University of Nebraska - Lincoln
DigitalCommons@University of Nebraska - Lincoln

Nutrition & Health Sciences Dissertations & Theses | Nutrition and Health Sciences, Department of

4-22-2011

Regulation of Sterol Transport by Dietary Phytosterol Esters

Trevor J. Carden
University of Nebraska – Lincoln, carden.tj@gmail.com

Follow this and additional works at: http://digitalcommons.unl.edu/nutritiondiss

Part of the Dietetics and Clinical Nutrition Commons, and the Molecular, Genetic, and Biochemical Nutrition Commons

Carden, Trevor J., "Regulation of Sterol Transport by Dietary Phytosterol Esters" (2011). *Nutrition & Health Sciences Dissertations & Theses.* Paper 24.
http://digitalcommons.unl.edu/nutritiondiss/24

This Article is brought to you for free and open access by the Nutrition and Health Sciences, Department of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Nutrition & Health Sciences Dissertations & Theses by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

REGULATION OF STEROL TRANSPORT BY DIETARY PHYTOSTEROL ESTERS

By

Trevor J. Carden

A THESIS

Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of the Requirements
For the Degree of Master of Science

Major: Nutrition

Under the supervision of Professor Timothy P. Carr

Lincoln, Nebraska

May, 2011

REGULATION OF STEROL TRANSPORT BY DIETARY PHYTOSTEROL ESTERS

Trevor J. Carden, M.S

University of Nebraska, 2011

Advisor: Timothy P. Carr

LDL cholesterol is associated with the development of atherosclerosis and is therefore considered an important target for intervention to prevent cardiovascular diseases. The inhibition of cholesterol absorption in the small intestine is an attractive approach to lowering plasma cholesterol, one that is exploited by drug therapy as well as dietary supplementation with plant sterols. The mechanism of action of plant sterol esters (PSE) is still incompletely understood, therefore this study was conducted to test the hypothesis that hydrolysis of plant sterol esters is necessary for their cholesterol-lowering effects to be realized.

Male Syrian hamsters were fed diets containing no PSE, PSE containing stearic acid, palmitic acid, oleic acid or plant sterol ethers containing stearic acid. Treatment compounds were added at 5% of the diet (g/g). Diets were high is cholesterol and saturated fat to induce hyperlipidemia. The treatments effectively created a spectrum of PSE hydrolysis across which cholesterol metabolism could be compared. Stearate ethers, Stearate Esters and Palmitate Esters were poorly hydrolyzed (1.69-4.12%), while oleate sters were hydrolyzed at 88.29%, and cholesterol absorption correlated negatively with percent hydrolysis with a correlation coefficient of -0.8504. These results suggest that

PSE hydrolysis plays a necessary role in the cholesterol-lowering effects of PSE. In addition, these data also suggest that poorly hydrolyzed plant sterol esters may act through an alternative mechanism than that of competition with cholesterol for micelle incorporation. We suggest that these PSE that are not well hydrolyzed may lower cholesterol by forming an oil phase into which cholesterol is solubilized making it unavailable for absorption into enterocytes.

In summary, our results demonstrated that PSE hydrolysis is necessary for cholesterol-lowering. Additionally, poorly hydrolyzed PSE may function through an alternative pathway than micelle competition with cholesterol.

Acknowledgements

DR. TIMOTHY CARR

Thank you for supporting my research and my education these past two years. I recognize your willingness to give of your time and energies as I had need, and I do not take your sacrifice lightly. I count it an honor to have been trained by a superior researcher and professor.

DR. ANDREW BROWN

Thank you for the countless hours you have spent training me in laboratory practice and imparting sound research philosophy. Apart from your efforts, a graduate education of the caliber that I have received would not have been possible.

JACQUE CARDEN

Perhaps the greatest acknowledgement belongs to my wife who has been with for the duration of my academic pursuits, and has played an integral role in my success. Your support and encouragement have been indispensible. A pursuit of this nature would be infinitely less significant to me without the end goal of providing for a woman of your integrity and devotion.

Table of Contents

	Page
List of Tables and Figures	vi
Introduction	1
Literature Review	3
I. Cardiovascular Disease and Atherosclerosis	3
II. Cholesterol Absorption and Transport	5
III. Mechanisms of Action of Plant Sterols	18
IV. Cholesterol Lowering Properties of Plant Sterols	21
Materials and Methods	32
Results	47
Discussion	67
References	76

List of Tables and Figures

		Page
Table 1	Diet Composition	35
Table 2	Hamster Body Weight	48
Table 3	Food Intake	49
Table 4	Cholesterol Absorption and Plasma Lipid	56
Table 5	Liver Weight and Lipids	56
Table 6	Fecal Sterols	57
Table 7a	Biliary Bile Acids	58
Table 7b	Biliary Bile Acids – Continued	59
Figure 1A-F	Chemical Structures	36
Figure 1G-I	Chemical Structures	37
Figure 2	Dual Isotope Formula	40
Figure 3	Hydrophobicity Index Formulas	46
Figure 4	Cumulative Weight Gain	48
Figure 5	Correlation: % Hydrolysis and Cholesterol Absorption	60
Figure 6	Correlation: %Hydrolysis and Fecal Cholesterol Output	61
Figure 7	Correlation: % Hydrolysis and Non-HDL Cholesterol	62
Figure 8	Correlation: % Hydrolysis and Liver Esterified Cholesterol	63
Figure 9	Correlation: Fecal Cholesterol Output and Non-HDL Cholesterol	64
Figure 10	Correlation: Fecal Cholesterol Output and Ln of Non-HDL Cholesterol	65
Figure11	Correlation: Cholesterol Absorption and Non-HDL Cholesterol	66

INTRODUCTION

Cardiovascular disease takes on many forms, but the form of cardiovascular disease that is responsible for the most deaths in the United States is Coronary Heart Disease (CHD). Atherosclerosis, or plaque buildup in blood vessels, is a major contributor to CHD and has been strongly associated with elevated levels of low density lipoprotein (LDL) cholesterol. Large efforts over decades to control the development and progression of atherosclerosis and CHD have yielded numerous therapeutic options for controlling LDL cholesterol including pharmaceutics targeted at reducing cholesterol absorption, cholesterol biosynthesis and bile acid re-absorption. In an effort to target LDL cholesterol absorption with fewer side effects than drug therapy, nutrition supplementation with plant sterols has become a large topic of interest.

Plant sterols have been suggested to work through a number of different mechanisms; 1.) competition with cholesterol for an esterification enzyme required for absorption 2.) co-crystallization with cholesterol or 3.) competition with cholesterol for entrance into micelles necessary for absorption. The latter two mechanisms have been shown to be viable explanations of plant sterol actions, although the third mechanism is thought to be quantitatively most important.

A number of parameters are important to consider for optimum cholesterol lowering effects of plant sterol supplementation. Plant sterols in contrast to their hydrogenated counterparts, plant stanols, have different chemical properties that appear to make them less efficient, particularly over long periods of supplementation, than plant stanols at lowering cholesterol. Also, the food matrix in which the plant sterol is

delivered is important. Plant sterols supplemented in bread or breakfast cereal, for example, were less effective than those supplemented in milk or yoghurt (Clifton et al 2004). Dose is another important consideration. Doses as low as 0.8g/day have been shown to lower cholesterol (Hendriks et al 1999), but it appears that the optimal maximum dose above which few additional benefits are realized is 2g/day ((Katan et al 2003). Additionally, some evidence suggests that plant sterols taken in multiple doses per day are more effective than one large dose per day.

Another parameter that has often been assumed to be required for effective cholesterol-lowering by plant sterol esters (PSE) is hydrolysis of the molecule to yield free plant sterols. Although the necessity of PSE hydrolysis has been assumed, it has not been unequivocally demonstrated. Therefore, this study was proposed to test the hypothesis that PSE hydrolysis is necessary for optimum cholesterol lowering effects. Male Syrian hamsters were fed either no PSE, PSE containing stearic, palmitic or oleaic acid, a plant sterol ether with stearic acid, or free sterol substituted into the diet at 3% plant sterol equivalent. Diets were designed to create a spectrum of hydrolysis to determine the dependence of the cholesterol-lowering effects of plant sterols on hydrolysis of the PSE. The study was carried out for 23 days. Plasma, liver, bile and feces were collected analysis.

LITERATURE REVIEW

I. Cardiovascular Disease and Atherosclerosis

Cardiovascular Disease (CVD) is an umbrella term that encompasses a variety of disease states including high blood pressure, coronary heart disease (CHD), stroke and heart failure. The etiology of CHD is largely attributable to atherosclerosis, or the build-up of plaques on blood vessel walls as a result of cholesterol and foam cell deposition. Damaged endothelial tissue triggers the formation of clots to prevent further damage. Increasing foam cell deposition, however, leads to an inflammatory state characterized by lipid oxidation and eventual rupture of the clots, likely blocking blood flow through the affected vessels and leading to the characteristic manifestations of CHD; myocardial infarction and angina pectoris.

Because of the profound influence that CHD has upon human health, it has become the focus of a great deal of research. There are a number of factors with which elevated incidence of CHD is associated. These include nonlipid parameters such as physical inactivity, smoking, and obesity, as well as lipid factors such as reduced high density lipoprotein (HDL)-cholesterol, elevated triglycerides (TG) and elevated low density lipoprotein (LDL)-cholesterol. Of these factors, an elevated level of LDL-cholesterol has, for years, been widely accepted as the most important for the development of CHD (NCEP 2002), and consequently therapeutic treatments targeted at lowering LDL-cholesterol are paramount.

LDL-cholesterol is responsible for the transport of the majority of plasma cholesterol in humans (Galeano et al 1998). LDL particles are removed by the liver facilitated by LDL apoB protein interaction with liver LDL receptors. However, significant quantities of the lipoprotein can still interact with tissues independently of LDL receptors, leading to the atherogenic nature of LDL in extra-hepatic tissues (Galeano et al 1998). Small, dense LDL, a sub-fraction of LDL-cholesterol, has been presented as even more atherogenic than its larger fraction counterparts due to its greater ability to be oxidized and its increased ability to enter blood vessel intima (Berneis and Krauss 2002, Galeano et al 1998, Tribble et al 1995). Furthermore, the oxidative modification of LDL-associated lipids is a direct initiator of atherogenesis (Rizzo et al 2009).

Restriction of dietary cholesterol has traditionally been recommended to reduce circulating cholesterol levels, and thereby reduce atherosclerotic risk. Recent research, however, has determined diet to be a less-than-significant contributor to atherosclerosis or cardiovascular risk (McNamara 2000). Hepatic cholesterol synthesis is, on average, nearly 1000 mg/day, making it a quantitatively more important target for lowering circulating cholesterol levels (McNamara 2000). Consequently pharmacological intervention has been focused on this endogenous cholesterol source. Statin therapy has been successful in lowering LDL cholesterol levels by 18-55% through the inhibition of 3-hydroxy-3-methyl-glutaryl-CoA reductase, the rate limiting enzyme in cholesterol synthesis (NCEP 2002). As is common with drug therapies, however, there are side effects to the use of statins. Most commonly, myopathy, elevated creatine kinase and alanine aminotransferase levels are seen with statin therapy (Joy and Hegele 2009,

Tolman 2002). Even with the efficiency of cholesterol-lowering seen with statin administration, new recommendations for optimal LDL levels are still often unattainable and require the addition of other drugs which add additional risk of drug-drug interaction and side effects (Katragadda et al 2010). This reality of side effects has created the need for the development of other cholesterol-lowering treatments. Because of their relative potency of action and paucity of side effects, dietary components, of which plant sterols rank among the most powerful, have become an increasingly attractive alternative.

II. Cholesterol Absorption and Transport

Intestinal Cholesterol Absorption

Dietary cholesterol intake for the average adult American is 400-500 mg per day (Grundy 1983), most of which is in the free form (Best and Duncan 1958). The small amount that enters the intestine as esters is quickly hydrolyzed by pancreatic cholesterol esterase (Carr and Jesch 2006), as only free sterol may be absorbed. Early perspectives of intestinal contents described a uniform emulsion from which lipid absorption occurred, but subsequent clarification of lipolysis and brush border anatomy suggested the need for re-evaluation (Hofmann and Borgstroem 1964). The current understanding is that intestinal contents consists of an oily phase and an aqueous phase existing above the critical micelle concentration (CMC) (Johnston and Borgstroem 1964, Miettinen and Siurala 1971, Nissinen et al 2002). The CMC is the surfactant concentration above which any added surfactant is, with high probability, in a micellar aggregate (Ruckenstein and Nagarajan 1975). The formation of micelles in the intestine during digestion occurs

through bile acid surfactant activity above the CMC that allows for spontaneous formation of these structures and incorporation of lipids, including free cholesterol. Cholesterol must be incorporated into micelles in order to pass through the small intestine brush border membrane and cross the unstirred water layer border where it enters the cytoplasm of the enterocyte (Simmonds 1972, Westergaard and Dietschy 1976). While previously thought to be a process of simple diffusion (Iqbal and Hussain 2009), it is now known that uptake of cholesterol from the micelle into the enterocyte is a protein mediated process (Thurnhofer and Hauser 1990), and that NPC1L1 is the likely the primary transporter responsible for its uptake (Altmann et al 2004). This complex system of sterol ester hydrolysis, micelle incorporation, and protein mediated transport allows for approximately 50% absorption efficiency of cholesterol from the intestine although a number of percent ranges have been observed (Altmann et al 2004, Borgstrom 1960).

Cellular Cholesterol Transport

Upon entrance into the enterocyte, and in order to target it towards nascent chylomicrons rather than toward excretion into the intestine, free cholesterol is esterified by the endoplasmic reticulum-localized enzyme, acyl-CoA:cholesterol acyltransferase 2 (ACAT2) (Lee et al 2000). The rate of esterification by this enzyme is influenced positively by substrate availability and negatively by product accumulation (Iqbal and Hussain 2009), affording the cell a mechanism for sensing the degree of cholesterol ester accumulation. In addition, ACAT2 serves as a determinate of whether free cholesterol is esterified and secreted into chylomicrons and other lipoproteins, or whether it is released from the cell back into the intestine. If ACAT2 is inhibited by a large pool of esterified

cholesterol, free cholesterol entering the cell is then likely to be directed out of the cell through the action of two heterodimeric, brush border transporters, ABCG5 and ABCG8. Transgenic mice over-expressing the human ABCG5/G8 proteins show a 50% reduction in cholesterol absorption supporting the hypothesis that they function to limit sterol absorption (Yu et al 2002). Free cholesterol that enters the enterocyte may also be secreted with the protein apoA-1 as nascent HDL particles via the ABC transporter ABCA1. Early work with the ABCA1 transporter describes the docking of the apoA-1 protein with ABCA1 leading to the subsequent formation of a phospholipid-apoA-1 complex which then facilitates cholesterol efflux from cells (Wang et al 2001). Mutations in the gene coding for ABCA1 leads to the disease known as Tangiers characterized by low HDL production and reduced cholesterol efflux from tissues. Genetic mutations in human populations, both those that increase and decrease gene function, have been shown to alter plasma lipid profiles and the progression of Coronary Artery Disease (CAD) (Clee et al 2001). More specifically, increased activity of ABCA1 reduces CAD progression, and the opposite is true with a reduced ABCA1 activity.

Lipoprotein Cholesterol Transport

Due to cholesterol's hydrophobic nature, its transport through the aqueous environment of the plasma necessitates a transport system. This lipid transport system is composed of a variety of lipoprotein molecules that carry cholesterol between enterocytes, hepatocytes, and peripheral tissues. Cholesterol taken up by enterocytes is either expelled back into the intestinal lumen by the action of the ABCG5/8 heterodimer or is esterified for subsequent lipoprotein incorporation. Two lipoprotein classes are

produced in the enterocyte into which cholesterol may be incorporated; high density lipoproteins (HDL) and chylomicrons.

The most quantitatively important of the two lipoproteins for cholesterol secretion from the enterocyte is the chylomicron. These largest vesicles of the lipoprotein family are synthesized within the enterocyte on the endoplasmic reticulum as apoB is synthesized and lipid, TG and cholesterol, is joined to it forming a stabilized structure (van Greevenbroek and de Bruin 1998). Upon formation the chylomicron is secreted via an exocytotic mechanism into the lymphatic system (Mansbach 2009). From the lymph, the chylomicron then travels to the thoracic duct where it enters the circulation through the subclavian vein (van Greevenbroek and de Bruin 1998). As chylomicrons travel to peripheral tissues through the circulation, they are rapidly degraded to chylomicron remnants as lipoprotein lipase (LPL) hydrolyzes TG, and tissues uptake the resultant free fatty acids, monoglycerides and glycerol. Chylomicron remnants then travel to the liver where they are taken up along with their cholesterol and remaining TG into hepatocytes by the action of chylomicron remnant receptors (Brown and Goldstein 1985).

HDL represents a much smaller intestinal cholesterol efflux pathway. This lipoprotein is formed through the action of ABCA1 in the presence of the protein, apoA-1. In fact, there is evidence that the primary function of intestinal ABCA1 is the basolateral efflux of unesterified cholesterol and phospholipids from the intestine cells into HDL (Brunham et al 2006). The enterocytes are in close proximity to both capillaries and lymphatic ducts, however, ABCA1-mediated cholesterol efflux in HDL preferentially enters that plasma rather than the lymph, although both are sites of HDL secretion (Brunham et al 2006). Early HDL particles are often described as discoidal, as

they have not yet taken on the more spherical shape of mature HDL. At this point, they are composed of apolipoproteins, phospholipids and a small amount of free cholesterol (Eisenberg 1984). As HDL travels through the plasma and lymph, the maturation process whereby the lipoproteins take on more cholesterol and become increasingly spherical, is mediated by the enzyme lecithin:cholesterol acyl transferase (LCAT). LCAT functions to esterify HDL membrane free cholesterol to fatty acids from membrane phospholipids, allowing for movement of the newly synthesized polar lipid into the HDL core, making room for more cholesterol uptake into the lipoprotein membrane (Kontush et al 2008). This process is dependent on apoA-1, of which other lipoproteins such as chylomicrons and VLDL are often a source (Sahoo et al 2004).

In addition to those of intestinal origin, other lipoproteins are produced by the liver. Chylomicrons, and to a lesser degree, HDL of intestinal origin are responsible for the movement of exogenous lipid from the intestinal cells to some peripheral tissues and to liver cells. In the liver, however, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and HDL of hepatic origin are then tasked with the transport of endogenous lipids from the liver to peripheral tissues and back.

VLDL, considered to be a TG-rich lipoprotein despite its relatively low TG content compared to chylomicrons and their remnants, are secreted from the liver cell to supply TG to peripheral tissues. The maturation process of VLDL takes place in the hepatocyte. After the apoB protein is synthesized, a neutral lipid particle including cholesterol esters esterified by ACAT2, is joined to the protein and a mature VLDL particle is then ready for secretion into the systemic circulation (Hebbachi and Gibbons 2001, Lee et al 2000). A major function of VLDL is the transport of TG to peripheral

tissues. As the lipoprotein reaches its target tissue, LPL acts on the particle to release free fatty acid. The removal of TG from VLDL particles then yields LDL. In fact, almost all of LDL is produced in this manner. LDL has fewer cholesterol esters per lipoprotein molecule compared to VLDL, evidencing a loss of cholesterol during the interconversion from VLDL to LDL. It is hypothesized that one source of loss may the action of LPL action on VLDL (Grundy 1983).

LDL particles are taken up by almost every tissue in the body (Spady et al 1985). Although other mechanisms may exist, there are primarily two by which this plasma LDL clearance takes place; receptor-dependent which comprises as much as 50-80% of the LDL clearance (Kesaniemi et al 1983, Shepherd et al 1979), and receptor-independent which comprises 20-35% of LDL clearance from the plasma (Kesaniemi et al 1983). Some discrepancies exist among literature accounts of the fractional catabolism of LDL, therefore the ranges above exclude these studies. For example, experiments using LDL particles modified with cyclohexanedione as tracers for receptor-independent LDL catabolism have been shown to underestimate receptor-dependent LDL catabolism and have thus been left out of the ranges noted above(Steinbrecher et al 1983). Receptor-dependent LDL catabolism is quantitatively the most important pathway for LDL clearance from plasma. The pathway relies on an LDL receptor on the cell surface that binds to a series of lysine and arginine residues of apolipoprotein B molecules on surface of LDL particles (Slater et al 1984). Upon binding to these particles, the receptor and LDL molecule are endocytosed allowing for the lipoprotein-transported cholesterol to be utilized by the cell. At the time of the discovery of the LDL receptor, it was thought that receptor mediated LDL uptake was the only method of clearance. Although the receptor

pathway does lend the greatest clearance quantitatively, it is now known that a second pathway exists; receptor-independent LDL uptake.

Receptor-independent LDL uptake is a secondary and quantitatively inferior pathway of LDL uptake. Rather than a specific, receptor-dependent mechanism, this pathway involves a non specific, receptor-independent mechanism that functions through bulk-phase pinocytosis, or non specific endocytosis (Goldstein and Brown 1974, Goldstein and Brown 1977). This mechanism is of less importance at low plasma LDL concentrations, but as the receptor mediated pathway can besaturated, the receptor-independent pathways increases in importance and its contribution to plasma LDL clearance increases linearly with plasma LDL concentrations (Goldstein and Brown 1977).

Familial hypercholesterolemia is known to arise from a genetic mutation in the LDL receptor that diminishes the existence of functional receptors by 50% (Goldstein and Brown 1977). In this case, or cases of diet-induced receptor down regulation, the functionality of the receptor-dependent pathway to remove LDL from the plasma is diminished, and the receptor-independent pathway must then function as the primary pathway for LDL uptake from plasma (Spady et al 1987).

Another LDL uptake pathway is suggested to exist that is mechanistically distinct from the two previously mentioned pathways. This LDL uptake is hypothesized to work through a structure known as the Lipoprotein Binding Site (LBS). The LBS has been shown to selectively uptake cholesterol esters from lipoproteins while not engulfing the entire lipoprotein such as occurs with the LDL receptor and bulk-phase pinocytosis (Brissette et al 1996). The LBS is suggested to be a protein and, as its name suggests, it is

not specific for only one lipoprotein. Rather, the LBS binds to all lipoprotein classes, and it does so by interaction with apoprotein component of the lipoprotein (Brissette et al 1996). The rate of selective lipoprotein cholesterol ester uptake by LBS appears to vary by animal model e.g., human < rat, and is present in fewer tissues than other LDL uptake pathways (Green and Pittman 1991). LBS has similar specificity as SR-B1 regarding its ability to also bind HDL (Acton et al 1994). However, no further research has confirmed the presence of this socalled LBS distinct from the better-characterized transport proteins (Truong et al 2000).

The great deal of study given to the topic of LDL cholesterol spurred interest and subsequent exploration into the topic of HDL metabolism. Early epidemiological data showed an inverse relationship between HDL and susceptibility to heart disease (Miller and Miller 1975). Further study also showed that levels of cholesterol present in peripheral tissues were inversely related to the levels of plasma HDL present (Miller et al 1976). HDL has since been implicated as an important particle in cholesterol transport and, in absence, leads to pathology. HDL, as mentioned previously, is synthesized to a small degree in the intestine. Although the mechanism of HDL synthesis in other extra-hepatic tissues is ABCA1 mediated and similar to the intestinal mechanism, a more detailed description of high density lipoprotein particles and of whole body HDL synthesis and transport is given here.

Because atherosclerosis results from a net influx of cholesterol into the intima of blood vessels, and LDL carries cholesterol to these tissues, it follows that a method for removing cholesterol from these tissues and returning it to the liver would be paramount. Because non-steroidogenic and non-hepatic tissues do not have a pathway for converting

excess cholesterol hormones or bile acids, they rely on an alternative method for cholesterol removal; high density lipoproteins (HDL). HDL contains apo A-1 as its primary lipoprotein. It is this protein that is responsible for the lipoprotein-binding of cell receptors and it is also the primary activator of the LCAT enzyme responsible for maturing the HDL particle (Stoffel et al 1983). apo A-1 is synthesized in the liver and the intestine in its pro form, after which it is secreted into the plasma to undergo its final conversion to active, pre-β-1 HDL by the action of a serum, serine protease (Favari et al 2009, Stoffel et al 1983). Pre-β-1 HDL describes a heterogeneous group of molecules that ranges from monomolecular, lipid free apo A-1 to a conglomerate molecule containing phospholipid, free cholesterol and two or three apo A-1 molecules (Favari et al 2009). Evidence exists that this heterogeneity plays a role in determining the destination of these molecules. It has been shown that the small, monomolecular apo A-1, pre-β-1 HDL interacts preferentially with ABCA1 whereas the larger conglomerate pre-β-1 HDL, rather, interacts only with ABCG1 and SR-B1 (Rye and Barter 2004). Other data suggest that there is a pre-β-1 HDL size and lipidation range, where increasing size and lipidation gradually reduce the particle's affinity for ABCA1 (Favari et al 2009).

In support of a lipid transport function of HDL from peripheral tissues back to the liver are data showing a large percentage of pre-β-1 HDL in LCAT deficient plasma, but only very little (about 4%), in normal plasma suggesting that pre-β-1 HDL quickly acquires cellular cholesterol from peripheral tissues (Francone and Fielding 1990). It has also been shown that HDL binding to peripheral tissues such as fibroblasts is increased when tissue cholesterol content in increased. Incubation of fibroblasts with non-

lipoprotein cholesterol increased HDL-high affinity binding several fold (Oram et al 1983).

The maturation of pre-β-1 HDL by acquisition of extra-hepatic tissue cholesterol, and transport of that cholesterol back to the liver is known as reverse cholesterol transport. As pre-β-1 HDL is secreted and facilitates the efflux of cholesterol from tissues via ABCA1, esterification by LCAT takes place whereby free cholesterol in the HDL exterior is joined to a phospholipid fatty acid, creating a non-polar cholesterol ester capable of migrating to the core of the lipoprotein particle, thus allowing for further cholesterol uptake (Glomset 1968). Another mechanism present to increase the cholesterol transport capacity of the plasma HDL fraction is an enzyme-mediated cholesterol transfer by way of Cholesterol Ester Transfer Protein (CETP). The role of CETP is the transfer of cholesterol esters in HDL produced by LCAT to apoB containing lipoproteins, LDL and VLDL, thus providing a method of returning peripheral tissue cholesterol to the liver and making available more transport capacity in HDL. Investigation into the function of CETP (Lagrost et al 1993) has shown that normolipidemic subjects with less variable LDL and VLDL size distributions, suggesting more completely matured apo B lipoproteins, had greater CETP activity. When plasma from these same individuals was incubated at 37^0C in the absence of LCAT activity, CETP activity was inversely correlated with HDL cholesterol. These data support the hypothesis that cholesterol esters from HDL are provided a transport route to apoB lipoproteins and back to the liver by action of CETP. Importantly, this study showed no increase in the cholesterol content of the VLDL+LDL plasma fraction. This may suggest

that, although cholesterol, via CETP, is transported to more atherogenic lipoproteins, this does not pose the risk of accelerated atherosclerosis.

In addition to the HDL-associated cholesterol ester recycling mediated by CETP, other mechanisms exist to return HDL cholesterol to the liver directly. One such mechanism, rather than a transfer to other lipoprotein molecules, involves HDL particle docking with receptors, after which cholesterol esters are selectively removed without degradation of the lipoprotein molecule. SR-B1 was identified as an HDL receptor working through this mechanism and has since been well characterized as a high affinity HDL-particle binding receptor that is highly expressed in the liver and nonplacental steroidogenic tissues (Acton et al 1996). In addition to being an important mechanism for cholesterol clearance from peripheral tissues, SR-B1 may be important for providing cholesterol as a hormone precursor to steroidogenic tissues especially during pregnancy (Landschulz et al 1996).

An additional direct route for HDL-cholesterol ester delivery to the liver involves protein-mediated, lipoprotein uptake whereby the entire molecule is taken into the cell non-selectively through endocytosis. The concept of an endocytotic mechanism of whole HDL particle uptake has been widely explored by a number of researchers returning nearly as many results as studies conducted. HDL binding proteins have been described ranging from 58 kDa (Ferreri and Menon 1990) to 210 kDa (Bond et al 1991) and numerous in between. Speculation suggests that many of the proteins of similar size actually represent the same molecule, but it's likely that there are a number of HDL binding proteins of varying sizes indicating that affinity may vary depending on HDL particles size and extent of lipidation.

Due to its great importance in the transport of cholesterol and its relation to HDL particle metabolism, macrophage cholesterol efflux deserves a measure of attention in the discussion of overall cholesterol metabolism. Macrophages derived from monoocytes function, in part, to collect cellular debris, of which cholesterol is a large component. Macrophages then efflux this cholesterol towards the continuation of reverse cholesterol transport. Aberrantly, however, macrophages in vascular endothelial cells may become engorged with cholesterol, forming foam cells leading the arterial plaques. Cholesterol efflux from these phagocytic cells has therefore become an important topic.

Study into the cholesterol metabolism of the macrophage began as a means of explaining the apparent ability of macrophages to take up cholesterol from lipoproteins and deposit it into tissues without a functioning LDL receptor, as seen in homozygotes for familial hypercholesterolemia. Early data showed a negligible ability of the macrophage to take up native LDL in its unmodified form. Subsequent studies then showed an efficient receptor-mediated uptake of acylated LDL or LDL complexed with dextran sulfate (Basu et al 1979, Goldstein et al 1979). In addition to modified LDL, macrophages have also been shown to uptake intact cholesterol esters from aortic plaques in a manner that is distinct from other modified-LDL receptors, and specific for aortic tissue (Goldstein et al 1981).

Once cholesterol esters are taken up into the macrophage, they require acceptor particles such as HDL in order to be effluxed from peripheral tissues and transported to the liver or to other apoB containing lipoproteins (Werb and Cohn 1972). The macrophage is another important stage for the action of the sterol efflux transporter, ABCA1. As macrophages take up cholesterol, ABCA1 expression is increased as a

protective mechanism to facilitate cholesterol efflux. As cell cholesterol increases the level of efflux, the ABCA1 efflux mechanism begins to fail (Feng and Tabas 2002). With the uptake of cholesterol in absence of sufficient acceptor molecules for its efflux, the formation of foam cells arises, leading the development of atherosclerotic plaques.

Cholesterol Excretion and Conversion

The liver is a central reservoir for cholesterol storage and it receives cholesterol, in addition to its own synthesis, through the many pathways mentioned above; chylomicron remnants, LDL, and HDL. In addition to receptor down regulation of these pathways as a method off regulating cellular cholesterol, the liver possesses other conversion and excretion pathways that are important for maintaining cholesterol homeostasis. Most straight forward is the direct excretion of cholesterol into bile. Working through the same mechanism as cholesterol efflux in the intestine, free cholesterol in the hepatocyte is effluxed through apical transporters ABCG5/G8 of the hepatocyte into the canalicular space for subsequent secretion into the bile duct (Graf et al 2003).The primary regulatory pool of cholesterol that determines the rate of cholesterol efflux into bile is plasma cholesterol, and primarily found in the HDL fraction (Botham and Bravo 1995). Normal human cholesterol excretion into the bile is 800-1400 mg/day (Carr and Jesch 2006). Another cholesterol outlet is that of enzymatic conversion of cholesterol bile acids, and eventual secretion through the feces. This enzymatic conversion is carried out by cholesterol 7α-hydroxylase, also known as Cytochrome P450 7A1 or CYP7A1. Mice with over expression of CYP7A1 were protected from diet-induced hypercholesterolemia, and exhibited increased fecal and bile acid excretion (Li et

al 2011). These data support the role of CYP7A1 in mediating cholesterol conversion to bile acids as a method of maintaining cholesterol homeostasis. While bile acid synthesis from cholesterol is quantitatively the most important conversion, producing around 400 mg/day, other less significant conversions include skins sterols at about 85 mg/day, and steroid hormones at about 50 mg/day (Arias 1988).

III. Mechanisms of Action of Plant Sterols

Plant sterols have been known for decades to lower circulating cholesterol levels upon incorporation into the diet. A number of theoretical mechanisms for how this cholesterol-lowering affect is imparted have been presented. Three primary mechanisms proposed include 1.) competition of plant sterols with cholesterol for an esterification enzyme required for absorption, 2.) co-crystallization of cholesterol and plant sterols forming insoluble sterol complexes, and 3.) competition with cholesterol for incorporation into micelles essential for transporting lipids to the mucosal membrane for absorption.

The first mechanism, esterification-enzyme competition, was based on early studies showing that sitosterol and other plant sterol mixes could serve as substrates for pancreatic cholesterol esterase in the production of long chain esters of fatty acids produced in the intestinal lumen (Swell et al 1956). Because this enzyme was also shown to act on cholesterol in the same manner (Swell et al 1950), it was further proposed that plant sterol metabolism occurs in the same manner as cholesterol metabolism, and that this common metabolism may suggest a competitive mechanism. Furthermore, the

finding that esterification of sterols did indeed occur in the intestine strengthened the hypothesis that esterification was necessary for absorption (Siperstein et al 1952).

Work exploring the differential absorption of sterols from esters compared to free sterols, however, showed no difference between the two, giving no support to the above hypothesis (Best and Duncan 1958). In addition, the demonstration of the existence of a cholesterol esterase in the intestinal mucosa, and evidence suggesting that esters are first hydrolyzed and then absorbed, further countered the enzyme competition hypothesis (Best and Duncan 1958, Swell et al 1950). Finally, it was reported that esterified sterols in the mucosa were sporadic and only present in trace amounts, although esterified sterols in the lymph exists at about 70%, further arguing for a post-luminal esterification of sterols (Glover et al 1959), and calling for an alternative hypothesis to explain the cholesterol-lowering effects of plant sterols.

A second hypothesis is that of a co-crystallization phenomenon whereby cholesterol and plant sterols form complexes that are unable to be solubilized in the oily or aqueous phases in the intestinal lumen. Free plant sterols and cholesterol are soluble in TG oils at a level of about 3% (Glover et al 1959, Jandacek et al 1977, Vaikousi et al 2007), and are negligibly soluble in water (Trautwein et al 2003). As lipid digestion progresses and the polar nature of the intestinal contents in increases due to lipolysis, there is less oily phase present for sterol solubilization, and thus co-crystallization of cholesterol and plant sterols is encouraged (Trautwein et al 2003). As plant sterols are increasingly incorporated into the diet, cholesterol is further outcompeted leading to increased crystallization and lower cholesterol absorption. The possibility for the existence of this mechanism has been demonstrated in a number of in-vitro environments;

co-crystallization of cholesterol and beta sitosterol in methanol has been demonstrated (Davis 1955), and similar findings were shown in ethanol over a broad range of sterol ratios. The question regarding this mechanism is not if it is possible, but rather does it happen to a significant degree so as to contribute to the overall cholesterol lowing effects of plant sterols. Recent work to answer this question explored the relative interactions of sitosterol and sitostanol with cholesterol in a model digestive system (Mel'nikov et al 2004). Because sitostanol and sitosterol have been shown to reduce cholesterol absorption equivalently, either the two sterols co-crystallize with cholesterol to the same degree or it must be assumed that the co-crystallization mechanism does not contribute in any great degree to cholesterol-lowering seen by plant sterols. This recent work demonstrated significant differences between cholesterol-sitosterol interactions versus cholesterol-sitostanol interactions, suggesting that co-crystallization is not a significant contributor to the mechanism of action of plant sterols.

A third cholesterol-lowering mechanism of greater quantitative importance than the one described above is that of competition for solubilization into dietary mixed micelles (DMM). Bile salts are known to solubilize large quantities of lipid in solution (Hofmann and Borgstroem 1964); a phenomenon attributed to their detergent properties that allow them to aggregate and micelle structures. These micelle structures then function to encase hydrophobic compounds in their lipophilic core. Early studies into cholesterol absorption showed equivalent amounts of cholesterol existing in the oily phase and aqueous phases of intestinal contents. Visible differences in the phases suggested that the aqueous phase was micellar due to its clear nature, further suggesting that lipid incorporation into micelles was limited, as not all cholesterol could be found in

the aqueous layer (Borgstrom 1960, Ponz de Leon et al 1981). It has been further supported that lipid absorption into enterocytes takes place from these micelle structures, and that lipid incorporation into micelles is limited by the amount of bile salt present, above which level lipid is emulsified in the oil phase (Hofmann and Borgstroem 1964). Given these conclusions and the current knowledge of the greater micelle affinity of plant sterols relative to cholesterol (Matsuoka et al 2008), a competitive mechanism for plant sterol mediated cholesterol-lowering seems most likely.

IV. Cholesterol Lowering Properties of Plant Sterols

Efficacy of Plant Sterol versus Plant Stanol

Plant sterols and plant stanols are steroid molecules with very similar structures to cholesterol. They are also very similar in relation to each other with the exception of the lack of a double bond between the fifth and sixth carbons in the steroid ring of the plant stanol. The difference in the structures between the two classes of molecules requires investigation into the possibilities that they may impart differential cholesterol-lowering effects.

The seemingly small difference between plant sterols and their hydrogenated counterpart has been shown to yield notable differences in their functions as bioactive substances. Plant stanols are negligibly absorbed, while plant sterols are absorbed as much as 4-10%. It has been suggested, although not shown conclusively, that the extent of hydrogenation of a mix of plant sterols is a factor in the cholesterol lowering efficacy of the plant sterols (Pritchard et al 2003).

Heinemann, et al compared the ability of sitosterol versus sitostanol to alter cholesterol absorption efficiency and found a greater ability of the stanol (Heinemann et al 1991). This difference, a reduction by 50% in sterol compared with an 85% reduction with the stanols, was attributed to the higher hydrophobicity exhibited by the stanol variety. Alternatively, comparison of the micellar solubilities of sitosterol compared to sitostanol showed now differences (Ikeda et al 1989). In addition, no differences were detected between the liver cholesterol of rats fed sterol or stanol, although there was a significantly elevated fecal cholesterol excretion seen with the stanol fed rats (Sugano et al 1977). An eight week feeding of plant sterols and plant stanols to apo E-deficient mice resulted in a greater reduction in plasma cholesterol with sterols. At week 14, however, this difference was attenuated and there were no differences between either group in plasma cholesterol levels or aortic lesion area (Pritchard et al 2003). In a study of the differential cholesterol lowering effects of plant sterols and stanols in a free living, hypercholesterolemic group of 14 people, both varieties were shown to be equally capable of lowering plasma LDL cholesterol, although the sterol variety more efficiently blocked cholesterol absorption, reducing cholesterol absorption efficiency by 56% compared to only 34% by stanols (Vanstone et al 2002). Additionally, a study of 34 hypercholesterolemic individuals in which plant sterol esters and stanol esters were consumed in a margarine vehicle at 2.01-2.04 g/day for 14 days as part of a low fat diet showed no differences between sterol and stanol cholesterol-lowering efficacy (Hallikainen et al 2000). Plant sterol and stanol treatments lowered LDL cholesterol by 12.7% and10% respectively. Interestingly, in the sterol ester group, plasma sitosterol and

campesterol increased by 0.83 and 2.77 mg/L respectively, while the stanol ester group only increased sitostanol and campestanol by 0.11 and 0.19 mg/L respectively.

A two month study (O'Neill et al 2005) explored the relative effectiveness of plant sterol esters versus plant stanol esters in familial hypercholesterolemic patients on lipid lowering medication and normal individuals with even ratios of normal to hypercholesterolemic in each treatment group. Subjects were randomized to three groups; sterol ester (1.6 g/day), stanol ester (1.6 g/day) and stanol ester (2.6 g/day). All groups were equal in their ability to lower LDL cholesterol up to two months; however, after two months, the sterol group's cholesterol-lowering effect was attenuated and no longer differed from baseline levels. In addition, a 27% decrease in bile acid synthesis from cholesterol, as measured by serum 7α-hydroxy-4-cholesten-3-one, a marker of bile acid synthesis, was seen after two months, suggesting a short term effectiveness of plant sterol ester compared to plant stanol ester.

In contrast, a study conducted with 15 hypercholesterolemic subjects using plant sterol or plant stanol esters showed a greater LDL-cholesterol lowering efficiency with sterols above stanols; -12.9 and -7.9 respectively (Jones et al 2000). Plant sterols lowered total cholesterol absorption by 13.4% compared with only 10.2% with plant stanols, and sterols lowered cholesterol absorption to a greater degree than stanols; -36.2% and -25.9% respectively. This could be evidence that plant sterols are more effective than plant stanols in lowering cholesterol at least in the short term. However, given the previous study, an extension of this study beyond 2 months may fail to maintain a significant difference due to the alleged inability of plant sterols to continue to elevate the conversion of cholesterol to bile acids.

Another long-term study of sterol versus stanol showed a difference in cholesterol-lowering ability, albeit the opposite of the previously mentioned study (de Jong et al 2008). A group of 37 hypercholesterolemic subjects on stable statin use were given a placebo or and 2.5 g/day of plant sterol or stanol ester in a margarine vehicle for 85 weeks. The plant sterol group showed an 8.7 % reduction in LDL-cholesterol, while the plant stanol group showed a superior reduction of 13.1%. In a 16 week study, three groups of 15 participants consumed 2.6 g/day of either a control margarine, a plant sterol ester-enriched margarine or a plant stanol ester-enriched margarine (Jong 2008). Plasma LDL cholesterol in both the stanol and sterol group were significantly lower than control, but did not differ from each other. A recent study (Weingartner et al 2011) administering plant sterol and stanol esters at 2% of diet by weight to apo E-deficient mice on a high cholesterol (1.25%) diet has shown lower plasma cholesterol levels in sterol ester mice compared with stanol ester fed mice. Liver cholesterol, however, was no different between the groups, and sterol ester mice showed elevated inflammatory nature of macrophages. In addition, stanol ester mice responded with reduced superoxide release and lipid hydroperoxides compared to other diets. Also compared with sterol ester group and control, stanol ester group reduced IL-6 expression in aortic tissue and TNF-alpha expression in plasma compared to other diets. In opposition to other studies, however, stanol ester treatment increased plasma plant stanol concentrations by 48 fold while plant sterol treatment only increased plasma plant sterol concentrations by 28 fold.

A meta-analysis examined randomized, controlled trials that compared plant sterols and plant stanols for their lipid altering effects (Talati et al). Of 146 abstracts, 14 were considered in the analysis for differences in total cholesterol, LDL cholesterol, HDL

cholesterol and triglycerides compared between groups treated with plant sterols and groups treated with plant stanols. Statistical analysis showed no significant differences in any of these parameters between plant sterol or stanol treatment. To further complicate the issue, another meta-analysis of 113 placebo-controlled, randomized studies considering the LDL cholesterol lowering abilities of free plant sterol, plant sterol esters, free plant stanols and plant stanol esters was recently published (Musa-Veloso et al 2011). The maximal cholesterol lowering effects of free plant stanols and plant stanol esters, 16.4% and 17.1% respectively, were shown to be significantly greater than that of free plant sterol and plant sterol ester, 8.3% and 8.4% respectively.

In summary, a great deal of research has explored the differences between plant sterol and stanol treatments and has shown, at times, conflicting results. Some studies point to a greater ability of plant sterols over stanols to lower cholesterol absorption, while others have shown a lower inflammatory response to stanols compared to sterols. Meta-analysis has also returned less than consistent results as well. One meta-analysis shows no differences between cholesterol-lowering efficacy, while the other reports showed differences favoring stanol. Those who support the equivalency of the two varieties note the importance of choosing between the two based on safety and economy.

Plant Sterols in Different Food Matrices

With the esterification of free sterols to form sterol esters of fatty acids, it became possible to solubilize plant sterols to a greater degree in a wider variety of food vehicles. It then became important to determine if the cholesterol-lowering effects of plant sterols could be influenced by the type of food in which they are delivered, e.g. high-fat versus

low-fat. While a large number of studies have determined that plant sterols and plant sterol esters are capable of lowering plasma cholesterol in a number of food vehicles, only more recently has there been any study to compare the efficacy of plant sterols between those food vehicles. In a study conducted with 58 free-living men and women, sterol esters were incorporated into bread, yoghurt, milk and breakfast cereal, and fed at a dose of 1.6 g/day of sterol ester for three weeks (Clifton et al 2004). Plant sterol ester in milk proved to be most effective at lowering cholesterol above all other matrices; total cholesterol was lowered by 9.7% and LDL cholesterol by 15.9%. Yoghurt, while not as efficient as milk, was more efficient than both bread and cereal, lowering total cholesterol by 5.6% and LDL cholesterol by 8.6% in comparison to bread (LDL cholesterol -6.5%) and cereal (LDL cholesterol -5.4%). There were no significant differences between the abilities of bread and cereal to lower total or LDL cholesterol. This was perhaps the first evidence that the food matrix in which the plant sterol ester is delivered may have an effect on its cholesterol-lowering properties.

Another study, however, has shown opposite results, in which 39 men and women with moderate hypercholesterolemia consumed 2 g/day plant sterol ester in milk, a spread or a combination of the two (Noakes et al 2005). After three weeks, all groups showed lower total and LDL cholesterol levels compared to control, although there were no differences between the different treatments' abilities to lower cholesterol.

A meta-analysis of 59 studies with over 4500 participants, analyzed as a component of the study, the differential effects of the food carriers in which plant sterol esters were delivered (AbuMweis et al 2006). The food products used for sterol ester incorporation were placed into categories based on their fat content, creating four

categories; fat spreads, mayonnaise and salad dressings, milk and yoghurt, and other foods. LDL cholesterol-lowering achieved through consumption of fat spreads, mayonnaise and salad dressings, milk and yoghurt were 0.33, .32 and 0.34 mmol/L respectively. These groups were all similarly and significantly more effective at lowering LDL compared with the other foods group which only lowered LDL by 0.2mmol/L. These results confirm that there may indeed be a significant difference in effectiveness of plant sterol in different food vehicles, and supports the findings s of Clifton et al (2004) who also showed also that milk and yoghurt were more effective carrier foods than other groups such as bread or breakfast cereal.

Plant Sterols and Background Diet

Another important factor to consider in understanding the effectiveness of plant sterols to lower cholesterol is the background diet. A number of studies have undertaken the task of determining the affects of high and low fat and cholesterol intake on the function of plant sterols. A study conducted in a population of 33 moderately hypercholesterolemic men consuming a low cholesterol, low fat diet tested the dietary cholesterol dependence of plant sterols to affect cholesterol-lowering (Denke 1995). Participants adhered to the American Heart Association, Step 1 diet (Ginsberg et al 1990) consisting of 30% fat of which 10% saturated, and less than 200 mg/day cholesterol for 30-days followed by a 30-day wash out period and then a 30-day period of the same diet with the addition of 3 g/day sitostanol followed again by an additional diet only 30-day period. At the end of the study, there were no differences between total cholesterol or LDL cholesterol between any of the treatment periods. Researchers proposed that a low-

fat, low-cholesterol diet may attenuate the cholesterol-lowering properties of sitostanol, and suggested that background diet may have a significant effect on plant sterol action.

In opposition to this study, another plant sterol trial was conducted again in subjects adhering to the Step 1 diet and receiving plant sterol esters in a low-fat spread margarine (Maki et al 2001). A total of 219 mild to moderate hypercholesterolemic subjects were divided among three treatment groups; a 50% fat margarine group with no plant sterol, a 50% fat margarine group delivering 1.1 g/day plant sterol ester and a 50% fat margarine group delivering 2.2 g/day plant sterol ester. Subjects consumed a control spread and adhered to the Step 1 diet for a four week lead in period followed by a five week intervention period where treatment groups received their sterol ester enriched spreads. At the end of five weeks, groups receiving 1.1 g/d and 2.2 g/d plant sterol ester showed total cholesterol reductions of 5.2% and 6.6% lower and LDL-cholesterol reductions of 7.6% and 8.1%, respectively. While previous reports suggest that a low fat, low cholesterol diet may attenuate plant sterol effects, Maki et al showed that plant sterol esters may still be able to reduce cholesterol levels in the presence of a low-fat, low-cholesterol diet.

A more recent study supports the findings of Maki et al, where 84 mildly hypercholesterolemic subjects were placed into one of three groups; a healthy diet group with plant sterol ester-free milk, a healthy diet group with plant sterol ester-enriched milk or a free diet group with plant sterol ester-enriched milk (Hernandez-Mijares et al 2010). Sterol ester-enriched milk delivered 2 g/day of plant sterol ester. After the intervention period of three months, the healthy diet group and free diet group treated with plant sterols showed similar total cholesterol lowering of 6.7% and 5.5% and LDL-cholesterol

lowering of 9.6% and 7%, respectively. These changes in the plant sterol ester groups were different from healthy diet alone and baseline, but didn't differ from each other, suggesting that plant sterol ester treatment works effectively to lower total and LDL cholesterol despite the background diet.

Plant Sterol Dose

Plant sterols and their esters have been studied at varying dosages, and have produced varying results as well concerning cholesterol absorption and lowering. Large plant sterol doses are highly effective, but are unpleasant due to a chalky taste and texture and may be uneconomical. In the interest of usability, economy and safety, it is important to determine the lowest effective does.

The ability of a range of plant sterol doses in their free form were explored to lower total and LDL cholesterol (Hendriks et al 1999). Using 0.83, 1.61, 3.24 g/d of free sterols in spreads, total cholesterol reduction for the doses was 4.9%, 5.9%, 6.8%. LDL cholesterol dropped by 4.9%, 5.9%, 6.8% for their respective doses. Each dose was equally effective as no statistical differences were found. 0.8 g/day of free sterol in a margarine vehicle was shown to reduce total cholesterol by 3.8% and LDL-cholesterol by 6% (Sierksma et al 1999). 13.4% reduction of total cholesterol and 12.9% reduction of LDL-cholesterol resulted from consumption of 1.84 g/day of plant sterol ester (about 1.1 g/day free sterol equivalent) (Jones et al 2000). In a study exploring the abilities of varying free plant sterol doses to lower cholesterol, 1.5 g/day and 3 g/day lowered total by 8.9% and 11.3% respectively, and LDL-cholesterol by 8.3% and 10.6% respectively. There were no significant differences between the two dosages (Christiansen et al 2001).

A study exploring the relative abilities of free plant sterols, free plant stanols or a mix of the two to lower total and LDL cholesterol showed a similar ability across all groups. Total cholesterol lowering for sterol, stanol and the 50/50 mix was 7.8%, 11.9% and 13.1% respectively, and LDL-cholesterol lowering was 11.3%, 13.4% and 16% respectively (Vanstone et al 2002). These previously mentioned data seem to show an optimal range of plant sterol intake between 1-2 g/day which is in accordance with the results and conclusions of a meta-analysis showing that about 2 g/day results in a 10% reduction in LDL cholesterol, but that doses higher than 2 g/day contribute very little additional benefit (Katan et al 2003). In addition, it has been suggested that 1 g/day may serve as a minimum requirement to achieve significant cholesterol reduction (Nguyen 1999).

Plant Sterol Serving Frequency

The topic of serving frequency in plant sterol administration was explored through a study that directly compared the cholesterol metabolism-altering effects of a single plant sterol dose with multiple smaller plant sterol doses (AbuMweis et al 2009). In a study conducted in adult men and women with moderate hypercholesterolemia, 19 subjects participated in three trial periods with different plant sterol treatments in a crossover study with 2 week washout periods. The periods consisted of a control margarine with no plant sterol, a plant sterol margarine dose in the morning containing 1.8 g and then one control margarine dose at lunch and one at dinner, or 1.8 g of plant sterol in three even doses at breakfast, lunch and dinner. The morning dose proved to be ineffective at lowering LDL-cholesterol levels while the three times daily treatment

lowered cholesterol significantly by 6%. These results were in line with a previous study conducted by this same group showing that a single morning dose of plant sterol was insufficient to achieve cholesterol-lowering (AbuMweis et al 2006). A meta analysis exploring the differential effects of multiple doses of plant sterol compared with only one dose of plant sterol showed a "more pronounced" LDL-cholesterol lowering effect with the multiple doses, although this differences did not reach statistical significance (p=0.054) (Demonty et al 2009). Although some research suggests multiple doses are superior for inducing cholesterol-lowering, more research is needed to confirm.

Plant Sterol Atherogenicity

Sitosterolemia is characterized by elevated plasma plant sterol concentrations, and is accompanied by premature CHD. This phenomenon has brought about controversy as to whether or not plant sterols may promote atherosclerosis and heart disease. To explore this topic, chow diets and western type diets were fed to female mice with inactivated ABCG5 and ABCG8 as a model of elevated plasma plant sterols. Mice had 20-fold increases in plasma plant sterols above normal, yet after 7 months of feeding showed no differences in aortic lesion area compared to wild type mice (Wilund et al 2004). In this same study, coronary calcium, a predictive measure of CHD in various major ethnicities, was assayed in over 2500 subjects (Detrano et al 2008). While cholesterol levels were elevated in subjects with elevated coronary calcium plant sterols showed no relation. Alternatively, large epidemiological studies have shown a relationship between the plasma plant sterol concentrations and negative cardiac events. The PROCAM-study

showed increased plasma plant sterol concentrations in subjects who experienced myocardial infarction or sudden cardiac death (Assmann et al 2006).

MATERIALS AND METHODS

Animals and Diets

Sixty male Syrian hamsters (BioBreeders, Watertown, MA) weighing 73-87g were divided into six experimental groups of 10. All hamsters were housed in polycarbonate cages with sawdust bedding, and were given free access to food for 29 days. They were maintained in a humidity, temperature controlled room at 25°C with a 12-hour light dark cycle. During the first four days, all animals were given a control diet for acclimation, after which treatment diets were administered for the duration of the study. The control diet was an AIN-93M, semi-purified diet consisting of 0.12% cholesterol (g/g), and 8% coconut oil (g/g) (Reeves et al 1993). Each treatment diet consisted of the control diet with replacement of cornstarch with an equal mass of plant sterol ester (5% g/g), ether (5% g/g) or free sterol (3% g/g) plus triglyceride (2%g/g) as follows: Palmitate Ester (PEs), Oleate Ester (OEs), Stearate Ester (SEs), Stearate Ether (SEt), and free sterol + high oleic sunflower oil (FS). Diet mixtures were produced manually in our laboratory by mixing ingredients in a least-to-greatest fashion to ensure a homogeneous mixture. After sufficient mixing, the powered diet was mixed with 15% of its weight in water and formed into approximately ¾ inch sheets using clean, plastic trays as a mold. The diets were manually compressed, scored with a razor blade into approximately 2x2 inch squares and placed into a drying oven until they returned to their

original dry weight. Each of the six treatment diets was broken into squares and stored in gallon-sized zip-lock bags at -80 degrees. The AIN-93 mineral and vitamin mixes, casein, dextrinized cornstarch, guar gum and solka flock and coconut oil were all purchased from Dyets, Inc. (Bethlehem, PA). Choline bitartrate, L-cystine, and cholesterol were purchases from Sigma Chemicals (St. Louis, MO). Cornstarch, sucrose, and soybean oil were purchased from a local grocery store. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Nebraska.

Feed consumption was tracked biweekly and body weight was recorded on a weekly basis. Hamsters were transferred to cages with new bedding each week, and the used bedding was discarded with the exception of the cage change on day 21 during which the bedding was collected into individual bags for each animal for subsequent collection of fecal pellets. On this same day and the next, the first and second doses of radiolabeled sterol were delivered to the hamsters orally, and the bedding was individually bagged on day 26 for recovery of radioactive pellets.

On day 29, hamsters were anesthetized by CO2 gas and the thoracic cavity was opened to allow for removal of blood by cardiac puncture using 10mL syringes. Blood was then placed into 10mg EDTA tubes and centrifuged to separate the plasma and red blood cells. Plasma was then placed into cryo-tubes and frozen at -80 until analysis. Bile was removed from gallbladders by aspiration with pre-weighed, 1mL insulin syringes, diluted with saline and transferred to micro centrifuge tubes for immediate freezing in dry ice. Livers were excised, weighed and quickly frozen in dry ice until being stored at -80 to await analysis.

Table 1. Diet Composition

	Athero Control	Palmitate Ester	Stearate Ester	Oleate Ester	Stearate Ether	Free Sterol
	g/kg	g/kg	g/kg	g/kg	g/kg	g/kg
Cornstarch	404.5	354.5	354.5	354.5	354.5	354.5
Dextrinized cornstarch	155.0	155.0	155.0	155.0	155.0	155.0
Casein	140.0	140.0	140.0	140.0	140.0	140.0
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0
Coconut oil	80.0	80.0	80.0	80.0	80.0	80.0
Soybean oil	20.0	20.0	20.0	20.0	20.0	20.0
Sterol Palmitate Ester		50.0				
Sterol Stearate Ester			50.0			
Sterol Oleate Ester				50.0		
Sterol Stearate Ether					50.0	
Free Plant Sterol						30.0
High Oleic Sunflower Oil						20.0
Insoluble fiber (Solka-Floc cellulose)	40.0	40.0	40.0	40.0	40.0	40.0
Soluble fiber (Guar gum)	10.0	10.0	10.0	10.0	10.0	10.0
Cholesterol	1.2	1.2	1.2	1.2	1.2	1.2
AIN-93 mineral mix (70% sucrose)	35.0	35.0	35.0	35.0	35.0	35.0
AIN-93 vitamin mix (98% sucrose)	10.0	10.0	10.0	10.0	10.0	10.0
L-Cystine	1.8	1.8	1.8	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
	g/kg	g/kg	g/kg	g/kg	g/kg	g/kg
Carbohydrate	693.8	643.8	643.8	643.8	643.8	643.8
Protein	140.0	140.0	140.0	140.0	140.0	140.0
Fat	100.0	100.0	100.0	100.0	100.0	100.0
	kcal/kg	kcal/kg	kcal/kg	kcal/kg	kcal/kg	kcal/kg
Carbohydrate	2775.2	2575.2	2575.2	2575.2	2575.2	2575.2
Protein	560.0	560.0	560.0	560.0	560.0	560.0
Fat	900.0	900.0	900.0	900.0	900.0	900.0
Total	4235.2	4035.2	4035.2	4035.2	4035.2	4035.2
	%energy	%energy	%energy	%energy	%energy	%energy
Carbohydrate	65.53	63.82	63.82	63.82	63.82	63.82
Protein	13.22	13.88	13.88	13.88	13.88	13.88
Fat	21.25	22.30	22.30	22.30	22.30	22.30

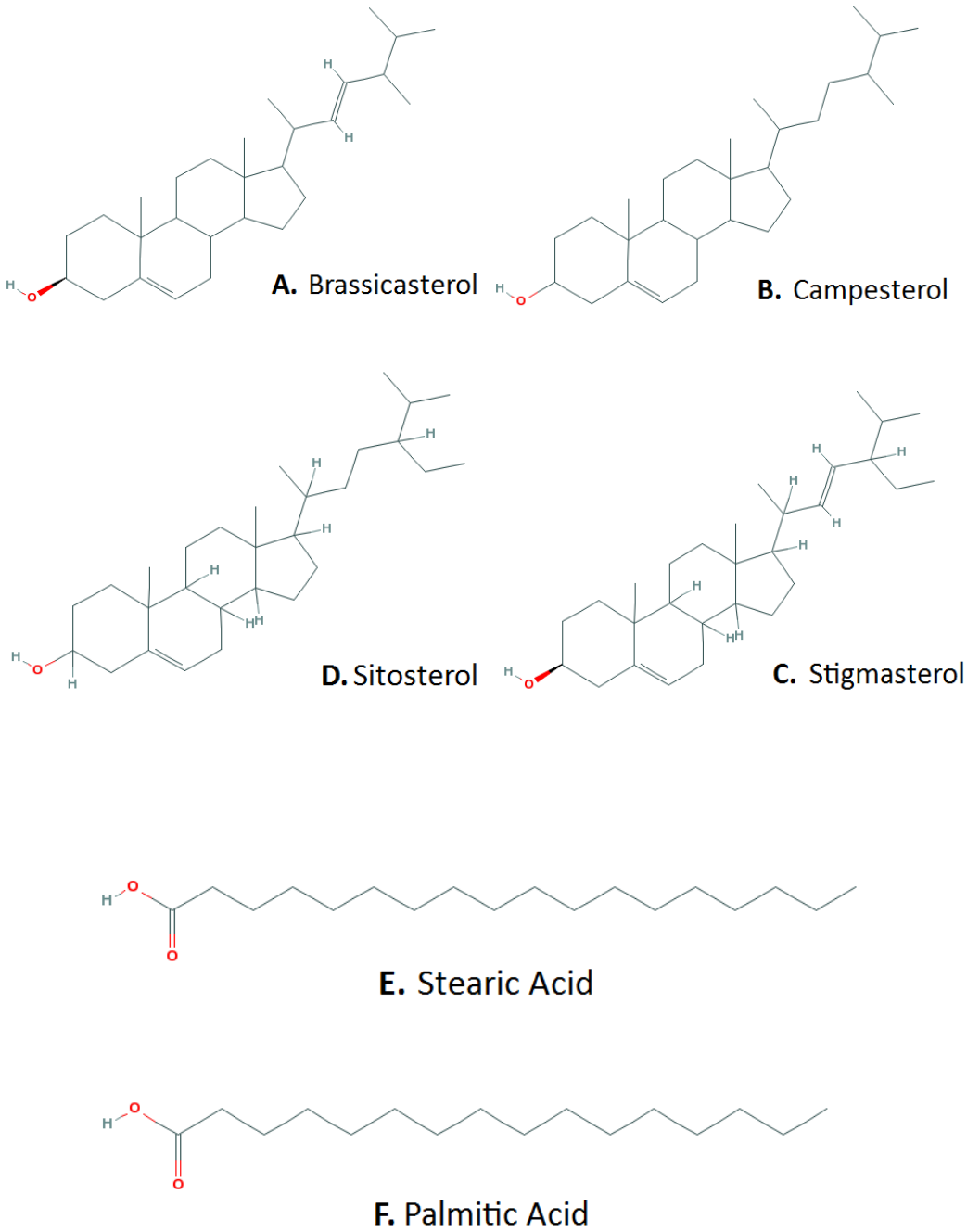
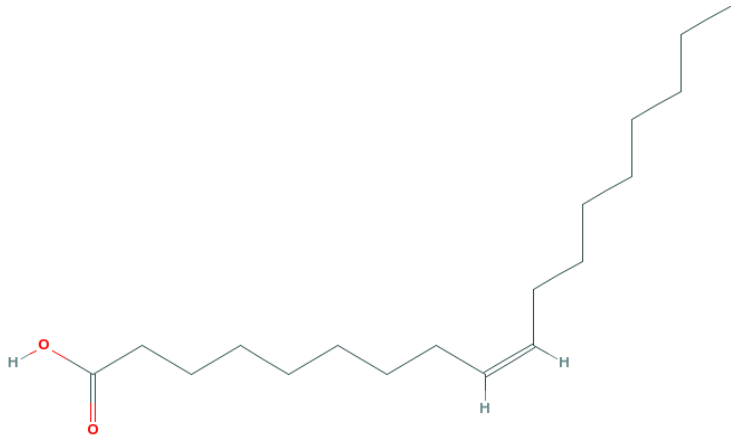
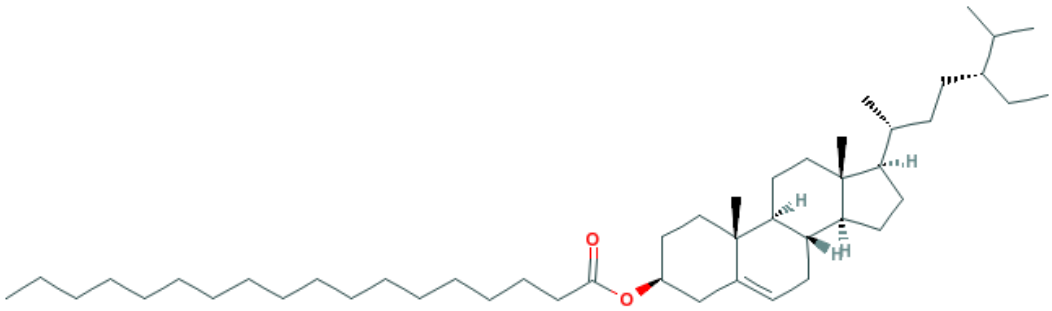


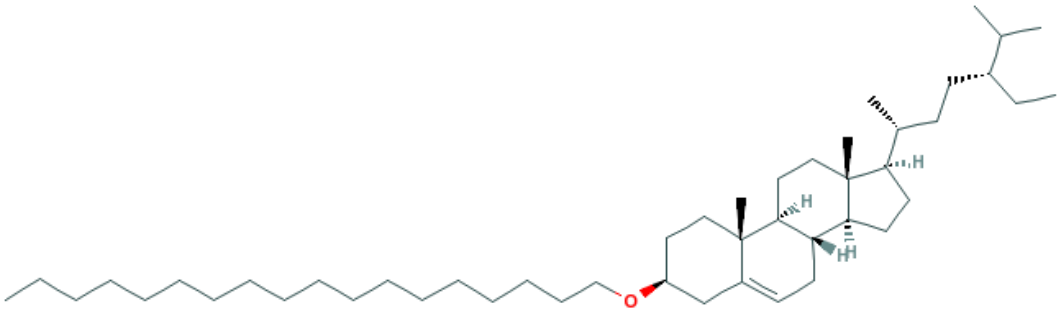
Figure 1A-F. Plant Sterol and Fatty Acid structures.



G. Oleic Acid



H. Sitosterol Stearate Ester



I. Sitosterol Stearate Ether

Figure 1G-I. Plant Sterol and Fatty Acid structures.

Sterol Ester and Ether Synthesis

The treatment group compounds consisted of the Palmitic, Oleic and Stearic acid esters of mixed soybean sterols, along with the octadecyl ether derivative of the mixed soy sterols. The esters and ether were synthesized by Mr. Jiliang Hang in the laboratory of Dr. Patrick Dussault (UNL Chemistry).

Esters were synthesized by first converting the free fatty acids into the corresonding acid chlorides by reaction with oxalyl chloride in benzene in the presence of *N,N*-dimethyl formamide (DMF) as a catalyst. Following removal of solvent under vacuum, the fatty acid chloride was slowly added to a solution of the free plant sterols and pyridine in ethanol-free chloroform. The reaction was stirred overnight after which the solution was washed with dilute aqueous HCl to remove pyridine. The organic layer was concentrated and the resulting solid was recrystallized from hot ethanol. The sterol esters were collected by vacuum filtration and analyzed for purity by thin-layer chromatography, as well as by proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR).

The octadecyl ether was prepared by a different procedure. An excess of sodium hydride (60% suspension in oil) was washed with a small volume of hexane under an atmosphere of nitrogen. The resulting pyrophoric powder was maintained at all times under an atmosphere of nitrogen. To a suspension of the sodium hydride in THF was cautiously added a solution of sterol in THF, followed by iodooctadecane. The reaction afforded a mixture of the desired octadecyl ether(s), octadecene as a byproduct of

elimination, and recovered sterol. The octadecyl ether was purified by multiple recrystallizations from ethyl acetate, and analyzed for purity as described for the esters.

Cholesterol Absorption by Fecal Dual-Isotope Method

Cholesterol absorption efficiency was measured by a dual isotope method described by Turley (Turley et al 1994) (Figure 2) whereby [^{3}H]-sitostanol and [^{14}C]-cholesterol were orally administered simultaneously. Because sitostanol is essentially unabsorbed in the intestine, it serves as an absorption reference. Radiolabeled cholesterol and sitostanol were purchased from American Radiolabeled Chemicals, St. Louis, MO. Radiolabeled sterols were dissolved in vegetable oil and given in a 50µl dose on two consecutive days. Each dose delivered approximately 1µCi [^{14}C]-cholesterol 2µCi [^{3}H]-sitostanol. Upon dosing, hamsters were placed into cages with new bedding, and this bedding was collected three days later for separation of radioactive pellets. In addition, five days after dosing, bedding was collected again to ensure complete containment of radioactivity.

The total collected mass of radiolabeled feces (2-4.5g) was placed into 20 x 150 glass tubes. 9mL of 100% ethanol was added to each tube, after which, 1mL of 50% KOH in water was added and the samples were capped and vortexed. The lipids were extracted and saponified over the course of two days in a 50^{0}C water bath with frequent vortexing. Samples were removed from the water bath and allowed to return to room temperature, after which 3mL of deionized water was added and the samples were vortexed. Nonsaponifiables were extracted by the addition of 7mL of hexane. Samples

were shaken vigorously, and the phases were separated by brief centrifugation at 1000 x g. The upper hexane layer was removed and placed into clean, 20 mL scintillation vials where it was allowed to dry completely under ultra violet light for one and a half weeks to remove pigmentation that could interfere with scintillation counting. Once the pigmentation was sufficiently diminished, 8 mL of scintillation cocktail, Bio-Safe II (Research Products International), was added and the samples were capped. In addition, three aliquots of the oil dose were placed into 20 mL scintillation vials and filled with 8mL of cocktail for quantification. Sample disintegrations per minutes were measured on a Packard 1900 TR liquid scintillation counter using a dual channel for counting [^{14}C] and [^{3}H]. The following equation was used to calculated cholesterol absorption efficiency using the ratio of two isotopes in the dose and the fecal samples to arrive at a percentage.

$$\frac{[^{14}C]/[^{3}H] \text{ dosing mixture} - [^{14}C]/[^{3}H]}{[^{14}C]/[^{3}H] \text{ dosing mixture}} \times 100$$

Figure 2. Dual Isotope Formula

Plasma Lipids

Total plasma cholesterol was quantified using a 96-well plate, reagent based, colorimetric assay as previously described (Carr et al 1993). Total plasma cholesterol was measured using a total cholesterol reagent (Roche Diagnostics, Indianapolis, IN). Apolipoprotein-B containing lipoproteins were precipitated from plasma by a 1:1 (v/v) addition of an Apo-B precipitating agent (Thermo Electron Corp., Melbourne, Australia).

After addition of the precipitating reagent, samples were centrifuged at 1000 x g for 10 minutes, and the supernatant was collected for analysis of the HDL Cholesterol fraction using the total cholesterol reagent. Non-HDL cholesterol was then determined by subtraction of HDL cholesterol from total cholesterol.

Liver Lipids

About 0.2g of frozen liver was placed into tarred tubes and minced. According to the Folch extraction method (Folch et al 1957), 5 mL Chloroform/Methanol (2:1, v/v) was added, and each sample was capped and allowed to sit overnight for extraction of lipids. After extraction, samples were poured through Whatman #41 filter paper into 15 mL graduated, glass conical tubes. Each extraction tube and filter paper was washed into the graduated tubes about three times with chloroform to ensure quantitative recovery, and to bring the final volume to 10 mL. Next 2 mL of 0.88% KCL was added, and tubes were capped and gently inverted several times, and phases were separated by centrifugation. The upper phase was aspirated and discarded, and the lower phase placed into a new tube. To solubilize lipids in water for reagent based, colorimetric assay, 10% Triton X-100 in cholorform (v/v) was added to 0.25 mL aliquots of liver extract upper phase. Chloroform was dried down under nitrogen and lipids were brought back up in 0.96mL of deionized water for a total sample volume of 1ml. Total cholesterol and triglyceride reagents were obtained from Roche Diagnostics (Indianapolis, IN), and free cholesterol and phospholipids reagents were purchased from Wako Chemicals

(Richmond, VA). Lower phase aliquots of 100 µL were analyzed in duplicate. Esterified cholesterol was calculated from the difference between total and free cholesterol values.

Fecal Bile Acids

Approximately 200mg of feces was weighed and the exact mass recorded. Total bile acids were extracted from the feces into 10mL of 2:1 (v/v) chloroform/methanol using the methods (Folch et al 1957). Samples were flushed with nitrogen and allowed to sit overnight at room temperature for extraction. 2 mL of 0.88% KCL was then added, and the samples were gently inverted three or four times, and centrifuged at 1000 x g for 10 minutes to separate phases. The upper phase was removed and placed into a graduated conical tube, and the lower phase was washed with chloroform/methanol/water (3:48:47, v/v), inverted several times to mix, and centrifuged at 1000 x g for 10 minutes. The upper phase was then added to the previously removed upper phase. The total upper phase volume was then quantified and recorded. Quantification of bile acids was achieved by addition of the enzyme 3-α Hydroxysteroid Dehydrogenase (Sheltawy and Losowsky 1975). 5mL aliquots of extraction upper phase were evaporated under nitrogen at 60^0C in standardized glass cuvets. Bile acids were resolubilized in 100 µL of methanol. 3.5mL of β-NAD in CAPS buffer (0.2 mg/mL; pH 10.8) was added to each tube, and a background absorbance reading was taken at 340nm. 0.4mL of 3-α Hydroxysteroid Dehydrogenase (0.75 units/mL of 0.01 M phosphate buffer, pH 7.2) was added to each sample and incubated at 37^0C for 30 minutes, after which the absorbance was measured again at 340 nm. Total bile acid concentration was calculated using the difference between the pre-

enzyme absorbance readings and the post-enzyme absorbance readings after correcting for the enzyme volume. Molar concentrations were derived from the absorbance differences using a cholic acid standard curve diluted to 0.1-0.5 mmol/assay. β-NAD, CAPS buffer, cholic acid, and 3-α hydroxysteroid dehydrogenase enzyme were purchases from Sigma-Aldrich (St. Louis, MO).

Free Neutral Sterols

Feces (50-60 mg) was weighed and the exact mass recorded, and 0.2 mL of 0.5 M HCL was added to acidify and optimize the extraction. Total fecal lipids were extracted from the feces into 5 mL of 2:1 (v/v) chloroform/methanol with 20 µg/mL of 5-α cholestane (Folch et al 1957). Samples were vortexed, flushed with nitrogen and allowed to sit overnight at room temperature for extraction. 1 mL of 0.88% KCL was then added, and the samples were gently inverted three or four times, and centrifuged at 1000 x g for 10 minutes to separate phases. The upper phase was aspirated and discarded, and the lower phase was decanted into clean tubes. Samples were then dried down at 50^0C under nitrogen. After solvent evaporation, 2 mL 1.0 M KOH in methanol was added to each tube, flushed with nitrogen and left in a water bath at 50^0C for 3 days to allow for complete saponification of lipids. Samples were removed from the water bath and allowed to cool to room temperature before the addition of 2 mL of dionized water and vortexing. Non-saponifiables were extracted into 3 mL of hexane by vigorous shaking for 1min and centrifugation at 1000 x g for 1 minute to separate phases. The upper hexane layer was transferred to a new tube, and the lower phase was washed with an additional 3

mL of hexane, centrifuged and the upper layer removed and combined with the first upper hexane layer. The samples were then dried down under nitrogen and brought back up in equivalent hexane volumes and placed into gas chromatography vials for analysis. Gas chromatography was conducted using an AT-5 capillary column (Alltech, Deerfield, IL), held initially at 270^0C for 1 minute with a 15.0^oC/minute temperature ramp up to 300^0C and held for 14 minutes. The inlet temperature was 270^oC, and the Flame Ionizing Detector was 300^0C.

Esterified Neutral Sterols

Feces (50-60 mg) was weighed, the exact mass recorded, and 0.2 mL of 0.5 M HCL was added to acidify and optimize the extraction. Total fecal lipids were extracted from the feces into 5 mL of 2:1 (v/v) chloroform/methanol with 20 µg/mL of 5-α cholestane (Folch et al 1957). Samples were vortexed, flushed with nitrogen and allowed to sit overnight at room temperature for extraction. 1 mL of 0.88% KCL was then added, and the samples were gently inverted four times, and centrifuged at 1000 x g for 10 minutes to separate phases. The upper phase was aspirated and discarded, and the lower phase was decanted into clean tubes. Aliquots of this lower phase were placed into GC vials for gas chromatographic analysis. Gas chromatography was conducted using an AT-5 capillary column (Alltech, Deerfield, IL), held initially at 270^0C for 1 minute with a 15.0^oC/minute temperature ramp up to 300^0C and held for 14 minutes. The inlet temperature was 270^oC, and the Flame Ionizing Detector was 300^0C.

Biliary Bile Acids

Bile samples were aspirated from hamster gallbladders in a 1mL syringe and immediately diluted with saline solution. Biliary bile acids were extracted by solid phase extraction and quantified by HPLC (Scalia 1988). The entire volume of bile recovered (10-40 µL bile diluted with 100-200 µL saline) from each gallbladder was transferred into 16 x 150mL tubes and diluted with 8mL of 0.1 M NaOH. The diluted bile was then heated for 15 minutes at 65^0C and allowed to cool to room temperature. Extraction of bile acids was then conducted by passing diluted bile through a sodium hydroxide/methanol-preconditioned, C-18, solid-phase extraction cartridge (Grace Davison Discovery Sciences, Deerfield, IL) in a drop-wise fashion. The cartridge was then washed with 20 mL of deionized water to elute highly polar compounds, and the remaining bile acids were then eluted and collected with 4mL of methanol. Methanol was then dried down and brought back up in 0.5mL of the initial HPLC mobile phase (15% mobile phase B, and 85% mobile phase A as described below) and filtered through a 0.4µm Teflon syringe filter (National Scientific, Rockwood, TN). 250 µL of filtered sample was drawn up in an HPLC injection syringe and injected onto a Hichrom Ultrasphere 5 ODS column (150 x 4.6 mm I.D. particle 5 micrometer) from via a 200 µL loop. The sample was eluted with a dual-mobile phase gradient initiated at 15% B for 25 minutes after which %B was increased to 50% over 15 minutes with a constant flow rate of 1 mL/min. Mobile phase A consisted of 65% methanol and 35% 0.04 M sodium acetate in water adjusted to pH 4.3. Mobile phase B consisted of 90% methanol and 10% 0.098 M sodium acetate in water adjusted to pH 4.3. Initial mobile phase used to solubilize standards and samples was a mixture of 15% B and 85%A. Bile acids were detected using a UV/Vis detector set at 210

nm, and bile acids were quantified with Chrom Perfect software. Bile acid data was compiled into a final hydrophobicity index for each sample using the formulas below (Armstrong and Carey 1982, Heuman 1989).

$$K'_x = (T_r - T_o) / T_o$$

$$HI = ln(k'_x)/ln(k'_{tlc})$$

Figure 3. *Hydrophobicity Index Formulas*

Where K'_x is the retention factor unique to each bile acid, 'r' is the migration of the individual bile acid in minutes and '$_o$' is the solvent peak front in minutes. K'_{tlc} is the retention factor of taurolithocholic acid calculated using the formula for K'_x. HI is the hydrophobicity index for the individual bile acid. The HI of each sample of bile is the sum of each bile acid's molar percent multiplied by its own HI.

Statistical Analysis

One way ANOVA analysis was used to compare study endpoints of the treatment groups. Mean values were analyzed for treatment differences using the Tukey multiple-comparison procedure, and mean values were considered significantly different at a p value of < 0.05. Association between experimental endpoints was analyzed by Pearson product-moment correlation analysis. Statistical procedures were conducted using JMP 8 (SAS Institute, Inc., Cary, NC).

RESULTS

Body Weight and Food Intake

Body weight and cumulative weight gain for the hamsters showed significant differences between some treatments at certain time points (Table 2, Figure 4). Regarding body weight, at Week 0 both the Palmitate Ester (PEs) (79.3 ± 0.8 g) and Stearate Ether (SEt) (79.6 ± 0.6 g) groups were significantly lighter than Control (83.6 ± 0.8 g). After Week 1, however, PEs weights were no longer different from Control, however SEt would be lighter than Control for the remainder of the study period, and would also differ from the Oleate Ester (OEt) group and the Stearate Ester (SEs) group during Week 3. Also, the free sterol (FS) group was lighter than Control for Weeks 1, 3 and 4. When considering cumulative weight gain, however, the only significant differences were during the first weigh point in which FS (2.5 ± 1.6 g) was lower than Control (6.1 ± 0.9 g) but neither was different compared to SEt (4.6 ± 0.6 g), SEs (5.4 ± 0.5 g), PEs (6.2 ± 0.6 g) and OEs (5.5 ± 0.5 g). The low average body weight in the FS groups was due to one animal that lost approximately 10 g in the first week. Removing this data point removed any significant difference. Concerning food intake, there were no differences between any groups during any week ($p > 0.05$) (Table 3).

Table 2. Hamster Body Weight

	Control	Stearate Ether	Stearate Ester	Palmitate Ester	Oleate Ester	Free Sterol
Week			g			
0	83.6 ± 0.8[a]	79.6 ± 0.6[b]	82.3 ± 1.2[ab]	79.3 ± 0.8[b]	81.2 ± 1.0[ab]	81.3 ± 0.8[ab]
1	89.6 ± .9[a]	84.2 ± 0.7[b]	87.7 ± 1.1[ab]	85.5 ± 1.0[ab]	87.7 ± 0.9[ab]	83. ± 1.8[b]
2	95.1 ± 1.2[a]	89.1 ± 1.0[b]	94.7 ± 1.2[a]	91.2 ± 1.3[ab]	94.9 ± 1.0[a]	90.2 ± 1.5[ab]
3	99.9 ± 1.3[a]	93.7 ± 0.9[b]	99.1 ± 1.0[a]	95.2 ± 1.3[ab]	97.7 ± 1.3[ab]	93.4 ± 1.4[b]
4	104 ± 2[a]	97.1 ± 0.9[bc]	102 ± 1[ab]	98.8 ± 1.4[abc]	102 ± 2[abc]	96.7 ± 1.1[c]

Values are means ± SEM, n = 10
Means within a row having different superscripts are statistically different ($P < 0.05$)

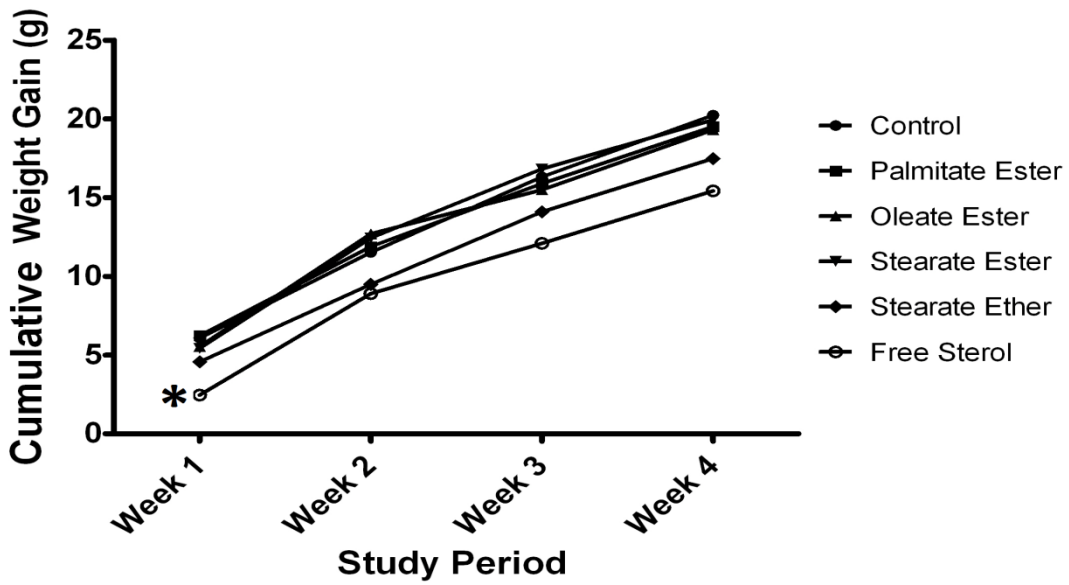


Figure 4. Cumulative body weight gain in hamsters during 23 day dietary plant sterol feeding.
* Statistically Lower compared to control during Week 1.

Table 3. Food Intake

	Control	Stearate Ether	Stearate Ester	Palmitate Ester	Oleate Ester	Free Sterol
			g/day			
Week 1	2.20 ± 0.11	2.33 ± 0.08	2.45 ± 0.11	2.36 ± 0.14	2.33 ± 0.08	2.30 ± 0.09
Week 2	5.57 ± 0.08	5.58 ± 0.11	5.62 ± 0.12	5.39 ± 0.26	5.46 ± 0.26	5.03 ± 0.17
Week 3	6.17 ± 0.11	6.53 ± 0.07	6.33 ± 0.11	6.41 ± 0.12	6.22 ± 0.14	6.24 ± 0.13
Week 4	6.86 ± 0.22	7.01 ± 0.19	6.86 ± 0.21	7.04 ± 0.19	7.37 ± 0.20	6.59 ± 0.15

Values are means ± SEM, n = 10
One-way Anova analysis showed no significant treatment differences.

Plasma Lipids and Cholesterol Absorption

After 23 days on treatment diets, hamsters showed statistically significant differences in non-HDL and HDL cholesterol levels (Table 4). OEs and FS non-HDL cholesterol levels were 2.81 ± 0.18, and 2.84 ± 0.15 mmol/L, respectively. Both were equivalently and significantly lower than Control (6.73 ± 0.35 mmol/L). SEt (6.15 ± 0.32 mmol/L), SEs (6.22 ± 0.33 mmol/L) and PEs (5.89 ± 0.26 mmol/L) non-HDL cholesterol levels were not significantly different compared to Control, but were greater than OEs and FS ($P < 0.0001$). SEs and PEs HDL cholesterol concentrations were 2.24 ± 0.08 mmol/L and 2.18 ± 0.06 mmol/L, respectively. Compared to Control, these groups were statistically identical. HDL cholesterol concentrations in SEt, OEs and FS groups were 1.68 ± 0.09, 1.67 ± 0.13, and 1.57 ± 0.07 mmol/L, respectively and were significantly

lower than Control, SEs and PEs ($P < 0.01$). Cholesterol absorption (Table 4), was decreased in all treatments compared to Control except for SEs. Percent absorption was 64.9 ± 2.1, 36.9 ± 0.8, 56.3 ± 1.8, 53.48 ± 3.7, 14.92 ± 3.8, 12.82 ± 1.7 for Control, SEt, SEs, PEs, OEs and FS respectively.

Liver Weight and Lipids

Liver weights (Table 5) for the Control group, SEs and SEt were 5.61 ± 0.1, 5.38 ± 0.2 and 5.61 ± 0.1 g, respectively and were no different from each other ($P > 0.05$). PEs liver weight was 4.94 ± 0.1 g and was lighter than Control and SEt but no different from SEs. OEs (3.90 ± 0.1 g) and FS (3.62 ± 0.1 g) were equivalently and significantly lower than all other groups ($P < 0.0001$).

Total cholesterol (Table 5) in the Control group (37.53 ± 2.2 µmol/g) was elevated above all other treatments. Significant reductions compared to the Control group were seen in PEs and SEs with concentrations of 24.8 ± 2.1 and 28.43 ± 2.0 µmol/g, respectively. SEt, OEs and FS were statistically no difference and lower than all other groups, with total cholesterol concentrations of 11.06 ± 0.6, 6.46 ± 0.3, and 6.09 ± 0.3 µmol/g, respectively. An identical trend of the same statistical relationships between groups was seen in esterified cholesterol levels (Table 5), whereby Control (30.92 ± 2.2 µmol/g) was greater than all groups, SEs (18.89 ± 2.0 µmol/g) and PEs (21.94 ± 2.0 µmol/g) were lower than Control, and SEt (5.38 ± 0.6 µmol/g), OEs (2.14 ± 0.3 µmol/g), and FS (1.79 ± 0.3 µmol/g) were the lowest and equivalent to each other. With the exception of PEs compared to Control ($P = 0.011$), all other pair-wise treatment

comparisons for esterified cholesterol showed P values less than 0.0001. Free cholesterol (Table 5) concentrations were highest in the Control group (6.61 ± 0.1 µmol/g) and PEs (5.60 ± 0.1 µmol/g). The SEt (5.69 ± 0.1 µmol/g) and SEs (5.91 ± 0.1 µmol/g) were equally less than Control and PEs. OEs and FS had the statistically lowest free cholesterol concentrations of 4.32 ± 0.04 µmol/g and 4.30 ± 0.1 µmol/g respectively. Free cholesterol treatment differences were significant at $P < 0.01$. The greatest concentrations of liver triglycerides (Table 5) were seen in equivalently across the Control group (1.91 ± 0.1 µmol/g), OEs (2.33 ± 0.2 µmol/g) and FS (1.98 ± 0.1 µmol/g). While no different than OEs or FS, the triglyceride concentrations of SEt (1.51 ± 0.1 µmol/g) SEs (1.61 ± 0.1 µmol/g) and PEs (1.67 ± 0.1 µmol/g) were statistically lower compared to Control at $P < 0.01$. Phospholipid concentrations (Table 5) did not differ among Control, SEt, SEs, PEs, and OEs, 17.03 ± 0.3, 16.86 ± 0.2, 17.06 ± 0.2, 17.18 ± 0.3, 17.92 ±0.2 µmol/g respectively. FS, however, was statistically lower than Control, SEt and SEs, but no different than PEs or OEs. Phospholipid differences were significant at $P < 0.05$.

Fecal Sterols

Fecal bile acids (Table 6) (µmol/day/100g BW) in the Control group (0.58 ± 0.04), SEt (0.57 ± 0.04), SEs (0.75 ± 0.05) and PE (0.71 ± 0.04) were equivalent ($P > 0.05$), while OEs was the lower compared to all groups except FS (0.37 ± 0.04), and FS was equivalent to the Control, SEt and OEs. Differences were observed with P values less than 0.05 for all treatments. Fecal neutral sterol content (Table 6) (µmol/day/100g BW) was greatest in the OEs and FS groups which were identical to one another; 0.37 ± 0.04

and 0.43 ± 0.05, respectively. SEt was the second highest (11.97 ± 0.3). SEs (6.72 ± 0.5) and PEs (6.72 ± 0.5) were lower than SEt, and no differences were observed between the two. The Control group was statistically the lowest at 2.84 ± 0.0. All differences were significant at $P < 0.0001$ with the exception of pair wise comparisons between PEs and Control, and SEs and Control, both of which returned P values of 0.0031. Total fecal plant sterol output (Table 6) (µmol/day/100g BW) was different only in the Control group (1.31 ± 0.1) as compared to all other treatments ($P < 0.0001$); SEt (362.4 ± 15.3), SEs (359.0 ± 16.0), PE (357.6 ± 16.0), OE (345.0 ± 10.6) and FS (386.7 ± 16.1). Percent of plant sterol ester hydrolysis was 1.69 ± 0.03 in SEt, 3.13 ± 0.2 in SEs, 4.12 ± 0.2 in PEs, 88.29 ± 0.8 in OEs and 100 ± 0.0 in FS. Differences were significant at $P < 0.0001$with the exception of the comparison of PEs to SEt which was significant at $P = 0.0002$.

Biliary Bile Acids

Treatment differences were only observed for four bile acids (Tables 7a-b). The molar percent of ursodeoxycholate in the Control group (1.1 ± 0.7%) was significantly lower than FS (29.4 ± 6.9%), but there is no evidence to suggest that a difference exists between these two groups and SEt (18.2 ± 5.3%), SEs (19.0 ± 6.5%), PEs (10.6 ± 4.7%) or OEs (13.7 ± 7.6%) with $P > 0.05$. Tauroursodeoxycholate was greater in OEs (7.60 ± 2.23%) compared to SEt (2.50 ± 0.78) and SEs (1.80 ± 0.24), but no differences were detected between OEs, Control (3.10 ± 1.05%), PEs (3.90 ± 0.65%) or FS (3.20 ± 0.46) with $P > 0.05$. Taurochenodeoxycholate was greatest in PEs (9.6 0± 1.44%) compared

with SEs (4.38 ± 0.78%), OEs (2.66 ± 0.77%) and FS (3.79 ± 0.82%), but no evidence of a difference was found between PEs, Control (5.3 ± 1.4%), SEt (4.8 ± 1.7), SEs, OEs or FS. Glycochenodeoxycholate was greater in PEs (10.4 ± 1.6%) than in FS (4.8 ± 0.9%), but no evidence of a difference was found when comparing PEs and FS to Control (8.3 ± 1.6%), SEt (7.6 ± 1.3%), SEs (6.9 ± 0.7%) or OEs (5.8 ± 1.1%) as all P values were greater than 0.05. All other bile acids were unchanged between treatments. The molar percent of hyodeoxycholic acid in the Control, SEt, SEs, PEs, OEs and FS groups was 10.9 ± 7.4%, 2.2 ± 0.4%, 2.4 ± 0.4%, 2.0 ± 0.3%, 5.2 ± .09% and 3.1 ± 0.5%, respectively. Across the same treatment groups, cholate molar percent was 19.2% ± 6.1%, 15.3% ± 3.3%, 19.3 ± 3.9%, 16.6 ± 3.2%, 10.3 ± 3.0% and 11.5 ± 3.2%, respectively. Chenodeoxycholicate molar percent was 12.4 ± 5.8%, 22.3 ± 6.9%, 18.6 ± 3.3%, 11.9 ± 3.4%, 13.7 ± 3.9%, 23.0 ± 3.7%, respectively and across the same groups, deoxycholate molar percent was 12.9 ± 5.1, 6.2 ± 5.1, 12.0 ± 4.0, 14.3 ± 3.7, 21.2 ± 7.6, 5.5 ± 1.9%, respectively. Lithocholate molar percent of Control, SEt, SEs, PEs, OEs and FS groups was 1.60 ± 0.70%, 1.30 ± 1.33%, 1.80 ± 0.72%, 0.40 ± 0.27%, 0.10 ± 0.10% and 1.05 ± 0.93%, respectively. Taurodeoxycholate molar percent of Control, SEt, SEs, PEs, OEs and FS was 9.37 ± 6.18%, 4.75 ± 1.39%, 2.31 ± 0.41%, 3.98 ± 0.69%, 7.30 ± 1.28% and 5.16 ± 1.15%, respectively. Taurocholate molar percent across these same treatments was 2.81 ± 1.94%, 0.85 ± 0.18%, 0.83 ± 0.17%, 1.14 ± 0.21%, 1.63 ± 0.35% and 0.87 ± 0.15%, respectively. Taurolithocholate molar percent across the same groups was 1.05 ± 0.35%, 3.15 ± 1.47%, 1.07 ± 0.18%, 1.96 ± 0.75%, 0.78 ± 0.19% and 0.87 ± 0.21%, respectively. Glycocholate molar percent in Control, SEt, SEs, PEs, OEs and FS was 1.51 ± 0.4%, 3.61 ± 2.53%, 1.42 ± 0.51%, 1.84 ± 0.62%, 2.64 ± 0.65% and 0.93 ±

0.28%, respectively. In the same groups, glycodeoxycholate molar percent was 10.5 ± 2.2%, 7.24 ± 2.08%, 8.21 ± 0.96%, 11.2 ± 1.9%, 7.36 ± 1.45% and 6.86 ± 1.25%, respectively. With the exception of ursodeoxycholate, tauroursodeoxycholate, taurochenodeoxycholate and glycochenodeoxycholate, no differences were detected in molar percents individual bile acids among treatments (P>0.05). Total molar percent of unconjugated bile acids showed no evidence of differences between the Control (58.1 ± 8.8%), SEt (65.5 ± 6.1%), SEs (73.1 ± 2.4%), PEs (55.9 ± 5.5%), OEs (64.2 ± 6.6%) or FS (73.6 ± 4.4% (P>0.05). Also, no differences were observed across the same treatments regarding total conjugated bile acids with Control, SEt, SEs, PEs, OEs and FS molar percents being 41.9 ± 8.8%, 34.5 ± 6.1%, 26.9 ± 2.4%, 44.1 ± 5.5%, 35.8 ± 6.6% and 26.4 ± 4.4%, respectively. The sum of tauroconjugated bile acids across all treatments showed no significant differences with Control, SEt, SEs, PEs, OEs and FS molar percents being 21.6 ± 7.9%, 16.3 ± 3.3%, 10.4 ± 1.0%, 20.6 ± 2.4%, 20.0 ± 4.1% and 13.9 ± 2.3%, respectively (P>0.05). Also, no evidence of differences for the sum of glycoconjugated bile acids was founds across the same treatments in with molar percents of 20.3 ± 4.0%,18.2 ± 3.9%,16.5 ± 2.0%,23.5 ± 3.3%,15.8 ± 2.9% and 12.6 ± 2.2% for Control, SEt, SEs, PEs, OEs and FS, respectively. The sum of primary bile acids molar percents was 49.4 ± 7.6%, 55.8 ± 6.1%, 51.4 ± 6.4%, 51.6 ± 3.3%, 36.7 ± 5.8% and 44.9 ± 5.1% for Control, SEt, SEs, PEs, OEs and FS, respectively with no differences observed between any groups (P>0.05). The sum of secondary bile acid molar percents was 50.6 ± 7.6%, 44.2 ± 6.1%, 48.6 ± 6.4%, 48.4 ± 3.3%, 63.3 ± 5.8% and 55.1 ± 5.1% for Control, SEt, SEs, PEs, OEs and FS, respectively with no differences observed between any groups (P>0.05). The ratio of primary to secondary bile acids was 1.47 ±

0.37, 1.82 ± 0.61, 1.47 ± 0.37, 1.14 ± 0.13, 0.70 ± 0.16 and 0.97 ± 0.20 for Control, SEt, SEs, PEs, OEs and FS respectively. No differences were observed between any treatment groups with all p values greater than 0.05. The ratio of glycoconjugated to tauroconjugated bile acids was no different between any groups with Control, SEt, SEs, PEs, OEs and FS ratios of 1.63 ± 0.24, 1.61 ± 0.57, 1.68 ± 0.21, 1.16 ± 0.08, 0.81 ± 0.10 and 0.93 ± 0.08, respectively. The ratio of free to conjugated bile acids was also no different across the same groups with Control, SEt, SEs, PEs, OEs and FS ratios being 3.36 ± 1.53, 2.81 ± 0.69, 3.02 ± 0.38, 2.19 ± 0.99, 3.45 ± 1.23 and 4.16 ± 1.16, respectively. The hydrophobicity index was no different across all treatment groups with relative units of 0.86 ± 0.1, 0.84 ± 0.1, 0.89 ± 0.03, 0.82 ± 0.04, 0.83 ± 0.1 and 0.83 ± 0.02 p values from all pair-wise comparisons above 0.05.

Correlations

Percent hydrolysis was correlated significantly with fecal cholesterol output ($r=0.9218$, $p<0.0001$), Liver esterified cholesterol ($r=-0.6843$, $p<0.0001$), and percent cholesterol absorption ($r=-0.8504$, $p<0.0001$). Fecal cholesterol output was significantly correlated with the natural log of non-HDL cholesterol ($r=-0.8383$, $p<0.0001$), and cholesterol absorption was correlated significantly with non-HDL cholesterol ($r=0.7653$, $p<0.0001$).

Table 4. Cholesterol Absorption and Plasma Lipids

	Control	Stearate Ether	Stearate Ester	Palmitate Ester	Oleate Ester	Free Sterol
			%			
Cholesterol Absorption	64.9 ± 2.1[a]	36.9 ± 0.8[c]	56.3 ± 1.8[ab]	53.5 ± 3.7[b]	14.9 ± 3.8[d]	12.8 ± 1.7[d]
			mmol/L			
Non-HDL Cholesterol	6.73 ± 0.35[a]	6.15 ± 0.32[a]	6.22 ± 0.33[a]	5.89 ± 0.26[a]	2.81 ± 0.18[b]	2.84 ± 0.15[b]
HDL Cholesterol	2.42 ± 0.09[a]	1.68 ± 0.09[b]	2.24 ± 0.08[a]	2.18 ± 0.06[a]	1.67 ± 0.13[b]	1.57 ± 0.07[b]

Values are means ± SEM, n = 10
Means within a row having different superscripts are statistically different ($P < 0.05$).

Table 5. Liver Weight and Liver Lipid Concentrations

	Control	Stearate Ether	Stearate Ester	Palmitate Ester	Oleate Ester	Free Sterol
			µmol/g			
Total Cholesterol	37.5 ± 2.2[a]	11.1 ± 0.6[c]	24.8 ± 2.1[b]	28.4 ± 2.0[b]	6.46 ± 0.3[c]	6.09 ± 0.3[c]
Esterified Cholesterol	30.9 ± 2.2[a]	5.38 ± 0.6[c]	18.9 ± 2.0[b]	21.9 ± 2.0[b]	2.14 ± 0.3[c]	1.79 ± 0.3[c]
Free Cholesterol	6.61 ± 0.14[a]	5.69 ± 0.13[b]	5.91 ± 0.10[b]	5.60 ± 0.11[a]	4.32 ± 0.05[c]	4.30 ± 0.10[c]
Triglyceride	1.91 ± 0.10[ab]	1.51 ± 0.11[b]	1.61 ± 0.11[b]	1.67 ± 0.15[b]	2.33 ± 0.19[a]	1.98 ± 0.07[ab]
Phospholipid	17.0 ± 0.3[b]	16.9 ± 0.2[b]	17.1 ± 0.2[b]	17.2 ± 0.3[ab]	17.9 ±0.2[ab]	18.3 ± 0.4[a]
			g			
Liver Weight	5.61 ± 0.10[a]	5.61 ± 0.13[a]	5.38 ± 0.15[ab]	4.94 ± 0.14[b]	3.90 ± 0.12[c]	3.62 ± 0.08[c]

Values are means ± SEM, n = 10
Means within a row having different superscripts are statistically different ($P < 0.05$).

Table 6. Fecal Sterols

	Control	Stearate Ether	Stearate Ester	Palmitate Ester	Oleate Ester	Free Sterol
				µmol/day/100g BW		
Bile Acids	0.58 ± 0.04[ab]	0.57 ± 0.04[ab]	0.75 ± 0.05[a]	0.71 ± 0.04[a]	0.37 ± 0.04[c]	0.43 ± 0.05[bc]
Neutral Sterol	2.84 ± 0.00[d]	12.0 ± 0.30[b]	6.72 ± 0.47[c]	6.72 ± 0.46[c]	21.1 ± 0.78[a]	21.7 ± 0.70[a]
Total Plant Sterol	1.31 ± 0.10[a]	362 ± 16[b]	359 ± 16[b]	358 ± 16[b]	345 ± 11[b]	387 ± 16[b]
Esterified Plant Sterol	0.00 ± 0.0[c]	348 ± 15[a]	42.5 ± 4.2[a]	346 ± 16[a]	339 ± 11[b]	0.00 ± 0.00[c]
Free Plant Sterol	1.31 ± 0.10[c]	5.81 ± 0.12[c]	11.2 ± 0.8[c]	14.9 ± 0.8[c]	317 ± 13[b]	387 ± 16[a]
				%		
Plant Sterol Ester Hydrolysis		1.69 ± 0.03[d]	3.13 ± 0.2[cd]	4.12 ± 0.2[c]	88.3 ± 0.8[b]	100 ± 0.0[a]

Values are means ± SEM, n = 10
Means within a row having different superscripts are statistically different ($P < 0.05$).

Table 7a. Biliary Bile Acids

	Control	Stearate Ether	Stearate Ester	Palmitate Ester	Oleate Ester	Free Sterol
			Mole %			
UNCONJUGATED	58.1 ± 8.8	65.5 ± 6.1	73.1 ± 2.4	55.9 ± 5.5	64.2 ± 6.6	73.6 ± 4.4
Ursodeoxycholate	1.1 ± 0.7[b]	18.2 ± 5.3[ab]	19.0 ± 6.5[ab]	10.6 ± 4.7[ab]	13.7 ± 7.6[ab]	29.4 ± 6.9[a]
Hyodeoxycholate	10.9 ± 7.4	2.20 ± 0.4	2.40 ± 0.4	2.00 ± 0.3	5.20 ± .09	3.10 ± 0.5
Cholate	19.2 ± 6.1	15.3 ± 3.3	19.3 ± 3.9	16.6 ± 3.2	10.3 ± 3.0	11.5 ± 3.2
Chenodeoxycholate	12.4 ± 5.8	22.3 ± 6.9	18.6 ± 3.3	11.9 ± 3.4	13.7 ± 3.9	23.0 ± 3.7
Deoxycholate	12.9 ± 5.1	6.20 ± 5.1	12.0 ± 4.0	14.3 ± 3.7	21.2 ± 7.6	5.5 ± 1.9
Lithocholate	1.60 ± 0.70	1.30 ± 1.33	1.80 ± 0.72	0.40 ± 0.27	0.10 ± 0.10	1.1 ± 0.93
TAURO-CONJUGATED	21.6 ± 7.9	16.3 ± 3.3	10.4 ± 1.0	20.6 ± 2.4	20.0 ± 4.1	13.9 ± 2.3
Urosdeoxycholate	3.10 ± 1.05[ab]	2.50 ± 0.78[b]	1.80 ± 0.24[b]	3.90 ± 0.65[ab]	7.60 ± 2.23[a]	3.20 ± 0.46[ab]
Deoxycholate	9.37 ± 6.18	4.75 ± 1.39	2.31 ± 0.41	3.98 ± 0.69	7.30 ± 1.28	5.16 ± 1.15
Cholate	2.81 ± 1.94	0.85 ± 0.18	0.83 ± 0.17	1.14 ± 0.21	1.63 ± 0.35	0.87 ± 0.15
Chenodeoxycholate	5.25 ± 1.40[ab]	4.78 ± 1.68[ab]	4.38 ± .78[b]	9.60 ± 1.44[a]	2.66 ± 0.77[b]	3.79 ± 0.82[b]
Lithocholate	1.05 ± 0.35	3.15 ± 1.47	1.07 ± 0.18	1.96 ± 0.75	0.78 ± 0.19	0.87 ± 0.21

Values are means ± SEM, n = 9-10
Means within a row having different superscripts are statistically different ($P < 0.05$).

Table 7b. Biliary Bile Acids - Continued

	Control	Stearate Ether	Stearate Ester	Palmitate Ester	Oleate Ester	Free Sterol
			Mole %			
GLYCO-CONJUGATED	20.3 ± 4.0	18.2 ± 3.9	16.5 ± 2.0	23.5 ± 3.3	15.8 ± 2.9	12.6 ± 2.2
Cholate	1.51 ± 0.43	3.61 ± 2.53	1.42 ± 0.51	1.84 ± 0.62	2.64 ± 0.65	0.93 ± 0.28
Chenodeoxycholate	8.27 ± 1.61[ab]	7.64 ± 1.33[ab]	6.89 ± 0.75[ab]	10.45 ± 1.62[a]	5.80 ± 1.07[ab]	4.80 ± 0.87[b]
Deoxycholate	10.5 ± 2.2	7.24 ± 2.08	8.21 ± 0.96	11.2 ± 1.9	7.36 ± 1.45	6.86 ± 1.25
ALL CONJUGATES	41.9 ± 8.8	34.5 ± 6.1	26.9 ± 2.4	44.1 ± 5.5	35.8 ± 6.6	26.4 ± 4.4
PRIMARY	49.4 ± 7.6	55.8 ± 6.1	51.4 ± 6.4	51.6 ± 3.3	36.7 ± 5.8	44.9 ± 5.1
SECONDARY	50.6 ± 7.6	44.2 ± 6.1	48.6 ± 6.4	48.4 ± 3.3	63.3 ± 5.8	55.1 ± 5.1
			Molar Ratio			
PRIMARY:SECONDARY	1.47 ± 0.37	1.82 ± 0.61	1.47 ± 0.37	1.14 ± 0.13	0.70 ± 0.16	0.97 ± 0.20
GLYCO:TAURO	1.63 ± 0.24	1.61 ± 0.57	1.68 ± 0.21	1.16 ± 0.08	0.81 ± 0.10	0.93 ± 0.08
FREE:CONJUGATED	3.36 ± 1.53	2.81 ± 0.69	3.02 ± 0.38	2.19 ± 0.99	3.45 ± 1.23	4.16 ± 1.16
			Relative Units			
HYDROPHOBICITY INDEX	0.86 ± 0.1	0.84 ± 0.1	0.89 ± 0.03	0.82 ± 0.04	0.83 ± 0.1	0.83 ± 0.02

Values are means ± SEM, n = 9-10
Means within a row having different superscripts are statistically different ($P < 0.05$).

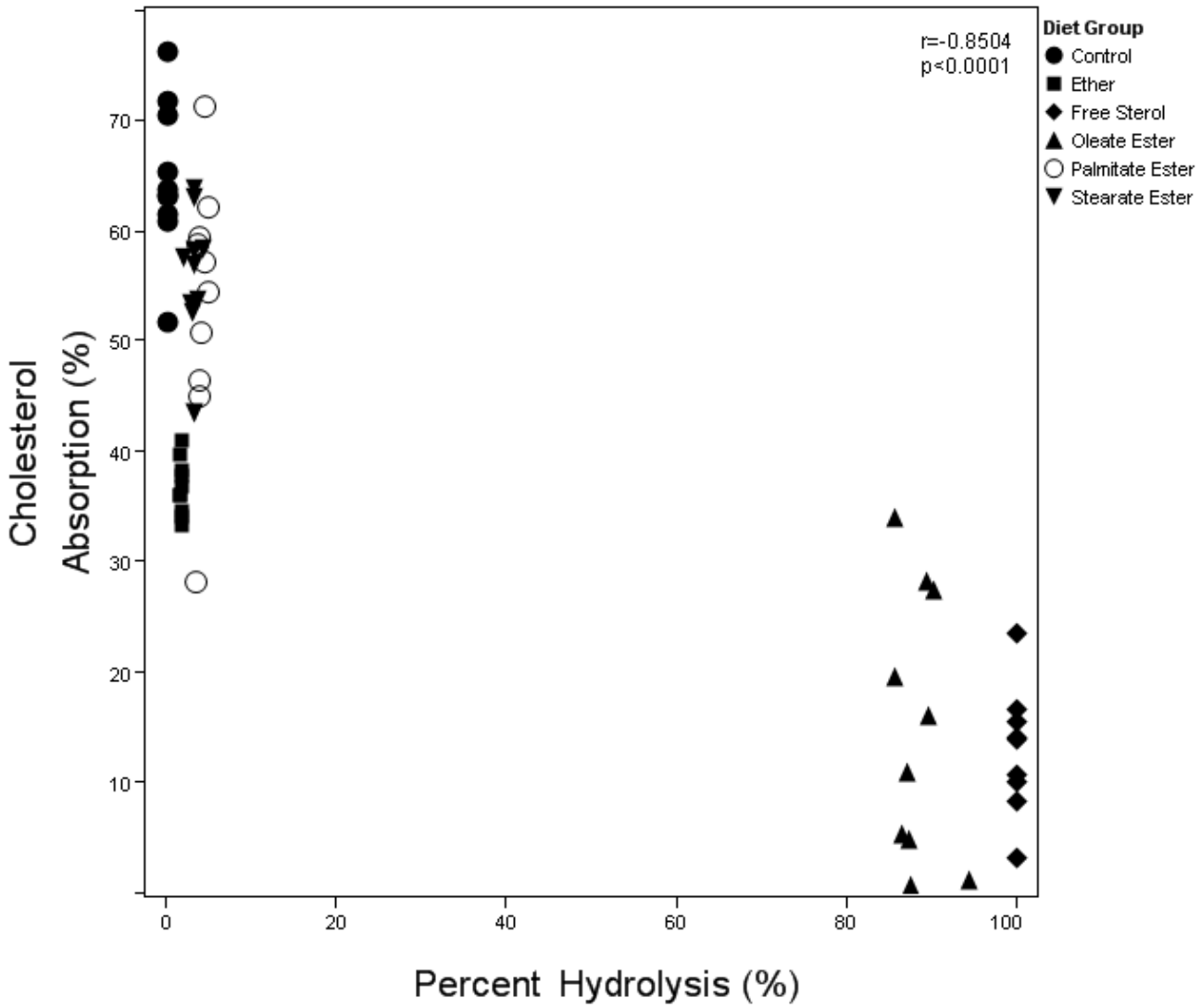


Figure 5. Correlation analysis of percent hydrolysis and cholesterol absorption.

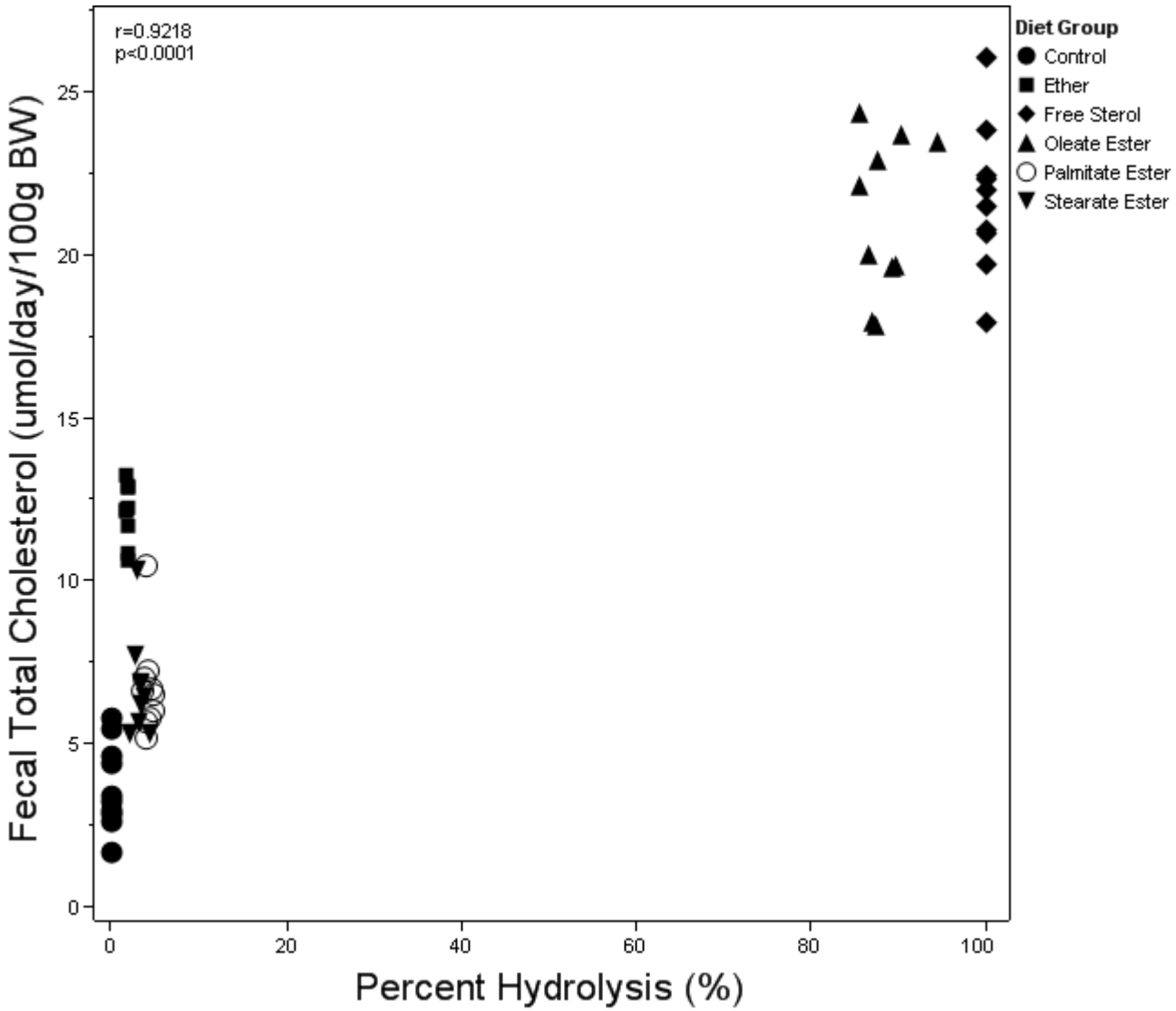


Figure 6. Correlation analysis of percent hydrolysis and fecal cholesterol output.

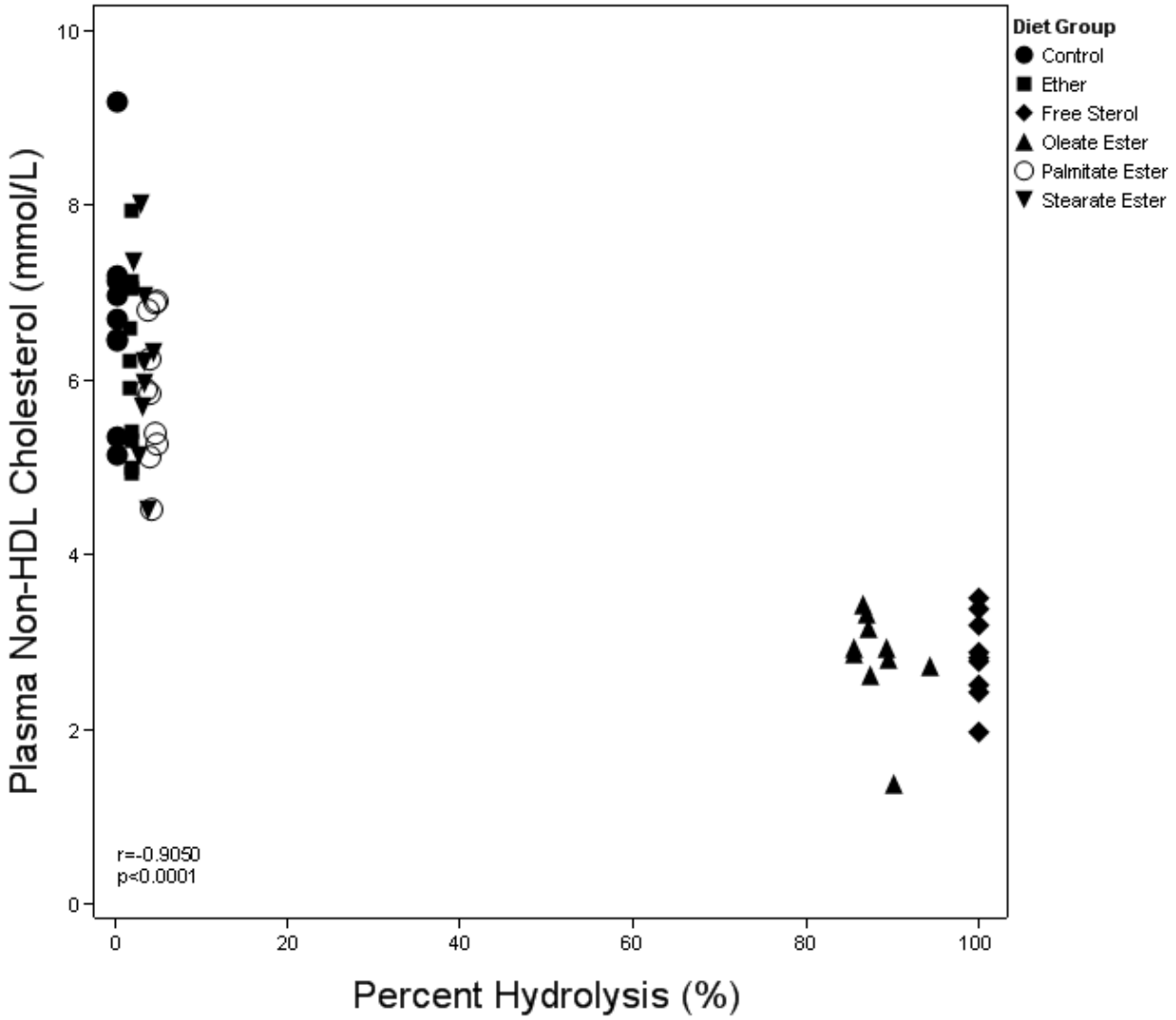


Figure 7. Correlation analysis of percent hydrolysis and Non-HDL cholesterol.

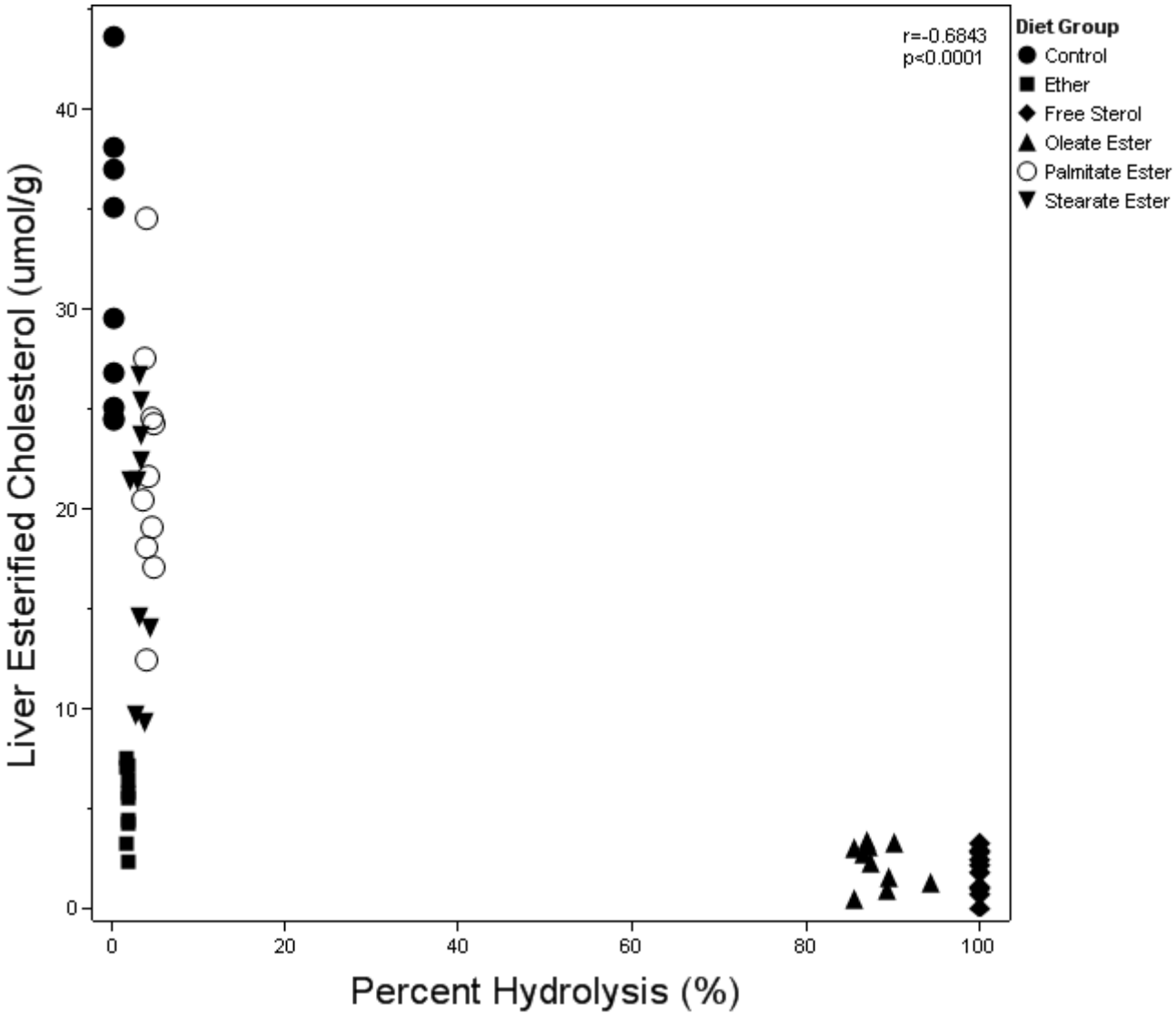


Figure 8. Correlation analysis of percent hydrolysis and liver esterified cholesterol.

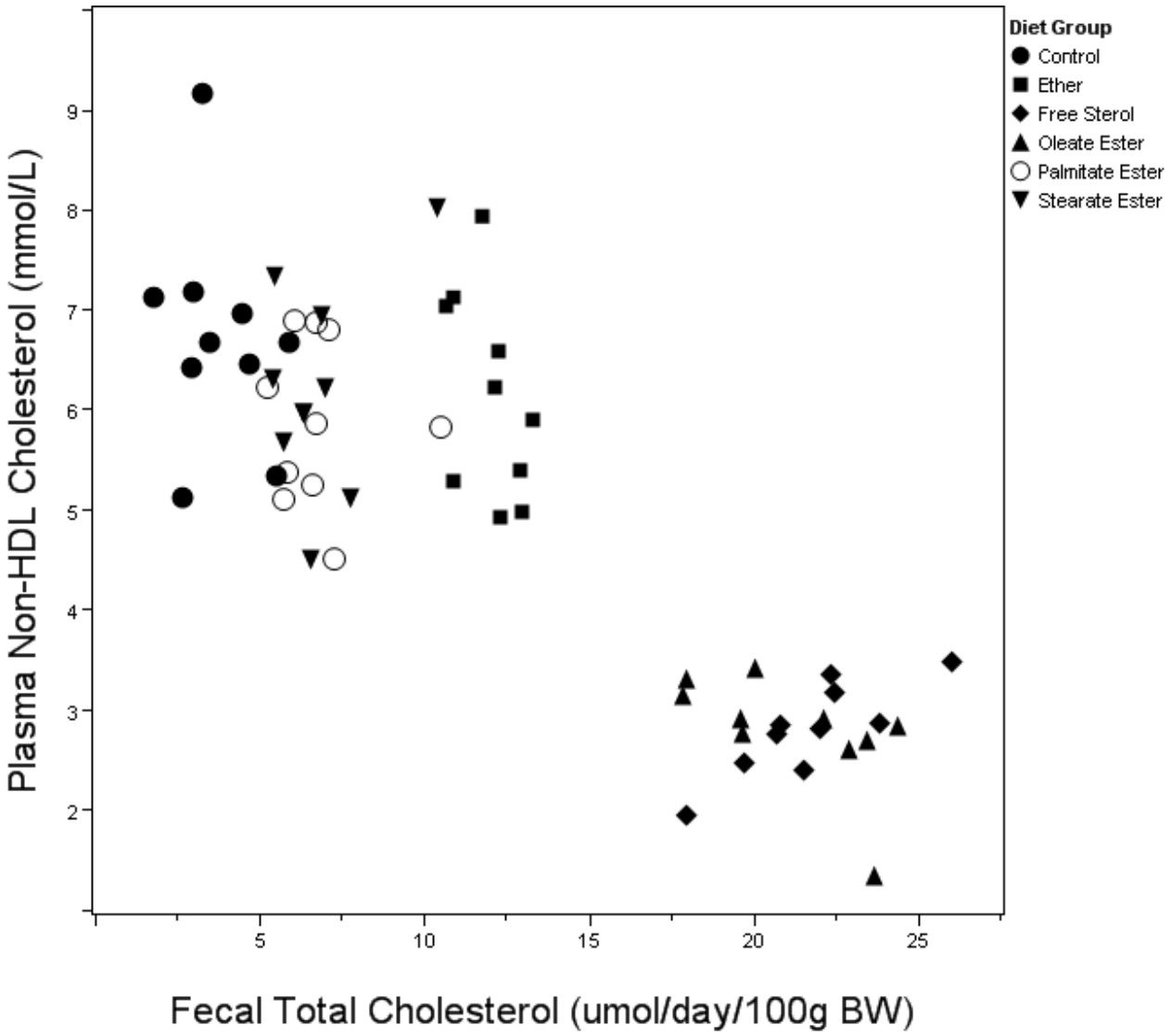


Figure 9. Correlation analysis of fecal cholesterol output and the Non-HDL cholesterol.

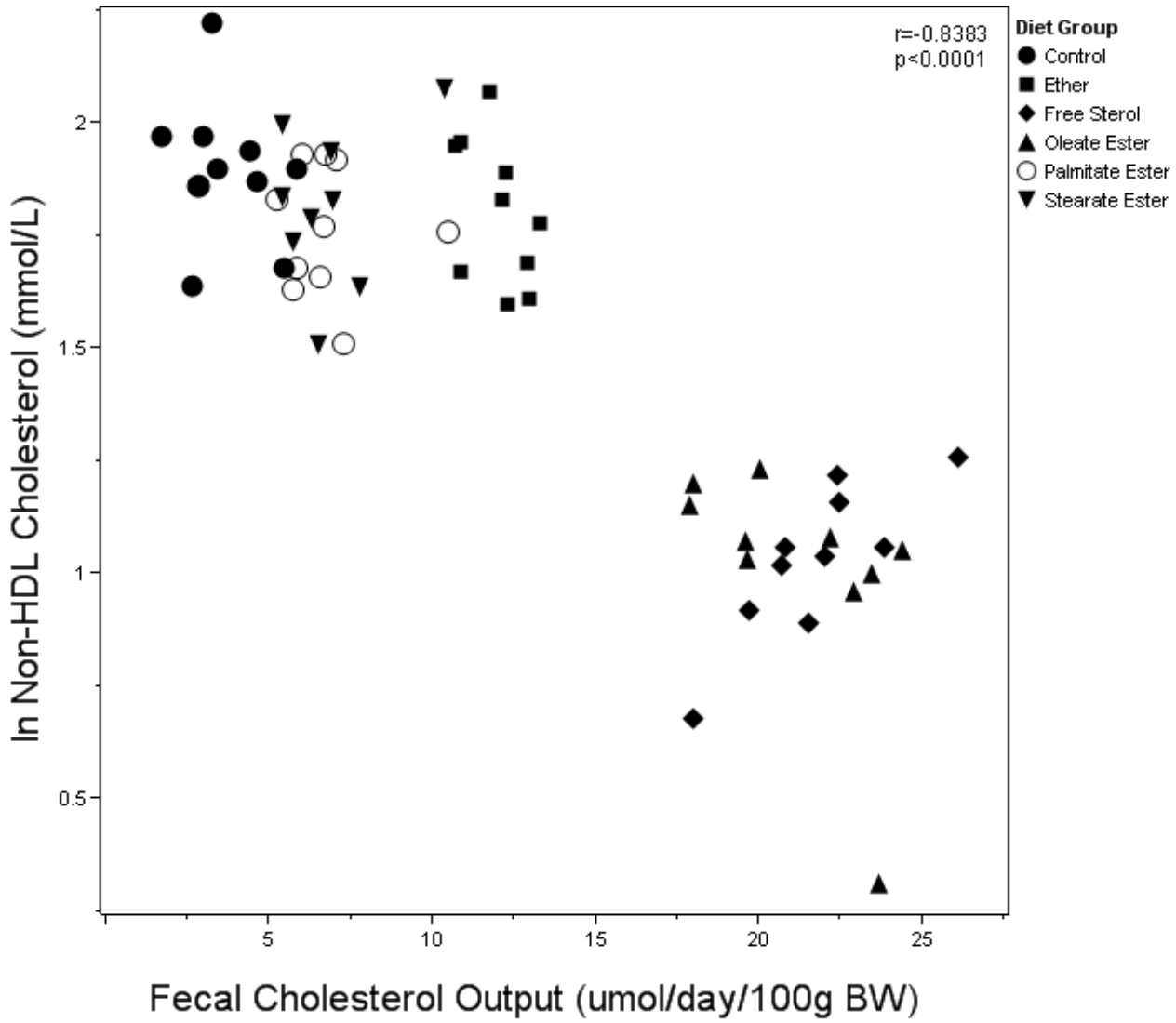


Figure 10. Correlation analysis of fecal cholesterol output and the natural log of Non-HDL cholesterol.

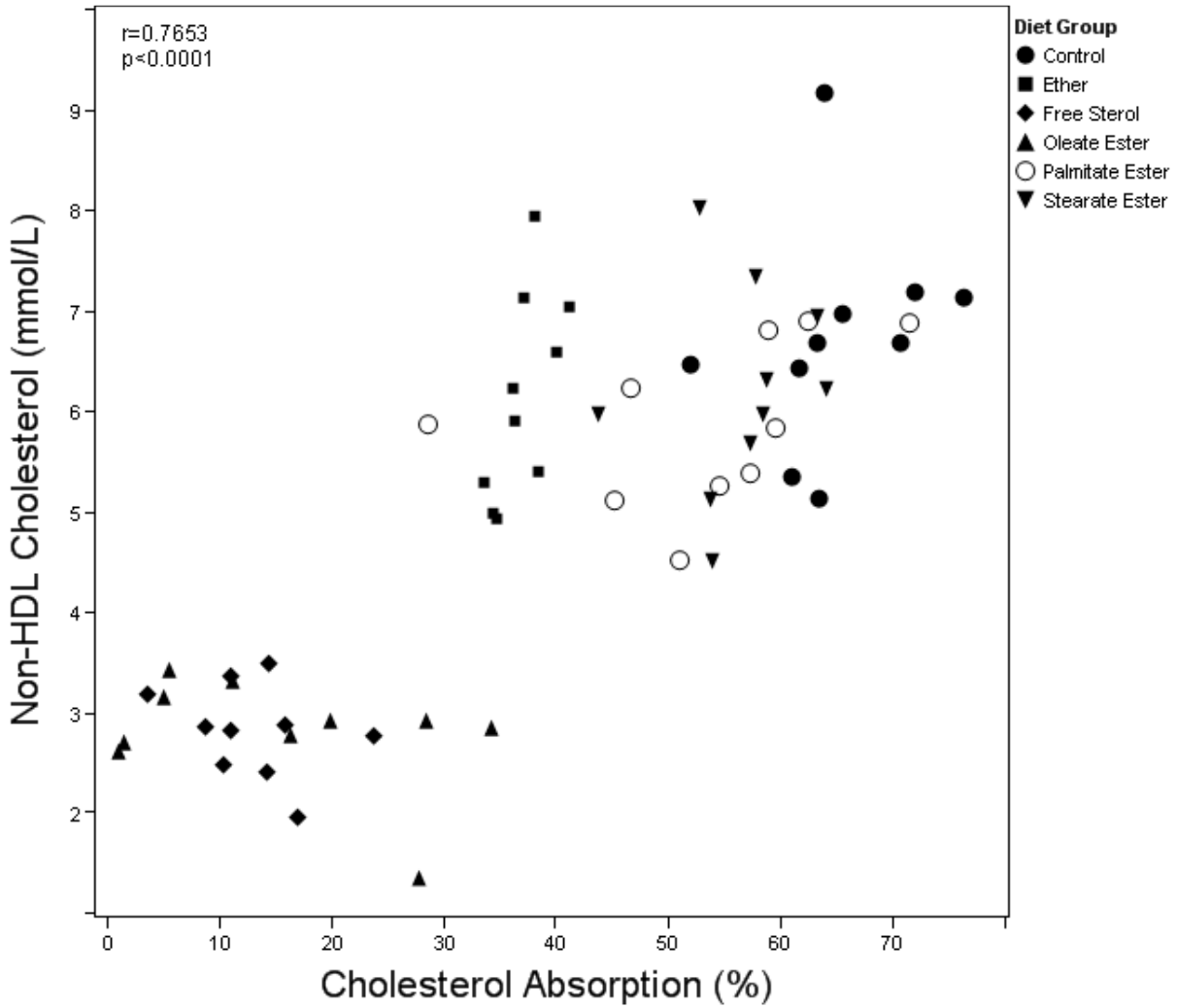


Figure 11. Correlation analysis of percent cholesterol absorption and Non-HDL cholesterol.

Discussion

The purpose of this study was to determine the relationship between the ability of plant sterol esters (PSE) to be hydrolyzed in the intestinal lumen and their cholesterol-lowering capabilities. Studies conducted as early as the 1950s (Best and Duncan 1958, Peterson et al 1953) and later (Mattson et al 1977, Normen et al 2006) demonstrated that it is the free plant sterol molecule that is active, and therefore in order for cholesterol-lowering to take place, the esterified molecule must be hydrolyzed in the lumen. However, few studies stating this hypothesis endeavored to quantify the degree to which the PSE were hydrolyzed. Furthermore, recent work from our laboratory has suggested that hydrolysis is indeed necessary, showing that phytosterol stearates fed to hamsters resulted in only 0.88-4.68% hydrolysis and no hypocholesterolemic effects (Carr and Ash). Therefore, the current study tested the hypothesis that hydrolysis of PSE is required to observe a cholesterol-lowering effect.

In this study, one sterol ether treatment and four sterol ester treatments were used to produce a spectrum of hydrolysis across which cholesterol metabolism may be compared. The relative degrees of hydrolysis of the treatment compounds were found to be SEs = SEt < PEs < OEs < FS with no significant difference between SEs and PEs (Table 6). These relative levels of hydrolysis aligned perfectly with the predicted spectrum conceptualized from in-vitro work previously conducted in this laboratory (Brown et al 2010). In the current study, however, the range of hydrolysis was much less evenly distributed across the entire spectrum, leaving SEs, SEt and PEs clustered at the lower end of the spectrum (1.69-4.12% hydrolyzed), and OEs and FS clustered at the upper end (88.29-100% hydrolyzed). Without coverage of the middle of the spectrum, we

were left to speculate about the cholesterol lowering efficiency of PSE that are only partially hydrolyzed. A correlation of fecal cholesterol output and non-HDL cholesterol (Figure 9) provided a more complete spectrum coverage and produced a hyperbolic shape with a sharp drop in non-HDL cholesterol as fecal cholesterol output increased, which seemed to level off towards the high end of the cholesterol output spectrum suggesting a saturation of the system. Considering that fecal cholesterol output was highly correlated with percent hydrolysis (Figure 6), it may follow that the mid-spectrum trend of correlations of percent hydrolysis with other parameters would be similar to that of cholesterol output. If this is true, plant sterol competition with cholesterol for micelle incorporation may reach its peak efficiency before reaching 88-100% hydrolysis, as was seen with OEs and FS respectively, suggesting that there may be a threshold of PSE supplementation above which few additional benefits are realized.

Despite the clustering of data points at each end of the hydrolytic spectrum, changes in cholesterol metabolism across treatment groups support our hypothesis that hydrolysis is vital to the cholesterol-lowering action of PSE. With the exception of SEt, percent cholesterol absorption closely matched the inverse of the pattern of hydrolysis noted above; relative cholesterol absorption: FS < OEs < PEs = SEs. Furthermore, percent hydrolysis was highly and inversely correlated with cholesterol absorption showing a correlation coefficient of -0.8504 (Figure 5). Percent hydrolysis was also correlated negatively (r=-0.6843) with liver esterified cholesterol (Figure 8), and positively (r=0.9218) with fecal cholesterol output (Figure 6). These data are also in line with early studies exploring the effects of plant sterols esterified to fatty acids of similar and dissimilar structure to our treatments. (Best and Duncan 1958) conducted

experiments in a rat model showing that free sitosterol and sitosteryl oleate were nearly identical in their abilities to lower liver cholesterol compared to Control. Palmitate Ester, on the other hand, was significantly less capable of lowering liver cholesterol, agreeing with our conclusions that palmitate and oleate esters undergo differential hydrolysis which accounts for unique cholesterol-lowering capabilities between the two. (Peterson et al 1953), using two-week old chicks, showed that a diet containing 1% cholesteryl caprate, an ester of a 10 carbon saturated fatty, resulted in lower plasma and liver cholesterol compared to a diet containing 1% free cholesterol only, suggesting that cholesterol from the caprate ester was unable to be absorbed due to a lack of hydrolysis, which is consistent with the lack of hydrolysis in our saturated fatty acid esters. Furthermore, a diet containing 1% free cholesterol and 1.38% soy sterol caprate resulted in elevated plasma and liver cholesterol compared to a diet containing 1% free cholesterol and 1.38% free soy sterols (Peterson et al 1953), further suggesting that sterol caprates are poorly hydrolyzed. These two experiments suggested that both cholesterol and plant sterols must be hydrolyzed in order to be absorbed or to compete for absorption, respectively. Conversely in a rat study, decanoate (caprate) and oleate esters of the same plant sterols possessed equivalent cholesterol lowering abilities, contrasting Peterson et al. who showed decanoate esters to be poor hypocholesterolemic agent (Mattson et al 1977). In further support of our findings was a study conducted in rats fed free sterols, oleate esters and stearate esters of plant sterols resulting in equivalently lowered liver cholesterol and equivalently increased fecal cholesterol excretion in oleate ester and free sterol groups compared to control, but no changes in liver cholesterol or fecal cholesterol excretion in the stearate ester group (Kobayashi et al 2008). Oleate

esters were then found to be 99.5% hydrolyzed while stearate esters were only 19.2 % hydrolyzed.

According to unpublished data from previous work in our laboratory (Carr and Ash), plant sterol stearates containing different plant sterol moieties fail to lower cholesterol when consumed at 2.5% of the diet in a hamster model of hypercholesterolemia. This was hypothesized to be a result of poor stearate ester hydrolysis. It was further hypothesized, then, that non-hydrolyzed PSE may, at high doses, lower cholesterol by forming an "oil phase" in the intestinal lumen that solubilizes cholesterol and prevents its micellar incorporation, thus lowering cholesterol absorption. Considering in this study that stearate esters were poorly hydrolyzed, yet still managed to lower liver esterified cholesterol and increase fecal neutral sterol excretion, the possibility exists that stearate esters at 5% of the diet succeeded in creating an oil phase capable of partitioning cholesterol away from micelles while 2.5% was previously shown to be insufficient. In opposition to this theory was the cholesterol absorption data from this current study in which stearate esters, regardless of changes in liver cholesterol and fecal neutral sterol output, were unable to lower cholesterol absorption in relation to the Control group. Furthermore, other unpublished data in our laboratory (Brown et al.) gave no evidence to suggest that phase-partitioning of cholesterol differs between treatments of this current study.

A unique strength of our study design, in addition to the ability of treatment groups to be compared across degrees of hydrolysis, was the ability to compare cholesterol metabolism between PSE containing different fatty acids. In the case of SEs and PEs, no differences were observed other than a slight lowering of liver free

cholesterol in PEs compared to SEs, a slightly lower liver weight in PEs compared to Control which was not observed in SEs, and a slight difference in the biliary concentrations of taurochenodeoxycholate between SEs and PEs. Given that, in this study, SEs and PEs were hydrolyzed to the same extent, with only the few differential metabolic effects noted above, it is likely that the fatty acid moiety of the PSE does not contribute greatly to cholesterol metabolism upon its liberation. This lends further credence to the hypothesis that the primary mechanism of PSE cholesterol-lowering effects takes place through micellar cholesterol displacement and is dependent on hydrolysis above any other physical property of the PSE.

The comparison of SEs and SEt groups was also informative in that there was no evidence of a difference in percent hydrolysis between the two, but their metabolic effects regarding cholesterol metabolism vary greatly. Notably, while SEs did not alter cholesterol absorption compared to Control, SEt lowered it by 43% as compared to Control. Also compared to Control, SEt lowered liver esterified cholesterol by 83% compared to SEs which only lowered it by 39%. The SEt treatment group was included to represent a PSE that was perfectly intact with no hydrolysis. Given our hypothesis that hydrolysis of the PSE molecule is required for cholesterol-lowering, the SEt group represents an unexpected anomaly. Given the similarity in hydrolysis of SEs and SEt with differential metabolic effects, it may be assumed that any differences in metabolism are due to a structural difference between the two molecules. In this case, the only structural difference was that of a carbonyl group present on the first carbon of the stearic acid molecule in SEs that is not present on the SEt molecule (Figures 1 H and I). As mentioned above, poorly hydrolyzed PSE such as SEs and PEs may work through an “oil

phase" mechanism. It may be possible that the SEt works through this mechanism as well, and may act as a more potent oil phase generator as a results of its unique chemistry. A mechanism that could account for the more efficient development of an oil phase is an enzymatic cross-interaction where the SEt molecule may interact with the active site of pancreatic triglyceride lipase (PTL), and competitively reduce its function. Evidence of in-vitro and in-vivo work has suggested that remodeling of luminal non-polar lipids, whereby triglycerides are hydrolyzed to free fatty acids, monoglycerides and diglycerides, is necessary for efficient cholesterol absorption. Utilizing IEC-6 cells, cholesterol from lipid emulsions was not transported to intestinal cells without lipase activity sufficient to increase the phospholipid to triglyceride ratio of the emulsion particle to greater than 0.3, further suggesting that PTL is vital for cholesterol absorption (Young and Hui 1999). (Huggins et al 2003), using PTL-/- knockout mice showed that, while triglyceride absorption was only delayed and minimally decreased in these animals compared to a wild type control, cholesterol absorption was significantly lower in the PTL-/- mice after a lipid bolus feeding, suggesting that cholesterol absorption is reliant on PTL activity for optimum efficiency. Because many lipases are considered to be promiscuous and will act on a variety of lipid substrates, it is possible that a SEt molecule may enter the lipase active site, and due to the inability of the enzyme to catalyze hydrolysis without a free carbonyl group to propagate the necessary catalytic triad in the PTL enzyme active site (Chen et al 1998, Lowe 1992), the molecule will not hydrolyze and thus may not efficiently leave the active site, blocking the entrance of other lipids. Lipids that enter the lipase active sites are immobilized during hydrolysis by a tetrahedral intermediate complex formed with the free carbonyl group. Because SEt lacks this

oxygen, it may be able to penetrate the active site further and, due to the hydrophobic nature of the lipase active sites, may not be easily released back into an aqueous environment. Taking together the data suggesting a compulsory role for PTL in cholesterol absorption and the hypothetical PTL-competition of SEt, as well as data from this current study that showed lower liver triglycerides in SEs, SEt and PEs compared to Control, an oil phase mechanism may be valid.

Biliary bile acid composition has been shown to be diet-labile. As a method of quantifying the biological significance of this compositional change, the relative hydrophobicity of each bile acid in a sample of bile, as calculated based on its migration on an HPLC column in a reverse phase system, may be used to arrive at a hydrophobicity index (Armstrong and Carey 1982, Heuman 1989). This index describes the thermodynamic favor with which a mixture of bile salts will aggregate into a micelle, with the rationale that a high index will result in greater micelle formation and greater intestinal absorption of lipid. Work involving our laboratory has shown PSE (Carr et al 2002) and free stearic acid (Cowles et al 2002) to be capable of inducing bile acid composition changes significant enough to alter the hydrophobicity index. Although the current study used the same 3% sterol equivalent of plant sterols as did Carr et al. 2002, the changes in bile acid metabolism were not great enough to alter the hydrophobicity index. This may be due to the use of a greater number of bile acids in the current analysis that may have balanced out any changes seen in the other bile acids used previously. Another difference existed between the methods used in the two studies. Currently, direct UV detection of bile acids with a different HPLC system in the current study was used as compared to the use of fluorometric detection of NAD as an enzymatic by-product,

previously. In addition, there seemed to be a high degree of variability within treatments of the current study that made detecting differences between treatments difficult. Although there were not large enough differences in bile acids to affect a hydrophobicity index change, there were differences in individual bile acids. In general, differences in individual bile acids could not be attributed to a single treatment, but it appears that ursodeoxycholate and chenodeoxycholate were most sensitive to PSE treatment as both free and tauro-conjugated ursodeoxycholate and glyco and tauro-conjugated chenodeoxycholate were altered by PSE. In general, however, this study provided no evidence that PSE supplementation works through changes in bile acid metabolism to bring about its cholesterol-lowering effects.

In summary, our findings indicate that hydrolysis of PSE was a necessary factor in order to induce an optimum cholesterol-lowering effect, suggesting that it is the free sterol molecule that is active in lowering cholesterol, and that competition with cholesterol for micellar incorporation is the primary mechanism through which plant sterols act. Additionally, poorly hydrolyzed PSE may contribute a cholesterol-lowering effect by causing the production of an oil phase either by their own aggregation, or by reducing the efficiency of other lipases. Finally, altering bile acid metabolism significantly enough to effect the thermodynamic properties of micelle formation may not be a significant mechanism through which PSE affects cholesterol-lowering.

AbuMweis SS, Vanstone CA, Ebine N, Kassis A, Ausman LM, Jones PJ *et al* (2006). 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* **136:** 1012-1016.

AbuMweis SS, Vanstone CA, Lichtenstein AH, Jones PJ (2009). Plant sterol consumption frequency affects plasma lipid levels and cholesterol kinetics in humans. *Eur J Clin Nutr* **63:** 747-755.

Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M (1996). Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* **271:** 518-520.

Acton SL, Scherer PE, Lodish HF, Krieger M (1994). Expression cloning of SR-BI, a CD36-related class B scavenger receptor. *J Biol Chem* **269:** 21003-21009.

Altmann SW, Davis HR, Jr., Zhu LJ, Yao X, Hoos LM, Tetzloff G *et al* (2004). Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science* **303:** 1201-1204.

Arias I (1988). *The Liver: biology and pathobiology*. Raven Press: New York.

Armstrong MJ, Carey MC (1982). The hydrophobic-hydrophilic balance of bile salts. Inverse correlation between reverse-phase high performance liquid chromatographic mobilities and micellar cholesterol-solubilizing capacities. *J Lipid Res* **23:** 70-80.

Assmann G, Cullen P, Erbey J, Ramey DR, Kannenberg F, Schulte H (2006). 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* **16:** 13-21.

Basu SK, Brown MS, Ho YK, Goldstein JL (1979). Degradation of low density lipoprotein . dextran sulfate complexes associated with deposition of cholesteryl esters in mouse macrophages. *J Biol Chem* **254:** 7141-7146.

Berneis KK, Krauss RM (2002). Metabolic origins and clinical significance of LDL heterogeneity. *J Lipid Res* **43:** 1363-1379.

Best MM, Duncan CH (1958). Effects of the esterification of supplemental cholesterol and sitosterol in the diet. *J Nutr* **65:** 169-181.

Bond HM, Morrone G, Venuta S, Howell KE (1991). Characterization and purification of proteins which bind high-density lipoprotein. A putative cell-surface receptor. *Biochem J* **279 (Pt 3):** 633-641.

Borgstrom B (1960). Studies on intestinal cholesterol absorption in the human. *J Clin Invest* **39:** 809-815.

Botham KM, Bravo E (1995). The role of lipoprotein cholesterol in biliary steroid secretion. Studies with in vivo experimental models. *Prog Lipid Res* **34:** 71-97.

Brissette L, Charest MC, Falstrault L (1996). Selective uptake of cholesteryl esters of low-density lipoproteins is mediated by the lipoprotein-binding site in HepG2 cells and is followed by the hydrolysis of cholesteryl esters. *Biochem J* **318 (Pt 3):** 841-847.

Brown AW, Hang J, Dussault PH, Carr TP (2010). Plant sterol and stanol substrate specificity of pancreatic cholesterol esterase. *J Nutr Biochem* **21:** 736-740.

Brown MS, Goldstein JL (1985). The receptor model for transport of cholesterol in plasma. *Ann N Y Acad Sci* **454:** 178-182.

Brunham LR, Kruit JK, Iqbal J, Fievet C, Timmins JM, Pape TD *et al* (2006). Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. *J Clin Invest* **116:** 1052-1062.

Carr TP, R.M C, Illston BJ, Stuefer-Powell CL, Gallaher DD (2002). Plant sterols alter bile acid metabolism and reduce cholesterol absorption in hamsters fed a beef-based diet. *Nutrition Research* **22:** 745-754.

Carr TP, Jesch ED (2006). Food components that reduce cholesterol absorption. *Adv Food Nutr Res* **51:** 165-204.

Chen JC, Miercke LJ, Krucinski J, Starr JR, Saenz G, Wang X *et al* (1998). Structure of bovine pancreatic cholesterol esterase at 1.6 A: novel structural features involved in lipase activation. *Biochemistry* **37:** 5107-5117.

Christiansen LI, Lahteenmaki PL, Mannelin MR, Seppanen-Laakso TE, Hiltunen RV, Yliruusi JK (2001). Cholesterol-lowering effect of spreads enriched with microcrystalline plant sterols in hypercholesterolemic subjects. *Eur J Nutr* **40:** 66-73.

Clee SM, Zwinderman AH, Engert JC, Zwarts KY, Molhuizen HO, Roomp K *et al* (2001). Common genetic variation in ABCA1 is associated with altered lipoprotein levels and a modified risk for coronary artery disease. *Circulation* **103:** 1198-1205.

Clifton PM, Noakes M, Sullivan D, Erichsen N, Ross D, Annison G *et al* (2004). Cholesterol-lowering effects of plant sterol esters differ in milk, yoghurt, bread and cereal. *Eur J Clin Nutr* **58:** 503-509.

Cowles RL, Lee JY, Gallaher DD, Stuefer-Powell CL, Carr TP (2002). Dietary stearic acid alters gallbladder bile acid composition in hamsters fed cereal-based diets. *J Nutr* **132:** 3119-3122.

Davis WW (1955). Symposium on sitosterol. III. The physical chemistry of cholesterol and beta-sitosterol related to the intestinal absorption of cholesterol. *Trans N Y Acad Sci* **18:** 123-128.

de Jong A, Plat J, Lutjohann D, Mensink RP (2008). Effects of long-term plant sterol or stanol ester consumption on lipid and lipoprotein metabolism in subjects on statin treatment. *Br J Nutr* **100:** 937-941.

Demonty I, Ras RT, van der Knaap HC, Duchateau GS, Meijer L, Zock PL *et al* (2009). Continuous dose-response relationship of the LDL-cholesterol-lowering effect of phytosterol intake. *J Nutr* **139:** 271-284.

Denke MA (1995). Lack of efficacy of low-dose sitostanol therapy as an adjunct to a cholesterol-lowering diet in men with moderate hypercholesterolemia. *Am J Clin Nutr* **61:** 392-396.

Detrano R, Guerci AD, Carr JJ, Bild DE, Burke G, Folsom AR *et al* (2008). Coronary calcium as a predictor of coronary events in four racial or ethnic groups. *N Engl J Med* **358:** 1336-1345.

Eisenberg S (1984). High density lipoprotein metabolism. *J Lipid Res* **25:** 1017-1058.

Favari E, Calabresi L, Adorni MP, Jessup W, Simonelli S, Franceschini G *et al* (2009). Small discoidal pre-beta1 HDL particles are efficient acceptors of cell cholesterol via ABCA1 and ABCG1. *Biochemistry* **48:** 11067-11074.

Feng B, Tabas I (2002). ABCA1-mediated cholesterol efflux is defective in free cholesterol-loaded macrophages. Mechanism involves enhanced ABCA1 degradation in a process requiring full NPC1 activity. *J Biol Chem* **277:** 43271-43280.

Ferreri K, Menon KM (1990). Detection of a 58-kilodalton high density lipoprotein-binding protein in the membrane fraction of luteinized rat ovaries. *Endocrinology* **126:** 2137-2144.

Francone OL, Fielding CJ (1990). Initial steps in reverse cholesterol transport: the role of short-lived cholesterol acceptors. *Eur Heart J* **11 Suppl E:** 218-224.

Galeano NF, Al-Haideri M, Keyserman F, Rumsey SC, Deckelbaum RJ (1998). Small dense low density lipoprotein has increased affinity for LDL receptor-independent cell surface binding sites: a potential mechanism for increased atherogenicity. *Journal of Lipid Research* **39:** 1263-1273.

Ginsberg HN, Barr SL, Gilbert A, Karmally W, Deckelbaum R, Kaplan K *et al* (1990). Reduction of plasma cholesterol levels in normal men on an American Heart Association Step 1 diet or a Step 1 diet with added monounsaturated fat. *N Engl J Med* **322:** 574-579.

Glomset JA (1968). The plasma lecithins:cholesterol acyltransferase reaction. *J Lipid Res* **9:** 155-167.

Glover J, Green C, Stainer DW (1959). Sterol metabolism. 5. The uptake of sterols by organelles of intestinal mucosa and the site of their esterification during absorption. *Biochem J* **72:** 82-87.

Goldstein JL, Brown MS (1974). Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J Biol Chem* **249:** 5153-5162.

Goldstein JL, Brown MS (1977). The low-density lipoprotein pathway and its relation to atherosclerosis. *Annu Rev Biochem* **46:** 897-930.

Goldstein JL, Ho YK, Basu SK, Brown MS (1979). Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci U S A* **76:** 333-337.

Goldstein JL, Hoff HF, Ho YK, Basu SK, Brown MS (1981). Stimulation of cholesteryl ester synthesis in macrophages by extracts of atherosclerotic human aortas and complexes of albumin/cholesteryl esters. *Arteriosclerosis* **1:** 210-226.

Graf GA, Yu L, Li WP, Gerard R, Tuma PL, Cohen JC *et al* (2003). ABCG5 and ABCG8 are obligate heterodimers for protein trafficking and biliary cholesterol excretion. *J Biol Chem* **278:** 48275-48282.

Green SR, Pittman RC (1991). Selective uptake of cholesteryl esters from low density lipoproteins in vitro and in vivo. *J Lipid Res* **32:** 667-678.

Grundy SM (1983). Absorption and metabolism of dietary cholesterol. *Annu Rev Nutr* **3:** 71-96.

Hallikainen MA, Sarkkinen ES, Gylling H, Erkkila AT, Uusitupa MI (2000). 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* **54:** 715-725.

Hebbachi AM, Gibbons GF (2001). Microsomal membrane-associated apoB is the direct precursor of secreted VLDL in primary cultures of rat hepatocytes. *J Lipid Res* **42:** 1609-1617.

Heinemann T, Kullak-Ublick GA, Pietruck B, von Bergmann K (1991). Mechanisms of action of plant sterols on inhibition of cholesterol absorption. Comparison of sitosterol and sitostanol. *Eur J Clin Pharmacol* **40 Suppl 1:** S59-63.

Hendriks HF, Weststrate JA, van Vliet T, Meijer GW (1999). 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* **53:** 319-327.

Hernandez-Mijares A, Banuls C, Rocha M, Morillas C, Martinez-Triguero ML, Victor VM *et al* (2010). Effects of phytosterol ester-enriched low-fat milk on serum lipoprotein profile in mildly hypercholesterolaemic patients are not related to dietary cholesterol or saturated fat intake. *Br J Nutr* **104:** 1018-1025.

Heuman DM (1989). Quantitative estimation of the hydrophilic-hydrophobic balance of mixed bile salt solutions. *J Lipid Res* **30:** 719-730.

Hofmann AF, Borgstroem B (1964). The Intraluminal Phase of Fat Digestion in Man: The Lipid Content of the Micellar and Oil Phases of Intestinal Content Obtained during Fat Digestion and Absorption. *J Clin Invest* **43:** 247-257.

Huggins KW, Camarota LM, Howles PN, Hui DY (2003). Pancreatic triglyceride lipase deficiency minimally affects dietary fat absorption but dramatically decreases dietary cholesterol absorption in mice. *J Biol Chem* **278:** 42899-42905.

Ikeda I, Tanabe Y, Sugano M (1989). Effects of sitosterol and sitostanol on micellar solubility of cholesterol. *J Nutr Sci Vitaminol (Tokyo)* **35:** 361-369.

Iqbal J, Hussain MM (2009). Intestinal lipid absorption. *Am J Physiol Endocrinol Metab* **296:** E1183-1194.

Jandacek RJ, Webb MR, Mattson FH (1977). Effect of an aqueous phase on the solubility of cholesterol in an oil phase. *J Lipid Res* **18:** 203-210.

Johnston JM, Borgstroem B (1964). The Intestinal Absorption and Metabolism of Micellar Solutions of Lipids. *Biochim Biophys Acta* **84:** 412-423.

Jones PJ, Raeini-Sarjaz M, Ntanios FY, Vanstone CA, Feng JY, Parsons WE (2000). Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters. *J Lipid Res* **41:** 697-705.

Joy TR, Hegele RA (2009). Narrative Review: Statin-Related Myopathy. *Annals of Internal Medicine* **150:** 858-868.

Katan MB, Grundy SM, Jones P, Law M, Miettinen T, Paoletti R (2003). Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. *Mayo Clin Proc* **78:** 965-978.

Katragadda S, Rai F, Arora R (2010). Dual inhibition, newer paradigms for cholesterol lowering. *Am J Ther* **17:** e88-99.

Kesaniemi YA, Witztum JL, Steinbrecher UP (1983). Receptor-mediated catabolism of low density lipoprotein in man. Quantitation using glucosylated low density lipoprotein. *J Clin Invest* **71:** 950-959.

Kobayashi M, Hamada T, Goto H, Imaizumi K, Ikeda I (2008). Comparison of effects of dietary unesterified and esterified plant sterols on cholesterol absorption in rats. *J Nutr Sci Vitaminol (Tokyo)* **54:** 210-214.

Kontush A, Guerin M, Chapman MJ (2008). Spotlight on HDL-raising therapies: insights from the torcetrapib trials. *Nat Clin Pract Cardiovasc Med* **5:** 329-336.

Lagrost L, Gandjini H, Athias A, Guyard-Dangremont V, Lallemant C, Gambert P (1993). Influence of plasma cholesteryl ester transfer activity on the LDL and HDL distribution profiles in normolipidemic subjects. *Arterioscler Thromb* **13:** 815-825.

Landschulz KT, Pathak RK, Rigotti A, Krieger M, Hobbs HH (1996). Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat. *J Clin Invest* **98:** 984-995.

Lee RG, Willingham MC, Davis MA, Skinner KA, Rudel LL (2000). Differential expression of ACAT1 and ACAT2 among cells within liver, intestine, kidney, and adrenal of nonhuman primates. *J Lipid Res* **41:** 1991-2001.

Li T, Matozel M, Boehme S, Kong B, Nilsson LM, Guo G *et al* (2011). Overexpression of cholesterol 7alpha-hydroxylase promotes hepatic bile acid synthesis and secretion and maintains cholesterol homeostasis. *Hepatology*.

Lowe ME (1992). The catalytic site residues and interfacial binding of human pancreatic lipase. *J Biol Chem* **267:** 17069-17073.

Maki KC, Davidson MH, Umporowicz DM, Schaefer EJ, Dicklin MR, Ingram KA *et al* (2001). Lipid responses to plant-sterol-enriched reduced-fat spreads incorporated into a National Cholesterol Education Program Step I diet. *Am J Clin Nutr* **74:** 33-43.

Mansbach CM (2009). The Formation and Intracellular Transport of Chylomicrons. *Immun, Endoc & Metab Agents in Med Chem* **9:** 55-59.

Matsuoka K, Nakazawa T, Nakamura A, Honda C, Endo K, Tsukada M (2008). Study of thermodynamic parameters for solubilization of plant sterol and stanol in bile salt micelles. *Chem Phys Lipids* **154:** 87-93.

Mattson FH, Volpenhein RA, Erickson BA (1977). Effect of plant sterol esters on the absorption of dietary cholesterol. *J Nutr* **107:** 1139-1146.

McNamara DJ (2000). Dietary cholesterol and atherosclerosis. *Biochim Biophys Acta* **1529:** 310-320.

Mel'nikov SM, Seijen ten Hoorn JW, Bertrand B (2004). Can cholesterol absorption be reduced by phytosterols and phytostanols via a cocrystallization mechanism? *Chem Phys Lipids* **127:** 15-33.

Miettinen TA, Siurala M (1971). Bile salts, sterols, sterol esters, glycerides and fatty acids in micellar and oil phases of intestinal contents during fat digestion in man. *Z Klin Chem Klin Biochem* **9:** 47-52.

Miller GJ, Miller NE (1975). Plasma-high-density-lipoprotein concentration and development of ischaemic heart-disease. *Lancet* **1:** 16-19.

Miller NE, Nestel PJ, Clifton-Bligh P (1976). Relationships between plasma lipoprotein cholesterol concentrations and the pool size and metabolism of cholesterol in man. *Atherosclerosis* **23:** 535-547.

Musa-Veloso K, Poon TH, Elliot JA, Chung C (2011). A comparison of the LDL-cholesterol lowering efficacy of plant stanols and plant sterols over a continuous dose range: Results of a meta-analysis of randomized, placebo-controlled trials. *Prostaglandins Leukot Essent Fatty Acids*.

NCEP (2002). Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation* **106:** 3143-3421.

Nguyen TT (1999). The cholesterol-lowering action of plant stanol esters. *J Nutr* **129:** 2109-2112.

Nissinen M, Gylling H, Vuoristo M, Miettinen TA (2002). Micellar distribution of cholesterol and phytosterols after duodenal plant stanol ester infusion. *Am J Physiol Gastrointest Liver Physiol* **282:** G1009-1015.

Noakes M, Clifton PM, Doornbos AM, Trautwein EA (2005). Plant sterol ester-enriched milk and yoghurt effectively reduce serum cholesterol in modestly hypercholesterolemic subjects. *Eur J Nutr* **44:** 214-222.

Normen L, Ellegard L, Janssen HG, Steenbergen H, Trautwein E, Andersson H (2006). Phytosterol and phytostanol esters are effectively hydrolysed in the gut and do not affect fat digestion in ileostomy subjects. *Eur J Nutr* **45:** 165-170.

O'Neill FH, Sanders TA, Thompson GR (2005). Comparison of efficacy of plant stanol ester and sterol ester: short-term and longer-term studies. *Am J Cardiol* **96:** 29D-36D.

Oram JF, Brinton EA, Bierman EL (1983). Regulation of high density lipoprotein receptor activity in cultured human skin fibroblasts and human arterial smooth muscle cells. *J Clin Invest* **72:** 1611-1621.

Peterson DW, Shneour EA, Peek NF, Gaffey HW (1953). Dietary constituents affecting plasma and liver cholesterol in cholesterol-fed chicks. *J Nutr* **50:** 191-201.

Ponz de Leon M, Loria P, Iori R, Carulli N (1981). Cholesterol absorption in cirrhosis: the role of total and individual bile acid pool size. *Gastroenterology* **80:** 1428-1437.

Pritchard PH, Li M, Zamfir C, Lukic T, Novak E, Moghadasian MH (2003). Comparison of cholesterol-lowering efficacy and anti-atherogenic properties of hydrogenated versus non-hydrogenated (Phytrol) tall oil-derived phytosterols in apo E-deficient mice. *Cardiovasc Drugs Ther* **17:** 443-449.

Rizzo M, Kotur-Stevuljevic J, Berneis K, Spinas G, Rini GB, Jelic-Ivanovic Z *et al* (2009). Atherogenic dyslipidemia and oxidative stress: a new look. *Transl Res* **153:** 217-223.

Ruckenstein E, Nagarajan R (1975). Critical Micelle Concentration. A Transition Point for Micellar Size Distribution. *The Journal of Physical Chemistry* **79:** 2622-2626.

Rye KA, Barter PJ (2004). Formation and metabolism of prebeta-migrating, lipid-poor apolipoprotein A-I. *Arterioscler Thromb Vasc Biol* **24:** 421-428.

Sahoo D, Trischuk TC, Chan T, Drover VA, Ho S, Chimini G *et al* (2004). ABCA1-dependent lipid efflux to apolipoprotein A-I mediates HDL particle formation and decreases VLDL secretion from murine hepatocytes. *J Lipid Res* **45:** 1122-1131.

Shepherd J, Bicker S, Lorimer AR, Packard CJ (1979). Receptor-mediated low density lipoprotein catabolism in man. *J Lipid Res* **20:** 999-1006.

Sierksma A, Weststrate JA, Meijer GW (1999). Spreads enriched with plant sterols, either esterified 4,4-dimethylsterols or free 4-desmethylsterols, and plasma total- and LDL-cholesterol concentrations. *Br J Nutr* **82:** 273-282.

Simmonds WJ (1972). The role of micellar solubilization in lipid absorption. *Aust J Exp Biol Med Sci* **50:** 403-421.

Siperstein MD, Chaikoff IL, Reinhardt WO (1952). C14-Cholesterol. V. Obligatory function of bile in intestinal absorption of cholesterol. *J Biol Chem* **198:** 111-114.

Slater HR, McKinney L, Packard CJ, Shepherd J (1984). Contribution of the receptor pathway to low density lipoprotein catabolism in humans. New methods for quantitation. *Arteriosclerosis* **4:** 604-613.

Spady DK, Turley SD, Dietschy JM (1985). Receptor-independent low density lipoprotein transport in the rat in vivo. Quantitation, characterization, and metabolic consequences. *J Clin Invest* **76:** 1113-1122.

Spady DK, Huettinger M, Bilheimer DW, Dietschy JM (1987). Role of receptor-independent low density lipoprotein transport in the maintenance of tissue cholesterol balance in the normal and WHHL rabbit. *J Lipid Res* **28:** 32-41.

Steinbrecher UP, Witztum JL, Kesaniemi YA, Elam RL (1983). Comparison of glucosylated low density lipoprotein with methylated or cyclohexanedione-treated low density lipoprotein in the measurement of receptor-independent low density lipoprotein catabolism. *J Clin Invest* **71:** 960-964.

Stoffel W, Knyrim K, Bode C (1983). A serum proteinase converts proapolipoprotein AI secreted by rat hepatocytes to the mature apolipoprotein. *Hoppe Seylers Z Physiol Chem* **364:** 1631-1640.

Sugano M, Morioka H, Ikeda I (1977). A comparison of hypocholesterolemic activity of beta-sitosterol and beta-sitostanol in rats. *J Nutr* **107:** 2011-2019.

Swell L, Byron JE, Treadwell CR (1950). Cholesterol esterases: IV. Cholesterol esterase of rat intestinal mucosa. *J Biol Chem* **186:** 543-548.

Swell L, Boiter TA, Field H, Treadwell JR, Treadwell CR (1956). The absorption of plant sterols and their effect on serum and liver sterol levels. *Journal of Nutrition* **58:** 385.

Talati R, Sobieraj DM, Makanji SS, Phung OJ, Coleman CI The comparative efficacy of plant sterols and stanols on serum lipids: a systematic review and meta-analysis. *J Am Diet Assoc* **110:** 719-726.

Thurnhofer H, Hauser H (1990). Uptake of cholesterol by small intestinal brush border membrane is protein-mediated. *Biochemistry* **29:** 2142-2148.

Tolman KG (2002). The liver and lovastatin. *Am J Cardiol* **89:** 1374-1380.

Trautwein EA, Duchateau GS, Lin Y, Mel'Nikov SM, Molhuizen HO, Ntanios FY (2003). Proposed mechanisms of cholesterol-lowering action of plant sterols. *Eur J Lipid Sci Technol* **105:** 171-185.

Tribble DL, Krauss RM, Lansberg MG, Thiel PM, van den Berg JJ (1995). Greater oxidative susceptibility of the surface monolayer in small dense LDL may contribute to differences in copper-induced oxidation among LDL density subfractions. *J Lipid Res* **36:** 662-671.

Truong TQ, Auger A, Denizeau F, Brissette L (2000). Analysis of low-density lipoprotein catabolism by primary cultures of hepatic cells from normal and low-density lipoprotein receptor knockout mice. *Biochim Biophys Acta* **1484:** 307-315.

Vaikousi H, Lazaridou A, Biliaderis CG, Zawistowski J (2007). Phase transitions, solubility, and crystallization kinetics of phytosterols and phytosterol-oil blends. *J Agric Food Chem* **55:** 1790-1798.

van Greevenbroek MM, de Bruin TW (1998). Chylomicron synthesis by intestinal cells in vitro and in vivo. *Atherosclerosis* **141 Suppl 1:** S9-16.

Vanstone CA, Raeini-Sarjaz M, Parsons WE, Jones PJ (2002). Unesterified plant sterols and stanols lower LDL-cholesterol concentrations equivalently in hypercholesterolemic persons. *Am J Clin Nutr* **76:** 1272-1278.

Wang N, Silver DL, Thiele C, Tall AR (2001). ATP-binding cassette transporter A1 (ABCA1) functions as a cholesterol efflux regulatory protein. *J Biol Chem* **276:** 23742-23747.

Weingartner O, Ulrich C, Lutjohann D, Ismail K, Schirmer SH, Vanmierlo T *et al* (2011). Differential effects on inhibition of cholesterol absorption by plant stanol and plant sterol esters in apoE-/- mice. *Cardiovasc Res*.

Werb Z, Cohn ZA (1972). Cholesterol metabolism in the macrophage. 3. Ingestion and intracellular fate of cholesterol and cholesterol esters. *J Exp Med* **135:** 21-44.

Westergaard H, Dietschy JM (1976). The mechanism whereby bile acid micelles increase the rate of fatty acid and cholesterol uptake into the intestinal mucosal cell. *J Clin Invest* **58:** 97-108.

Wilund KR, Yu L, Xu F, Vega GL, Grundy SM, Cohen JC *et al* (2004). No association between plasma levels of plant sterols and atherosclerosis in mice and men. *Arterioscler Thromb Vasc Biol* **24:** 2326-2332.

Young SC, Hui DY (1999). Pancreatic lipase/colipase-mediated triacylglycerol hydrolysis is required for cholesterol transport from lipid emulsions to intestinal cells. *Biochem J* **339 (Pt 3):** 615-620.

Yu L, Li-Hawkins J, Hammer RE, Berge KE, Horton JD, Cohen JC *et al* (2002). Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. *J Clin Invest* **110:** 671-680.