterols**

Investigator: Peter Jones, PhD
Richardson Centre Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive, Smartpark
Winnipeg, Manitoba R3T 6C5
Phone: (204) 474-9787

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 doctor or 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 "Genetic Basis for Heterogeneity in Response of Plasma Lipids to Plant Sterols", we would like to extract DNA and perform a genetic analyses using a laboratory technique that augments and recognizes specific genes to determine why some people decrease their cholesterol levels better than others when consuming plant sterols. 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 that perform specific biological functions in your body.

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

Initials of Subject: _____

this time, all samples will be destroyed. Your DNA samples will only be used for the purpose of this research project.

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 is no additional invasive procedure to undergo and no physical risk to you. While there may be no direct benefits to you for taking part in these additional analyses, we hope that these results will provide us with the information on genetic characteristics of people in which the intake of plant sterols provided an enhanced or inferior cholesterol-lowering therapy.

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 not be utilized for any additional analyses, nor stored for any prolonged period, nor shared with any other group, other than is indicated in the protocol, without my specific consent.

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.

_____ _____

Signature of Subject Date

Initials of Subject: _____

Participant baseline information form

UNIVERSITY OF MANITOBA | Richardson Centre for Functional Foods and Nutraceuticals

Room 106
196 Innovation Drive
Winnipeg, Manitoba
Canada R3T 2N2
Telephone (204) 474-8883
Fax (204) 474-7552
peter_jones@umanitoba.ca

Genetic Basis for Heterogeneity in Response of Plasma Lipids to Plant Sterols

Day 1 Information Form

To be filled out by participant:

Circle appropriate YES/NO responses

Name:			
Date of Birth:	Month	Day	Year
Sex:	Male:	Female:	Postmenopausal: YES NO
Contact Information			
Street Address			
Postal Code			
City			
Home Phone:			
Cell Phone:			
Email:			

Medical History			
Diabetes mellitus	YES	NO	If Yes to Other, please specify:
Thyroid disease	YES	NO	
Kidney disease	YES	NO	
Liver disease	YES	NO	
Heart disease	YES	NO	
Other	YES	NO	

Cholesterol lowering medication? *(in the last 3 months)*		YES	NO	
Other medications	YES	NO	If Yes, specify :	
			Are the doses of these medications stable? YES...NO	
Vitamin, Mineral supplement	YES	NO	If Yes, specify:	
Herbal, food supplement	YES	NO	If Yes, specify:	
Laxatives	YES	NO		
Fiber	YES	NO		
Allergies (food such as corn)	YES	NO	If Yes, specify:	
Vegetarian	YES	NO		
Any metallic bone components	YES	NO		

Lifestyle		
Smoker?	YES	NO
If Yes, how many per day?		
Drink Alcohol?	YES	NO
If Yes, how many drinks/week		

To be filled out by a study coordinator:

Screening Information		
Weight	lbs:	kg:
Height	' "	m
BMI (kg/m^2)		
Waist circumference (inches)		
Activity Level	1 2 3 4 5	

Is subject fasted for blood sampling? YES NO

Sample study calendar

CIHR PS Participant Calendar November 9th start

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
		November 9th **Start day** 630-900 AM Fasted Blood Sampling	November 10th 630-900 AM Fasted Blood Sampling	November 11th **430-700 PM** **Evening meal**	November 12th 430-700 PM Evening meal	November 13th 430-700 PM Evening meal
November 14th 430-700 PM Evening meal	November 15th 430-700 PM Evening meal	November 16th 430-700 PM Evening meal	November 17th 430-700 PM Evening meal	November 18th 430-700 PM Evening meal	November 19th 430-700 PM Evening meal	November 20th 430-700 PM Evening meal
November 21st 430-700 PM Evening meal	November 22nd 430-700 PM Evening meal	November 23rd 430-700 PM Evening meal	November 24th 430-700 PM Evening meal	November 25th 430-700 PM Evening meal	November 26th 430-700 PM Evening meal	November 27th 430-700 PM Evening meal
November 28th 430-700 PM Evening meal	November 29th 430-700 PM Evening meal	November 30th 430-700 PM Evening meal	December 1st 430-700 PM Evening meal	December 2nd **630-900 AM** **Fasted** **Blood Sampling**	December 3rd 630-900 AM **Fasted** Blood Sampling	December 4th 630-900 AM **Fasted** Blood Sampling
December 5th 630-900 AM **Fasted** Blood Sampling	December 6th **Stop Day** 630-900 AM **Fasted** Blood Sampling	December 7th Washout period begins for minimum 28 days	Washout period	Washout period	Washout period	Washout period

Appendix 4: Book chapter publication of thesis relevance

The following book chapter is published in:

"Bioactive Food as Dietary Interventions for Cardiovascular Disease"

Editors: Ronald Ross Watson and Victor R. Preedy

Elsevier Inc, Amsterdam, NL

Phytosterols and Cardiovascular Disease

D.S. MacKay, P.J.H. Jones

University of Manitoba, Winnipeg, Manitoba, Canada

Summary

Phytosterols, which comprise plant sterols and their saturated stanol forms, naturally occur in the diet. Supplemental phytosterols in the 2–3 g day−1 level have been repeatedly shown to reduce total and LDL cholesterol in the 5–15% range in randomized, blinded, placebo controlled clinical trials. This cholesterol lowering is also seen in individuals already taking statin medication to treat their elevated cholesterol levels, further reducing LDL levels. Numerous factors such as dose, formulation, and timing may affect the efficacy of phytosterols, and these factors should be considered when incorporating phytosterols into a hypercholesterolemic regime. Phytosterols are a natural and healthy alternative or adjunct to current pharmaceutical strategies to control cholesterol levels.