Efficacy of Plant Sterol Treatment in Individuals with High or Low Baseline Levels of Circulating Plasma Plant Sterols

Adrielle H. Houweling

School of Dietetics and Human Nutrition, McGill University,

Ste-Anne-de-Bellevue, Québec, Canada

Submitted October 2006

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science in Human Nutrition

Your file Votre référence
ISBN: 978-0-494-32720-3
Our file Notre référence
ISBN: 978-0-494-32720-3

Table of Contents

ABSTRACT VI

RÉSUMÉ VII

ACKNOWLEDGEMENTS VIII

CONTRIBUTION OF AUTHORS IX

LIST OF ABBREVIATIONS X

1 INTRODUCTION 1

1.1 **Background** 1

1.2 **Objectives and Rationale** 4

1.3 **Null Hypotheses** 4

2 REVIEW OF THE LITERATURE 5

2.1 **Plant Sterols** 5
2.1.1 History 5
2.1.2 Chemical Structure 5
2.1.3 Dietary Consumption 6
2.1.4 Mechanism of Action 6

2.2 **Plasma Campesterol & Sitosterol Levels** 8
2.2.1 Determinants of Circulating Levels 8
2.2.1.1 Diet 8
2.2.1.2 Genetics 8
2.2.2 Surrogate Markers of Absorption 9
2.2.2.1 Plant Sterol Absorption 9
2.2.2.2 Cholesterol Absorption 9

2.3 **Plant Sterol Supplementation** 11
2.3.1 Safety 11
2.3.2 Anti-Hypercholesterolemic Effects 17
2.3.3 Anti-Inflammatory Effects 21

3 MANUSCRIPT 1 25

3.1 **Abstract** 26

3.2 **Introduction** 27

3.3 **Subject and Methods** 29

3.3.1 Subjects 29
3.3.2 Recruitment Strategy 30
3.3.3 Experimental Design 30
3.3.4 Blood Protocol 31
3.3.5 Analyses 32
3.3.5.1 Lipid and C - reactive protein concentrations 32
3.3.5.2 Lipid and C - reactive protein concentrations 32
3.3.5.3 Statistics 32

3.4 Results 34
3.4.1 Subject Baseline Characteristics 34
3.4.2 Effect of Plant Sterols on Baseline Plasma Lipid and C – Reactive Protein Levels 34
3.4.3 Effect of Plant Sterols on Baseline Circulating Plasma Plant Sterol Levels 36
3.4.4 The Relationship between Percent Change in Lipid and Plasma Plant Sterols Levels following Plant Sterol Therapy 38
3.4.5 The Relationship between Percent Change in Lipid and Plasma Plant Sterols Levels following Plant Sterol Therapy 38

3.5 Discussion 40

4 SUMMARY AND CONCLUSIONS 45

5 BIBLIOGRAPHY 67

APPENDIX 72

A. ETHICS APPROVAL 72

B. WAIVER FORM 81

List of Tables

Manuscript I

Table 1: Plant sterol content of foods 24
Table 2: Levels of plasma plant sterols associated with CVD risk 24
Table 3: Baseline characteristics for the total study population and for subjects above the 75th (HIGH) and below the 25th (LOW) percentile of screening sum of plasma plant sterol levels 52
Table 4: Post-treatment and percent change in lipid and CRP results for subjects above the 75th(HIGH) and below the 25th (LOW) percentile of screening sum of plasma plant sterol levels (n=42) 53
Table 5: Post-treatment and percent change in plant sterol concentrations for subjects above the 75th (HIGH) and below the 25th (LOW) percentile cut-off of screening sum of plant sterol levels (n=42) 55
Table 6: Composition of background diet 56
Table 7: Composition of plant sterol spread 56
Table 8: Post-treatment and percent change in lipid and CRP levels for total study population (n=82) 57
Table 9: Post-treatment and percent change in plant sterol levels for total study population (n=82) 57

List of Figures

Introduction

Figure 1: Chemical structure of cholesterol, campesterol and sitosterol 24
Figure 2: Distribution of the sum of campesterol and sitosterol levels for the total study population with the 25th, 50th and 75th percentile cut-offs (n=82) 58

Manuscript I

Figure 3: Regression of baseline plasma sum of campesterol and sitosterol concentrations vs. percent change in lipid levels following plant sterol therapy, as compared to control 59
Figure 4: Regression of baseline plasma sum of campesterol and sitosterol levels vs. percent change in plant sterol levels following plant sterol therapy, as compared to control. 60
Figure 5: Regression of percent change in lipid vs. campesterol levels following plant sterol therapy, as compared to control. 61
Figure 6 : Regression of percent change in lipid vs. sitosterol levels following plant sterol therapy, as compared to control... 62
Figure 7: Post-treatment and percent change in lipid levels for total study population (n=82) 63
Figure 8: Post-treatment and percent change in lipid levels for subjects above the 75th (HIGH) and below the 25th (LOW) percentile cut-off of screening sum of plasma plant sterol levels (n=42) 64
Figure 9: Post-treatment and percent change in plant sterol levels for the total study population (n=82) 65
Figure 10: Post-treatment and percent change in plant sterol levels for subjects above the 75th (HIGH) and below the 25th (LOW) percentile cut-off of screening sum of plant sterol levels (n=42) 66

Abstract

Plant sterols are effective cholesterol-lowering agents; however, recent evidence suggests that this treatment may not be safe and beneficial in all individuals. This study determined whether high and low baseline circulating plasma campesterol and sitosterol are related to subsequent changes in plasma LDL-C, plant sterol or CRP levels, after accounting for plant sterol supplementation in hypercholesterolemic men (n=82). This trial was a 2-phase randomized cross-over design consisting of a controlled diet with and without a dose of 2.0 g/d of plant sterols over 4 weeks. There was no significant difference in plasma LDL-C, in the elevation of plasma plant sterol or in the changes of CRP levels for high and low groups, respectively. In view of these data, a supplement of 2.0 g/d of plant sterols should be viewed as a safe and beneficial cholesterol-lowering therapy for all individuals, with respect to their baseline plasma plant sterol levels.

Résumé

Les stérols de plantes (PS) sont des agents hypocholestérolémiants efficaces. Cependant, de récentes études ont démontré que, dépendant des individus recevant ce traitement, les PS ne sont pas toujours un moyen sûr et bénéfique pour réduire le cholestérol sanguin. La présente étude a examiné la relation entre les taux de base de campestérol et sitostérol dans le plasma d'une part, et la variation du taux de cholestérol de type LDL, des PS et de CRP dans le plasma de l'autre, et cela prenant en compte un traitement de PS donné à des hommes hypercholestérolémiques (n=82). La présente étude est de type randomisé croisé comprenant deux phases au cours desquelles les sujets ont consommé un régime contrôlé supplémenté de 2.0 g de PS par jour, pendant 4 semaines. Aucune différence significative n'a été notée au niveau des taux de cholestérol de type LDL, des PS ainsi que de CRP entre les groupes ayant des taux de base élevés et bas de PS dans le plasma. En conclusion, nos résultats montrent qu'un supplément de 2.0 g de PS par jour peut être considéré comme un traitement hypocholestérolémiant bénéfique et sûr, et cela indépendamment du taux de base de stérols de plantes dans le plasma.

Acknowledgements

First and foremost, I would like to extend my thanks to my supervisor Dr. Peter Jones who provided me with the opportunity to do my masters project in such a unique and interesting field of research. I am also grateful that I was able to work with Catherine Vanstone, who was the clinical coordinator at the Mary Emily Clinical Nutrition Research Unit at the time of my studies. Although the project required a mass amount of work and dedication, I was never left with the impression that things would fall apart since she always kept the clinical trial running smoothly. I must also thank Dr. Elke Trautwein and Dr. Guus Duchateau who conceptualized the study and provided feedback throughout the project. I would also like to express my gratitude to my co-supervisor, Dr. Hope Weiler. Although she only became involved in my project towards the end of my graduate studies, the help she provided during those remaining months was crucial for the completion of the masters. I must also thank Donna Leggee, Evan Nitschmann, Lam Chu, and Behnam Azadi who were always available for consult when I encountered problems in the lab. I would also like to thank Dr. Stan Kubow for providing me with the equipment I needed in for my analysis. I would also especially like to thank Krista Varady, Suhad AbuMweis, Christopher Marinangeli and the other members of my lab group who made the many laborious days of lab work manageable and even entertaining. Finally, I am forever indebted to my family but more specifically to my parents, who have always provided me with constant support and feedback throughout my education.

Contribution of authors

The manuscript included in the thesis was written and revised in whole, by Adrielle Houweling. She played an active role in the organization of the human clinical trial. Additionally, she was responsible for the collection and analysis of data, which appear in this document.

Manuscript 1: *Baseline plasma plant sterol concentrations do not predict changes in lipids, plasma plant sterols and C-reactive protein following intake of a plant sterol-enriched food*

Dr. Peter J.H Jones was the principal investigator for the study. He was responsible for the experimental protocol and design of the study. He also offered invaluable feedback in weekly meetings, at every step of the study. Dr. Jones additionally provided a significant amount of input in the manuscript and the thesis as a whole.

Catherine Vanstone was the clinical coordinator for this study. She was responsible for organizing and orchestrating the clinical portion of the overall project.

Dr. Elke A. Trautwein and Dr. Guus SMJE Duchateau conceptualized the study and provided feedback at several points for the whole project.

List of Abbreviations

ACBG – Artery coronary bypass graft operation

BMI – Body mass index

CVD – Cardiovascular disease

C – Control

CRP – C-reactive protein

GC – Gas chromatograph

HDL-C – High-density lipoprotein cholesterol

HPS – High plant sterol

IL – 6 – Interleukin - 6

LDL-C – Low-density lipoprotein cholesterol

LPS – Low plant sterol

NCEP – National Cholesterol Education Program

Non HDL-C – Non high-density lipoprotein cholesterol

PS – Plant sterols

TC – Total cholesterol

TG – Triglycerides

TNF – α – Tumour necrosis factor alpha

Tx – Treatment

1 Introduction

1.1 Background

Cardiovascular disease (CVD) is a leading cause of morbidity and mortality worldwide (1). As hypercholesterolemia is a major risk factor for the progression of CVD, effective treatment of this condition is essential (1). Plant sterols, reduce LDL-C by approximately 10% (1-3), offer an effective and natural alternative to achieving cholesterol reduction (1, 3). The cholesterol-lowering effect of these plant-derived components is achieved through a competition with cholesterol for micelle uptake and subsequent absorption across the intestinal epithelial barrier (1-4). As a result, plant sterols cause a reduction in cholesterol levels and minimally elevate circulating plasma plant sterol concentrations. In view of their beneficial health effects, the National Education Cholesterol Adult Treatment Program (NCEP) recommends the use of plant sterols in combination with lifestyles changes as a cholesterol-lowering therapy (4).

Plant sterol use is not completely favored; however, since elevated plasma concentrations of 290-966 μmol/L are associated with sitosterolemia, a disease characterized by early onset of atherosclerosis (5). Plant sterols should be viewed as safe since treatment elevates plasma plant sterol levels to 14-48 μmol/L, which is not comparable to values expressed in sitosterolemia (5). In fact a recent study demonstrated that there is no relationship between plasma plant sterol levels and CVD risk in non-sitosterolemic patients (6). The use of plant sterols in hypercholesterolemic but otherwise healthy individuals remains controversial, however, as other studies have demonstrated that moderate levels of plasma plant sterols ranging from 15 to 23 μmol/L may be associated with increased CVD risk (7-

11). Since elevations in plasma plant sterols after plant sterol treatment are highly variable (12-16), individuals showing the greatest elevation in plasma plant sterols may be at risk for the development of CVD. At this time, however, no factor has been identified as an indicator of response to plant sterol treatment. Baseline campesterol and sitosterol levels, as surrogate markers of plant sterol absorption (10), may be predictive of changes in plasma plant sterol levels. Therefore a thorough understanding of the role baseline plasma plant sterol concentrations in plant sterol therapy is required as the association between plasma plant sterols and CVD risk is controversial (6-11) .

Although plant sterols lower circulatory (LDL-C) by 10 % (2, 3), response to this cholesterol-lowering therapy is variable (17). Baseline campesterol and sitosterol levels, as surrogate markers for cholesterol absorption (18), may be predictive of changes in plasma cholesterol levels. Two non-metabolically controlled studies have already demonstrated that reductions in LDL-C levels only occur in individuals with high but not low baseline plasma plant sterol (15, 19). Replication of the data obtained from those studies in a larger metabolically controlled study, however, would provide stronger evidence that baseline plasma plant sterol levels determine the cholesterol-lowering response to plant sterol therapy. In doing so, the role of baseline plasma plant sterols in the cholesterol-lowering response to plant sterol therapy would be thoroughly established.

Plant sterols have also been shown to function as anti-inflammatory agents. A previous study showed that plant sterols significantly reduced plasma concentrations of IL-6 and TNF-alpha (20). As other studies have demonstrated that CRP is

increased by IL-6 and TNF-alpha (21, 22), it may be hypothesized that plant sterols reduce levels of this CVD promoting factor as well. Consequently, plant sterols, despite being associated with CVD risk, may be additionally favorable for heart health if this therapy reduces concentrations of CRP.

1.2 Objectives and Rationale

Plant sterol use remains controversial as moderately elevated concentrations of plasma plant sterols have inconsistently been associated with an increase in CVD risk (6-9, 11) and treatment with this therapy is not effective in all individuals (15, 19, 20). Therefore the objective of the present study was to determine whether a difference exists in a response to plant sterol supplementation in hypercholesterolemic men with high or low baseline plasma sum of campesterol and sitosterol levels, as measured by percent change and post-treatment plant sterols (campesterol and sitosterol). In addition, this trial examined the anti-hypercholesterolemic and anti-inflammatory effects of plant sterols, which were measured using percent change and endpoint in lipid (TC and LDL-C) and CRP values, respectively.

1.3 Null Hypotheses

1) That no difference in campesterol and sitosterol changes between subjects with high and low baseline sum of campesterol and sitosterol, would be evident following plant sterol supplementation.

2) That no difference in total cholesterol, low-density lipoprotein cholesterol changes between subjects with high and low baseline sum of campesterol and sitosterol concentrations, would be evident following plant sterol supplementation.

3) That no difference in C-reactive protein (CRP) changes between subjects with high and low baseline sum of campesterol and sitosterol concentrations, would be evident following plant sterol supplementation.

2 Review of the Literature

2.1 Plant Sterols

2.1.1 History

The cholesterol-lowering effects of plant sterols were first noted in 1951 in an animal feeding trial (23). Soon after in 1957, a cholesterol lowering agent termed "Cytellin" was developed and marketed as a pharmaceutical agent. The low bioavailability and poor water solubility of this product, however, required that large doses of these plant sterols be consumed. Consequently, this pharmaceutical agent was not profitable and the product was eventually abandoned. Since then, however, improvements in the manufacturing of plant sterols have been made so that low doses of these plant-derived components are capable of achieving cholesterol-reduction. As a consequence, plant sterols were recommended for use by NCEP in 2001 in combination with lifestyle changes as a cholesterol-lowering therapy (4).

2.1.2 Chemical Structure

Plant sterols are classified as sterol compounds in that they are all composed of a fused cyclopentanophenanthrine ring structure specific to cholesterol with an added alcohol moiety (3). These compounds are plant-derived substances that are structurally and functionally similar to cholesterol (3), playing a key role in cell membrane function (4). The majority of plant sterols differ structurally from cholesterol by changes in their side chain; campesterol has an additional methyl group at the carbon-24 position, whereas sitosterol has an additional ethyl group at the carbon-24 position (3). As many as 200 phytosterols exist, however, campesterol and sitosterol are the two most abundant of these plant derived components (24).

2.1.3 Dietary Consumption

Plant sterols are consumed at approximately 200-400 mg/day in the standard North American diet (25). Levels can increase 2-fold in populations where there is a greater emphasis on vegetable consumption (26). All plant-derived foods contain appreciable amounts of plant sterols, however, they are principally found in oils and to a lesser extent in nuts, breads and whole vegetables (3) (Table 1). It is the specie of origin which characterizes the distribution of plant sterols (4); campesterol and sitosterol are primarily found in oils (3), whereas shellfish is the main source of dietary brassicasterol (27).

2.1.4 Mechanism of Action

The mechanism by which plant sterols mediate their cholesterol-lowering action is not fully understood (23). It has been proposed that the subtle difference in their structure causes an associated steric asymmetry (Figure 1), which allows plant sterols to compete effectively with cholesterol for uptake into micelles and eventual sterol transfer across the intestinal epithelial cell. At present, the Neimann Pick CL1 protein (NPCL1) is the proposed transporter, which is thought to mediate the transfer of the sterol-containing micelle across the intestinal barrier (23). In the presence of phytosterols, less cholesterol will be transferred across this barrier since incorporation into micelle is essential in this process. As a consequence of this action, cholesterol absorption and plasma levels decrease; in compensation, cholesterol synthesis may increase while plant sterol absorption and plasma levels increase. The rise in plasma plant sterol levels, however, is minimal for two reasons. First, absorption of campesterol and sitosterol occurs at 1.9 % and 0.5 %, respectively (28, 29). Second,

plant sterols may additionally be shuttled back to the intestine via the phytosterols-specific transporter protein ABCG5/G8, located at the apical membrane of the intestinal epithelial cells (23), thus preventing the majority of plant sterols from reaching the plasma compartment.

2.2 Plasma Campesterol & Sitosterol Levels

2.2.1 Determinants of Circulating Levels

2.2.1.1 Diet

Plant sterol consumption, as mentioned above, is dependent on intake of oils and to a lesser extent on breads, nuts and whole vegetables. Consequently, circulating plasma plant sterol levels may be determined by diet as well (3). In a study by Kempen et al, however, it was demonstrated that dietary plant sterol intake, indirectly estimated from fibre consumption, was not significantly correlated with plasma plant sterol levels (30). Moreover, in a 1 year study by Berge et al, it was observed that plasma plant sterol concentrations, taken at 24-48 week intervals, were very stable intra-individually with variation in replicate samples drawn 48 weeks apart ranging from 0.20-2.6% (26). Baseline plasma plant sterol levels, unaffected by changes in natural diet, must thus have an underlying biological component (26).

2.2.1.2 Genetics

Baseline plasma plant sterol levels vary extensively in the population (26). Campesterol and sitosterol levels are skewed in distribution within an American population, where plasma concentrations vary approximately between 3.7-16 μmol/L and 2.4- 12 μmol/L, respectively (26). Baseline plasma plant sterol levels from a Dutch population showed a similar skewed distribution with campesterol and sitosterol plasma concentrations approximately between 3.0-30 μmol/L and 3.1-19 μmol/L, respectively (30). The intra-individual stability of plant sterols and variability of plant sterols between individuals and populations suggests that sterol absorption is genetically controlled (26). In fact Berge et al demonstrated that the heritability of

plasma plant sterol concentration is greater than 80 % for sitosterol and campesterol (26). Given the fact that baseline plasma plant sterol levels are largely determined by genetics and relatively unaffected by diet, circulating plasma plant sterol concentrations may provide insight into understanding certain aspects of intestinal absorption. In fact current research suggests that cholesterol absorption can vary significantly between individuals who express different polymorphisms of the ABCG5/G8 and NPCL-1 transporter proteins, which control plant sterol absorption (31, 32).

2.2.2 Surrogate Markers of Absorption

2.2.2.1 Plant Sterol Absorption

Campesterol and sitosterol are plant derived. Therefore circulating plasma levels reflect plant sterol absorption since mammalian synthesis of phytosterols is not possible (5). As a result elevations in plasma plant sterols, which follow plant sterol use may be predicted by plasma campesterol and sitosterol concentrations, however, no study thus far has examined response to plant sterol therapy in this context.

2.2.2.2 Cholesterol Absorption

Plasma plant sterols may additionally be used to indirectly measure cholesterol absorption (33). Cholesterol absorption efficiency has previously been shown to correlate well with both baseline campesterol ($r = 0.75$; $p < 0.05$) and sitosterol ($r = 0.81$; $p < 0.05$) levels (34). Determination of cholesterol absorption efficiency using this method has previously been demonstrated using absolute plasma plant sterol levels or plasma plant sterol to lathosterol, a precursor in cholesterol synthesis, or plasma plant sterol to cholesterol ratios (15, 19). In this manner, cholesterol

absorption is measured while accounting for hepatic cholesterol synthesis (19). Whether plasma plant sterols are expressed as absolute values or ratios, they can only assess cholesterol absorption under static conditions where dietary plant sterol intake is fixed and baseline levels of plant sterols are measured (35); this no longer exists under dynamic conditions where plant sterol treatment is given. In such cases, plant sterols compete with cholesterol uptake thus altering cholesterol absorption significantly and only showing a 15-48 μmol/L increase in plant sterol absorption. As a result, reductions in cholesterol concentrations after plant sterol therapy may be predicted by baseline measurements of plasma plant sterol concentrations (35). In fact two recent trials, which assessed cholesterol absorption using this technique, confirmed that plasma plant sterols reflect cholesterol absorption as individuals with the greatest baseline levels of plasma plant sterols show a greater cholesterol-lowering response after plant sterol use (15, 19). Although this method is fast and relatively inexpensive, data cannot be considered quantitatively since cholesterol absorption efficiency is determined indirectly (18). Additionally, there has been concern that the variability of plant sterol intake might affect interpretation of results in spite of the fact that it has been clearly shown that dietary intake of phytosterols is not a determinant of baseline plasma plant sterol levels (26, 30). Consequently assessment of cholesterol absorption using baseline plasma plant sterol levels is acceptable, however, data may be further confirmed using more stringent techniques such as the dual isotope method (18).

2.3 Plant Sterol Supplementation

2.3.1 Safety

Plant sterol supplementation favorably alters the lipid profile while minimally elevating plasma plant sterol levels (1). Concerns regarding this treatment have recently surfaced since plasma plant sterols, if present in high enough levels, are potentially atherogenic and ultimately serve as cardiovascular risk inducing agents (5). This situation presents itself in the rare autosomal recessive disorder, sitosterolemia (5). The first two cases of sitosterolemia were described by Bhattacharyya and Connor in 1974 (23). Two normocholesterolemic sisters, who had developed xanthomas as children, were found with plasma sitosterol levels that were above the normal range of 15-48 μmol/L and exceeded 411μmol/L. The lethality of sitosterolemia was fully realized when its association with premature CVD was established in a 5-yr old atherosclerotic girl with exceedingly high plasma sitosterol levels. Since then, other cases of the disease have been reported suggesting that elevated plasma plant sterol levels are detrimental to health (23). From the reported cases, it has been established that the major clinical symptoms of this disease are development of xanthomas of the Achilles tendon, the extensor tendons of the hands and possibly tuberous xanthomas at an early age as well as angina pectoris, myocardial infarction and sudden death as a result of premature atherosclerosis. In spite of these findings, recent studies indicate that plant sterol treatment increases plasma plant sterol levels to only 15-48 μmol/L in hypercholesterolemic but healthy individuals (5, 6, 36).

In a cross-over placebo controlled trial, Nestel et al demonstrated that a 4-week supplementation of 2.4 g/d of plant sterols in margarine caused an overall 51 % increase in plasma campesterol in 22 hypercholesterolemic subjects (16). Post sterol treatment campesterol concentrations for the study group were approximately 13.0 ± 6.0 and 17.39 ± 9.42 μmol/L for placebo and plant sterol phases of the study, respectively.

In a trial by Clifton et al, 105 % and 45 % increases in campesterol and sitosterol levels were observed in 35 mildly hypercholesterolemic subjects after a 6-week supplementation with 6.6 g/d of plant sterols in bread, cereal and spread, as compared to control (13). The post treatment campesterol and sitosterol levels for the plant sterol supplementation phase of the trial were 15.75 ± 5.50 and 11.35 ± 4.35 μmol/L, respectively. Baseline campesterol and sitosterol levels for the study were 7.75 ± 3.75 and 7.97 ± 3.62 μmol/L, respectively.

In a parallel-arm placebo-controlled study, Christiansen et al examined the effect of plant sterol dose of 3.0 g/d in a spread for hypercholesterolemic subjects over the course of 6 months (12). Percent increases in sitosterol levels were 75 %, as compared to control. There were no significant increases in campesterol levels, as compared to control. Post sterol treatment campesterol and sitosterol levels were 9.50 ± 4.00 and 7.00 ± 2.90 μmol/L, respectively. Post-treatment campesterol and sitosterol levels for the placebo phase of the study were 9.75 ± 3.75 and 3.86 ± 1.21 μmol/L, respectively.

In a placebo-controlled parallel arm trial, Davidson et al considered the effect of a 3.0 g/d of plant sterols in a fat spread and salad dressing for 84 free-living

subjects over an 8-week period (14). Campesterol significantly increased by 43 % while no elevations were noted for sitosterol. Post sterol treatment campesterol and sitosterol concentrations ranged between 12.12 - 42.40 and 1.42 – 20.85 μmol/L, respectively.

Finally, Mussner et al demonstrated in a cross-over trial, that supplementation of 1.82 g/d of plant sterols to 63 healthy individuals elevated plasma campesterol and sitosterol levels by 78 and 35 %, respectively (15). This corresponds to post-treatment campesterol and sitosterol concentrations of 9.00 ± 3.75 and 4.35 ± 1.93 μmol/L, for the control phase and 16.60 ± 5.50 and 6.04 ± 2.42 μmol/L for the plant sterol phase, respectively.

From the data presented above, it is evident that plasma plant sterol level elevations following plant sterol supplementation vary between individuals (12-16). However, the greatest increases observed from these studies conducted in healthy individuals do not compare to concentrations expressed in sitosterolemic individuals (12-16). In fact, post sterol treatment concentrations of sitosterol and campesterol observed in the Christiansen et al study did not even exceed the moderate increase in plasma plant sterols that are associated with plant sterol therapy (12). Moreover, in this same study, changes in campesterol levels were not significantly changed following plant sterol therapy. Similarly, Davidson et al demonstrated that plasma sitosterol concentrations are not significantly affected by plant sterol therapy (14). Consequently, many researchers believe that plant sterol use should still be regarded as safe (1, 4, 37). Supplementation of plant sterols, however, may not be appropriate for individuals with high baseline plasma plant sterol levels. Recent studies have

demonstrated that even modest levels (15-24 μmol/L) of plasma plant sterols may be risk factors for CVD (7, 9) (Table 2).

In a study by Glueck et al, plasma phytosterol levels were assessed in 595 hypercholesterolemic individuals with and without a personal or family history of CVD (7). Twenty-four percent of individuals with campesterol levels in the upper 5 % of the study group were found have to premature CVD. On the other hand, none of the individuals with campesterol levels in the lower 5 % of the study group were found to have premature CVD. The author stated that the association between CVD and campesterol was established independently of serum cholesterol, as the median level of this lipid parameter was not different between the upper and lower group. Serum cholesterol and campesterol, however, were significantly correlated suggesting that the association, which was established between CVD risk and campesterol, was confounded by cholesterol ($r=0.15$, $p=0.0003$). Since plasma cholesterol is a major risk factor for CVD this lipid parameter should have been controlled for using multiple regression analysis so that the true association between plasma plant sterols and CVD could have been established. No significant association between sitosterol and CVD risk was found in this study.

Sudhop et al compared the levels of plasma plant sterols in patients with and without a family history of CVD who had been admitted to the hospital for elective artery coronary bypass graft (ACBG) operation (10). From the 42 men and 11 women with a proven personal history of CVD, serum campesterol and sitosterol concentrations were approximately 30 % higher in patients with a positive family history of CVD. When expressed as a ratio to cholesterol, serum campesterol and

sitosterol remained higher for patients with a positive history of CVD. However, as illustrated in a recent review by Chan et al (38), it is difficult to interpret these results and make generalizations for the population as a whole because this study did not include a true control group.

Rajaratnam et al examined the relationship between plasma plant sterol levels and CVD in a population of post-menopausal women consisting of the cases who had been successfully treated for CVD and aged-matched controls (9). Serum campesterol and sitosterol concentrations were found to be significantly higher in the cases than the controls. Introduction of baseline cholesterol levels into the regression model did not affect the association between CVD and plasma plant sterol concentrations.

In a nested case-control study by Assmann et al, plasma sitosterol and campesterol levels were compared between cases of myocardial infarction or major coronary event and age and smoking status matched controls (11). It was concluded that sitosterol concentrations were significantly higher in the cases than the controls; however, no difference in concentrations were noted once sitosterol was expressed as a ratio to cholesterol. Concentrations of campesterol showed a non-significant trend that was higher in the cases than controls, which remained non-significant even when values were expressed as a ratio to cholesterol. They did note, however, that the hazard ratio for CVD risk was significantly increased a 3-fold once data was stratified into quartiles in terms of sitosterol or the sitosterol/cholesterol ratio.

Miettinen et al considered the association between serum plant sterol levels and the progression of atherosclerosis, as measured by the cholesterol content of atheromatotic plaques in 25 patients undergoing carotid endarterectomy (8).

Individuals were ranked into triads based on the cholesterol content of their atherosclerotic tissues. No association between serum and tissue plant sterol to cholesterol ratios were noted between the tissue triads. Across individuals within each triad, however, it was observed that the ratio of campesterol to cholesterol in the serum was significantly correlated with the ratio observed in the carotid wall ($r = 0.56$, $p < 0.01$, $r = 0.80$, $p < 0.001$, $r = 0.68$, $p < 0.001$ for the 1^{st}, 2^{nd} and 3^{rd} triad, respectively). The relationship between serum plant sterols and the progression of atherosclerosis would have been more accurately examined had a comparison of all individuals been undertaken.

As a result of improved technology, a recent study considered the role of plant sterols in CVD using a more advanced techniques that quantify their association with atherosclerotic plaques. Wilund et al assessed the role of plasma plant sterols in young atherosclerotic adults, by associating levels with coronary calcium, as assessed using electron beam computer tomography and with family history of CVD (6). Although cholesterol was significantly associated with coronary calcium ($r = 0.12$, $p < 0.001$), this relationship was not apparent for either campesterol ($r = -0.01$, $p = 0.63$) or sitosterol ($r = -0.03$, $p = 0.27$). No significant relationship was found between family history and plasma plant sterol concentrations. Cholesterol, however, was found to be significantly higher in women with a family history of CVD, as compared to control. Further studies using Electron Beam Computerized Tomography (EBCT) are required, however, as this is the only study that assesses the association between CVD risk and plasma plant sterols in such a novel manner.

From these five studies, it is plain to see that there is no conclusive evidence linking plasma plant sterols levels to CVD risk (7-11). While Glueck et al showed a significant association of CVD risk with campesterol and not sitosterol, Assmann et al showed reciprocating results (7, 11). Furthermore, in both studies the association between plasma plant sterols and CVD risk was potentially confounded as the relationship was not established independently from plasma cholesterol levels. Although Sudhop et al concluded a significant association between plasma plant sterols and CVD risk, which was independent of plasma cholesterol, a lack of a true control makes it difficult to apply to a CVD free population (10). Rajartnam et al, however, did include a proper control in their study and established a significant association between plasma plant sterols and CVD risk which was independent of cholesterol concentrations (9). Furthermore, Miettinen et al demonstrated that plant sterol content in atherosclerotic plaques reflects levels in the blood, suggesting that plant sterols may play a role in the progression of atherosclerosis (8). Their findings, however, are not supported by a recent study by Wilund et al (6), in which advanced techniques show that plasma plant sterols do not associate with the progression of atherosclerosis. As no consensus can be reached with regards to the association between moderate plasma plant sterol levels and CVD risk in these studies (6-11), debate over plant sterol use continues.

2.3.2 Anti-Hypercholesterolemic Effects

There have been several reviews which summarize the cholesterol-lowering effects of all plant sterol research to date (1-3). Overall these reviews indicate that plant sterol treatment achieves beneficial dose-dependent cholesterol lowering effects

that are maximally effective at treatment doses of approximately 2.0-2.5 g/day. At the recommended dose, these studies collectively demonstrate an approximate 10 % decrease in plasma LDL-C levels, and no change in plasma TG and plasma HDL-C levels with respect to control levels. Within and between studies, however, alterations in lipid profile do not necessarily follow this pattern.

In a cross-over design trial, Hallikainen et al demonstrated that a 1.5 g/d dose of plant sterol supplemented margarine decrease LDL-C levels significantly overall by 10.7 %, as compared to control over 4 weeks (39). Response to supplementation of LDL-C levels within their study group, however, was more variable and ranged between -6.7 and -15 %. HDL-C and TG levels showed a 3.3 % and -8.9 % change, respectively, which was not significant when compared to control.

Clifton et al similarly demonstrated in a cross-over study that 6.6 g/d of plant sterols supplemented in bread, cereal and margarine spread favorably decrease LDL-C levels by -12.4 %, as compared to control, in 35 mildly hypercholesterolemic adults over a 6-week period (13). They noted as well that this response was variable and ranged between an increase and decrease in LDL-C levels of 1.05 mmol/L and 1.64 mmol/L, respectively. HDL-C and TG levels showed non-significant increases and decreases of 2.2 and 5.5 %, respectively, when compared to control.

In a cross-over study, Noakes et al demonstrated that the average LDL-C level of a moderately hypercholesterolemic group decreased by 10 % when receiving a 2.0 g/d dose of plant sterols supplemented into a spread, as compared to the control diet over a 3-week period (40). Individually, however, the LDL-C lowering response to plant sterol supplementation ranged between 0.32-0.66 mmol/L. Non-significant

decreases in HDL-C and TG levels were 1.6 and 3.6 % respectively following plant sterol supplementation, as compared to control.

In a 1.61 g/d supplementation trial, Hendriks et al established that plant sterols in spread reduce LDL-C levels by 9.2 %, as compared to control in healthy and mildly hypercholesterolemic individuals over a 3.5-week period (41). The LDL-C lowering-response ranged between 0.15-0.36 mmol/L. The range of response may have been smaller for this trial than the ranges presented from the aforementioned studies since the dose of plant sterols was not as great for this study. Non-significant decreases in HDL-C and TG concentrations were noted to be -0.6 and -7.1 %, respectively as compared to control.

Vissers et al demonstrated that an approximate 2.0 g/d dose of plant sterol supplemented margarine reduced group LDL-C levels by 8.5 % over a 3-week period, which is similar to those studies mentioned above (42). As with the trial mentioned beforehand, the range in the reduction of LDL-C within the group was small (0.10-0.30 mmol/L), suggesting that response to plant sterol supplementation does not show large inter-variability. Changes in HDL-C and TG concentrations were -0.7 and 4.9 %, respectively, as compared to control.

In a recent cross-over trial, Yoshida et al found that 1.8 g/d of plant sterols supplemented in bars reduced LDL-C levels non-significantly by -6.1 %, as compared to control over a 3-week period (43). This percent change, in spite of being smaller in magnitude than that of the aforementioned studies, may have achieved significance if its range had been less variable (3.1-7.7 %). The changes to HDL-C and TG were 1.0 and -4.1 %, respectively, as compared to control.

In a cross-over trial by Mussner et al, 1.82 g/d was supplemented into margarine for 63 healthy subjects over a 3 week period (15). LDL-C levels were reduced by -6.5 %, which is similar to the results achieved by Yoshida et al. Unlike the previous trial, however, plant sterol supplementation achieved significant reductions in LDL-C levels. In contrast to the studies mentioned above, HDL-C was significantly reduced by 3.5 %. No significant changes in TG were observed.

In a cross-over study by Volpe et al, plant sterols were supplemented at a 1.0-2.0 g/d dose of plant sterol supplemented yoghurt in subjects with primary to moderate hypercholesterolemia over a 4-week period (44). Following plant sterol supplementation, LDL-C levels dropped by 11.1 %, as compared to baseline. The range in the LDL-C reductions during the plant sterol phase was 8.6-31.2 %, indicating a large inter-individual response to treatment. Changes in TG and HDL-C levels were non-significant.

Independently, these studies demonstrate that plant sterols do not strictly lower LDL-C levels by 10 %; rather reductions in this parameter are more variable and range between 6-15 % (13, 15, 39-44). Although the study design may be the most probable explanation for the disparity in percent reductions between studies, one cannot exclude the possibility that response to plant sterol treatment may be variable between individuals. This explanation becomes more favorable when one recognizes that percent changes in LDL-C levels after plant sterol supplementation are highly variable for individuals within studies where compliance was good. Since plasma plant sterol levels show inter-individual variability (30) and reflect cholesterol

absorption efficiency (18), it is not surprising that the cholesterol-lowering response of this therapy varies between individuals.

In 2002, Mussner et al showed that plasma campesterol to cholesterol ratios were associated with the cholesterol-lowering response to a 1.82 g/d dose of plant sterol supplemented into margarine over a 3-week period (15). LDL-C levels were significantly reduced by 7.4 % in individuals with high plasma campesterol levels. Conversely, individuals with low plasma campesterol levels showed non-significant reductions in their plasma LDL-C levels. Changes across all participants in the study, however, were not assessed.

More recently, Thuluva et al demonstrated that plasma lathosterol to campesterol was equally predictive of the cholesterol-lowering response of a 1.0 g/d dose plant stanols supplemented into margarine over a 4-week period (19). Study participants in the high lathosterol to campesterol ratio and low lathosterol to campesterol ratio groups showed changes of 4.3 and -13.8 %, respectively. This study additionally demonstrated a significant relationship between lathosterol to campesterol ratio and percent change in lipid levels for all individuals following plant sterol supplementation. The limitation of this study, however, was that it lacked a control.

2.3.3 Anti-Inflammatory Effects

Plant sterols, in addition to lowering-cholesterol, have anti-inflammatory properties. A study by Bouc et al. showed that plant sterols reduced levels of TNF-alpha and IL-6 (20) . The reduction in these inflammatory markers is beneficial since TNF-alpha and IL-6 activate C-reactive protein (21, 22) which has been associated with elevated risk for CVD. Despite the fact that plant sterols have been shown to

reduce TNF-alpha and IL-6, few studies to date have considered the effects of this agent on CRP levels (45, 46).

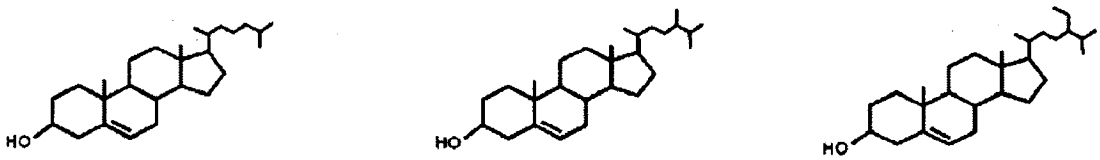
In a study by Jenkins et al, 2.0 g/d of plant sterols significantly reduced CRP levels in study participants below the 75th percentile (3.5 mg/L) for CRP, as compared to control (46). The anti-inflammatory action, however, could not be firmly established in this trial; the test diet also contained other components such as soy protein, almonds and viscous fibres, which may have contributed to the reduction of CRP levels.

In a study by AbuMweis et al, consumption of 1.7 g/d of plant sterols resulted in non-significant changes to CRP levels (45). In addition, plant sterols did not significantly lower TC, LDL-C or plasma plant sterol levels either. The cholesterol-lowering effect of plant sterols may not have been apparent, however, as treatment was supplemented at a dose given once a day. As part of the study design of previous trials that showed that plant sterols reduce LDL-C, treatment had been given at multiple time points per day. Therefore, it could not be concluded that plant sterols do not alter CRP levels since they did not alter any lipid parameter either.

The two studies mentioned above did not conclusively establish whether plant sterols mediate their anti-inflammatory effects through CRP (45, 47). It is difficult, however, to establish an association between plant sterols and CRP, as this inflammatory marker also strongly correlated with exercise (48), smoking and alcohol consumption (49, 50) and influenza (51). Therefore, it is difficult to isolate whether acute changes in its levels are due to changes in cholesterol levels, or other potential mediators of inflammatory response (46). Consequently many factors must be

considered when using CRP as a marker for CVD related studies so that accurate conclusions can be established.

Figure 1: Chemical structure of cholesterol, campesterol and sitosterol (adapted from Ostlund et al, 2002 (3))



Cholesterol Campesterol Sitosterol

Table 1: Plant sterol content of foods (adapted from Ostlund et al, 2002 (3))

Food	Plant Sterol Content (mg/100 mg edible portion)
Corn oil	952
Sunflower oil	725
Safflower oil	444
Soybean oil	221
Olive oil	176
Almonds	143
Beans	76
Corn	70
Wheat	69
Lettuce	38
Tomato	7

Table 2: Levels of plasma plant sterols associated with elevated CVD risk

Study	Plasma Concentration (μmol/L)		
	Campesterol	Sitosterol	Sum of Plant Sterols
Assmann et al, 2001	11.2	5.25	16.4
Glueck et al, 1991	6.40	9.10	15.5
Sudhop et al, 2002	12.5	9.70	22.2
Rajaratnam et al, 2000	15.3	8.03	23.3

3 Manuscript 1

Baseline plasma plant sterol concentrations do not predict changes in lipids, plasma plant sterols and C-reactive protein following intake of a plant sterol-enriched food

Adrielle H. Houweling[1], Catherine A. Vanstone [1], Elke A. Trautwein[2], Guus S.M.J.E. Duchateau[2], Peter J.H. Jones[1]

[1]School of Dietetics and Human Nutrition, McGill University, Macdonald Campus, 21,111 Lakeshore, Ste-Anne-de-Bellevue, QC, Canada H9X 3V9

[2]Unilever-Food and Health Research Institute, Vlaardingen, The Netherlands

In preparation for submission to the Journal of Lipid Research

3.1 Abstract

Background: Plant sterols have been shown to effectively lower cholesterol levels. However, their use remains controversial as moderately elevated plasma concentrations have been associated with an increase in CVD risk. **Objective:** To examine whether baseline plasma sum of campesterol and sitosterol concentrations were associated with ensuing changes in plasma campesterol and sitosterol and TC and LDL-C and CRP following plant sterol therapy in otherwise healthy hypercholesterolemic men. **Design:** This single-blinded, randomized, controlled study, consisted of two four-week phases separated by a 4-week wash-out, where participants consumed the placebo or the 2.0 g/d plant sterol supplemented diet. **Results:** Plant sterol supplementation increased ($p<0.0001$) campesterol and sitosterol levels by 24.3 and 28.2 %, respectively, for the total study population. Changes in plasma concentrations, however, were not different between high and low plant sterol individuals. TC and LDL-C levels were decreased ($p<0.0001$) by 6.3 and 7.8 %, respectively, for all individuals. Changes in lipid parameters were not different between high and low plant sterol individuals. No changes in CRP for total and high and low plant sterol individuals were noted. **Conclusions:** Baseline plasma sum of campesterol and sitosterol do not predict changes in campesterol, sitosterol, TC, LDL-C and CRP, following plant sterol therapy. Consequently, baseline plasma plant sterol levels may not be indicative of the safety and efficacy of plant sterol therapy.

3.2 Introduction

Hypercholesterolemia is a major yet modifiable risk factor for the development of coronary heart disease (CHD) (41). While statins are primarily prescribed to treat this condition, with consumption of plant sterol-enriched foods, cholesterol is reduced in a natural yet effective manner in subjects with more modestly elevated serum cholesterol concentrations (52). Results from a meta-analysis of 41 trials show that the intake of 2 g/d of plant sterols (PS) reduces low-density lipoprotein cholesterol (LDL-C) by on average 10 % (1). In accordance with their beneficial health effects, plant sterols and stanols are recommended as part of the therapeutic lifestyle changes aimed at reducing LDL-C in the National Cholesterol Education Program (NCEP) Adult Treatment Program (ATP) III guidelines (4).

Plant sterols mediate their cholesterol-lowering effect by competing with cholesterol uptake by dietary mixed micelles and subsequent uptake into intestinal epithelial cells resulting in an inhibition of intestinal cholesterol absorption (1, 2, 4, 53) . Consequently, PS cause positive changes in the serum lipid profile, while minimally elevating plasma PS concentrations.

In the general population, plasma PS concentrations range from 6.9 to 27.9 μmol/L and 2.8 to 16.0 μmol/L for campesterol and sitosterol, respectively (38). After dietary intake of 2 g/d of plant sterols, plasma plant sterol concentrations are modestly elevated to 14.5 - 48.3 μmol/L in hypercholesterolemic, yet otherwise healthy individuals (5). Plasma plant sterol concentrations of 290 - 966 μmol/L, however, are associated with sitosterolemia, a rare genetic disorder, characterized by premature atherosclerosis and CHD events (5). Certain observational studies also suggest that

slightly elevated concentrations of PS might be associated with an increased risk of cardiovascular events; however, this relationship has not been consistently shown in all studies (6-11, 54). Plasma plant sterol concentrations may reflect PS absorption efficiency (5), thus there is a need to address whether individuals with high baseline plasma plant sterols (HPS) hyperabsorb PS and show elevations in plasma levels which are greater than what has previously been established in the literature.

In addition, earlier studies have reported that plant sterols may only show beneficial cholesterol-lowering effects in individuals with high baseline plasma plant sterol concentrations (15, 19). Plasma plant sterol concentrations may also reflect cholesterol absorption efficiency (18). Accordingly, individuals with higher plasma plant sterol levels may hyperabsorb cholesterol and thus benefit more from an inhibition of intestinal cholesterol absorption. However, these results have yet to be replicated in the context of a diet-controlled study.

Plant sterols may additionally play an important role in the anti-inflammatory response. Plant sterols have been shown to reduce plasma concentrations of the inflammatory markers, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (20). IL-6 and TNF-α have been further shown to increase concentrations of the CVD risk marker, C-reactive protein (CRP) (21, 22). Thus plant sterols may affect CRP via a reduction in IL-6 and TNF-α; however, data to this effect is limited (45, 46, 55).

Plasma plant sterol concentrations reflect sterol absorption (18). Thus, HPS individuals may show a greater elevation in plasma plant sterols after intake of plant sterol-enriched foods and plasma concentrations could even exceed the 14.5 - 48.3

μmol/L previously established in the literature. In addition, consumption of plant sterols may reduce serum cholesterol concentrations the most in individuals with high baseline plasma plant sterol concentrations. Also, plant sterols may beneficially lower CRP. Therefore, the aim of this study was to determine i) whether baseline plasma concentrations of plant sterols (the sum of sitosterol and campesterol) and ii) whether consumption of a plant sterol-enriched spread for 4 weeks would result in a different response in serum total (TC) and LDL-cholesterol, (LDL-C), in plasma campesterol, sitosterol and CRP in male hypercholesterolemic individuals, with either high (HPS) or low (LPS) baseline plasma plant sterol concentrations.

3.3 Subject and Methods

3.3.1 Subjects

Ninety male subjects were recruited by advertisement in local newspapers, clinics and community centers in the West Island and surrounding area of Montreal. Inclusion criteria required that participants were between 35 – 70 yrs of age, normal to moderately overweight (as defined by a 25 kg/m^2 < BMI < 32 kg/m^2), moderately hypercholesterolemic (as defined by LDL-C > 3.0 mmol/L), with no medical history of diabetes or uncontrolled hypertension or uncontrolled hypothyroidism; patients taking any cholesterol-lowering medication within the last 6 months were excluded from the study. Eighty-two of the 90 participants enrolled in the study completed the trial successfully; two of the subjects withdrew from the study because they moved and 6 of the subjects withdrew from the study due to personal reasons. All study participants gave their written informed consent and the study protocol was approved

by the Ethics Committee of the Faculty of Medicine at McGill University. No adverse effects to the treatment margarine were noted over the course of the study.

3.3.2 Recruitment Strategy

In order to effectively recruit a sufficient number of men with high and low baseline plant sterol concentrations, a recruitment strategy was undertaken in the form of a plant sterol acceptance protocol. A distribution curve for the sum of plasma campesterol plus sitosterol concentrations of the first 40 subjects enrolled into the study was realized by averaging the 1st and 2nd screening plasma plant sterol results of each participant. The 25th and 75th percentiles of the distribution curve was determined and used as the cut-off limits for further screening. Entrance criteria for any new potential subjects required that they either had a high (above the 75th percentile) or a low (below the 25th percentile) plasma sum of campesterol plus sitosterol concentrations.

3.3.3 Experimental Design

During the two 4-week phases of the randomized, single-blinded, controlled trial subjects only consumed the test diets provided by the metabolic kitchen of the Mary Emily Clinical Nutrition Research Unit. The controlled diet contained 25 g of a regular low-fat margarine or the same margarine, which provided an amount of 2.0 g/day of plant sterols (prepared by Unilever Foods, Purfleet, UK) (Table 7). The daily spread serving was divided into three equal portions and provided with each of the three daily meals in a three-day rotating menu. The two phases of the study were separated by a 4-week washout period, where subjects consumed their regular uncontrolled home diet and did not come to the clinic. The diets were equicaloric and

followed the guidelines for a Heart Healthy Diet where dietary intake of fat, carbohydrate and protein represented 30 %, 55 % and 15 % of the energy, respectively (Table 6). Both food and beverages were provided in amounts to maintain a stable body weight. Caloric intake for each participant was determined using the Mifflin equation (56), where small adjustments were made if there were fluctuations in body weight over the course of the first week into the study. Body weights continued to be monitored daily throughout the remainder of the study. Subjects consumed their breakfast each morning at the clinical unit under supervision of the staff. The other two meals were prepared and packed for consumption at work or at home. Subjects were required to consume all foods and beverage provided by the unit and instructed to refrain from all other food and beverage consumption, including coffee and alcohol. Post-treatment plant sterol concentrations served as an indicator of compliance since absolute and percentage changes of plasma campesterol and sitosterol increase in subjects consuming a plant sterol supplemented diet (35).

3.3.4 Blood Protocol

On days 1, 2, 28 and 29, blood was drawn after a 12 hour fast and collected in EDTA and serum tubes. Serum tubes were allowed to sit for 30 minutes to ensure proper coagulation. Blood was then centrifuged at 520 x *g* for 20 minutes at 4° C to ensure proper separation of plasma and serum, which was then stored in microcentrifuge tubes at -80° C until further analysis of circulating concentrations of lipids, CRP and plant sterols.

3.3.5 Analyses

3.3.5.1 Lipid and C - reactive protein concentrations

TC, triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) were measured from serum samples using respective reagent Flex on a multianalyzer (Dimension RxL Max, Behring Diagnostics, Marburg, Germany) (57-59). LDL-C was indirectly calculated using the equation by Friedwald et al (60). Serum CRP was assessed using a highly sensitivity CRP assay (Dade Behring Diagnostics, Marburg, Germany).

3.3.5.2 Lipid and C - reactive protein concentrations

Plant sterols were extracted following the procedure set by Wolthers et al (61). After the addition of the internal standard, 5-α cholestane, 0.3 ml of plasma was saponified with methanolic KOH for 2 hrs at 100° C. Addition of doubly distilled water and petroleum ether enabled extraction of the non-saponifiable neutral sterols which were dried under nitrogen, derivitized with TMSi reagent [pyridine-hexamethyldisilazan-trimethylchlorosilane (9:3:1, vol:vol)] and quantified using gas chromatographic analysis of plant sterol peak areas (62). Duplicate samples were injected into a gas chromatograph (Varian 3400, Varian, Palo Alto, CA) fitted with a 30-m capillary column (SAC-5; Supelco, Bellefont, PA) and a FID detector. Plasma plant sterols were identified using authentic standards purchased from Sigma-Aldrich Canada Ltd., Oakville, Ontario.

3.3.5.3 Statistics

Sample size for this study was set at 96 with a 20 % drop-out rate. The trial was designed with a power of 80 %, an alpha of 5 % and the assumption that 2.0 g/d

of PS would reduce LDL-C by approximately 10 %. The data were analyzed using SAS software (version 9.1 SAS Institute Inc., NC). All data presented in tables are expressed in means ± SEM. A repeated measures ANOVA design was used to establish an association between baseline plasma plant sterol concentrations, (individuals with high (HPS) vs. low (LPS) baseline plasma plant sterols) and treatment (plant sterol intake vs. placebo). High and low groups were designated using both the 25^{th} and 75^{th} percentile, as well as the 50^{th} percentile as cut-off points (Figure 2). Values presented in the tables include data obtained using both grouping methods where groupings established using the 50^{th} percentile appear in brackets. In addition, regression analysis was used to assess whether baseline plasma plant sterol concentrations predict changes in serum cholesterol as well as campesterol and sitosterol concentrations across all individuals. Spearman correlations were used to show a relationship between the sum of baseline plant sterol concentrations with serum lipid and CRP concentrations across all individuals ($n = 82$). Shapiro-Wilks statistics were used to test for normality. Transformation was carried out on CRP data to correct for non-normality. Statistical significance was set at $p < 0.05$.

3.4 Results

3.4.1 Subject Baseline Characteristics

Subject baseline characteristics are presented in Table 3. The study population consisted of middle aged (51.3 ± 1.0 yr), moderately overweight (BMI = 28.4 ± 0.4 kg/m^2), hypercholesterolemic (as defined by a LDL-C of 3.71 ± 0.09 mmol/L) male participants. Average circulating concentrations of campesterol, and sitosterol and campesterol plus sitosterol for this study group were 12.8, 6.7 and 19.5 μmol/L, respectively. By design of the study, the HPS group possessed higher ($p < 0.0001$) mean campesterol, sitosterol and sum of plant sterol concentrations compared with levels of the LPS group. The HPS group was found to have higher ($p < 0.05$) mean TC, LDL-C, and HDL-C concentrations than the LPS group. The LPS group also had a higher mean ($p = 0.041$) BMI than that of the HPS sterol group, but only when data were expressed in terms of the $25^{th}/75^{th}$ percentile groupings. No differences in baseline TG or CRP concentrations were found between groups. Across all individuals, the sum of baseline plant sterol concentrations were found to correlate with TC ($r = 0.44$, $p < 0.0001$), LDL-C ($r = 0.38$, $p = 0.0004$), HDL-C ($r = 0.32$, $p = 0.0037$) and non-HDL-C ($r = 0.39$, $p = 0.0003$). No relationship could be established for baseline circulating plant sterols with CRP or TG.

3.4.2 Effect of Plant Sterols on Baseline Plasma Lipid and C – Reactive Protein Levels

The post-treatment and percent change in lipid and CRP levels for plant sterol and placebo phases of the study are presented in Table 4 and 8 and Figures 7 and 8.

For the total study population, intake of 2.0 g/day of plant sterols reduced ($p < 0.0001$) the absolute post-treatment concentration and the percent change in TC by 0.32 mmol/L and 6.3 %, respectively, as compared to control (Figure 7). For HPS and LPS groups established from the $25^{th}/75^{th}$ percentiles, absolute post-treatment TC concentrations were reduced by 0.24 and 0.26 mmol/L, respectively; percent decreases for these two groups were 2.9 and 2.7 %, respectively, as compared to control (Figure 8). For HPS and LPS groups established using the 50^{th} percentile, TC was reduced by 0.34 and 0.26 mmol/L; percent decreases for these two groups were 7.6 and 5.0 %, respectively, as compared to control. No significant interaction between treatment and group was observed. The effect of group was only significant when baseline concentrations were not controlled for in the ANOVA model ($p < 0.01$).

Across all individuals, LDL-C was reduced ($p < 0.0001$) by 0.28 mmol/L, or 7.8 % as compared to control (Figure 7). For HPS and LPS groups established from the $25^{th}/75^{th}$ percentiles, absolute post-treatment LDL-C concentrations decreased by 0.26 and 0.27 mmol/L; percent decreases for these two groups were 4.6 and 4.9 %, respectively, as compared to control (Figure 8). For the high and low plant sterol groups established from the 50^{th} percentile, LDL-C was reduced by 0.30 and 0.25 mmol/L, respectively; percent decreases for these two groups were 9.7 and 5.9 % (Figure 2), respectively, as compared to control. No significant interactions were observed between treatment and group. The effect of group on LDL-C was only significant when baseline concentrations were not controlled for in the ANOVA model ($p < 0.01$).

For all subjects, HDL-C concentrations were reduced ($p < 0.03$) by 3.1 % or 0.02 mmol/L, respectively, as compared to control (Figure 7). For the high and low plant sterol groups established from the 25^{th}/ 75^{th} percentiles, HDL-C was changed by 0.01 and -0.02 mmol/L, respectively; percent decreases for these two groups were 0.89 and 1.5 %, respectively, as compared to control. For HPS and LPS groups established from the 50^{th} percentile, post-treatment HDL-C concentrations decreased by 0.01 and 0.02 mmol/L; percent reductions for these two groups were 2.3 and 3.8 % (Figure 8), respectively, as compared to control. There was no significant interaction between treatment and group.

For the study population, TG was decreased ($p = 0.034$) by 7.7 %, as compared to control (Figure 7). There was no change, however, in post-treatment concentrations of this lipid parameter. Additionally, no differences were observed in percent change of TG concentrations between high and low plant sterol individuals (Figure 8). No significant association was noted for baseline circulating plant sterol concentrations and C-reactive protein.

3.4.3 Effect of Plant Sterols on Baseline Circulating Plasma Plant Sterol Levels

The post-treatment and percent change in plasma plant sterol levels for the plant sterol and placebo phases of the study are presented in Table 5 & 9 and Figures 9 & 10.

For all individuals, plasma campesterol concentrations were elevated ($p < 0.0001$) by 24.3 % or 3.7 μmol/L following intake of 2.0 g/d of plant sterols (Figure 9). The mean post plant sterol intervention campesterol concentration was 19.8 μmol/L. For HPS and LPS groups established from the 25^{th}/75^{th} percentiles, the

absolute post-treatment concentrations of campesterol were elevated from 22.1 to 29.2 μmol/L and from 10.1 to 13.2 μmol/L; percent increases were 31.9 and 49.1 %, respectively, as compared to control (Figure 10). For HPS and LPS groups established from the 50th percentile, the absolute post-treatment campesterol concentrations were elevated from 20.2 to 24.5 μmol/L and from 12.0 to 15.2 μmol/L; percent increases were 13.8 and 34.9 %, respectively, as compared to control. There was no interaction between treatment and group.

Across all study participants, sitosterol concentrations were increased ($p < 0.0001$) by 28.2 % or 2.7 μmol/L (Figure 9). The mean post plant sterol intervention sitosterol concentration was 12.7 μmol/L. For HPS and LPS groups established from the 25th/75th percentiles, the post-treatment concentrations of campesterol were elevated from 12.5 to 16.9 μmol/L and from 6.4 to 7.6 μmol/L; percent increases were 51.8 and 46.0 %, respectively, as compared to control (Figure 10). For HPS and LPS groups established from the 50th percentile, post-treatment campesterol concentrations were elevated from 12.0 to 14.8 μmol/L and from 7.8 to 10.5 μmol/L; percent increases were 28.8 and 27.6 %, respectively, as compared to control. There was no interaction between treatment and group.

Across all study participants, lathosterol and desmosterol concentrations were not changed following plant sterol supplementation, as compared to control.. There was no significant interaction between group and treatment for either lathosterol or desmosterol.

3.4.4 The Relationship between Percent Change in Lipid and Plasma Plant Sterols Levels following Plant Sterol Therapy

For lipid levels, no significant relationship was observed between baseline campesterol plus sitosterol concentrations and the percent changes in TC and LDL-C following PS intake across all study participants, as compared to control ($r_{adj.}$ = -0.059, p = 0.399; $r_{adj.}$ = 0.109, p = 0.811, for TC and LDL-C respectively) (Figure 3). Similarly, no significant relationship was apparent when baseline campesterol plus sitosterol concentrations were plotted against end of PS phase lipid values across all individuals, as compared to control ($r_{adj.}$ = -0.130, p = 0.722; $r_{adj.}$ = 0.151, p = 0.150, for TC and LDL-C respectively).

For plant sterol levels, baseline campesterol plus sitosterol concentrations were not found to be significantly correlated with percent changes in campesterol and sitosterol following PS intake across all study participants, as compared to control ($r_{adj.}$ = -0.10, p = 0.69; $r_{adj.}$ = -0.11, p = 0.86, for campesterol and sitosterol, respectively) (Figure 4). Similarly, no significant correlation was apparent when baseline sum of plant sterol concentrations were plotted against the absolute post plant sterol treatment PS concentrations for all individuals, as compared to control ($r_{adj.}$ = -0.08, p = 0.48; $r_{adj.}$ = -0.13, p = 0.73, for campesterol and sitosterol respectively).

3.4.5 The Relationship between Percent Change in Lipid and Plasma Plant Sterols Levels following Plant Sterol Therapy

No significant relationships were observed between the percent change in TC vs. campesterol, TC vs. sitosterol, LDL-C vs. campesterol or LDL-C and sitosterol

following plant sterol therapy (r= -0.034 , p=0.94; r = -0.018, p=0.66; r=-0.034, p=0.96; r=-0.0096, p=0.53, respectively) (Figure 5 and 6).

3.5 Discussion

This study demonstrates that the sum of baseline circulating PS concentrations does not predict or associate with elevations in plasma plant sterols, following a dietary intake of 2.0 g/d of plant sterols. Although elevations in circulating PS concentrations were highly variable between individuals, changes occurred independently of the sum of baseline circulating plant sterol concentrations.

The baseline circulating plant sterol concentrations were, however, significantly related to the baseline lipid concentrations (TC, LDL-C and HDL-C) and the BMI of the participants enrolled in the study. Miettinen et al have demonstrated that HPS and LPS individuals, established from the cholestanol to cholesterol ratios, have a significantly different average BMI; however, their groups did not differ in terms of their TC, LDL-C and HDL-C (63). The contradictory data obtained from these studies may be attributed to a difference in genetic background of the populations under investigation; the apoE and ABCG5/G8 genes, which are known to influence cholesterol absorption and BMI, are thought to account for 16.4 to 22.7 % of the variation in plasma plant sterol concentrations (38). On the other hand, the present study compared lipid concentrations of HPS and LPS groups established from the absolute plant sterol levels of study participants; thus it is possible that a difference of BMI between HPS and LPS groups would have been noted if groups had been established using PS ratios, as demonstrated in a recent study by Pinedo et al (54). The mean baseline campesterol and sitosterol concentrations of 12.8 ± 1.2 and 6.7 ± 0.6 μmol/L were found to fall within the range of concentrations established in a recent meta-analysis by Chan et al (6.9 to 27.9 μmol/L and 2.8 to 16.0 μmol/L), respectively.

Excessively elevated concentrations of plasma plant sterols are associated with the rare yet lethal autosomal-recessive disorder, sitosterolemia (5). Since elevations in plasma concentrations following treatment are not comparable to the 290-966 μmol/L concentrations expressed in sitosterolemic individuals, plant sterol should be viewed as safe (5). Mussner et al demonstrated in a cross-over trial, that supplementation of 1.8 g/d of plant sterols to 63 healthy individuals elevated plasma campesterol plus sitosterol concentrations from 9.0 to 16.6 μmol/L and 4.4 to 6.0 μmol/L (15). Clifton et al, showed that supplementation with a greater dose of 6.6 g/d in 35 mildly hypercholesterolemic subjects increased campesterol and sitosterol concentrations to a similar extent (7.8 to 15.8 μmol/L and 8.0 to 11.4 μmol/L, respectively) (13). No studies thus far, however, have considered the extent to which PS intake elevates concentrations of plasma plant sterols in HPS and LPS individuals. Circulating PS concentrations reflect plant sterol absorption (5). Accordingly, HPS individuals may show a higher elevation in circulating PS concentrations than what has already been established in the literature, if they hyperabsorb plant sterols.

The results presented here conclusively demonstrate that an intake of 2.0 g/d of plant sterols does not elevate plasma plant sterols concentrations to levels characterized by sitosterolemia. The elevations in plasma plant sterols which were observed in this trial are comparable to the 14.5 - 48.3 μmol/L concentrations previously established in the literature.

Certain studies suggest that moderately elevated plasma plant sterol concentrations are associated with a greater CHD risk (7-11). Glueck et al were the first to recognize the relationship between campesterol but not sitosterol

concentrations and CHD in a study of 565 hypercholesterolemic patients (7). Similarly, Assmann et al found that the sitosterol, but not campesterol, concentrations were significantly higher in cases of myocardial infarction or major coronary event than in age and smoking status matched controls (11). Although Assmann et al and Glueck et al both concluded that plasma plant sterols are associated with CHD risk, the relationship was not established independently from serum cholesterol, which on its own is a major risk factor for CHD (7, 11). Additionally, these studies cannot be used together as supporting evidence that CHD risk is associated with moderately elevated plasma plant sterol concentrations, as the relationship of campesterol and sitosterol with CHD was inconsistent between studies. Data obtained by Rajaratnam et al and Sudhop et al (9, 10) do indicate, however, that the association between plasma plant sterols and CVD risk exists for both campesterol and sitosterol. These findings are strongly supported by data from Miettinen et al that demonstrated that the campesterol and sitosterol content in atherosclerotic plaques reflect concentrations in serum (8). Nevertheless the proportion of cholesterol to plant sterol concentrations in serum and plaque tissue and the ratio plant sterols/cholesterol remains the same, suggesting no preferential uptake of plant sterol into plaque tissue and thus no accelerated accumulation.

Overall, these studies show limited evidence to suggest that slightly elevated plasma plant sterol concentrations are associated with a greater CHD risk; however, PS use may be perceived as less favorable for HPS individuals since they may have higher intestinal plant sterol absorption efficiencies. Results from this study, however,

demonstrate that HPS individuals, despite having indigenously high PS levels, do not show greater elevations in circulating PS concentrations after plant sterol treatment.

Providing further evidence that moderately elevated plasma plant sterol concentrations are not associated with an increased risk of CHD are two recent case-control studies. Wilund et al demonstrated that plasma plant sterol concentrations do not differ between individuals with a positive history of heart disease and case-matched controls (6). Baseline plasma plant sterol concentrations did not associate with atherosclerosis based on arterial calcium scores, a marker of the degree of atherosclerosis. More recently Pinedo et al also demonstrated that baseline plasma plant sterol concentrations were no different among healthy individuals who developed CHD during a 6-year follow-up and case-matched controls (54).

Baseline plasma plant sterol concentrations were further examined in the context of their role in the cholesterol-lowering response to plant sterol intake. Contrary to findings by Mussner et al and Thuluva et al (15, 19), the present study demonstrates that baseline plant sterols are not predictive of changes in TC and LDL-C, following plant sterol supplementation. For both those studies, however, significant associations between baseline plasma plant sterol concentrations and the cholesterol-lowering response were established in free-living individuals. Furthermore the linear relationship established in the trial by Thuluva et al may have been confounded by clustering of the data, as only HPS and LPS individuals were included in the regression analysis (19). Nonetheless, it is difficult to make cross-comparisons between these studies, as cholesterol absorption efficiency was determined using plasma plant sterol to cholesterol or lathosterol ratios in the previous

studies. Ultimately, the relationship between baseline plasma plant sterol concentrations and cholesterol absorption efficiency would have most accurately been captured not with plasma plant sterol concentrations or ratios but rather with direct isotopic techniques.

To summarize, this study demonstrates that the baseline plasma plant sterol concentrations are not associated with or predictive of changes in plant sterols, lipid or CRP concentrations following plant sterol supplementation. Additionally, elevations in plasma plant sterol concentrations for this study are by far not comparable to values expressed in sitosterolemia. In view of these data, a supplement of 2.0 g/d of plant sterols should be viewed as a beneficial cholesterol-lowering therapy that minimally elevates circulating PS concentrations to the same extent in all individuals, irrespective to their baseline plasma plant sterol concentrations. Further studies examining the effect of long-term use of plant sterols are required, however, to clearly define the relationship between dietary intake of plant sterols and CHD risk.

4 Summary and Conclusions

The aim of this thesis was to determine whether the baseline sum of plant sterols (campesterol plus sitosterol) levels can predict the changes in plasma plant sterols, CRP and lipid levels, which follow plant sterol supplementation. The following paragraphs discuss the rationale, the key results of the study and the significance of those findings. In addition, the following sections examine the challenges encountered over the course of the project as well as the direction taken to resolve these problems.

Plant sterol supplementation is not completely favoured as a cholesterol-lowering therapy since extremely elevated plasma concentrations are associated with the rare yet lethal autosomal disorder, sitosterolemia (5). Several trials, however, have demonstrated that the 14-48 μmol/L elevations in plasma campesterol and sitosterol levels following plant sterol therapy do not compare to levels expressed in sitosterolemic patients (12-16). Nonetheless, plant sterol use remains a concern as moderately elevated levels of plasma campesterol and sitosterol have been associated with CVD risk (7-11). Consequently, supplementation of plant sterols in individuals with elevated baseline plasma plant sterols may not be appropriate. Evidence by Wilund et al and Pinedo et al demonstrate, however, that moderately elevated plasma plant sterols are not associated with the progression of atherosclerosis or CVD risk (6, 54). Accordingly, plant sterol supplementation is controversial for individuals with high baseline plasma plant sterol levels, with the majority of studies suggesting that intake may not be appropriate.

Treatment of hypercholesterolemia with plant sterol therapy is additionally controversial as the anti-hypercholesterolemic effects of this agent are not apparent in all individuals. In fact two previously conducted studies in free-living populations, noted that the cholesterol-lowering effect of plant sterol supplementation was only beneficial for individuals with elevated plasma plant sterol concentrations (15, 19). The positive cholesterol-lowering effects, however, may not outweigh the risks that have been associated with plant sterol supplementation for this subset of the population.

Still, plant sterols may prove beneficial for heart health despite the fact that moderately elevated plasma plant sterols levels have been implicated in CVD risk. Previous research has demonstrated that plant sterols reduce plasma concentrations of the inflammatory markers, IL-6 and TNF-alpha (20), which elevate levels of the CVD promoting factor C-reactive protein (21, 22). Consequently plant sterols may improve CVD risk if they reduce CRP levels as well.

In view of the controversy surrounding the safety of plant sterol therapy and its efficacy as a cholesterol-lowering agent, a human clinical trial was conducted to examine the effect of plant sterol supplementation in male hypercholesterolemic individuals with high and low baseline levels of plasma campesterol and sitosterol. As specific values for high and low baseline concentrations of plasma plant sterols have not yet been ascertained in the literature, high and low cut-offs needed to be established in order to effectively address the objectives of this study. From the 40 individuals enrolled in the trial, high and low baseline plasma plant sterol levels were taken as the 75^{th} and 25^{th} percentile cut-offs of that distribution, respectively.

Consequently, this trial assumed that the distribution of baseline plasma plant sterol levels of the forty individuals initially enrolled in the study accurately reflected the distribution for the population.

There are several techniques, which may be used to assess plasma plant sterol concentrations. However, for the purpose of this study, gas chromatography was used to measure plasma plant sterol levels. Using this technique, plasma campesterol and sitosterol concentrations are assessed by integrating peak size obtained in the GC analysis. The sitosterol peak was easily integrated with GC; however, the campesterol peak was less so as it tended to be adjoined with an earlier peak. This earlier peak may in fact be another plant sterol, however, at the present time it remains unidentified. Consequently, campesterol peak size could not accurately or precisely be determined and a certain amount of estimation was required in the integration process. As a result, stronger data might have been obtained in the analysis had the campesterol measurements been more accurate and precise. Still, measurements of plasma campesterol were consistent as the interference of the unknown peak was consistent in size across samples of the respective study subject. Nonetheless, the need to obtain accurate and precise plant sterol measurements was stressed in a recent review by Chan et al, which showed that bio-analytical techniques might account for 22.5 % of the variation in plasma plant sterol concentrations. Ultimately, gas chromatography- mass spectrometry (GC-MS) could have been used to accurately and precisely measure plasma campesterol concentrations as this technique allows for separation and resolution of the campesterol peak. Nonetheless, plasma campesterol concentrations obtained using GC and GC-MS is comparable suggesting that either

technique is acceptable for making plasma campesterol measurements or (REFERENCE). Furthermore the intra-analysis CV was noted to be less than 3 %. Accordingly campesterol measurements were made with a limited amount of imprecision.

A comparison of baseline characteristics between groups showed that individuals with high baseline plasma plant sterol levels had significantly higher TC, HDL-C and LDL-C but lower BMI values than individuals with low baseline plasma plant sterol levels. While previous research supports the results obtained here (10, 11), other studies indicate that there is no difference in the baseline cholesterol concentrations between individuals with high and low baseline levels of plasma plant sterols (9, 63). A review by Chan et al recently showed that genetics is an important factor in determining baseline plasma plant sterol concentrations (38). Accordingly, it may be difficult to cross compare studies that assess baseline characteristics in different populations.

The results of this single-blinded, 2-phase, cross-over trial demonstrated that individuals with high and low plasma plant sterol concentrations showed the same changes in plasma campesterol, sitosterol, TC, LDL-C and CRP concentrations after a 4-week supplementation of 2.0 g/d of plant sterols, as compared to control. Similarly, across all individuals no linear relationship was apparent for baseline plasma plant sterol levels and change in plasma plant sterols, lipids and CRP levels, as compared to control.

Plant sterol supplementation may have been perceived as less favorable for individuals with high baseline plasma plant sterol concentrations due to their high

plant sterol absorption efficiency. Results from these studies, however, demonstrate that HPS individuals, despite being hyper absorbers of PS do not show greater elevations in circulating PS concentrations after plant sterol treatment. Thus, the findings of this study suggest that plant sterol therapy is safe as plant sterol supplementation resulted in similar changes in plasma plant sterols for individuals with high and low plasma plant sterol concentrations. Still, the final plasma concentrations were higher for individuals with high baseline plasma plant sterol levels as compared to individuals with low baseline plasma plant sterol levels, after plant sterol supplementation. Accordingly, full justification of the safety of plant sterols can only be established once it has been fully established that moderate levels of plasma plant sterol concentrations are not associated with CVD risk.

The conclusions drawn from this study are limited, however, as it was assumed that only individuals with high and low baseline plasma plant sterol concentrations had been included in the trial. On the other hand, many potential study candidates were excluded from the study as the recruitment protocol specified that individuals express certain plasma plant sterol concentrations.

Contrary to other research (15, 19), this trial also demonstrates that plant sterols benefit all individuals regardless of their baseline plasma plant sterol levels. Cholesterol absorption efficiency, however, between trials was assessed using different indirect measurement techniques thus making it difficult to cross compare data. In the end, the assessment of cholesterol absorption efficiency in high and low plasma plant sterol individuals would have best been ascertained using direct techniques, such as the single or dual isotope method (18).

Nonetheless, there were several strengths to this study. A sufficient number of individuals with high and low baseline plasma plant sterol levels were recruited in order to achieve a power of 80 %. In addition, this trial was metabolically controlled. Accordingly, the anti-hypercholesterolemic and anti-inflammatory effects of plant sterols could be investigated without consideration of the background diet.

Results from this trial also show that plant sterols do not alter CRP levels for the whole study group or in high and low plasma plant sterol individuals. Changes in CRP may not have reached significance, as values were highly variable and ranged from -12 to 45 g/L following plant sterol treatment, as compared to the control phase. On the other hand, the present trial was adequately powered, thus, it is unlikely that changes in CRP would have reached significance with a larger population size. The large variability in the data set may be attributed to the fact that CRP is affected not only by CVD risk but also other factors such as exercise (48), smoking and alcohol consumption (49, 50) and influenza (51). Consequently assessment of CVD risk with CRP may not be appropriate. Still, some researchers have shown a consistent relationship between CRP and CVD risk (64). Regardless of the lack of association between CRP reduction and plant sterols, the approximate 10 % reductions in LDL-cholesterol still demonstrate that this therapy is beneficial for heart health.

Hypercholesterolemia is a major risk factor for CVD, the leading cause of morbidity and mortality worldwide (1). Plant sterols may be used as a cholesterol-lowering therapy, however, until this point questions of safety and efficacy surrounded its use in individuals with high and low plasma plant sterol individuals. Plant sterols may now be considered as a natural and cost-effective therapy, as baseline plasma

plant sterols concentrations may not be indicative of the safety and efficacy of this treatment.

Future directions in research still require that the effects of plant sterol supplementation be examined in a long-term study; this would clearly demonstrate that baseline plasma plant sterol levels do not associate with CVD risk. The efficacy and safety of plant sterol treatment should be also investigated by measuring cholesterol and plant sterol absorption using direct techniques.

Table 3: Baseline characteristics for the total study population and for subjects above the 75th (HIGH) and below the 25th (LOW) percentile of screening sum of plasma plant sterol levels (n=42)

Variable	Total (n=82)	25th and 75th percentile cut-off (n=42)	
		High	Low
Age (yrs)	51.3 ± 1.0	51.7 ± 2.08	52.1 ± 1.9
Weight (kg)	88.6 ± 1.4	85.3 ± 2.3	91.2 ± 2.8
BMI (kg/m^2)	28.4 ± 0.4	27.5 ± 0.6 [a]	29.7 ± 0.9 *[b]
Total cholesterol (mmol/L)	5.82 ± 0.10	6.27 ± 0.14 [a]	5.42 ± 0.13 †[b]
HDL cholesterol (mmol/L)	1.21 ± 0.03	1.30 ± 0.29 [a]	1.12 ± 0.04 †[b]
LDL cholesterol (mmol/L)	3.71 ± 0.09	4.00 ± 0.10 [a]	3.34 ± 0.12 †[b]
non-HDL cholesterol (mmol/L)	4.75 ± 0.10	4.97 ± 0.13 [a]	4.29 ± 0.12 †[b]
Total:HDL cholesterol	4.94 ± 0.10	4.98 ± 0.14	4.92 ± 0.12
Triglycerides (mmol/L)	2.03 ± 0.10	2.18 ± 0.15	2.31 ± 0.22
Campesterol (µmol/L)	12.76 ± 1.21	19.51 ± 1.17 [a]	6.02 ± 0.26 †[b]
Sitosterol (µmol/L)	6.72 ± 0.64	10.18 ± 0.67 [a]	3.26 ± 0.16 †[b]
Sum of Campesterol and Sitosterol (µmol/L)	19.48 ± 1.83	29.68 ± 1.79 [a]	9.28 ± 0.31 †[b]
C-Reactive Protein (mg/L)	2.46 ± 0.31	2.44 ± 0.35	2.22 ± 0.25

Different superscript letters indicate significance between groups.
*$p<0.05$, †$p<0.01$, using one-way ANOVA
All values are expressed as Means ± SEM

Table 4: Post-treatment and percent change in lipid and CRP results for subjects above the 75th (HIGH) and below the 25th (LOW) percentile cut-off of screening sum of plant sterol levels (n=42)

Lipid parameter	Group/Intervention	Post-Treatment				Percent Change (%)			
		Mean	SEM	Group	Sterols	Mean	SEM	Group	Sterols
Total cholesterol	High PS[3]/PS[3]	5.42	0.22	0.0052	0.0006	-13.1 ‡	2.2	0.059	0.024
	High PS[3]/placebo	5.66	0.23			-10.2 ‡	2.2		
	Low PS[3]/PS[3]	4.55	0.19			-15.0 ‡	2.2		
	Low PS[3]/placebo	4.81	0.18			-12.3 †	2.9		
HDL cholesterol[2]	High PS[3]/PS[3]	1.15	0.2	0.0068	0.54	-9.9 ˙	2.7	0.0084	0.52
	High PS[3]/placebo	1.14	0.04			-9.0 ˙	2.5		
	Low PS[3]/PS[3]	0.96	0.05			-14.2 ‡	2.2		
	Low PS[3]/placebo	0.98	0.04			-12.7 †	3.5		
LDL cholesterol[1]	High PS[3]/PS[3]	3.45	0.15	0.0052	<0.0001	-12.8 †	3.0	0.074	0.011
	High PS[3]/placebo	3.71	0.18			-8.2 ˙	3.1		
	Low PS[3]/PS[3]	2.81	0.15			-12.4 †	3.8		
	Low PS[3]/placebo	3.08	0.14			-7.4 ˙	4.3		
Triglycerides	High PS[3]/PS[3]	1.81	0.21	0.62	0.79	-10.2	7.7	0.46	0.28
	High PS[3]/placebo	1.83	0.19			-10.8 *	6.7		
	Low PS[3]/PS[3]	1.74	0.17			-23.6 ˙	5.3		
	Low PS[3]/placebo	1.66	0.14			-11.8	9.3		
Non-HDL cholesterol[2]	High PS[3]/PS[3]	4.27	0.21	0.013	0.0003	-13.6 ‡	2.4	0.13	0.017
	High PS[3]/placebo	4.52	0.21			-10.2 ‡	2.3		
	Low PS[3]/PS[3]	3.59	0.16			-15.2 ‡	2.4		
	Low PS[3]/placebo	3.83	0.16			-12.1 †	2.9		

Cholesterol/HDL[2]	High PS[3]/PS[3]	4.77	0.19	0.92	0.0004	-2.4	2.6	0.61	0.073
	High PS[3]/placebo	5.01	0.18			-0.7	2.0		
	Low PS[3]/PS[3]	4.84	0.18			-0.6	2.0		
	Low PS[3]/placebo	4.99	0.19			1.2	2.0		
C-Reactive Protein	High PS[3]/PS[3]	1.96	0.30	0.63	0.63	-1.7	9.4	0.12	0.29
	High PS[3]/placebo	2.35	0.46			17.0	17.3		
	Low PS[3]/PS[3]	2.37	0.42			35.1	24.0		
	Low PS[3]/placebo	2.93	0.66			26.0	24.9		

*$p<0.05$, †$p<0.001$, ‡$p<0.0001$ significant from baseline, paired t-test.
[1] Low density lipoprotein, LDL
[2] High density lipoprotein, HDL
[3] Plant Sterol
All lipid values are expressed in mmol/L, except for Cholesterol/HDL which is expressed as a ratio. CRP is expressed in mg/L
All data are presented as means ± SEM. CRP data was log-transformed before statistical analysis
For post-treatment data, ANOVA model was used.
For percent change data, ANCOVA model was used with baseline lipid levels as covariates.

Table 5: Post-treatment and percent change in plant sterol concentrations for subjects above the 75th (HIGH) and below the 25th (LOW) percentile cut-off of screening sum of plant sterol levels (n=42)

Plasma Sterols	Group/Intervention	Post-treatment				Change (%)			
		Mean	SEM	Group	Tx	Mean	SEM	Group	Tx
Campesterol	High PS/PS	29.24	2.35	<0.0001	<0.0001	24.6‡	11.9	0.044	0.0006
	High PS/placebo	22.12	2.65			-7.3‡	10.6		
	Low PS/PS	13.22	0.92			39.7*	15.5		
	Low PS/placebo	10.05	0.60			-9.4*	9.42		
Sitosterol	High PS/PS	16.93	1.56	<0.0001	0.0021	33.6‡	17.9	0.15	0.0028
	High PS/placebo	12.47	1.74			-18.2‡	11.8		
	Low PS/PS	7.58	0.68			32.0‡	17.6		
	Low PS/placebo	6.40	0.62			-14.0‡	10.3		

*$p<0.05$, †$p<0.001$, ‡$p<0.0001$ significant from baseline, paired t-test.
All sterol values are expressed in µmol/L.
All data are presented as means ± SEM.
For post-treatment data, ANOVA model was used.
For percent change data, ANCOVA model was used with baseline sterol levels as covariates.

Table 6: Composition of background diet

Nutrient	Amount/3000Cal	Percentage (%)
Protein	113-116 g	15
Carbohydrates	419-426 g	55
Fats	101-103 g	30
Saturated	26-30 g	7-8
Monounsaturated	38-41 g	11-12
Poly unsaturated	24-30 g	7-9
Cholesterol	240-268 mg	
Fiber	36-39 g	

Table 7: Composition of plant sterol spread

	Amount(g)/25g of Margarine	
	Control Diet	Sterol supplemented Diet
Fat	0.8	0.35
Total sterols		2
Sitosterol		1.174
Campesterol		0.44
Brassicasterol		0.06
Campestanol		0.012
Stigmasterol		0.192
D5-Avenasterol		0.018
Sitostanol		0.068
Others		0.04

Table 8: Post-treatment and percent change in lipid and CRP levels for total study population (n=82)

Lipid parameter	Intervention	Post-Treatment			Percent Change (%)		
		Mean	SEM	Tx	Mean	SEM	Tx
Total cholesterol	Plant Sterols	4.92	0.10	<0.0001	-14.9‡	1.03	<0.0001
	Placebo	5.24	0.10		-8.6‡	1.29	
HDL cholesterol[2]	Plant Sterols	1.05	0.02	0.029	-12.1‡	1.27	0.024
	Placebo	1.07	0.02		-9.0‡	1.48	
LDL cholesterol[1]	Plant Sterols	3.13	0.08	<0.0001	-13.7‡	1.51	<0.0001
	Placebo	3.41	0.09		-5.9‡	1.77	
Triacylglycerols	Plant Sterols	1.65	0.09	0.18	-18.1‡	3.13	0.034
	Placebo	1.71	0.09		-10.4†	3.44	
Non-HDL Cholesterol[2]	Plant Sterols	3.87	0.09	<0.0001	-15.5‡	1.12	<0.0001
	Placebo	4.17	0.10		-8.4‡	1.34	
Cholesterol/HDL[2]	Plant Sterols	4.82	0.11	<0.0001	-2.4*	1.17	0.0015
	Placebo	5.01	0.11		1.1	1.02	
C-Reactive Protein	Plant Sterols	3.39	0.67	0.26	214.7	201	0.48
	Placebo	2.74	0.38		10.2	8.9	

*p<0.05, †p<0.001, ‡p<0.0001 significant from baseline, paired t-test.
[1] Low density lipoprotein, LDL
[2] High density lipoprotein, HDL
All lipid values are expressed in mmol/L, except for Cholesterol/HDL which is expressed as a RATIO.
All data are presented as means ± SEM. CRP data was log-transformed before statistical analysis.
For post-treatment data, ANOVA model was used.
For percent change data, ANCOVA model was used with baseline lipid levels as covariates.

Table 9: Post-treatment and percent change in plant sterol levels for total study population (n=82)

Plasma Sterol	Intervention	Post-Treatment			Percent Change (%)		
		Mean	SEM	Tx	Mean	SEM	Tx
Campesterol	Plant Sterols	19.84	1.01	<0.0001	26.5	6.6	0.016
	Placebo	16.10	1.04		2.2	9.3	
Sitosterol	Plant Sterols	12.65	0.97	<0.0001	23.2	7.6	0.004
	Placebo	9.92	0.79		-5.0‡	7.1	

*p<0.05, †p<0.001, ‡p<0.0001 significant from baseline, paired t-test.
All sterol values are expressed in µmol/L.
All data are presented as means ± SEM.
For post-treatment data, ANOVA model was used.
For percent change data, ANCOVA model was used with baseline sterol levels as covariates.

Figure 2: Distribution of the sum of campesterol and sitosterol levels for the total study population with the 25th, 50th and 75th percentile cut-offs (n=82). The 25^{th}, 50^{th}, 75^{th} percentile values were 11.5, 15.3, 21.6 µmol/L, respectively.

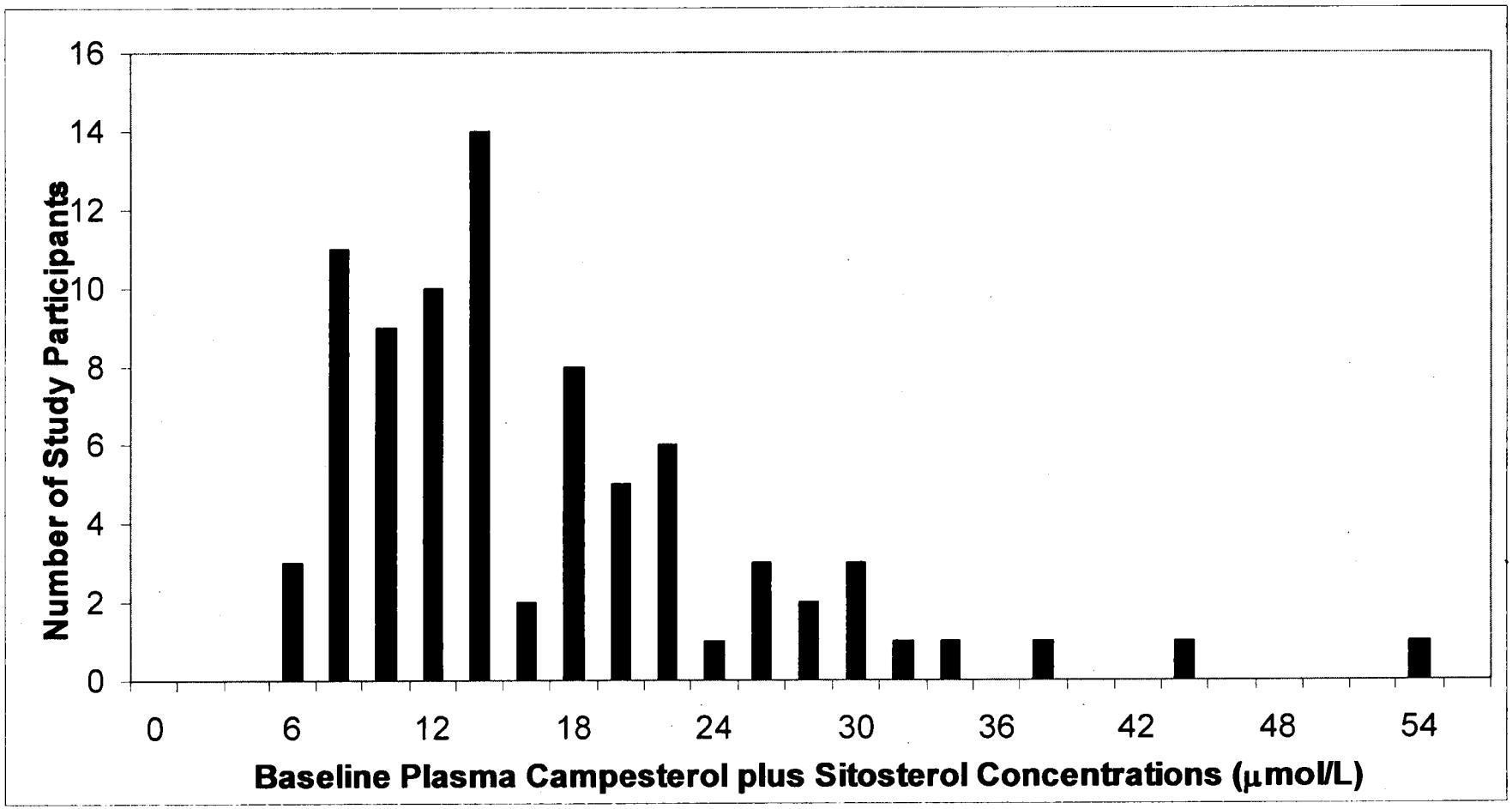
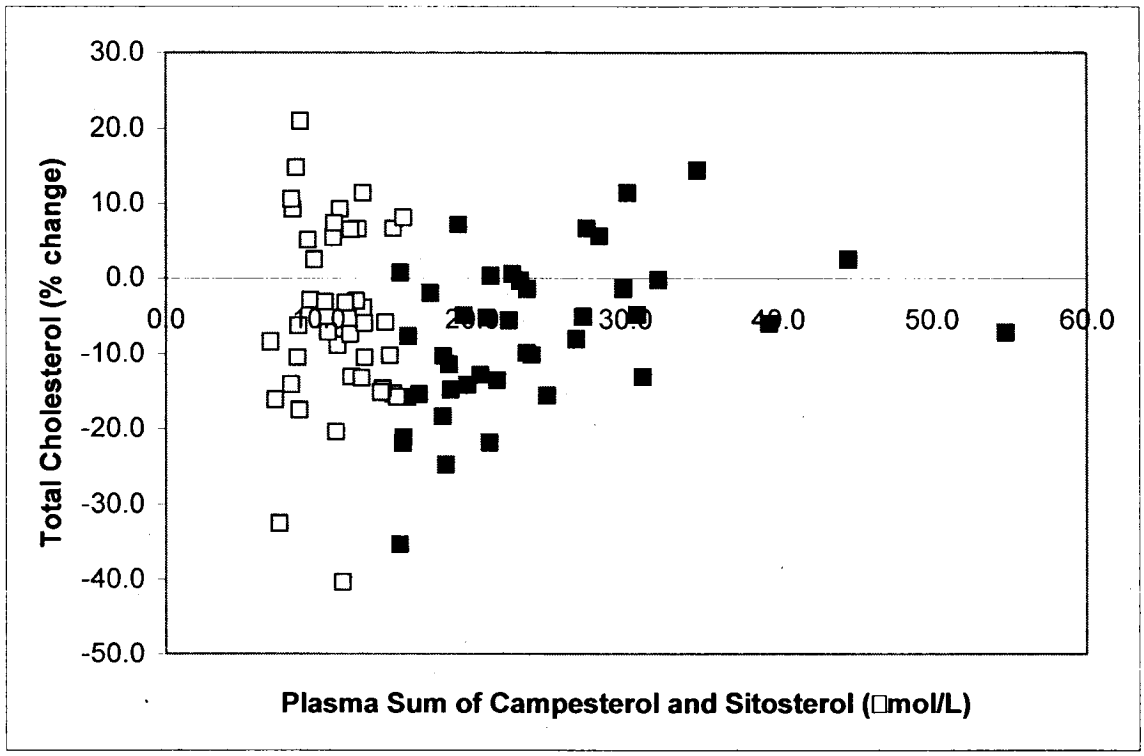


Figure 3: Regression of baseline plasma sum of campesterol and sitosterol concentrations vs. percent change in lipid levels following plant sterol therapy, as compared to control. HIGH (♦) and LOW (▪) plant sterol group ($r_{adj.}$ = -0.059, p = 0.399; $r_{adj.}$ = 0.109, p = 0.811, for TC and LDL-C respectively)



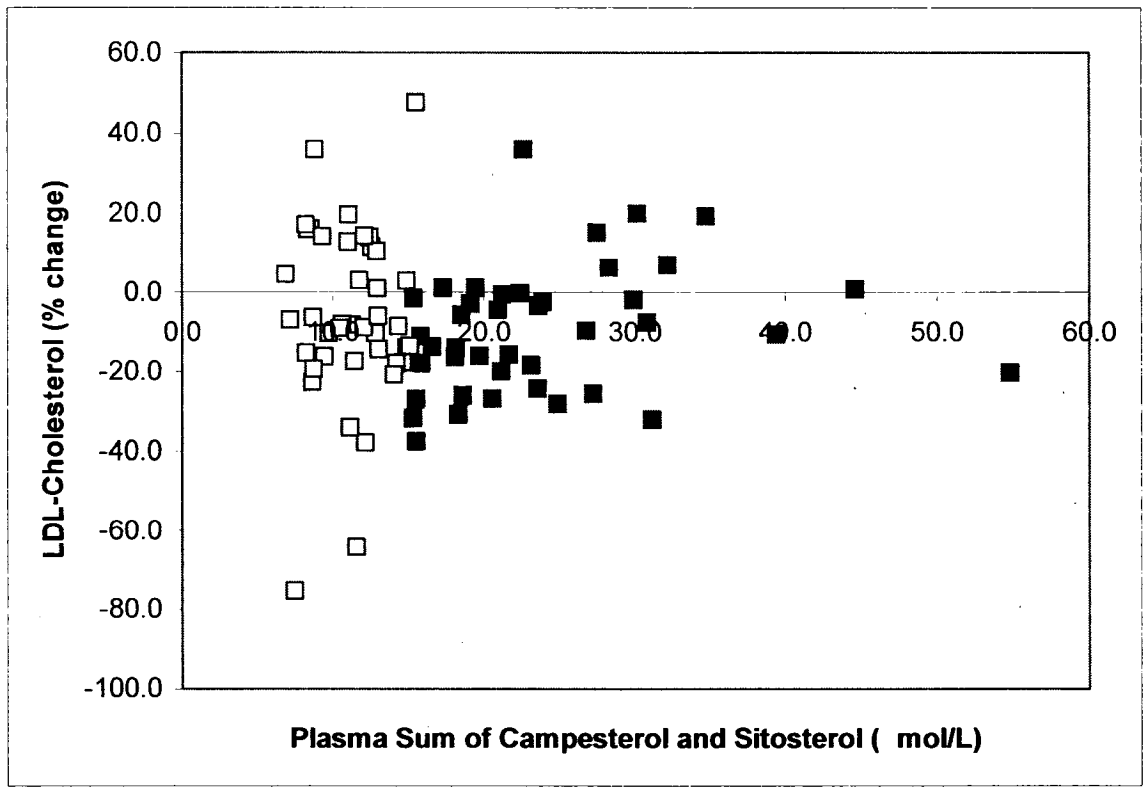
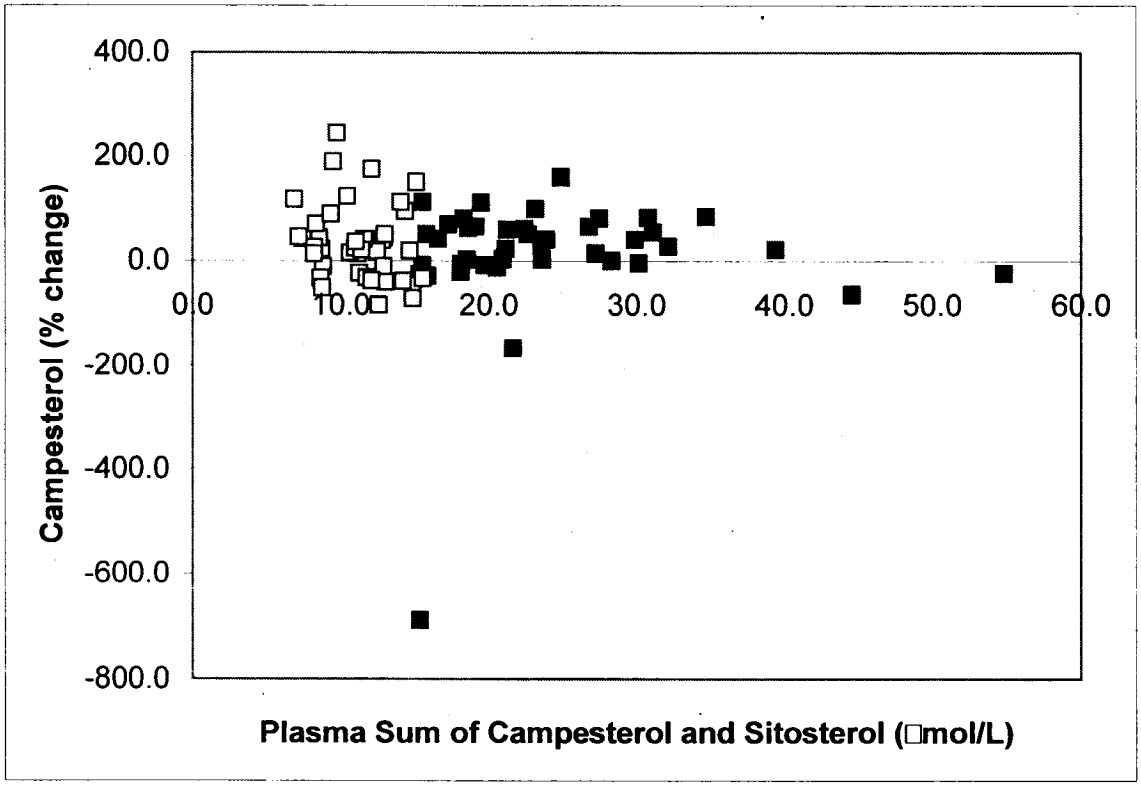


Figure 4: Regression of baseline plasma sum of campesterol and sitosterol levels vs. percent change in plant sterol levels following plant sterol therapy, as compared to control. HIGH (◆) and LOW (■) plant sterol group ($r_{adj.}$ = -0.10, p = 0.69; $r_{adj.}$ = -0.11, p = 0.86, for campesterol and sitosterol respectively)



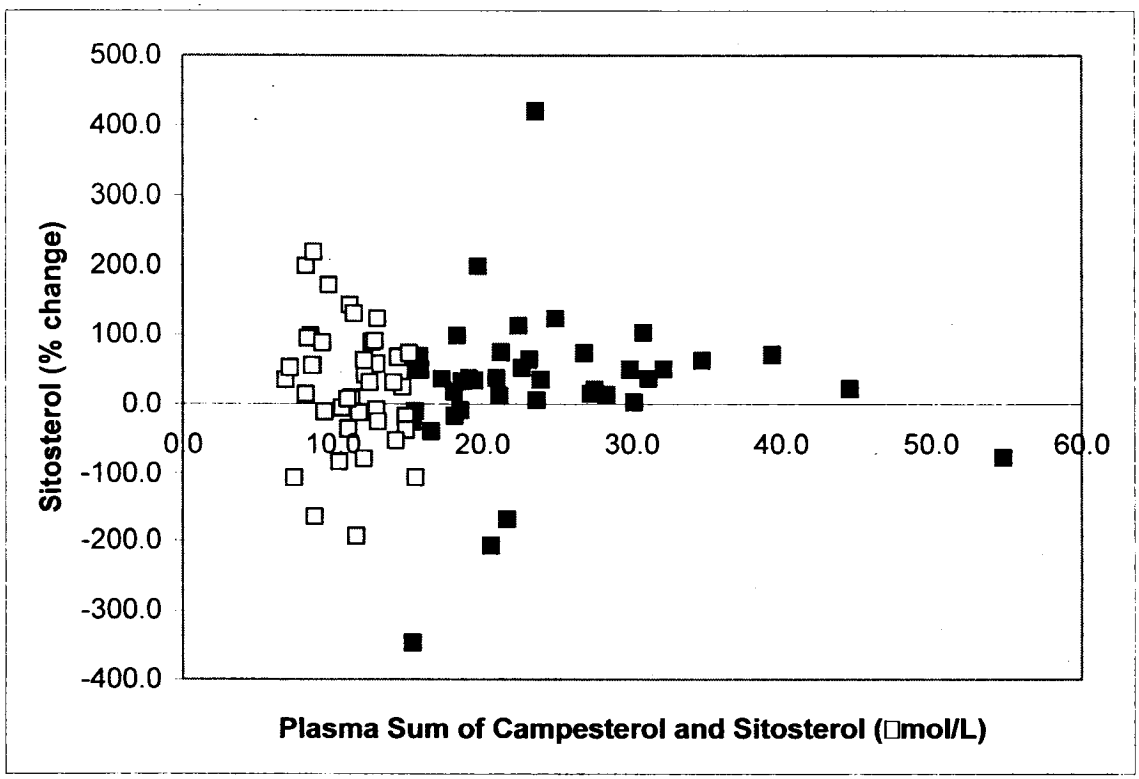
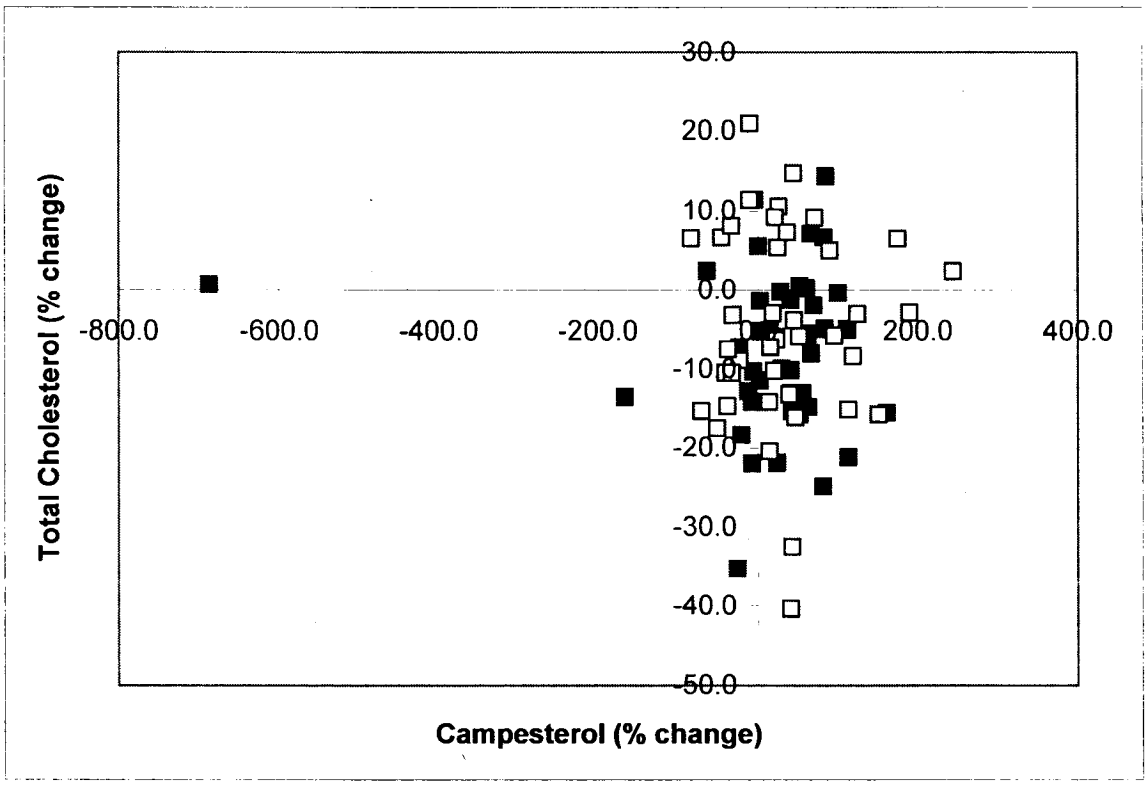


Figure 5: Regression of percent change in lipid vs. campesterol levels following plant sterol therapy, as compared to control. HIGH (◆) and LOW (▪) plant sterol group (r= -0.0334, p=0.9430; r = -0.0178, p=0.6643, for Total and LDL-cholesterol, respectively.)



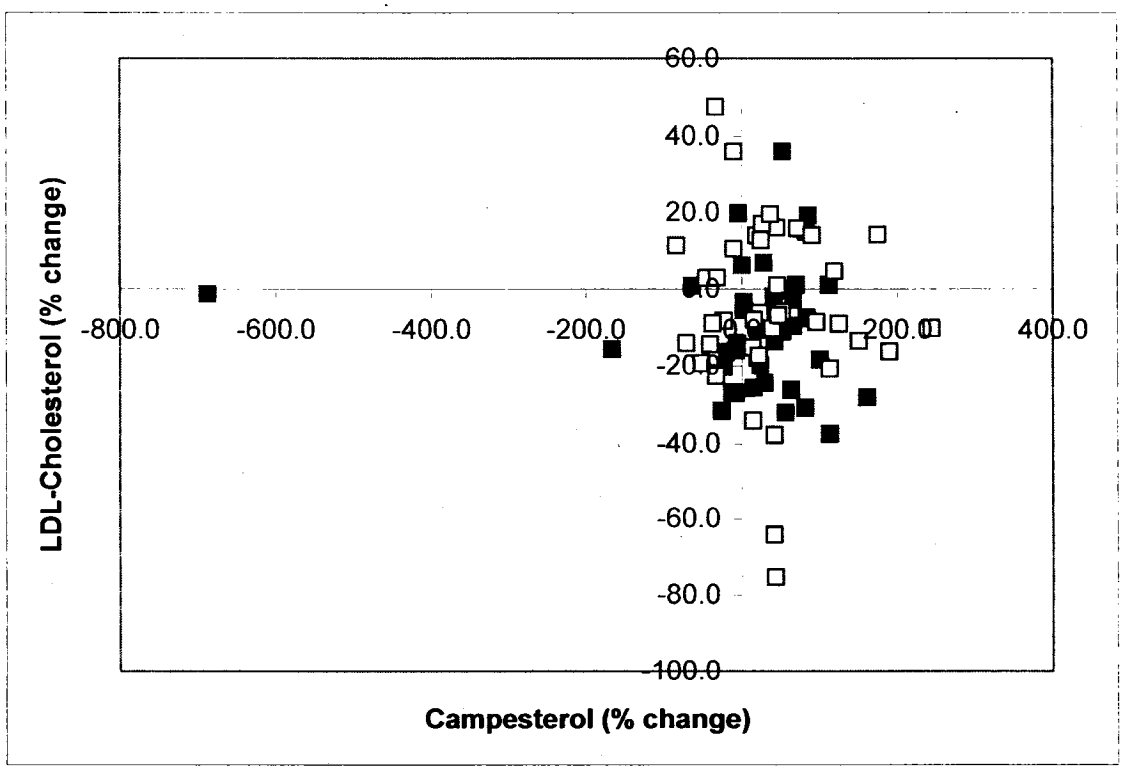
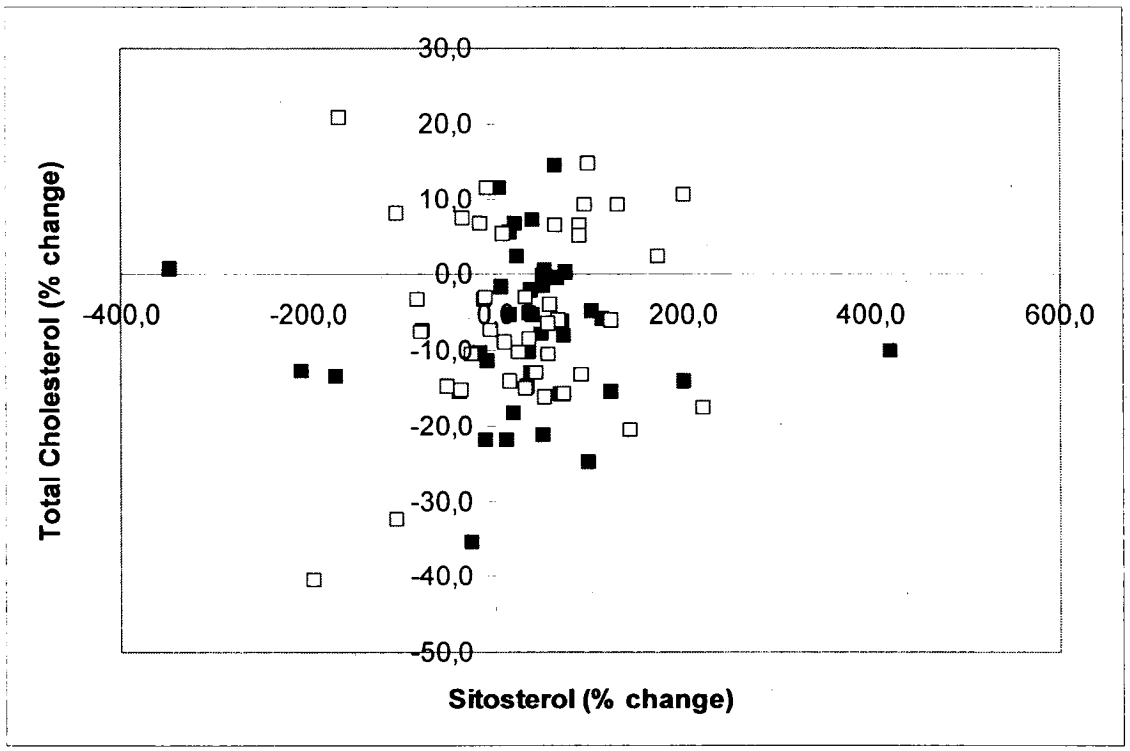


Figure 6: Regression of percent change in lipid vs. sitosterol levels following plant sterol therapy, as compared to control. HIGH (◆) and LOW (▪) plant sterol group (r=-0.0344, p=0.9588; r=-0.0096, p=0.5292, for Total and LDL-cholesterol, respectively).



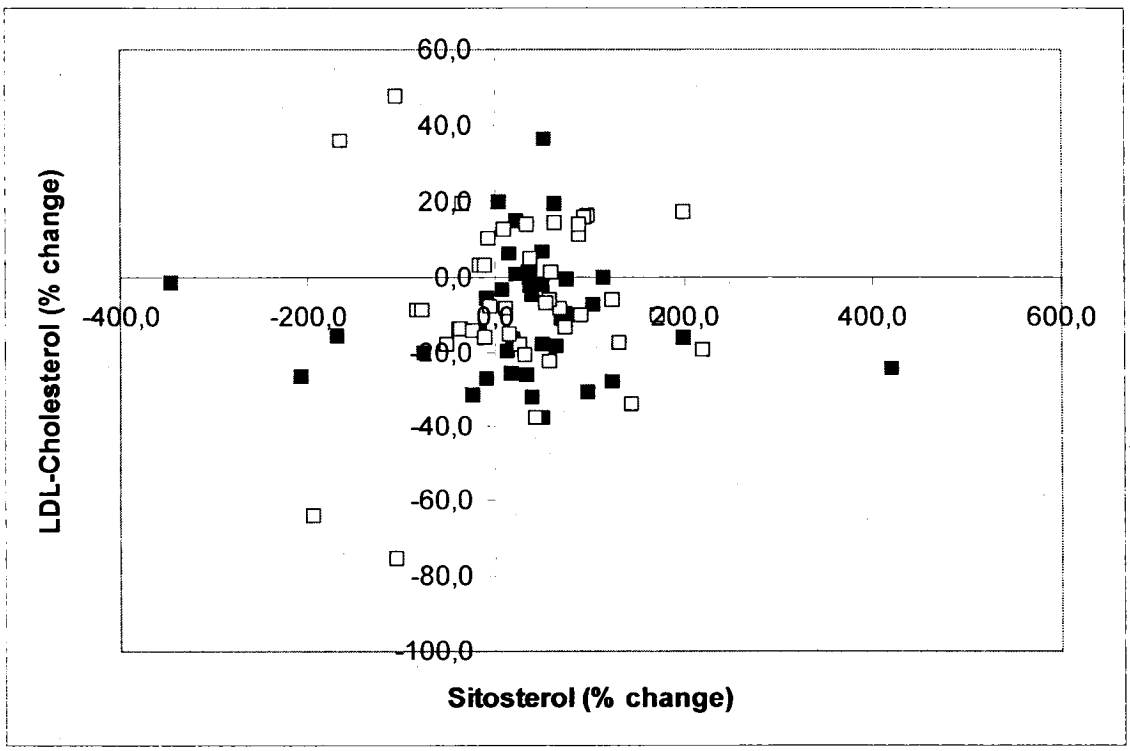
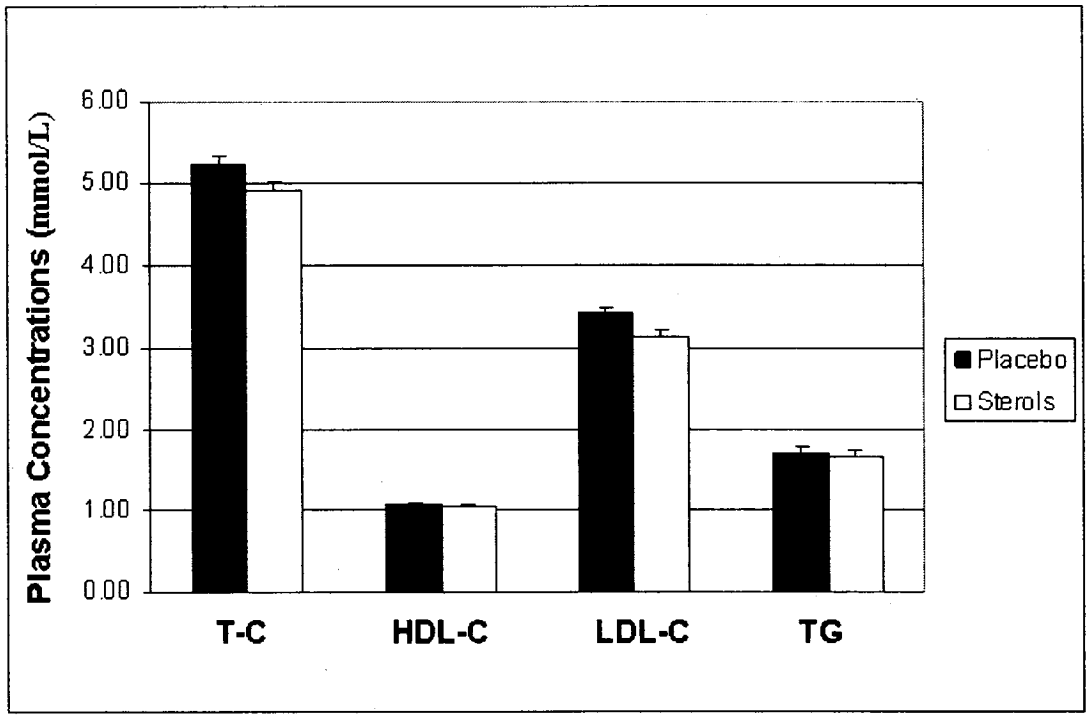
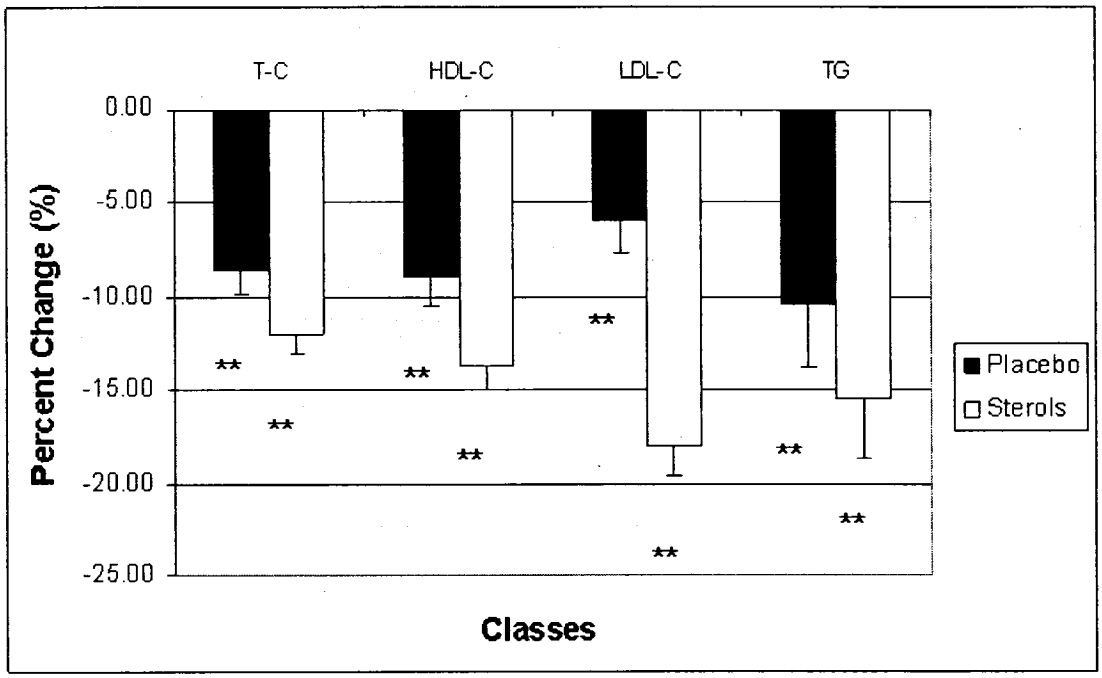


Figure 7: Post-treatment and percent change in lipid levels for total study population (n=82)



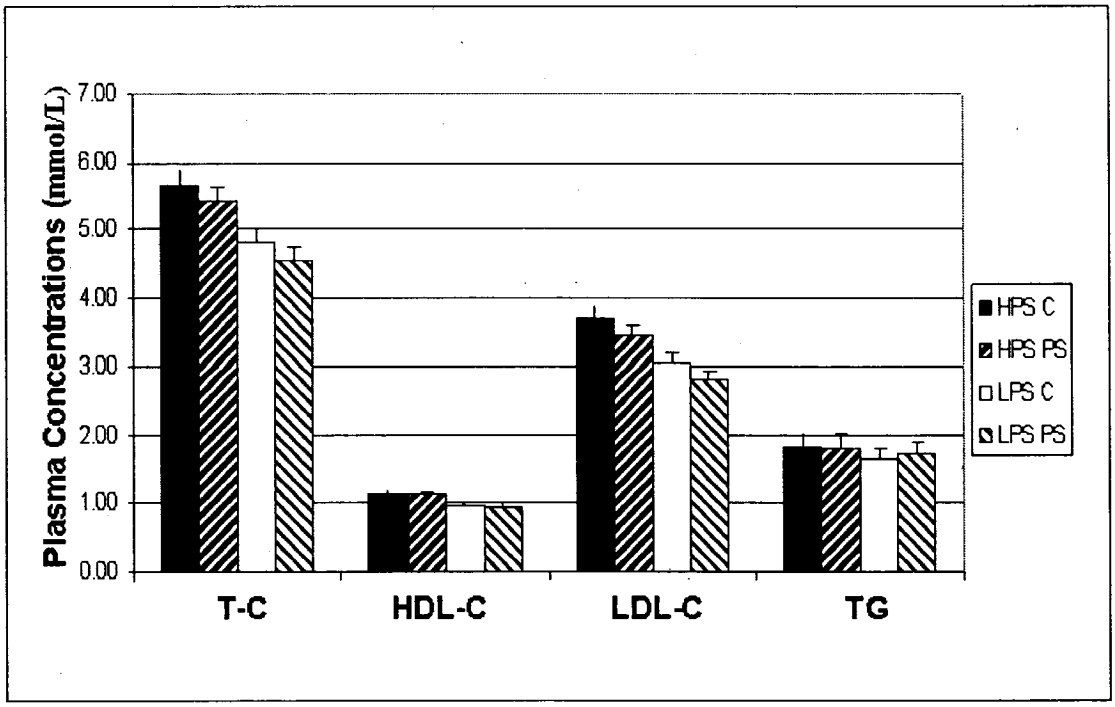
Main effect of treatment P<0.0001, P<0.0291, P<0.0001: ANOVA for T-C, HDL-C and LDL-C, respectively.



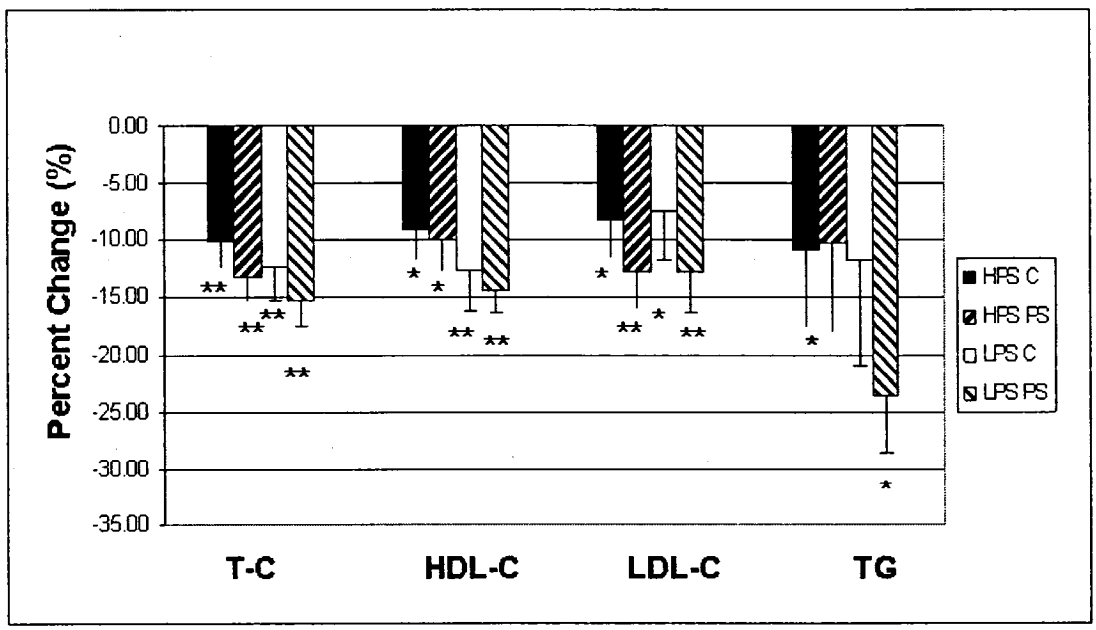
Main effect of treatment P<0.0001, P=0.0238, P<0.0001, P=0.0341: ANCOVA for T-C, HDL-C, LDL-C and TG, respectively. Baseline lipid levels were used as covariates

** Significantly different (P<0.001) from baseline, respectively: Student's paired t-test.

Figure 8: Post-treatment and percent change in lipid levels for subjects above the 75th (HIGH) and below the 25th (LOW) percentile cut-off of screening sum of plasma plant sterol levels (n=82)

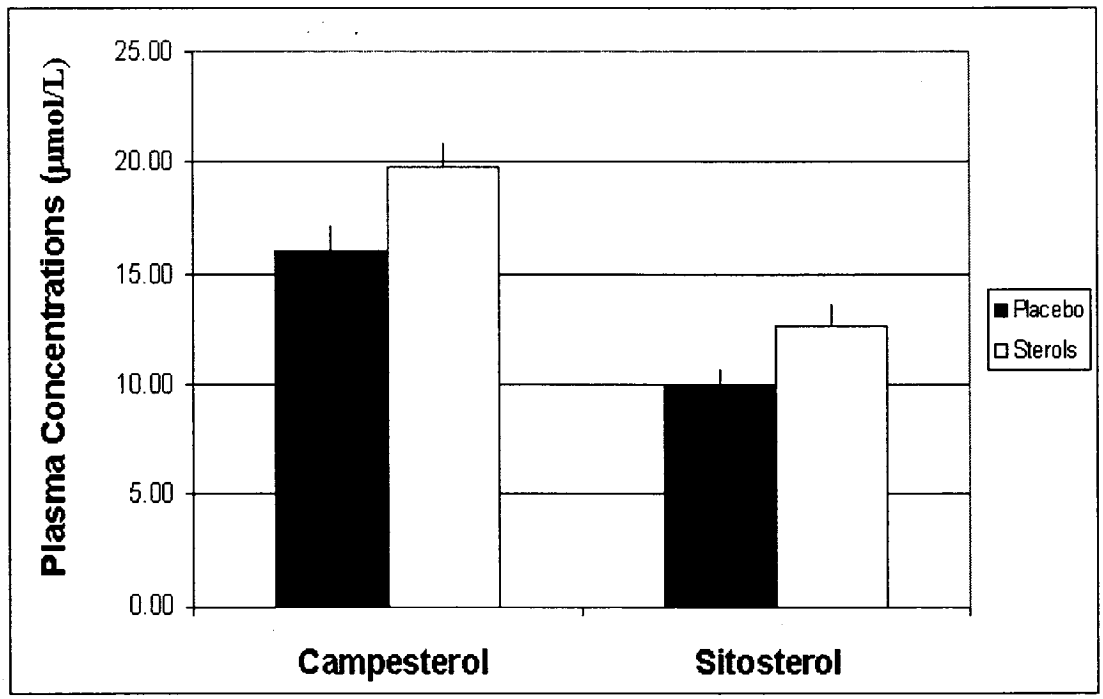


Main effect of treatment P=0.0006, P<0.0001: ANOVA for T-C, LDL-C, respectively.
Main effect of group P=0.0052, P=0.0068, P=0.0052: ANOVA for T-C, HDL-C and LDL-C, respectively.

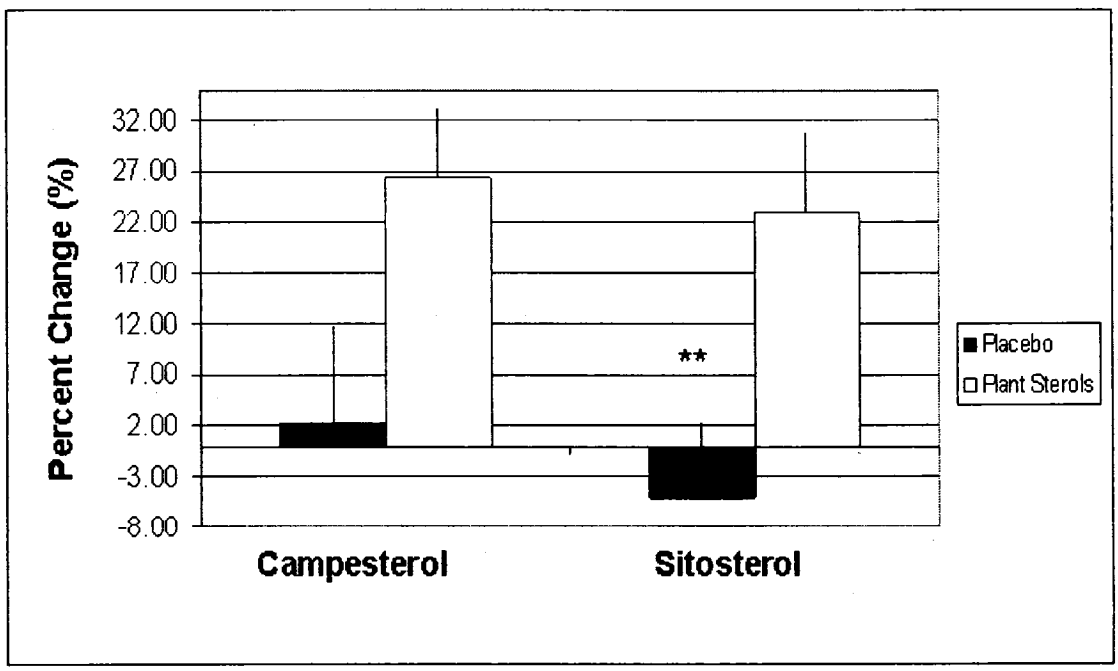


Main effect of treatment P=0.0236, P=0.0110: ANOVA for T-C, LDL-C, respectively.
Main effect of group P=0.0084 for HDL-C.
* And ** significantly different ($P < 0.05$) and (P<0.001) from baseline, respectively: Student's paired t-test.

Figure 9: Post-treatment and percent change in plant sterol levels for the total study population (n=82)

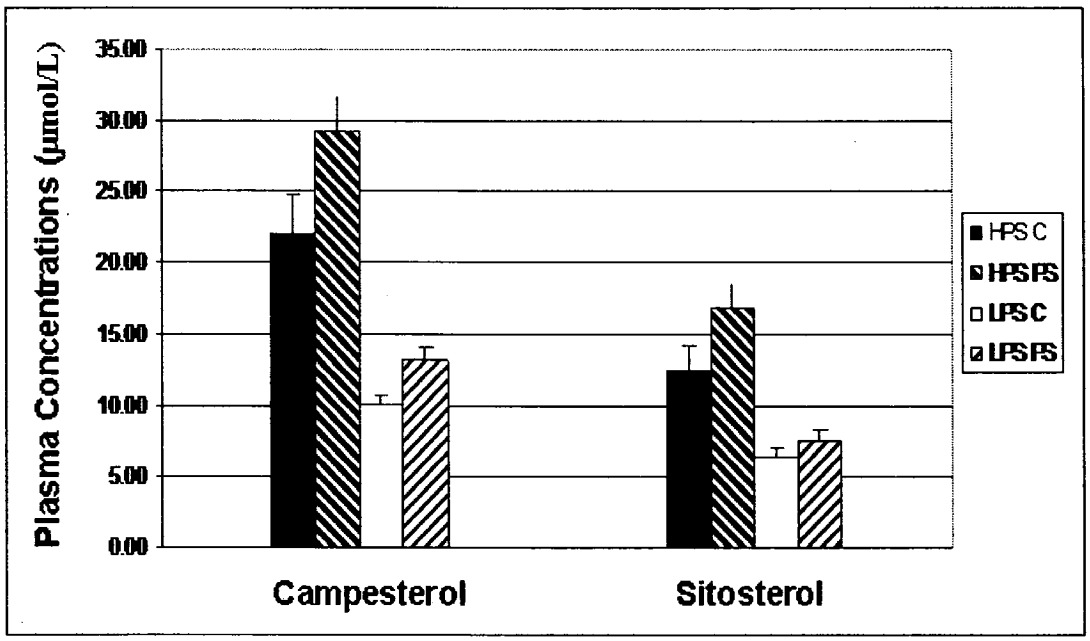


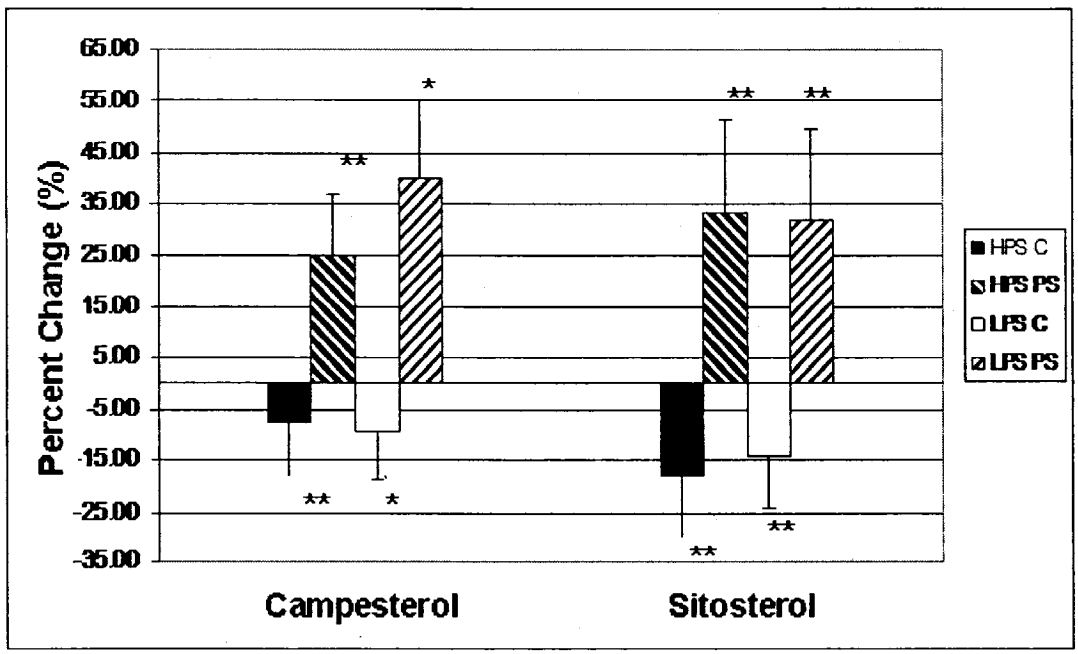
Main effect of treatment P<0.0001: ANOVA for Campesterol and Sitosterol, respectively



Main effect of treatment P=0.0163, P=0.0040: ANOVA for Campesterol and Sitosterol, respectively.
** Significantly different (P<0.001) from baseline, respectively: Student's paired t-test.

Figure 10: Post-treatment and percent change in plant sterol levels for subjects above the 75th (HIGH) and below the 25th (LOW) percentile cut-off of screening sum of plant sterol levels (n=42)





Main effect of treatment P=0.0006, P=0.0028: ANOVA for Campesterol and Sitosterol, respectively.
Main effect of group P=0.043: ANOVA for Campesterol. No interactions were significant.
* And ** significantly different ($P < 0.05$) and (P<0.001) from baseline, respectively: Student's paired t-test.

5 Bibliography

1. Katan MB, Grundy SM, Jones P, Law M, Miettinen T, Paoletti R. Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. Mayo Clin Proc 2003;78:965-78.
2. Law M. Plant sterol and stanol margarines and health. Bmj 2000;320:861-4.
3. Ostlund RE, Jr. Phytosterols in human nutrition. Annu Rev Nutr 2002;22:533-49.
4. Qullez J, Garcla-Lorda P, Salas-Salvado J. Potential uses and benefits of phytosterols in diet: present situation and future directions. Clin Nutr 2003;22:343-51.
5. Sudhop T, von Bergmann K. Sitosterolemia--a rare disease. Are elevated plant sterols an additional risk factor? Z Kardiol 2004;93:921-8.
6. Wilund KR, Yu L, Xu F, et al. No association between plasma levels of plant sterols and atherosclerosis in mice and men. Arterioscler Thromb Vasc Biol 2004;24:2326-32.
7. Glueck CJ, Speirs J, Tracy T, Streicher P, Illig E, Vandegrift J. Relationships of serum plant sterols (phytosterols) and cholesterol in 595 hypercholesterolemic subjects, and familial aggregation of phytosterols, cholesterol, and premature coronary heart disease in hyperphytosterolemic probands and their first-degree relatives. Metabolism 1991;40:842-8.
8. Miettinen TA, Railo M, Lepantalo M, Gylling H. Plant sterols in serum and in atherosclerotic plaques of patients undergoing carotid endarterectomy. J Am Coll Cardiol 2005;45:1794-801.
9. Rajaratnam RA, Gylling H, Miettinen TA. Independent association of serum squalene and noncholesterol sterols with coronary artery disease in postmenopausal women. J Am Coll Cardiol 2000;35:1185-91.
10. Sudhop T, Gottwald BM, von Bergmann K. Serum plant sterols as a potential risk factor for coronary heart disease. Metabolism 2002;51:1519-21.
11. Assmann G, Cullen P, Erbey J, Ramey DR, Kannenberg F, Schulte H. Plasma sitosterol elevations are associated with an increased incidence of coronary events in men: results of a nested case-control analysis of the Prospective Cardiovascular Munster (PROCAM) study. Nutr Metab Cardiovasc Dis 2006;16:13-21.
12. Christiansen LI, Lahteenmaki PL, Mannelin MR, Seppanen-Laakso TE, Hiltunen RV, Yliruusi JK. Cholesterol-lowering effect of spreads enriched with microcrystalline plant sterols in hypercholesterolemic subjects. Eur J Nutr 2001;40:66-73.
13. Clifton PM, Noakes M, Ross D, Fassoulakis A, Cehun M, Nestel P. High dietary intake of phytosterol esters decreases carotenoids and increases plasma plant sterol levels with no additional cholesterol lowering. J Lipid Res 2004;45:1493-9.
14. Davidson MH, Maki KC, Umporowicz DM, et al. Safety and tolerability of esterified phytosterols administered in reduced-fat spread and salad dressing to healthy adult men and women. J Am Coll Nutr 2001;20:307-19.

15. Mussner MJ, Parhofer KG, Von Bergmann K, Schwandt P, Broedl U, Otto C. Effects of phytosterol ester-enriched margarine on plasma lipoproteins in mild to moderate hypercholesterolemia are related to basal cholesterol and fat intake. Metabolism 2002;51:189-94.
16. Nestel P, Cehun M, Pomeroy S, Abbey M, Weldon G. Cholesterol-lowering effects of plant sterol esters and non-esterified stanols in margarine, butter and low-fat foods. Eur J Clin Nutr 2001;55:1084-90.
17. Bjorkhem I, Miettinen T, Reihner E, Ewerth S, Angelin B, Einarsson K. Correlation between serum levels of some cholesterol precursors and activity of HMG-CoA reductase in human liver. J Lipid Res 1987;28:1137-43.
18. Matthan NR, Lichtenstein AH. Approaches to measuring cholesterol absorption in humans. Atherosclerosis 2004;174:197-205.
19. Thuluva SC, Igel M, Giesa U, Lutjohann D, Sudhop T, von Bergmann K. Ratio of lathosterol to campesterol in serum predicts the cholesterol-lowering effect of sitostanol-supplemented margarine. Int J Clin Pharmacol Ther 2005;43:305-10.
20. Bouic PJ. The role of phytosterols and phytosterolins in immune modulation: a review of the past 10 years. Curr Opin Clin Nutr Metab Care 2001;4:471-5.
21. Cottone S, Mule G, Nardi E, et al. Relation of C-reactive protein to oxidative stress and to endothelial activation in essential hypertension. Am J Hypertens 2006;19:313-8.
22. Luo Y, Jiang D, Wen D, Yang J, Li L. Changes in serum interleukin-6 and high-sensitivity C-reactive protein levels in patients with acute coronary syndrome and their responses to simvastatin. Heart Vessels 2004;19:257-62.
23. Patel MD, Thompson PD. Phytosterols and vascular disease. Atherosclerosis 2006;186:12-9.
24. Lagarda MJ, Garcia-Llatas G, Farre R. Analysis of phytosterols in foods. J Pharm Biomed Anal 2006.
25. von Bergmann K, Sudhop T, Lutjohann D. Cholesterol and plant sterol absorption: recent insights. Am J Cardiol 2005;96:10D-14D.
26. Berge KE, von Bergmann K, Lutjohann D, et al. Heritability of plasma noncholesterol sterols and relationship to DNA sequence polymorphism in ABCG5 and ABCG8. J Lipid Res 2002;43:486-94.
27. Connor WE, Lin DS. The effect of shellfish in the diet upon the plasma lipid levels in humans. Metabolism 1982;31:1046-51.
28. Salen G, Batta AK, Tint GS, Shefer S, Ness GC. Inverse relationship between plasma cholestanol concentrations and bile acid synthesis in sitosterolemia. J Lipid Res 1994;35:1878-87.
29. Heinemann T, Axtmann G, von Bergmann K. Comparison of intestinal absorption of cholesterol with different plant sterols in man. Eur J Clin Invest 1993;23:827-31.
30. Kempen HJ, de Knijff P, Boomsma DI, van der Voort HA, Gevers Leuven JA, Havekes L. Plasma levels of lathosterol and phytosterols in relation to age, sex, anthropometric parameters, plasma lipids, and apolipoprotein E phenotype, in 160 Dutch families. Metabolism 1991;40:604-11.

31. Miwa K, Inazu A, Kobayashi J, et al. ATP-binding cassette transporter G8 M429V polymorphism as a novel genetic marker of higher cholesterol absorption in hypercholesterolaemic Japanese subjects. Clin Sci (Lond) 2005;109:183-8.
32. Davis HR, Jr., Zhu LJ, Hoos LM, et al. Niemann-Pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. J Biol Chem 2004;279:33586-92.
33. Miettinen TA, Tilvis RS, Kesaniemi YA. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. Am J Epidemiol 1990;131:20-31.
34. Gylling H, Miettinen TA. Inheritance of cholesterol metabolism of probands with high or low cholesterol absorption. J Lipid Res 2002;43:1472-6.
35. Vanstone CA, Raeini-Sarjaz M, Parsons WE, Jones PJ. Unesterified plant sterols and stanols lower LDL-cholesterol concentrations equivalently in hypercholesterolemic persons. Am J Clin Nutr 2002;76:1272-8.
36. Fernandez ML, Vega-Lopez S. Efficacy and safety of sitosterol in the management of blood cholesterol levels. Cardiovasc Drug Rev 2005;23:57-70.
37. Berger A, Jones PJ, Abumweis SS. Plant sterols: factors affecting their efficacy and safety as functional food ingredients. Lipids Health Dis 2004;3:5.
38. Chan YM, Varady KA, Lin Y, et al. Plasma concentrations of plant sterols: physiology and relationship with coronary heart disease. Nutr Rev 2006;64:385-402.
39. Hallikainen MA, Sarkkinen ES, Gylling H, Erkkila AT, Uusitupa MI. Comparison of the effects of plant sterol ester and plant stanol ester-enriched margarines in lowering serum cholesterol concentrations in hypercholesterolaemic subjects on a low-fat diet. Eur J Clin Nutr 2000;54:715-25.
40. Noakes M, Clifton PM, Doornbos AM, Trautwein EA. Plant sterol ester-enriched milk and yoghurt effectively reduce serum cholesterol in modestly hypercholesterolemic subjects. Eur J Nutr 2005;44:214-22.
41. Hendriks HF, Weststrate JA, van Vliet T, Meijer GW. Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolaemic and mildly hypercholesterolaemic subjects. Eur J Clin Nutr 1999;53:319-27.
42. Vissers MN, Zock PL, Meijer GW, Katan MB. Effect of plant sterols from rice bran oil and triterpene alcohols from sheanut oil on serum lipoprotein concentrations in humans. Am J Clin Nutr 2000;72:1510-5.
43. Yoshida M, Vanstone CA, Parsons WD, Zawistowski J, Jones PJ. Effect of plant sterols and glucomannan on lipids in individuals with and without type II diabetes. Eur J Clin Nutr 2006;60:529-37.
44. Volpe R, Niittynen L, Korpela R, et al. Effects of yoghurt enriched with plant sterols on serum lipids in patients with moderate hypercholesterolaemia. Br J Nutr 2001;86:233-9.
45. AbuMweis SS, Vanstone CA, Ebine N, et al. Intake of a single morning dose of standard and novel plant sterol preparations for 4 weeks does not

dramatically affect plasma lipid concentrations in humans. J Nutr 2006;136:1012-6.
46. Jenkins DJ, Kendall CW, Marchie A, et al. Direct comparison of dietary portfolio vs statin on C-reactive protein. Eur J Clin Nutr 2005;59:851-60.
47. Jenkins DJ, Kendall CW, Marchie A, et al. Direct comparison of a dietary portfolio of cholesterol-lowering foods with a statin in hypercholesterolemic participants. Am J Clin Nutr 2005;81:380-7.
48. Cushman M, Legault C, Barrett-Connor E, et al. Effect of postmenopausal hormones on inflammation-sensitive proteins: the Postmenopausal Estrogen/Progestin Interventions (PEPI) Study. Circulation 1999;100:717-22.
49. Albert MA, Glynn RJ, Ridker PM. Alcohol consumption and plasma concentration of C-reactive protein. Circulation 2003;107:443-7.
50. Kohut ML, McCann DA, Russell DW, et al. Aerobic exercise, but not flexibility/resistance exercise, reduces serum IL-18, CRP, and IL-6 independent of beta-blockers, BMI, and psychosocial factors in older adults. Brain Behav Immun 2006;20:201-9.
51. Ledue TB, Rifai N. Preanalytic and analytic sources of variations in C-reactive protein measurement: implications for cardiovascular disease risk assessment. Clin Chem 2003;49:1258-71.
52. Normen L, Holmes D, Frohlich J. Plant sterols and their role in combined use with statins for lipid lowering. Curr Opin Investig Drugs 2005;6:307-16.
53. Ostlund RE, Jr. Phytosterols and cholesterol metabolism. Curr Opin Lipidol 2004;15:37-41.
54. Pinedo S, Vissers MN, von Bergmann K, et al. Plasma levels of plant sterols and the risk of future coronary artery disease in apparently healthy men and women; the prospective EPIC-Norfolk population study. J Lipid Res 2006.
55. Devaraj S, Autret BC, Jialal I. Reduced-calorie orange juice beverage with plant sterols lowers C-reactive protein concentrations and improves the lipid profile in human volunteers. Am J Clin Nutr 2006;84:756-61.
56. Mifflin MD, St Jeor ST, Hill LA, Scott BJ, Daugherty SA, Koh YO. A new predictive equation for resting energy expenditure in healthy individuals. Am J Clin Nutr 1990;51:241-7.
57. Nagele U, Hagele EO, Sauer G, et al. Reagent for the enzymatic determination of serum total triglycerides with improved lipolytic efficiency. J Clin Chem Clin Biochem 1984;22:165-74.
58. Esterbauer H, Gebicki J, Puhl H, Jurgens G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. Free Radic Biol Med 1992;13:341-90.
59. Harris N, Galpchian V, Rifai N. Three routine methods for measuring high-density lipoprotein cholesterol compared with the Reference Method. Clin Chem 1996;42:738-43.
60. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972;18:499-502.
61. Wolthers BG, Walrecht HT, van der Molen JC, Nagel GT, Van Doormaal JJ, Wijnandts PN. Use of determinations of 7-lathosterol (5 alpha-cholest-7-en-3

beta-ol) and other cholesterol precursors in serum in the study and treatment of disturbances of sterol metabolism, particularly cerebrotendinous xanthomatosis. J Lipid Res 1991;32:603-12.

62. Lutjohann D, Meese CO, Crouse JR, 3rd, von Bergmann K. Evaluation of deuterated cholesterol and deuterated sitostanol for measurement of cholesterol absorption in humans. J Lipid Res 1993;34:1039-46.

63. Gylling H, Hallikainen M, Pihlajamaki J, et al. Polymorphisms in the ABCG5 and ABCG8 genes associate with cholesterol absorption and insulin sensitivity. J Lipid Res 2004;45:1660-5.

64. Tuomisto K, Jousilahti P, Sundvall J, Pajunen P, Salomaa V. C-reactive protein, interleukin-6 and tumor necrosis factor alpha as predictors of incident coronary and cardiovascular events and total mortality. A population-based, prospective study. Thromb Haemost 2006;95:511-8.

****
*** TX REPORT ***
****

TRANSMISSION OK

TX/RX NO	1039
CONNECTION TEL	5307
SUBADDRESS	
CONNECTION ID	
ST. TIME	03/15 11:03
USAGE T	00'42
PGS. SENT	3
RESULT	OK

McGill Faculty of Medicine

Institutional Review Board

- Continuing Review Form -

Principal Investigator: Peter Jones Department/Institution: Dietetics and Human Nutrition

IRB Review Number: Study Number (if any): A04-M31-04A Review Interval:

Title of Research Proposal: Evaluation of plant sterol and cholesterol absorption in overweight, hypercholesterolemic men with or without coronary heart disease

INTERIM REPORT (PLEASE CHECK OR SPECIFY)

Current Status of Study: Active Study ☒ On Hold ☐ Closed to Enrolment ☒

Interim Analysis ☐ Final Analysis ☒ Study Not Activated ☐*

*If the study has not become active at McGill, please enclose correspondence to explain or provide explanation.

McGill hospital(s) where study is being conducted and has received acceptance of local Research Ethics Board(s) (if applicable):

DouglAS ☐ JGH ☐ MUHC/MCH (Mtl Children's) ☐ MUHC/MCI (Mtl Chest Inst.) ☐ MUHC/RVH ☐

MUHC/MNH-WW ☐ MUHC/RVH ☐ Shriners Hospital ☐ SMH ☐ Other ☒ Many Family Clinic

McGill hospital(s) where study is being conducted and has NOT received acceptance of local Research Ethics Board(s) (if applicable):

In the case of a clinical trial, has the sponsor registered the study in the WHO Clinical Trials Registry www.wrcgh.com ? Yes ☐ No ☒

If study sponsorship or financial support has changed, please provide correspondence to explain enclosed: N/A

Total number of subjects to be enrolled in the study: 80 Number of subjects to be enrolled at McGill sites: 80

Number of subjects enrolled by McGill PI to date: 80 Number of subjects enrolled by McGill PI since the last review: Nil

Have any of these subjects withdrawn from the study, and if yes, how many? Yes ☒ 8 No ☐

Has the study been revised since the last review? Yes ☐ No ☒

Has the consent form been revised since the last review? Yes ☐ No ☒ Date of current consent form: JANUARY 2005

McGill Faculty of Medicine

Institutional Review Board

- Continuing Review Form -

Principal Investigator: Peter Jones — Department/Institution: Metabolic and Molecular Nutrition

IRB Review Number: — Study Number (if any): A04 M31 04A — Review Interval:

Title of Research Proposal: Evaluation of plant sterol and cholesterol absorption in overweight hypercholesterolemic [illegible] with or without coronary heart disease

INTERIM REPORT (PLEASE CHECK OR SPECIFY)

Current Status of Study: Active Study ☑ On Hold ☐ Closed to Enrolment ☑

Interim Analysis ☐ Final Analysis ☑ Study Not Activated ☐*

*If the study has not become active at McGill, please enclose correspondence to explain or provide overview

McGill hospital(s) where study is being conducted and has received acceptance of local Research Ethics Board (REB) application:

Douglas ☐ JGH ☑ MUHC/MCH (Montreal Children's) ☐ MUHC/MNH (Montreal Neurological) ☐ Montreal West ☐

MUHC/MNH Mini ☐ MUHC/RVH ☐ Shriners Hospital ☐ SMH ☐ Other: ☐ Mary Emily Clinic

McGill hospital(s) where study is being conducted and has NOT received acceptance of local Research Ethics Board (REB) application:

Is the sponsor of a clinical trial, has the sponsor registered the study in the WHO Clinical Trials Registry: Yes ☐ No ☐

If this is a multicentre trial and the protocol has changed, please provide correspondence to explain or provide overview

Total number of subjects to be enrolled in the study: 90 — Number of subjects to be enrolled at McGill site: 90

Total number of subjects enrolled by McGill to date: 90 — Number of subjects enrolled by McGill since last review: 90

Have any of these subjects withdrawn from the study, and if yes, how many? Yes ☑ 8 No ☐

Has the study been revised since the last review? Yes ☐ No ☑

Has the consent form been revised since the last review? Yes ☐ No ☐ Date of current consent form: [illegible]

Have the study and consent form revisions been submitted and approved by the IRB? Yes ☐ No ☐ n/a

Are there any new data since the last review that could influence a subject's willingness to provide continuing consent? No

Have there been any Serious Adverse Experiences (SAE's)? Yes ☐ No ☑

Have all Serious Adverse Experiences (SAE's) and Safety Reports relevant to the study been reported to the IRB? Yes ☐ No ☐

SIGNATURES

Principal Investigator: _____ Date: March 15, 2005

IRB Chair: _____ Date: _____