ASSESSMENT OF STEROL METABOLISM IN SITOSTEROLEMIA

By

Rgia A. Othman

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Human Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA
FACULTY OF GRADUATE STUDIES

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ABSTRACT

Sitosterolemia (STSL) is a sterol storage disorder characterized by extremely high plasma and tissue plant sterols (PS) and 5α-stanols. The clinical consequences of expanded body pools of PS in STSL can lead to premature atherosclerosis, xanthomas, macrothrombocytopenia and endocrine disruption. Ezetimibe (EZE) is a sterol absorption inhibitor that shows its potential as a new treatment for STSL. However, EZE effects on tissue pool of PS and cholesterol homeostasis have not been investigated yet. The objectives of this research were to determine if EZE reduces whole body sitosterol and cholesterol pool sizes, and improves whole body cholesterol homeostasis in STSL using stable isotopes. We also determined if EZE improves platelet indices and red blood cells (RBC) hemolysis markers as well as reduces plasma and tissue 5α-stanols of endogenous origin (cholestanol) relative to those of dietary source (sitostanol). To delineate the origin of cholestanol in STSL, we studied EZE effect on cholestanol precursors (cholesterol and bile acid derivative 7α-hydroxy-4-cholesten-3-one, 7α-H-C4). Lastly, we sought to find if a relationship exists between 5α-stanols and serum thyroid hormones in STSL, and to determine whether EZE normalizes disrupted thyroid hormones in STSL *via* decreasing plasma and tissue 5α-stanol levels.

STSL patients (n=8) were taken off EZE for 14 wks. After 4 wks off EZE, they received IV doses of D_7-sitosterol and ^{18}O-cholesterol for sterol pool sizes assessments, and oral doses of ^{13}C-cholesterol and deuterium oxide to measure fractional cholesterol absorption and synthesis rates. EZE treatment (10 mg/day) was resumed and stable isotope testing repeated. Blood was collected over 10 wks to measure sterol enrichment. Moreover, complete blood cell count, plasma and RBC levels of PS, cholesterol, 5α-stanols and

cholesterol precursor lathosterol as well as plasma levels of 7α-H-C4 and serum thyroid hormones were measured 14 wks off and on EZE.

The results showed that EZE progressively reduced plasma sitosterol, which led to substantial decreases in the whole body sitosterol pool size but slightly reduced plasma total cholesterol (TC), which caused a small decline in the size of cholesterol pool. EZE reduced fractional cholesterol absorption and increased cholesterol fractional synthesis, production and metabolic clearance rates. EZE increased platelet count and decreased platelet size without affecting RBC indices or hemolysis markers. Progressive reductions in sitostanol levels in plasma and tissues, reflected by RBC, were sustained over time with EZE therapy. However, reductions in plasma cholestanol levels were not maintained over time, and therefore moderate decrease was noted after 14 wks on EZE. EZE did not affect cholestanol precursor 7α-H-C4 but increased lathosterol, cholesterol precursor. EZE-induced change in plasma cholestanol linearly correlated with that in TC but not with 7α-H-C4, suggesting that cholestanol in STSL is mostly synthesized from cholesterol. When patients were off EZE, serum thyroid hormone free triiodothyronine (T3) tended to decrease but EZE reversed this decrease, increasing free T3 to free thyroxine (T4) ratio and thyroid-stimulating hormone. EZE-induced changes in serum free T3 and T4 inversely correlated with those in 5α-stanols.

Overall, EZE therapy at 10 mg/d reduces whole body stores of PS and cholesterol as well as improves cholesterol turnover by reducing cholesterol absorption and enhancing cholesterol synthesis and clearance. Thereby, EZE may reduce the risk of developing premature atherosclerosis and early mortality in STSL. EZE decreases PS deposition in tissues and improves platelet indices, and therefore may potentially ameliorate bleeding

tendency in STSL. EZE gradually reduces accumulation of dietary 5α-stanols than those of endogenous origin. Endogenous cholestanol in STSL is mostly synthesized from cholesterol but not through bile acid synthesis pathway. 5α-stanols are inversely correlated with thyroid hormones, and EZE reverses thyroid disruption by reducing plasma and tissue 5α-stanols, and may reduce risk of developing hormone related diseases in STSL. Overall, this research shows the efficacy and possible clinical benefit of EZE, and therefore strengthens the rationale for the use of EZE in treatment of STSL.

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DEDICATION

This dissertation is dedicated to my husband, Ali, my kids, Qutaibah and Nuha, and my parents, Salma and Ali. I give my deepest expression of love and appreciation for the encouragement that you gave and the sacrifices you made during this graduate program. Thank you for your continuous love, support and encouragement during the challenges of graduate school and life.

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ABBREVIATIONS

7α-H-C4	7α-Hydroxy-4-cholesten-3-one
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ABCG5/8	ATP-binding cassette transporters G5/G8
ABCG5-KO	ABCG5-knock out
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
Apo-A	Apolipoprotein A
Apo-B	Apolipoprotein B
Apo-E	Apolipoprotein E
ASR	Absolute synthesis rate
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AUC	Area under the curve
BMI	Body mass index
BUN	Blood urea nitrogen
BW	Body weight
CAD	Coronary artery disease
CD36	Cluster of differentiation 36
CE	Cholesteryl esters
CHD	Coronary heart disease
CK	Creatine kinase
CM	Chylomicron
CMR	Chylomicron remnants
CTX	Cerebrotendinous xanthomatosis
CYP27A1	27-Hydroxylase
CYP7A1	Cholesterol 7 alpha-hydroxylase
DAPP	Disodium ascorbyl phytostanyl phosphate
ER	Endoplasmic reticulum
EZE	Ezetimibe
FC	Free cholesterol
FDA	Food and drug administration
FL	Femtoliters
FPE	Ferulate phytostanyl esters
FSR	Fractional synthesis rate
GC	Gas chromatography
GC/P/IRMS	Gas chromatography-pyrolysis-isotope ratio mass spectrometry
GGT	Gamma-glutamyltransferase
Hb	Hemoglobin
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein cholesterol
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A

IHD	Ischemic heart disease
IL	Interleukin
LCPUFA	Long-chain polyunsaturated fatty acid
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein cholesterol
LDL-R	LDL-receptor
LRP	LDLR-related protein
LXR	Liver X receptor
MCF	Metabolic clearance fraction
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCR	Metabolic clearance rate
MCV	Mean corpuscular volume
MPV	Mean platelet volume
MRM	Multiple reaction-monitoring
MTTP	Microsomal triglyceride transfer protein
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NCEP	National cholesterol education
NPC1L1	Niemann-Pick C1-Like 1
oxLDL	Oxidized low-density lipoprotein
PR	Production rate
PS	Plant sterols
RBC	Red blood cells
RDW-CV	Red cell distribution width coefficient of variation
RDW-SD	Red cell distribution width standard deviation
SA	Stearic acid
SMC	Smooth muscle cells
SOAT2	Sterol-o-acyltransferase 2
SR-B1	Scavenger receptor class B member 1
SREBP-2	Sterol regulatory element binding protein 2
STSL	Sitosterolemia
T3	Triiodothyronine
T4	Thyroxine
TAG	Triacylglyceride
TC	Total cholesterol
TNF-α	Tumor necrosis factor-α
TODPM	Tall oil-derived PS mixture (TODPM)
TSH	Thyroid-stimulating hormone
UPLC-MS/MS	Ultra-performance liquid chromatography tandem mass spectrometry
VLDL	Very low-density lipoprotein
WBC	White blood cells
WKY	Wild-type Kyoto
WTD	Western-type diet

CHAPTER I

OVERALL INTRODUCTION

1.1 INTRODUCTION

Elevated plasma total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) are positively associated with cardiovascular disease (CVD) (1). Plant sterols (PS) and stanols, collectively called phytosterols or non-cholesterol sterols, have been shown to reduce blood levels of TC and LDL-C (2, 3), and sometimes triacylglyceride (TAG) (4, 5), with no significant effect on high-density lipoprotein cholesterol (HDL-C), and thereby may reduce progression of CVD. Increased plasma PS levels associated with PS supplementation have raised questions about the safety of prolonged use. The concern is that increases in circulating PS levels in individuals consuming PS may increase CVD risk (6-9). This concern arose primarily from observational data among sitosterolemia patients who typically have extremely elevated plasma and tissue PS levels (10, 11) and suffer premature atherosclerosis (12-15).

Sitosterolemia (STSL), also known as phytosterolemia, is a rare autosomal recessive sterol storage disease caused by homozygous or compound heterozygous mutations in either one of the two adenosine triphosphate binding cassette (ABC) transporters genes, *ABCG5* and *ABCG8*, tandemly located in a head-to-head orientation on chromosome 2p21 (16-18). *ABCG5* and *ABCG8* encode ABC transporters, ABCG5 and ABCG8 (also called sterolin-1 and -2, respectively), which are expressed in both the intestine and liver, forming heterodimers that rapidly remove absorbed PS and cholesterol. STSL is primarily characterized by elevated plasma levels of PS, mainly sitosterol, campesterol

and stigmasterol as well as 5α-stanols (e.g., sitostanol, campestanol and cholestanol) but with normal to moderately elevated plasma TC levels (19, 20). However, in some cases TC can be extremely high (21). Clinical manifestations of STSL include tendon xanthomas, premature coronary artery disease, and in some cases chronic hemolytic anemia, thrombocytopenia (22), and bleeding (23-28). Endocrine disruption has also been noted in STSL (29); for instance, underactive thyroid has been noted in some STSL patients. The true prevalence of STSL is not known, with less than 100 patients described worldwide (30). This is likely due to the fact that STSL is influenced by both genetic and dietary components. For instance, macrothrombocytopenia phenotype in STSL is strongly dependent on diet and this phenotype would converge if all patients adopted the same diet (i.e PS rich diets) (22). Furthermore, sophisticated technique such as gas-liquid chromatography (20) is required to differentiate between cholesterol and PS, which is not feasible with the routine enzymatic methods used for measuring serum cholesterol.

Kinetic studies performed in STSL patients using radioisotopes showed sluggish turnover and elimination of PS, 5α-stanols and, more variably, cholesterol from the body leading to enormously enlarged PS body pools (31, 32). Furthermore, data from studies using techniques such as sterol balance and mevalonic acid excretion, suggest that decreased endogenous cholesterol synthesis is associated with reduced sterol efflux in STSL (31, 33-37). Failure of cholesterol biosynthesis in the intestinal mucosa may encourage partition of extracellular sterols, including PS (38) into the mucosal cell membrane, consequently, increasing sterol retention and thus contributing further to whole body sterol accumulation. Many attempts have been made to modulate endogenous cholesterol

synthesis with either a low sterol diet or bile acid malabsorption (cholestyramine or ileal bypass surgery), but both approaches have failed to modulate cholesterol biosynthesis despite the declines observed in circulating sterol levels (33). To date, to our knowledge, no study has attempted to measure cholesterol turnover and trafficking (i.e., absorption and synthesis) in STSL patients before and after specific treatment regimens.

Ezetimibe (EZE) is a sterol absorption inhibitor which lowers plasma TC and LDL-C levels in hypercholesteremia (39) and plasma PS levels in STSL (30). EZE localizes to the brush border of the small intestine (40) and blocks the transport of cholesterol and PS from dietary and biliary sources by inhibiting the intestinal Niemann-Pick C1 Like 1 protein (41, 42) without affecting intestinal absorption of bile acids, fatty acids, fat-soluble vitamins, or TAG (39). Despite EZE therapy the levels of PS remain above the normal range, and reached a long-term plateau in STSL (43) even with higher doses of EZE (40 mg/d) (44). Despite the wide use of EZE in clinical practice its effects on whole body PS and cholesterol homeostasis have not yet been characterized in STSL.

Sterols, primarily cholesterol, play a significant role in regulating membrane fluidity, permeability, cellular trafficking and signal transduction (45). Cholesterol is also a precursor of steroid hormones, which govern a range of physiological functions, and bile salts, which are essential for the intestinal absorption of cholesterol, fats and lipid soluble vitamins (46). Red blood cells (RBC) cannot synthesize sterols, and so must get them from the plasma since they are freely exchanged between plasma and RBC. Accumulation of PS in blood cells in STSL may displace cholesterol in cell membranes

(47), altering physical properties of the membranes (48) and thus reduces deformability and increases fragility, leading to episodes of hemolysis (49-51). Hematologic abnormalities, including large platelets with low platelet counts (macrothrombocytopenia) abnormal red blood cells (RBC) morphology (stomatocytes) and chronic hemolytic anemia, have been reported in some STSL patients (22, 52, 53), linking hematologic abnormalities to disruption of sterol metabolism. Incorporation of PS into the inner layer of RBC and platelet membranes caused stomatocytic hemolysis (54) and macrothrombocytopenia (55). The accumulation of PS in RBC and its contribution to hemolytic events have also been described in individuals who do not have STSL. For example, diets rich in olive oils, a high source of PS, caused macrothrombocytopenia in Mediterranean migrants who reside in Australia and do not have STSL (56). Furthermore, children on long-term total parenteral nutrition containing plant-based emulsions, which are high in PS, have been reported to develop hematologic disorders including macrothrombocytopenia (57). These data suggest that blood cells could be a target for the toxic effect of plasma PS even in patients with intact ABCG5/8 transporters. Blocking intestinal absorption of PS with EZE has been shown to restore platelet counts in ABCG5-knockout mice (ABCG5-KO) (28) but this effect was inconsistent in humans (58, 59).

In addition to PS levels, STSL patients have also elevated levels of 5α-stanols, saturated derivatives of sterols, in plasma and various tissues, including xanthomas and arteries (60). Diets provide ample amounts of cholesterol and PS, but small quantities of 5α-stanols such as sitostanol and cholestanol (61, 62). Plasma sitostanol is a plant stanol that

is derived from diet (63) whereas cholestanol is a 5α-saturated analog of cholesterol, which is derived from diet and endogenous synthesis (61, 62). Endogenous cholestanol is synthesized in the liver from cholesterol by two possible ways: direct conversion of cholesterol to cholestanol (64) or indirect transformation of 7α-hydroxycholesterol, a bile acid precursor, or its immediate enzymatic product 7α-hydroxy-4-cholesten-3-one (7α-H-C4) to cholestanol (65, 66). Normally circulating cholestanol levels are low (0.1 to 0.4 mg/dl) (67), but they can be elevated (1 to 4 mg/dl) in cerebrotendineous xanthomatosis (CTX), an autosomal recessive disorder of bile acid synthesis. CTX is caused by mutations in *CYP27A1* gene, which codes for sterol 27-hydroxylase, an essential enzyme for bile acid synthesis (68). Deficiency of this enzyme results in pathological induction of cholesterol 7α-hydroxylase (CYP7A1) to increase 7α-hyroxycholesterol and derivative 7α-H-C4, which is converted to cholestanol that accumulates in plasma and tissues of affected patients (65, 69, 70). In STSL, plasma cholestanol levels ranged from 1 to 2.3 mg/dl (67), which might be due to unrestricted sterol absorption (61). Given that cholesterol and bile acid syntheses are low in untreated STSL (34, 71), it is not clear whether cholestanol in STSL patients is mostly synthesized endogenously or comes from diet. If the endogenous production of cholestanol is possible, then is it coming from cholesterol or through induction of the bile acid pathway? Thus far, no studies have explored these questions in STSL patients.

In general, plasma cholestanol is an accepted marker of exogenous cholesterol absorption (67), suggesting that individuals with high cholestanol levels (high cholesterol absorbers) are more likely to benefit from EZE than those of low levels (high cholesterol

synthesizers). Previous studies showed that EZE treatment for 12 wks decreased (-13%) plasma cholestanol levels compared in hypercholesterolemic subjects with type II diabetes mellitus compared with baseline (0.26 vs 0.3 mg/dl) (72). However, in healthy subjects, plasma cholestanol levels were decreased by 37% (0.24 vs 0.3 mg/dl) after 4 wks of EZE relative to baseline (73). Thus far, EZE has been shown to reduce plasma PS levels but its effect on plasma and tissue cholestanol levels have not yet been investigated in STSL.

Steroid hormones govern a range of physiological functions, and bile salts, which are essential for the intestinal absorption of cholesterol, fats and lipid soluble vitamins (46). These hormones are generally synthesized from cholesterol. PS and 5α-stanols may disrupt normal endocrine functions by altering normal hormone levels or inhibiting the production of hormones. Accumulation of PS and 5α-stanols in STSL inhibited cholesterol biosynthesis, which may disrupt hormone production (29, 74-76). Furthermore, synthesis of thyroid hormones appears to be deranged in STSL (77). Increases of serum cholestanol levels have been found in patients with hypothyroidism, an endocrine disorder in which the thyroid gland produces inadequate quantities of the thyroid hormones (78), which has also been found in CTX and STSL (77-80), suggesting that high 5α-stanol levels may affect the secretion of thyroid hormones.

1.2 RATIONAL

Despite the wide use of EZE in clinical practice, its effects on whole body sitosterol and cholesterol homeostasis have not been characterized in STSL. Whether EZE reduces expanded tissue stores of PS and cholesterol in STSL remains to be seen. Furthermore,

there are no published data regarding the effects of EZE on whole-body sterol metabolism in STSL including cholesterol absorption, synthesis and turnover. Whether macrothrombocytopenia and hemolysis would be reversed with EZE treatment, as an indication of reduced tissue stores of PS, remain to be seen.

While EZE may reduce plasma levels of exogenous PS and 5α-stanols (sitostanol), it is not known if EZE reduces those of endogenous origin (cholestanol). If endogenous synthesis of cholestanol is possible in STSL, is it mostly synthesized from cholesterol or through induction of the bile acid pathway? This is important because STSL patients appear to have complete failure of sterol efflux and increased plasma and tissue level of cholestanol but reduced endogenous syntheses of cholesterol and bile acids.

Elevated plasma 5α-stanols (i.e cholestanol) levels are found in patients with endocrine disturbances such as hypothyroidism. Moreover, synthesis of thyroid hormones appears to be deranged in STSL. Many studies have been published concerning the efficacy of pharmaceutical agents to reduce plasma PS mainly, sitosterol and campesterol; however, effects of EZE on circulating levels of 5α-stanols in STSL patients have not yet been explored. Furthermore, whether accumulation of these compounds could interrupt endocrine function is not known. Therefore, it is important to seek to find if there is a relationship between 5α-stanols and serum thyroid hormones in STSL. Furthermore, we need to determine whether EZE decreases plasma and tissue 5α-stanol levels and subsequently normalizes disrupted thyroid hormones in STSL.

1.3 OBJECTIVES

The present research has 5 specific objectives:

1. Investigate whether EZE reduces whole body sitosterol pool size in STSL patients.

2. Determine whether EZE decreases whole body cholesterol pool size, decreases fractional cholesterol absorption, increases cholesterol fractional and absolute synthesis rates (FSR and ASR), and increases whole body cholesterol turnover by increasing metabolic clearance rate of cholesterol from plasma.

3. Investigate whether reductions of plasma and RBC PS levels with EZE therapy are associated with improved macrothrombocytopenia and RBC hemolysis markers in STSL patients.

4. Evaluate whether EZE decreases plasma and RBC cholestanol levels in comparison with sitostanol (exogenous 5α-stanol), and assess the levels of cholestanol precursors (cholesterol and 7α-H-C4) in STSL in order to delineate whether cholestanol is derived from cholesterol or through induction of the bile acid pathway.

5. Examine if there is a relationship between 5α-stanols and serum thyroid hormones in STSL, and determine whether EZE decreases plasma and tissue 5α-stanol levels and subsequently normalizes disrupted thyroid hormones in STSL.

1.4 HYPOTHESES

The hypotheses to be tested include:

1. EZE treatment will decrease whole body sitosterol pool size in STSL patients.

2. EZE will decrease whole body cholesterol pool size, decrease fractional cholesterol absorption, increase FSR and ASR and increase cholesterol turnover by increasing metabolic clearance rate of cholesterol.

3. EZE will increase platelet count, reduce platelet volume, improve RBC indices and decrease RBC hemolysis markers in STSL.

4. EZE will progressively decrease plasma and tissue 5α-stanols, which come from diet (e.g., sitostanol) but moderately reduce those of endogenously synthesis (e.g., cholestanol). Cholestanol in STSL is mostly synthesized from cholesterol not through induction of the bile acid pathway (7α-H-C4). When absorption of dietary cholesterol is blocked with EZE, distance cholestanol precursor (lathosterol, cholesterol precursor) will increase.

5. Plasma 5α-stanols will be inversely correlated with serum thyroid hormones, and EZE treatment will normalize disrupted thyroid hormones in STSL by reducing 5α-stanols.

The first manuscript will discuss the plasma cholesterol-reducing effect of PS and evaluate the relationship between PS and coronary heart disease in animal and clinical studies. The second manuscript will critically assess the existing body of evidence surrounding PS and cholesterol metabolism in STSL. The third manuscript examines the effect of EZE on the sizes of whole body pools of sitosterol and cholesterol and cholesterol synthesis, absorption and turnover in STSL using stable isotopic techniques. The fourth manuscript presents an investigation of the effects of EZE on macrothromobocytopenia and hematologic parameters in STSL patients. The fifth manuscript will address the impact of EZE on plasma and RBC 5α-stanols, and delineate whether cholestanol is derived from cholesterol or through induction of the bile acid pathway as well as seek to find if there is a relationship between 5α-stanols and thyroid disruption in STSL.

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CHAPTER II

MANUSCRIPT 1: LITERATURE REVIEW

This chapter is excerpt from the published book chapter "Functional Foods and Cardiovascular Disease" by CRC Press Taylor & Francis Group (July 9 2012) ISBN: 978-1-4200-7110-8. (Appendix IV)

EXPERIMENTAL AND CLINICAL EVIDENCE OF CARDIOVASCULAR BENEFITS OF PLANT STEROLS

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2.1 INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the Western societies. High total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels are considered the major risk factors for coronary heart disease (CHD) (1). Plasma TC levels are positively associated with ischemic heart disease (IHD) (2); for each 1% decline in TC the risk of CHD is expected to decrease by 2.7%. Similarly, a 10% decrease in LDL-C is associated with about 12% to 20% decline in the population incidence of IHD over 5 years (3). Hence, reducing blood cholesterol levels may be needed to lower risk of CVD. Therapeutic life-style changes, such as inclusion of dietary plant sterols (PS), may reduce cardiovascular risk. PS have been shown in numerous studies to reduce the levels of TC, LDL-C (4), apolipoprotein B (apo-B) (5), and non-high-density lipoproteins cholesterol (non-HDL-C) (6), and fibrinogen (7-9). On average, consuming 2-3 g PS/d reduces

serum LDL-C by 10 - 15%, which may translate into 10-30% risk reduction of cardiovascular events. Increased plasma PS levels associated with PS supplementation have raised questions about the safety of prolonged use. The concern is that increases in circulating PS levels in individuals consuming PS may increase CVD risk (10-13). This concern arose primarily from observation data among sitosterolemia patients who typically have over 30 fold elevated PS levels (14, 15), and premature atherosclerosis (16-19). This chapter reviews animal and clinical studies related to cholesterol-lowering effects of PS and cardiovascular associated-benefits of PS.

2.2 PLANT STEROLS

Plant sterols (PS) or phytosterols collectively indicate plant-derived sterols and stanols. PS are structurally related to cholesterol but the side chain contains a methyl (campesterol) or an ethyl (sitosterol and stigmasterol) group (20) (**Figure 2.1**).

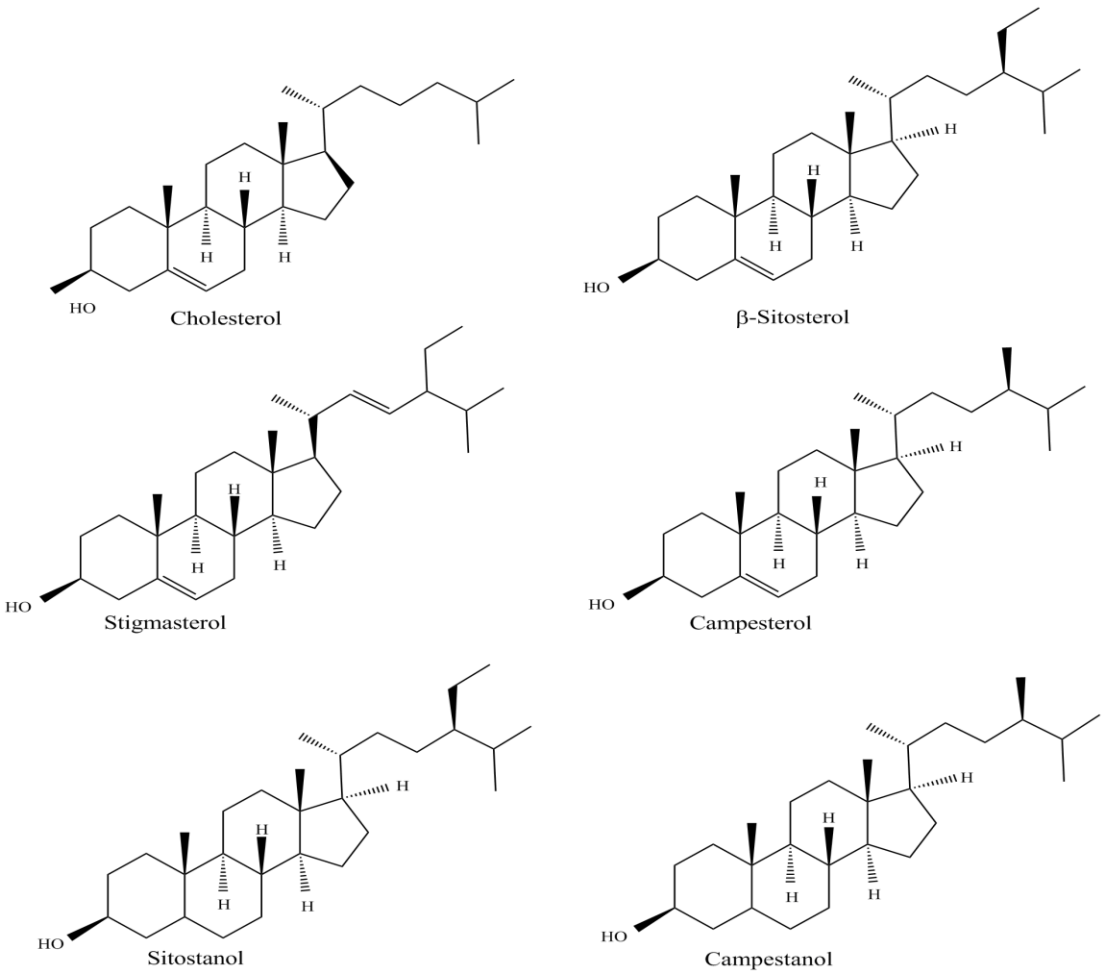

Figure 2. 1*: Chemical structure of the most common plant sterols and stanols.*

Sitosterol, campesterol, and stigmasterol are the principal molecular forms, while only sitosterol and campesterol are considered to be the most frequent PS in food constituting ~60% and 35%, respectively (21). Saturated forms of PS are called stanols. Humans cannot synthesize PS; thus, they always originate from the diet. While maximum amounts of PS can be sourced from plant oils, moderate and minimal levels can be found in nuts and seeds, and fruits and vegetables, respectively. Tall oil, derived from the process of paper production from wood, is considered to be a high source of plant stanols than vegetable oils (22). With intestinal absorption rates ranging from 0.4% to 5% and 0.02%

to 0.3%, respectively, PS and stanols are poorly absorbed (23) compared to dietary cholesterol (24). Dietary intakes of PS do not exceed 200 mg/d in neither the American diet (25) nor in British diet but to be upward of 373 mg/d in Japanese diet and 500 mg/d in vegetarian (25) and Mediterranean diets (26). PS in foods compete with cholesterol for uptake into mixed micelles, displacing cholesterol molecule from the micelles (23) and reducing intestinal cholesterol absorption (27). Usually, once phytosterols enter the enterocytes they are very efficiently pumped back into intestinal lumen by phytosterol transporter proteins sterolin 1 and 2; encoded by the adenosine triphosphate (ATP) binding cassette transporter genes, (*ABCG5* and *ABCG8*), respectively **Figure 2.2.** These proteins are expressed in intestinal mucosal cells and the bile canaliculus system, therefore, re-secreting PS also from the liver to bile. Thus, the intestinal reabsorption of cholesterol is reduced by ~60%, and subsequently fecal output is increased (27).

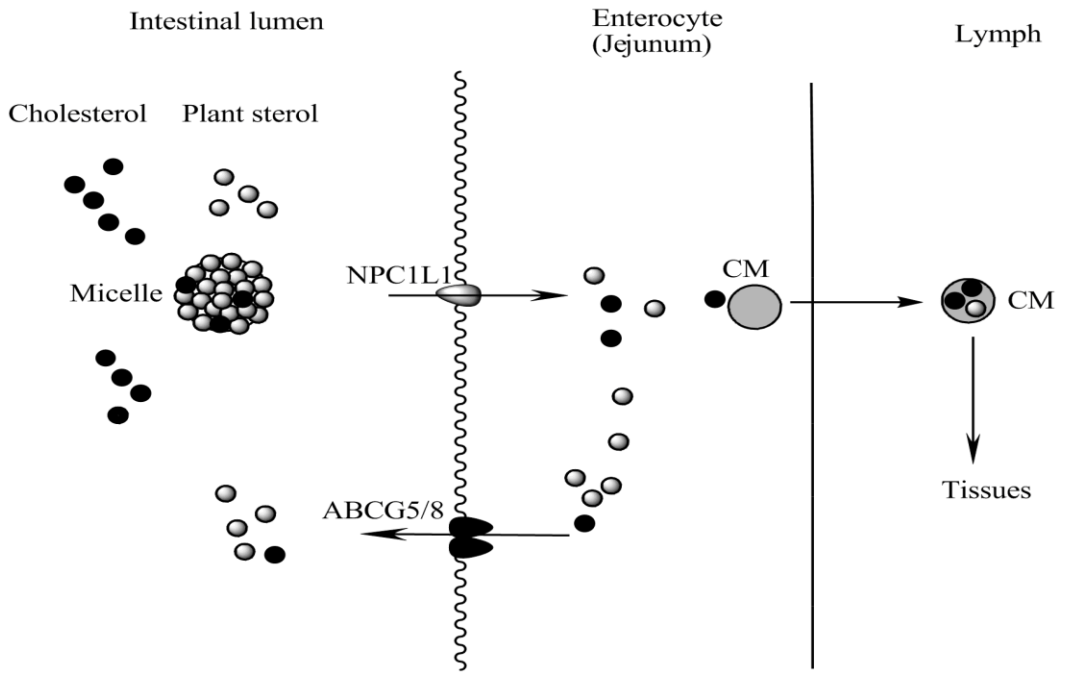

Figure 2. 2*: Cholesterol-lowering mechanism of plant sterols.*
PS bind to the mixed micelles, with a greater affinity than cholesterol, and are transported into the intestinal enterocyte through an NPC1L1-mediated pathway and efficiently pumped back out into the intestinal lumen by the ABCG5/8.Cholesterol is esterified and packaged into CM that are exported in the lymph. NPC1L1, Niemann-Pick C1 Like 1; ABCG5/8, ATP-binding cassette transporter G5 and 8; CM, chylomicrons.

Niemann-Pick C1 like 1 (NPC1L1) is a protein believed to be involved in cellular sterol influx, whereas ABC transporters ABCG5/G8, ABCA1, and ABCG1 are important for sterol efflux (28). Low absorption rate of PS is due to the active re-secretion of PS back into the intestinal lumen and bile, which is mediated by the ATP-binding cassette (ABC) half-transporter ABCG5 and ABCG8 in the intestine and liver (29). Increased PS absorption is extremely high in sitosterolemia patients, where the either *ABCG5* or *ABCG8* genes are mutated (30). After an extensive review of the scientific literature, a dose-response relationship was found with PS and a maximum effect of about 10% reduction in LDL-C levels at a dose of 2 g/d (3, 20). This has been the impetus for the United States National Cholesterol Education Program (NCEP) (31) to recommend the

inclusion of PS/stanols (2 g/d) to the diet as a part of the therapeutic lifestyle changes to lower LDL-C in patients with elevated levels by about 10%. In similar, the Food and Drug Administration (FDA) recommends a minimum of 800 mg/d of PS/stanols to assist reducing CHD risk through decreasing LDL-C (32). Moreover, the FDA allowed a health claim stating that products containing PS may reduce risk of CHD (32, 33).

2.3 CHOLESTEROL LOWERING EFFECTS

2.3.1 Experimental Evidence

Addition of PS alone or in combination with lipid lowering agent to atherogenic diet reduced plasma TC levels in apo-E-deficient mice (9, 34-39). Plant stanols, in a dose-dependent manner, reduced serum lipids by 10% to 33% in apo-E-deficient mice (40). Feeding wild-type Kyoto (WKY) rats diets containing PS (1%, w/w) for 4 weeks decreased plasma TC (-21%) and hepatic cholesterol levels (-34%) and increased the activity of the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (41). Diet supplemented with stigmasterol (0.5%, w/w) for 6 weeks decreased plasma TC levels by 11% in Wistar and WKY rats (42). Stigmasterol decreased cholesterol synthesis, activated the liver X receptor (LXR) in a cell-based reporter assay, and suppressed SREBP-2 processing in cultured adrenal cells from ABCG5/8 knockout mice (43). PS esters (PSE) (~2.5 mg/d free PS) reduced TG by 36% and TC by 21% in apo-E-deficient mice (44). Decline in TG levels could be related to the higher dosage of 1, 3-diacylglycerol in PS of canola oil mixture. The reduction in TG levels associated with PS treatment was also previously reported in mice (36) and hamsters (45). Diet supplemented with PS mixture containing no sitostanol failed to reduce plasma TC levels in either hamsters (46) or rats (47). Decreases in plasma TC and

LDL-C were attained when sitostanol was added to PS mixture (47). Ntanios et al. (48) suggested that plant stanols might be more efficient in lowering TC in rodents than PS. Both PS and plant stanols have comparable cholesterol-lowering effects (49). Addition of ferulate phytostanyl esters (FPE) or sitostanol to (0.1%, wt/wt) cholesterol diet reduced plasma TC levels by 15% in hamsters after 6 weeks (45).

Esterified plant stanols may exert more potent cholesterol-lowering effects than unesterified. In this regards, feeding disodium ascorbyl phytostanyl phosphate (DAPP) at (1.4%, w/w) decreased plasma TG levels by 49% in Golden Syrian hamsters fed compared to unesterified stanol (50). Moreover, DAPP (1.4%) decreased body weights relative to non-cholesterol and stanol diets and body fat mass strongly correlated with plasma TG (51). Reduced TG levels after DAPP treatment may be because of its lowering effect on body weight. Administration of DAPP by either food or drinking water decreased TC levels by 75% apo-E-deficient mice (36). Diet containing PS esterified with fish oil fatty acids (25 g/kg diet) reduced plasma TG and TC compared to those fed no PS or fish oil (52). Insulin resistant rats fed PS-fish oil esters (2.6 g/kg/d) showed declines in serum TG and TC compared to rats fed diet devoid of PS or fish oil (53). In contrast, PS esterified to fish oil [1.8% eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) sterol esters, providing 1% PS] reduced plasma TC levels by 20% and non-HDL-C by 29% in hamsters fed (0.25%, w/w) cholesterol diet for 5 weeks (54). Rasmussen et al. (55) investigated the impact of the fatty acid moiety on cholesterol metabolism in male hamsters for 4 weeks, where the PSE esterified with fatty acids from soybean oil (SO), beef tallow (BT), or purified stearic acid (SA). Hamsters fed

BT and SA had low cholesterol (LC) absorption and decreased levels of plasma non-HDL-C and liver cholesterol esters (CE), and greater fecal sterol excretion compared to SO and control hamsters fed no PSE. Hayes et al. (56) showed that non-esterified (free) PS were as effective as esterified sterols and stanols in lowering plasma TC when compared to no PS-fed group.

PS compete with dietary and biliary cholesterol for intestinal absorption in mixed micelles (57). PSE may bind to cholesterol esterase, suppressing its activity (58) and preventing esterification of free cholesterol (59). If they remained intact in the intestine PSE could act as a lipophilic pool to attract intestinal cholesterol to the lower part of the intestine where absorption is restricted (58). Increasing bile salt excretion has also been suggested (60). Because of their competition with cholesterol for solubilization in dietary mixed micelles, PS co-crystallize with cholesterol forming insoluble, complex crystal, which impedes the process of hydrolysis by lipases and cholesterol esterases (58). PS may not necessarily be in the intestinal lumen with cholesterol to reduce plasma TC levels. Cholesterol-lowering effects of PS in chicks (61) and hamsters (62) were achieved with a subcutaneous (s.c.) injection. PS can be converted into a LXR agonist, which activates the expression of ABC proteins (63). Mixed micelles enriched with sitostanol were potent inducers of ABCA1 expression compared to free cholesterol study in Caco-2 cells (64). ABCA1 was found to pump free cholesterol out of the enterocytes back into the lumen (28). **Table 2.1** summarizes recent experimental studies that have been conducted regarding lipid-lowering effects of PS.

Table 2. 1*: Recent animal studies of lipid-lowering effects of plant sterol treatment.*

Reference	Model	Dose of sterols/stanols/ duration	Major changes
(40)	12-week-old female apoE *3-Leiden transgenic (n=7)	Cholesterol containing diet (0.25%, w/w) plus 0.0%, 0.25%, 0.5%, 0.75%, or 1.0%, w/w) plant stanols (sitostanol 88%, campestanol 10%)(9 weeks)	↓ 10% to 33% (P<0.05), mainly in VLDL-, IDL-& LDL-C; ↓ 62% in CE, ↓ 31% in free cholesterol & ↓ 38% in TG (hepatic contents) (all P<0.05)
(41)	7-week-old male wild-type Kyoto (WKY) rats (n=6)	Chow diet supplemented with (1%, w/w) PS with (4 weeks)	↓ 21 % in TC; ↓ 34% hepatic cholesterol levels in WKY vs untreated rats (P<0.05)
(42)	6-week-old male Wistar or WKY rats (n=6)	Chow diet enriched with (0.5%, w/w) stigmasterol (6 weeks)	↓ 11% in plasma TC (P<0.05); ↓ 23 & 30 % and ↓ 12 & 23% in cholesterol & sitosterol absorption (WKY & Wistar rats, respectively)
(44)	8-week-old apo E-deficient mice (n=5)	PSE of canola oil fatty acids chow diet; 2.5 mg/d free PS mice were gavaged once in 3 or 4 days (45 μL/3days/mouse or 60 μL/4 days/mouse) (10 weeks)	↓ in TC 21% (P<0.0517); ↓ 36% in TG (P<0.05)
(45)	Male Golden Syrian hamsters weighing 80–110 g n=10-12	Cholesterol containing diet (0.1%, w/w) plus sitostanol (0.5%, w/w) or ferulate phytostanyl esters (FPE) (0.73%, w/w) (4 weeks)	↓ 14% in TC (sitostanol) (P<0.05); ↓ 15% in TC (FPE) (P<0.02); ↓ in TG (sitostanol)(P=0.04) & FPE (P=0.05); ↓ 39% in HDL-C (sitostanol) (P=0.001) & ↓ 22% (FPE) (P=0.01)
(50)	Male Golden Syrian hamsters weighing 90–110 g n=10	Cholesterol containing diet (0.25,w/w) plus free phytostanols (1%, w/w), DAPP (0.7%, w/w) or DAPP (1.4%, w/w) (5 weeks)	↓ 45% in TG (DAPP 1.4%) (P<0.05) vs cholesterol-control; ↓ 42% in TG (DAPP 0.7%) (P<0.05) & ↓ 49% (P<0.01) in TG in DAPP 1.4% vs stanol
(54)	5- to 6-week-old male Golden Syrian hamsters weighing 80–100 g (n=10)	Cholesterol containing diet (0.25%, w/w) plus EPA & DHA sterol esters (1.76%), providing 1% PS) (5 weeks)	↓ 20% in plasma TC levels (P=0.001); ↓ 29% non-HDL-C (P<0.0001) vs nonesterified PS
(55)	Male F1B Syrian hamsters weighing 90–100 g (n=8-9)	Diets containing 50 g/kg PSE esterified with fatty acids from soybean oil (SO), beef tallow (BT), or purified stearic acid (SA)(4weeks)	↓ 90% (SA) & 59 % (BT) in cholesterol absorption; ↓ 99% (SA) & 96% (BT) in liver cholesterol; ↓73% (SA) & 64.4% (BT) in plasma non-HDL-C (all P<0.05)
(56)	5- to 9-week-old male Mongolian gerbils (n=5-8)	Cholesterol containing diet (0, 0.05, 0.1, 0.15, or 0.5%, g/100 g) plus 0.5 or 0.75% nonesterified (free), esterified sterols, esterified stanols (4-5 weeks)	↓ 58%, 54% & 52% in plasma TC; ↓ 80%, 77% &76% in liver TC; ↓ 91%, 88% & 88% (all P<0.05) in liver EC in free sterols, esterified sterols & esterified stanols, respectively vs no PS fed group

2.3.2 Clinical Evidence

The role of PS in lowering LDL-C levels has been the subject of numerous controlled clinical studies. Combination of low-fat margarine and milk enriched with PS (2.3 g/day) with NCEP step 1 diet for 4 weeks decreased TC (6%), LDL-C (8%) and apo-B (5%) levels (5). Decrease in LDL-C was observed after adding 2 g of plant stanol ester into the meat, pasta, low-fat yogurt drink (65), and low-fat hard cheese (66). In contrast, low-fat yoghurt enriched with PS (1-2 g/day) failed to reduce TC and LDL-C (67). Chocolate snack bars containing 1.5 g PSE twice a day for 6 weeks reduced TC and LDL-C in mild hypercholesterolemic subjects (68). Hansel et al. (69) reported reduced plasma oxidized LDL (oxLDL) and cholesterol levels without any changes to serum β-carotene levels after consuming low-fat yogurt containing (1.6 g/day) of PS for 6 weeks. Dietary PS may reduce LDL-C and therefore reduce number of circulatory LDL particles that are prone to oxidation. PS-enriched drinkable yogurt (1.6 g/day) along with the main meal for 42 days reduced LDL-C levels by 11% (70). In contrast, incorporating PS (1.8 g/day) into beverages containing either no fat or 1 g of fat failed to reduce TC and LDL-C levels (71). Similarly, consumption of PS-supplemented low-fat beverages for 3 weeks did not modulate the LDL particle size (72). Availability of PS in adequate amounts and sufficient dispersion in the fat phase of the digested food for best for solubilization in mixed micelles may be crucial for cholesterol-lowering effect. Incorporating of PS (1 g/MJ) into portfolio diet, a diet high in PS, soy protein, viscous fiber, and almonds, reduced all the LDL fractions including small dense LDL (73). Milk tea supplemented with 2.3 or 1.5 g PS with the two fatty meals of the day reduced TC and LDL-C, however, to a lesser extent than was anticipated (74). In disagreement, Weidner et al. (6)

showed significant diminutions in plasma levels of LDL-C (7%) and TC (4%) after 8 weeks of consuming soy drink enriched with 2.6 g PSE. Furthermore, linking the time of ingestion to a main meal is critical (75); single-dose of PS (3 g/day) attenuated LDL-C when taken with a meal independent of its fat content (76). AbuMweis et al. (77) showed in meta-analysis study that the timing of administration of a single dose of PS might influence their cholesterol-lowering actions since consuming of single dose with main meal or lunch lowered LDL-C levels but not prior or with breakfast. Similarly, consumption of ground beef supplemented with 2.7 g of soybean PS as a single dose for 1 month attenuated the levels of TC (9%) and LDL-C (15%) in mildly hypercholesterolemic men compared to those of baseline (78). Daily consumption of a relatively low-fat product enriched with 2 g of plant stanols esters decreased TC by 4% and LDL-C by 5% (79). The small decline in the LDL-C may be due to low baseline LDL-C value as the magnitude was more pronounced when a single-shot yoghurt drink was consumed with lunch and the baseline LDL-C level was ≥3.4 mmol/L. Plant stanol esters at lunch time may inhibit the absorption of both dietary and biliary cholesterol (57), and coincide with the diurnal rhythm of cholesterol and bile acid synthesis as cholesterol levels peaked at night and declined during day time. The intake of PS capsules with meal for 12 weeks reduced LDL-C (5%) (80). In contrast, low dose of PS PSE decreased plasma LDL-C (5%) in hypercholesterolemic subjects (81). Decreases in plasma LDL-C increase with PS doses, in the range from 0.7 to 2.5 g/day; high doses have no additional benefits as to reducing plasma cholesterol (75). However, dose-dependent reduction in TC by PS can be observed when dietary cholesterol intake increased (40, 56). Increased baseline LDL-C values were related to greater LDL-C

reductions. The effects of the dose-response curves established for PS versus stanols, fat-based versus nonfat-based food formats and dairy versus nondairy foods were not significantly different (82). Solid foods compared to liquid foods tended to have a larger influence only at high doses of PS (>2 g/day) (82). Given that liquid portion of the meal undergoes fast gastric evacuating compared to solid portion (83), delivering PS in solid portion of the meal may delay gastric emptying and prolong time for micelle formation. PS compete with cholesterol for micellar incorporation, and concurrent presence of PS, cholesterol, and bile acids in repeated occasions during the day could lead to a larger efficacy than a single intake. Cholesterol content of the background diet does not seem to influence the efficacy of PS (84). PS may have an additive effect on reducing LDL-C levels when combined with lipid-lowering agent. Incorporation of PS into the portfolio diet in hyperlipidemic subjects decreased LDL-C levels by 15% (85). Additional reductions (15%) in LDL-C levels were observed when plant stanols combined with statins for long-term (86, 87). Combination of PS with n-3 long-chain polyunsaturated fatty acids (LCPUFA) decreased TC and LDL-C by 13% (88). The efficacy of PS may vary among individuals due to genetic variation. PSE decreased serum levels of TC and LDL-C only in apo-E phenotype E2 and E3 subjects (89). Genetic variations including the ATP-binding cassette transporter G5 and G8 polymorphism affect the PS cholesterol-lowering response of subjects (29). Variations observed in the LDL-C responses to PS may be related to the diverse doses, the rate of esterification of sterols, the timing of administration and ingestion of PS with or without meal besides genotype variation of the subjects. **Table 2.2** summarizes recent clinical studies that have been conducted regarding lipid-lowering effects of PS.

Table 2. 2*: Recent clinical studies of lipid-lowering effects of plant sterol treatment*

Reference	Study design	Subjects	Dose of plant sterol /duration	Major findings
(6)	Randomized, placebo-controlled double-blind	Untreated moderate hypercholesterolemic (19-65) yrs (n=25)	200 ml of soy drink enriched with 2.6 g PSE (8 weeks)	↓ 4% in TC; ↓ 7 % in LDL-C; ↓ 6 % in non-HDL-C (P<0.05)
(67)	Meta-analysis of two randomized double-blind crossover or parallel	Moderately hyperlipidemic; study I: (41 yrs, mean)(n=15); study II: (54 yrs, mean) (n=27)	Study I: yoghurt containing 1 g PS 1X/d (4 weeks)-2-week run-in & 2-week wash-out periods; Study II: yoghurt containing 2 g PS 2X/d (8 weeks)	↓ 5.2% in TC P<0.2; ↓ 5.8% in LDL-C (P<0.3)
(68)	Randomized, double-blind parallel arm	Hypercholesterolemic; (49 yrs, mean) (n=32)	Cocoa flavanol-enriched snack bar containing 1.5 g PS(6 weeks) (2X/d)	↓ 4.7% in TC (P<0.01); ↓ 6% in LDL-C (P<0.01); ↓ 7.4% in TC: HDL-C ratio (P<0.001)
(69)	Randomized double-blind, parallel	Hypercholesterolemic (n= 95)	Low-fat fermented milk enriched with 0.8 g PSE/portion (2X/d) (6 weeks)	↓ 4.7 % in TC; ↓7.8% in LDL-C (P<0.05); ↓ in oxLDL vs control(-1.7 vs 1.4 U/L; P<0.03)
(70)	Randomized, double-blind, placebo-controlled, parallel	Hypercholesterolemic (18 -75 yrs) (n=44)	100 ml PS enriched (1.6 g 1X/d) drinkable yogurt along with the main meal for 42 days	↓ 10.6% in LDL-C; ↓ 14% in TG; ↑ in sterols: TC ratio
(79)	Randomized, placebo, controlled, double-blind	Hypercholesterolemic (n= 49.5 ± 13.1 yrs, mean) (n=102)	2 g/d of stanols as stanol-ester-enriched low fat milk product (5 weeks)	↓ 3.8% in TC, P<0.001;↓ 4.9% in LDL-C, P=0.002)
(84)	Semirandomize, double-blind, crossover	Hypercholesterolemic (45-85 yrs) (n=22)	22 mg PS/kg of BW with either low-cholesterol (LC) or high- cholesterol (HC) diets; four 28-day feeding phases each separated by a 4-week washout	↓ 12.8% in TC; ↓ 11.9 % in LDL-C (LC); ↓ 4.0 % in TC; ↓ 2.5% in LDL-C (HC)
(85)	Intervention, single-phase	Hyperlipidemic (59.7± 1.5 yrs, mean) (n=42)	PS-enriched margarine (1g/1000 kcal) with portfolio diet (80 weeks); eliminating PS from the diet between weeks 52 and 62 weeks	↓ 15.4% in LDL-C (P<0.001) ↓ 9.0% in LDL-C (P<0.001)
(88)	Randomized, double-blind, placebo-controlled	Hyperlipidemic (55.4 ± 1.0 yrs, mean) (n=60)	25 g/d of a spread containing 2 g/d with 1.4 g/d (n-3) LCPUFA (3 weeks)	↓13.3%(P<0.001) in TC; ↓ 12.5% (P< 0.002) in LDL-C; ↑ 8.6% (P<0.04) in HDL-C
(89)	Randomized, double-blind, controlled	Hypercholesterolemic with varied apo-E genotype (21-75 yrs) (n=120)	50% fat reduced spread as part of NCEP-I diet with PSE (1.1 g or 2.2 g/d	↓ TC, LDL-C, apo-B TC: LDL-C & LDL: HDL-C ratios in only E2 & E3 subjects

2.4 PLANT STEROLS AND CARDIOVASCULAR DISEASE

2.4.1 Experimental Evidence

Dietary supplementation with (0.8%, w/w) sitostanol reduced lesion developments in coronary arteries in atherogenic diet-fed rabbits (46). PS may decrease buildup of fatty streaks by reducing circulating levels of atherogenic lipoproteins including secretion of apo-B100 and apo-B 48 (90). Imbalance between vascular smooth muscle cells (SMC) proliferation and apoptosis accelerates the development of atherosclerosis. Maintaining a balance in proliferation and apoptosis of endothelial cells may play a role in the atherosclerosis prevention. Sitosterol had a stronger cytotoxic activity than cholesterol, apoptotic influence and reduced cell viability of human abdominal aorta endothelial cells (91) and decreased growth of vascular SMC. PS reduced the number of foam cells in the atherosclerotic plaque (92). LDL oxidation stimulates macrophages to engulf oxLDL, which induce the secretion of proinflammatory cytokines and promote the recruitment of monocytes and accumulation of foam cells (93). Feeding female apoE*3-Leiden transgenic mice diets containing (1%, w/w) different derivatives of plant stanol esters for 38 weeks reduced atherosclerotic lesion area by 90% and severity of atherosclerotic lesions (94). Addition of PS to statin, for 35 weeks, lowered serum TC by 39%, and atherosclerotic lesion size by 99% in LDL-r+/− mice (95). Tall oil-derived PS mixture (TODPM) reduced the formation of atherosclerotic lesions in apo-E-deficient mice (35, 96). A strong positive correlation ($r=0.69$, $P<0.01$) was found between plasma TC levels and lesion area in the aortic sinuses (96). PS alone (9) or combined with nicotinamide (vitamin B3) (37), probucol (lipid-lowering agent) (39), cyclosporine A (immunosuppressant agent) (38) reduced atherosclerotic lesion size in apo-E-deficient mice. However, when compared with ezetimibe (EZE), a sterol absorption inhibitor, PSE

tended to have more pronounced atherosclerotic lesion in apo-E-deficient mice (97). It should be mentioned that additional effects of EZE such as raising HDL-C, lowering TG, non-HDL-C, apo-B, and remnant-like particle cholesterol, reducing circulating PS, oxysterols, oxidative derivatives of cholesterol levels on atherogenesis cannot be excluded (98). After induction of atherosclerosis in apo-E-deficient mice by Western-type diet (WTD), PS failed to decrease atherosclerotic lesions (99). In parallel, dietary PSE decreased foam cell area by 70% regardless of increase in plasma PS levels in hamsters fed an atherogenic diet for 12 weeks (100). The exact mechanism by which PS reduce atherosclerosis in animals is not fully understood. PS may lower cholesterol absorption and decrease hepatic lipase activity and plasma fibrinogen levels (96). Low accumulation of oxLDL in macrophages, reduced amount of serum peroxides (44) and preserved serum paraoxonase1, an enzyme located on HDL activity, and reduced LDL oxidation were noted after PS (44). Incorporation of PS into macrophages may protect against atherosclerosis by reducing prostaglandin release (101), increasing prostacyclin production, and reducing vascular SMC proliferation (102), and consequently atherosclerotic plaque formation. **Table 2.3** summarizes recent experimental studies regarding anti-atherosclerotic effects of PS.

Table 2. 3*: Recent animal studies of anti-atherosclerotic effects of plant sterol treatment*

Reference	Model	Dose of sterols/stanols/ duration	Major changes
(9)	4-wk-old male apo-E deficient mice	Cholesterol-enriched diet (0.2% , w/w) plus (2%, w/w) soybean-derived PS mixture (14 wks)	↓ 62% (PS) in lesion size; ↓ 59.4% (PS) in atherosclerotic lesion: lumen ratio vs control (P<0.05)
(37)	4-wk-old male apo-E deficient mice (n=7-8)	Cholesterol-enriched diet (0.2%, w/w) plus (2%, w/w) soybean-derived PS mixture with or without (0.1%, w/w) fenofibrate or (0.5%, w/w) niacin (14 wks)	↓ 50% (PS &PS + niacin) in lesion size; ↓ 59 & 50% (PS & PS+niacin) in lesion:lumen ratio
(38)	4-wk-old male apo-E-deficient mice (n=8)	Cholesterol-enriched diet (0.2%, w/w) plus (2%, w/w) soybean-derived PS mixture with or without CA (0.02%, w/w) (14 weeks)	↓ (64, 63 %) (PS); ↓ (47, 46 %) (PS+CA) in atherosclerotic lesion size ratios; ↓(59,58%)(PS); ↓ (44, 42%) (PS+CA) in lesion:artery lumen ratios vs control & CA groups (all P<0.05)
(39)	4-wk-old male apo-E-deficient mice (n=7-8)	Cholesterol-enriched diet (0.2%, w/w) plus (2%, w/w) soybean-derived PS mixture with or without (1%, w/w) probucol (14 weeks)	↓ 60% in atherosclerotic lesion area; ↑ 66% in HDL-C& ↓ 47% in TC/HDL ratio (PS); ↓ 59% in TC (PS+probucol) (all P<0.05 vs control)
(46)	4-6-wk- old male New Zealand White rabbits 80–100 g(n=6)	Cholesterol-enriched diet (0.5%, w/w) plus PS mixtures (0.01, 0.2 or 0.8%, w/w) (2 months)	↓ 48.6% in TC; ↓ 63% in VLDL-C; ↓ plaque accretion in coronary arteries & ascending aorta in (0.8%, w/w) (p<0.005 vs control)
(94)	12-wk-old female apo-E*3-Leiden transgenic Mice (n=10)	Cholesterol-enriched diet (0.25%, w/w) plus (1.0%,w/w) plant stanol esters derived from either vegetable oil, wood or a mixture of both (38 wks)	↓ 91% (vegetable oil), ↓ 97% (wood) & ↓ 78% (vegetable oil/wood) (P<0.0001) in the atherosclerotic lesion area
(95)	8-wk-old female & male heterozygous LDLr +/- (n=6-12)	Study I, Western diets enriched with atorvastatin (0.003%, w/w) plus (1%, w/w) PS or stanols (35 wks); Study II, Western diets (33 wks& addition of atorvastatin (0.005%, w/w), (2%, w/w) PS or (2%, w/w) plant stanols for the subsequent 12 wks	Study I: ↓ 99% (atorvastatin + PS); ↓ 98 % (atorvastatin+ stanol) in atheroma lesion size (all P<0.05) vs controls Study II: ↓66 %(PS); ↓ 64% (plant stanol) in atheroma lesion size vs control (P<0.03)
(96)	5-wk-old male apo-E-deficient mice (10)	Cholesterol-enriched diet (0.15%,wt/wt) plus TODPM (2%,wt/wt)(18 wks)	↓ >50% in the average area of atheromata vs control (1.96±0.8 vs 4.08±0.3 mm2; P<0.0001)
(97)	8-12 -wk-apo-E deficient mice weighing 20-25 g (n =10)	WTD containing either (2%, w/w) PSE, (0.005%, w/w) ezetimibe or the combination (24 wks)	↑ 20.4% in plaque sizes (WTD+ PSE) vs.10.0 % (WTD+ ezetimibe); p<0.05
(100)	11-wk- old male F1B hybrid Syrian golden hamsters weighing ~106 g (n=20)	Basal diet containing (30%) of energy as fat enriched with (0.12%, w/w) cholesterol plus PSE (0, 0.2, 0.5, 1, 2 & 3 %, w/w) (12 wks)	↓ ~ 70, 90 &100% in lipid-filled foam cell areas (0.24, 0.48 & 2.84% PSE); ↓19, 36, 51, 52 & 55% in TC (0.24, 0.48, 0.96, 1.92 & 2.84% PSE); ↓77, 50 & 47% in VLDL-, LDL- & HDL-C (0.96% PSE), respectively (all P<0.001 vs control)

2.4.2 Clinical Evidence

PS treatment has been shown to reduce the cluster of differentiation 36 (CD36), a member of the scavenger receptor family involved in the uptake of oxLDL from the bloodstream, and increase LDL-receptor affinity expressions among polygenic hypercholesterolemic subjects, suggesting potential anti-atherogenic effect (103). PS may attenuate atherogenesis through different mechanisms than cholesterol-lowering effects (104). The activity of antithrombin-III, a potent inhibitor of coagulation, tended to increase in healthy subjects after plant stanol ester relative to control. PS prolonged the adhesion and aggregation time of blood platelets after collagen-epinephrine activation among 42 healthy male subjects, suggesting antiplatelet activity of PS in the prevention of CVD (105). Despite reduction in TC and LDL-C levels plant stanols failed to improve endothelial function (106, 107).

Increased serum campesterol and sitosterol were noted after PS supplements (67, 87) but such levels were much lower than those seen in patients with sitosterolaemia (108). Serum campesterol levels were higher than those of sitosterol (109) suggesting that campesterol is preferentially absorbed over sitosterol despite low levels of campesterol in diet (109). There have been concerns with respect to the possible atherogenicity of PS in some people. These issues were brought up after reporting premature atherosclerosis in sitosterolemia patients (110). Increased serum PS levels are thought to be associated with a family history of coronary artery disease (CAD) in patients with hypercholesterolemia (11), suggesting that slightly higher PS were a heritable factor for increased CAD risk. However, no causal relationship can be proven from any observational study. A nested

case-control analysis also found that elevated sitosterol levels or sitosterol to cholesterol ratios were associated with approximately threefold increase in the CHD risk (13). Given that only univariate analysis was performed in this study, it is not clear if the positive correlation between plasma sitosterol levels and CHD risk would have still been significant if multivariate analyses had been used. Furthermore, PS have been identified in atherosclerotic plaques biopsies (111) and stenotic aortic valves (112) obtained from patients with apparently normal PS absorption. These findings raised the possibility that PS could be an independent risk marker for CHD. It should be mentioned that these observational studies are not comparable and the results are not conclusive (113). Weingätner et al. (21) suggested that increased plasma levels of PS could be an atherogenic and reported that increases in plasma PS in patients was associated a fivefold increment in sterol concentration in aortic valve tissue (97). However, no evidence of selective accumulation of PS in diseased tissue has been found. Indeed, the distribution of PS across body tissues is virtually the same with that of cholesterol.

Helske et al. (112) found no significant differences in the valvular sterol contents between the stenotic and control valves. In parallel, no evidence supporting associations between plasma levels of PS and family history of CHD was found after comparing plasma cholesterol, sitosterol and campesterol levels in 2,542 subjects who underwent coronary artery calcification scoring (114). Windler et al. (115) found no association between CHD and plasma PS levels in women with incident symptomatic CHD, indicating that plasma PS levels in women with CHD were not higher than those of healthy women of the same age. Similarly, in a cohort of 1,242 subjects older than 65

years, participating at the Longitudinal Aging Study Amsterdam (LASA), increased plasma levels of sitosterol were associated with reduced risk for CHD, suggesting neutral or even protective effects of PS on development of CHD (116). This was further supported by the observation that PS levels and their ratio to cholesterol were increased in patients without CHD. Furthermore, neither stanols nor markers of cholesterol synthesis differed in subjects with or without CHD (116). The relationship between plasma PS and the risk of CHD not only was examined in subjects with CHD but also in apparently healthy subjects. In this regard, Pinedo et al. (117) have shown in a prospective study that increased levels of PS, at least in the physiological range, were not adversely related to CAD in apparently healthy men and women. Silbernagel et al. (118) measured plasma cholestanol, a metabolite of cholesterol, lathosterol, a cholesterol precursor, campesterol, and sitosterol levels in 2,440 subjects who underwent angiography, and Friesinger scoring (FS) to assess the severity of CAD. The results revealed that while campesterol to cholesterol ratio was associated with the FS, sitosterol to cholesterol ratio showed no correlation with the FS in the whole group. The authors concluded that plasma PS seem less likely to be atherogenic in the absence of sitosterolemia. Overall data from the available clinical research showed no clear evidence suggesting involvement of PS in development of atherosclerotic plaque (119). Indeed, epidemiological studies have revealed that 4 to 5% reduction of LDL-C correlates with 5 to 10% reduction in CHD risk in the first 5 years, and by 10% over lifetime (3). The potential of PS being atherogenic seems less likely and LDL-C-lowering benefits of PS may far outweigh possible risks. However, more large epidemiological studies are needed

to confirm the effect of PS on the CHD risk. **Table 2.4** summarizes clinical studies reporting relationship between plasma PS and cardiovascular risk factors.

Table 2. 4*: Relationship between plant sterol and cardiovascular risk factors based on clinical trials.*

Reference	Study design	Subjects	Dose of sterols/stanols/ duration	Major findings
(13)	Nested case-control analysis of the prospective cardiovascular münster (PROCAM)	Patients with CHD in age, smoking status, and date of investigation-matched population based control (16-65 yrs) followed for 10 yrs (n=318)	_	↑ in serum sitosterol levels or sitosterol:cholesterol ratio correlated with ~ 3-fold ↑ in the risk of CHD (P<0.030)
(103)	Single-blind randomized crossover	Patients with polygenic Hypercholesterolemia(54±7 yrs, mean) (n=15)	Yogurt containing 1.6 g/d esterified phytosterols (equivalent to 1.0 g free phytosterol)(4 weeks)	↓ 4.3% in LDL-C (P=0.03); ↑ ~ 10 % in LDL-receptor affinity (P=0.01); ↓18.2% in CD36 expression (p=0.01)
(106)	Double-blind placebo-controlled crossover	Pre-pubertal children with familial hypercholesterolemia (FH) (7-12 yrs) (n=20)	500 mL of a low-fat yogurt enriched with 2.0 g/d of plant stanols (4 weeks)	↓ 7.5% in TC; ↓ 9.2% in LDL-C (P<0.001); ↔ in FMD of the brachial artery vs placebo
(107)	Randomized, double-blind, parallel controlled	Mildly to moderately hypercholesterolaemic (25-70 yrs) (n=282)	Plant stanol or PSE (2 g/d) containing spread (1-yr)	↓ 4.2 % (stanol ester) (P<0.01) & ↓ 4.4 % (sterol ester) in TC (P<0.01) group vs control; ↔ in endothelial function
(112)	Cross-sectional	Patients with clinically symptomatic aortic valve stenosis (AS)(67±10 yrs, mean) (n=82)	_	Correlation of serum ratios of campesterol (r=0.88; P<0.0001 & sitosterol (r=0.84;P<0.0001): cholesterol with those of aortic valve campesterol & sitosterol
(115)	Case-control study of CAD (CORA) study	Women with CHD in age-matched population-based controls (30-80 yrs)(n=200)	_	no correlation between CHD and plasma phytosterol levels
(116)	Cross-sectional	Subjects with CHD participating at the Longitudinal Aging Study Amsterdam (LASA)(65-89 yrs) (n=1242)	_	Correlation between ↑ in plasma sitosterol levels & ↓ in the risk for CHD (odd ratio 0.78, CI 0.62-0.98, p<0.05)
(118)	Cohort study	Subjects with CAD participating in the Ludwigshafen Risk and Cardiovascular health aged (LURIC) study; mean age 58±12 to 65±9 yrs (n=2,440)	_	Correlation between high FS & ↑ in cholestanol & campesterol (P<0.006 & P<0.026): cholesterol ratios, but not with sitosterol; ↑ in campesterol, sitosterol & cholestanol to lathosterol ratios correlated with high FS (P<0.001)

2.5 CONCLUSION

PS-enriched products appear to be effective at reducing serum TC and LDL-C levels whether used alone in the diet, or as an adjuvant to drug therapy. Evidence from animal and clinical trials strongly supports the notion that PS effectively lower LDL-C levels. The effects of PS or plant stanols on lipid parameters are not different; therefore, the use of which should be based on safety considerations that need to be resolved upon further research. Evidence from animal studies strongly supports the notion that PS decrease atherosclerosis. Beyond their anti-hyperlipidemic properties, additional health benefits of PS including anti-inflammatory and anti-cancer properties have also been observed from experimental studies (120).

Increased plasma PS levels associated with PS supplementation have raised questions about the safety of prolonged use. Evidence from clinical studies does not support the notion that PS proatherogenic in non-sitosterolemic subjects. Therefore, the concept that increases in circulating PS levels in individuals consuming PS may increase CVD risk is still debatable as direct assessment between PS and atherosclerosis has not been proven. Whether noted decrease in LDL-C levels related to PS may be translated into decreases in CVD morbidity and mortality remains unknown. Future controlled, randomized clinical interventions are needed to assess long-term effects of PS on CVD outcomes and to evaluate their possible additive effects with drugs on health outcomes in order to explore their new areas of therapeutic applications.

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CHAPTER III

MANUSCRIPT 2: LITERATURE REVIEW

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NON-CHOLESTEROL STEROLS AND CHOLESTEROL METABOLISM IN SITOSTEROLEMIA

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3.1 ABSTRACT

Sitosterolemia (STSL) is a rare autosomal recessive disease, manifested by extremely elevated plant sterols (PS) in plasma and tissue, leading to xanthoma and premature atherosclerotic disease. Therapeutic approaches include limiting PS intake, interrupting enterohepatic circulation of bile acid using bile acid binding resins such as cholestyramine, and/or ileal bypass, and inhibiting intestinal sterol absorption by ezetimibe (EZE). The objective of this review is to evaluate sterol metabolism in STSL and the impact of the currently available treatments on sterol trafficking in this disease. The role of PS in initiation of xanthomas and premature atherosclerosis is also discussed. Blocking sterols absorption with EZE has revolutionized STSL patient treatment as it reduces circulating levels of non-cholesterol sterols in STSL. However, none of the available treatments including EZE have normalized plasma PS concentrations. Future studies are needed to: (i) explore where cholesterol and non-cholesterol sterols accumulate, (ii) assess to what extent these sterols in tissues can be mobilized after blocking their absorption, and (iii) define the factors governing sterol flux.

Keywords: Plant sterols; non-cholesterol sterol; cholesterol; HMG CoA reductase, ABCG5/8, NPC1L1; SOAT2; atherosclerosis; xanthoma

3.2 INTRODUCTION

Sitosterolemia (STSL) is a rare autosomal recessive lipid storage disease that is caused by homozygous or compound heterozygous mutations in either one of the two adenosine triphosphate binding cassette transporters genes, *ABCG5* and *ABCG8.* These proteins are expressed in both the intestine and liver and form heterodimers that normally function to rapidly remove absorbed plant sterols (PS) and cholesterol. STSL is characterized by elevated plasma levels of the major PS including sitosterol and campesterol, but with normal to moderately elevated plasma cholesterol levels (1). It is clinically characterized by presentation of xanthomas, accelerated atherosclerosis, and occasionally arthritis, arthralgias (2, 3), hemolysis, and thrombocytopenia (4-9). The presentation of these clinical signs varies based on the level of PS retention in tissues. The exact prevalence of STSL is unknown with only 100 known cases identified worldwide (10). The diagnosis of this disease relies on confirming elevated plasma levels of sitosterol, which are normally below 0.4 mg/dl (11), through the use of high-performance liquid chromatography or gas chromatography. In the past 8 years, 5 pedigrees of STSL who presented with hemolysis and an abnormal platelet count were identified in one location, indicating that the disorder is not as rare as initially recognized (12).

The accumulation of dietary plant and other non-cholesterol sterols appears to disrupt the endocrine system and cause infertility in ABCG5/8- knockout **(**KO) mice (13), although in humans endocrine disruption has only been reported once (14), and infertility has never been reported. Mannucci et al. (15) reported a proband that was homozygous for a single-nucleotide mutation in the *ABCG5* gene and later found that her mother and

brother were homozygous for the same mutation, suggesting that sitosterolemia patients are able to have children who can also be affected. Complete failure of sterol efflux, and down-regulated cholesterol biosynthesis along with increased expression of low-density lipoprotein receptor (LDL-R) underpins the etiology of this disease (16). This review, aims to update recent findings concerning sterol metabolism in STSL, and discusses the impact of available therapeutic approaches on sterol trafficking and homeostasis in this disorder. Moreover, the role of PS in development of xanthomas and atheromatous cardiac disease will also be reviewed.

3.3 PLANT STEROLS

Plant sterols (PS), also called phytosterols, are structurally related to cholesterol but differ from cholesterol in having an extra ethyl group (sitosterol and stigmasterol) or methyl (campesterol) group at the 24-carbon atom of the sterol side chain. Saturated sterols, termed stanols, are less abundant in nature compared with PS. PS are principally found in wheat germs, vegetable oils, nuts, and seeds; certain shellfish contain non-cholesterol sterols, including brassicasterol and 24-methylene cholesterol (17). The average person diet contains PS (180 to 450 mg/day) in amounts that are similar to those of cholesterol (200-500 mg/d), with intakes increasing in vegetarians (18). Non-cholesterol sterols including PS, their 5α-saturated stanols or shellfish sterols are absorbed in the gut with absorption rates that differ based on the length of the side chain, hydrophobicity and micellar solubility of sterols. The longer the side-chain of the sterol, the less is absorbed because of its increased hydrophobicity and decreased micellar solubility (17, 19-23). Moreover, hydrogenation of the nucleus double bond of a sterol decreases absorbability (24-26) (**Table 3.1)**. Differences in absorption patterns amongst varying sterols suggest

unselective intestinal sterol uptake, and that the body has the ability to differentiate cholesterol from PS at the level of sterol efflux from the enterocyte, which appears to be lost in STSL.

Table 3. 1*: Molecular structure, absorbability and cellular effects of non-cholesterol sterols*

Sterol	Molecular structure	Absorbability	Cellular effects
Campesterol	Steroid structure with extra methyl group at C-24	<20% or 9% -18% (20, 22)	↔ ABCA1 or ABCG1 levels; ↓ cholesterol efflux to HDL; ↔ TNF-α, IL-6 and IL-1β secretion (77);↓ SREBP-2 processing and ↓ cholesterol synthesis (62)
Sitosterol	Steroid structure with extra ethyl group at C-24	<8% or 4% to 8% (20, 22)	↔ ABCA1 or ABCG1 levels or efflux of cholesterol to apoA-I and HDL (77); ↓ macrophage cholesterol biosynthesis; ↑ pro-oxidant activity (70) and the oxidised derivatives of sitosterol were the most cytotoxic and apoptotic (103)
Stigmasterol	Steroid structure with extra ethyl group at C-24; double bonds at C-22 and C-23	5% (19)	↑ Expression of ABCA1 and ABCG1;↑ efflux of cholesterol to Apo AI and HDL (77); ↓TNF-α, IL-6 and IL-1β secretion in macrophage foam cells (77); ↓ SREBP-2 processing and ↓ cholesterol synthesis; activates the liver X receptor (62)
Brassicasterol	Steroid structure with extra methyl group at C-24; double bonds at C-22 and C-23	4.8% (17)	↓ Cholesterol biosynthesis; cause damage to adrenal gland due to disrupting cholesterol homestasis influence steroid hormone synthesis (66)
Plant stanols e.g.,campestanol and sitostanol	Steroid structure with extra methyl or ethyl groups at C-24; no double bonds between C-5 and C-6 in B-ring*	0.1%-2%; (24-26)	↓ Plaque accretion in coronary arteries (84); ↔ apoptosis; ↓ both the viability and growth of intestinal cells (104)
PS esters	Hydroxyl (OH) group of steroid structure at C-3 is esterified with a fatty acid	4-18%; similar to free form; PS esters are hydrolysed to free sterols in the intestine	↑ Endothelial dysfunction (85), ↑ pro-atherogenic' monocyte subpopulation, ↑ the amount of atherosclerotic lesion formation in mice (82)
Plant stanol esters	Hydroxyl (OH) group of steroid structure at C-3 is esterified with a fatty acid	0.1-2%; similar to free form; plant stanol esters are hydrolysed to free stanols in the intestine	↑ Regenerative monocytes; ↓ vascular superoxide release;↓ lipid hydroperoxides;↓ inflammatory cytokines in aortic tissue, in plasma, and in circulating monocytes (82)

↑, increase; ↓, decrease; ↔, no significant; * one of the four (A, B, C, D) carbon rings in a steroid skeleton

3.4 STEROL ABSORPTION

Dietary and biliary free cholesterol (FC), and non-cholesterol sterols primarily PS and stanols, are absorbed from the intestinal lumen *via* the Niemann Pick C1 Like 1 (NPC1L1) transporter (**Figure 3.1)**. NPC1L1 is a sterol influx transporter found in the brush border of the intestinal epithelium and hepatobiliary interface to facilitate sterol absorption by enterocytes. After uptake in the enterocytes, about 50-60% intestinal FC is esterified by the endoplasmic reticulum acetyl- Sterol O-acyltransferase 2 (SOAT2) and packaged into chylomicrons (CM) that transport lipids to the liver (27). Unesterified cholesterol can also be secreted back to the intestinal lumen by ABCG5/8 transporters. SOAT2 is exclusively expressed in hepatocytes and enterocytes (27), and may help with sorting of dietary sterols in the enterocytes by differentiating among them as substrate candidates. PS are poor substrates of SOAT2 compared with cholesterol (28), which allows ABCG5/8 transporters to shuttle out these sterols from the enterocytes into the intestinal lumen (29), and therefore predominant incorporation of cholesterol ester (CE) into CM (30). Non-cholesterol sterols not pumped back to the intestine *via* ABCG5/8 transporters become part of the CM, entering circulation (31); however, they will be returned to the intestine by ABCG5/8 transporters at the hepatobiliary interface. Free PS may be mobilized *via* ATP-binding cassette transporter A1 (ABCA1) located at the basolateral membrane of enterocytes and become a part of apolipoprotein (apo) A-I, containing high-density lipoprotein (HDL) particles (32-34).

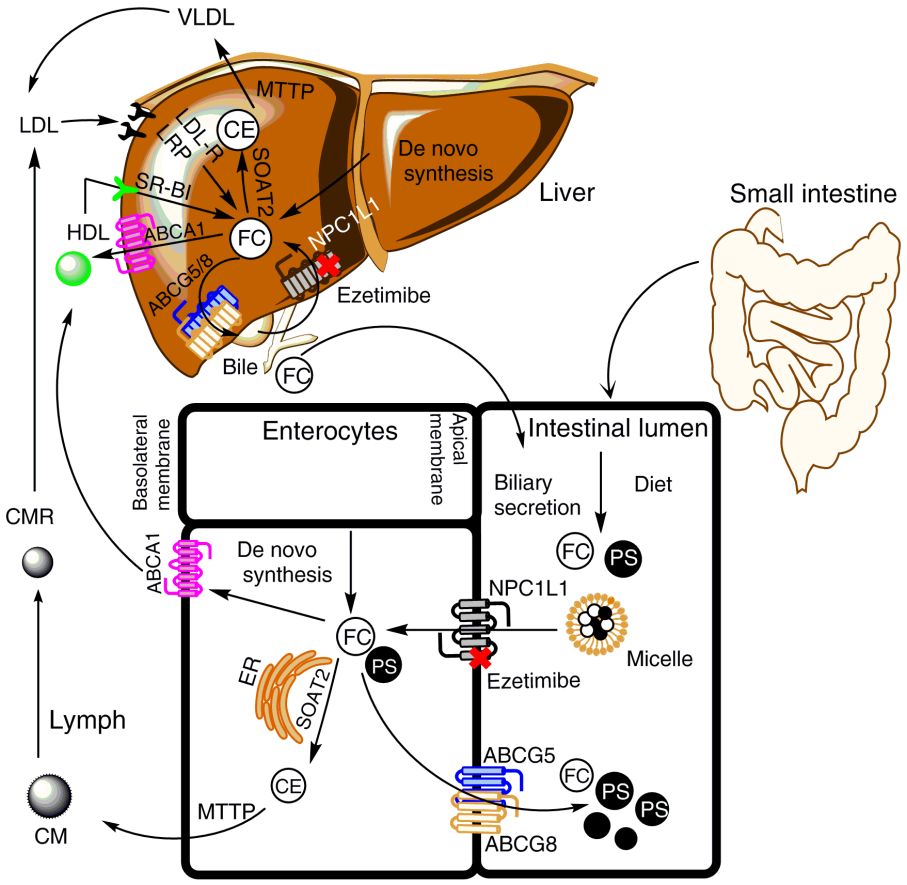

Figure 3. 1*: Schematic description of the main routes of sterol trafficking in intestine and liver. FC and PS are absorbed from the intestinal lumen by NPC1L1, and secreted back to the lumen by ABCG5/8. Intracellular FC can be esterified by SOAT2 in the endoplasmic reticulum. PS are poor substrates for SOAT2 and are preferentially secreted back into the intestinal lumen through the ABCG5/8. A portion of FC is also selectively secreted back into the lumen through theABCG5/8. Cholesteryl ester (CE) is packaged into CM by MTTP, and secreted into intestinal lymph, entering the bloodstream. FC and free PS may be mobilized by ABCA1 in apoA-I containing HDL particles that enter the lymphatic system. The liver can synthesize cholesterol locally or take it up from circulating lipoproteins such as LDL and CMR via LDL-R and LRP, and HDL-cholesterol by SR-B1. In the liver SOAT2 esterifies cholesterol to CE that are repackaged into VLDL particles and delivered to the circulation and periphery. Majority of FC in the liver is converted into bile acids for biliary secretion. Hepatic NPC1L1 can facilitate reuptake of biliary cholesterol. Abbreviations; ABCA1, ATP-binding cassette transporter A1; ABCG5/8, ATP-binding cassette transporters G5/G8; apoA-I, apolipoprotein A-I; CE, cholesteryl esters; CM, chylomicron; CMR: chylomicron remnants; ER, endoplasmic reticulum; FC, free cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very low-density lipoprotein; LDL-R, LDL-receptor; LRP: LDLR-related protein; MTTP: microsomal triglyceride transfer protein; NPC1L1, Niemann-Pick C1-Like 1; PS, plant sterols; SR-B1, scavenger receptor class B member 1; SOAT2, sterol O-acyltransferase 2.*

In HDL, PS are esterified by lecithin:cholesterol acyltransferase almost as efficiently as cholesterol and can also be taken up from HDL in a comparable manner to that of CE (28). Hepatocytes can produce cholesterol locally or take it up from circulating lipoproteins such as low-density lipoprotein (LDL) and chylomicron remnants (CMR) *via* LDL-R and LDLR-related protein (LRP), and *via* scavenger receptor class B type I (SR-BI) (35). In liver, FC can be esterified to CE by SOAT2 and repackaged into very low-density lipoprotein (VLDL) particles and delivered to the peripheral tissues; VLDL is partially cleared by the hepatic LDL-R. Majority of FC in the liver is converted into bile acids for biliary secretion. If it is not metabolized, FC is secreted to bile *via* ABCG5/8 transporter localized at the canalicular membrane of hepatocytes (32). Hepatic NPC1L1 can also facilitate reuptake of biliary cholesterol back to hepatocytes. The subcellular localization of the two opposing sterol transporters NPC1L1 and ABCG5/8 in liver and intestine indicates the importance of these gatekeepers in regulation of whole body sterol homeostasis.

3.4.1 Sterols Absorption in Sitosterolemia

Defective forms of the ABCG5/8 transporters lead to PS accumulation and development of xanthomas and premature atherosclerosis. Given that cholesterol is synthesized *in vivo*, no absolute need exists for dietary intake; however, cholesterol is tightly regulated in order to maintain a stable body cholesterol pool. When cholesterol biosynthesis is reduced, absorption is increased. Hypercholesterolemia has been noted early in the breastfed infant, whereas STSL was only evident after consuming plant containing foods (36). The absorption of sitosterol increases by 40-60% in STSL approaching the absorption of cholesterol (37). Some sitosterolemic patients also present with pseudo-

homozygous familial hypercholesterolemia (FH) (38-40), which is due to a complete failure of cholesterol efflux into bile (36). Despite normal LDL-R activities, some children were initially diagnosed with pseudo-homozygous FH based on elevated cholesterol levels, which seem to fall but remain higher than those of healthy individuals (41). Given that LDL-R activity is normally decreased in hypercholesterolemia, it is possible that hepatic retention of PS leads to potential inhibition of LDL-R activity mediated by the sterol regulatory element binding protein (SREBP) pathway (42). Increased LDL-R activity, coupled with reduced of 3-hydroxy-3-methyl-glutaryl-CoA (HMG CoA) reductase, the rate-controlling enzyme in cholesterol synthesis pathway, has been found in hepatic tissues in STSL (43), revealing dissociation between regulation of LDL-R expression and HMG CoA reductase. The increased LDL-R activity promotes accumulation of sitosterol in the tissues and therefore leads to greatly expanded body sitosterol pools due to the sluggish sterol turnover and elimination from the body (37, 44-47). In contrast, heterozygotes of STSL show moderately increased PS levels (48), but these values are still within the normal range for a general population (49, 50). This is due to the rapid clearance, resulting in less PS deposition in tissues and thus a small body pool (49).

3.4.2 Involvement of NPC1L1 in Sitosterolemia

NPC1L1 protein seems to be involved in both cholesterol and PS absorption. *In vitro* studies suggest that NPC1L1 may be able to discern PS from cholesterol since NPC1L1 can efficiently transport FC but not sitosterol in hepatoma cells (51). Overexpression of NPC1L1 facilitated sitosterol uptake; however, it was 60% lower than that of cholesterol in CaCo-2 cells (52). This implies that NPC1L1 has a lower affinity to transport PS, and

thus may represent the first line of defense against intestinal absorption of diet-derived PS. Structurally, NPC1L1 senses cholesterol through a direct binding by its N-terminal domain (NTD), probably owing to a closed cholesterol binding pocket that possesses high cholesterol binding affinity (53). In contrast, sitosterol cannot bind to NPC1L1-NTD, presumably due to the unfavorable steric clash caused by the ethyl group (54). Nguyen et al. (55) have suggested that excessive accumulation of PS over time, as seen in STSL, could be due to the assumption that NPC1L1 system might be potentially leaky. Thus, ABCG5/8 transporter fails to work as a secondary clean-up mechanism for eliminating PS molecules that bypass NPC1L1 (55). Inactivation of NPC1L1 has prevented STSL in ABCG5/8- KO mice, suggesting that NPC1L1 must be essential for the intestinal absorption of dietary PS (56). Inhibition of NPC1L1-dependent transport of dietary PS into the body, by the cholesterol-lowering drug EZE (**Figure 3.1**), represents the best available treatment currently for sitosterolemic patients (57).

3.5 CHOLESTEROL SYNTHESIS IN SITOSTEROLEMIA

Serum levels of the cholesterol precursor, lathosterol, is linked with overall cholesterol synthesis but negatively associated with dietary absorption of cholesterol (58). Circulating levels of sitosterol and campesterol are positively correlated with fractional and absolute absorption of dietary cholesterol, but are inversely related to overall rates of whole body cholesterol synthesis (58).

The *ABCG5* and *ABCG8* genes are found only in vertebrates that are capable of *de novo* synthesis of cholesterol, which may indicate that assiduous exclusion of non-cholesterol sterols, may be needed to maintain normal cholesterol homeostasis (59). Accumulation of

non-cholesterol sterols may account for the very low rates of cholesterol synthesis in STSL (60). Yu et al. (61) found that ABCG5/8-KO mice retained more PS in their plasma and liver, and had an approximately 50% decline in cholesterol levels due to low hepatic cholesterol synthesis relative to wild-type mice (61). Likewise, extreme accumulation of PS noticeably reduced adrenal cholesterol content (-91%), reflecting a profoundly perturbed cholesterol homeostasis (62) with an immense decline in the removal of non-cholesterol sterols into bile (63). Hepatic conversion of [^{14}C] mevalonic acid, a cholesterol precursor, to cholesterol was reduced by 50% in STSL (64). Several key enzymes involving cholesterol biosynthesis were suppressed by at least 40% in STSL compared with control subjects (16), representing the main abnormality that thought to be inherited (64, 65). Nevertheless, Patel et al. (43) found that the structural genes involving cholesterol biosynthesis, HMG-CoA reductase and synthase, regulation, (SREBP-1 and -2), esterification, ACAT, as well as gene mediated dietary lipid absorption, microsomal triglyceride transfer protein (MTTP) did not segregate with the disease and were not the sites of defect. Thus, identifying defective genes in STSL may lead to a better understanding of molecular mechanisms that regulate sterol influx and efflux in the body. The coordinated down-regulation of the entire pathway of cholesterol biosynthesis is likely not aligned with enhanced LDL-R activity in this disorder (64). Increased LDL-R function in STSL may be related to the assumption that LDL-R may supply cells with sterols that could not be synthesized *in situ* since cholesterol synthesis is suppressed.

Sitosterol was less effective than cholesterol in suppressing HMG-CoA reductase activity (16). In fact, sitosterol did not inhibit cholesterol biosynthesis in either *in vivo* (16) or *in*

vitro studies (62) (66). Sharpe et al. (67) showed that *de novo* cholesterol synthesis was not disrupted after exposing the gonads of goldfish to higher concentration of sitosterol. Conversely, as with cholesterol and other PS (campesterol, stigmasterol (62) and brassicasterol) (66), sitosterol inhibited cholesterol synthesis in intestinal (68), hepatic (69), and macrophage foam cells (70).

Increasing sitosterol levels to 7% of cellular cholesterol levels increased HMG-CoA reductase activity by 23%, whereas addition of the same amount of cholesterol reduced the activity by 46% in sitosterolemic skin fibroblasts (71). These cells may be capable of distinguishing between the effects of cholesterol and sitosterol on the activity of HMG-CoA reductase. Reduced HMG-CoA reductase activity in this disease may not be caused by the accrued sitosterol or feedback inhibition due to high cellular sitosterol levels. Whether PS or their metabolites, oxidized PS contribute to reduced cholesterol synthesis in STSL remains unknown.

Several attempts have been made to stimulate endogenous cholesterol synthesis with either a low sterol diet or bile acid malabsorption (cholestyramine or ileal bypass surgery), but both approaches have failed to stimulate cholesterol biosynthesis despite a marked decline in circulating sterol levels (16). Feeding mevalonic acid failed to modulate cholesterol synthesis in subjects with STSL (16). Blocking the absorption of dietary and biliary sterols with EZE has been shown to increase endogenous cholesterol synthesis as evidenced by a rise in plasma ratio of lathosterol to cholesterol in STSL patients (57) and ABCG5/8-KO mice (62). Tracer kinetic studies may help characterize

changes in cholesterol and PS metabolism under specific therapeutic strategies, providing further insight to understanding sterol metabolic mechanisms in STSL patients.

3.6 XANTHOMAS AND ATHEROSCLEROSIS IN SITOSTEROLEMIA

Xanthomas and atherosclerotic plaques are lesions of lipid storage that are filled with foamy macrophages. These lesions are most commonly found in soft tissues such as blood vessels, skin and tendon (2). Patients with STSL are more prone to xanthomatosis than others who have similar circulating levels of cholesterol. Indeed, PS appear to contribute to xanthomatosis even at low plasma levels (30-40 mg/dl), whereas concentrations greater than 400 mg/dl of cholesterol are required to induce tendon xanthomas in FH (10).

Both elevated plasma cholesterol and PS levels in STSL (36) may contribute to premature vascular disease in early childhood (72) or later in life (73). It is difficult to conceptualize why some sitosterolemic cases present with xanthomas or accelerated heart disease (74), while others do not show typical features of STSL (75). However, some sitosterolemics showed no evidence of xanthomas while they had elevated plasma sitosterol but lower cholesterol concentrations (15). Accumulation of PS in plasma lipoproteins has no impact on the particles' chemical structure or appearance (37) but it influences the stability of both cholesterol and PS in lipoproteins, favoring the accumulation of these sterols within tissues, initiating inflammatory reactions, and may cause premature coronary artery disease (76). Like cholesterol, free sterols such as PS may penetrate arterial walls, and stimulate recruitment of monocytes into the tissues. Once inside tissues, monocytes differentiate into macrophages and become engorged

with sterols, which give them a foamy appearance, referred to as foam cells, a hallmark feature of atherosclerotic lesion as well as xanthoma **(Figure 3.2).**

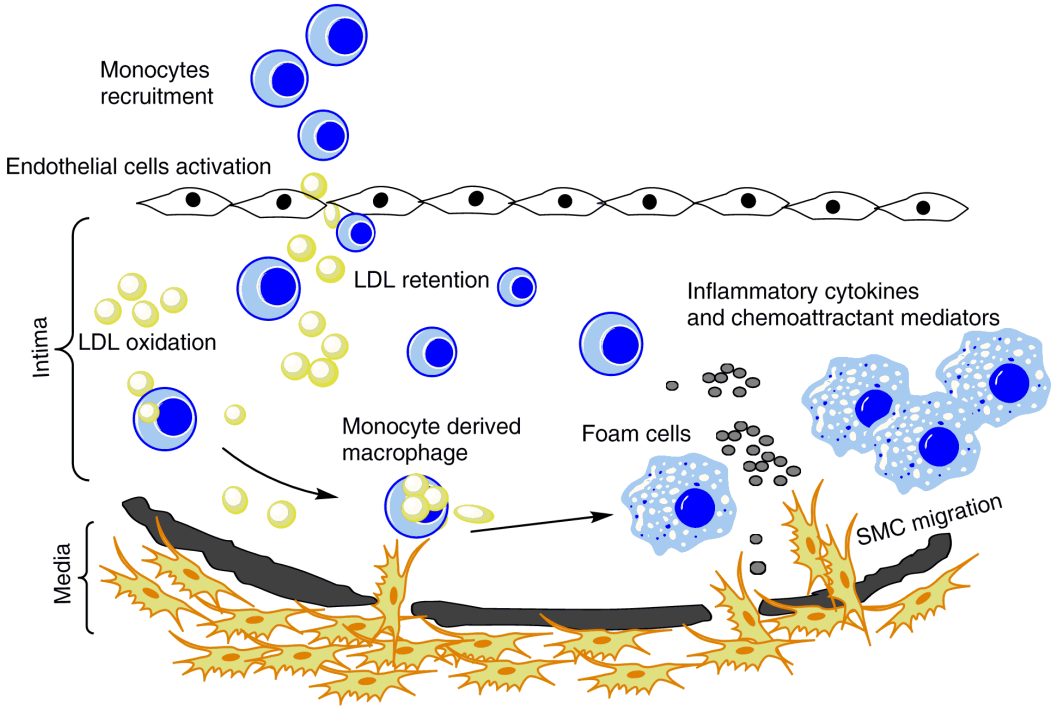

Figure 3. 2*: The initial steps of atherosclerosis.*
Atherogenesis steps include recruitment of blood monocytes to the activated endothelial monolayer of the normal artery wall by the action of chemoattractants and adhesion molecules. Subsequently, monocytes differentiate into macrophages, which engulf oxidized LDL and form foam cells. The macrophages secrete inflammatory cytokines that attract additional monocytes. Oxidized LDL stimulates SMC migration and proliferation, which also contribute to intimal thickening and plaque formation. SMC, smooth muscle cells; LDL, low-density lipoprotein.

Local accumulation of each individual sterol and heterogeneity in PS may influence a number of signaling and trafficking pathways, with implications for arteriosclerosis. For instance sterols containing a double bond at C-22 in the side chain (e.g., stigmasterol) reduced cytokine secretion and increased cholesterol efflux to HDL in macrophage foam cells while PS that have saturated side chain (e.g., sitosterol) exacerbated inflammatory response and tend to decrease cholesterol efflux (77). Furthermore, stigmasterol and C-22- and C-24-unsaturated cholesterol biosynthetic intermediates were shown to be liver X

receptor ligands that promote cholesterol efflux from macrophage foam cells *via* the ABCA1 and ABCG1 and thus prevent cholesterol accumulation in tissues (59, 62).

Plaque rupture and coronary thrombosis is the major cause of acute myocardial infarction and sudden cardiac death (78). Macrophage death plays a pivotal role in plaque disruption, leading to generation of the necrotic or lipid core (78, 79). Sitosterol exerts a robust cytotoxic tendency inducing apoptosis in human endothelial cells, revealing detrimental effects on vascular cells (80). Indeed, sitosterol-containing lipoproteins were strong inducers of macrophage death and plaque necrosis, a precursor to atherothrombosis, linking STSL with premature atherosclerosis (79). PS are susceptible to oxidative processes, and indeed may be more so than cholesterol (81) since they have side chain substitutions at carbon 24 with an extra double bond between C-22 and C-23. Plant stanols lack a double bond in the sterol ring, which makes their oxidation unlikely. In fact, PS esters have been shown to impair endothelial function (82) and produce severe atherosclerotic lesion whereas those of plant stanols showed less atherosclerotic lesion in apoE-KO mice (82). The apparent biological effects of PS or their oxides have been shown to be to be both cell-type and sterol specific (77, 83) that may be either beneficial (84) or deleterious (85) on atherosclerosis.

Absence of ABCG5/8 transporters leads to development of severe cardiac lesions (86) while their overexpression did not prevent or mitigate these lesions in LDL-R-KO mice (87). Whether accelerated atherosclerosis observed in STSL is a direct result of disturbed cholesterol trafficking or a secondary effect of PS accumulation in tissues is still

unknown. Another question that remains is whether PS can initiate inflammation and thus contribute to high cardiovascular risk in general population. However, the data would argue in favor of an amelioration effect of PS on the inflammatory process (88) with no evidence of an association between circulating levels of PS and risk of cardiovascular disease found in healthy people (89). Consumption of PS has shown to be safe and effective in reducing circulating LDL-cholesterol level in heterozygous carriers of STSL, and was not associated with major increases in serum PS that were different from the general population (50).

3.7 CURRENT THERAPEUTIC STRATEGIES FOR SITOSTEROLEMIA

The currently available treatment for STSL includes low sterol diets, bile acids binding resins, ileal bypass surgery or plasmapheresis, and sterol absorption inhibitors (90). Low sterol diets should include low cholesterol and PS contents and restrict intakes of nuts, seeds, olives, avocados, vegetable oils, shortening, margarine, shellfish, and chocolate. Low cholesterol diet normalizes plasma cholesterol and eliminates cutaneous xanthoma with no impact on plasma PS levels (38). Therefore, STSL should be suspected when plasma cholesterol levels are seen to be rapidly decreasing (91) and xanthoma regressing after a low sterol diet (92). Limiting PS intake from the diet for 6 months has been shown to reduce individual and total plasma PS levels by at least 30% (92). The adherence to a low sitosterol diet (26 mg sitosterol) for 3 weeks failed to reduce circulating levels or modulate cholesterol biosynthesis in STSL (93). Thus, low sterol diets alone do not seem to be effective in reducing or maintaining sustained declines in plasma PS levels. Most STSL patients are poor responders to HMG-CoA reductase inhibitors since *de novo* cholesterol synthesis is already suppressed (94).

Bile acid binding resins, e.g., cholestyramine, have been shown to reduce serum total sterols by about 50% in most STSL patients (92, 95, 96), despite a lack of effectiveness in some cases (48). Relative to diet alone, cholestyramine (8 g/d) for 10 days suppressed plasma total PS by 45% and cholesterol by 52%; these decreases remained evident for 8 weeks of cholestyramine therapy (24 g/d) but reached a long-term plateau (92). Addition of low sterol diet and cholestyramine rapidly attenuated individual and total PS levels, as well as LDL-cholesterol values and xanthoma size in STSL (15, 74, 96), suggesting an effective means of treating this disorder. However, compliance to cholestyramine remains a challenge because of the gastrointestinal side effects (97).

EZE blocks dietary and biliary cholesterol absorption by inhibiting cholesterol transporter NPC1L1 (57). This sterol absorption inhibitor has revolutionized STSL patient care since it directly hinders sterol absorption (90). Several studies with EZE conducted over 8 weeks show reductions in LDL-cholesterol and sitosterol levels by 13% and 30%, respectively, in STSL subjects (57, 98, 99). Introducing EZE treatment to a newly diagnosed 10-year-old Iranian girl with STSL caused marked diminutions in plasma sitosterol (-40%) and LDL-cholesterol (-50%) values (15). These findings were aligned with those of Niu et al. (97), who reported complete regression of xanthomas with a greater than 45% reduction in plasma cholesterol levels with EZE in children with STSL. Relative to bile acid resins or statins, the use of EZE has contributed to the greatest improvements in lipoprotein and sitosterol levels (100). Combining EZE with bile acid resins, statins and/or low sterol diets further attenuated plasma sitosterol and campesterol levels, but such levels remain significantly above the normal range (57, 101) even after

extending its use to 3 years (97). EZE has been shown to be ineffective in children less than 2 years of age (97). The lack of efficacy may be due to the immature hepatic glucuronidation system in infants (97). It should be mentioned that sterol reduction with EZE may not provide substantial reductions in hard clinical endpoints such as mortality, even after long-term use (102). Despite a non-functional intestinal ABCG5/8 system in STSL, restoring ABCG5/8 activity *via* liver transplant can normalize plasma PS levels (75). The biliary removal of PS appears to be sufficient to limit tissue deposition of PS, representing a significant role in regulating plasma PS than previously thought (75). **Table 3.2** shows the effects of therapeutic approaches on plasma lipids and PS in sitosterolemic patients.

Table 3. 2*: Impact of therapeutic means on plasma lipids and plant sterols in sitosterolemic patients.*

Patient/mutation/	Main symptoms	Baseline blood lipids (mg/dl)	Therapy	Major outcomes*	Reference
11-month-old Caucasian girl-Romanian parents; ABCG5	Severe hypercholesterolemia; Achilles tendon xanthomas	TC=1023 LDL=837 HDL=54 Sitosterol=2.4	Statins (40 mg) + EZE (10 mg/d); while breast-fed Statins (2.5 mg); (weaned off breast milk)	↓ 41% in TC ↓ 75% in TC; ↑72% in sitosterol	(36)
6-month-old; 11-month-old Japanese girl; ABCG5	Pseudo-homozygous FH; cutaneous and tendon xanthomas; premature atherosclerosis	TC= 704; 527 HDL=39; Sitosterol=15.6; 4.7	Low cholesterol diet (<250 mg/d)	↔ in serum sitosterol; levels remained high levels (after 3 years; >50↓ in TC	(38)
70-year-old Japanese–Canadian female; ABCG5; 14-year-old Hispanic girl; ABCG8 (n=2)	Xanthomas	TC=180,153 Total PS= 25,36.5	Sterol-free diet + Benecol margarine contained sitostanol; 2g/d for patient#1; 7 weeks; 1.7g/d for patient#2; 10 weeks	↓ 7% and 26% TC; ↑ 531% and ↑ 182% in plasma sitostanol; ↓ 28.6% and ↓36% in campesterol; ↓ 31.3% and ↓ 18.9% in plasma sitosterol	(45)
41-year-old Caucasian female; ABCG8	Tuberous xanthomas; accelerated atherosclerosis;	TC=118 Sitosterol=18.5 Campesterol=6.7	Low sterol diet [cholesterol (225), sitosterol (150), campesterol (20) sitostanol (20); all mg/d]	↑ 80% in absorption rate of campestanol vs. 14.3%)in heterozygote; ↑ 10 and 20 -fold in campestanol pool size in the homozygote vs. the heterozygote, and controls	(47)

7-year-old girl (no race or ethnicity reported)	Tuberous xanthomas	TC= 441 LDL=374 HDL=57 Sitosterol=16.5 Campesterol=9.8	Low fat diet (~ 30% of calories from fat) + 2g cholestyramine; 6 months	↓ 59.4% in TC; ↓ 24.6% in HDL;↓ 69% in LDL; xanthomas slightly reduced	(91)
48-year-old Caucasian man; ABCG8	Xanthomas; angina pectoris; claudication	TC=228.15	Low sterol diet (95-125 mg PS/d); cholesterol (160-200)/d); 6 months	↓ 37% in total PS, 59% in VLDL and 32% in LDL-PS; ↔ TC, LDL, VLDL-cholesterol; ↑ 31% in sito sterol; ↑42% in campesterol;↑ 64% in stigmasterol	(92)
48-year-old Caucasian man; ABCG8	Xanthomas; anginapectoris; claudication	TC=228.15	Low sterol diet+ cholestyramine (24g/d); 12 months	↓ 67% in TC and ↓ 76% in LDL;↓ 77% in LDL-PS; ↔ VLDL-PS; ↓ 89% in apoB;↑ 39% in apoA; ↓ xanthoma, ↓ angina pectoris; ↓ intermittent claudication	(92)
10-year-old Caucasian girl; ABCG8	Tuberous xanthomas	TC=303.2 LDL=264 Sitosterol=37	Low sterol diet; fat (30%); sit sterol (120 mg); cholesterol (<300 mg)/d	↔ TC ↔ sitosterol	(93)
11-year-old African American boy; ABCG8	Diffuse tendinous and tuberous xanthomas	TC= 14.35 LDL=12.34 Sitosterol=31.3	Low sterol diet; 1 month Low sterol diet + cholestyramine (8 g/d)	13 weeks; ↓ 60% in plasma total sterols; ↓ in xanthoma ↓ 69% in plasma total sterols; ↓ in xanthoma	(96)
4-year-old Chinese boy; ABCG5	Tuberous xanthomas;	Total sterols=707.7 LDL=634.6	Low cholesterol diet (low in shellfish) +	↓~73% in TC ↓ 75% in LDL	(74)

	thrombocytopenia	HDL=51.4 Sitosterol=49 Campesterol=24.4	cholestryamine (4g/d); 2 months		
10-year-old Iranian girl; ABCG5	Tuberous xanthomas; atherosclerosis	TC =380 LDL=270 HDL=87 Sitosterol=15.76 Campesterol=3.03	Low-fat diet + cholestyramine (4 g/d) EZE (10 mg/d); 12 weeks	↓ 61% in TC; ↓ 79.66% in LDL ↓ 50% in LDL; ↓ 40% in sitosterol	(15)
11-year-old girl; ABCG8 (no race or ethnicity reported)	Tuberous xanthomas; thrombocytopenia	TC=196 LDL=126 HDL=60 Sitosterol=33 Campesterol=14	Cholestyramine (2 g/d) + EZE (10 mg/d); 1 year	↓ 52% in sitosterol; ↓ 52.6% in campesterol; carotid bruit and thrombocytopenia resolved; complete regression of xanthomas	(101)
19-year-old Japanese man; ABCG5	Acute myocardial infarction with obstruction of one coronary artery	TC=310 LDL=243 HDL=50 Sitosterol=9.9	Angioplasty and coronary atherectomy; after discharge; low PS diet + cholestyramine	Amelioration all of the stenosis	(105)

***** *indicates significant difference compared to control unless otherwise stated; ↑, increase; ↓, decrease; ↔, no significant change.*

3.8 CONCLUDING REMARKS

The ABCG5/8 transporter system regulates sterol homeostasis and mediates the excretion of sterols in both liver and intestine. Sterol transporters have complex cellular and regulatory pathways that remain to be completely understood. Disruptions in these transporters lead to accrual of neutral sterols in plasma and tissues. Compared with low sterol diet and bile acid binding resins, EZE has been shown as efficacious in lowering plasma PS and has the potential to up-regulate low cholesterol biosynthesis in STSL; however, plasma PS concentrations remain above normal levels. This sterol storage disease underpins the necessity for future kinetic sterol trafficking studies to explore where non-cholesterol sterols accumulate and to what extent tissue sterols levels can be mobilized through limitation of sterol intake and /or inhibition of sterol absorption. Factors that modulate circulating and tissue cholesterol and non-cholesterol sterols levels may have major impacts on initiation, progression, and regression of xanthoma and accelerated heart disease found in this disease.

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CHAPTER IV

MANUSCRIPT 3

MODULATION OF WHOLE BODY SITOSTEROL AND CHOLESTEROL POOL SIZES AND CHOLESTEROL ABSORPTION AND SYNTHESIS BY EZETIMIBE IN PATIENTS WITH HOMOZYGOUS SITOSTEROLEMIA

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4.1 ABSTRACT

Background and aims: Sitosterolemia (STLS) is a rare autosomal recessive disease caused by mutations in one of the adenosine triphosphate-binding cassette (ABC) transporter genes *ABCG5* or *ABCG8* gene, which encodes transporter proteins for excretion of plant sterols (PS) from enterocytes. Increased retention and delayed excretion of PS lead to their deposits in tissues and tendon xanthomas and premature atherosclerosis. Ezetimibe (EZE), a sterol absorption inhibitor, has been shown to reduce plasma PS levels in STSL but the levels of PS remain above normal levels despite long-term use. Studies on the effect of EZE on whole body sitosterol and cholesterol pool sizes, and cholesterol synthesis and absorption, have not been performed so far in STSL.

Methods and results: Effects of EZE (10 mg/d) on whole body sitosterol and cholesterol pool sizes as well as cholesterol absorption and synthesis, were investigated in a crossover study with 8 Caucasian STSL patients homozygous for the ABCG8 S107X mutation. Patients were first taken off EZE for 14 wks. After 4 wks without EZE, they received 1) an intravenous infusion of D_7-sitosterol (1 mg/kg body weight, BW) and ^{18}O-cholesterol (1.4 mg/kg BW) to measure sitosterol and cholesterol turnover, respectively, 2) an oral dose of deuterium oxide and ^{13}C-cholesterol to measure fractional cholesterol synthesis and cholesterol absorption, respectively. Blood samples were serially collected over 10 wks. Afterward, patients resumed EZE therapy and the experiment repeated. EZE decreased ($p<0.0001$) plasma sitosterol (-35±4%) and campesterol (-47±4%) compared with off EZE. EZE reduced (-28±11%, $p=0.02$) whole body sitosterol pool size (151±13 vs 210±25 mg/kg BW).

Likewise, EZE decreased (-9±3%, p=0.02) whole body cholesterol pool size (572±38 vs 632±49 mg/kg BW). EZE decreased serum TC (-13±4%) and LDL-cholesterol (-23±6%) and increased HDL-cholesterol (+26±8%). In patients on EZE, plasma sitosterol and TC positively correlated with whole body sitosterol (r=0.84, p=0.02) and cholesterol pool sizes (r=0.70, p=0.06). EZE inhibited (p<0.01) cholesterol absorption as measured by single and dual-isotope tracer methods (-62±13 and -57±11%, respectively) and increased (p<0.01) cholesterol fractional and absolute synthesis rates (3±0 fold). The metabolic clearance of cholesterol from plasma, expressed as volume or fraction, was increased (p=0.02) (2±0-fold) from 331±59 to 593±156 ml/d, and from 11±2 to 19±3%/d, respectively, after EZE, reflecting an increase in cholesterol efflux.

Conclusions: The results suggest that EZE progressively reduces plasma PS levels and whole body PS pool size. EZE slightly reduces plasma TC and whole body cholesterol pool size. EZE inhibits cholesterol absorption, increases metabolic clearance of cholesterol from plasma and increased whole body cholesterol synthesis rates.

EZE limits PS deposition in tissues and thus may reduce risk of developing premature atherosclerosis and early mortality in STSL. The current study strengthens the rationale for the use of EZE in treatment of STSL.

Keywords: whole body sitosterol pool size; whole body cholesterol pool size; sitosterol; cholesterol; cholesterol absorption; cholesterol synthesis; plant sterols

4.2 INTRODUCTION

Sitosterolemia (STSL) is a rare autosomal recessive disease caused by homozygous or compound heterozygous mutations in either one of the two adenosine triphosphate binding cassette transporters genes, *ABCG5* and *ABCG8*, located in a head-to-head organization on human chromosome 2p21 (1-3). They are expressed in both the intestine and liver, and form heterodimers that normally function to rapidly remove absorbed plant sterols (PS) and cholesterol. STSL is characterized by increased plasma PS and normal to moderately elevated plasma total cholesterol (TC) levels (4). The clinical features of STSL include xanthomas, accelerated atherosclerosis, and occasionally arthritis, arthralgias, hemolysis, and thrombocytopenia (5-10). STSL patients have complete failure of sterol efflux, and down-regulated cholesterol biosynthesis, along with increased expression of low-density lipoprotein receptor (LDL-R) (11).

Several studies, using techniques such as sterol balance, isotope kinetic and mevalonic acid excretion, show that whole body cholesterol synthesis is reduced in STSL patients while PS accumulate (12-15). Many attempts have been made to increase endogenous cholesterol synthesis with either a low sterol diet or bile acid malabsorption (cholestyramine or ileal bypass surgery), but both approaches have failed to increase cholesterol biosynthesis despite decline in circulating sterol levels (11). The down regulation of cholesterol synthesis may in turn increase cholesterol absorption, thus contributing further to whole body sterol accumulation.

Ezetimibe (EZE) is a sterol absorption inhibitor that lowers plasma TC and LDL-cholesterol levels in hypercholesterolemic patients (16). EZE localizes to the brush border of the small intestine (17) and blocks the transport of sterols from diet and biliary sources by inhibiting intestinal Niemann-Pick C1 Like 1 (NPC1L1) protein (16, 18, 19). EZE has been shown to reduce plasma PS levels in STSL (20), and has become the primary treatment choice for STSL patients. However, the levels of PS with EZE therapy remained above the normal range and plateaued (21) with higher doses of EZE (40 mg/d) (22). Despite the wide use of EZE in clinical practice, its effects on whole body PS and cholesterol homeostasis have not yet been characterized in STSL. The objective of the study was therefore to determine if treatment of EZE reduces whole body sitosterol pool size in STSL patients. Secondary study objectives were to determine if treatment with EZE 1) decreases whole body cholesterol pool size, 2) decreases cholesterol absorption, and 3) increases cholesterol fractional synthesis in STSL patients.

4.3 EXPERIMENTAL METHODS

4.3.1 Patients

Eight patients, between 16 and 56 years of age, were recruited from Hutterite colonies in Manitoba, Canada (n=4) and South Dakota, United States (n=4) (5 males and 3 females). All patients were identified as having homozygous ABCG8 S107X mutation and related to a proband previously reported by Mymin et al. (23, 24) and Chong et al. (25). All procedures involving human patients were approved by the Data Management and Coordinating Center, the National Institute of Child Health and Human, the Development Data and Safety Monitoring Board, and the University of Manitoba Biomedical ethics

board. A written informed consent was obtained from all the patients. The trial was registered with www.clintrials.gov under number NCT01584206.

4.3.2 Study Design

The study was a one-site pilot interventional study of 8 patients with STSL. The study was conducted at the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba and was designed as two 14-wk phases, off and on EZE. The consented patients (n=8) were taken off EZE for 14 wks and baseline blood samples were collected for plasma sterols and lipid profile. After 4 wks, patients received stable isotope testing and blood specimens were collected at weekly interval for 10 wks. Whole body sitosterol and cholesterol pool sizes were calculated from the isotopic enrichment curves of plasma sitosterol and cholesterol following an intravenous injection of labeled sitosterol and cholesterol, and cholesterol synthesis and absorption were determined after oral doses of 3, 4-^{13}C-cholesterol and deuterium oxide (D_2O), respectively. These procedures were performed before EZE therapy, and repeated after 10 wks of EZE therapy (10 mg/d) while continuing on therapy.

4.3.3 Isotope Protocol and Blood Sampling

After 4 wks off EZE, study participants were admitted to the Lipid Clinic in the Health Science Centre, University of Manitoba. At 0700 h, after a 12-h overnight fast blood samples were collected directly before the start of infusion. Thereafter, an intravenous catheter was placed for the infusion of sitosterol-25,26,26,26,27,27,27-D_7-sitosterol (1.0 mg/kg BW) and ^{18}O-cholesterol (1.4 mg/kg BW) to measure whole body sitosterol and cholesterol pool sizes, respectively. Infusates were prepared by pharmacist under sterile conditions by dissolving D_7-sitosterol and ^{18}O-cholesterol (C/D/N Isotopes Inc., Pointe-

Claire, QC, Canada) into ethanol USP, 100% (0.285 ml/kg BW), which was mixed slowly into 20% Intralipid® (0.7 ml/kg BW) (Baxter Healthcare Corporation, Mississauga, ON, Canada). Infusates were adminstered using Medfusion® 3500 syringe pump at rate of (1 ml/min) and given piggy-backed into normal saline over 1 hour. After infusion, blood samples were serially drawn at 12 h, 24 h, 48 h, 72 h, 96 h, 1 wk, 3 wks, 5 wks, 8 wks and 10 wks, while still off EZE. Serum, plasma and RBC fractions were separated by centrifugation at 3000 rpm for 20 min at 4ºC, and immediately stored at -80ºC until further analysis. To measure cholesterol synthesis and absorption, oral doses of D_2O (0.5 g/kg BW) and ^{13}C-cholesterol (1.0 mg/kg BW) were given to all participants immediately after intravenous infusions of D_7-sitosterol and ^{18}O-cholesterol. 3, 4-^{13}C-cholesterol (Cambridge Isotope Laboratories Inc., Tewksbury, MA, USA) was dissolved in butter (75 mg in 5g) and spread on a small bun. The D_2O was provided in 1⁄2 cup of apple juice (Cambridge Isotope Laboratories Inc., Tewksbury, MA, USA). On the last blood draw (10 wks), subjects resumed their EZE treatment. After 4 wks on EZE they received another infusion of D_7-sitosterol and ^{18}O-cholesterol and oral doses of D_2O and ^{13}C-cholesterol. Blood draws were performed for 10 wks following the same schedule as when they were off EZE. For the duration of the study, subjects were instructed to follow their usual diet.

4.3.4 Plasma Plant Sterols and Cholesterol Levels Analysis

Plasma sitosterol, campesterol, stigmasterol and cholesterol concentrations were measured by using gas-liquid chromatography equipped with a flame ionization detector and an auto sampler system using a Varian gas chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA). For sterol quantification, an internal standard, 5α-

cholestane, was used in combination with sterol standard curve for each individual sterol, using authentic standards (Sigma-Aldrich Ltd., Oakville, ON, Canada) and (MJS BioLynx Inc., Brockville, ON, Canada). Briefly, 5α-cholestane (50 ug) was added to each 0.5 ml plasma or 0.5 g red blood cells (RBC) samples, and saponified with 4 ml methanolic potassium hydroxide for 2 h at 100°C. The unsaponifiable portion (sterols) was extracted twice from the mixture with 4 ml petroleum ether, derivatized with 0.1 ml TMS reagent (pyridine–hexamethyldisilazane–trimethylchlorosilane; 9:3:1 v/v), resuspended in 0.4 ml hexane and injected (1 μl) onto a 30-m SAC-5 column (Sigma-Aldrich Ltd., Oakville, ON, Canada). The column temperature was 280°C and isothermal running conditions were maintained for 30 min. The injector and detector were set at 295°C and 300°C, respectively. The carrier gas (helium) flow rate was 1 ml/min, with the inlet split set at 40:1.

4.3.5 Serum Lipids Analysis

Serum TC, and high-density lipoprotein (HDL)-cholesterol and triacylglyceride (TAG) levels were determined by automated enzymatic methods on a Vitros-350 chemistry analyzer (Ortho-Clinical Diagnostics, Markham, ON, Canada). Serum LDL-cholesterol levels were calculated by the Friedewald equation (26). Non-HDL-cholesterol was calculated by subtracting the HDL value from the total cholesterol value.

4.3.6 Stable Isotope Sitosterol and Cholesterol Analysis

The stable isotope sterols were measured by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) adapted from Honda et al. (27) with minor modification. Briefly, total sterols were extracted from RBC using Folch's method (28) and derivatized into their picolinyl ester. The derivatizing reagent mixture consisted of 2-

methyl-6-nitrobenzoic anhydride (100 mg), 4-dimethylaminopyridine (30 mg), picolinic acid (80 mg), pyridine (1.5 ml) and triethylamine (0.2 ml). The freshly prepared reagent mixture (0.17 ml) was added to the dried sterol extract, and incubated for 2 h at 25°C. Thereafter, 0.5 ml of n-hexane was added to the mixture and mixed by vortexing for 15 s and centrifuged at 12000 g for 3 min. The clear supernatant was collected and evaporated under nitrogen. The residue was dissolved in 0.15 ml of acetonitrile containing 0.1% formic acid and then passed through a 4 mm syringe filter (Phenex 0.2 µm PTFE membrane; Phenomenex Inc., CA, USA) before 1 µl was injected into the Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA). The column used for the separation was a Kinetix XB-C18 column (2.1 x 100mm, particle size 1.7 µm; Phenomenex Inc., CA, USA) with an in line filter (KrudKatcher ULTRA HPLC In-Line Filter, 0.5 µm Depth Filter x 0.004in; Phenomenex Inc., CA, USA), and isocratically eluted with acetonitrile (Optima grade) containing 0.1% formic acid at a flow rate of 0.30 mL/min. The column temperature was maintained at 35°C. The total run time for each sample analysis was 10 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 Corporation, Milford, MA, USA) equipped with an electrospray ionization interface. Measurement of sitosterol and cholesterol stable isotopes was performed using multiple reaction-monitoring (MRM) mode using peak areas. The MS parameters were as follows: capillary voltage 3.50 kV; source temperature 120°C; desolvation temperature 400°C; nitrogen gas with flow rates of desolvation and cone gas of 600 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 ^{18}O-cholesterol

were 494.20>369.30 m/z, ^{13}C-cholesterol were 494.20>371.30 m/z, cholesterol were 492.00>369.10 m/z, sitosterol were 520.30>397.30 m/z and D_7-sitosterol were 527.20>404.30 m/z.

4.3.7 Whole Body Sitosterol and Cholesterol Pool Sizes

Estimates of whole body sitosterol and cholesterol pool sizes for each subject were obtained from analysis of the stable isotope enrichment curves following intravenous injections of D_7-sitosterol and ^{18}O-cholesterol, respectively. Isotopic enrichments expressed as tracer to tracee ratios were obtained and corrected for naturally occurring stable isotopes within the body prior to tracer infusion, and fitted to a three-compartment model by Goodman et al. (29-31). The three-pool model provided the best description of turnover curves for sitosterol and cholesterol. The time dependent disappearance of the stable isotope D_7-sitosterol and ^{18}O-cholesterol enrichment in RBC in pool A, following its initial introduction to pool A, was expressed by the sum of two exponential curves **(Figure 4.1)** using the equation: $C= C_1e^{k1t} + C_2e^{k2t} + B$, where C is the enrichment of the stable isotope in pool A; C_1, C_2, alpha (K_1), beta (K_2) and B are constants; e is the base of the natural logarithm; and t= time. Pool size was calculated as shown in the following equation = (infusate enrichment x dose)/C_0 (29-31).

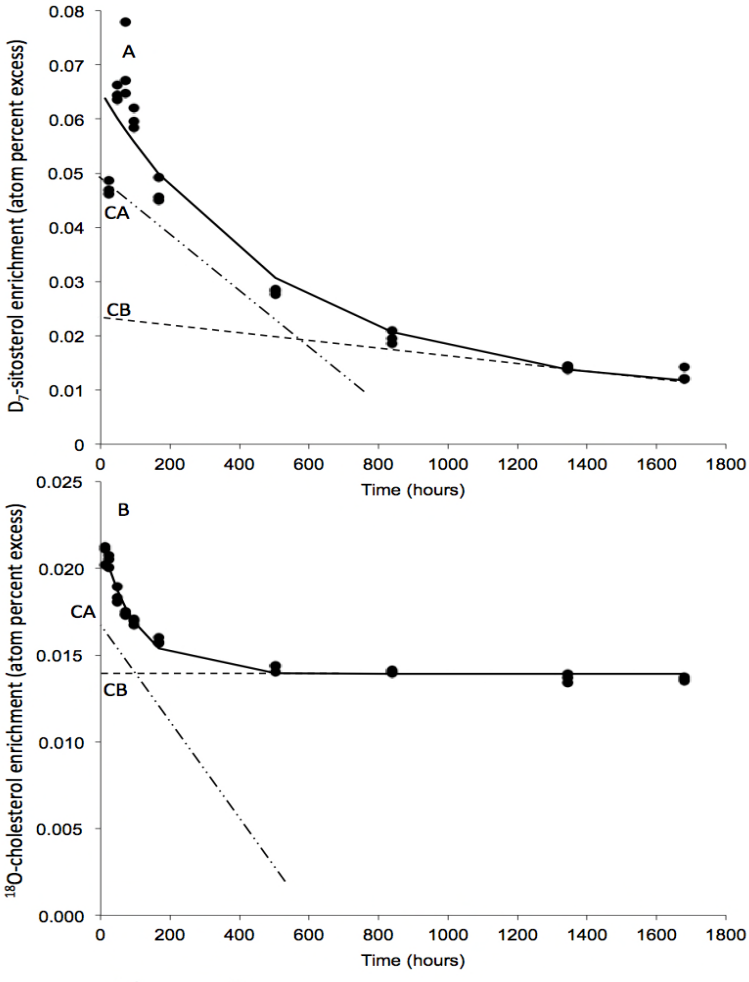

Figure 4. 1*: Schematic stable isotope enrichment curves for red blood cell sitosterol (A) and cholesterol (B) for red blood cell following intravenous infusions of D_7-sitosterol and ^{18}O-cholesterol in one sitosterolemic patient over 10 wks (1,680 hours).*
The dark circles, connected by a solid line, represent the experimental values observed during the 10 wks study. Extrapolation of the linear portion of the curve to zero time (dashed line) provides the intercept CB. Subtraction of this extrapolated line from the experimental points provides intercept CA at zero time, which is the difference values shown by an interrupted (dash-dot-dot) line.

4.3.8 Cholesterol Absorption

The single and dual-stable isotopes labeled cholesterol tracer methods were used to determine cholesterol intestinal absorption (32). The single isotope-labeled cholesterol tracer method is based on measuring isotope enrichment in the cholesterol pool following an oral dose of labeled cholesterol. The plasma dual-isotope ratio method involves an

intravenous injection of labeled cholesterol along with oral administration of differently labeled cholesterol. Measurement of plasma cholesterol isotope ratios at a set point in time: by definition, it assumes "100% absorption" of the intravenous dose. Briefly, lipid was extracted from RBC using Folch's method (28) and free cholesterol derivatized into picolinyl ester. Measurements of ^{13}C-cholesterol and ^{18}O-cholesterol enrichments were simultaneously carried out using UPLC-MS/MS as described above (27). Isotopic enrichments were measured for 0h, 24h, 48h and 72h, and the area under the curve (AUC) was used to compare cholesterol absorption before and after EZE. Fractional cholesterol absorption rates were also calculated by multiplying the ratio of the RBC enrichment of cholesterol administered orally and enrichment of cholesterol injected intravenously by the ratio of the intravenous dose of cholesterol and the oral dose administered to the subject, and this total was then multiplied by 100. Mass absorption of dietary cholesterol was calculated by multiplying the daily cholesterol intake by the percent cholesterol absorption and expressed as mg cholesterol/kg BW/d. A 3-d food record was collected weekly following each phase for quantifying cholesterol intake (mg/d) (Food Processor SQL software, version 10.9, 2011, ESHA Research).

4.3.9 Cholesterol Synthesis

Cholesterol synthesis rate was determined based on the rate of deuterium incorporation from body water into newly synthesized RBC membrane free cholesterol over 0 h to 24 h each visit (33). Deuterium enrichments were measured in both RBC and plasma water. RBC cholesterol deuterium enrichment was determined after extraction of total RBC lipids (28) and analyzed for deuterium content using online gas chromatography–pyrolysis-isotope ratio mass spectrometry (GC/P/IRMS). Isotope abundances, expressed

in δ ‰, were calculated by using H2 as a reference gas. Deuterium enrichment was measured in both RBC free cholesterol and plasma water. Lipids were extracted from RBCs. Enrichments were expressed relative to standard mean ocean water and a series of standards of known enrichment. Aliquots of plasma water sample were analyzed by a temperature conversion elemental analyzer (TC/EA)-IRMS. Cholesterol fractional synthesis rate (FSR) over 24 h represents RBC free cholesterol deuterium enrichment values relative to the corresponding mean plasma water sample enrichment after correction for the free cholesterol pool (33, 34), and was then calculated using the following equation: FSR = (deuterium enrichment of free cholesterol above baseline/deuterium enrichment of plasma water above baseline) x 0.478 x 24 h x 100% x t, where t represents the actual measurement period. The factor 0.478 represents the fraction of hydrogen atoms per cholesterol molecule that may become enriched by deuterium, that is, 22 of the 46 hydrogen atoms derived from H_2O or NADPH (33). Absolute synthesis rate (ASR) of the rapidly exchanging free cholesterol was also calculated using TC pool size M1 and FSR (33, 34). That is, ASR (g/d) =FSR (%/d)/100%x M1x0.33, where M1 is TC pool, derived from ^{18}O-cholesterol.

4.3.10 Cholesterol Production and Clearance Rates

Cholesterol production rate (PR) was also calculated by adding absorbed and synthesized cholesterol. The metabolic clearance rate (MCR) of cholesterol from the rapidly exchangeable pool was calculated by dividing the PR by the plasma TC concentrations and expressed as a volume of plasma (MCR) or the fraction (MCF) of plasma cleared daily (35).

4.4 STATISTICAL ANALYSES

Effects of EZE treatment were examined using a mixed model ANOVA procedure with treatment, phase, and genders were included as fixed factors and subject, site, age and BW as a random factor in the model. For plasma sterols, repeated measures were used to examine the existence of effects of treatment, time and interaction of treatment and time. Significant treatment effects were examined by using Bonferroni post hoc tests for multiple comparisons. Baseline values were inserted into the model as covariates for plasma PS and serum lipids. Pearson correlation analyses were conducted to examine the associations between sterol levels and whole body sterol pool sizes. Statistical significance was set at $p<0.05$ for all the analyses. Statistical analyses were performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, US). Results were expressed as means with their standard errors unless otherwise noted. Data not normally distributed were transformed to fit the model assumptions before statistical analysis as determined by a Shapiro–Wilk test.

4.5 RESULTS

4.5.1 Patients' Characteristics

Baseline characteristics of the patients are shown in **Table 4.1**. Eight patients were initially recruited, and all completed the trial. There were 5 males and 3 females, with a mean age of 28±6 years (range 16 to 56 years), BW of 72±6 kg (range 43 to 101 kg), and body mass index of 26±2 kg/m^2 (range 18 to 34). All patients were Caucasians with homozygous ABCG8 S107X mutation (NM_022437.2:c.320C>G) and had confirmed diagnosis of STSL at the time of enrollment in the study.

Table 4. 1*: Baseline characteristic of subjects.*

Variable	Mean	SEM
Age (year)	28	5.5
Gender (male/female)	5/3	
Body weight (kg)	72.1	6.4
Height (cm)	166.1	2.4
Body mass index (kg/m²)	25.9	1.9
Plasma lipids and sterols		
Total cholesterol (mg/dl)	210.5	17.3
LDL-cholesterol (mg/dl)	131.4	13.9
HDL-cholesterol (mg/dl)	54.7	2.6
TAG (mg/dl)	121.5	27.2
VLDL (mg/dl)	24.3	5.4
Non-HDL (mg/dl)	155.8	16.4
Non-HDL/HDL	2.9	0.3
TC/HDL	3.9	0.3
LDL/HDL	2.4	0.2
Sitosterol (mg/dl)	6.2	0.7
Campesterol (mg/dl)	1.9	0.3
Stigmasterol (mg/dl)	0.8	0.1
Total plant sterols (mg/dl)	9.1	1.1
Total plant sterols/cholesterol ratio	0.06	0.01
Cholesterol (GC, mg/dl)	155.8	12.7
Prior treatment of sitosterolemia, n		
EZE	3	

*Note: Data are mean±SEM; *n=8 for demographic data. EZE, ezetimibe; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TAG, triacylglyceride; TC, total cholesterol; VLDL, very low-density lipoprotein.*

Mean values of serum TC and LDL-cholesterol indicated mild hypercholesterolemia. Serum TC levels were normal (<200 mg/dl) in three, border high (200-239 mg/dl) in two, and high (>240 mg/dl) in three patients. Serum LDL-concentrations were optimal (<100 mg/dl) in two, above optimal (100-129 mg/dl) in one, border high (160-189 mg/dl) in three and very high (>190 mg/dl) in two patients. All patients had elevated levels of sitosterol (>1 mg/dl) **(Table 4.1)**. Three patients were on EZE (10 mg/d) for treatment of STSL at the time of recruitment; one of them was taking fiber laxative therapy 500 mg, cod liver oil, lecithin 400 mg and calcium 1200 mg besides his EZE regimen.

4.5.2 Plasma Plant Sterols and Total Cholesterol Response to Treatment

Plasma PS concentrations significantly changed over 14 wks of EZE treatment. EZE progressively reduced plasma sitosterol (-35±4%), campesterol (-47±4%), and stigmasterol (-20±5%, all $p<0.0001$) after14 wks on EZE compared to off EZE. The reductions in plasma levels of sitosterol and stigmasterol after EZE treatment tended to increase with time ($p=0.06$ and $p=0.08$, respectively), whereas those of campesterol were affected by treatment-time interaction ($p=0.02$). After adjusting for plasma TC, reductions in plasma sitosterol levels (-26±5) were affected by treatment-time interaction ($p=0.04$) and comparable to those of campesterol levels (-39±4%, $p=0.01$) **(Figure 4.2A, B, C, E** and **F)**. The decreases in plasma TC measured by GC-FID were observed as early as 2 wks ($p=0.001$) after EZE, with greatest reductions noted at 6 wks (-37±3%) but were not sustained to 14 wks. In fact, cholesterol levels rose on the last 4 wks of 14 wks, and only 12±6% decrease was observed at the end of EZE phase compared with off EZE ($p=0.01$) **(Figure 4.2D)**.

4.5.3 Serum Lipids Response to Treatment

Percentage changes in serum lipids after 14 wks on EZE compared with off EZE and baseline are presented **(Figure 4.3A** and **B).** EZE reduced ($p<0.05$) serum TC measured enzymatically, and LDL-cholesterol levels (-13±4 and -23±6%) at 14 wks compared with off EZE. Effect of EZE on TC measured enzymatically was in a good agreement with that of GC-FID (-13±4% vs -12±6%). Serum HDL-cholesterol levels gradually increased after EZE starting at 2 wks (+8±8%, $p=0.28$), 8 wks (+17±6%, $p=0.01$), and 14 wks (+26±8, $p=0.01$) compared with off EZE. The endpoint increases in HDL were also higher (+11.6±4.7%, $p=0.04$) than those of baseline **(Figure 4.3B)**.

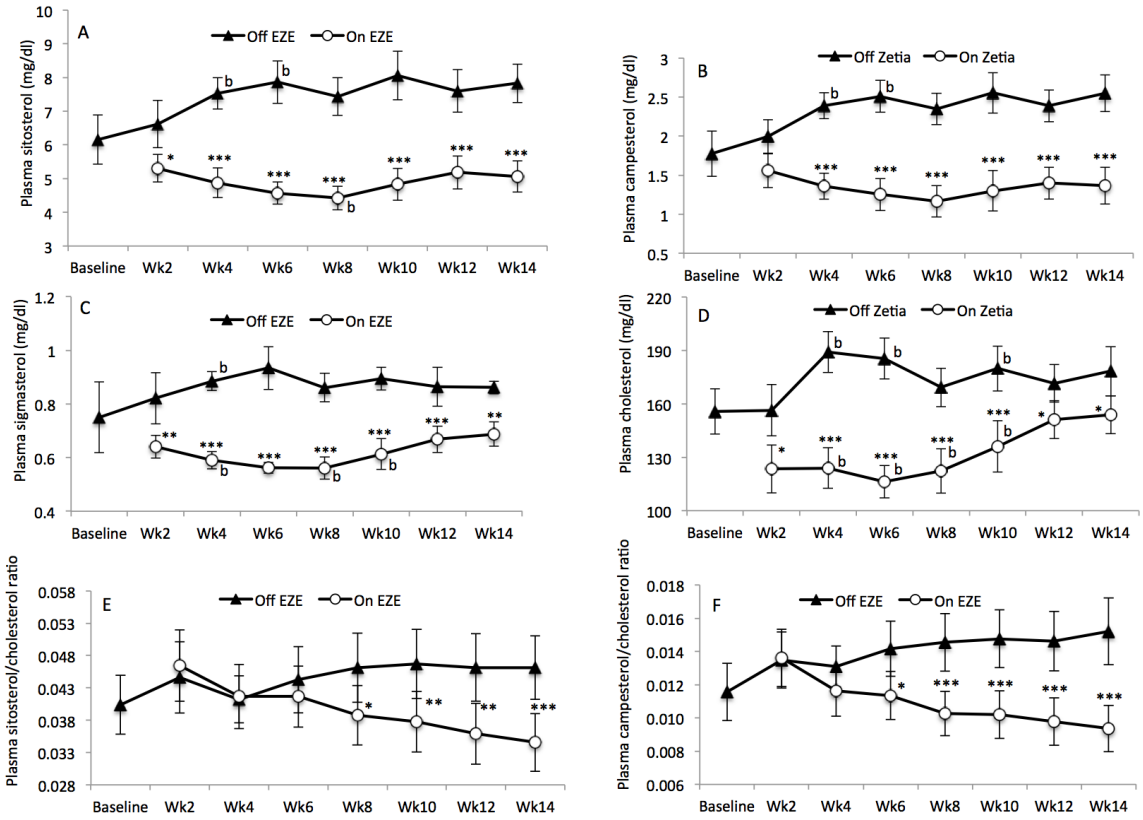

Figure 4. 2*: Plasma concentrations of the major plant sterols and their ratio to cholesterol and cholesterol over time off and on ezetimibe.*
*Plasma concentrations of the major plant sterols and their ratio to cholesterol and cholesterol over time before and after ezetimibe (A, B, C, D, E and F). P values for the treatment, time or interaction were analyzed using a linear-mixed-model ANOVA (Bonferroni post hoc test for multiple comparisons) by including treatment and time as main effects and the interaction term into the linear mixed-model ANOVA. All values are means±SEM; n=8. *p<0.05, **p<0.01, ***p<0.0001, asterisk signifies significant differences compared to off EZE.*

While serum TAG remained unchanged (-11±8, p=0.54), other lipid measures including non-HDL-cholesterol (-22±5%), and the ratios of TC/HDL (-28±6%), non-HDL/HDL (-35±6%) and LDL/HDL (-36±7 %) were decreased (p<0.05) after 14 wks of EZE compared with off EZE **(Figure 4.3A)**, but were not statistically different relative to baseline **(Figure 4.3B).**

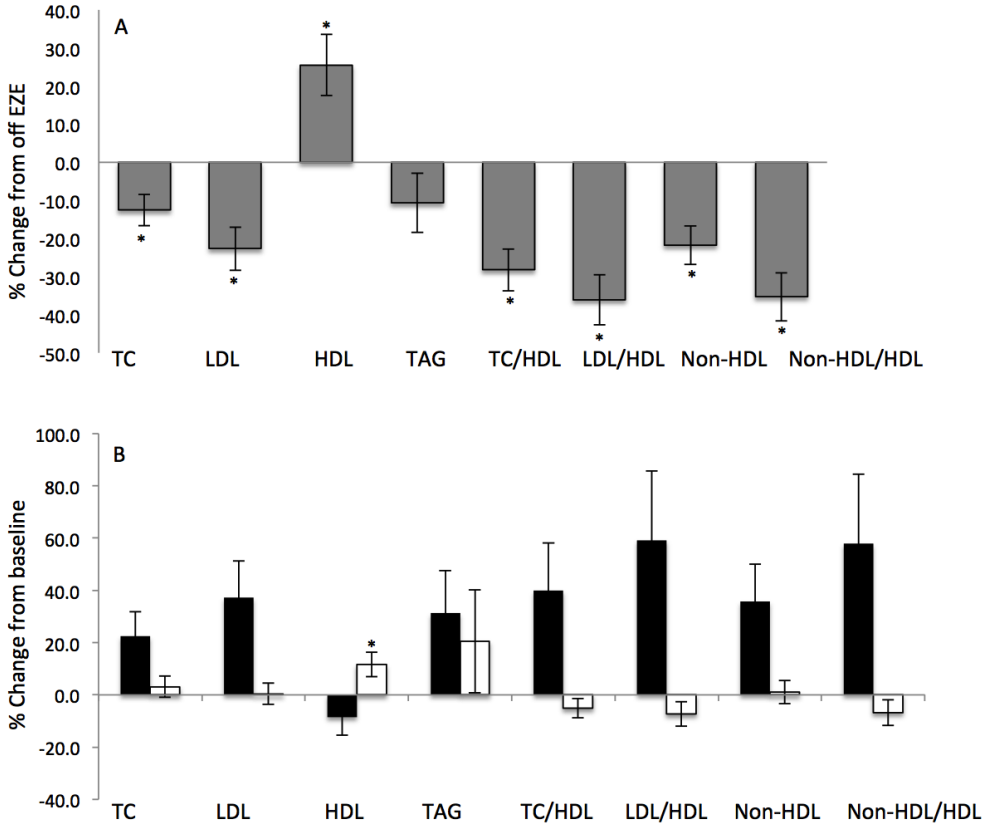

Figure 4. 3*: Percentage changes in serum lipids relative to off ezetimibe (A) and baseline (B). All values are means±SEM (n=8). *p<0.05 (two-tailed paired-Student's t test), mean values significantly different when compared with baseline. Differences when off (black bars) and on (white bars) EZE period.*

4.5.4 Analysis of Red Blood Cell Sitosterol and Cholesterol Turnover Curves

Kinetic studies showed that turnover of cholesterol are determined after an intravenous injection of labeled cholesterol. Semilogarithmic plotting of sterol enrichment against the time is a curvilinear during the first 4-6 wks. After this time the plot is linear, which may reflect the turnover of the total body pool exchangeable cholesterol. The equilibration of cholesterol across plasma and tissue pools is slow and once attained, it would be maintained indefinitely (30). In the current study, the turnover of D_7-sitosterol and ^{18}O-cholesterol in RBC following intravenous infusion of D_7-sitosterol and ^{18}O-cholesterol

were obtained in all patients and followed for up to 10 wks. Representative results from one of the 8-sitosterolemic patients are shown in **(Figure 4.1A** and **B)**. The solid curve drawn in each figure represents the isotopes enrichment in RBC for both sterols and resolved into two exponentials and fitted into a three-pool model (29). The equilibration of sitosterol between the two pools, in the patient, occurred between 8 and 10 wks as compared with 5 to 8 wks for that of cholesterol. This may indicate even a slower turnover for sitosterol than that of cholesterol. Furthermore, the enrichment curve of sitosterol remained constant, which is expected as sitosterol is not synthesized in the body.

4.5.5 Whole Body Sitosterol and Cholesterol Pool Sizes Responses to Treatment

Whole body sitosterol and cholesterol pool sizes before and after EZE are shown in (**Figure 4.4A** and **B)**. EZE decreased (-28±11%, p=0.02) the whole body sitosterol pool size after 10 wks from 210±25 to 151±13 mg/kg BW. The mean percentage change in sitosterol pool size after EZE varied widely from +7 to -60%. It is worth noting that two patients, who had sitosterol pool size lower than 133 mg/kg/BW, had a slight increase in the pool size after EZE, whereas those with pool size greater than 233 showed at least 50% reduction. After EZE, whole body sitosterol pool size positively correlated with plasma sitosterol levels (r=0.84, p=0.02), indicating that progressive reductions in plasma sitosterol levels may reflect a depletion of sitosterol tissue stores **(Figure 4.4A** and **C)**.

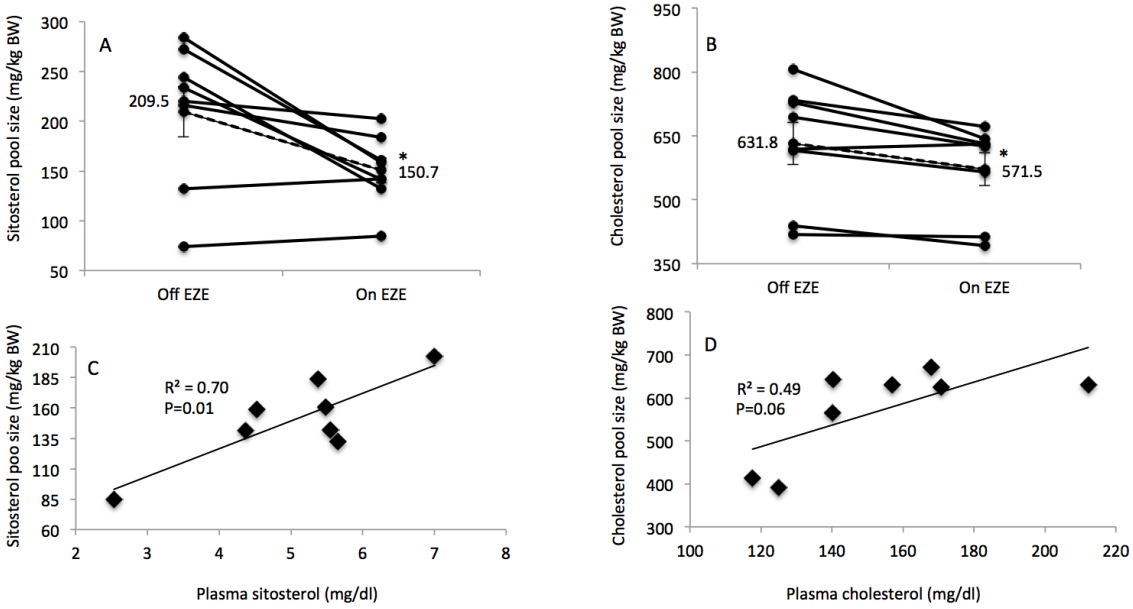

Figure 4. 4*: Whole body sitosterol and cholesterol pool sizes off and on ezetimibe (A and B), and their relationships with plasma sitosterol and cholesterol levels in homozygous sitosterolemia patients (C and D). Data were analyzed using a Pearson correlation.*

Likewise, EZE reduced cholesterol pool size (-9±3%, p=0.02) from 632±49 to 572±38mg/kg BW relative to off EZE. Whole body cholesterol pool size tended to positively correlate with plasma TC levels. However, after removing an outlier, correlation became stronger (r=0.86, p=0.01) than it was in presence of the outlier (r=0.70, p=0.06) **(Figure 4.4B** and **D).** Furthermore, whole body cholesterol pool size positively correlated with BW (r=0.79, p=0.02) and excess weight (r=0.77, p=0.03), suggesting that the size of cholesterol pool increases as BW increases.

4.5.6 Cholesterol Absorption Response to Treatment

Figure 4.5 shows cholesterol absorption data measured using single (**A** and **B**) and dual stable-isotope ratio methods (**C** and **D**). The mean AUC for RBC cholesterol ^{13}C enrichment across 72h is illustrated in (**Figure 4.5A)**. At 24h, measured cholesterol ^{13}C

enrichments tended to decrease (-43±17%, p=0.05) with EZE therapy from AUC 0.03±0.0 to AUC 0.02±0.0 compared with off EZE. At 48h and 72h, cholesterol enrichment values on EZE were further reduced (-62±13 and -68±12%, p<0.01). Individual cholesterol absorption measured by the total AUC for ^{13}C-cholesterol RBC enrichment over 72 h decreased (-61±13%, p=0.004) from AUC 0.19±0.02 to AUC 0.10±0.01 compared with off EZE (**Figure 4.5B)**. Fractional cholesterol absorption was also determined by comparing isotope ratios of RBC cholesterol following oral and intravenous administration. EZE reduced (-57±11, p=0.002) fractional cholesterol absorption from 65±7 to 35±3% compared with off EZE (**Figure 4.5C)**. The amount of absorbed cholesterol was reduced (p=0.005) after EZE from 3±0.5 to 2±0.2 mg/kg/d (222±46 to 122±26 mg/d per total BW) compared with off EZE **(Figure 4.5D)**. Daily cholesterol intake remained stable throughout off and on EZE treatment periods (323±48 and 341±55 mg/d, p=0.12).

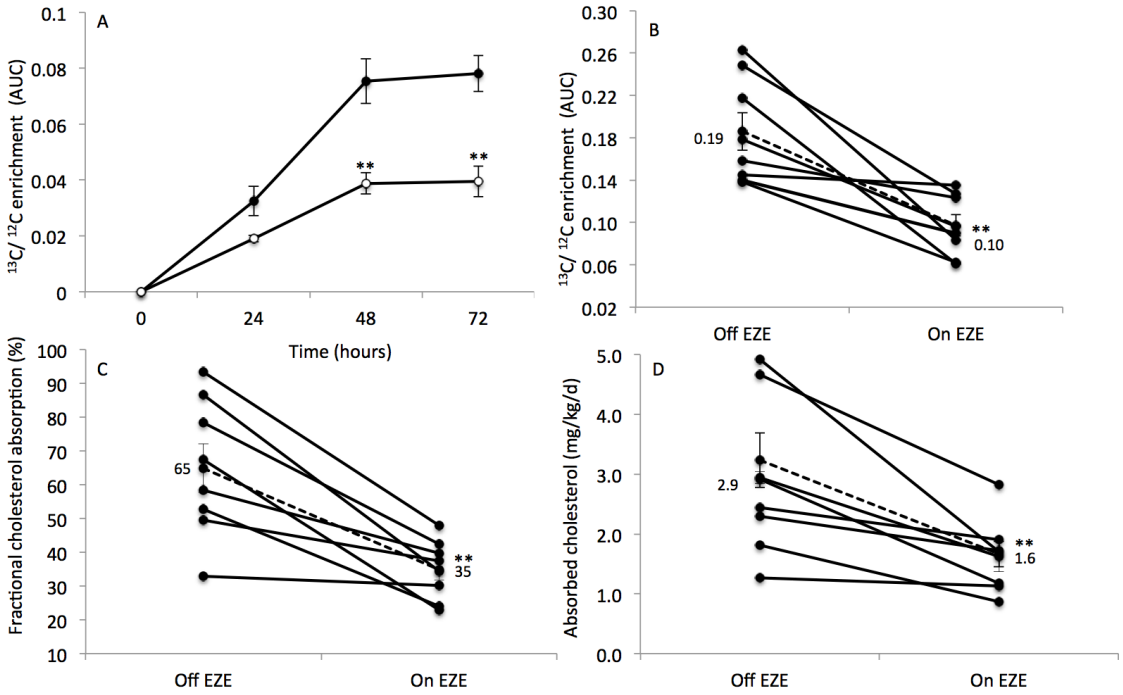

Figure 4. 5*: Cholesterol absorption data measured using single and dual stable-isotope ratio methods. Cholesterol ^{13}C enrichments in RBCs at different time points after isotope administration off and on EZE groups (n=8). Black circle: off EZE, empty circle: on EZE. AUC off EZE = 0.19; AUC on EZE = 0.10, **p=<0.001(A). Individual changes in cholesterol absorption as measured by area under the ^{13}C-cholesterol red blood cell enrichment curve over 72 h after ingestion of 3, 4-^{13}C- cholesterol before and after ezetimibe treatment in homozygous sitosterolemia (B) (n=8). Individual changes in cholesterol absorption rates measured by dual-isotopic ratio of oral ^{13}C-cholesterol and intravenous ^{18}O-cholesterol enrichment in RBC over 72 h off and on ezetimibe treatment in homozygous sitosterolemia (C). Individual changes in mass absorption of dietary cholesterol calculated by multiplying the individual daily cholesterol intake by cholesterol absorption rates off and on ezetimibe treatment in homozygous sitosterolemia (D) (n=8).*

4.5.7 Cholesterol Synthesis Response to Treatment

Cholesterol synthesis was determined as the FSR of the rapid-turnover body cholesterol pool, and calculated as an estimate of ASR/d is shown in **(Figure 4.6)**. FSR increased (+2.7±0.4%, p=0.002) from 2.3±0.4 to 5.3±1.0% pool/d **(Figure 4.6A)**. Most of the patients had a 2-to 4-fold increase in cholesterol synthesis except one patient who showed about a 0.8-fold decrease. The cholesterol ASR increased (+2.5±0.4-fold, p=0.02) from 0.4±0.1 to 0.8±0.3 g/d after EZE compared with off EZE **(Figure 4.6B)**. When expressed per kg BW the ASR values remained higher (10±2 vs 5±1 mg/kg BW/d, p=0.004) after EZE relative to off EZE.

4.5.8 Cholesterol Production and Metabolic Clearance Rates in Responses to Treatment

Cholesterol PR slightly increased (59±18%, p=0.04) from 0.6±0.1 to 0.9±0.3 g/d after EZE compared with off EZE **(Figure 4. 6C)**. EZE increased (2±0.3-fold, p=0.02) MCR of plasma cholesterol from 331±59 to 593±156 ml/d, suggesting that adequate excretion is needed to prevent development of hypercholesterolemia **(Figure 4.6D)**. The fraction of plasma cholesterol cleared daily (MCF) was also increased (2±0.3-fold, p=0.02) from 11±2 to 19±3% plasma volume/d after EZE compared with off EZE **(Figure 4. 6E)**.

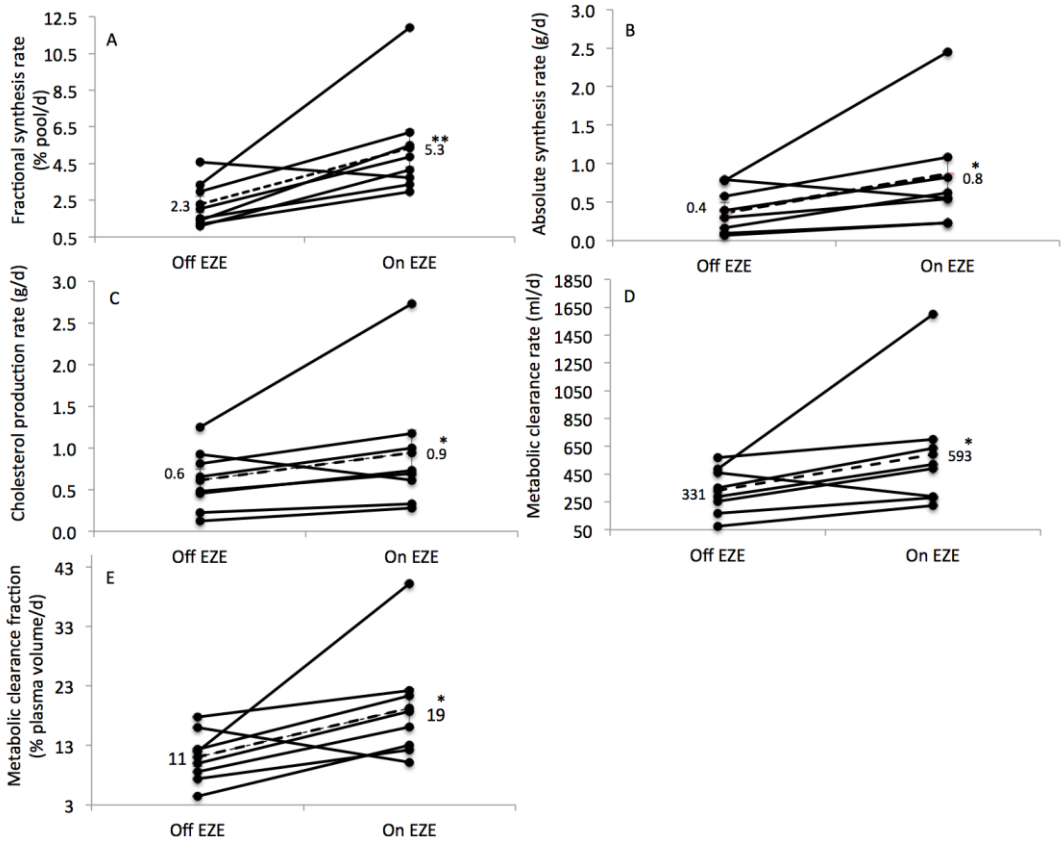

Figure 4. 6*: Fractional and absolute synthesis rates of cholesterol in response to treatment.*
The fractional synthesis rate of cholesterol measured at 24 h by deuterium incorporation after deuterium oxide consumption (A), and absolute synthesis rate of cholesterol synthesized per day (B) off and on ezetimibe treatment in homozygous sitosterolemia (n=8).

4.6 DISCUSSION

The results showed that EZE reduced whole body sitosterol and cholesterol pool sizes in STSL patients. EZE inhibited intestinal absorption of cholesterol and presumably PS, leading to decreases in their circulating levels and consequently whole body pool sizes of these sterols in STSL. EZE improves whole body cholesterol turnover by reducing cholesterol absorption and enhancing cholesterol synthesis and clearance. EZE is effective at decreasing plasma PS levels and their deposition in tissues. The current study strengthens the rationale for the use of EZE in treatment of STSL.

EZE has been shown to lower plasma TC and LDL-cholesterol, and PS levels in hypercholesterolemia (36) and STSL (20), respectively. However, despite the ability to lower PS levels in STSL patients, long-term use of EZE therapy at 10 mg/d failed to further decrease plasma PS (21), and such levels plateaued after higher doses (40 mg/d) (22). A few reports noticed a complete regression of xanthoma (37, 38) and improved ominous cardiac findings in STSL after EZE (38), suggesting a diminution in plasma and tissue PS levels.

The current study revealed dramatic reductions in plasma sitosterol and campesterol levels as early as 4 wks; these decreases were sustained over time. EZE reduced ($p=0.02$) whole body sitosterol pool size (-28±11%) from 210±25 to 151±13 mg/kg BW. Marked reductions in plasma PS with EZE reflected a decline in their absorption. Given that the human body cannot synthesize PS, EZE progressively reduced circulating levels of sitosterol, and lead to further decreases in whole body sitosterol pool size. In the current study, whole body sitosterol pool size varied widely from 6 to 23 g (15±2 g). The wide variations in the pool sizes in these patients (74 to 284 mg/kg BW) are likely to be related to dietary intake since a similar variation was also noted in plasma sitosterol concentrations (3 to 9 mg/dl). Quantitatively, whole body sitosterol pool size values in the present study are comparable to those (4.1g and 9.6 g) previously reported in STSL patients by Salen et al. (39) and Lin el al. (40) using radioisotope tracer methods and were at least 30 times larger than previously reported in heterozygous STSL (12, 39).

EZE decreased plasma TC as early as 2 wks; however, this decrease was not sustained over 14 wks, and only 13% was noted at the endpoint of EZE phase. In agreement, Salen et al. found no further decreases in plasma TC levels after 2 wks of EZE in STSL (20). After 14 wks off EZE, HDL-cholesterol levels seemed to decrease (-8.6%, p=0.12), although not significant, compared with baseline. However, EZE normalized these decreases in HDL-cholesterol levels, which agreed with previous studies in which EZE contributed to approximately 5 to 7% increases in HDL-cholesterol (41, 42) and promoted reverse cholesterol transport, a major atheroprotective property of HDL (43, 44). We showed that EZE reduced (-9±3%) whole body cholesterol pool size from 47±7 to 42±6 g. In patients on EZE, cholesterol pool size linearly correlated with plasma TC levels. The estimated cholesterol pool size values are comparable to those previously observed in STSL (40) and were not different from those reported in normal subjects or subjects heterozygous for STSL (39). Furthermore, whole body cholesterol pool size correlated directly with BW and excess weight, suggesting that the size of cholesterol pool increases as BW increases.

Increased tissue retention of sterols, decreased removal, and expanded body pools compensate for reduced cholesterol synthesis in STSL (12, 45). EZE inhibits cholesterol and presumably PS absorption in hypercholesterolemia (46) and STSL (20, 38). To our knowledge, the present study was the first to directly test the effect of EZE on intestinal cholesterol absorption in STSL patients using stable isotopes. EZE reduced cholesterol absorption efficiency (-62±13 and -57±11%) as determined by single and dual-isotope ratio methods, respectively. As previously confirmed (32), a good agreement was found

between percentage changes in the area under the ^{13}C enrichment curve and the cholesterol absorption rate measured by the dual-stable isotope ratio method after EZE ($r=0.87$, $p=0.01$, $n=7$). Herein, the fractional cholesterol absorption rates are consistent with those in hypercholesterolemic subjects, in which EZE inhibited absorbed cholesterol by 57% (36, 46). Salen et al. used radiolabeled techniques and observed cholesterol absorbed at rates 49 and 69% in two STSL patients (12). In the current study, the fractional cholesterol absorption rates varied widely from 33 to 93% before EZE and from 23 to 48% after EZE. Although we reported a high upper limit for cholesterol absorption rate before EZE, daily absorbed cholesterol mass (1.5 to 5 mg/kg BW/d) are similar to those of Salen et al. (2 and 5.6 mg/kg BW/d) (12). These values confirm inter-individual variation in cholesterol absorption (29 to 80%) observed in healthy subjects (47). It should be noted that absorbed cholesterol is derived from both dietary cholesterol and biliary cholesterol. If biliary cholesterol is low, as in STSL, dietary cholesterol would not be diluted and fractional absorption would be relatively high (48). These findings are consistent with variations in cholesterol absorption found in hypercholesterolemic subjects, and may be due to the differences in responsiveness to EZE (46). EZE inhibits cholesterol absorption and consequently reduces delivery of intestinal cholesterol to the liver, reducing hepatic cholesterol content and increased removal of cholesterol from circulation.

STSL patients and mice have deficits in cholesterol biosynthesis and subsequently low cholesterol content in tissues such as liver (49) and adrenal glands (50, 51). Several key enzymes involving cholesterol synthesis such as 3-hydroxy-3-methyl-glutaryl-CoA

(HMG CoA) reductase and synthase were inhibited by at least 40% in STSL (11, 49). Previous attempts including feeding cholesterol precursor mevalonic acid, low sterol diet (11), ileal bypass surgery or bile acid binding resins (52), have failed to stimulate *de novo* cholesterol synthesis in STSL. EZE treatment was effective to increase hepatic HMG CoA reductase activity in mice (53). The current study was the first to use the D_2O method and directly measure whole body *in vivo* cholesterol synthesis from D_2O in STSL. EZE increased (2.7±0.4 and 2.5±0.4%) whole body *in vivo* cholesterol synthesis as measured by FSR and ASR. In accord, increases in plasma indirect markers of cholesterol synthesis (i.e. lathosterol) were observed after EZE observed in STSL (20) and hypercholesterolemia (46, 54, 55), reflecting reciprocal increases in hepatic cholesterol synthesis due to inhibited cholesterol absorption (39). The absolute amount of *de novo* cholesterol synthesized daily in the current patients are similar to those calculated following subtracting absorbed cholesterol from turnover in STSL (12) and were 3, 5 and 9 times lower than those of heterozygous STSL (56), hypercholesterolemia (57) and normal subjects (12), respectively.

The daily cholesterol PR (assumed to be equivalent to total turnover rate consists of both absorbed plus synthesized cholesterol) ranged from 0.7 to 2 g/d in humans (31) with 1.1 g/d for a 70 kg person (35). Herein cholesterol PR was 0.6±0.1 g/d before EZE and 0.9±0.3 g/d after EZE. EZE increased cholesterol PR (59±18%). Such increases did not obviously lead to increased cholesterol pool size after EZE. Cholesterol PR values are similar to those previously reported in STSL (12, 39) and lower than those reported in healthy or hypercholesterolemic subjects (35).

The turnover of cholesterol in the whole system can also be expressed as metabolic clearance, either as the volume (MCR) or the fraction (MCF) of plasma cleared daily (31). Approximately, 23 and 12% of the plasma volume were cleared daily when the serum TC levels were 200 and 400 mg/dl in hypercholesterolemia (31). In the current study, MCF values ranged from 4 to 18% before EZE, and were about two-fold lower than those of hypercholesterolemia, suggesting a slower rate of cholesterol clearance in STSL patients. EZE increased MCR and MCF for TC, reflecting an increase in cholesterol efflux and cholesterol turnover. The EZE-induced changes in MCR and MCF correlated with those in PR ($r=+0.98$ and $r=+0.96$, all $p<0.0001$) and TC ($r=-0.70$, $p=0.05$ and $r=-0.77$, $p=0.02$), suggesting that increased excretion of cholesterol from plasma after is needed to prevent hypercholesterolemia.

4.7 CONCLUSION

EZE progressively reduced circulating levels of PS and decreased whole body sitosterol pool size in STSL. Moreover, EZE decreased plasma TC and LDL-cholesterol and whole body cholesterol pool size in STSL by inhibiting cholesterol absorption and increasing its total rate of clearance from the body. Inhibiting cholesterol absorption with EZE increases whole body cholesterol synthesis rates. EZE is effective at decreasing plasma PS levels and their deposition in tissues. In STSL, EZE not only reduces PS accumulation and but improves whole body cholesterol turnover by reducing cholesterol absorption and enhancing cholesterol synthesis and clearance. The current study strengthens the rationale for the use of EZE in treatment of STSL. Whether progressive reductions in plasma and

whole body stores of PS with EZE improve devastating clinical consequences of STSL including premature atherosclerosis remain unknown. The efficacy and possible clinical benefit of longer-term treatment warrants further investigation.

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BRIDGE TO CHAPTER V

The data presented in Chapter IV demonstrate PS-lowering efficacy of EZE therapy. EZE reduces tissue stores of PS over 14 wks of treatment. Moreover, body pool size of PS was directly correlated with circulating PS concentrations after EZE treatment. The clinical consequences of expanded tissue stores of PS in STSL include premature atherosclerosis and tendon xanthomas. Other clinical independent features such as chronic hemolytic anemia and macrothrombocytopenia have also been reported. Whether complications of STSL including low platelet count, large platelet volume and hemolysis would be reversed with EZE treatment as indication of reduced tissue stores of PS remains to be seen. As such, it can be suggested that reducing plasma and tissue PS with EZE would improve hematologic abnormalities. However, no studies have explored these questions in STSL patients. The purpose of the following study was to simultaneously investigate if EZE, a sterol-absorption inhibitor, improves platelet indices and reduces serum red blood cell hemolysis markers in STSL patients.

CHAPTER V

MANUSCRIPT 4

This manuscript is in print for publication in the

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REDUCING CIRCULATING LEVELS OF PLANT STEROLS BY EZETIMIBE FAVORABLY INCREASES PLATELET COUNTS IN SITOSTEROLEMIA PATIENTS

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Running title: Ezetimibe improves thrombocytopenia in sitosterolemia

5.1 ABSTRACT

Objective To determine if ezetimibe (EZE), a sterol-absorption inhibitor, improves platelet indices and reduces red blood cell (RBC) hemolysis in sitosterolemia (STSL) patients.

Study design 8 STSL patients were first taken off EZE for 14 wks followed by EZE (10 mg/d) treatment for another 14 wks. Blood samples were collected at baseline and on EZE. Plasma and RBC sterol levels were analysed by gas chromatography. Complete blood cell count including platelet indices and serum markers of hemolysis were determined by automated analyzers.

Results EZE increased platelet count (23±9%) and decreased mean platelet volume (MPV; 10±3%, all $P<0.05$). In patients off EZE, plasma and RBC total PS to cholesterol ratio inversely correlated with platelet counts ($r=-0.96$, $P=0.001$ and $r=-0.91$, $P=0.005$) and positively correlated with MPV ($r=0.91$, $P=0.03$ and $r=0.93$, $P=0.02$). EZE reduced absolute plasma and RBC sitosterol (-35±4 and -28±3%), and total PS (-37±4 and -28±3%, all $P<0.0001$) as well as their ratios to cholesterol (-27±4 and -28±4%, $P<0.01$). The change in the plasma total PS to cholesterol ratio was strongly correlated ($r=0.99$, $P<0.0001$) with the change in total PS to cholesterol ratio in RBC, suggesting that PS tissue stores represented by RBC PS were reduced by EZE. EZE showed the desired therapeutic effects of increased platelet count (+23±9%) and decreased MPV (-10±3%, all $P<0.05$), without affecting RBC hemolysis markers or other RBC indices. In patients off EZE, RBC TC was negatively correlated to RBC sitosterol ($r=-0.77$, $P=0.03$), total PS ($r=-0.79$, $P=0.02$) and their ratios to cholesterol ($r=-0.86$, $P=0.006$), suggesting reciprocity between cholesterol and PS.

Conclusions EZE had beneficial effects on platelet indices in STSL patients prone to macrothrombocytopenia including increased platelet count and decreased MPV. These effects were noted with concomitant decreases in plasma and RBC PS levels. By lowering plant sterol levels in STSL, EZE treatment improves platelet count and size, thereby potentially ameliorating bleeding tendency in STSL.

5.2 INTRODUCTION

Sitosterolemia (STSL) (also known as phytosterolemia, MIM number 210250) is a rare autosomal recessive condition caused by mutations in either of the adenosine triphosphate-binding cassette (ABC) transporter genes *ABCG5* or *ABCG8,* tandemly located in a head-to-head orientation on chromosome 2p21. [1] *ABCG5* and *ABCG8* encode ABCG5 and ABCG8 transporters, are expressed in the intestine and liver, to rapidly remove absorbed sterol and prevent sterol accumulation. [1-3] STSL is characterized by elevated plasma levels of plant sterols (PS), mainly sitosterol, campesterol and stigmasterol with normal to moderately elevated plasma total cholesterol (TC) levels.[4, 5] However, in some cases TC can be extremely high.[6] STSL patients frequently develop tendon and cutaneous xanthomas and, above all, are at risk of developing premature coronary artery disease. [2, 7-9] Hematologic abnormalities including abnormal red blood cells (RBC) morphology (stomatocytes) [10] and large platelets present in reduced quantity (macrothrombocytopenia) were also reported. Some patients often show clinically problematic bleeding episodes, presumably caused by the macrothrombocytopenia. [11, 12] Rees et al. [11] and Su et al.[13] successively reported STSL co-existing macrothrombocytopenia and stomatocytic hemolysis. Severe bleeding episodes have been reported in STSL, which necessitated platelet transfusion. [11]

Ezetimibe (EZE) is a sterol absorption inhibitor that blocks the intestinal transporter protein Niemann-Pick C1-like 1 [14] thereby, reducing dietary and biliary sterols absorption. EZE decreases TC, low-density lipoprotein (LDL)-cholesterol, [15] and PS levels in STSL.[16] EZE improved thrombocytopenia in ABCG5-knockout (KO) mice, [12,

[17] but this effect was inconsistent in STSL patients. [18, 19] EZE is considered safe and effective in the treatment of STSL, [16, 20, 21] but abnormal liver function was reported in one patient. [22] Herein, we sought to investigate if EZE improves platelet count and size, and reduces markers of RBC hemolysis in STSL patients and if so, whether this occurs *via* reduction in circulating PS levels. The safety of EZE on liver function was also examined.

5.3 EXPERIMENTAL METHODS

5.3.1 Patients

Eight patients, between 16 and 56 years of age, were recruited from Hutterite colonies in Manitoba, Canada (n=4) and South Dakota, US (n=4) (5 males and 3 females). All patients were identified as having homozygous *ABCG8* S107X mutation and related to a proband previously reported by Mymin et al.[7, 23] and Chong et al.[24] All procedures involving human patients were approved by the Data Management and Coordinating Center, the National Institute of Child Health and Human, the Development Data and Safety Monitoring Board, and the University of Manitoba Biomedical ethics board. Written informed consents were obtained from all patients. The trial was registered with www.clintrials.gov under number NCT01584206.

5.3.2 Study Design

The study was a single-site pilot interventional study of 8 patients with STSL. The study was conducted at the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba and was designed as two 14-wk phases, off and on EZE. After consent, study patients (n=8) were taken off EZE for 14 wks and baseline blood samples were collected. On the last day of blood draw, patients were instructed to resume EZE

(10 mg/d) for another 14 wks and follow their usual diet. Blood was collected at on the end of the 14 wk treatment period with EZE. Plasma and RBC sterols, lipids, complete blood counts and blood chemistries as measures of liver were measured at baseline and 14 wks after EZE. Serum, plasma and RBC fractions were separated by centrifugation at 3000 rpm for 20 min at 4ºC, and immediately stored at -80ºC until further analysis.

5.3.3 Blood Sampling

Blood was collected at baseline and endpoint off and on EZE phase. Serum, plasma and erythrocyte fractions were separated by centrifugation at 3000 rpm for 20 min at 4ºC, and immediately stored at -80ºC until further analysis.

5.3.4 Quantitative Analysis of Plasma and Red Blood Cell Sterol Levels

The levels of plasma and RBC TC and PS were measured by gas-liquid chromatography equipped with a flame ionization detector and an auto sampler system (Varian 430-GC; Agilent Technologies, Santa Clara, CA, US). For sterol quantification, an internal standard, 5α-cholestane, was used in combination with sterol standard curve for each individual sterol, using authentic standards (Sigma-Aldrich Ltd. Oakville, ON, Canada) and (MJS BioLynx Inc, Brockville, ON, Canada). Briefly, 5α-cholestane (50 ug) was added to each 0.5 ml plasma or 0.5 g RBC sample, and saponified with 4 ml methanolic potassium hydroxide for 2 h at 100°C. The unsaponifiable portion (sterols) were extracted twice from the mixture with 4 ml petroleum ether, derivatized with 0.1 ml TMS reagent (pyridine–hexamethyldisilazane–trimethylchlorosilane; 9:3:1 by volume), re-suspended in 0.4 ml hexane and injected (1 μl) onto a 30-m SAC-5 column (Sigma-Aldrich Ltd. Oakville, ON, Canada). The column temperature was set to 280°C, with isothermal running conditions maintained for 30 min per sample. The injector and

detector temperatures were set at 295°C and 300°C, respectively. The carrier gas (helium) flow rate was 1 ml/min, with the inlet split set at 40:1. [25]

5.3.5 Hematologic Parameters and Serum Markers of Hemolysis

Complete blood counts were performed on blood samples collected on ethylenediaminetetraacetic acid (EDTA) (COULTER® LH 750 Hematology Analyzer, Beckman Coulter Inc. Gamma Dynacare Medical Laboratories, Winnipeg, MB, Canada) and (Sysmex XT-2000i Automated Hematology Analyzer, Avera Queen of Peace Hospital, Mitchell, SD, US). Serum markers of hemolysis including lactate dehydrogenase (LDH) levels and total bilirubin were measured enzymatically on the Dimension RXL (Siemens, Gamma Dynacare Medical Laboratories, Winnipeg, MB, Canada).

5.3.6 Serum Lipids Profile

Baseline serum TC, and HDL-cholesterol, and triacylglyceride levels were determined by automated enzymatic methods on a Vitros-350 chemistry analyzer (Ortho-Clinical Diagnostics, Markham, ON, Canada). Serum LDL-cholesterol levels were calculated by the Friedewald equation (26).

5.3.7 Liver Function and Muscle damage marker Creatine Kinase

Serum levels of liver enzymes including aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and gamma-glutamyltransferase (GGT), and blood urea nitrogen (BUN) and indirect indicator of muscle damage (creatine kinase, CK) were measured using automated enzymatic methods on a Vitros-350 chemistry analyzer (Ortho Clinical Diagnostics, Markham, ON, Canada).

5.4 STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS 21.0 (SPSS, Inc., Chicago, IL, US). All data are presented as mean±SEM. Statistical significance was set at $p<0.05$. Linear mixed-model analysis was used where treatment, gender and site were specified as fixed factors, and age was specified as covariate in the model. Significant values for treatment effect were analyzed by including the treatment term as main effect into the linear mixed-model ANOVA. Percentage change from baseline for each phase was analysed using two-tailed paired Student's t test. Relationships between two variables were assessed with stepwise multiple linear regression analysis unless otherwise stated. Data that were not normally distributed, as determined by a Shapiro-Wilk test, were log or inverse transformed before statistical analysis.

5.5 RESULTS

5.5.1 Patients' Characteristics

Baseline characteristics of the patients are shown **(Table 5.1)**. Eight patients were initially recruited, and all completed the trial. There were 5 males and 3 females, with a mean age of 28.0±5.5 years. All patients had confirmed diagnosis of STSL at the time of enrollment in the study; identified as homozygous *ABCG8*S107X mutation (NM_022437.2:c.320C>G) as previously genotyped by Mymin et al.[7, 23] and Chong et al.[24] Mean values of serum TC and LDL-cholesterol indicate mild hypercholesterolemia. Serum TC levels were normal (<200 mg/dl) in three patients, borderline high (200-239 mg/dl) in two patients, and high (>240 mg/dl) in three patients. Serum LDL concentrations were optimal (<100 mg/dl) in two patients, above optimal (100-129 mg/dl) in one patient, borderline high (160-189 mg/dl) in three patients and very high

(>190 mg/dl) in two patients. Baseline TC concentrations were lower (p=0.003) in RBC than plasma **(Table 5.1)**. All patients had elevated plasma and RBC levels of PS **(Table 5.1)** Sitosterol and total PS levels were comparable between plasma and RBC. Patients with STSL have elevated plasma and RBC levels of PS **(Table 5.1).** Individual PS (sitosterol) and total PS levels were comparable between plasma and RBC. However, total PS to cholesterol ratio was higher in RBC (p=0.08) than plasma. Baseline RBC indices including hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width-standard deviation (RDW-SD) and red cell distribution width-coefficient of variation (RDW-CV), white blood cells (WBC), neutrophils, lymphocytes, monocytes, eosinophils, and basophils are presented in **(Table 5.2)**. Three patients had decreased platelet counts ($\leq 140x10^3$/ul) while four had increased platelet size (MPV >12 fl). Two patients had both large and decreased platelets (macrothrombocytopenia). One patient had Hb<12 g/dl, below normal RBC, HCT, and MCHC levels, and another patient had increased stomatocytes. All of the patients had no hemolytic anemia and hemolysis. Patients lacked evidence of abnormal liver function except two patients who had mild elevations in one liver enzyme (GGT) or all liver enzymes (ALT, AST, GGT, and ALP) and CK. Nevertheless, all patients had normal bilirubin levels (0.42±0.06 mg/dl) **(Table 5.2)**.

Table 5. 1*: Baseline characteristics of patients.*

Variable	Mean	SEM
Age (year)	28.0	5.5
Body weight (kg)	72.1	6.4
Height (cm)	166.1	2.4
BMI (kg/m²)	25.9	1.9
TC (mg/dl)	210.5	17.3
LDL-cholesterol (mg/dl)	131.4	13.9
HDL-cholesterol (mg/dl)	54.7	2.6
Triacylglyceride (mg/dl)	121.5	27.2
Plasma TC (GC, mg/dl)	155.8	12.7
RBC TC (mg/dl)	100.8	6.2
Plasma sitosterol (mg/dl)	6.2	0.7
RBC sitosterol (mg/dl)	4.6	0.5
Plasma total PS (mg/dl)	9.1	1.1
RBC total PS (mg/dl)	7.9	0.7
Plasma total PS to cholesterol ratio	0.06	0.01
RBC total PS to cholesterol ratio	0.08	0.01

*Data are mean±SEM; n=8 for demographic data; *n=7.*

***Table 5. 2**: Baseline complete blood count and biochemical profile.*

Variable	Mean	SEM
WBC ($x10^3$/ul)	5.6	0.5
RBC ($x10^6$/ul)	4.8	0.1
Hb (g/dl)	14.2	0.5
Hematocrit (%)	42.6	1.3
MCV (fl)	89.7	0.8
MCH (pg)	30.0	0.4
MCHC (g/dl)	33.4	0.4
PLT ($x10^3$/ul)	164.3	11.9
MPV (fl)	13.0	0.4
RDW-SD (fl)	43.3	0.8
RDW-CV (%)	13.6	0.1
Neutrophils (%)	55.5	2.2
Lymphocytes (%)	32.2	2.8
Monocytes (%)	9.7	1.4
Eosinophils (%)	2.2	0.5
Basophils (%)	0.4	0.1
AST (IU/l)	23.3	2.8
ALT (IU/l)	32.0	7.3
ALP (IU/l)	84.7	9.9
CK (IU/l)	142.1	40.1
GGT (IU/l)	38.3	7.6
Total bilirubin (mg/dl)	0.4	0.1
LDH (IU/l)	147.4	10.1
BUN (mg/dl)	32.0	2.4

Data are mean±SEM; n=7; Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CK, creatine kinase; fl, femtoliters; GGT, gamma-glutamyltransferase; Hb, hemoglobin; LDH, lactate dehydrogenase; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; RBC, red blood cells; RDW-CV, red cell distribution width coefficient of variation; RDW-SD, red cell distribution width standard deviation; WBC, white blood cells.

5.5.2 Effect of Ezetimibe on Plasma and Red Blood Cells Total Cholesterol and Plant

Sterols Levels

The effects of EZE on TC and PS levels in plasma and RBC are shown **(Figure 5.1)**. EZE tended to lower plasma TC (-12±6%, P=0.09) after 14 wks but did not affect RBC TC (0.3±3%, P=1.0) **(Figure 5.1A** and **B)**. In patients off EZE, RBC TC was negatively correlated to RBC sitosterol (r=-0.77, P=0.03), total PS (r=-0.79, P=0.02) and their ratios to cholesterol (r=-0.86, P=0.006), suggesting reciprocity between cholesterol and PS. Regardless of EZE, RBC consistently had lower (P<0.001) TC levels than plasma (-39±3 and -30±4%) off and on EZE. EZE dramatically reduced plasma and RBC sitosterol (-35±4 and -28±3%, all P<0.0001) and total PS (-37±4 and -28±3%, all P<0.0001) **(Figure 5.1C**, **D**, **E** and **F)**. When total PS was expressed against cholesterol concentrations in plasma and RBC, reductions in PS were significant (-27±4%, P=0.002 and -28±4%, P=0.003). Furthermore, the decrease in the total PS to cholesterol ratio in plasma after EZE treatment mirrored that of tissue (RBC). After removing an outlier, the correlation became significantly stronger (r=0.99, P<0.0001) than when the outlier was included (r=0.72, P=0.05).

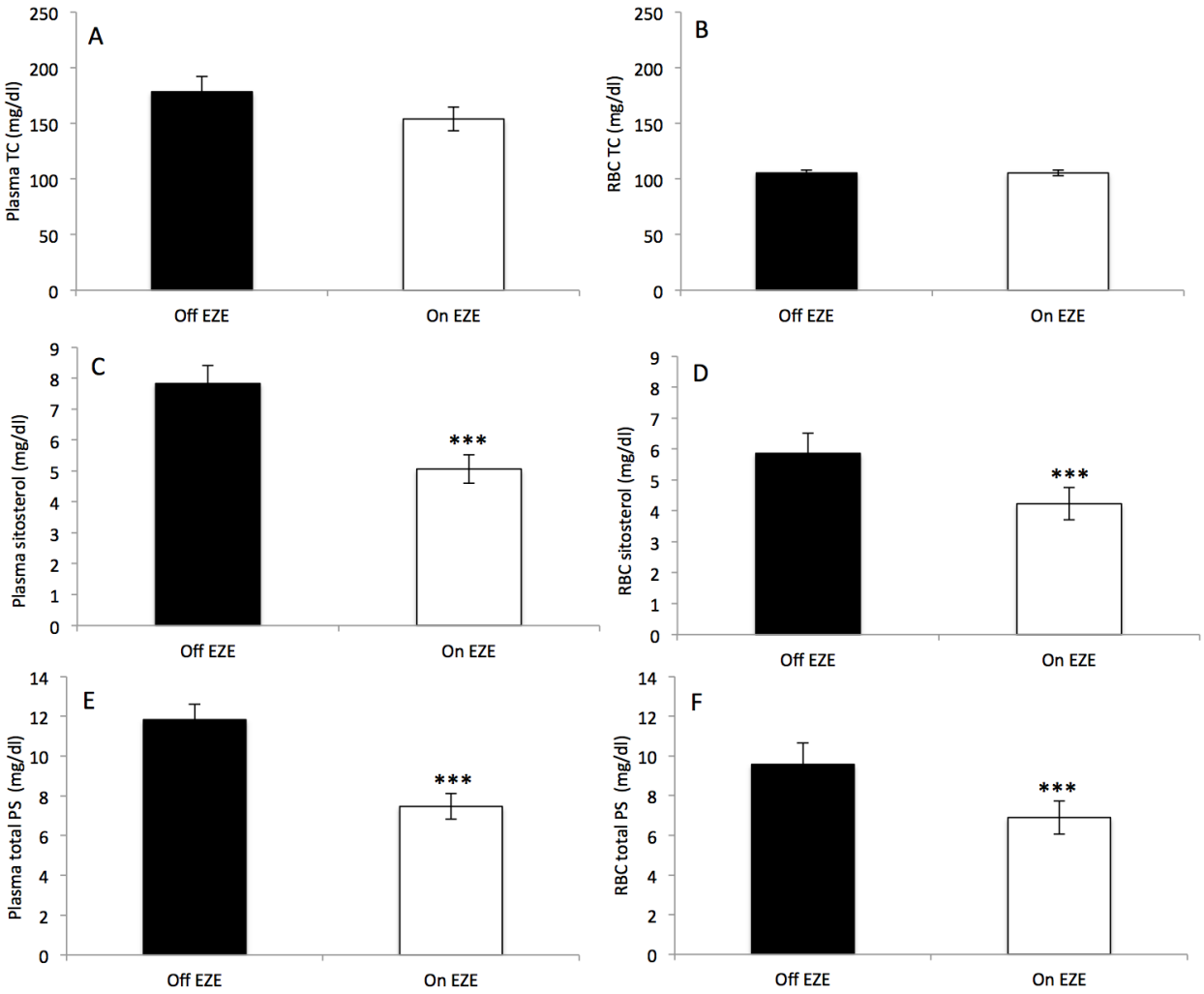

Figure 5. 1*: Plasma and red blood cells cholesterol, sitosterol and total plant sterols at 14 weeks off and on ezetimibe in sitosterolemia patients.*
*Plasma and red blood cells cholesterol (A, B), sitosterol (C, D) and total plant sterols (E, F) at 14 weeks off and on ezetimibe. Mean values were significantly different when compared with off EZE: *p<0·05, **p<0·01, ***p<0·0001). Abbreviations: EZE; ezetimibe, RBC; red blood cells; TC, total cholesterol*

5.5.3 Effect of Ezetimibe on Platelet Count and Size

Figure 5.2A and **B** shows platelet count and size at 14 weeks off and on EZE therapy. In patients off EZE, platelet count showed insignificant inverse relationships with plasma and RBC PS (r=-0.47, P=0.24 and r=-0.68, P=0.06) and PS to cholesterol ratio (r=-0.64, P=0.09 and r=-0.68, P=0.06). However, after removing an outlier, these correlations became statistically significant for PS (r=-0.77, P=0.04 and r=-0.91, P=0.004) and PS to

cholesterol ratio (r=-0.96, P=0.001, and r=-0.91, P=0.005) **(Figure 5.2C)**. Baseline plasma and RBC PS to cholesterol ratio were positively correlated with MPV (r=0.91, P=0.03 and r=0.93, P=0.02, n=5) **(Figure 5.2D)**. EZE increased (P=0.03) platelet count (23±9%) compared with 14 weeks off EZE (170±14 vs 144±16 $x10^3$/ul) **(Figure 5.2A)**. Likewise, EZE reduced MPV (-10±3%) compared with baseline (12±0.4 vs 13±0.4 fl, P=0.04) **(Figure 5.2B)**. Noted, at 14 weeks off EZE the hematology analyzer could not read the MPV values for 6 patients due to abnormal distribution and large sizes of the platelet, therefore statistical comparison between 14 weeks off and 14 weeks on EZE was not feasible. Instead, comparison was made between 14 weeks on EZE and baseline.
At baseline, plasma and RBC total PS to cholesterol ratio were positively correlated with MPV (r=0.93, n=5, P=0.02) **(Figure 5.2D)**.

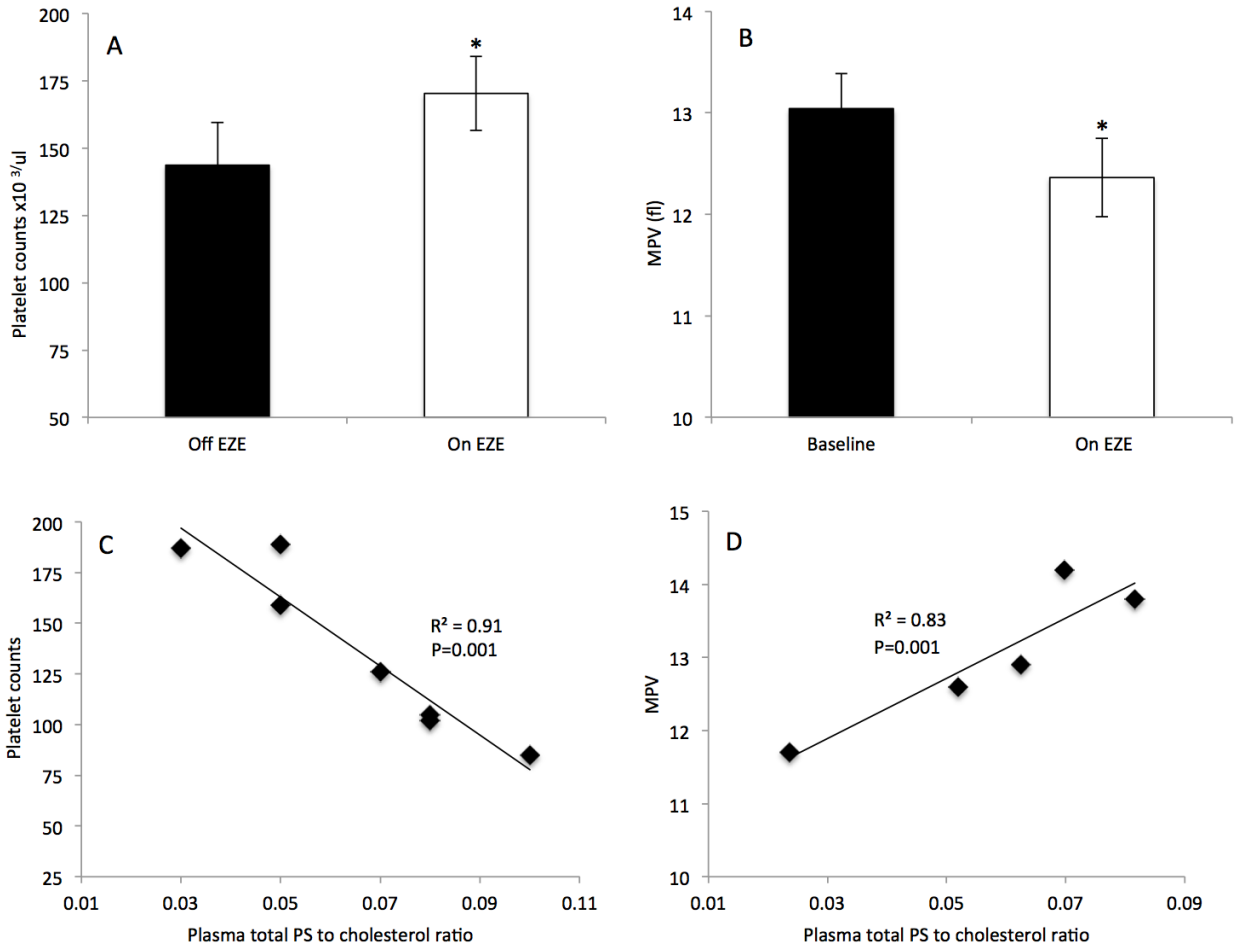

Figure 5. 2*: Platelets count and mean platelet volume in sitosterolemia patients 14 weeks off and on ezetimibe and their relationships with total plant sterols to cholesterol ratio.*
*Data are mean±SEM and significantly different when compared with off EZE;*P<0.05, n=8. Linear regression coefficients (r2) for relationships between platelet count (C, n=7) and MPV (D, n=5) and total plant sterols to cholesterol ratio off EZE. Abbreviations: EZE; ezetimibe, RBC; red blood cells.*

5.5.4 Effect of Ezetimibe on Red Blood Cell Indices

After 14 wks on EZE there were no statistically significant differences in RBC indices such as Hb, HCT, MCV, MCH, RDW-SD and RDW-CV, WBC, neutrophils, lymphocytes, monocytes, eosinophils and basophils compared with off EZE **(Table 5.3)**. However, MCHC, which reflects the proportion of Hb in RBC, slightly increased (P=0.03) after EZE relative to off EZE (34±0.2 vs 33±0.3 g/dl). MCHC inversely associated with RBC sitosterol (r=-0.86, P<0.01), total PS (r=-0.91, P<0.01), and total PS to cholesterol ratio (r=-0.88, P<0.01) before EZE. Similarly, plasma sitosterol (r=-0.51, P=0.19), total PS (r=-0.68, P=0.07) and total PS to cholesterol ratio (r=-0.90, P=0.003) were negatively correlated with MCHC in patients off EZE, suggesting that when plasma PS increase in patients off EZE, MCHC decreases. Furthermore, upon resuming EZE, all RBC indices MCV (r=-0.77), MCH (r=-0.82), and MCHC (r=-0.85, all P<0.05) inversely correlated with plasma PS, in particular, campesterol, suggesting that RBC size and Hb concentration increase with the decreases in plasma PS. Likewise, RDW-CV, which reflects variation in RBC size (anisocytosis) and is elevated during anemia, was positively related to plasma and RBC campesterol levels (r=0.71, P<0.05 and r=0.70, P=0.05) after EZE.

Table 5. 3*: Complete blood count and serum hemolysis markers at 14 weeks off and on ezetimibe in sitosterolemia patients*

Parameter	14 wk off EZE	14 wks on EZE	Mean % change	P-value
WBC (10^3/ul)	5.6±0.3	6.3±0.5	+11.2±5.3	0.07
RBC (10^6)	4.6±0.2	4.7±0.1	+1.0±1.4	0.57
Hb (g/dl)	13.8±0.5	14.0±0.6	+1.3±1.0	0.24
Hematocrit (%)	41.4±1.5	41.4±1.5	+0.1±1.1	0.98
MCV (fl)	89.5±0.9	88.8±1.2	-0.8±0.7	0.32
MCH (pg)	29.8±0.4	29.9±0.6	+0.4±0.8	0.61
MCHC (g/dl)	33.3±0.3	33.7±0.2	+1.3±0.5	**0.03**
RDW-SD (fl)	45.0±0.9	44.6±0.7	-0.8±1.0	0.37
RDW-CV (%)	14.2±0.2	14.1±0.4	-0.8±2.0	0.86
Neutrophils (%)	56.2±2.0	53.7±3.0	-4.4±4.0	0.31
Lymphocytes (%)	33.3±2.1	34.5±3.1	+3.4±5.9	0.59
Monocytes (%)	8.1±0.8	8.7±0.9	+10.5±9.7	0.42
Eosinophils (%)	2.1±0.6	2.6±0.6	+20.4±10.7	0.07
Total bilirubin (mg/dl)	0.44±0.06	0.42±0.04	+7.6±13.3	0.45
LDH (IU/l)	155.6±9.4	144.1±10.0	-6.9±4.8	0.15

Abbreviations: Hb, haemoglobin; fl, femtoliters; LDH, lactate dehydrogenase; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RBC, red blood cells; RDW-CV, red cell distribution width coefficient of variation; RDW-SD, red cell distribution width standard deviation; WBC, white blood cells; n=8.

5.5.5 Effect of Ezetimibe on Liver Enzymes and Creatine Kinase

There were no significant differences among serum hepatic enzymes (AST, ALT, ALP and GGT) after 14 wks on EZE compared with those off EZE. Serum levels of CK, an indirect muscle damage indicator, were decreased (-18.1±10.1%) after 14 of EZE treatment but were not statistically significant (p=0.50). Serum BUN concentrations remained comparable between the two phases **(Table 5.4)**.

Table 5. 4*: Liver function and muscle damage marker 14 weeks off and on ezetimibe.*

Parameter	14 wks off EZE	14 wks on EZE	Mean % change	P value
AST (IU/l)	23.1±3.2	30.1±4.3	+10.4±9.3	0.18
ALT (IU/l)	31.4±5.0	43.8±10.9	+1.9±16.5	0.24
ALP (IU/l)	84.6±9.0	85.3±8.4	+0.9±11.3	0.74
GGT (IU/l)	31.5±7.2	40.1±10.5	-20.4±2.3	0.12
CK (IU/l)	93.3±16.6	85.0±14.2	-18.1±10.1	0.50
BUN (mg/dl)	33.5±3.0	30.9±1.6	-5.2±5.4	0.23

*Abbreviations: AST, aspartate aminotransferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; CK, creatine kinase; EZE, ezetimibe; GGT, gamma-glutamyltransferase; * indicates significance vs baseline; n=8.*

5.6 DISCUSSION

The major findings of this study were that blocking intestinal absorption of PS with EZE in STSL patients increased platelet number and decreased platelet size *via* reductions in PS concentrations. EZE had no effect on serum hemolytic biomarkers, including LDH and total bilirubin, and RBC indices, but slightly increased MCHC, which inversely correlated with total PS and their ratios to cholesterol. EZE tended to lower plasma TC levels without affecting RBC TC concentrations. RBC TC levels negatively correlated with total PS and their ratios to cholesterol, suggesting reciprocity between cholesterol and PS. EZE treatment restored platelet count in STSL and reduced platelet size and likely PS content without affecting RBC indices and serum markers of hemolysis. Mutations in ABCG5/8 cause STSL characterized by increased absorption of PS. We previously showed that the presence of intact ABCG5/8 protected against PS accumulation and hemolysis in hamsters [27] or hypercholesterolemic subjects. [28] In the absence of STSL, total PS represented 0.2% of total plasma sterols. [29] These levels

increase 30-fold in STSL. The *ABCG5* and *ABCG8* genes are not expressed in blood cells and therefore these cells could be target for the toxic effect of PS. [30]

Macrothrombocytopenia has been identified as a hematologic presentation of STSL. [12, 31, 32] The mechanisms behind these hematologic abnormalities are not clear. PS have been found to incorporate directly into the platelet membrane of sitosterolemic mice, disrupting lipid asymmetry, and leading to the formation of hyperactivatable platelets that lose the ability to aggregate.[12] PS may displace cholesterol in the RBC membrane, [33] weaken interactions between the molecules, modifying cell morphology [34] and increasing cell fragility. [10, 11]

In Chinese STSL patients who presented with moderate bleeding, bile acid sequestrants reduced PS levels but did not normalize macrothrombocytopenia. [31] EZE has been used successfully in STSL, [8, 16] but little data on improvement in hematologic parameters has been reported. [18] In a mouse model of STSL, EZE reversed macrothrombocytopenia [35] compared with bone marrow transplantation, indicating that low platelet count in STSL is caused by the build-up of PS rather than intrinsic hematopoietic defects. In the current study, when patients were off EZE, platelet count inversely correlated with total PS to cholesterol ratio and MPV positively correlated with plasma total PS to cholesterol ratio. Total PS constituted 7 and 8% of total sterols in plasma and RBC; however, these percentages were equally decreased by 26% with EZE in both compartments. EZE increased platelet count by 23% and decreased MPV by 10%.

Stomatocytosis and hemolytic anemia were reported in some STSL. [10, 11] Recently, EZE failed to decrease plasma PS levels but improved hemolysis after one year in patient with STSL, suggesting that improved hemolysis after EZE may be due to modulation of lipid content at the RBC, thus increasing their resistance to lysis. [18] Herein, EZE tended to lower plasma TC but did not affect RBC TC levels. When current patients were off EZE RBC TC concentrations inversely correlated with RBC sitosterol and total PS, suggesting reciprocity between cholesterol and PS.

In the current study, EZE had no impact on serum markers of hemolysis and RBC indices, RDW, MCV and MCH but slightly increased MCHC. In patients on EZE, plasma and RBC total PS (r=-0.74, P=0.04 and r=-0.70, P=0.06) were negatively correlated with MCHC, suggesting that EZE may have increased MCHC by reducing PS levels. A high RDW reflects an increased variability in the size of RBC (anisocytosis) and indicates body's iron status. RDW increased with increased plasma PS levels. [32, 36] Plasma and RBC total PS levels in patients on EZE tended to positively correlate (r=0.64, P=0.09 and r=0.67, P=0.07) with anisocytosis, denoting a possible lower variation in RBC size after EZE. EZE had no impact on serum markers of hemolysis, which may be related to the fact that current patients did not have hemolytic anemia compared with others. [18]

Cholesterol plays a substantial role in regulating membrane fluidity, permeability, and cellular trafficking [37] with imbalance in cholesterol level in RBC resulting in hemolysis. [38, 39] Cholesterol concentrations in RBC are similar to plasma in healthy humans. [40] Despite EZE treatment, STSL subjects' RBC consistently had at least 30% lower TC

levels than plasma in both phases. Decreased TC levels in RBC are likely due to increased RBC membrane PS incorporation [38] and may also indicate decreased tissue cholesterol content in STSL subjects. Miettinen et al. showed that RBC and plasma free and TC values were not related to each other in hypercholesterolemic children compared with the high respective correlations of PS. [41] In the current study, RBC and plasma TC after EZE tended to correlate ($r=0.71$, $P=0.05$), while PS in RBC and plasma were strongly correlated ($r=0.90$, $P=0.003$) and had a perfect linear relation when adjusted for cholesterol ($r=1.00$, $P<0.0001$). Comparable levels of PS in plasma and RBC suggest a rapid exchange of PS between two compartments, suggesting that large amounts of PS in plasma could probably replace cholesterol in RBC membrane. In addition, strong correlation between decreases in plasma and RBC total PS to cholesterol ratio suggests tissue stores of PS represented by RBC sterols were also reduced with EZE treatment. As previously described by Salen et al., [19] EZE therapy over 14 wks was well-tolerated and with no adverse effect liver functions and serum marker of muscle damage. Previous studies have suggested that liver dysfunction is correlated with increased PS levels. [16, 42, 43] Herein, we found no correlations between plasma and RBC PS and liver enzymes in either phase. A number of limitations need to be noted regarding the present study. The numbers of patients were relatively small. However, it should be noted that STSL is an extremely rare disease with fewer than 100 cases reported worldwide. Furthermore, this study included patients of only a single ethnic group, the Hutterites, all with a single genotype and, as such, the results from this study cannot be generalized to other populations. The current investigation was also limited by short duration. Long-term follow-up is needed to fully assess the ability of EZE to further reduce plasma and tissue

PS levels. Given that RBC have a long life span (e.g., 120 d) compared with platelet, which have an estimated turnover of 9-10 days [44] longer duration of treatment may be required to see a marked effect on RBC indices. Also three patients were on EZE prior to study, which may have blunted the effect despite the washout period. Notwithstanding these limitations, the study suggests that EZE decreased plasma and RBC PS concentrations as expected, but most noteworthy treatment also increased platelet count and decreased platelet size in STSL patients.

5.7 CONCLUSION

PS incorporation into the platelet membrane leads to macrothrombocytopenia, which can result in severe bleeding episodes. Blocking the absorption of PS with EZE may EZE decreased plasma and RBC PS concentrations, increased platelet count and decreased platelet size in STSL patients. Therefore, EZE treatment may decrease risk of bleeding in STSL patients who are more prone to macrothrombocytopenia. The study also suggests that the ABCG5/8 genes and plasma plant sterols should be investigated in patients with unexplained hematologic abnormalities.

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RAO initiated research question and conception, performed analysis/interpretation of the data, and wrote the first draft of the paper. RAO is grateful to the Libyan Scholarship Program and the Manitoba Health Research Council Graduate Student Fellowship for financial support.

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BRIDGE TO CHAPTER VI

Given that the human body cannot synthesize PS, EZE progressively reduces their accumulation in plasma and tissue and thus improves platelet indices in STSL as shown in Chapter V. STSL patients have also elevated 5α-stanols, 5α-saturated derivatives of sterols, including sitostanol and cholestanol in plasma and tissues such as atherosclerotic lesions and xanthoma. Sitostanol, the saturated derivative of sitosterol, is derived from diet, while cholestanol, saturated derivative of cholesterol, is derived from diet and endogenous synthesis. Cholesterol in the gut can be hydrogenated by anaerobic bacteria to cholestanol. Endogenous cholestanol is synthesized in the liver from direct conversion of cholesterol to cholestanol or indirect transformation of bile acid precursor 7α-hyroxycholesterol and derivative 7α-hydroxy-4-cholesten-3-one (7α-H-C4), to cholestanol. STSL patients have increased level of 5α-stanols but reduced endogenous syntheses of cholesterol and bile acids. Thus, it is not clear whether cholestanol in STSL patients is mostly synthesized endogenously or comes from diet. If cholestanol is endogenously produced, is it derived from cholesterol or bile acid pathway? Endocrine disruption has also been reported in STSL. In fact, synthesis of thyroid hormone appears to be deranged in STSL, and elevated plasma cholestanol levels are found in patients with hypothyroidism. EZE effect on plasma cholestanol levels in STSL has not been investigated yet. In **Chapter V**, EZE improves macrothrombocytopenia in STSL. However, so far, it is not known if EZE reverses endocrine disruption in STSL patients. The following study will determine the effects of EZE on plasma and RBC cholestanol relative to sitostanol. Cholestanol precursor levels (cholesterol and 7α-H-C4) will be measured to delineate whether cholestanol in STSL is derived from cholesterol or

through induction of the bile acid pathway. Furthermore, the following chapter will seek to find if a relationship exists between 5α-stanols and serum thyroid hormones in STSL, and to determine whether EZE normalizes disrupted thyroid hormones in STSL *via* decreasing plasma and tissue 5α-stanol levels.

CHAPTER VI

MANUSCRIPT 5

EZETIMIBE EFFECTS ON CIRCULATING 5α-STANOLS AND THYROID HORMONES IN SITOSTEROLEMIA

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6.1 ABSTRACT

Sitosterolemia (STSL) is a sterol storage disorder characterized by very high plasma plant sterols (PS) levels and their 5α-saturated derivatives (5α-stanols) in plasma and tissues. Plasma sitostanol is a 5α-stanol derived from diet while cholestanol is a 5α-stanol analogue of cholesterol derived from diet and endogenous synthesis: cholestanol precursors (cholesterol and bile acid precursor 7α-hydroxycholesterol and the derivative 7α-hydroxy-4-cholesten-3-one, 7α H-C4. Cholestanol levels are usually high in STSL patients while cholesterol and bile acid synthesis rates are low. It is not clear if plasma cholestanol in STSL is mostly endogenously synthesized or derived from diet. If cholestanol is endogenously produced, is it from cholesterol or 7α H-C4?

Endocrine disruption has been reported in STSL, and synthesis of thyroid hormones appears to be deranged as well. Plasma 5α-stanols (i.e. cholestanol) were elevated in patients with underactive thyroid. We aimed to investigate whether plasma cholestanol is mostly from diet or endogenously synthesized, and determine if endogenous cholestanol is derived from cholesterol or 7α-H-C4. We examined the effect of ezetimibe (EZE), a sterol absorption inhibitor, on plasma and tissue, represented by red blood cells (RBC), cholestanol, and cholesterol synthesis precursor lathosterol and 7α-H-C4 relative to sitostanol (exogenous 5α-stanol). Further, we evaluated the relationship between 5α-stanols and serum thyroid hormones in STSL.

METHODS AND RESULTS

STSL patients (n=8) received no EZE for 14 wks followed by EZE (10 mg/d) for another 14 wk. Blood was collected during 14 wks off and on EZE for measuring circulating levels of 5α-stanols, total cholesterol (TC), cholestanol precursors, and serum thyroid

hormones. EZE reduced plasma (-38±6%, p<0.0001) and RBC (-20±4%, p=0.001) sitostanol levels over 14 wks compared with off EZE. EZE reduced plasma cholestanol at wk6 (-40±3%, p<0.0001); however, levels rose during the last 4 wks of 14 wks study, only a decrease of 18±6% (p=0.001) was noted at wk14. EZE reduced RBC cholestanol levels (-13±3%, p<0.01). EZE did not affect serum 7α-H-C4 (1±23%, p=0.31) but increased plasma lathosterol levels over 14 wks. All changes in plasma lathosterol after EZE were similar to those of the RBC. Absolute change in plasma cholestanol at wk14 on EZE linearly correlated with the absolute change in TC (r=0.88, p=0.004) but not with 7α-H-C4 (r=0.18, p=0.70), suggesting that endogenous cholestanol in STSL is mostly synthesized from cholesterol. In patients off EZE, serum free triiodothyronine (T3) tended to decrease (-10%, p=0.05) at wk8 relative to baseline. Furthermore, decreases in serum thyroid-stimulating hormone (TSH) from baseline correlated with decreases in plasma lathosterol (r=0.94, p=0.006, n=6). When EZE resumed, the decrease in serum free T3 was normalized (13%). The ratio of free T3 to free thyroxine (T4) (10%) and thyroid-stimulating hormone were increased (TSH, 2-fold) (all, p<0.05). Absolute changes in serum free T3 and T4 on EZE, inversely correlated with absolute changes in cholestanol (r=-0.67, p=0.05 and r=-0.78, p=0.04) and sitostanol (r=-0.82 and r=-0.74, p<0.05), suggesting inverse associations between 5α-stanols and thyroid hormones.

CONCLUSION

EZE progressively decreased plasma and tissue levels of sitostanol over time but only moderately reduced cholestanol. Cholesterol synthesis precursor lathosterol increased after EZE but not 7α-H-C4. These results, taken together with the correlation between cholestanol and cholesterol levels, suggest that cholestanol in STSL is mostly synthesized

from cholesterol. It also appears that when absorption of dietary cholesterol is restricted with EZE cholesterol synthesis is upregulated. Inverse correlations between 5α-stanols and thyroid hormones suggest that these non-cholesterol sterols may be endocrine disruptors. EZE normalized disrupted serum thyroid hormones by reducing plasma and tissue 5α-stanols. EZE may reduce risk of developing some hormone related diseases in STSL.

KEYWORDS: Cholestanol, sitostanol, lathosterol, 7α-hydroxy-4-cholesten-3-one, thyroid hormones

6.2 INTRODUCTION

Sitosterolemia (STSL) is a rare cholesterol storage disease in which large amounts of plant sterols (PS) and their respective 5α-saturated derivatives (5α-stanols) accumulate in plasma and tissues due to failure of sterol efflux (1). It is caused by mutations in either genes encoding ATP-binding cassette, subfamily G5 (*ABCG5*) or G8 (*ABCG8*), and clinically characterized by presentation of xanthomas, accelerated atherosclerosis. Chronic hemolytic anemia, macrothrombocytopenia and endocrine disruption are also reported (2). Accumulation of dietary PS and 5α-stanols appears to disrupt the endocrine system and cause infertility in ABCG5/8-knockout (KO) mice (3), although in humans endocrine disruption has only been reported once (4).

Diets provide ample amounts of cholesterol and PS but small quantities of their respective 5α-stanols (5-7). Normally plasma levels of sitostanol are derived solely from the diet (7) while cholestanol, the 5α-stanol analog of cholesterol, is mostly derived from endogenous synthesis (~12 mg/d) and to a small extent from the diet (<2 mg/d from animal products) (5, 6). Cholestanol is synthesized in the liver from cholesterol by two possible pathways: direct conversion of cholesterol to cholestanol (8) or indirect transformation of bile acid precursor 7α-hydroxycholesterol and the derivative 7α-hydroxy-4-cholesten-3-one (7α H-C4) to cholestanol **(Figure 6.1)** (9, 10). Normally plasma cholestanol levels are low (0.1 to 0.4 mg/dl) (11), but they can be elevated (1 to 4 mg/dl) in cerebrotendineous xanthomatosis (CTX), an autosomal recessive disorder of bile acid synthesis. CTX is caused by mutations in *CYP27A1* gene, which codes for sterol 27-hydroxylase, an essential enzyme for bile acids synthesis (12). Deficiency of this

enzyme results in pathological induction of cholesterol 7α-hydroxylase (CYP7A1) to increase 7α-hyroxycholesterol and 7α-H-C4, which is converted to cholestanol that accumulates in plasma and tissues of affected patients (9, 13, 14).

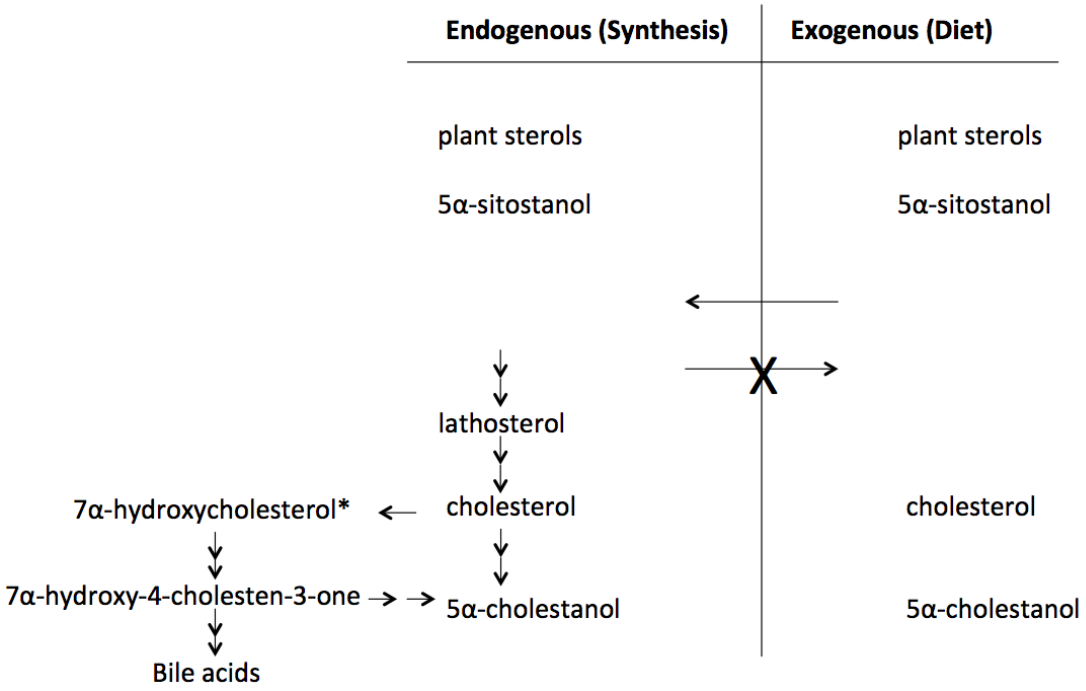

Figure 6. 1*: Cholestanol biosynthesis. Cholestanol is synthesized in the liver from either direct conversion of cholesterol to cholestanol or indirect transformation of bile acid precursor 7α-hyroxycholesterol and derivative 7α-hydroxy-4-cholesten-3-one (7α-H-C4), to cholestanol.*Most cholestanol in CTX is synthesized through 7α-hydroxy-4-cholesten-3-one. 5α-sitostanol is solely derived from diet.*

Elevated cholestanol levels are also found in STSL (1 to 2.3 mg/dl) (11), which may be due to unrestricted sterol absorption (5). Given that cholesterol and bile acid syntheses are low in untreated STSL (15, 16), it is not obvious whether plasma cholestanol arises from dietary cholesterol and cholestanol or endogenous synthesis. Elevated serum cholestanol has been found in patients with hypothyroidism, an endocrine disorder in which the thyroid gland does not produce enough thyroid hormones (17). Hypothyroidism has also been observed in CTX (17-19) and STSL patients (20),

suggesting high cholestanol levels might contribute to hypothyroidism. Ezetimibe (EZE) is a sterol absorption inhibitor that interferes with Niemann-Pick-like 1 enterocyte receptor, reducing intestinal uptake of cholesterol and PS (21, 22). In general, plasma cholestanol is an accepted marker of exogenous cholesterol absorption (11); this may suggest that individuals with high plasma cholestanol levels (absorbers) are more likely to benefit from EZE than those with low levels (synthesizers). EZE treatment for 12 wks decreased (-13%) plasma cholestanol levels in hypercholesterolemic subjects with type II diabetes mellitus (0.26 vs 0.3 mg/dl (23). However, in healthy subjects, plasma cholestanol levels were decreased by 37% (0.24 vs 0.3 mg/dl) after 4 wks of EZE (24). EZE has been shown to decrease plasma PS in STSL (22) but its effect on circulating cholestanol has not yet been investigated. The objectives of this investigation were to examine the effect of EZE on plasma and RBC cholestanol, and cholestanol precursors (cholesterol synthesis precursor lathosterol and 7α-H-C4) in STSL in comparison with sitostanol (exogenous 5α-stanol) in order to determine the origin of cholestanol. We also sought to find if there is a relationship between 5α-stanols and serum thyroid hormones in STSL.

6.3 EXPERIMENTAL METHODS

6.3.1 Patients

Eight patients, between 16 and 56 years of age, were recruited from Hutterite colonies in Manitoba, Canada (n=4) and South Dakota, US (n=4) (5 males and 3 females). All patients were identified as having homozygous ABCG8 S107X mutation and related to a proband previously reported by Mymin et al. (20, 25) and Chong et al. (26). All procedures involving human patients were approved by the Data Management and

Coordinating Center, the National Institute of Child Health and Human, the Development Data and Safety Monitoring Board, and the University of Manitoba Biomedical ethics board. Written informed consents were obtained from all patients. The trial was registered with www.clintrials.gov under number NCT01584206.

6.3.2 Study Design

The study was a single-site pilot interventional study of 8 patients with STSL. It was conducted at the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba and designed as a two 14-week phase (off and on EZE) study. After consent baseline blood samples were collected and study patients (n=8) were taken off EZE for 14 weeks during which blood was collected at wk2, wk4, wk6, wk8, wk10, wk12 and wk14 off. On the last day of blood draw, patients were instructed to resume EZE (10 mg/d) for another 14 wks and follow their usual diet. Blood was collected throughout the wk14 on EZE treatment. Fasting blood samples were centrifuged for 20 min at 3000 rpm and separated aliquots of plasma, serum and RBC were frozen at -80°C until analysis. All sterols, precursor sterols and 5α-stanols in plasma were measured at baseline and biweekly up to 14 wks off and on EZE. RBC sterols and 5α-stanols, and serum levels of 7α-H-C4 were determined at baseline, wk4, wk8, wk10 and wk14 off and on EZE. Serum levels of thyroid hormones were measured at baseline, wk8 and wk14 off and on EZE.

6.3.3 Blood Sampling Protocol

Twelve-hour fasting blood samples were collected throughout study period and centrifuged for 20 min at 3000 rpm. The separated aliquots of plasma, serum and RBC were frozen at -80°C until analysis.

6.3.4 Plasma and Red Blood Cells Sterols and Stanols

The concentrations of plasma and RBC sterols and 5α-stanols, were measured by using a gas-liquid chromatography equipped with a flame ionization detector (GC-FID) and an auto sampler system (Varian 430-GC; Agilent Technologies, Santa Clara, CA, US). For sterol quantification, an internal standard, 5α-cholestane was used in combination with sterol standard curve for each individual sterol, using authentic standards (Sigma-Aldrich Ltd. Oakville, ON, Canada) and (MJS BioLynx Inc, Brockville, ON, Canada). Briefly, 5α-cholestane (50 ug) was added to each 0.5 ml plasma or 0.5 g RBC sample, and saponified with 4 ml methanolic potassium hydroxide for 2 h at 100°C. The unsaponifiable portion were extracted twice from the mixture with 4 ml petroleum ether, derivatized with 0.1 ml TMS reagent (pyridine hexamethyldisilazane–trimethylchlorosilane; 9:3:1 by volume), re-suspended in 0.4 ml hexane and injected (1 μl) onto a 30-m SAC-5 column (Sigma-Aldrich Ltd. Oakville, ON, Canada). The column temperature was set to 280°C, with isothermal running conditions maintained for 30 min per sample. The injector and detector temperatures were set at 295°C and 300°C, respectively. The carrier gas (helium) flow rate was 1 ml/min, with the inlet split set at 40:1. (27).

6.3.5 Cholesterol Synthesis

Plasma and RBC concentration of the cholesterol precursor lathosterol was used as a surrogate marker of *de novo* cholesterol synthesis (11) and determined by GC-FID. Lathosterol levels were also adjusted for the TC concentration and expressed as a ratio (μg/mg) (11).

6.3.6 Serum 7α-Hydroxy-4-Cholesten-3-One Levels

Serum levels of 7α-H-C4 were analyzed by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) adapted from Steiner et al. (28). Briefly, 0.5 ml of serum sample were mixed with 0.1 ml of ddH20 and 0.05 ml of the 40 ng/ml deuterated internal standard 7-hydroxy-4-cholesten-3-one-25,26,26,26,27,27,27-d7 (C4-d7) (Avanti Polar Lipids Inc., Alabaster, AL, US) and 0.2 ml of methanol. Pre-conditioning the cartridges (HF Bond Elut-C18200 mg/3 ml, Agilent Technologies, Mississauga, ON, Canada) was achieved by activation with 2 ml methanol followed by 2 ml ddH20, after which the sample was loaded onto the cartridge and allowed to pass through it by gravity. The cartridge was washed twice with 2 ml ddH20 and briefly dried under vacuum. The retained compounds were eluted with 2 ml of methanol using gravity, and the samples were evaporated under nitrogen at 30°C. The dried residue was dissolved in 0.08 ml methanol and passed through a 4 mm syringe filter (Phenex 0.2 µm PTFE membrane; Phenomenex, Torrance, CA, US) before 3 µl 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 kept at 35°C; the gradient system was used with a mobile phase A (0.1% formic acid in ddH20) and a mobile phase B (0.1% formic acid in acetonitrile, Optima grade), a 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 and ran in duplicate. Quantification of 7α-H-C4 was performed using multiple reaction-monitoring (MRM) mode using peak areas. The MRM transitions for 7α-H-C4 were 401.4 > 383.4 m/z and for C4-d7 were 408.4 > 390.4 m/z. The positive mode ESI-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.

6.3.7 Serum Thyroid Hormones

Serum concentrations of thyroid hormones including free triiodothyronine (T3), and free thyroxine (T4) and thyroid-stimulating hormone (TSH), were measured by automated enzymatic methods on the ARCHITECT, Abbott Diagnostics, Gamma Dynacare Medical Laboratories, Winnipeg, Canada).

6.4 STATISTICAL ANALYSIS

Effect of EZE was examined using a mixed model ANOVA procedure with treatment, time, and site as fixed factors and age as a random factor in the model. Significant treatment effects were examined with the Bonferroni adjustment for multiple comparisons. Both treatment and time (with time representing the different time periods) were entered into the model. When a significant treatment effect, but no significant treatment-by-time interaction, was observed, the interpretation was that the treatment effect was essentially consistent over the different time periods. Statistical analysis was performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, US). Results are expressed as

means with their standard errors unless otherwise noted. Data that were not normally distributed, as determined by a Shapiro-Wilk test, were log or inverse transformed before statistical analysis. Relationships between two variables were assessed with stepwise multiple linear regression analysis unless otherwise stated.

6.5 RESULTS

6.5.1 Patients' Characteristics

Baseline characteristics of the patients are shown **(Table 6.1)**. There were eight Caucasian patients (5 males and 3 females) with homozygous ABCG8 S107X mutation (NM_022437.2:c.320C>G). Eight patients were initially recruited, and all completed the trial. There were 5 males and 3 females, with a mean age of 28.0±5.5 years. All patients had confirmed diagnosis of STSL at the time of enrollment in the study; identified as homozygous ABCG8S107X mutation (NM_022437.2:c.320C>G) as previously genotyped by Mymin et al. (20, 25) and Chong et al. (26). Patients with STSL usually have elevated plasma and RBC levels of PS and 5α-stanols (i.e sitostanol and cholestanol) **(Table 6.1)**. PS and 5α-stanols levels in plasma were similar to those in RBC, suggesting a free exchange of sterols between plasma lipoproteins and RBC. RBC indicates a longer-term average of plasma levels and a better reflection for tissue stores. Cholestanol to TC ratio tended (p=0.08) to be greater in RBC than plasma. Lathosterol and its ratio to TC were higher in RBC compared with plasma (5±2-fold, p=0.02) and (10±3-fold, p=0.004), respectively. All sterols in the RBC membrane must be obtained from the plasma since RBC do not synthesize sterols. Like PS, lathosterol can replace a small amount of cholesterol in RBC (29) because it has a low esterification rate compared with cholesterol, and thus free form is available to be taken up by the RBC (30).

Serum thyroid hormones including free T3, free T4, T3/T4 ratio and TSH are shown in **(Table 6.1)**. All patients were euthyroid based on serum TSH, which is the key hormone for diagnosing hyperthyroidism and hypothyroidism. The mean TSH levels were 3±0.3 mU/l ranging from 1.4 to 4.2 mU/l. Serum free T3 levels were lower than the normal range for one patient (0.2 ng/dl) and were within lower limits of the normal range (0.3-0.7 ng/dl) for the rest of the patients (n=6). Serum free T4 levels ranged from 0.7 to 1.2 ng/dl and were within normal range (0.7-1.8 ng/dl). The levels were within the lower limits (0.7 and 0.9 ng/dl) of the serum free T4 normal range for two patients. None of the patients had serum free T4 levels within the upper limits (1.4-1.8 ng/dl) of the normal range. Serum bile acid intermediate 7α-H-C4 varied widely (5-40 ng/ml) with mean of 16±4 ng/ml **(Table 6.1)**. Serum 7α-H-C4 levels were reported to be 63±5 ng/ml in healthy subjects (31) but extremely high in untreated CTX patients (4149± 2370 ng/ml) (32).

Table 6. 1*: Baseline sterols, 5α-stanols and serum derivative of bile acid synthesis and thyroid hormones.*

Sterol	Plasma	RBC
TC (mg/dl)	155.8±12.7	100.8±6.2
Lathosterol (ug/dl)	325.8±156.1	766.2±86.6
Lathosterol/TC ratio (ug/mg)	1.9±0.6	7.9±1.0
Cholestanol (mg/dl)	1.4±0.1	1.2±0.1
Cholestanol/TC ratio (mg/mg)	0.010±0.0	0.013±0.0
Sitosterol (mg/dl)	6.2±0.7	4.6±0.5
Total PS (mg/dl)	9.1±1.1	7.9±0.7
Total PS/TC (mg/mg)	0.06±0.01	0.08±0.01
Sitostanol (mg/dl)	1.1±0.1	0.9±0.1
Sitostanol/TC (mg/mg)	0.008±0.0	0.010±0.0
7α-H-C4/TC ratio (ng/mg)	0.10±0.02	
Free T3 (ng/dl)	0.30±0.02	
Free T4 (ng/dl)	1.0±0.06	
Free T3/Free T4	0.30±0.01	
TSH (mU/l)	2.9±0.3	

Data are mean±SEM; n=8 for demographic data; GC, gas chromatography; PS, plant sterols; TC, total cholesterol; RBC, red blood cells; 7α-H-C4, 7α-hydroxy-4-cholesten-3-one. Data are mean±SEM; n=7, biochemical data was not available for one patient.

6.5.2 Changes in Plasma and RBC Cholestanol and its Ratio to Cholesterol in Response to Ezetimibe

Plasma cholestanol levels were affected by treatment, time (all, $p<0.0001$) and treatment-by-time interaction ($p=0.003$). Plasma cholestanol levels fell (-40±3%, $p<0.0001$) sharply at wk6. However, the levels steadily increased during the last 4 wks of 14 wks on EZE, and only a decrease of 18±6% ($p=0.001$) was observed at wk14 compared with off EZE. A similar observation was also noted for plasma TC; decreases in plasma TC after EZE was noted at 6 wks (-37±3%, $p<0.0001$) but the levels rose at the last 4 wks of 14 wks, and only a decrease of 12±6% ($p=0.01$) was noted at wk14 on EZE compared with no treatment **(Figure 6.2A** and **B)**.

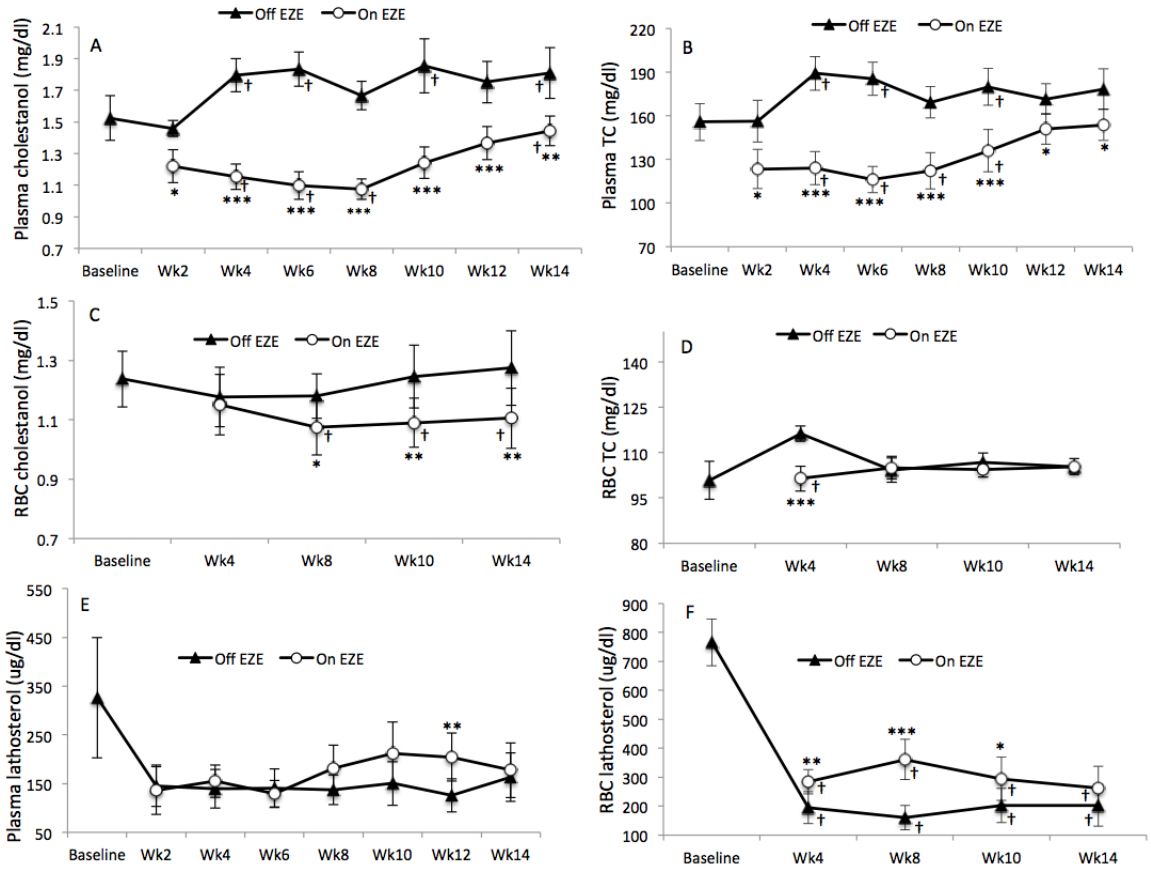

Figure 6. 2*: Plasma and red blood cells cholestanol, cholesterol, lathosterol throughout study period off and on ezetimibe.*
*A, C, cholestanol; B, D, cholesterol; E, F, lathosterol. Asterisk denotes significantly different compared with off EZE. Data are mean± SEM; n=8. *p<0.05, **p<0.01, ***p<0.0001). P values are shown for the treatment effect analyzed by mixed model ANOVA (with Bonferroni adjustment for multiple comparisons). Special character (†) denotes significantly different when compared within treatment group from baseline p<0.05 (two-tailed paired-Student's t test). TC, total cholesterol; RBC, red blood cells.*

When plasma cholestanol was adjusted for TC, the decrease was not sustained at wk14 (-6±2%, p=0.05) compared with off EZE. RBC cholestanol levels were affected only by EZE treatment (p<0.0001) with decreases sustained at wk14 on EZE compared with off EZE (-13±3%, p<0.01) **(Figure 6.2C)**. RBC TC remained comparable after 14 wks off and on EZE (105±3 vs 105±2 mg/dl, p=1.0) **(Figure 6.2D).** When RBC cholestanol levels were adjusted for RBC TC, the decrease was maintained at wk14 compared with off EZE (-13±3%, p<0.01).

6.5.3 Changes in Plasma and Red Blood Cells Cholesterol Synthesis Marker Lathosterol in Response to Ezetimibe

Treatment (all, p=0.04) and treatment-by-time interaction effects were observed on plasma lathosterol (p=0.01). Increases in plasma lathosterol were evident at wk 12 (+84±23%, p=0.004) but became insignificant at wk14 (p=0.64). Likewise, EZE and time had significant effects (p<0.0001) on RBC lathosterol, but not their interaction (p>0.29), suggesting a consistent EZE effect on RBC lathosterol. RBC lathosterol increased (2-fold, p<0.01) at wk4 on EZE and remained elevated until wk10. However, lathosterol levels dropped and thus increases became insignificant at wk14 (263±75 vs 203±72 ug/dl, p=0.13) compared with off EZE **(Figure 6.2E** and **F)**. Similar results were also noted when plasma and RBC lathosterol levels were adjusted for TC levels.

6.5.4 Changes in Serum 7α-Hydroxy-4-cholesten-3-one Levels in Response to Ezetimibe

Serum 7α-H-C4 levels were not affected by treatment (p=0.32), time (p=0.49) or treatment-by-time interaction (p=0.31) with a change of 1±23% noted at wk14 on EZE compared with no treatment. This suggests that EZE had no effect on cholestanol precursor **(Figure 6.3)**. A similar result was noted when serum 7α-HC levels were adjusted for plasma TC (p=0.63).

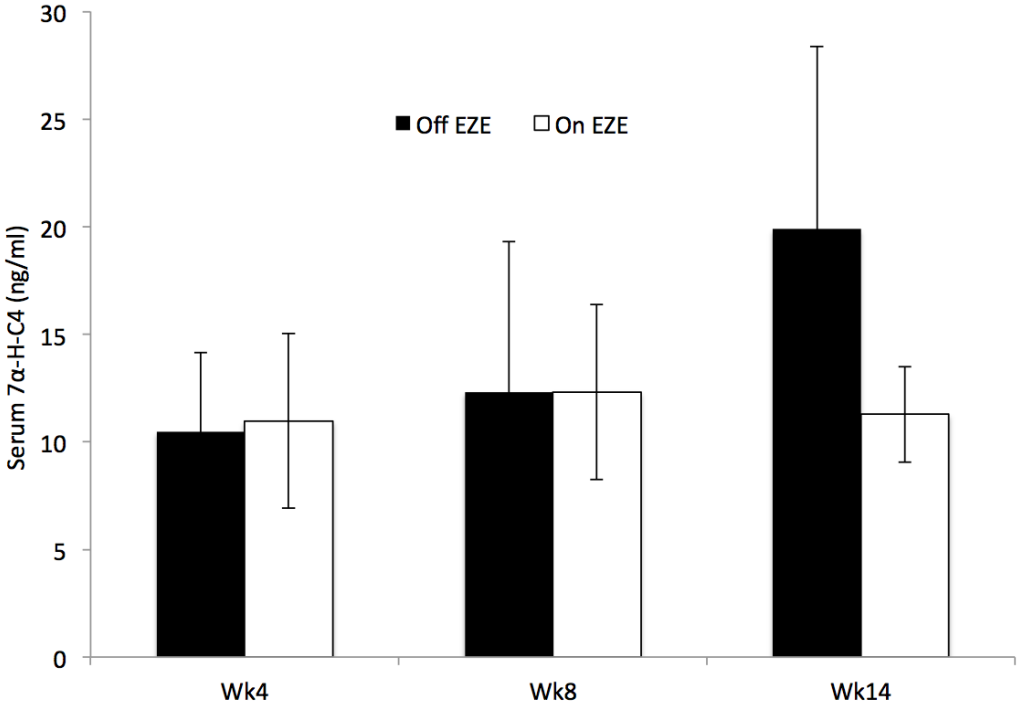

Figure 6. 3*: Serum 7α-hydroxy-4-cholesten-3-one levels throughout study period off and on ezetimibe. Data are mean± SEM; n=8. The treatment effect analyzed by mixed model ANOVA (with Bonferroni adjustment for multiple comparisons). 7α-H-C4, 7α-hydroxy-4-cholesten-3-one.*

6.5.5 Changes in Plasma and Red Blood Cells Sitostanol and its Ratio to Cholesterol in Response to Ezetimibe

Significant treatment ($p<0.0001$), time ($p=0.002$) and treatment-by-time interaction ($p=0.02$) effects were observed on plasma sitostanol. Plasma sitostanol levels were reduced as early as 6 wks (-23±9%, $p=0.001$), with further decreases noted at 14 wks (-38±6%, $p<0.0001$) compared with off EZE **(Figure 6.4A)**. Plasma sitostanol to TC ratio was affected by time ($p=0.005$) and its interaction with treatment ($p<0.0001$) but not treatment alone ($p=0.31$), with a decrease of 28±7% ($p<0.0001$) noted after 14 wks on EZE. Likewise, RBC sitostanol levels were affected by treatment ($p=0.001$) and time ($p<0.0001$) and there was a tendency for their interaction ($p=0.08$) with a decrease of 21±2%, $p<0.0001$ noted at wk14 on EZE compared with off EZE **(Figure 6.4B)**. When

RBC sitostanol levels were normalized for TC levels, the effect of interaction of treatment-by-time was significant (p=0.004) with a decrease of 20±4% (p=0.001) noted at 14 wks on EZE compared with off EZE, suggesting that EZE needs time to produce further reduction in plasma and RBC sitostanol levels.

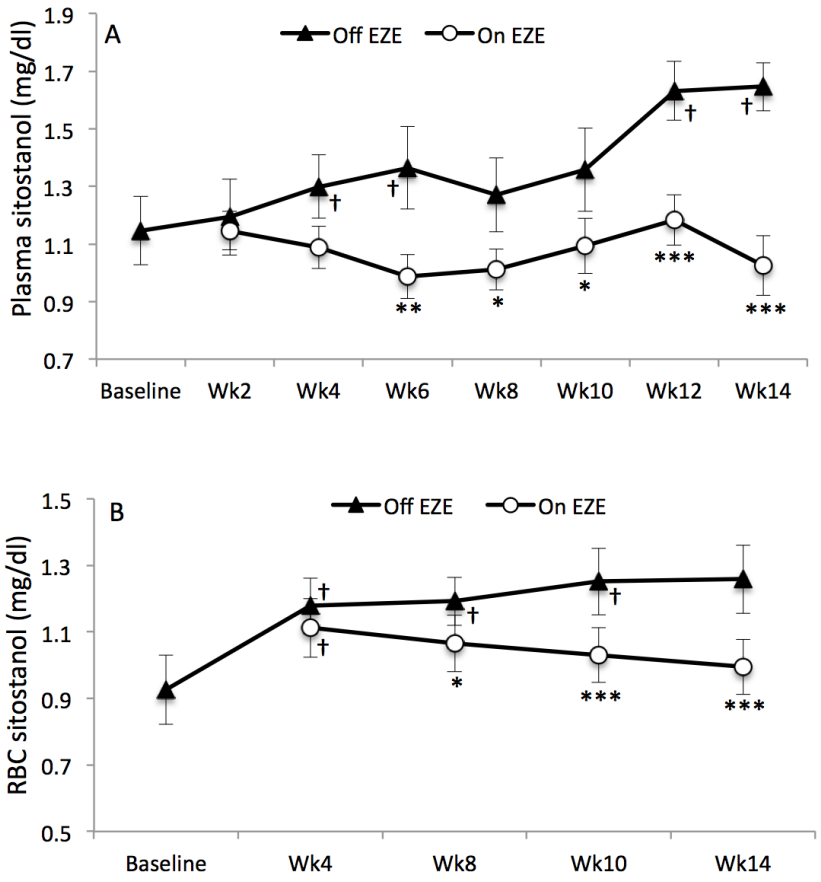

Figure 6. 4*: Plasma and red blood cells sitostanol levels throughout study period off and on ezetimibe. A, plasma;B, RBC. Data are mean± SEM; n=8. Asterisk denotes significantly different compared with off EZE: *p<0.05, **p<0.01, ***p<0.0001). P values are shown for the treatment effect analyzed by mixed model ANOVA (with Bonferroni adjustment for multiple comparisons). Special character (†) denotes significantly different when compared within treatment group from baseline p<0.05 (two-tailed paired-Student's t test). RBC, red blood cells.*

6.5.6 Changes in Serum Thyroid Hormones in Response to Ezetimibe

Serum thyroid hormones (free T3, free T4, free T3/T4 ratio and TSH) at wk8 and wk14 off and on EZE are shown in **(Figure 6.5)**. At wk8 off EZE, serum free T3 tended to decrease (-10±7%) compared with baseline (0.27±0.02 vs 0.30±0.02 ng/dl, p=0.05) **(Figure 6.5A)**. Decreases in serum free T3 from baseline (r=0.77, p=0.07) tended to correlate with decreases in lathosterol to TC ratio. At wk14 off EZE, plasma cholestanol and sitostanol were inversely correlated with serum free T3 (r=-0.92, p=0.001 and r=-0.65, p=0.08) and T3/T4 ratio (r=-0.74, p=0.04 and r=-0.89, p=0.003). Moreover, decreases in serum TSH at wk14 off EZE from baseline correlated with decreases in plasma lathosterol (r=0.94, p=0.006, detectable in n=6) and its ratio to TC (r=0.86, p=0.03).

There was a treatment effect (p=0.01) but neither time (p=0.56) nor treatment-by-time interaction (p=0.82) on serum free T3. At 8 wks on EZE, serum free T3 increased (13±5%, p=0.04) from 0.27±0.02 to 0.30±0.02 ng/dl; however, increases became insignificant (p=0.07) at wk14 compared with off EZE (0.31±0.02 vs 0.28±0.02 ng/dl). Serum free T4 levels were not affected by treatment (p=0.25), time (p=0.50) or treatment-by-time interaction (p=0.67), and remained comparable between off and on EZE (0.95±0.05 vs 1.0±0.06 ng/dl, p=0.24) **(Figure 6.5B)**. There was a treatment effect (p=0.001) but neither time (p=0.17) nor treatment-by-time interaction (p=0.87) on serum free T3/T4 ratio, suggesting a consistent effect of EZE on free T3/T4 ratio **(Figure 6.5C)**. Serum free T3/T4 ratio increased (10±4%, p=0.02) at 8 wk on EZE and this increase was sustained at wk14 on EZE compared with no treatment (0.32±0.03 vs 0.29±0.02, p=0.02).

After 8 wks on EZE, absolute changes in plasma cholestanol and sitostanol inversely correlated with absolute changes in serum free T3 and T4 (r=-0.67, p=0.05 and r=-0.78, p=0.04) and (r=-0.82 and r=-0.74, p<0.05), respectively. Effect of EZE on serum TSH levels was consistent (p=0.008) over the time period with a two-fold increase (p=0.008) noted at wk8 on EZE; however, these increases were insignificant at wk14 compared with off EZE (3.5±0.5 vs 2.6±0.4 mU/l, p=0.09) **(Figure 6.5D)**.

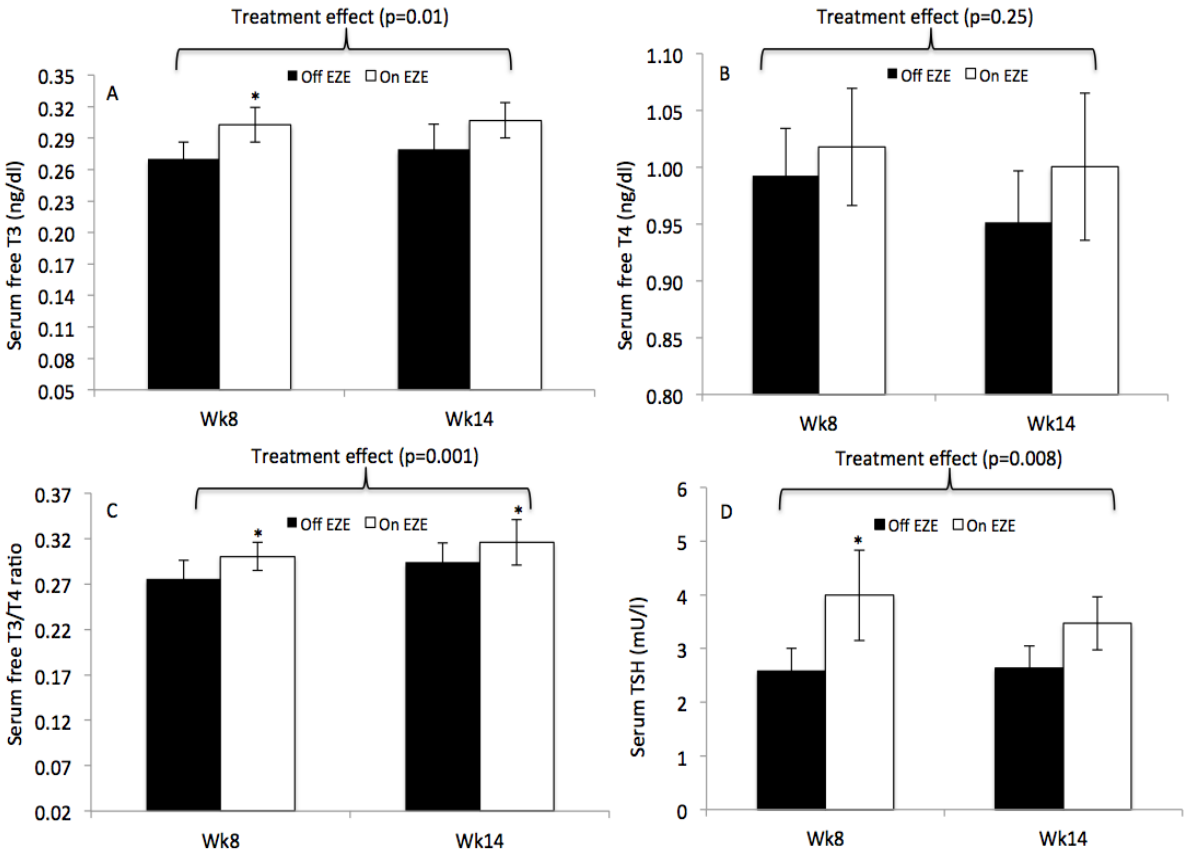

Figure 6. 5*: Serum levels of thyroid hormones at weeks 8 and 14 off and on ezetimibe*
*A, free T3; B, free T4, C, free T3/T4 ratio; D, TSH. Data are mean± SEM; n=8. Asterisk denotes significantly different compared with off EZE: *p<0.05. Linear mixed model ANOVA determined significant treatment effect but no time effect, or treatment-by-time interaction. P values are shown for the treatment effect analyzed by mixed model ANOVA (with Bonferroni adjustment for multiple comparisons). EZE, ezetimibe; T3, triiodothyronine; T4, thyroxine; TSH, thyroid-stimulating hormone.*

6.6 DISCUSSION

The results showed that all patients with untreated STSL had increased 5α-stanols in plasma and tissue, represented by RBC and low endogenous cholesterol synthesis as evidenced by low lathosterol levels, cholesterol synthesis marker. EZE decreased 5α-stanols in plasma and RBC with progressive declines sustained over time for sitostanol, which is derived from diet, but moderate reductions for cholestanol, which is derived from diet and endogenous synthesis. EZE did not affect serum cholestanol precursor 7α-H-C4 but increased plasma and RBC distance cholestanol precursor lathosterol over 14 wks, and thus precursor availability for synthesis of cholestanol. These results, taken together with the correlation between plasma cholestanol and TC suggest that cholestanol in STSL is mostly synthesized from cholesterol rather than 7α-H-C4. In STSL, 5α-stanols inversely correlated with thyroid hormones; this may suggest that these non-cholesterol sterols may be endocrine disruptors. By reducing plasma and tissue 5α-stanols EZE normalized disrupted serum thyroid hormone balance, and may therefore, reduce risk of developing some hormone related diseases in STSL.

Diets contain ample amount of cholesterol and PS, but provide small quantities of 5α-stanols (5, 6). Plasma cholestanol in healthy individuals is mostly derived from endogenous synthesis (~12 mg/d): cholestanol precursors (cholesterol and 7α-H-C4), and to a small extent from the diet (<2 mg/d from animal products) (5, 6). Increased plasma cholestanol levels are found in a condition of disturbed bile acid metabolism such seen in cerebrotendinous xanthomatosis (CTX), an autosomal recessive disorder of bile acid synthesis. CTX is caused by mutations in *CYP27A1* gene, which codes for sterol 27-

hydroxylase, an essential enzyme for bile acids synthesis (12). The major part of the cholestanol formed in CTX is derived from conversion of 7α-hydroxylated intermediates in bile acid synthesis, such as 7α-hydroxy-4-cholesten-3-one or its precursor, 7α-H-C4, into cholestanol (9, 10). STSL is another autosomal recessive disorder characterized by increased plasma and tissue level of PS and 5α-stanols, and caused by mutation in either *ABCG5* or *ABCG8* genes. It is clinically characterized by presentation of xanthomas, accelerated atherosclerosis, and occasionally arthritis, arthralgias, hemolysis and macrothrombocytopenia, and endocrine disruption (2).

Plasma cholestanol levels are also elevated in STSL (1.8 mg/dl), which might be due to unrestricted sterol absorption (5). In some heterozygous STSL cholestanol levels can also be higher (0.8 mg/dl) than normal range (0.1-0.5 mg/dl) (33, 34). In the current study, all untreated STSL patients had increased plasma cholestanol (1.1 to 2 mg/dl) as previously reported (15). These STSL homozygotes had higher plasma cholestanol levels than their heterozygotes and wild-type (1.8±0.2 vs 0.5±0.03 and 0.4±0.03 mg/dl, $p<0.0001$, unpublished data).

Lathosterol is a surrogate marker for cholesterol synthesis, which is transformed into to cholestanol. Thus, lathosterol is a distant precursor of cholestanol (35). Cholesterol biosynthesis is reduced in untreated STSL patients. In fact, baseline plasma lathosterol levels in current patients ranged from 0.08 to 0.9 mg/dl with mean of 0.3±0.1 mg/dl, confirming reduced cholesterol biosynthesis in STSL as previously reported (36). Given that cholesterol and bile acid syntheses are low in untreated STSL (15, 16), it is not clear

whether cholestanol in STSL patients is mostly synthesized endogenously or comes from diet. If the endogenous production of cholestanol is possible, then is it coming from cholesterol or through induction of the bile acid pathway?

EZE is a sterol absorption inhibitor that interferes with Niemann-Pick-like 1 enterocyte receptor, reducing intestinal uptake of cholesterol and PS (21, 22). Previous studies showed that EZE treatment for 12 wks decreased (-13%) plasma cholestanol levels in hypercholesterolemic subjects with type II diabetes mellitus (0.26 vs 0.3 mg/dl (23). In healthy subjects, EZE treatment for 4 wks decreased plasma cholestanol levels by 37% (0.24 vs 3 mg/dl) (24). Thus far, effect of EZE on circulating cholestanol levels in STSL has not been investigated yet. Herein, in patients off EZE, plasma cholestanol increased ($p=0.03$) by 35±11% compared with baseline. After resuming EZE, plasma cholestanol levels were reduced by 40% at wk 6 but the levels rose during the last 4 wks of the 14 wks, and only a decrease of 18% was noted at wk14. When adjusted for plasma TC level the decrease in plasma cholestanol was small (-6%, $p=0.05$). It is possible that EZE reduces plasma cholestanol at wk6 presumably by lowering the gastrointestinal absorption of cholestanol and cholestanol precursor cholesterol. Steadily increases in plasma cholestanol levels observed during the last 4 wks of the 14 wks suggest increases in precursor availability for synthesis of cholestanol. Measurement of plasma sterols reflects most recent meals while that of RBC indicates a longer-term average of plasma levels and a better reflection for tissue stores. In contrast to plasma, the decrease in RBC cholestanol was consistent over time, and sustained when normalized for TC (-13%). This observation is likely to result from slow incorporation of cholestanol from plasma

into RBC membrane since RBC do not make cholesterol or cholestanol (29). Plasma sitostanol, an exogenous 5α-stanol, is also elevated in STSL (7). When patients were 14 wks off EZE, plasma and RBC sitostanol levels were increased ($p<0.01$) by 60 and 35%, respectively, compared with baseline. EZE progressively reduced plasma (-38%) and RBC (-20%) levels of sitostanol over 14 wks. These reductions were maintained over 14 wks of EZE treatment.

Compared with progressive decreases in sitostanol, modest decreases in plasma cholestanol in current STSL patients may be due to increasing precursor availability for cholestanol synthesis. Herein, EZE increased circulating cholesterol precursor lathosterol and its ratio to TC over 14 wks. These increases remained significant up to wk12 on EZE compared with off EZE but not at wk 14. This may be due to the negative feedback of cholesterol or cholestanol on lathosterol levels. In patients on EZE, plasma lathosterol and its ratio positively correlated with plasma TC ($r=0.70$, $p=0.05$) and TC ($r=0.81$, $p=0.02$), respectively. In contrast, EZE did not affect serum cholestanol precursor 7α-H-C4 levels over 14 wks. Unlike CTX, cholestanol levels in STSL are more likely to be derived from cholesterol, and not 7α-H-C4 (15), and that increases in cholesterol marker lathosterol increase precursor availability for synthesis of cholestanol. This was also supported by the correlations noted between plasma cholestanol and cholesterol before and after EZE ($r=0.96$ and $r=0.88$, $p<0.01$) but not with 7α-H-C4 ($r=0.23$ and $r=0.18$ $p>0.60$). Taken together, these results suggest that cholestanol in STSL is mostly synthesized from cholesterol and not through induction of the bile acid pathway, which appears to be fairly specific to CTX.

Accumulation of dietary PS and 5α-stanols appears to disrupt the endocrine system and cause infertility in ABCG5/8-knockout (KO) mice (3), although in humans endocrine disruption has only been reported once (4). Deposition of these non-cholesterol sterols was associated adrenal insufficiency and lower tissue cholesterol content in STSL (2, 3, 37). This endocrine insufficiency was normalized by EZE treatment in a mouse model of STSL (37, 38). Moreover, synthesis of thyroid hormones appeared to be deranged in STSL (20). Elevated serum cholestanol levels have also been observed in patients with hypothyroidism, an endocrine disorder in which thyroid gland does not produce enough thyroid hormones (17), which has also been reported in CTX (17-19) and STSL (20), suggesting that 5α-stanols may be endocrine disruptors. Thyroid glands predominantly secrete the pro-hormone T4 that is converted to the active hormone T3 in response to TSH, which is produced by the pituitary gland. EZE has no effect on the metabolism of thyroid hormones in healthy subjects (39, 40). Herein, serum free T3 levels tended to decrease (-10%, p=0.05) at wk8 off EZE relative to baseline. Serum free T3 and T3/T4 ratio inversely correlated with plasma cholestanol and sitostanol, and directly correlated with absolute changes in plasma lathosterol to TC ratio. After EZE, decrease in serum free T3 was normalized along with increases in T3/T4 ratio (10%) and TSH (2-fold) at wk8 compared with off EZE. EZE-induced changes in serum free T3 and T4 inversely correlated with that in cholestanol and sitostanol levels. Only plasma 5α-stanols showed significant inverse correlation with serum free T3 while sitosterol and total PS inversely correlated with serum free T4 (data not shown). Increases in serum free T3 and its ratio to T4 after EZE may be due to increased peripheral conversion of T4 to T3, which is controlled by TSH to fulfill the increased demand in the peripheral tissues for thyroid

hormones. These results, taken together, with direct correlations between TSH and lathosterol, and inverse correlations between free T3 and 5α-stanols suggest that EZE may normalize disrupted thyroid hormone balance by reducing plasma and tissue 5α-stanol and increasing cholesterol biosynthesis. Unlike, adrenal hormones, which are synthesized from cholesterol, thyroid hormones are synthesized from the amino acid tyrosine. Thus, it is unclear therefore, why thyroid hormone levels are altered in STSL. Research shows that estradiol, steroid sex hormone, directly affects the thyroid by thwarting its ability to produce thyroid hormones (41). Some studies reported that PS and stanols have estrogenic effect (42-44), and have been shown to bind to estrogen receptor (ER) (45) and possibly affecting the secretion of thyroid hormones. There is also the potential for PS and stanols to decrease *de novo* steroid synthesis through reductions in cholesterol levels, and therefore PS and stanols may indirectly affect estrogen levels through means other than ER binding. EZE may indirectly improve thyroid hormones by reducing 5α-stanols and PS and increasing cholesterol biosynthesis in STSL patients.

6.7 CONCLUSION

STSL patients have increased 5α-stanols in plasma and tissues. EZE treatment decreases 5α-stanols with progressive decreases sustained over time for those derived from diet (i.e. sitostanol) but moderate reductions for those of endogenous origin (cholestanol). EZE increased lathosterol, cholesterol precursor but did not affect serum 7α-H-C4. The results, taken together with the correlation between cholestanol and cholesterol levels, suggest most cholestanol in STSL is synthesized from cholesterol. It also appears when absorption of dietary cholesterol is restricted with EZE cholesterol synthesis is increased. Negative correlations between 5α-stanols and thyroid hormones suggest that these non-

cholesterol sterols may be endocrine disruptors. EZE may reverse endocrine disruption in STSL by reducing plasma and tissue levels of PS and 5α-stanols and increasing cholesterol biosynthesis.

6.8 ACKNOWLEDGMENTS AND AUTHORS' CONTRIBUTIONS

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RAO initiated the research question and the conception, and performed analysis/interpretation of the data, and wrote the first draft of the paper. RAO is grateful to the Libyan Scholarship Program and the Manitoba Health Research Council Graduate Student Fellowship for financial support. The authors declare that there are no conflicts of interest.

6.9 REFERENCES

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CHAPTER VII

OVERALL CONCLUSION

7.1 SUMMARY AND IMPLICATIONS

The results of the present research have important implications for the clinical efficacy of ezetimibe (EZE) in sitosterolemia (STSL). STSL is considered as a rare disease with only about 100 cases reported worldwide; however, in the past 8 years 13 patients from 8 separate families were identified in one location, indicating that the disorder is not as rare as initially recognized (1, 2). Increased tissue retention of plant sterols (PS) in STSL, due to a complete failure of sterol efflux along with low cholesterol biosynthesis indicates impaired whole body sterol homeostasis (3). Low intestinal cholesterol synthesis may in turn enhance cholesterol absorption and encourage partition of PS into in several body compartments, thus contributing further to whole body sterol accumulation. Low PS diets provide only limited reductions in plasma PS levels with no impact on cholesterol biosynthesis. Medical treatments, such as bile salt binding resins, are poorly tolerated and insufficiently effective to reduce plasma PS levels as well as failed to modulate cholesterol biosynthesis (2). Ezetimibe (EZE) emerges as a new sterol absorption inhibitor that reduces plasma PS and cholesterol (4, 5). However, the available reports have questioned the efficacy of EZE to treat STSL since plasma PS levels reached a long-term plateau (5, 6) even with higher doses of EZE (40 mg/d) (6). In response to significant controversy surrounding the effectiveness of EZE to reduce whole body PS tissue stores, data in **Chapter IV** provide an evidence that EZE is capable of providing progressive decreases in plasma sitosterol levels (-35%) that led to a decrease (-28%) in whole body sitosterol pool size in STSL patients. With EZE therapy at 10 mg/d, whole

body sitosterol pool size linearly correlated with plasma sitosterol levels (r=0.84, p=0.02), reflecting a depletion of sitosterol tissue stores with EZE. Decreases in plasma PS levels are sustained over time due to the absence of endogenous synthesis. For the first time, we quantified total body sitosterol pool size using stable isotope labeled sterols. The values of whole body sitosterol pool size were similar to those previously reported in STSL patients using radioisotope tracer methods (7, 8), and were at least 30 times larger than those in heterozygous STSL (7, 9).

As discussed in **Chapter IV**, decreases in plasma TC levels were not sustained over time due to the endogenous synthesis. EZE decreased plasma TC (-12.5%), LDL-cholesterol (-22.7%) and whole body cholesterol pool size (-9.4%). Whole body cholesterol pool size tended to correlate with plasma TC (r=0.70, p=0.06), suggesting that slight reduction in plasma caused a small decrease in cholesterol pool size. The current patients were normo- and mildly hypercholesterolemic, and their cholesterol pool sizes were not different from those of normal subjects or subjects heterozygous for STSL (7).

As shown in **Chapter IV**, in patients off EZE, HDL-cholesterol levels seemed to decrease (-9%, p=0.12), although not significantly, compared with baseline. EZE normalizes decreases in HDL-cholesterol after 14 wks (+26%). EZE inhibits cholesterol absorption by at least 50% as measured using single and dual stable-isotope ratio methods. Endogenous synthesis of cholesterol in current STSL patients was lower than those of heterozygous STSL (10), hypercholesterolemia (11) and normal subjects (9). Increased PS retention, slow sterol turnover and expanded body pools may compensate

for reduced cholesterol synthesis in STSL (9); thus, increasing cholesterol biosynthesis with EZE might limit cholesterol absorption and reduce whole body sterol accumulation. EZE increased (~2-fold) the fractional and absolute amount of *de novo* cholesterol synthesized daily in these patients, providing the first direct evidence that whole body cholesterol synthesis rate in STSL is increased by EZE. Cholesterol production rate (PR) (sum of absorbed and synthesized cholesterol) increased after EZE. The turnover of cholesterol in the whole system increased by 2-fold by EZE treatment, suggesting that adequate excretion could be of importance to prevent hypercholesterolemia. The EZE-induced changes in MCR and MCF of cholesterol correlated with those in cholesterol PR ($r=+0.98$ and $r=+0.96$, all $p<0.0001$) and TC ($r=-0.70$, $p=0.05$ and $r=-0.77$, $p=0.02$). The data suggest that in STSL, EZE not only reduces PS accumulation but also improves whole body cholesterol turnover by reducing cholesterol absorption and enhancing cholesterol synthesis and clearance. PS are abnormally deposited in tissues causing xanthomas and premature atherosclerotic disease in STSL (12). Resolution of carotid bruits, improvement in cardiac murmur (13) and complete regression of xanthoma were observed after EZE (13, 14). The present research supports these findings, indicating that EZE decreases PS deposition in tissues, and thus EZE may reduce risk of developing premature atherosclerosis and early mortality in STSL.

The clinical consequences of tissue PS accumulation in STSL include chronic hemolytic anemia and macrothrombocytopenia (15, 16). As discussed in **Chapter V,** plasma and tissue total PS, and total PS to cholesterol ratio inversely correlated with platelet count and platelet size in patients off EZE. In the current study, only PS (sitosterol, campesterol

and stigmasterol) correlated with platelet numbers, sizes and RBC indices but not their saturated forms (stanols). Platelets isolated from ABCG5-KO mice had PS but not stanols (17). This observation may be related to their concentrations in the tissues since diets provide abundant amounts of unsaturated but little saturated sterols. As discussed in **Chapter V**, EZE reduced all plasma and red blood cells (RBC) total PS and total PS to cholesterol ratio, and increased platelet count (+23%) and decreased platelet size (-10%). These effects of EZE correspond well with the estimated turnover of platelets in human (e.g., 9-10 d) (18), indicating that reducing circulating PS levels with EZE must have reversed macrothromobocytopenia in these patients. Moreover, no changes in RBC hemolysis markers (serum lactate dehydrogenase and bilirubin) and RBC indices were observed after EZE but slightly increased mean corpuscular hemoglobin concentration (MCHC). The MCHC, which reflects the proportion of hemoglobin (Hb) in RBC, had negative strong associations with all PS in RBC, suggesting that accumulation of PS may cause hematologic abnormalities observed in STSL.

Plasma campesterol levels were inversely correlated with all RBC indices [mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and MCHC) and positively correlated with variation in RBC size after EZE, suggesting that decreases in circulating PS levels may improve RBC indices. A single report in STSL suggested that EZE may improve hemolysis, despite its failure to reduce plasma PS, by modulating RBC lipid content (19). In **Chapter V**, EZE did not affect RBC TC content (+0.3±3%); RBC had less TC levels than plasma (-39±3 and -30±4 %) before and after EZE. RBC TC negatively related to RBC sitosterol, total PS and total PS to cholesterol ratio before

EZE, suggesting that PS in STSL may replace a proportion of cholesterol in the RBC membranes in STSL. Total PS contributed to 7±0.7 and 8±1.0% of total sterols in plasma and RBC in current STSL patients, while they do not exceed 1% of total sterols in healthy subjects (20, 21). TC levels are similar between plasma and RBC in healthy subjects (22). Increased cholesterol in RBC membrane caused hemolysis in hypercholesterolemia (23), and that any decrease or increase in RBC membrane cholesterol can alter cell membrane physical properties and increase osmotic fragility of RBC. Lack of EZE effect on serum markers of hemolysis and RBC indices in current study may be due to: 1) inability of EZE to correct low RBC cholesterol content, 2) RBC have a long life span (e.g., 120 d) compared with platelets, and thus, longer duration of treatment may be required to see a marked effect on RBC indices and 3) current patients did not have prominent signs of hemolysis compared with others (19). Results from **Chapter V** suggest that blocking intestinal PS absorption with EZE provides an effective means to normalize macrothrombocytopenia. Moreover, EZE treatment may decrease risk of bleeding in STSL patients who are more prone to macrothrombocytopenia. The data also suggest that the *ABCG5/8* genes and plasma PS should be investigated in subjects with unexplained hematologic defects. This is important for the correct management and successful prognosis of STSL patients.

As discussed in **Chapter V**, EZE was safe and well-tolerated with no serious adverse events related to liver function observed over 14 wks. Previous studies observed elevated liver enzymes with elevated plasma PS (4, 24). Circulating PS did not correlate with liver enzymes in current patients. Of note, most current patients had normal liver function, and

that liver disease has not been reported to be a common feature in STSL compared with premature atherosclerosis and tendon xanthomas.

Like PS, all patients with untreated STSL had increased plasma and RBC 5α-stanols as previously reported. Data shown in **Chapter VI** suggest that EZE progressively reduces plasma and tissue 5α-stanols of dietary sources come (i.e sitostanol) but moderately decreases those of endogenous origins (i.e cholestanol). EZE increases distant precursor of cholestanol (lathosterol, a marker of cholesterol biosynthesis) without affecting cholestanol precursor (bile acid derivative 7α-hydroxy-4-cholesten-3-one, 7α-H-C4). Furthermore, plasma cholestanol directly correlated with cholesterol before and after EZE ($r=0.96$ and $r=0.88$, $p<0.01$) but not with 7α-H-C4 ($r=0.23$ and $r=0.18$ $p>0.60$). These results suggest that cholestanol in STSL is mostly synthesized from cholesterol, and not through induction of the bile acid pathway, which appears to be fairly specific to cerebrotendineous xanthomatosis (CTX), an autosomal recessive disorder of bile acid synthesis. CTX is caused by mutations in *CYP27A1* gene, which codes for sterol 27-hydroxylase, an essential enzyme for bile acids synthesis (25). Deficiency of this enzyme results in pathological induction of cholesterol 7α-hydroxylase (CYP7A1) to increase 7α-hyroxycholesterol and 7α-H-C4, which is converted to cholestanol that accumulates in plasma and tissues of affected patients (26-28). EZE treatment increased cholesterol biosynthesis and thus precursor availability for cholestanol production. In **Chapter VI**, plasma and RBC lathosterol levels were used as surrogate markers for whole body cholesterol synthesis, and in **Chapter IV** cholesterol fractional synthesis rate was directly measured. We found that plasma and tissue lathosterol levels were strongly correlated

(r=0.83, p=0.01 and r=0.95, p=0.02) with fractional synthesis rate of cholesterol, suggesting a good agreement between surrogate markers and direct measures of cholesterol synthesis in STSL.

The unrestricted accumulation of PS and 5α-stanols in STSL because of complete failure of sterol efflux may cause interference with cholesterol metabolism, leading to endocrine disturbances. In fact, build-up of these sterols caused infertility in a mouse model of STSL (29), although in humans endocrine disruption has only been reported once (30). Owing to the rarity of this disease, little research has been carried out on interference of PS and 5α-stanols with cholesterol metabolism in STSL. Synthesis of thyroid hormones appears to be deranged in STSL (31). Elevated serum cholestanol has been observed in hypothyroidism, an endocrine disorder in which thyroid gland does not produce enough thyroid hormones (32), which has also been reported in CTX (32-34) and STSL (31), suggesting that elevated cholestanol levels may contribute to hypothyroidism. Data shown in **Chapter VI** suggest that accumulation of these non-cholesterol sterols may disrupt thyroid hormone balance, and treatment with EZE reveres disturbances in thyroid hormones by reducing circulating levels of non-cholesterol sterols and increasing cholesterol synthesis. The data support the only report in STSL patients, in which adrenal insufficiency is linked with non-cholesterol sterols (30). Of all studied non-cholesterol sterols, cholestanol and sitostanol had significant inverse correlations with serum free triiodothyronine, which has greater biological activity. Unlike, adrenal hormones, which are synthesized from cholesterol, thyroid hormones are synthesized from the amino acid

tyrosine. It is unclear therefore, why thyroid hormone levels are altered in STSL. The reasons behind this observation are not clear.

Estradiol, the steroid sex hormone, directly affects the thyroid by thwarting its ability to produce thyroid hormones (35). Some studies reported that PS and stanols have estrogenic effect (36-38), and have been shown to bind to estrogen receptor (ER) (39) and possibly affecting the secretion of thyroid hormones. There is also the potential for PS and stanols to decrease *de novo* steroid synthesis by reducing cholesterol levels and therefore PS and stanols may indirectly affect estrogen levels through means other than ER binding. Indeed, the effect of PS, 5α-stanols and their metabolites on cholesterol synthesis is more likely to be tissue and sterol specific (3, 40-46). EZE may indirectly improve thyroid hormones by reducing 5α-stanols and PS and increasing cholesterol biosynthesis in STSL patients.

7.2 LIMITATIONS AND FUTURE DIRECTIONS

The metabolism of PS and cholesterol was determined in STSL patients off and on EZE. By paired comparisons of the off and on EZE, the influence of interindividual variation was minimized, and main effect of treatment was evaluated. Unrestricted retention of PS and reduced biliary excretion in STSL resulted in increased plasma and tissue levels of PS. The clinical consequences of tissue PS accumulation include premature atherosclerosis and coronary heart disease at a young age, tendon xanthomas, hematologic sequelae including chronic hemolytic anemia and macrothrombocytopenia. A major aim of the present thesis was to investigate if EZE reduces tissue retention of PS in STSL. The current study was the first to show that tissue stores of PS were reduced by EZE treatment. Moreover, EZE reduces tissue stores of cholesterol, reduces fractional

cholesterol absorption and increases cholesterol fractional and absolute synthesis rates as well as increases cholesterol turnover by increasing clearance rate of cholesterol from plasma. The current investigation was also limited by short duration. Long-term studies are needed to fully assess the ability of EZE to further reduce plasma and tissue PS levels, and whether such a change with EZE can be substantiated with more dramatic reductions in atherosclerosis and cardiovascular events. In the current study, we have no evidence for atherosclerotic manifestations in any of the 8 subjects. Thus, future long-term studies should investigate EZE effect on vascular health through measuring surrogate markers of atherosclerosis such as carotid intima-media thickness and carotid plaques. Furthermore, EZE reduced plasma TC levels rapidly, but reduction was moderate due to increased cholesterol synthesis. It would be interesting to determine to what extent cholesterol synthesis is increased and if the increase in cholesterol synthesis is a continuous, and if so whether can be prevented with the use of other cholesterol-lowering agents. The present study demonstrated that EZE produced a modest decrease in plasma cholesterol and cholestanol while decreasing PS and plant-derived sitostanol levels. Future investigations using stable isotopes should investigate the impact of EZE on whole body cholesterol and cholestanol pool sizes in comparison with other lipid-lowering agents, such as colesevelam hydrochloride and cholestyramine, bile acid-binding resins, and statins, HMG-CoA reductase inhibitors. These future studies will provide more information if the combination of EZE with these agents will produce greater reductions in plasma and tissue levels of PS, cholesterol and cholestanol, and their body stores, and subsequently dramatic regressions in xanthoma and cardiovascular events in STSL. In the current study, one patient had his/her xanthoma removed before

the study started, and only two patients presented with Achilles tendon xanthomas, which appeared to regress with EZE (Appendix). It would be interesting to obtain periodic biopsies from xanthoma tissues, presumably very slowly turning over mass of cholesterol, and study the relationship between plasma and tissue sterol turnover kinetics after EZE treatment.

The numbers of patients in this investigation were relatively small. However, it should be noted that STSL is an extremely rare disease with fewer than 100 cases reported worldwide. The current STSL patients had the same mutation in the *ABCG8* gene and, as such, the results from this study cannot be generalized to other populations or STSL patients who have different mutations in either *ABCG8* or *ABCG5* genes. Thus, it would be interesting to investigate if EZE reduces plasma PS levels and whole body PS pool size in STSL patients who present with different mutations in either *ABCG8* or *ABCG5* genes. Data from this thesis may help us identifying polymorphisms in the *ABCG5* or *ABCG8* genes in general population who may retain higher plasma PS and cholesterol levels, and show disrupted sterol metabolism.

Some STSL patients may present with hematologic abnormalities while others show the most common clinical features such as premature atherosclerosis and tendon xanthomas. Future studies should investigate whether possible differences in gene mutations could account for differences in presenting clinical consequences of STSL or differences in responses to EZE. Serum indirect RBC hemolysis markers were measured in the current study; it would be interesting to examine the effect of EZE on the osmotic fragility of

RBC. Herein, we measured the newly synthesized bile acids out of cholesterol, which is different from the bile acids found in the circulation after reabsorption from the intestine. We measured serum levels of 7α-H-C4, which is the immediate enzymatic product of 7α-hydroxycholesterol, reflecting the activity of cholesterol 7α-hydroxylase, the rate-limiting enzyme in one of the pathways of bile acid biosynthesis. Serum levels of 7α-H-C4 are not formed by lipid peroxidation, while plasma levels of 7α-hydroxycholesterol may be formed from cholesterol as an artifact due to lipid peroxidation and therefore a sample might contain undefined amounts of 7α-hydroxycholesterol of non-enzymatic origin. To investigate estrogenic potential of PS and 5α-stanols, we examined the effect of EZE on serum thyroid hormones. It would be more interesting to investigate the impact of reducing plasma PS levels and 5α-stanols with EZE therapy on cholesterol synthesis and serum steroid hormone levels (i.e adrenal and sex hormones), which are derived from cholesterol.

7.3 FINAL CONCLUSION

The present research is the first to discern the underlying mechanism of action of EZE in STSL, providing additional insight to understanding sterol metabolic mechanisms. The totality of the present research is the first to show that EZE progressively decreases plasma PS, causing a drain on the whole body PS pool size in STSL. Furthermore, EZE improves whole body cholesterol turnover by reducing cholesterol absorption and enhancing cholesterol synthesis and clearance. It is also the first reported direct measure of cholesterol synthesis and absorption in STSL using stable isotopes STSL. EZE is the current available for management of plasma PS in STSL. STSL patients have a strong propensity toward premature coronary atherosclerosis. Thus reducing PS deposition by

limiting absorption, mobilizing sterols from plasma, and promoting excretion may beneficially impact atherosclerosis. High levels of PS in STSL adversely affect platelet number and size. These defects can be normalized upon decreasing plasma PS levels by EZE treatment. In addition, the present research supports the link between hematologic abnormalities and disturbed sterol metabolism and suggests that EZE treatment may decrease risk of bleeding in STSL patients who are more prone to macrothrombocytopenia. Moreover, the data also suggest that the *ABCG5/8* genes and plasma PS should be investigated in patients with unexplained hematologic abnormalities. This is important for the correct management and successful prognosis of STSL patients.

EZE progressively reduced plasma and tissue 5α-stanols that come from diet (i.e sitostanol) but small reductions for those formed from endogenous production and from dietary sources (i.e cholestanol). The results suggest that most cholestanol in STSL is synthesized from cholesterol. It also appears when absorption of dietary cholesterol is restricted with EZE cholesterol synthesis is upregulated (supported by the increase in cholesterol synthesis marker lathosterol. EZE decreases plasma and tissue 5α-stanols, increases cholesterol synthesis marker lathosterol and normalizes disrupted thyroid hormone balance, thereby EZE may reduce tendency for hormonal imbalance in STSL. This research shows that EZE does not affect plasma levels of fatty acids **(Appendix III)** and triacylglyceride in STSL patients throughout 14 wks. EZE was safe and well tolerated with no serious adverse events related to liver function observed over 14 wks.

This research shows the efficacy and possible clinical benefit of EZE, and therefore strengthens the rationale for the use of EZE in treatment of STSL.

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APPENDICES

APPENDIX I

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

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. S. Myrie / Dr. P. Jones
Sponsor: NIH

Ethics Reference Number: B2011:051
Date of REB Meeting: May 30, 2011
Date of Approval: November 18, 2011
Date of Expiry: May 30, 2012

Protocol Title: Assessment of Sterol Metabolism in Sitosterolemia: A Pilot Study of Patients Treated with Ezetimibe

The following is/are approved for use:

- **Protocol, Version dated 17Aug2011**
- **Research Subject Information and Consent Form, Version 2 dated 17-Aug-11**
- **Assent Form for Children 16-17 Years Old, Version 2 dated 17-Aug-11**
- **Introductory Participant Letter, Version 1 dated 04-Apr-11**
- **Study Diary submitted April 4, 2011**

The above was approved by Dr. Ian Maclean, Acting Chair, Biomedical Research Board, Bannatyne Campus, University of Manitoba on behalf of the committee per your letter dated November 14, 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 for one year from the date of the meeting at which it was reviewed. 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]

Ian Maclean, Ph.D.
Acting 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

APPENDIX II

FORMS CORRESPONDING TO STUDIES DESCRIBES IN CHAPTERS IV, V, AND VI

STUDY ADVERTISEMENT – RARE DISEASES RESEARCH WEBSITE AD

Clinical Studies

7002: Assessment of Sterol Metabolism in Sitosterolemia: A Pilot Study of Patients Treated with Ezetimibe

Status: Closed to Accrual

Study Summary

Background

Sitosterolemia is an inherited condition in which plant sterols (chemicals from plants) are not broken down properly in the body. In sitosterolemia, plant sterols build up, causing damage to body tissues.

Ezetimibe is a drug that is often used to treat sitosterolemia. Ezetimibe reduces the amount of cholesterol (a fat-like substance) and plant sterol in the blood. The purpose of this study is to determine if ezetimibe improves whole body plant sterol and cholesterol balance. The results of this study will help us to learn more about the howsitosterolemia develops and and how ezetimibe works in patients with the disease.

The goal of this study is to use stable isotope techniques (measuring the level of tagged chemicals in your blood over time) to learn about the effect of ezetimibe on whole body plant sterol and cholesterol pool sizes and cholesterol trafficking (absorption and synthesis). Specific objectives are to determine if treatment with ezetimbe:

1. Reduces whole body sitosterol pool size Decreases whole body cholesterol pool size
2. Decreases cholesterol absorptioIncreases cholesterol fractional synthesis

About this Study

This is a pilot interventional study of 8 subjects. The purpose of the study is to know more about how ezetimibe changes plant sterol and cholesterol sitosterolemia on and off ezetimibe therapy.

After consent, study participants will be taken off ezetimibe for 14 weeks. After these 14 weeks, participants will be asked to take ezetimibe at the dose of 10 mg/day for 14 more weeks. Then, participants will be put back on pre-study ezetimibe treatment.

Participants will undergo 3 different testing procedures while off ezetimibe. These procedures will be repeated while you are on ezetimibe. The three procedures are:

1. Intravenous (i.v.) infusion of tagged sitosterol (D7-sitosterol) to measure whole body plant sitosterol pool size, and tagged cholesterol (18O-cholesterol) to measure whole body cholesterol pool size;
2. Oral administration of tagged water (2H2O) to measure whole body cholesterol synthesis;
3. Oral administration of tagged cholesterol (13C-cholesterol) to measure dietary cholesterol absorption.

Four weeks after you have started ezetimibe, you will be asked to come back to the Lipid Clinic. The procedures you will undergo will then be exactly the same as those described above.

During both phases (that is 14 weeks off ezetimibe and 14 weeks on ezetimibe) you will be asked to:

- Keep a log of what you eat
- Talk to the study coordinator about what you eat
- Have a physical exam
- Give blood (up to 4 teaspoons per session)
- Give urine sample (some visits)
- Give fecal sample (some visits)

Participation

You are eligible to participate if:

- You have a confirmed diagnosis of homozygous sitosterolemia as established by genotyping and clinical parameters
- You are age 16 and older.
- You are receiving a supplementary treatment regimen so that ezetimibe treatment can be stopped for a short-term without harmful health effects.

You are not eligible to participate if:

- You are not able to travel to a STAIR site
- You have (or have had) cancer, renal failure, diabetes, major infectious diseases, or immunodeficiency
- You are pregnant.
- You are unwilling or unable to comply with study procedures
- You are unable to give consent to participate.
-

Be notified when new locations open for this study!

Join the Contact Registry for: Sitosterolemia!

SUBJECT CONSENT FORM

Appendix II: Subject Consent Form

STAIR 7002: Sterol Metabolism in Sitosterolemia

Richardson Centre for Functional Foods and Nutraceuticals

University of Manitoba

RESEARCH SUBJECT INFORMATION AND CONSENT FORM

Title of Study: Assessment of Sterol Metabolism in Sitosterolemia: A Pilot Study of Patients Treated with Ezetimibe

Principal Investigator: Dr. Peter Jones
University of Manitoba
Richardson Centre for Functional Foods and Nutraceuticals
196 Innovation Drive, Smartpark
Winnipeg, Manitoba, R3T 6C5
Tel: 204-474-8883

Co-investigator: Dr. Semone Myrie
University of Manitoba
Richardson Centre for Functional Foods and Nutraceuticals
196 Innovation Drive, Smartpark
Winnipeg, Manitoba, R3T 6C5
Tel: 204-474-7290

Co-investigator: Dr. David Mymin
University of Manitoba
Department of Cardiology
Lipid Clinic
Health Sciences Centre
MS755D-820 Sherbrook Street
Winnipeg, Manitoba R3A 1R9
Tel: 204-787-1659

INTRODUCTION

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form carefully. You are encouraged to discuss any questions that 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 FOR THE STUDY

You are being asked to take part in this study because you have sitosterolemia. We hope to include 8 individuals in this study.

The purpose of the study is to know more about how ezetimibe changes plant sterol and cholesterol in people with sitosterolemia.

Ezetimibe is also known as Zetia or Ezetrol. Ezetimibe is a widely used drug to lower blood cholesterol in patients with high blood cholesterol levels. Ezetimibe has also been shown to lower blood plant sterol levels and is now used to treat people with sitosterolemia. To provide the best treatment for people with sitosterolemia we need to understand how ezetimibe affect cholesterol and plant sterol in the human body.

For this, we will administer a small amount of tagged cholesterol and tagged sitosterol by i.v. (that is, a needle in your arm) and give you tagged cholesterol and tagged water to ingest. Tagged sitosterol, cholesterol and water are non-radioactive and not toxic chemicals. They are almost identical to regular sitosterol, cholesterol and water except that they are slightly heavier. This makes them easy to be recognized one from another by our instruments.

We will then measure the level of tagged chemicals in your blood over time. These measurements will allow us to determine how much and where cholesterol and plant sterols are distributed in your body. They will also help us to determine how much cholesterol your body absorbs from the diet and how much is being made by your body.

The measurements will be made while you are off ezetimibe and compared to measurements made while you are on ezetimibe.

The study will consist of 2 sets of 14 weeks study periods and one month of follow-up. All together your participation in the study will last approximately 32 weeks.

STUDY PROCEDURES

If you agree to take part in this study, you will be asked to stop taking any ezetimibe for 14 weeks. After these 14 weeks, we will be asked to take ezetimibe at the dose of 10 mg/day for 14 more weeks. Then, you will be put back on your pre-study ezetimibe treatment.

Throughout the entire study you will continue to eat your usual diet. You will be asked not to consume alcohol during the study.

If you are taking other medications such as cholestyramine, colestipol, niacin, clofibrate, gemfibrozil, probucol, do not change the doses or stop taking these medicines while you are in the study, unless it is requested by your doctor. If your doctor changes the dose on any of your medications, we ask you to report this information to us as soon as possible.

We also ask you to report to us any change in your health at any time during the study.

You will undergo 3 different testing procedures while you are off ezetimibe. These procedures will be repeated while you are on ezetimibe. The three procedures are:

1) Intravenous (i.v.) infusion of tagged sitosterol (D_7-sitosterol) to measure whole body plant sitosterol pool, and tagged cholesterol (^{18}O-cholesterol) to measure whole body cholesterol pool size;

2) Oral administration of tagged water (^2H_2O) to measure whole body cholesterol synthesis;
3) Oral administration of tagged cholesterol (^{13}C-cholesterol) to measure dietary cholesterol absorption.

Procedures while you are off ezetimibe

On the day you are taken off ezetimibe, approximately 4 teaspoons of blood will be drawn from your arm to measure plant fat and cholesterol in your blood and your blood chemistry.

Two weeks later, 4 teaspoons of blood will be drawn to monitor changes in plant fat and cholesterol in your blood and changes in your blood chemistry while you are off ezetimibe.

After 4 weeks off ezetimibe, you will be asked to come back to the Lipid Clinic. You will be asked to fast overnight (nothing to eat or drink for 12 hours) before coming to the centre.

On the morning of this visit (6am), an i.v. catheter will be placed in your arm by the attending nurse and approximately 2 teaspoons of blood will be drawn. You will then receive a slow infusion of tagged sitosterol and cholesterol dissolved in ethanol and Intralipid.

Once the i.v. infusion is finished, you will be asked to eat a slice of toast or English muffin with a little margarine containing tagged cholesterol. You will also be asked to drink a cup of orange juice which contains approximately three tablespoon of tagged water. Additional breakfast will then be provided.

You will receive a lunch meal at the centre and asked either to stay or come back for another blood collection at the end of the day (6pm). You will be instructed not to eat anything for about 4-5 hours before the 6pm blood collection. Approximately 2 teaspoons of blood will be collected.

Additional fasted blood samples will be taken 24 hours, 48 hours, 72 hours, 92 hours, 1 week, 3 weeks, 5 weeks, 8 weeks and 10 weeks after the administration of tagged compounds. You will continue with your usual diet during this time. Blood collections will be performed by at the RCFFN, University of Manitoba. About 2 teaspoons of blood will be drawn each time and each blood collection will take approximately 5 minutes.

Procedures while you are on ezetimibe

After 14 weeks off ezetimibe, you will be instructed to take 10 mg/day of ezetimibe for another 14 weeks.

Two weeks after you have started ezetimibe, we will ask you to come to the research center for a blood draw to monitor changes in plant fat and cholesterol in your blood and changes in your blood chemistry while you are on ezetimibe.

Four weeks after you have started ezetimibe, you will be asked to come back to the Lipid Clinic. The procedures you will undergo will then be exactly the same as those described above while off ezetimibe.

You will be asked to fast overnight (nothing to eat or drink for 12 hours) before coming to the centre. Early morning (6am) an i.v. catheter will be placed in one of your arms and you will receive an infusion of tagged sitosterol and cholesterol.

Once the i.v. infusion is finished, you will be asked to eat a slice of toast or English muffin with a little margarine containing tagged cholesterol. You will also be asked to drink a cup of orange

juice which contains approximately three tablespoon of tagged water. Additional breakfast will then be provided.

You will receive a lunch meal at the centre and asked either to stay or come back for another blood collection at the end of the day (6pm). You will be instructed not to eat anything for about 4-5 hours before the 6pm blood collection. Approximately 2 teaspoons of blood will be collected.

Additional fasted blood samples will be taken 24 hours, 48 hours, 72 hours, 92 hours, 1 week, 3 weeks, 5 weeks, 8 weeks and 10 weeks after the administration of tagged compounds. You will continue with your usual diet during this time. Blood collections will be performed by at the RCFFN, University of Manitoba. About 2 teaspoons of blood will be drawn each time and each blood collection will take approximately 5 minutes.

After 10 weeks, you will be instructed to resume the ezetimibe treatment you had before you joined the study. One month later, we will ask you to come for a final study visit to follow up with you. At this visit we will draw blood (approximately 4 teaspoons) to check plant fat and cholesterol in your blood and your blood chemistry and ask you about any problem you may have experienced during the study.

Urine and stool collection

Urine and stool samples will be collected from you twice during the study- once while you are off ezetimibe and again while you are back on ezetimibe. These samples will be collected at home and we will ask you to bring them to the research center. Each stool sample collection period will occur over 5 days and urine over a 24 hour period. These samples will be collected and stored frozen for later studies.

The researcher may decide to take you off the study if it is in your medical best interest, funding is stopped, new information becomes available, failure to take treatment as described or another yet unknown reason.

You can stop participating at any time without any consequences. However, if you decide to stop participating in the study, we encourage you to talk to the study staff and your regular doctor first.

Result of the study will be provided to you once all data analysis has been completed.

RISKS AND DISCOMFORTS

1. **Blood draw –** Blood sampling is a very routine and safe procedure but may cause some pain due to placing a needle into a vein. Blood draw may cause a small risk of bleeding, bruising and/or infection at the puncture site.

2. **Administration of tagged cholesterol, sitosterol and deuterium oxide –** You will be given low doses of tagged cholesterol (^{18}O-cholesterol) and plant sterol (D_{7-} sitosterol) by i.v., and tagged cholesterol and water to ingest. There are no known risks associated with the administration of the isotopes to be used in this study. ^{18}O-cholesterol and D_{7-} sitosterol will be dissolved in 0.285 ml/ kg body weight absolute ethanol, suspended in 0.7 ml/ kg body weight Intralipid, and inf used immediately. There is no signif icant risk

associated with the infusion of this dose of ethanol. Stinging or burning sensation in vein may occur due to the presence of ethanol in the i.v. mixture.

3. **Ezetimibe –** Stopping your ezetimibe for a period of time may increase blood cholesterol and plant sterol levels. So, we will closely monitor the blood levels of these sterols throughout the study. We will measure your blood cholesterol and plant sterol levels before you stop taking ezetimibe and these measurements will be used as your baseline blood sterol levels. It is expected that both your blood cholesterol levels and blood plant sterol levels will increase when you are taken off ezetimibe. If these levels increase too much, that is your blood cholesterol level increases by more than 24% or your plant sitosterol level increases by more than 92%; we will request a confirmatory blood draw within 15 days.. Your usual ezetimibe treatment will be resumed and you will be excluded from the study if the high blood cholesterol or plant sterol levels are confirmed. If the increase is not confirmed, you will continue the study. However, your usual ezetimibe treatment will be resumed and you will be excluded from the study if we observe another high increase in your blood cholesterol or plant sterol level at any time while you are off ezetimibe.

   Taking ezetimibe or being taking off ezetimibe may carry a risk to the embryo or fetus if you are or may become pregnant.

4. **Ezetimibe safety and tolerability –** Research has shown that ezetimibe is generally well-tolerated in sitosterolemia patients. Some patients have reported adverse reactions including upper respiratory infection (4% of patients), diarrhea (4%), joint pain (3%), sinus infection (3%) and pain in limb (3%).

There are no known serious side effects for any of the procedures proposed in this study. However, as with any clinical trial, there might be as yet unknown or unforeseen risks of taking part.

If you experience any adverse reaction or notice any unusual sign or symptom you must contact Dr. Mymin at 204 787-1659.

Alternative treatment options for sitosterolemia patients include bile salt resins. This option should be discussed with your primary care physician.

BENEFITS

By participating in this study, you will be providing useful information that may lead to a better understanding of the effect of ezetimibe on whole body cholesterol and plant sterol deposit over time in the body of people with sitosterolemia. There may or may not be direct medical benefit to your from participating in this study. You will also receive access to your test results when they become available. If during your participation in this study new knowledge concerning sitosterolemia is discovered, these findings will be made known to you in a timely manner.

All clinic and professional fees, diagnosis and laboratory tests, which will be performed as part of this study are provided at no cost to you.

PAYMENT FOR PARTICIPATION

You will receive reimbursement for travel expenses related to taking part in this study

VOLUNTARY PARTICIPATION/WITHDRAWAL FROM THE STUDY

Your decision to take part in this study is voluntary. You may refuse to participate or you may withdrawal from the study at any time. Your decision not to participate or to withdraw from the study will not affect your other medical care. If your study doctor feels that it is your best interest to withdraw you from the study, your study doctor will remove you without your consent.

Your participation in this study may be terminated without your consent by the study coordinators, physician or principle 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's staff's instructions.

We will tell you about any 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 OF 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. Mymin at 204 787-1659 or go to your nearest emergency room to receive necessary medical treatment. You are not waiving any of your legal rights by signing this consent form or releasing the investigator or the sponsor from their legal and professional responsibilities. If any health abnormalities are identified in the clinical tests conducted during this experiment, Dr. Mymin will be contacted, who will inform you of the results.

CONFIDENTIALITY

Information gathered in this research study may be published and presented in public forums. However, your name and other identifying information will not be used or revealed.

Personal information such as your name, address, telephone number and/or any other identifying information will be protected. If the results of the study are published, your identity will remain confidential. No information revealing any personal information such as your name, address, telephone number will be made publicly available. Personal health identification numbers will not be collected in this study. The signed Consent Form and medical record which contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. The Health Research Ethics Board, University of Manitoba and the sponsor (National Institutes of Health) may review your research record as part of their auditing program.

Despite efforts to keep your personal information confidential, absolute confidentially cannot be guaranteed. Your personal information may be disclosed if required by law. All records will be kept in a locked secure area and only those persons identified will have access to these records. If any of your medical/research records need to be copied to, any of the above, your name and all identifying information will be removed. No information revealing personal information such as your name, address or telephone number will leave the Richardson Centre for Functional Foods and Nutraceuticals.

The clinical information collected for this study will be stored at the Data Management and Coordinating Center at the University of South Florida in Tampa, FL. The data management center uses several layers of protection for the clinical data stored there. It meets all of the local and federal security requirements for research datacenters. Your information is stored only using a study ID.

Blood samples will be stored for 5 years in case the need arises for further testing for research or medical purposes. This could happen as the result of new discoveries by us or other researchers.

Your blood samples will only be used for the assessment of blood fat levels, plant sterol levels and blood fat metabolism. Also, we would be prepared to make samples available to other researchers should there be a good reason to do so, in which case the sample would have no personal identification.

By checking one of the boxes below, please indicate whether or not you are willing to let us share biological specimens collected during the study with other researchers.

☐ Yes, I am willing to have the biological specimens collected during the study be shared with other researchers.

☐ No, I am not willing to have the biological specimens collected during the study be shared with other researchers.

QUESTIONS OR CONCERNS

You are free to ask any questions that you may have about the study procedures and your rights as a research participants. If any questions come up during or after the study you can contact the researchers in the study (see page 1) or the research staff.

For questions about your rights as a research participant you may contact the University of Manitoba, Biomedical Research Ethics Board Office at **204-789-3389**.

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

STATEMENT OF 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 ☐

STAIR 7002: Sterol Metabolism in Sitosterolemia

I agree to being contacted in relation to this study. Yes ☐ No ☐

1. I have read and understand 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 purposes 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 my 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.

   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.

ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE

_____ _____
Participant signature D ate

_____
Participant Printed name

_____ _____
Signature of clinical coordinator Date

_____
Clinical coordinator Printed name

ASSENT CONSENT ADDITIONAL SIGNATURE SHEET

By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

I, the undersigned, attest that the information in the Participant Information and Consent Form was accurately explained to and apparently understood by the participant or the participant's legally acceptable representative and that consent to participate in this study was freely given by the participant or the participant's legally acceptable representative.

Parent/legal guardian's signature: _____ Date: _____

Parent/legal guardian's printed name: _____

Child's signature: _____ Date: _____

Child's printed name:_____

Parent/ Legal guardian's signature: _____ Date: _____

Parent/Legal guardian's printed name: _____

Witness signature: _____ Date: _____

Witness printed name: _____

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: _____

Printed name of above:_____ Role in the study_____

PARTICIPANT INFORMATION SHEET

Appendix II

Participant Information Sheet

Date(DD/MM/YYYY):	
Participant first name:	Last name:
Date of birth(DD/MM/YYYY):	Gender: (M/F)
Body weight(in pounds):	Height(in feet):
Home address	Mailing address, if different from home address:
Home telephone number:	
Are you taking any medication now? (Yes/No)	If yes, please write name of medications:

If you have any questions please call

Peter J. Jones, PhD
Professor, Departments of Food Science and Human Nutritional Sciences
University of Manitoba
196 Innovation Drive
Winnipeg, MB R3T 6C5
Canada
Phone: 204-474-8883
Fax: 204-474-7552
peter_jones@umanitoba.ca

INSTRUCTIONS FOR TAKING OFF EZETIMIBE

Appendix II

Sterol Metabolism in Sitosterolemia

Instruction for taking off Zetia or any cholesterol-lowering medications for 14-weeks

Once you agree to participate in the study, we will ask you if you are taking Zetia or any cholesterol-lowering medication, not to take them for the first 14 weeks of the study. During this period, we will recommend you to follow low cholesterol and low plant fat diet. This diet is low in foods such as nuts, seeds, olives, avocados, vegetable oils, shortening, margarine and chocolate as well as shellfishes.

Alternatively, we will collect fasting blood sample every two weeks to closely monitor your cholesterol and plant sterols concentrations as well as liver, kidney and thyroid functions while you are off medication.

You will be asked to monitor and report any adverse experiences including headaches, chest pain, or dizziness.

A comprehensive clinical evaluation will be performed by your physician each month. During each visit, the size of fat deposits (called xanthomas) in the skin as well as in tendons, if you have, will also be measured.

If you have any questions please call

Peter J. Jones, PhD
Professor, Departments of Food Science and Human Nutritional Sciences
University of Manitoba
196 Innovation Drive
Winnipeg, MB R3T 6C5
Canada
Phone: 204-474-8883
Fax: 204-474-7552
peter_jones@umanitoba.ca

SUPPLIES NEEDED FOR STUDY PROCEDURES

Appendix II

Items needed for I.V. Infusion

1. Body Weighing scale
2. Analytical scale to weigh syringes
3. Sheet for information (Patients).To be created
4. Saline bags
5. Y. Connector
6. Syringes
7. Dual channel i.v. pumps
8. Intralipid to check with Pharmacy.
9. Bufferflies
10. Gauze
11. Arrange with compounding company to send the Isotope stock solution to Tache pharmacy.

Items needed for oral isotopes

1. Margarine
2. Juices
3. Cups
4. English muffin or Buns
5. Toaster
6. Plates
7. Scale to weight margarine.
8. Portable appetizers
9. Containers for

Items needed for blood draws

1. Vac needle W/ADAP(21GX3X4''X12 or 23Gx3X4''X12) butterfly
2. Vacutainer blood collection tubes
3. Blood tubes adaptors
4. Alcohol wipes
5. Tourniquet
6. Gauze

Appendix II: Supplies Needed For Study Procedures

7. Micropore paper tape
8. Band-acids
9. Latex gloves(medium)
10. Test tube racks
11. EDTA, Serum tubes
12. Urine containers
13. Stool containers
14. Ice packs

INSTRUCTIONS FOR PREPARING I.V INFUSION

Appendix II

Stable isotope Tests

- Stable isotope tests involve the Research Laboratory, the Research Pharmacy and the RCFFN (or equivalent) Bionutrition and nursing team (for infusion).
- The stock solutions containing the stable isotopes need to be ordered and prepared weeks in advance.
- The working solutions (to be administered i.v. or per os) need to be prepared the day of the test, hence the need to add the test(s) to the orders and work closely with Research Pharmacy.
- Four tests are considered: 1) sitosterol pool size measurement, 2) cholesterol pool size measurement, 3) cholesterol absorption, and 4) cholesterol synthesis. Pool size measurements are the top priority in this list. But optimally all four measurements should be performed together.

Pharmacy – Preparation of Stable Isotope of Cholesterol and Sitosterol

The Role of the Research Pharmacy:

1. The preparation, testing and storage of a stock solution of the stable isotope of cholesterol and sitosterol dissolved in ethanol.
2. The preparation of the intravenous solution on the morning of the infusion and setting aside two aliquots for send-out. Aliquots will be sent to University of Manitoba at Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) lab to measure the precise **concentration** of the stable isotope in the intravenous solution (necessary for the calculation of each dose administered) and the second aliquot will be used to measure the **enrichment** of cholesterol (amount of stable isotope of cholesterol (tracer)/ amount of unlabeled cholesterol (tracee)). The dose of stable isotope administered and the enrichment of the cholesterol (and sitosterol) are both critical values in the calculation of the pool size and cholesterol (and sitosterol) absorption.

Stock solution of the Stable Isotope of Cholesterol and Sitosterol

The first step is to prepare a stock solution. Cholesterol and sitosterol are insoluble in water, so it is necessary to prepare the stock solution of the stable isotope in a solvent that will be safe for the eventual intravenous injection. Ethanol is a reasonable choice, but even the solubility of cholesterol (and sitosterol) in ethanol is limited; only about 15 mg/ml at room temperature. Almost all of the published methods for stable isotope infusion, start by preparing a stock of 5 mg to 20 mg cholesterol per ml of ethanol(16-20). After the cholesterol is dissolved, the solution is passed through a 0.22-micron solvent resistant filter. The sterile solution must be tested for sterility and pyrogenicity before it can be administered to patients.

1.1 Preparation of ^{18}O-cholesterol or $^{2}H_7$-sitosterol-ethanol stock solutions

- Prepare the stock of ^{18}O-cholesterol or $^{2}H_7$-sitosterol (5.5 mg/mL) for sterile injection. One gram will supply about 20 injections of stable isotope depending on the weight of subject and assuming about 25% loss during preparation (including the amounts needed for sterility, pyrogen testing, 15 to 45 mg per injection).
    - Pharmacy Background for background information about preparing the cholesterol-ethanol stock solution.
    - NIH stock solution preparation form
- <u>Chemicals required:</u>
    - ^{18}O-cholesterol: C/D/N Isotopes Inc. Pointe-Claire, Quebec, Canada cat no W-6988.
    - $^{2}H_7$-sitosterol: C/D/N Isotopes Inc. Pointe-Claire, Quebec, Canada cat no
    - Ethanol USP (100%)
    - 0.2 micron solvent resistant Teflon filter (Millex-FG, Millipore, Bedford MS)
    - Sterile vials (can be obtained from Allergy Labs) to place the aliquots of cholesterol-ethanol stock
    - Glassware and syringes as needed

- Procedures:
- Complete the following procedure using aseptic technique in an appropriate sterile environment (Laminar Air Flow Work bench)

1. Add the following to a sterile glass container (for example a graduated cylinder dry heat sterilized for pyrogens): 160 mL (for 1 g of sterol), or 35 mL (for 0.25 g of sterol) of ethanol (USP, 100%)
2. Quantitatively transfer the contents of the primary vial 1 g (or 0.25 g). After transferring the crystals, obtain complete transfer of the cholesterol from the primary vial with 5 or 6 rinses of ethanol.
3. Make up to final volume of 182 mL (for 1 g of sterol) or 45 mL(for 0.25 g of sterol) with ethanol (USP 100%)
4. Mix until the cholesterol dissolved; it may take at least 6 hr to overnight (may use a spin bar, dry heat sterilized). Cover the glass container to prevent evaporation of ethanol.
5. Set up sterile vials (to contain 1 to 4 mL) to receive the aliquots of the cholesterol-ethanol stock solution.
6. Withdraw the solution out of the bottle using a 60 mL syringe.
7. Attach a 0.22 micron filter (solvent resistant) and filter the cholesterol-ethanol stock solution directly into sterile vials and seal.
8. Divide the solution such that each vial contains approximately 1 to 4 mL of solution
9. Send a vial (or vials) for sterility and pyrogenicity testing.
10. Store most of the solution in vials under sterile conditions at - 80 °C until use
11. Send 1 mL aliquot to Semone Myrie for cholesterol analysis to determine the precise concentration of the stock. This will be the concentration used to calculate the volume for the intravenous dose (see below). Label vial with site number, approximate concentration, lot number and date prepared. Send samples packed with dry ice.

    Semone Myrie, RD, Ph.D.
    Assistant Professor
    196 Innovation Drive,
    Richardson Centre for Functional Foods and Nutraceuticals
    University of Manitoba
    Winnipeg, MB R3T 2N2
    Phone: 204-272-1555

Intravenous solution of the Stable Isotope of Cholesterol and Sitosterol
The intravenous solution is prepared by mixing the cholesterol-ethanol stock with Intralipid®. Presumably the Intralipid® prevents the cholesterol come coming out of solution in the aqueous medium by providing triglycerides and phospholipids for association. Both 10% Intralipid® (16, 18, 21) and 20% Intralipid® (22) have been used. The infusate is filtered before administration with a 0.8 micron filter (16, 17) or a 1.2 micron filter(23, 24).

1.2 Preparation of stable isotope solutions for i.v. administration

- The intravenous solution is to be prepared on the morning of the infusion. UM nursing to administer IV $^{2}H_{7}$-sitosterol and ^{18}O-cholesterol immediately after oral isotope administration. .

- <u>Chemicals, supplies and equipment required:</u>
    - 20% Intralipid®
    - Syringes of various sizes depending on the dose
    - Water bath
    - Water proof zip lock bag
    - Filter: 1.2 micron (alcohol resistant PALL Corp cat no HP4648)
    - Microfuge tubes (1.0 mL) with screw top caps
- Obtain the subject body weight, site #, and subject code # from your Study Coordinator (See the Appendix: Pharmacy Background to see a sample calculation and information about the dose of cholesterol, the volume of the Intralipid ®, and the maximum safe volume of ethanol).

1. Set up a 37°C water bath
2. Bring cholesterol-ethanol stock vial(s) to room temperature
3. Calculate the dose (mg) of stable cholesterol (1.4 mg/kg times body weight (kg)= dose (mg))
4. Calculate the volume of cholesterol-ethanol stock for the dose (ml), (dose(mg) divided by the concentration of stock (mg/kg)= ml)
5. Calculate the maximum safe volume of ethanol (body weight(kg) times 0.285= max safe volume (ml) to verify that this volume is less than the volume of stable cholesterol-ethanol dose (in step #5)
6. Calculate the volume of cholesterol-ethanol stock needed that includes the approximate loss during preparation: (= volume of sample stock), (Volume of stable isotope dose plus 0.2 ml)
7. Calculate the volume of 20% Intralipid® (Volume of sampled stock (ml) times 3)
8. Calculate the total volume of the mixture (Volume of cholesterol-ethanol stock needed (ml) plus volume of Intralipid®)

9. Select syringes to contain the total volume of the preparation **(A),** the volume of the stock sampled **(B)**, the volume received after filtering **(C)** and two 1-ml syringes for two 0.1ml aliquots.
10. Withdraw _____ml into syringe **A** from a bag of 20% Intralipid® Lot#_____ exp date _____
11. Remove the needed volume _____of cholesterol-ethanol stock solution with syringe **B** and inject it slowly into the diluted Intralipid®. Mix by inversion 20 times.
12. Warm the mixture for 5 min at 37°C. Time in_____ Time out _____
13. Let sit at room temperature for 15 minutes. Time start _____Time finish_____
14. Weigh Syringe **C** (empty weight) _____g
15. Pass contents of Syringe **A** through a 0.8 micro filter into Syringe **C**.
16. Remove a 0.1 mL aliquot to send to RCFFN for concentration measurement, place in a microfuge tube with a screw top cap. Label with site #, subject code#, and date.
17. Remove a 0.1 mL aliquot for enrichment measurement place in a microfuge tube with a screw top cap. Label with site #, subject code#, and date. Give this aliquot to your Study Coordinator to send to RCFFN with the plasma and red blood cell samples.
18. Reweigh the syringe **C** syringe prior to infusion (final weight)_____g
19. Send empty weight and final weight of Syringe **C** to Semone Myrie with the 0.1 mL aliquot. Freeze the aliquot and ship with dry ice to:

    Semone Myrie, RD, Ph.D.
    Assistant Professor
    196 Innovation Drive,
    Richardson Centre for Functional Foods and Nutraceuticals
    University of Manitoba
    Winnipeg, MB R3T 2N2
    Phone: 204-272-1555

- Note: Stable isotopes do not require an IND from the FDA to be used in research since stable isotopes are not drugs. Please see this document for more guidance on this issue: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM229175.pdf

ISOTOPES ADMINISTRATION INFORMATION SHEET

Appendix II

!

Date:.....'

Isotope Administration Information Sheet

Participant ID;…..…… **Body Weight**…...… …… (kg)

Blood Pressure……/……(mm Hg) **Pulse**:…….....……(mm Hg)

Time of blood draw (0h)……

Intravenous infusion

Dose of D_7-sitosterol ……..... (mg/kg bwt) **Volume**…….....…. (ml)

Dose of O^{18}-cholesterol …..... (mg/kg bwt) **Volume** ……...……..... (ml)

Total infused volume plus intralipid ..... …... (ml) **Infusion rate** ....… (ml/kg bwt)

Infusion START at……..…..…… **and STOP at** …...……..

Time of second blood draw at……

Oral administration

Dose of C^{-13} cholesterol ……..…….... (mg) **Amount of vehicle** .. …..... (g)

Dose of deutered water ….....(ml) V**olume juice**=…… ..(ml)

Time of consumption of C^{-13} cholesterol……..

Time of consumption Deutered water…….

Comments:

……
……
……

STUDY CALENDARS

Appendix II

April 2012(Manitoba)

Sun	Mon	Tue	Wed	Thu	Fri	Sat
1	*2*	*3*	*4*	*5*	*6*	*7*
8	*REMINDER TO FAST*	*10 No Zetia* **Fasting Blood draw**	*11 No Zetia*	*12 No Zetia*	*13 No Zetia* *24 h-food record*	*14 No Zetia* *24 h-food record*
15 No Zetia *24 h-food record*	*16 No Zetia*	*17 No Zetia*	*18 No Zetia*	*19 No Zetia*	*20 No Zetia*	*21 No Zetia*
22 No Zetia	*23 No Zetia* *REMINDER TO FAST*	*24 No Zetia* **Fasting Blood draw**	*25 No Zetia*	*26 No Zetia*	*27 No Zetia*	*28 No Zetia* *24 h-food record*
29 No Zetia *24 h-food record*	*30 No Zetia* *24 h-food record*					

May 2012

Sun	Mon	Tue	Wed	Thu	Fri	Sat
		1 No Zetia *24 h-food record*	*2 No Zetia* *24 h-food record*	*3 No Zetia* *24 h-food record*	*4 No Zetia*	*5 No Zetia*
6 No Zetia	7 No Zetia *REMINDER TO FAST*	**8 No Zetia** TEST DAY **Fasting blood (d0)** **Stool sample**	**9 No Zetia** **Fasting blood (d1)** **Stool/24 h-urine sample**	**10 No Zetia** **Fasting blood (d2)** **Stool sample**	**11 No Zetia** **Fasting blood (d3)** **Stool sample**	**12 No Zetia** **24 h-food record** **Fasting blood (d4)** **Stool sample**
13 No Zetia	*14 No Zetia* *REMINDER TO FAST*	*15 No Zetia* **Fasting Blood draw-wk1**	*16 No Zetia*	*17 No Zetia*	*18 No Zetia*	*19 No Zetia*
20 No Zetia	*21 No Zetia*	*22 No Zetia*	*23 No Zetia*	*24 No Zetia*	*25 No Zetia*	*26 No Zetia* *24 h-food record*
27 No Zetia *24 h-food record*	*28 No Zetia* *24 h-food record* *REMINDER TO FAST*	*29* **Fasting Blood draw wk-3**	*30*	*31*		

April 2012(SD)

Sun	Mon	Tue	Wed	Thu	Fri	Sat
1	*2*	*3*	*4*	*5*	*6 REMINDER TO FAST*	*7* **Fasting Blood** *24 h-food record*
8 No Zetia 24 h-food record	*9 No Zetia 24 h-food record*	*10 No Zetia*	*11 No Zetia*	*12 No Zetia*	*13 No Zetia*	*14No Zetia 24 h-food record*
15 No Zetia 24 h-food record	*16 No Zetia 24 h-food record*	*17 No Zetia*	*18 No Zetia*	*19 No Zetia*	*20 No Zetia 24 h-food record*	*21 No Zetia 24 h-food record*
22 No Zetia 24 h-food record	*23 No Zetia 24 h-food record*	*24 No Zetia*	*25 No Zetia*	*26 No Zetia*	*27 No Zetia REMINDER TO FAST*	*28 No Zetia* **Fasting Blood draw-PS**
29 No Zetia 24 h-food record	*30 No Zetia 24 h-food record*					

May 2012

Sun	Mon	Tue	Wed	Thu	Fri	Sat
		1 No Zetia	*2 No Zetia*	*3 No Zetia*	*4 No Zetia*	*5 No Zetia* *24 h-food record*
6 No Zetia *24 h-food record* *Drive to Winnipeg*	**7 No Zetia** **Fasting blood (d0)** **Stool sample** **24 h-food record** **Winnipeg**	**8 No Zetia** **Fasting blood (d1)** **Stool sample** **Winnipeg**	**9 No Zetia** **Fasting blood (d2)** **Stool sample** **24 h-urine sample** **Winnipeg**	**10 No Zetia** **Fasting blood (d3)** **Stool sample** **Winnipeg**	**11 No Zetia** **Fasting blood (d4)** **Stool sample** *REMINDER TO FAST*	*12 No Zetia* **Fasting Blood draw-wk1** *24 h-food record*
13 No Zetia *24 h-food record*	*14 No Zetia* *24 h-food record*	*15 No Zetia*	*16 No Zetia*	*17 No Zetia*	*18 No Zetia* *REMINDER TO FAST*	*19 No Zetia* *24 h-food record* **Fasting Blood draw-PS**
20 No Zetia *24 h-food record*	*21 No Zetia* *24 h-food record*	*22 No Zetia*	*23 No Zetia*	*24 No Zetia*	*25 No Zetia* *REMINDER TO FAST*	*26 No Zetia* *24 h-food record* **Fasting Blood draw wk-3**
27 No Zetia *24 h-food record*	*28 No Zetia* *24 h-food record*	*29*	*30*	*31*		

SCHEDULE OF EVENTS WHILE OFF EZETIMIBE

Appendix II

Study weeks	Recruitment	0*	2	4	5	7	9	12	14
Isotope Administration time points				24 hr, 48 hr, 72 hr, 92 hr	wk 1	wk 3	wk 5	wk 8	wk 10
Informed Consent Process	X	(X)							
Subject taken off ezetimibe		X	X	X	X	X	X	X	X
Blood draw for plasma sterol and lipid levels		X	X	X		X		X	X
Comprehensive clinical		X		X		X		X	
Biochemical evaluation		X		X		X		X	
Physical exam		X		X		X		X	
Measure tuberous and tendinous xanthomata		X		X		X		X	X
Blood draw for blood chemistries (liver, kidney, and thyroid function)		X				X			X
Intravenous infusion of D_7-sitosterol				X					
Intravenous infusion of ^{18}O-cholesterol				X					
Oral administration of $^{2}H_2O$				X					
Oral administration of ^{13}C-cholesterol				X					
Blood draw after isotope to monitor D_7-sitosterol, ^{18}O-cholesterol, ^{13}C-cholesterol and deuterated cholesterol enrichment levels				X	X	X	X	X	X

Study weeks	Recruitment	0*	2	4	5	7	9	12	14
Isotope Administration time points				24 hr, 48 hr, 72 hr, 92 hr	wk 1	wk 3	wk 5	wk 8	wk 10
Informed Consent Process	X	(X)							
Blood, urine and fecal samples obtained for biorepository					X**				X**
Adverse event review			X	X	X	X	X	X	X
Subject reinstated on ezetimibe									X

SCHEDULE OF EVENTS WHILE ON EZETIMIBE

Appendix II

Study weeks	14***	16	18	19	21	23	26	28	32
Isotope Administration time points			24 hr, 48 hr, 72 hr, 92 hr	wk 1	wk 3	wk 5	wk 8	wk 10	
Subject on ezetimibe	X	X	X	X	X	X	X	X	
Blood draw for plasma sterol and lipid levels		X	X		X		X	X	X
Comprehensive clinical			X		X		X		
Biochemical evaluation			X		X		X		
Physical exam			X		X		X		
Measure tuberous and tendinous xanthomata			X		X		X	X	X
Blood draw for blood chemistries (liver, kidney, and thyroid function)					X			X	X
Intravenous infusion of D_7-sitosterol			X						
Intravenous infusion of ^{18}O-cholesterol			X						
Oral administration of $^{2}H_2O$			X						
Oral administration of ^{13}C-cholesterol			X						
Blood draw after isotope to monitor D_7-sitosterol, ^{18}O-cholesterol, ^{13}C-cholesterol and deuterated cholesterol enrichment levels			X	X	X	X	X	X	X
Blood, urine and fecal samples obtained for biorepository				X**				X**	
Adverse event review		X	X	X	X	X	X	X	X

Study weeks	14***	16	18	19	21	23	26	28	32
Isotope Administration time points			24 hr, 48 hr, 72 hr, 92 hr	wk 1	wk 3	wk 5	wk 8	wk 10	
Subject reinstated on pre-study ezetimibe								X	

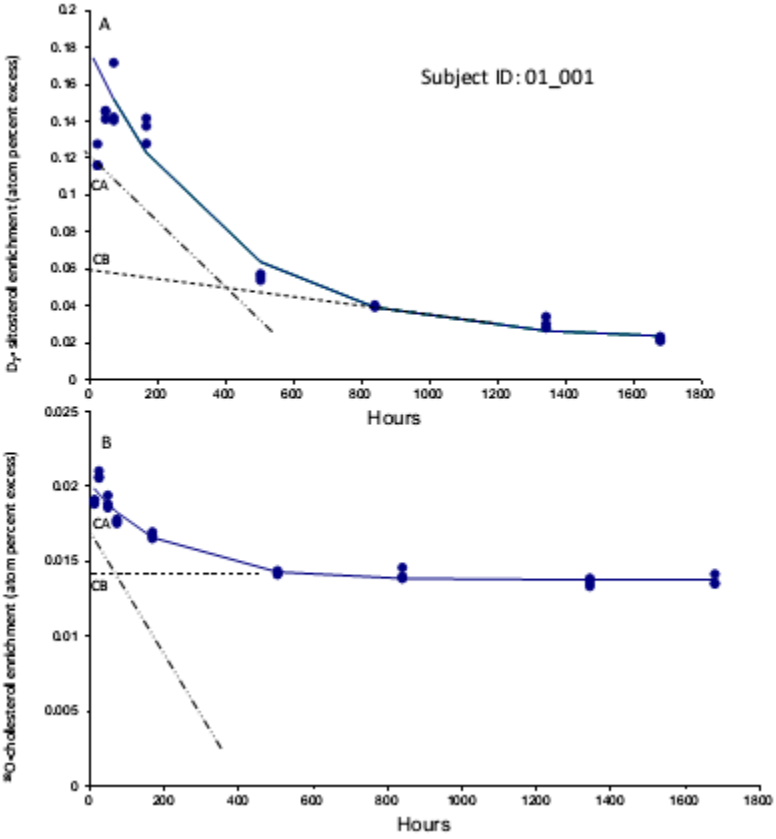
BLOOD DRAWS THROUGHOUT THE STUDY

Appendix II

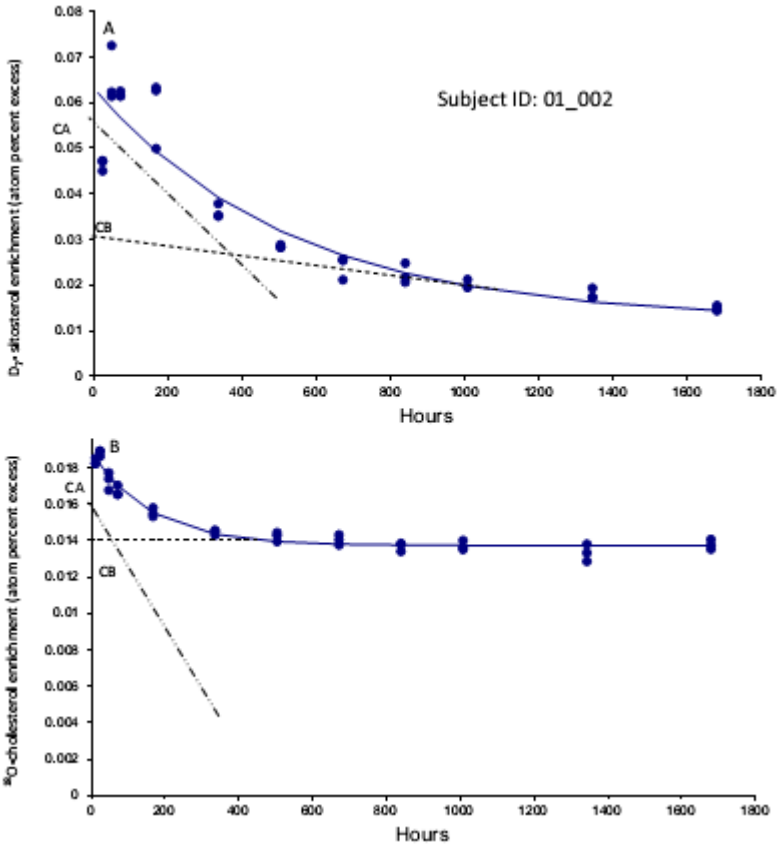
Study weeks	Purpose	Volume of blood
Week 0	Baseline evaluation: plasma plant sterols and cholesterol levels; comprehensive chem. panel for liver, kidney, and thyroid function.	20 ml
Week 2	Monitoring visit off ezetimibe: plasma plant sterols and cholesterol levels; comprehensive chem. panel for liver, kidney, and thyroid function.	20 ml
Weeks 4-14	Stable isotope testing OFF ezetimibe	
0 hr (pre-stable isotope i.v. infusion)	Measurement of whole body sitosterol and cholesterol pool sizes, cholesterol absorption and synthesis. These measurements require kinetic analysis of the enrichment of blood samples in stable isotopes given orally or i.v.	10 ml
12 hrs post infusion		10 ml
24 hrs post infusion		10 ml
48 hrs post infusion		10 ml
72 hrs post infusion		10 ml
96 hrs post infusion		10 ml
Wk 1 post infusion		10 ml
Wk 3 post infusion		10 ml
Wk 5 post infusion		10 ml
Wk 8 post infusion		10 ml
Wk 10 post infusion		10 ml
Week 16	Monitoring visit ON ezetimibe: plasma plant sterols and cholesterol levels; comprehensive chem. panel for liver, kidney, and thyroid function)	20 ml
Weeks 18-28	Stable isotope testing ON ezetimibe	
0 hr (pre-stable isotope i.v. infusion)	Measurement of whole body sitosterol and cholesterol pool sizes, cholesterol absorption and synthesis. These measurements require kinetic analysis of the enrichment of blood samples in stable isotopes given orally or i.v.	10 ml
12 hrs post infusion		10 ml
24 hrs post infusion		10 ml
48 hrs post infusion		10 ml
72 hrs post infusion		10 ml
96 hrs post infusion		10 ml
Wk 1 post infusion		10 ml
Wk 3 post infusion		10 ml
Wk 5 post infusion		10 ml
Wk 8 post infusion		10 ml
Wk 10 post infusion		10 ml
Week 32	Follow-up visit: plasma plant sterols and cholesterol levels; comprehensive chem. panel for liver, kidney, and thyroid function.	20 ml

APPENDIX III

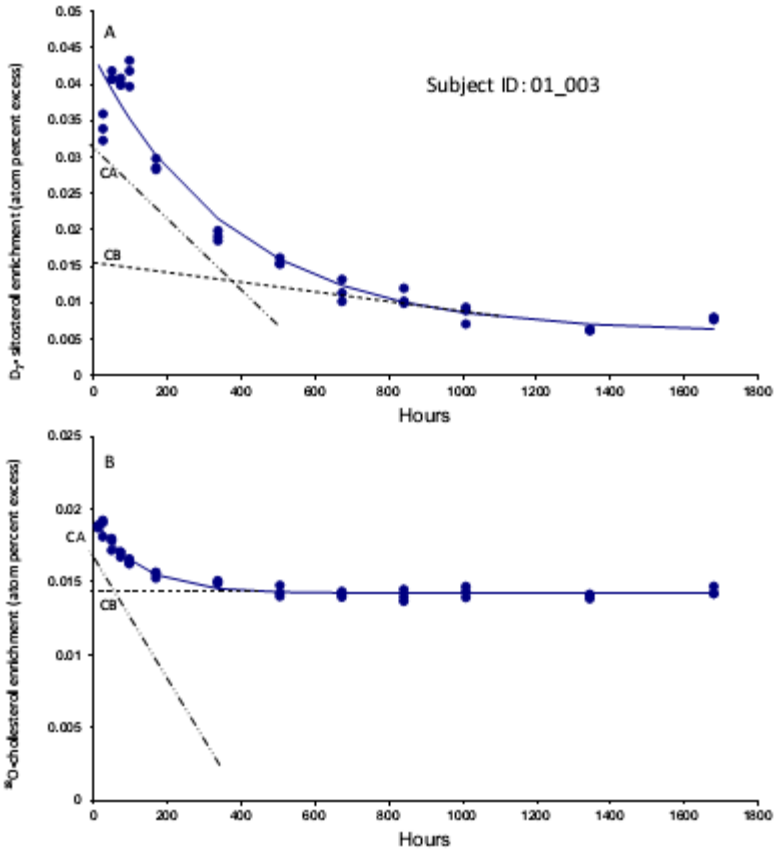
ADDITIONAL RESULTS AND TABLES CORRESPONDING TO STUDIES DESCRIBED IN CHAPTER IV, V AND VI.



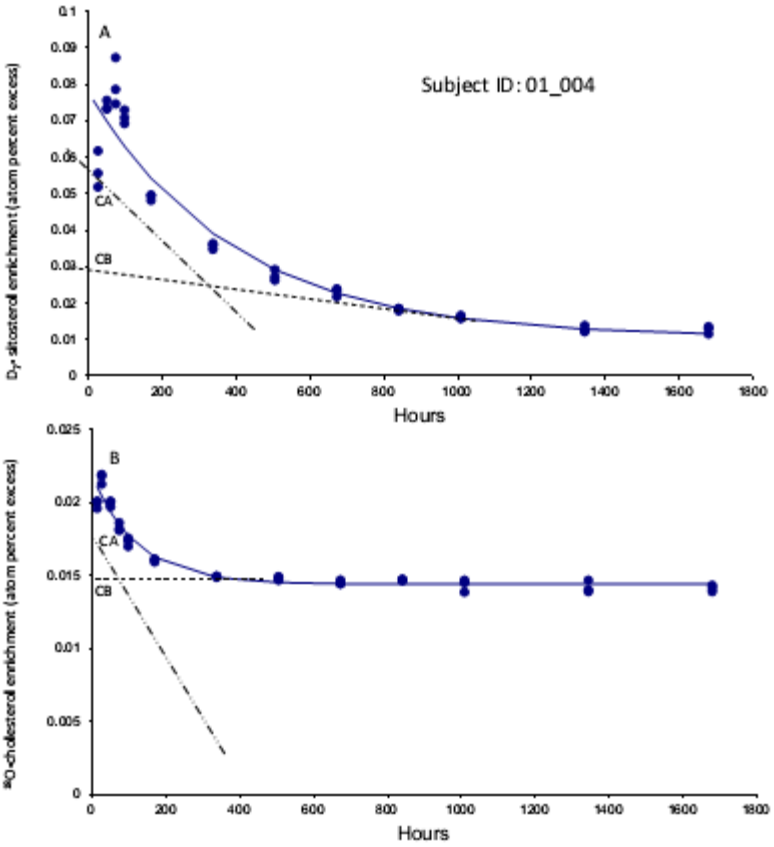
Individual D_7-sitosterol (A) and ^{18}O-cholesterol (B) enrichment curves for red blood cell following intravenous infusions of D_7-sitosterol and ^{18}O-cholesterol in one sitosterolemia patient over 10 wks (1,680 hours). The blue circles, connected by a solid line, represent the experimental values observed during the 10 wks study. Extrapolation of the linear portion of the curve to zero time (dashed line) provides the intercept CB. Subtraction of this extrapolated line from the experimental points provides intercept CA at zero time, which is the difference values shown by an interrupted (dash-dot-dot) line.



Individual D_7-sitosterol (A) and ^{18}O-cholesterol (B) enrichment curves for red blood cell following intravenous infusions of D_7-sitosterol and ^{18}O-cholesterol in one sitosterolemia patient over 10 wks (1,680 hours). The blue circles, connected by a solid line, represent the experimental values observed during the 10 wks study. Extrapolation of the linear portion of the curve to zero time (dashed line) provides the intercept CB. Subtraction of this extrapolated line from the experimental points provides intercept CA at zero time, which is the difference values shown by an interrupted (dash-dot-dot) line.



Individual D_7-sitosterol (A) and ^{18}O-cholesterol (B) enrichment curves for red blood cell following intravenous infusions of D_7-sitosterol and ^{18}O-cholesterol in one sitosterolemia patient over 10 wks (1,680 hours). The blue circles, connected by a solid line, represent the experimental values observed during the 10 wks study. Extrapolation of the linear portion of the curve to zero time (dashed line) provides the intercept CB. Subtraction of this extrapolated line from the experimental points provides intercept CA at zero time, which is the difference values shown by an interrupted (dash-dot-dot) line.



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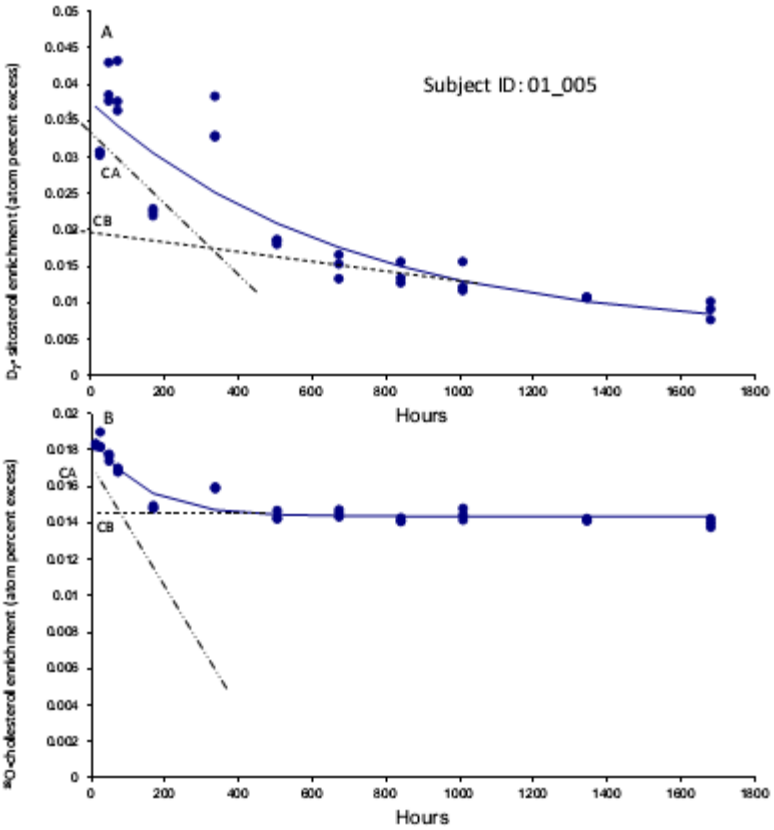

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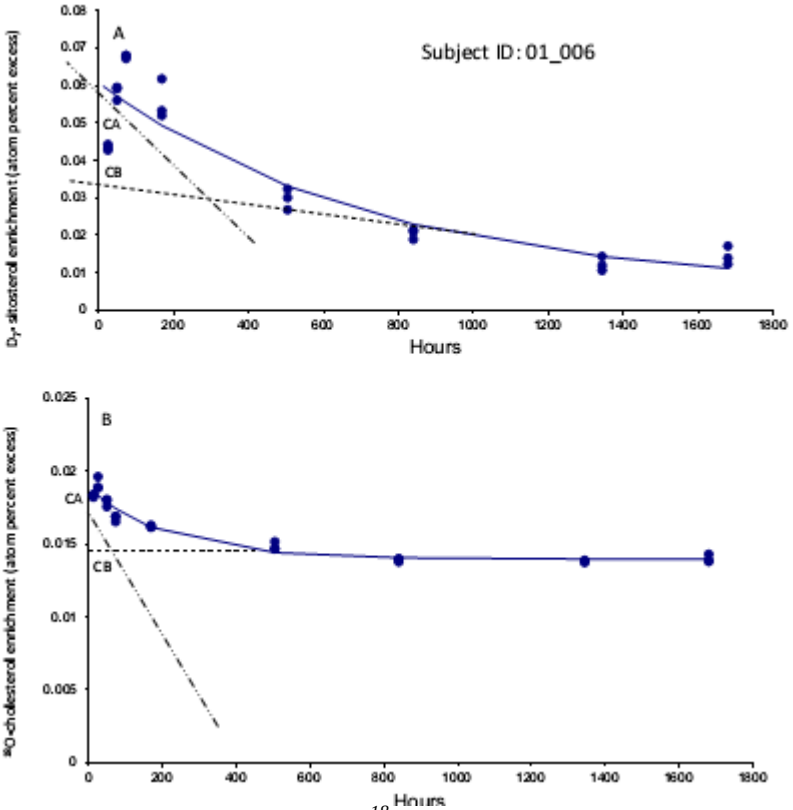

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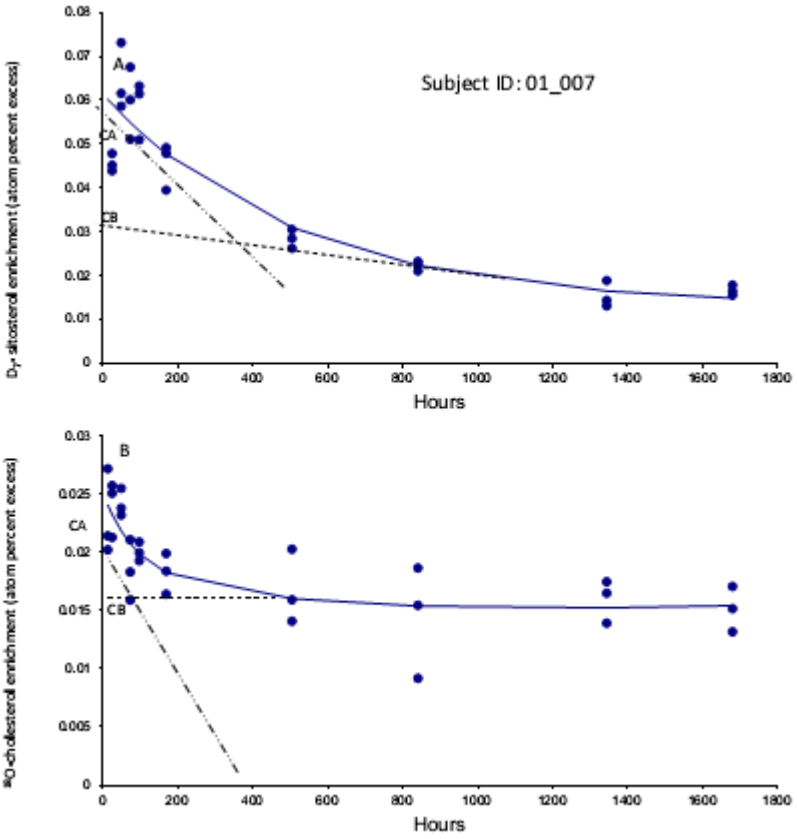
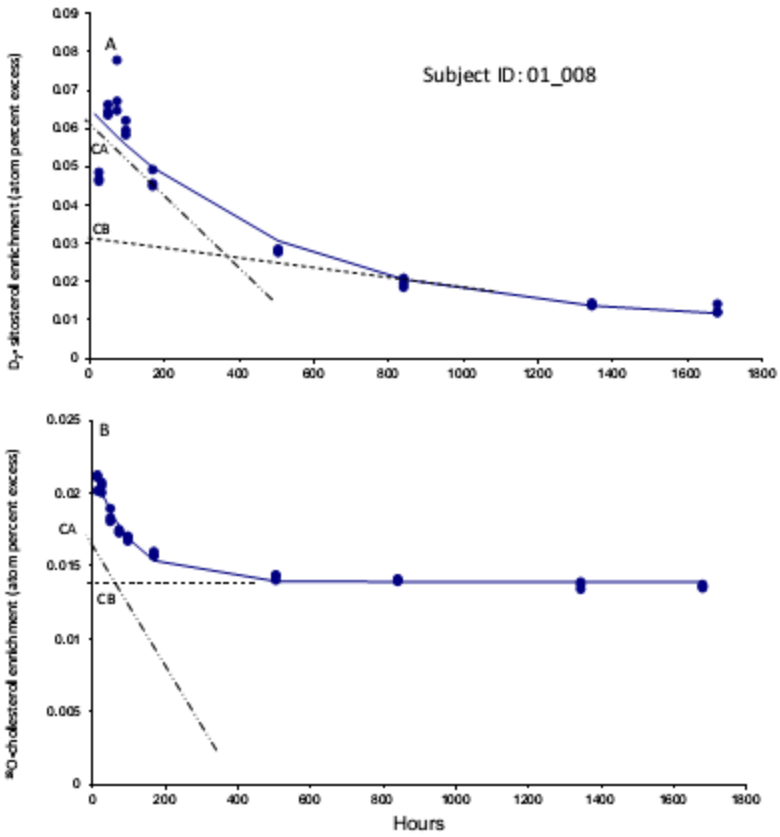


Individual D_7-sitosterol (A) and ^{18}O-cholesterol (B) enrichment curves for red blood cell following intravenous infusions of D_7-sitosterol and ^{18}O-cholesterol in one sitosterolemia patient over 10 wks (1,680 hours). The blue circles, connected by a solid line, represent the experimental values observed during the 10 wks study. Extrapolation of the linear portion of the curve to zero time (dashed line) provides the intercept CB. Subtraction of this extrapolated line from the experimental points provides intercept CA at zero time, which is the difference values shown by an interrupted (dash-dot-dot) line.



Individual D_7-sitosterol (A) and ^{18}O-cholesterol (B) enrichment curves for red blood cell following intravenous infusions of D_7-sitosterol and ^{18}O-cholesterol in one sitosterolemia patient over 10 wks (1,680 hours). The blue circles, connected by a solid line, represent the experimental values observed during the 10 wks study. Extrapolation of the linear portion of the curve to zero time (dashed line) provides the intercept CB. Subtraction of this extrapolated line from the experimental points provides intercept CA at zero time, which is the difference values shown by an interrupted (dash-dot-dot) line.

Table III. 1*: Changes in xanthoma size after 4 weeks off and on ezetimibe in homozygous sitosterolemia*

Subjects n=8	Xanthoma size (cmXcm) 4 Wks off EZE		4 Wks on EZE		Type
	Left	Right	Left	Right	
Subject #1	13 X 4	12X4	10 X4	9X3	Achilles tendon
Subject #2	-	-	-	-	-
Subject #3	-	-	-	-	-
Subject #4	-	-	-	-	-
Subject #5	-	-	-	-	-
Subject #6	-	-	-	-	-
Subject #7	4 X 2.75	3X2.5	3.25 X2.5	3.25X5	Achilles tendon
Subject #8	-	-	-	-	-

Table III. 2*: RELATIONSHIPS BETWEEN PLASMA CHOLESTEROL, PLANT STEROLS AND FRACTIONAL CHOLESTEROL SYNTHESIS*

Sterol/5α-stanol (n=8)	Pearson correlation r	P-value
Cholesterol (plasma)	r=0.56	0.15
Cholesterol (RBC)	r=0.60	0.11
Sitosterol (plasma)	r=-0.43	0.29
Sitosterol (RBC)	r=-0.57	0.14
Sitostanol (plasma)	r=-0.65	0.08
Sitostanol (RBC)	r=-0.55	0.16
Campesterol (plasma)	r=-0.60	0.12
Campesterol (RBC)	r=-0.60	0.11
Cholestanol (plasma)	r=-0.80	0.02
Cholestanol (RBC)	r=-0.86	0.006
Total PS (plasma)	r=-0.46	0.25
Total PS (RBC)	r=-0.55	0.16
Total PS/cholesterol (plasma)	r=-0.61	0.11
Total PS/cholesterol (RBC)	r=-0.56	0.15
Lathosterol (plasma)	r=0.83	0.01
Lathosterol (RBC), n=5	r=0.95	0.02
Lathosterol/cholesterol (plasma)	r=0.76	0.03
Lathosterol/cholesterol (RBC)	r=0.95	0.01

EZETIMIBE EFFECTS ON PLASMA FATTY ACIDS THROUGHTOUT STUDY

EZE inhibits intestinal sterol absorption, and it may also affect the absorption of other nutrients such as fatty acids. Fatty acids are not absorbed directly with cholesterol, which requires *via* the NPC1L1 transporter. The present study found no significant effect of EZE on plasma levels of fatty acids throughout 14 wks, suggesting that EZE did not influence the absorption of fatty acids in STSL (**Table III, 3)**.

Table III. 3: *Dietary fats, fatty acids and cholesterol intake over 14 weeks off and on ezetimibe in homozygous sitosterolemia patients.*

	Off EZE			On EZE			p value		
Fat (g)	Baseline (n=8)	Wk9 (n=6)	Wk14 (n=5)	Baseline (n=5)	Wk9 (n=5)	Wk14 (n=5)	Treatment	Time	Interaction
Total fat	124.8±28.3	105.5±37.3	82.4±18.5	93.6±18.4	88.6±23.8	85.9±19.6	0.33	0.51	0.70
Total SFA	52.2±11.9	41.3±13.1	30.2±6.4	35.8±7.3	35.5±10.8	29.5±7.4	0.17	0.27	0.56
14:0	0.9±0.5	1.3±0.4	0.7±0.2	0.8±0.3	0.9±0.2	1.1±0.2	0.87	0.88	0.72
16:0	5.1±1.7	11.2±3.8	8.9±2.7	8.1±3.1	9.4±2.8	8.7±2.4	0.90	0.51	0.76
18:0	2.0±0.7	6.0±1.5	3.5±0.8	2.8±0.9	4.1±1.0	3.8±0.9	0.86	0.38	0.75
20:0	0.02±0.0	0.03±0.0	0.1±0.0	0.04±0.0	0.03±0.0	0.01±0.01	0.68	0.77	0.04
22:0	0.7±0.1	0.8±0.4	1.1±0.1	0.4±0.1	0.9±0.2	0.8±0.2	0.33	0.16	0.97
24:0	0.01±0.0	0.03±0.0	0.03±0.0	0.02±0.0	0.02±0.0	0.0±0.0	0.52	0.88	0.09
Total MUFA	12.2±3.1	21.6±8.7	16.7±4.2	13.4±3.9	16.9±4.0	16.6±5.0	0.83	0.50	0.84
18:1	7.8±1.9	18.6±7.4	13.9±3.6	11.6±3.3	15.1±3.7	14.7±4.6	0.92	0.33	0.74
Total PUFA	6.1±1.6	7.5±4.3	9.6±2.6	8.8±3.3	8.2±1.6	7.7±2.3	0.86	0.86	0.66
Total n-6	4.0±0.8	5.8±3.6	7.8±2.1	7.5±2.9	6.9±1.5	6.6±2.2	0.60	0.74	0.57
18:2	3.9±0.8	5.7±3.6	7.6±2.1	7.5±2.9	7.1±1.4	6.4±2.2	0.56	0.74	0.57
20:3	0.02±0.0	0.03±0.0	0.01±0.0	0.01±0.0	0.01±0.0	0.01±0.0	0.10	0.20	0.46
20:4	0.1±0.0	0.1±0.0	0.2±0.03	0.08±0.02	0.07±0.03	0.2±0.0	0.55	0.07	0.77
Total n-3	0.6±0.2	1.1±0.5	0.74±0.2	0.7±0.3	0.8±0.2	0.5±0.1	0.29	0.44	0.72
18:3	0.4±0.1	0.6±0.2	0.70±0.2	0.7±0.3	0.8±0.2	0.5±0.1	0.67	0.63	0.48
20:5n-3(EPA)	0.02±0.0	0.2±0.1	0.01±0.0	0±0.0	0±0.0	0±0.0	0.07	0.45	0.45
22:5n-3(DPA)	0.02±0.0	0.1±0.0	0.01±0.0	0±0.0	0.0±0.0	0.01±0.0	0.05	0.30	0.16
22:6n-3(DHA)	0.1±0.1	0.1±0.1	0.03±0.0	0.01±0.0	0.01±0.0	0.03±0.0	0.18	0.69	0.53
n-6:n-3	11.3±1.0	10.3±3.6	13.5±2.3	12.0±2.0	9.4±0.9	10.2±1.7	0.42	0.44	0.55
Cholesterol(mg)	443.6±69.7	458.9±116.6	563.0±145.4	376.2±98.0	339.8±92.9	498.9±131.8	0.18	0.55	0.92

Data are mean are means ± SEM. P values are shown for the treatment, time, and interaction effects analyzed by mixed model ANOVA (with Bonferroni adjustment for multiple comparisons). DHA, docosahexaenoic acid; DPA, docospentaenoic acid; EPA, eicosapentaenoic acid; EZE, ezetimibe; MUFA, monounsaturated fatty acids; n-3 PUFA, omega-3 polyunsaturated fatty acids; n-6 PUFA, omega-6 polyunsaturated fatty acids; SFA, saturated fatty acids; Wk, week.

Table III. 4: *Plasma fatty acid concentrations over 14 weeks off and on ezetimibe in homozygous sitosterolemia patients*

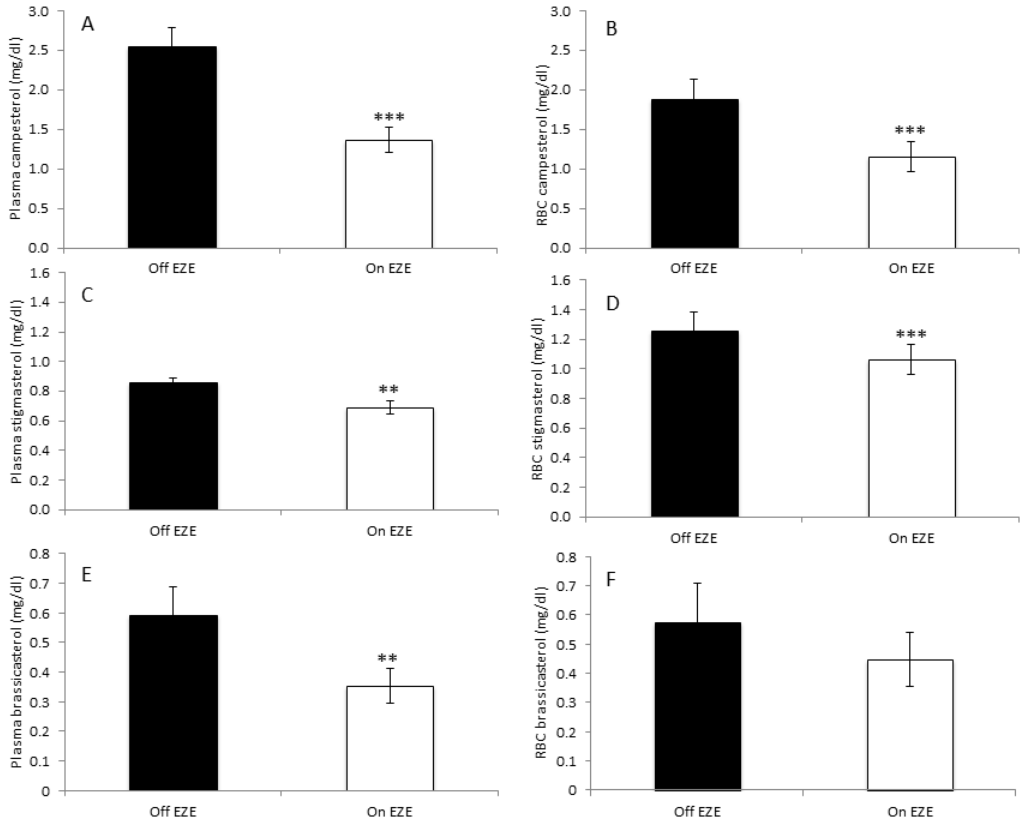
	Off EZE			On EZE			p value		
Total fatty acid (%)	Wk4	Wk9	Wk14	Wk4	Wk9	Wk14	Treatment	Time	Interaction
Total SFA	46.9±1.0	46.6±1.1	47.2±0.9	45.6±0.8	46.1±0.8	46.3±0.5	0.64	0.28	0.69
14:0	0.8±0.0	0.8±0.1	0.9±0.1	0.8±0.1	0.8±0.1	0.9±0.1	0.38	0.11	0.17
16:0	31.8±0.8	30.6±1.2	32.0±1.0	30.8±0.7	35.5±0.7	31.7±0.6	0.62	0.24	0.64
18:0	12.2±0.5	11.0±0.4[a]	12.4±0.4[b]	12.0±0.3	11.9±0.3	11.9±0.4	0.29	0.04	0.21
20:0	0.6±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.4±0.1	0.73	0.29	0.23
22:0	1.2±0.1	1.1±0.1	1.2±0.1	1.0±0.2	1.1±0.1	1.1±0.1	0.33	0.79	0.54
24:0	0.4±0.1	0.4±0.1	0.4±0.1	0.4±0.1	0.2±0.1	0.2±0.1	0.40	0.40	0.59
Total MUFA	14.4±1.7	15.5±1.0	13.6±1.6	15.7±0.8	14.1±0.8	14.9±1.0	0.79	0.93	0.36
14:1	0.04±0.0	0.2±0.1	0.1±0.0	0.04±0.0	0.04±0.0	0.03±0.0	0.23	0.25	0.38
16:1n-7	1.2±0.1	1.3±0.2	1.2±0.2	1.19±0.1	1.1±0.2	1.3±0.2	0.11	0.89	0.50
18:1n-9	11.9±1.8	12.4±0.9	10.9±1.6	12.9±0.6	11.5±0.7	12.2±0.8	0.43	0.96	0.39
24:1n-9	1.2±0.3	1.6±0.4	1.3±0.2	1.4±0.2	1.3±0.2	1.3±0.2	0.20	0.46	0.67
20:1n-9	0.2±0.0[a]	0.1±0.0[b]	0.1±0.0	0.1±0.0[a]	0.1±0.0[b]	0.1±0.0	0.72	0.03	0.24
Total PUFA	38.7±1.5	37.9±1.2[a]	39.2±1.2	38.7±1.3	39.8±1.3[b]	38.8±1.3	0.21	0.74	0.09
T. n-6 PUFA	33.8±1.4	33.1±1.3[a]	34.2±1.3	33.8±1.4	35.0±1.5[b]	34.0±1.6	0.10	0.79	0.08
18:2n-6	20.5±1.3	19.1±0.8	21.5±1.4	19.6±1.2	20.9±1.4	20.0±1.9	0.27	0.54	0.20
18:3n-6	0.1±0.0	0.1±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.14	0.42	0.73
20:2n-6	0.2±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.2±0.1	0.82	0.44	0.42
20:3n-6	2.5±0.2	2.6±0.2	3.1±0.2	2.6±0.1	2.7±0.1	2.5±0.1	0.68	0.64	0.08
20:4n-6	9.9±1.7	10.5±1.0	9.8±1.1	10.5±1.7	10.5±1.9	10.7±1.8	0.71	0.17	0.90
22:4n-6	0.7±0.2	0.5±0.2	0.6±0.2	0.6±0.2	0.6±0.2	0.4±0.1	0.34	0.82	0.36
T. n-3 PUFA	4.9±0.6	4.8±0.4	5.0±0.6	4.9±0.4	4.9±0.4	4.9±0.4	0.68	0.81	0.55
18:3n-3	0.3±0.0	0.6±0.3	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.1	0.21	0.46	0.32
20:5n-3(EPA)	0.8±0.2	0.7±0.2	0.8±0.2	0.8±0.2	0.8±0.1	0.9±0.2	0.85	0.59	0.51
22:5n-3(DPA)	0.9±0.1	0.9±0.1	1.1±0.2	1.0±0.1	1.0±0.1	0.9±0.1	0.74	0.95	0.11
22:6n-3(DHA)	2.9±0.4	2.6±0.3	2.8±0.3	2.8±0.3	2.8±0.3	2.7±0.3	0.79	0.43	0.05
n-6:n-3	7.7±1.0	7.4±1.0	7.6±0.9	7.4±0.91	7.7±0.9	7.5±1.0	0.47	0.92	0.68

Data are mean are means ± SEM. [a,b,c] *Mean values within a row with unlike superscript letters were significantly different between treatment groups ($p<0.05$). P values are shown for the treatment, time, and interaction effects analyzed by mixed model ANOVA (with Bonferroni adjustment for multiple comparisons). DHA, docosahexaenoic acid; DPA, docospentaenoic acid; EPA, eicosapentaenoic acid; EZE, ezetimibe; MUFA, monounsaturated fatty acids; n-3 PUFA, omega-3 polyunsaturated fatty acids; n-6 PUFA, omega-6 polyunsaturated fatty acids; SFA, saturated fatty acids; Wk, week.*

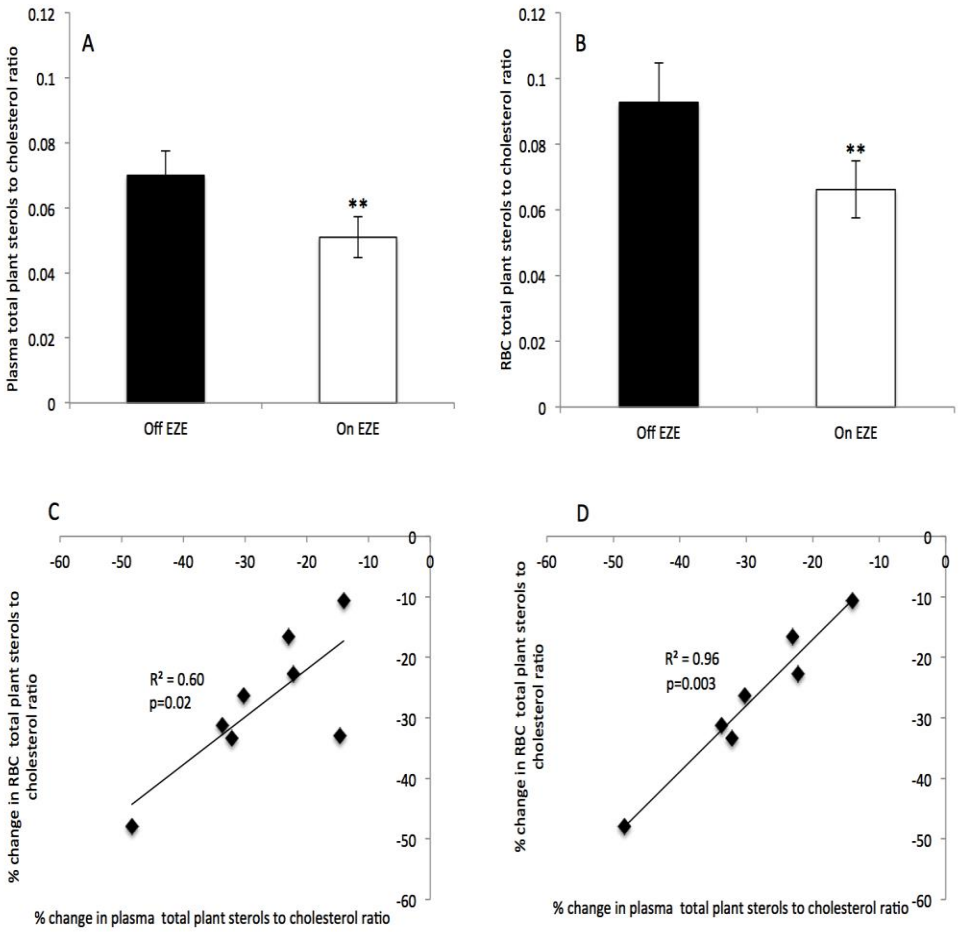
Table III. 5*: Changes in serum lipids throughout the study period in sitosterolemia patients*

Lipids (mg/dl)	Wk2	Wk4	Wk8	Wk10	Wk12	Wk14	Treatment P-value	Time P-value	Interaction P-value
TC									
Off EZE	218.9±13.4	238.9±11.2	223.8±10.0	244.5±16.5	235.9±11.3	241.2±14.0	<0.0001	0.01	0.13
On EZE	207.9±14.8	196.3±16.2***	200.1±15.2*	205.4±13.8***	220.4±17.1	209.3±12.1**			
TAG									
Off EZE	110.9±11.6	97.2±14.5	117.4±29.7	121.0±17.3	127.1±22.7	136.1±19.7	0.08	0.05	0.61
On EZE	92.7±14.8	92.0±16.1	121.7±29.8	86.3±11.01	135.3±32.3	112.9±21.3			
VLDL									
Off EZE	22.2±2.3	19.4±2.9	23.5±5.9	24.2±3.5	25.4±4.5	27.2±3.9	0.08	0.05	0.61
On EZE	18.5±3.0	18.4±3.2	24.3±6.0	17.3±2.2	27.1±6.5	22.6±4.3			
HDL									
Off EZE	51.7±2.7	54.1±3.4	52.2±1.9	53.7±3.9	51.24±3.2	49.8±4.0	<0.0001	0.38	0.63
On EZE	55.6±5.1	58.0±5.6	61.4±4.6*	63.8±5.8**	59.0±4.1*	60.9±3.4**			
LDL									
Off EZE	144.9±14.0	165.1±10.5	148.0±11.0	166.6±14.0	159.1±10.3	164.0±13.4	<0.0001	0.003	0.12
On EZE	133.6±11.5	119.7±12.9***	114.3±14.9**	124.3±13.4***	134.2±14.5*	125.7±12.05***			
TC/HDL									
Off EZE	4.35±0.40	4.52±0.32	4.31±0.20	4.6±0.3	4.7±0.4	5.1±0.5	<0.0001	0.007	0.09
On EZE	3.88±0.34	3.47±0.22**	3.37±0.31**	3.4±0.3***	3.9±0.3**	3.5±0.3***			
LDL/HDL									
Off EZE	2.91±0.4	3.2±0.3	2.9±0.2	3.2±0.3	3.2±0.3	3.5±0.4	<0.0001	0.003	0.15
On EZE	2.51±0.27	2.1±0.2***	1.9±0.3**	2.1±0.3***	2.4±0.3**	2.1±0.2***			
Non-HDL									
Off EZE	167.3±14.0	184.7±11.3	171.6±9.7	190.9±15.2	184.7±11.7	191.4±14.5	<0.0001	0.003	0.04
On EZE	152.3±13.3	138.3±13.5***	138.7±15.4**	141.6±14.2***	161.5±16.8*	148.4±12.5***			
Non-HDL/HDL									
Off EZE	3.4±0.4	3.5±0.3	3.3±0.2	3.6±0.3	3.7±0.4	4.1±0.5	<0.0001	0.007	0.09
On EZE	2.9±0.3	2.5±0.2**	2.4±0.3**	2.4±0.3***	2.9±0.3**	2.5±0.3***			

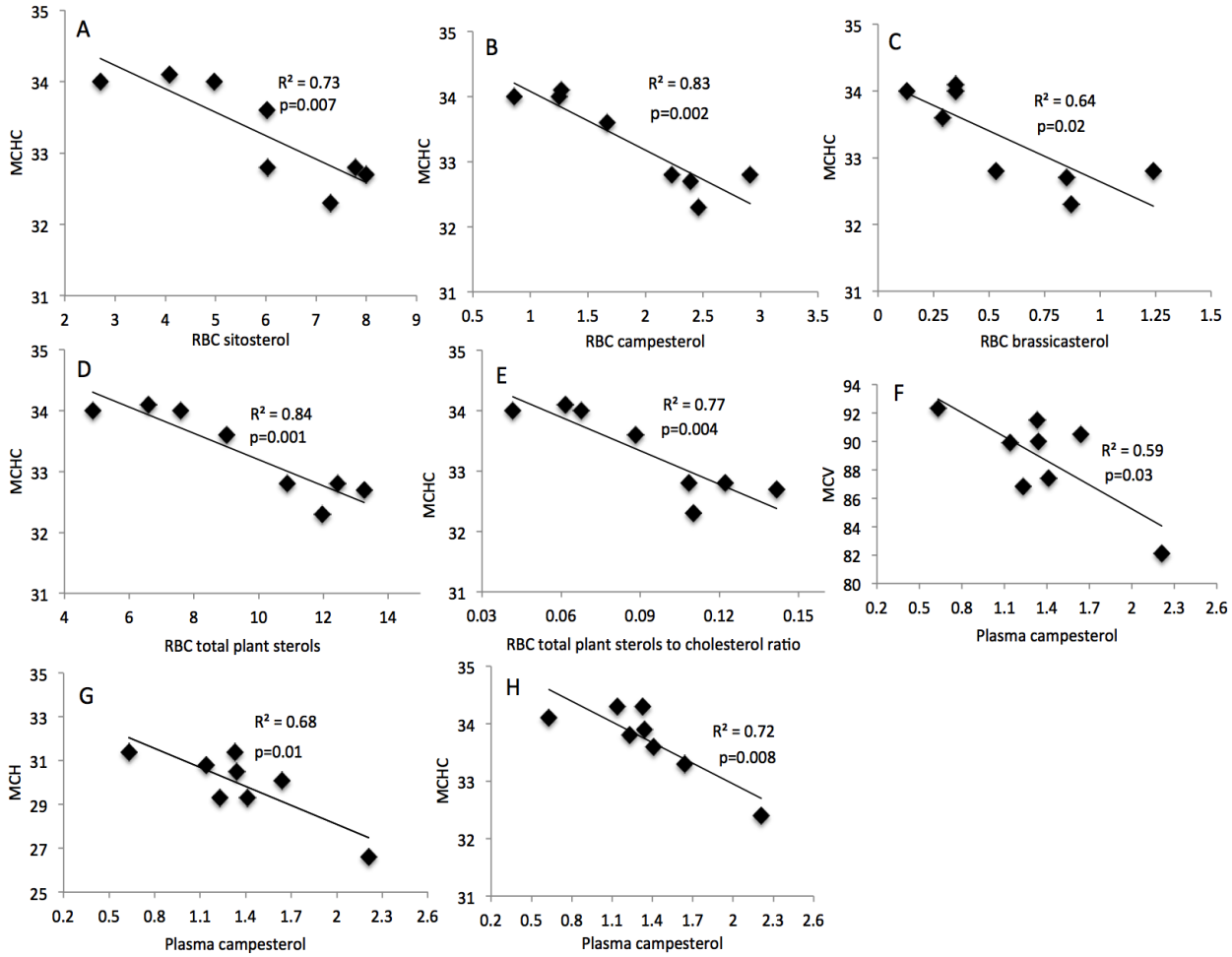
*All values are means±SEM (n=8). Mean values within a row with asterisk are significantly different between off EZE and on EZE treatments. *P<0.05, **P<0.01, ***P<0.0001. P values for the treatment, time or interaction were analyzed using a linear-mixed-model ANOVA (Bonferroni post hoc test for multiple comparisons) by including treatment and time as main effects and the interaction term into the linear mixed-model ANOVA. HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol; VLDL, very low-density lipoprotein.*



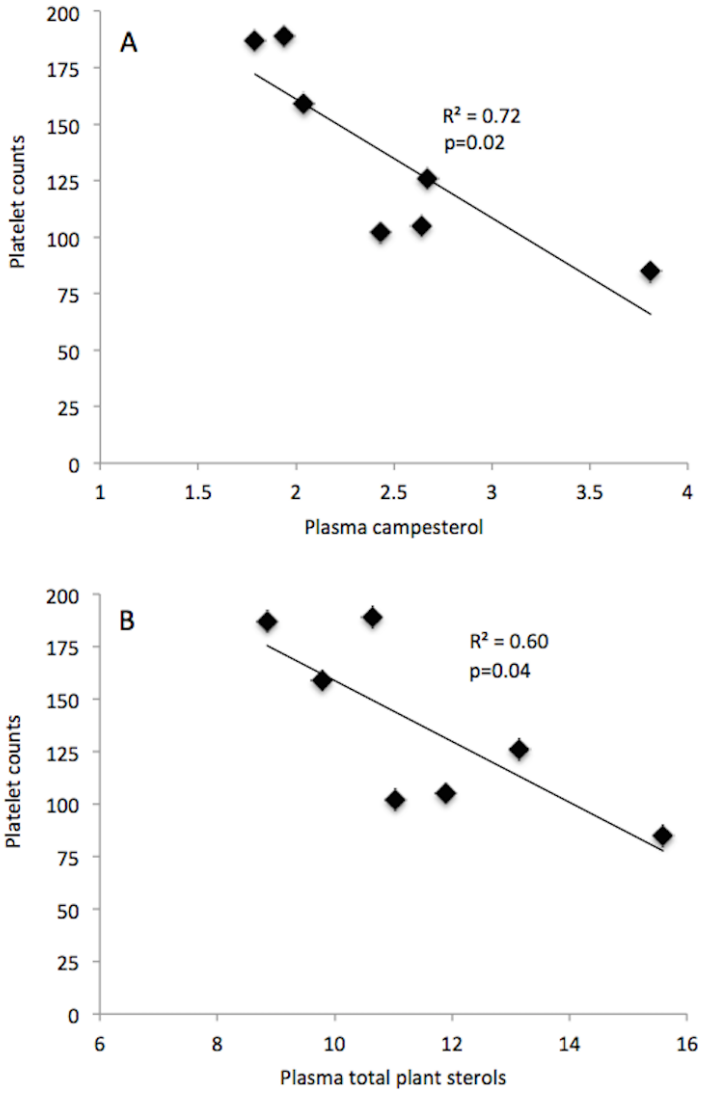
*Plasma and red blood cells campesterol, stigmasterol and brassicasterol at 14 weeks off and on ezetimibe in sitosterolemia patients. Plasma and red blood cells campesterol(A, B), stigmasterol (C, D) and brassicasterol (E, F) at 14 weeks off and on ezetimibe in sitosterolemia patients. Mean values were significantly different when compared with off EZE: *p<0·05, **p<0·01, ***p<0·0001), n=8. Abbreviations: EZE; ezetimibe, RBC; red blood cells.*



*Plasma and red blood cells total plant sterols to cholesterol ratio at 14 weeks off and 14 weeks on ezetimibe in sitosterolemia patients and their relationships. Plasma and red blood cells total plant sterols to cholesterol ratio (A, B) at 14 weeks off and 14 weeks on ezetimibe in sitosterolemia patients and their relationships. Data are mean±SEM and significantly different when compared with off EZE; *p<0·05, **p<0·01, ***p<0·0001, n=8. Relationship between plasma and RBC total plant sterols to cholesterol ratio (C, n=8, D, n=7) after ezetimibe. Abbreviations: EZE; ezetimibe, RBC; red blood cells.*



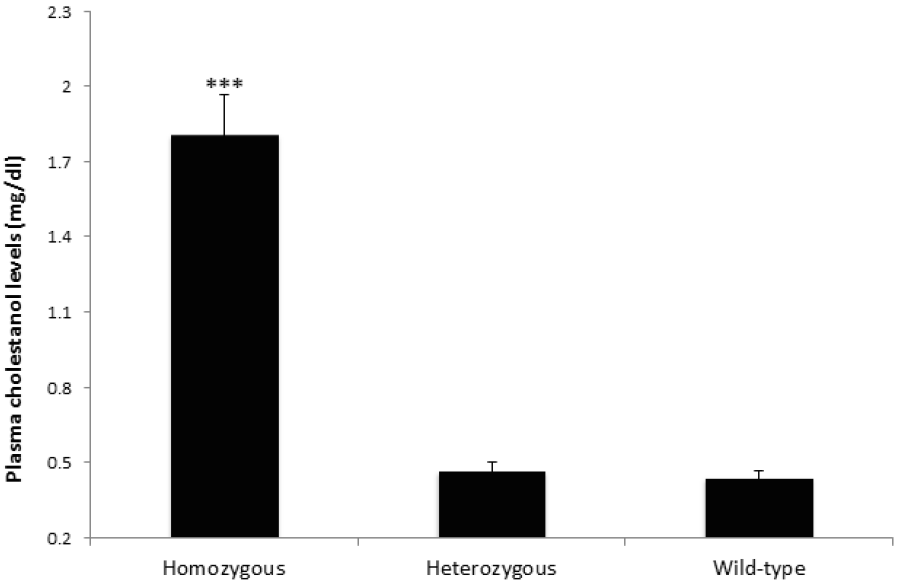
Linear regression coefficients (r2) for relationships between plant sterols and red cell indices (MCV, MCH and MCHC) before ezetimibe (A, B, C, D and E), and between plasma and red cell indices (MCV, MCH and MCHC) after ezetimibe (F, G and H) in sitosterolemia patients (n=8). MCV; mean corpuscular volume, MCH; mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.



Linear regression coefficients (r2) for relationships between campesterol and plant sterols and platelet count. Relationships between campesterol and platelet count (A) and between total plant sterols and platelet count before ezetimibe (B).

Table III. 6*: Correlation coefficients between changes in serum thyroid hormones and cholesterol, lathosterol plant sterols and 5α-stanols in homozygous sitosterolemia after 8 wks of ezetimibe therapy.*

Plasma sterol/stanol (n=8)	T3	T4	T3/T4	TSH
Cholesterol	-0.30	-0.46	-0.06	0.60
Lathosterol	-0.13	-0.05	0.12	0.53
Lathosterol to cholesterol	0.13	0.41	-0.20	-0.0
Cholestanol (n=7)	-0.67	-0.78*	-0.23	0.54
Sitostanol	-0.82*	-0.74*	-0.49	0.55
Stigmasterol	-0.51	-0.87**	-0.10	0.41
Sitosterol	-0.65	-0.88**	-0.12	0.35
Total PS	-0.57	-0.87**	-0.01	0.36
Total PS/cholesterol	-0.26	-0.63	0.24	0.18



*Plasma cholestanol concentrations in homozygous (n=8), heterozygous (n=10) sitoterolemia and wide-type control (n=15). One-way analysis of variance (ANOVA) followed by Dunnett's posttest was used to determine significance. ***, p< 0.0001.*

SERUM TRIACYLGLYCERIDE AND BILE ACID SYNTHESIS LEVELS IN HOMOZYGOUS AND HETEROZYGOUS SITOSTEROLEMIA

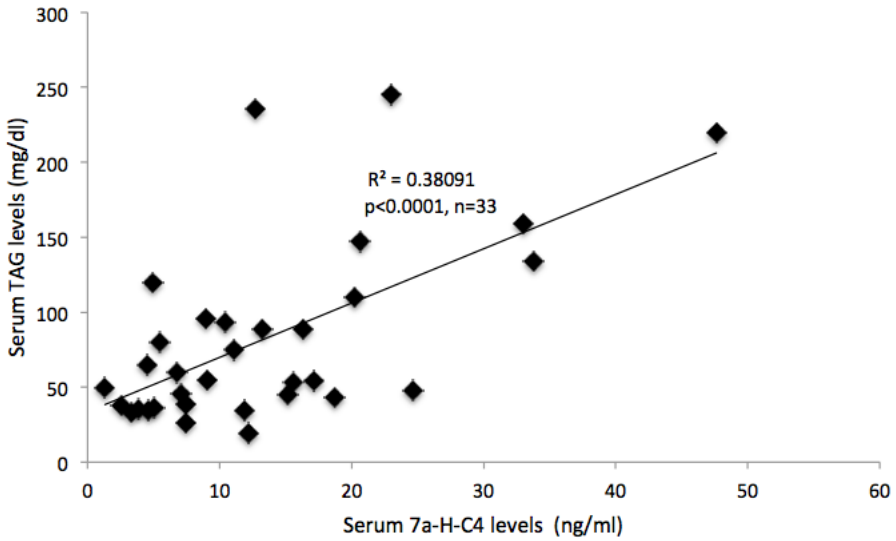
Conversion of cholesterol into bile acids is quantitatively the most important way for elimination of cholesterol from the body. Like PS, EZE inhibits intestinal cholesterol absorption. Previously, a PS consumption study was conducted at the Richardson Centre for Functional Foods and Nutraceuticals at the University of Manitoba, in which heterozygous STSL and wild-type subjects received 1.5 g of PS for 29 days. PS treatment resulted in a 10% reduction ($p<0.05$) in serum LDL cholesterol levels compared with placebo. However, effect of PS on bile acid synthesis is not known. Thus, we investigated whether treatment of PS would modulate serum marker of bile acid synthesis in heterozygous STSL. Similar to EZE treatment in homozygotes, PS did not impact serum bile acid biosynthesis marker, 7α-H-C4, 7α-hydroxy-4-cholesten-3-one (7α-H-C4) in STSL heterozygotes (8.3±1.4 vs 10.2±2.5 ng/ml, $p=0.51$) and wild-type controls (17.8±3.3 vs 20.5±7.0 ng/ml, $p=0.73$) compared with placebo. The upper level of the reference limits of serum 7α-HC seemed to be lower in heterozygous (4.5-14.6 ng/ml) and homozygous STSL (4.6-40.4 ng/ml) compared with wild-type controls (2.5-119.0 ng/ml) but was not statistically different ($p=0.15$) (**Table III, 7)**. Overall, serum 7α-H-C4 levels in homozygous and heterozygous STSL were comparable to the previously published levels in healthy adults (1, 2) and children (3). Furthermore, Miettinen et al. found that the bile acid synthesis was quantitatively normal in a subject with STSL compared with controls (4).

Table III. 7*: Changes in serum bile acid synthesis and triacylglyceride in homozygous and heterozygous Sitosterolemia*

7α-H-C4 (ng/ml)	Homozygous n=8	Heterozygous n=10	Wild-type n=15	Group	#Treatment	Interaction
Day 1-off	16.2±4.4	6.9±1.4	28.0±8.1	0.13	0.29	0.09
Day 1-on	11.0±4.1	13.6±3.3	12.5±1.9			
Endpoint off	19.9±8.5	10.2±2.5	20.5±7.0	0.15	0.32	0.82
Endpoint on	11.3±2.2	8.3±1.4	17.8±3.3			
TAG (mg/dl)						
Day 1-off	117.6±25.5*	115.7±10.2*	257.3±40.0	<0.0001	0.80	0.68
Day 1-on	92.7±14.8*	143.7±20.6	232.8±38.9			
Endpoint off	136.1±19.7**	137.4±25.4**	221.9±33.1	0.001	0.73	0.72
Endpoint on	128.5±28.3*	134.3±22.2*	262.8±42.6			

*Data are the mean±SEM.Two-way analysis of variance (ANOVA) followed by Dunnett's posttest to determine significance. Asterisks indicate significant differences: *, p< 0.05; **, p< 0.01; ***, p<0.0001 vs wild-type. TAG, triacylglyceride; 7α-H-C4, 7α-hydroxy-4-cholesten-3-one. #Treatment (i.e ezetimibe in homozygous and plant sterols in heterozygous sitosterolemia).*

Some previous reports observed a direct relationship between bile acid and TAG synthesis in patients with primary hyperlipoproteinemia (5). Low bile acid formation was accompanied with decreased TAG production (5) whereas increasing bile acids synthesis with bile acid resins increased hypertriglyceridemia (6-8). This suggests a link between degradation of cholesterol into bile acids and TAG production. In agreement, we found a positive association between plasma TAG and serum 7α-H-C4 ($r=0.64$, $p<0.0001$, $n=33$) that was consistent over time.



The relationship between serum triacylglyceride and serum 7α-hydroxy-4-cholesten-3-one levels in heterozygous (n=10), homozygous (n=8), wild-type (n=15) subjects. TAG, triacylglyceride; 7α-H-C4, 7α-hydroxy-4-cholesten-3-one.

Furthermore, we observed that serum TAG levels were at least 50% lower ($p<0.001$) in homozygous and heterozygous STSL than wild-type subjects (117.6±25.5, 115.7±10.2 and 257.3±40.0 mg/dl) **(Table III, 7)**. In disagreement, ABCG5/G8 deficiency in mice fed a chow diet markedly raises TAG levels by impairing TAG catabolism and increasing liver and intestinal TAG secretion (9). Cholesterol 7α-hydroxylase deficiency in APOE*3-Leiden mice decreased the VLDL production as a result of reduced bile acid biosynthesis, leading to a decrease in plasma TAG (10). These data underscore the close relationship between bile acid biosynthesis and TAG levels. Future kinetic studies with orally and intravenously administered labeled TAG are needed to determine its absorption, secretion and clearance, and establish whether low serum TAG in STSL is due to a decreased TAG absorption and synthesis or increased catabolism.

RELATIONSHIP BETWEEN PLASMA AND TISSUE PLANT STEROLS AND STANOLS, AND BILE ACID SYNTHESIS MARKER LEVELS IN HOMOZYGOUS AND HETEROZYGOUS SITOSTEROLEMIA

Increased plasma and tissues PS is related to low bile acids synthesis in STSL (11). We also tissue PS strongly and inversely correlated with bile acid synthesis intermediate 7α-H-C4, indicating that elevation of these sterols may inhibit bile acids synthesis; therefore, cholesterol cannot be converted to bile acids and then become available as substrate for cholestanol formation (12, 13). In the current research, serum 7α-H-C4 concentrations correlated positively with plasma and RBC cholesterol levels (r=0.86, p=0.006 and r=0.79, p=0.02) but inversely associated with RBC sitosterol (r=-0.74, p=0.03), campesterol (r=-0.72, p=0.05), sitostanol (r=-0.74, p=0.04), total PS (r=-0.73, p=0.04) and their cholesterol ratios (r=-0.73, p=0.04) and had marginal correlation with cholestanol (r=-0.66, p=0.08) (**Table III.8**). PS and 5α-stanols levels in RBC showed strong and significant associations with serum 7α-H-C4 levels compared with those of plasma; this may be due to the fact that plasma PS and 5α-stanols levels reflect the most recent meals whereas those of RBC probably indicate a longer-term average of plasma levels. The relationship between PS and 7α-H-C4 was not found in in heterozygous STSL and wild-type control subjects. Taken together, these results are of significance, further supporting interference of non-cholesterol sterols with cholesterol metabolism in STSL (**Table III.9**).

Table III. 8*: Correlation coefficients between serum 7α-hydroxy-4-cholesten-3-one and circulating levels of cholesterol, plant sterols and 5α-stanols in homozygous sitosterolemia before ezetimibe.*

Sterol/5α-stanol (n=8)	Pearson correlation r	P-value
Cholesterol (plasma)	0.86	0.006
Cholesterol (RBC)	0.79	0.02
Cholestanol (plasma)	-0.22	0.60
Cholestanol (RBC)	-0.66	0.08
Sitosterol (plasma)	-0.60	0.11
Sitosterol (RBC)	-0.74	0.03
Campesterol (plasma)	-0.63	0.10
Campesterol (RBC)	-0.72	0.05
Sitostanol (plasma)	-0.40	0.33
Sitostanol (RBC)	-0.74	0.04
Total PS (plasma)	-0.63	0.09
Total PS (RBC)	-0.73	0.04
Total PS/cholesterol (plasma)	-0.79	0.02
Total PS/cholesterol (RBC)	-0.73	0.04

RBC, red blood cells; PS, plant sterols

Table III. 9*: Correlation coefficients between serum 7α-hydroxy-4-cholesten-3-one, 7α-H-C4) and cholesterol and non-cholesterol sterols in heterozygous sitosterolemia (n=10) and wild-type control (n=15)*

Sterol (n=8)	Pearson correlation r	P-value
Cholesterol	0.13	0.54
LDL	0.35	0.09
HDL	-0.24	0.25
TAG	0.51	0.009
Cholestanol	0.11	0.61
Campesterol	-0.044	0.84
Sitosterol	-0.09	0.68
Sum Campesterol+sitosterol	-0.16	0.44
Lathosterol	-0.02	0.94
Desmosterol	0.25	0.24

TAG, triacylglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

RELATIONSHIP BETWEEN MONOCYTES AND PLANT STEROLS IN SITOSTEROLEMIA

Atherosclerosis is a chronic inflammatory disease, where monocytes play a crucial role in all stages of atherosclerotic lesions formation (14). Recently plasma brassicasterol co-segregated with atherosclerosis at a novel atherosclerosis locus and correlated with lesion size in mice (15). Moreover, low plasma brassicasterol levels have been found in patients with Down syndrome DS (16), who are recognized as an "atheroma-free model" (17) despite unfavorable lipid profile and accelerated aging (18, 19). Using data from Chapter 4, a robust positive association was found between monocytes and plasma brassicasterol ($r=0.89$, $p=0.003$) in current STSL patients. Thus far, there is no consensus on the role of specific PS in atherogenesis. Brassicasterol is found in canola oils and shellfish, and is structurally similar to cholesterol but it has a double bond at C-22. PS are susceptible to oxidative processes, and indeed may be more so than cholesterol (20) since they have side chain substitutions at carbon 24 with an extra double bond between C-22 and C-23 like brassicasterol. Plasma brassicasterol levels in the current STSL patients were higher (0.2 to 0.8 mg/dl) than those of healthy subjects (0.03 mg/dl) and DS patients (0.01 mg/dl) (16). Accumulation of brassicaterol in STSL might influence a number of signaling and trafficking pathways that play a special role in plaque formation or other processes related to atherogenesis. This finding generates interesting observation as whether individual PS behaves differently and thus has distinct effects. Future studies should investigate whether PS would induce different inflammatory reactions in macrophages and macrophage cell lines.

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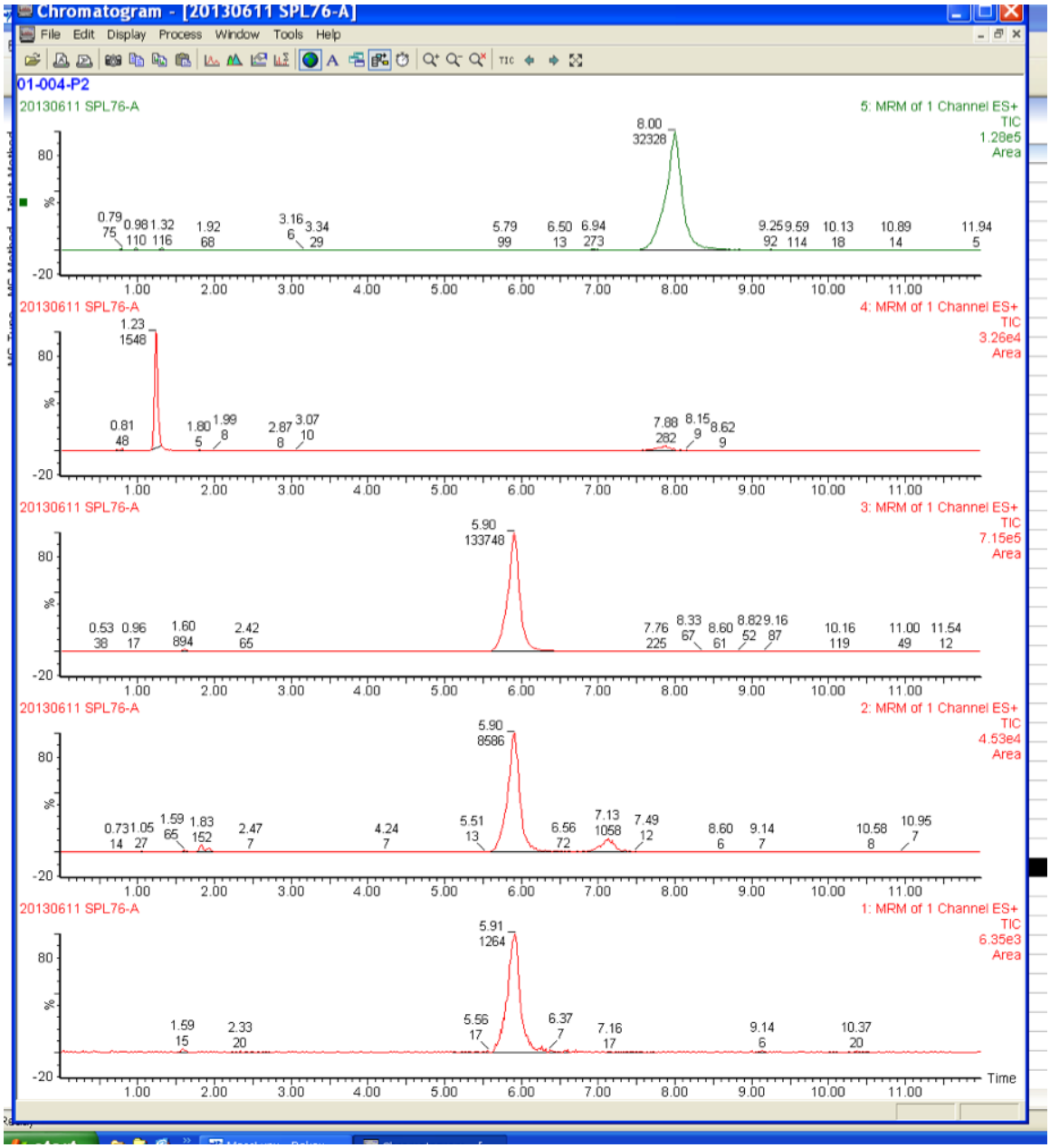
UPLC-MS/MS CHROMATOGRAMS FOR TRIPLE ISOTOPES (SAMPLE)



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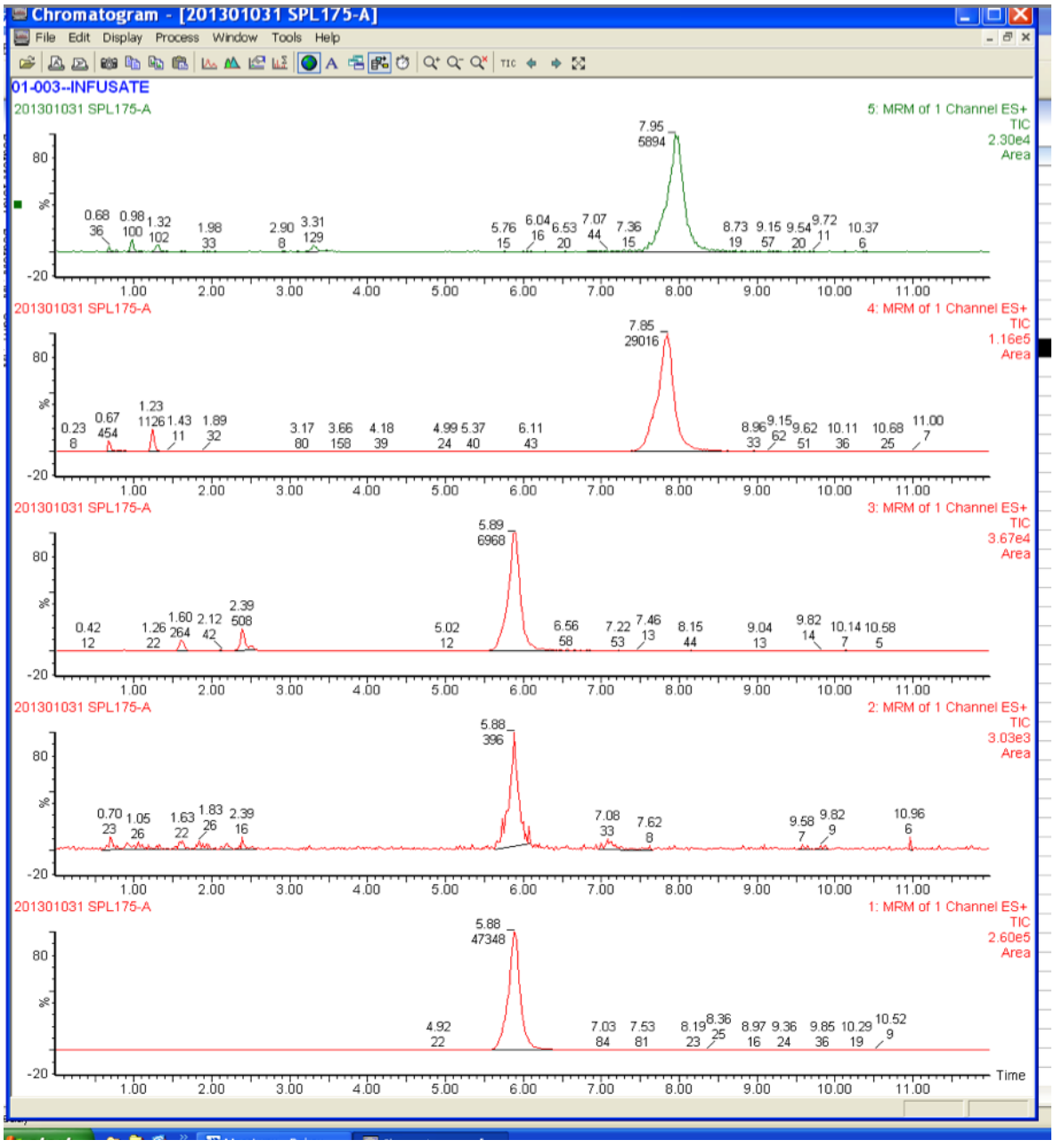

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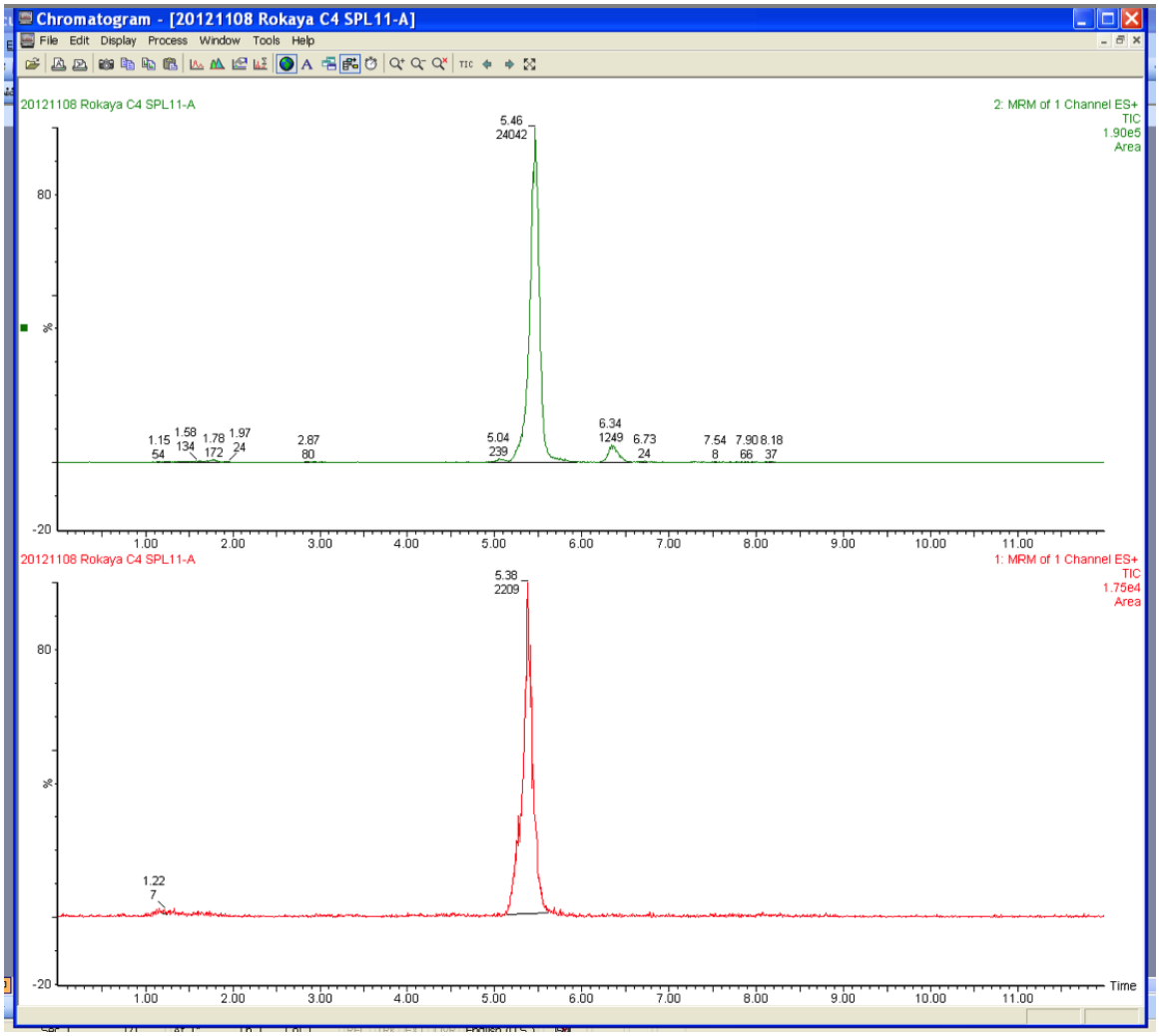

GC-CHROMATOGRAMS FOR PLANT STEROLS AND STANOLS

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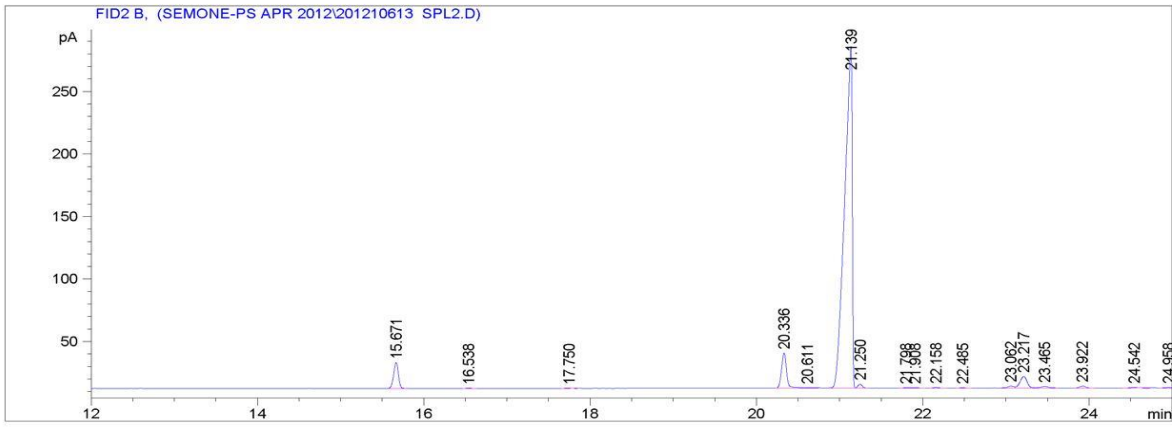


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APPENDIX IV

PUBLISHED LIERATURE REVIEWS AND BOOK CHAPTERS OF THESIS RELEVANCE

EXPERIMENTAL AND CLINICAL EVIDENCE OF CARDIOVASCULAR BENEFITS OF PLANT STEROLS

Appendix IV

6 Experimental and Clinical Evidence of Cardiovascular Benefits of Plant Sterols

Rgia A. Othman and Mohammed Hassan Moghadasian
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6.1 INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the Western societies. High total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels are considered the major risk factors for coronary heart disease (CHD) (Steinberg and Gotto, 1999). Current evidence has determined a positive association between lifestyle and dietary factors as they correlate with blood lipid concentrations and CHD (Van Horn et al., 2008). It is well-documented that plasma TC levels are positively associated with ischemic heart disease (IHD) (Lewington et al., 2007), and a 1% decline in TC could be expected to reduce the risk of CHD by 2.7%. Similarly, a 10% decrease in LDL-C is associated with about 12% to 20% decline in the population incidence of IHD over 5 years (Katan et al., 2003). Hence, reducing blood cholesterol levels may be needed to lower risk of CVD. Therapeutic life-style changes, such as inclusion of dietary phytosterols, may reduce cardiovascular risk.

Plant sterols (PS) have been shown in numerous studies to reduce the levels of TC, LDL-C, (Demonty et al., 2009), apolipoprotein B (apo-B) (Madsen et al., 2007), and nonhigh-density lipoproteins cholesterol (non-HDL-C) (Weidner et al., 2008), and C-reactive protein (CRP),

Rgia A. Othman *and Mohammed H. Moghadasian. (2012). Experimental and clinical evidence of cardiovascular benefits of plant sterols. In Mohammed H. Moghadasian and N. A. Michael Eskin(Eds.), Functional Foods and Cardiovascular Disease (Chap.3). CRC Press. ISBN: 978-1-4200-7110-8.*

BEYOND CHOLESTEROL-LOWERING EFFECTS OF PLANT STEROLS: CLINICAL AND EXPERIMENTAL EVIDENCE OF ANTI-INFLAMMATORY PROPERTIES

Appendix IV

Beyond cholesterol-lowering effects of plant sterols: clinical and experimental evidence of anti-inflammatory properties

Rgia A Othman and Mohammed H Moghadasian

Inflammation is a strong risk factor for cardiovascular disease. Dietary plant sterols are known to reduce plasma cholesterol levels and thereby reduce cardiovascular risk. Recent observations from animal and human studies have demonstrated anti-inflammatory effects of phytosterols. For example, several animal and human studies report reductions in the levels of proinflammatory cytokines, including C-reactive protein, after consumption of dietary plant sterols. Although the cholesterol-lowering effects of phytosterols in humans are well documented, studies on the effects of phytosterols on inflammatory markers have produced inconsistent results. This review summarizes and discusses findings from recent animal and human studies with regard to the potential anti-inflammatory effects of dietary phytosterols. Findings on the effects of plant sterols on inflammation remain limited and confounding. Future research using better-designed and well-controlled laboratory studies and clinical trials are needed to fully understand the mechanisms through which phytosterols influence inflammation. Additional well-designed placebo-controlled studies are needed to better understand how and to what extent dietary plant sterols may modify the immune system and the production of inflammatory markers.

INTRODUCTION

Inflammation plays a major role in the process of atherogenesis, from the development of fatty streaks to plaque rupture and thrombosis.[1,2] Atherogenic inflammation is initiated as a vascular response to injury and is commonly followed by lipid peroxidation and accumulation.[3,4] Leukocytes mediate localization and recruitment of monocytes into the subendothelial space; in turn, monocytes release a number of inflammatory mediators, including cytokines, chemokines, and adhesion molecules.[5] This can alter endothelial function, enhance expression of adhesion molecules, increase leukocyte adhesion, and impair endothelium-dependent vasodilator responses.[6,7] Once monocytes differentiate to macrophages, they will engulf oxidized lipids and transform into lipid-laden foam cells within the arterial wall, forming a fatty streak.[1,2] In addition to inflammatory cells, visceral adipose tissue is thought to secrete several inflammatory mediators, including tumor necrosis factor-α (TNF-α), plasminogen activator inhibitor-1 (PAI-1), interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-15, complement factors, and prostaglandin E_2 (PGE_2)[8]; these mediators stimulate the secretion of the acute-phase protein, C-reactive protein (CRP), by the liver.[9,10] Thus, the presence of CRP in plasma is recognized as a marker for low-grade systemic inflammation and is positively correlated with the risk of future cardiovascular events.[11] Whether there is a causal relationship between CRP and coronary heart diseases remains unclear; however, CRP levels have been shown to be markedly increased in patients with myocardial infarction.[12] Phytosterols are natural compounds found in

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ANTI-INFLAMMATORY PROPERTIES OF PLANT STEROLS AND PHYTOESTROGENS

Appendix IV

12 Anti-Inflammatory Properties of Plant Sterols and Phytoestrogens *Experimental and Clinical Evidence*

Rgia A. Othman and Mohammed H. Moghadasian

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12.1 INTRODUCTION

Dietary phytosterols and phytoestrogens may reduce the incidence of cardiovascular diseases through their anti-inflammatory effects in adipose tissue. Phytosterols are plant sterols similar in structure to cholesterol. Studies show that phytosterol-based diets reduce the levels of the inflammatory mediators including C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor (TNF-α), phospholipase A-1 (PLA-1), and fibrinogen. These mediators are elevated in adipose tissue inflammation (Bouic et al., 1999; Devaraj et al., 2006; Jones et al., 2007; Nashed et al., 2005). In addition, phytosterols exhibit immunomodulatory effects, increasing T-cell proliferation and modifying cytokine profiles in favor of T-helper type 1 (Th1-type) response and decreasing cytokines associated with the Th2-type cells (Bouic et al., 1996; Bouic et al., 1999; Breytenbach et al., 2001).

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Appendix IV

CHOLESTEROL-LOWERING EFFECTS OF NUTRACEUTICALS: PLANT STANOLS/ STEROLS VERSUS POLICOSANOLS

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ABSTRACT

Functional food components including plant sterols (PS) and stanols, and policosanols have been studied primarily for their anti-hyperlipidemic properties. Since the 1950s, PS have been shown to reduce total cholesterol and low-density lipoprotein cholesterol (LDL-C), and current research continues to affirm the benefits of PS on circulating lipid levels. On average, consuming 2-3 g PS/d reduces serum LDL-C by 10 - 15%, which may translate into a 10 - 30% risk reduction of cardiovascular events. However, a few studies have raised the possibility that circulating PS are a novel atherosclerotic risk factor.

Overall, evidence on the relationship between PS intake and the risk of developing atherosclerosis is still debatable. However, a direct assessment between PS and atherosclerosis has not been proven. Besides their cholesterol-lowering effect, evolving data also provide evidence for anti-inflammatory and anti-cancer activities of PS. Policosanols are a natural mixture of aliphatic alcohols derived primarily from sugarcane. Trials conducted in Cuba in the 1990's reported cholesterol-lowering effect of policosanol, with the suggestion that it may be more potent than PS and even statins. However, recent studies conducted outside of Cuba failed to replicate the efficacy of *policosanol* observed earlier.

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