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The Effects of Phytosterols on Lipoprotein Particle Size

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The Effects of Phytosterols on Lipoprotein Particle Size

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Abstract

Phytosterols (PS) have become a recent popular medication alternative for treatment of hypercholesterolemia and have proven effective. Whether PS decrease risk of cardiovascular disease through other mechanisms, such as lipoprotein particle size and inflammatory markers, remains unclear. PURPOSE: The primary aim of this study was to examine the effects of two forms of PS in milk on lipoprotein particle size, inflammatory markers and fat-soluble vitamins. METHODS: Twenty subjects (13 males, 7 females; age; 55±6.1 years, height; 169±10 cm, weight; 77.9±16.9 kg, BMI; 27.3) consumed 16oz of cow's milk daily for 12 weeks. The three sequential four-week phases consisted of 2% cow's milk, 2 grams/day of 'unaided' PS in skim milk, and 2 grams/day of 'aided' triglyceride recrystallized PS (TRP) in fat-free milk. Blood was taken after each phase for NMR lipoprotein particle size analysis, lipid panel, glucose, insulin, inflammatory markers, and fat-soluble vitamins. RESULTS: Subjects maintained body weight and composition, habitual diet, and physical activity throughout the twelve weeks ($p>0.05$). Total LDL particle concentrations from NMR decreased to a greater extent with TRP (-15%) compared to unaided (-5%) PS ($p<0.05$). Serum IL-8 and MCP-1 significantly decreased with TRP ($p<0.05$) but not unaided PS. Serum tocopherols and carotenoids were found to decrease with PS, but when normalized to cholesterol, only beta-carotene was significantly decreased ($p<0.05$) but still within normal blood range limits. CONCLUSION: These data suggest that supplementation with TRP and unaided PS in milk decrease serum concentrations of LDL, but TRP may further reduce CVD risk by decreasing LDL particle concentration and inflammatory markers.

Introduction

Hypercholesterolemia is associated with increased cardiovascular disease risk, a common problem among American adults. The National Cholesterol Education Program focuses on recommendations of diet, lifestyle, medications and phytosterols to lower cholesterol, in particular LDL cholesterol ((NCEP). 2002). However, there is increasing awareness of the importance of distinguishing between different forms of LDL-C. Specifically, small dense LDL particles have been shown to be more atherogenic than larger LDL particles (Cromwell WC, 2004). Phytosterols (PS) have been shown to consistently decrease LDL cholesterol concentration by as much as 13% (Law M. 2000); however, its effects on lipoprotein particle size are inconclusive.

Phytosterols are safe, effective, plant cholesterols that have been theorized to act on lipoprotein metabolism by displacing dietary cholesterol at the site of intestinal absorption. During digestion, PS are incorporated into micelles, packaged into chylomicrons, and either absorbed back into the intestinal lumen or transported to the lymph system. A minimal amount of PS is re-esterified by ACAT inside the mucosa then absorbed PS is carried by the CM (Ikeda I 1988). The liver then secretes PS into the blood in the form of CM remnants through VLDL particles (Bhattacharyya KE 1974). PS are incorporated into VLDL particles where cholesteryl ester transfer protein (CETP) ultimately helps to determine lipoprotein size and phenotype. An increase in CTEP activity is associated with smaller, more dense particles, linked to CVD risk (Guerin M 2001).

Dietary PS have been shown most effective in reducing cholesterol when dispersed in a fat-suspension ((NCEP). 2002), consumed with meals that include fat (AbuMweiss SS 2008), and taken in doses of 1.6g/day or more (St-Onge MP 2003). A recent method of incorporating PS

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into the fat molecule of a food product has been patented, resulting in triglyceride recrystallized PS (TRP) (Perlman D. 2003). TRP has proven effective in lowering LDL cholesterol (Perlman D. 2003) but its effects on lipoprotein particle size and numbers are inconclusive.

Although PS-induced decreases in serum LDL cholesterol are consistently observed, variable results have been reported on lipoprotein subfractions with some studies showing a positive effect (Charest, Vanstone et al. 2005) and other no effect (Charest A 2004). These results may be due to the dose of PS, background dietary macronutrient intake, or PS matrix. In addition, since fat-soluble vitamins are absorbed within chylomicrons, there is a concern that PS intake may decrease their absorption and bioavailability. Moreover, once absorbed most fat-soluble vitamins circulate in the plasma in association with lipoprotein particles, which decrease with PS intake. PS effects on inflammation markers are sparse in the literature but may add benefit to lowering CVD risk. Thus, the primary purpose of this study was to examine the effects of two forms of PS, 'unaided' and 'aided (i.e., TRP), incorporated into milk on lipoprotein particle concentration. Secondary objectives of this study were to examine the effects of PS on circulating inflammation markers and fat-soluble vitamins.

Review of Literature

Low-density lipoprotein cholesterol (LDL-C) is associated with risk for heart disease ((NCEP). 2002). Dietary approaches to decrease LDL-C have focused on reducing total fat content in diets, and have proven effective ((NCEP). 2002). However, in prospective studies, lowering LDL is usually not associated with a decreased risk for heart disease (Cromwell WC 2004). The disconnect between changes in LDL-C and heart disease outcome may be due to the heterogenic nature of LDL-C. LDL-C is comprised of particles varying in size and composition that reflect intravascular processing of lipoproteins and ultimately disease risk. Therefore when examining dietary strategies to reduce heart disease, it is important to examine both concentration and qualitative characteristics of LDL-C (Cromwell WC 2007). Phytosterols (PS) are increasingly being incorporated into various foods as a method to reduce LDL-C concentration (Pedersen, Baumstark et al. 2000; Matvienko OA 2002; Devaraj S 2006; Shrestha, Volek et al. 2006), but their impact on particle size remains unclear.

Phytosterols

Phytosterols (PS) are plant cholesterols, which include a wide variety of molecules structurally similar to cholesterol through the inclusion of an extra hydrophobic carbon chain at the C-24 position. PS play a key role in cell membrane function and may exist in different forms including a saturated form called stanols, in a free form, esterified with fatty acids, or as glycosides. For many years plant sterols have been noted for their beneficial health effects, specifically blood cholesterol reducing effects (St-Onge MP 2003).

The National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATPIII) ((NCEP). 2002) and the American Heart Association have set forth dietary guidelines as a primary therapy to maintain desired LDL-C concentrations through the use of soluble fiber and PS. Since PS are more hydrophobic than cholesterol and are known to have poor solubility in water and oil (Ostlund 2002), the NCEP recommends consuming about 2 grams each day of PS

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as part of a healthy diet ((NCEP). 2002) to control hypercholesterolemia. PS are naturally found in vegetable products and is most abundant in vegetable oils, but are also found in nuts, seeds, and dried fruits. PS are not synthesized in the body and therefore need to supplied via one's diet (Ostlund 2002). The typical Western diet contains about 160mg of PS/day, whereas vegetarian and Japanese diets can contain up to 400mg of PS/day, and therefore supplementation is required to reach the recommended 2 grams per day in people with hypercholesterolemia. Dietary PS are most commonly found in the specific forms of beta-sitosterol, campesterol, and stigmasterol (Berger A 2004) comprising about 65%, 30% and 5% of intake, respectively (Ostlund 2002).

Phytosterol Mechanism of Action

Similar to cholesterol, PS are also incorporated into mixed micelles through solubilization and cross the brush border membrane by passive diffusion or by receptor mediated process (Swell L 1956). Research findings suggest that ABCG5 and ABCG8, two half-transporters of the ABC transporter family are involved in the absorption process of PS into mucosal cells. ABCG5 and ABCG8 have also been shown as full active transporters in enterocytes, involving them in the efflux of absorbed PS back to the intestinal lumen (Berge KE 2000). A minimal amount of PS is re-esterified by ACAT inside the mucosa then absorbed PS is carried by the CM (Ikeda I 1988). The liver then secretes PS into the blood in the form of CM remnants through VLDL particles (Bhattacharyya KE 1974). Four hypotheses have been put forth to explain PS-mediated cholesterol lowering: micellar solubilization, co-crystallization of cholesterol and PS from micelles, inhibition of lipases, esterases, and other enzymes involved in the sterol absorption process, and effects of activity on the brush border transport (Trautwein, Duchateau et al. 2003).

Cholesteryl ester transfer protein (CETP) facilitates intravascular lipoprotein remodeling by promoting the equimolar exchange of cholesteryl esters with triglycerides and HDL-C and apo-B lipoproteins, ultimately affecting lipoprotein phenotype and composition. Increased CETP

activity is associated with formation of small, dense LDL particles (Guerin M 2001). These changes in lipoprotein metabolism may partially explain the hypolipidemic effects of PS, however changes in lipoprotein morphology and subfraction distribution caused by PS are inconclusive.

Effects of Phytosterols on LDL-C Concentration

In appropriate concentrations, PS can be efficiently incorporated into the micelles in the intestinal lumen, displace cholesterol, and lead to precipitation with other non-solubilized PS. Incorporation of PS into lipoproteins has been shown to not affect the density gradient (Salen G 1989). Cholesterol absorption, including free cholesterol, dietary cholesterol, and cholesterol from enterohepatic circulation is strongly reduced in the presence of PS mediated through the ABC transporters and elimination in feces. A meta-analysis of 18 clinical trials incorporating phytosterols into spreads showed an average LDL-C concentration decrease of 0.33-0.50 mmol/L with variations of dose and subject population. This absolute reduction is equivalent to an 8-13% decrease in LDL concentration and improved risk for cardiovascular disease (Law M. 2000). This shows that PS are effective in lowering quantity of LDL cholesterol, however, quality of the lipoprotein particle sizes are not accounted for within these changes.

PS have been shown most effective in LDL-C concentration reduction through suspension in high-fat foods such as margarines or oils because of their strong hydrophobic nature ((NCEP). 2002). Since PS should be supplemented daily to receive maximum benefits, taste and convenience need to be taken into account when manufacturing an ideal dietary vehicle for PS delivery. More recently, PS have been incorporated into milk, yogurts, juices and baked goods so comparisons can be made between fat vs. non-fat, and solid vs. liquid mediums.

In addition to how PS are incorporated into the diet, dosing and timing needs to be taken into account. PS have been shown to impair the absorption of both biliary and dietary cholesterol, and also been shown to be most effective when consumed at the same mealtime as

foods with cholesterol (Berger A 2004). Optimal dose for cholesterol reduction results are inconclusive, but have been shown effective in doses as little as ~1.6g/day (St-Onge MP 2003). No research has concluded an optimal dose for LDL-particle size improvements to date. Some studies suggest a daily PS dose broken into multiple portions consumed throughout the day may result in greater decreases of LDL-C, however changes in LDL- particle size have not been considered (AbuMweiss SS 2008; Demonty I 2009).

LDL-C and Its Association with Heart Disease

Serum LDL-C concentration is weakly associated with heart disease (Austin MA 1990) indicating there are other factors involved. When high-density lipoproteins (HDL-C) are in optimal concentration ranges, cholesterol is transported back to the liver for excretion or re-utilization, thus acting in a protective manner. It is well accepted that HDL-C concentrations are inversely proportional to risk for disease, although a low HDL level often indicates the presence of other atherogenic factors ((NCEP). 2002). Besides chylomicrons and very low-density lipoproteins (VLDL) which also contribute to cholesterol concentration, elevated levels of serum triglycerides (TG) are an independent risk factor for atherogenesis most commonly caused by obesity and physical inactivity (Health 1998). In the presence of high TG levels, the sum of VLDL and LDL levels become a better indicator of atherogenesis than does LDL alone ((NCEP). 2002).

In addition to LDL-C, LDL particle size and LDL subfractions are now being considered as clinical markers of risk for coronary heart disease (CHD) as they are highly heterogeneous in terms of size, density, and composition (Krauss RM 1982). For example, the predominance of small, dense LDL particles, independent of LDL concentration, is associated with higher incidences of CHD (St-Pierre AC 2001) and metabolic syndrome (Reaven 2002). Specifically, these diseases are characterized by increased plasma triglycerides, apolipoprotein B, decreased HDL cholesterol, insulin resistance, high plasma glucose and insulin, and hypertension, as well as

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atherogenesis, such as long residence time in plasma, increased susceptibility to oxidation, and permeability through the endothelial barrier. Small LDL particles may be more atherogenic because they are formed as a response to elevated serum TG ((NCEP). 2002). Therefore, subtyping cholesterol particles helps to improve disease risk assessment and evaluate specific cholesterol responses to lipid therapies.

Fat-Soluble Vitamins and Phytosterol Supplementation

Many of the fat-soluble vitamins, especially carotenoids, are incorporated into a micelle, passed into the intestine, and packaged into a chylomicron. Chylomicrons are absorbed into the lymph system where they enter the systemic circulation. In a meta-analysis (Katan MB 2003) reported that plasma vitamins A and D were not affected by sterols or stanols, and vitamin levels remained stable when their concentration was expressed relative to LDL-C concentration, the primary carrier of most fat soluble vitamins in circulation. Beta-carotene levels decreased (mean change -12% when adjusted for reductions in LDL) in 14 studies, but this was not associated with any adverse health related outcomes so the clinical implications of this relative reduction in beta-carotene remain uncertain. Vitamin supplementation may be a point of consideration when implementing a cholesterol reduction regimen involving PS.

Inflammatory Markers and Phytosterol Supplementation

Although the LDL-lowering effects of phytosterols have been well documented, studies of the effects of phytosterols on inflammation markers have been sparse in the literature with inconsistent results. Inflammation is a strong risk factor for cardiovascular disease and recently animal and human studies have demonstrated some anti-inflammatory effects of phytosterols. A PS-enriched orange juice drink consumed as part of a hypocaloric diet resulted in an average 12% decrease in C-reactive protein (Devaraj S 2006). However, a study using a PS-enriched spread

for 16 weeks found no effects on antioxidant stress, endothelial dysfunction, and low-grade inflammation (de Jong A 2008).

Cholesterol Metabolism and Lipoprotein Particle Size

Cholesterol in the intestine is incorporated into a mixed micelle comprised of fatty acids, phospholipids, bile salts, and free and esterified cholesterol. Once packaged into the micelle, the cholesterol can be absorbed from the lumen. This process of cholesterol absorption is a protein-mediated process involving scavenger receptor class B type 1 (SR-B1) and cell cluster determinant antigen 36 (CD36) as facilitators. The ATP binding cassette A1 (ABCA1) also serves to efflux free cholesterol back from the enterocyte into the intestinal lumen, ultimately contributing to the change of net cholesterol absorption efficiency. Once in the mucosal cell, most cholesterol is re-esterified by Acyl-CoA cholesterol acyltransferase (ACAT) into cholesterol esters. Before being secreted to the lymph, cholesterol is incorporated into chylomicrons (CMs). CMs circulate through the thoracic lymph duct and eventually are hydrolyzed by lipoprotein lipase (LPL) forming CM remnants and taken up into the liver by LDL receptors. CMs have a short half-life and are thus cleared by LDL quickly. The liver removes circulated CM remnants and the cholesterol they carry, which modulates hepatic cholesterol biosynthesis through feedback suppression of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. The liver secretes very low-density lipoprotein (VLDL) containing triacylglycerols (TAGs) and cholesterol. TAGs from the VLDL particles are hydrolyzed by LPL leading to the formation of LDL particles. Cholesterol can then be excreted from the body through processes in the liver after steps of hydroxylation into bile acids. Through intimate interactions between triglyceride (TG)-rich lipoproteins and a series of hydrolytic and lipid exchange enzymes within the intravascular bed, small dense LDL particles are formed (Berneis KK 2002).
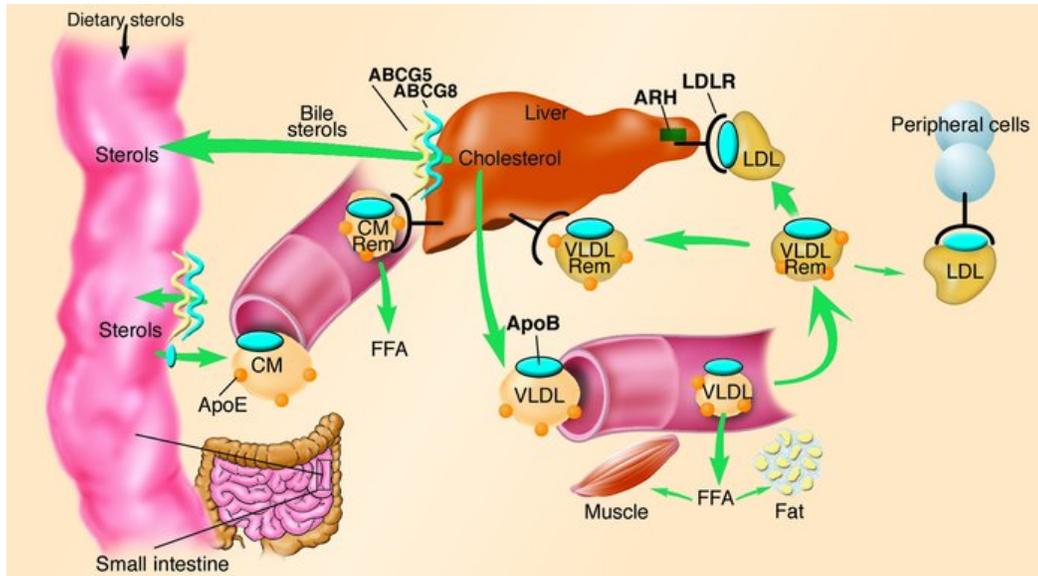
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Figure 1. Overview of LDL metabolism. (Rader DJ 2003)

TG-rich lipoproteins are heterogeneous and VLDL-1 subfractions (larger and less dense) are found in a greater proportion in people with hypertriglyceridemia or following a low-fat/high-carbohydrate diet because VLDL-1 subfractions are considered the primary precursor of the smallest LDL subfractions (Desroches and Lamarche 2004). VLDL-2 (most abundant in normolipidemic people) are thought to be the precursor of the larger LDL subfractions (Desroches and Lamarche 2004). The liver is most important in determining LDL particle size. Krauss found that increases in dietary carbohydrates from replacing dietary fat in low-fat isocaloric diets are associated with deleterious reductions in LDL size (Krauss 2001). Conversely, low-carbohydrate/ketogenic diets have reported significant increases in LDL-PPD and LDL-I levels with decreases in LDL-III subfractions and TG levels (Sharman MJ 2002; Sharman MJ 2004). This helps to explain why elevated TG levels are frequently associated with reduced LDL- PPD and increased proportion of small, dense LDL particles (Reaven 2002). Small, dense LDL particles are also commonly found in abdominally obese subjects, which is one of several dyslipidemic features of metabolic syndrome (Reaven 2002). Overall, research suggests particle sizes are easily interchangeable and highly dependent on diet.

Effects of Phytosterols on Lipoprotein Particle Size

Matvienko *et al.* (Matvienko OA 2002) showed in 34 mildly hypercholesterolemic men that the daily consumption of 2.7g/day of PS added to ground beef had no effect on LDL-Peak Particle Diameter (PPD) compared with placebo, suggesting the decrease in LDL-C was primarily due to a decrease in particle number. Charest *et al.* (Charest, Desroches et al. 2004) incorporated unesterified plant sterols and stanols into the butter component of diets and found the treatments did not induce significant changes in LDL-PPD analyzed by GGE, although a decrease in LDL-C was found. Charest also noted that the significant reduction in LDL cholesterol levels with stanol supplementation was largely attributed to a reduction in the cholesterol levels within the large LDL subfraction (Charest, Desroches et al. 2004). Varady et al. (Varady, St-Pierre et al. 2005) supplemented PS for 8 weeks and found significant decreases in the estimated cholesterol concentrations within small, medium, and large LDL particles but found no effect on the distribution of cholesterol among various LDL particle sizes. Since TG and LDL are mechanistically linked and correlate at high level, research has attributed evidence of no change in plasma TG levels with consumption of phytosterols (Matvienko OA 2002; Vastone CA 2002) as the lack of effect on LDL-PPD (Matvienko OA 2002; Charest A 2004).

Even with the lack of effect of phytosterols on LDL size phenotypes (Charest A 2004; Charest, Vanstone et al. 2005), Desroches et al. found a method of dietary manipulation using PS may cause a shift in LDL particles towards phenotype A particles (Desroches S 2004), thus reducing risk of CVD. St-Onge et al. (St-Onge, Lamarche et al. 2003) compared the effects of consuming a functional oil rich in PS and a medium-chain TG oil versus olive oil. A significant reduction in plasma cholesterol levels with the functional oil was complemented by a higher LDL-PPD when compared with the olive oil control. Additionally, a reduction in the cholesterol content of mid-sized and small LDL particles was found in the functional oil when compared with the olive oil control. Shrestha, *et al.* studied PS in combination with psyllium, a dietary fiber. Results found significant decreases in the number of smaller subfractions of LDL and HDL

particles, resulting in a less atherogenic lipoprotein profile in a free-living human trial using treatment cookies containing 2.6g/day of PS (Shrestha, Volek et al. 2006).

In a combination effect study, Lamarche et al. (Lamarche, Desroches et al. 2004) assessed the effects of plant sterols, soy protein, soluble fibers, and almonds on LDL size and phenotype in 12 mildly LDL-hypercholesterolemic people. Along with a 30% reduction in plasma LDL-C levels, concurrent reductions of cholesterol concentrations of large (-30%), medium sized (-29%) and small LDL particles (-21%) were found. These favorable changes, through consuming combination of foods and dietary components, can potentially contribute to an overall lower risk of CVD (Lamarche, Desroches et al. 2004).

Although only a limited number of studies have tested the effects of PS on lipoprotein characteristics, current evidence supports the hypothesis that sterol supplementation has no effect on LDL particle size or distribution, although research supports a decrease in concentration of cholesterol in the large subfractions. More research is needed looking at ranges of PS supplementation doses and particle characteristics, especially using the more novel technique of NMR analysis. In addition, multiple methods of PS incorporation into commercial products have been patented, yet their effects on lipoprotein particle sizes are not yet defined.

HDL and VLDL Particle Size

In addition to assessment of LDL subfractions, there may be value in determining sizes of HDL and VLDL. Through nuclear magnetic resonance spectroscopy (NMR) developed by Otvos and colleagues, HDL particles can be classified as large (10-13nm), intermediate (8.2 to 10nm) and small (7.3 to 8.2nm) particles. These NMR determined levels closely correlate with those determined by GGE or electron microscopy measurements separating HDL particles into classes largest to smallest or HDL_{2b}, HDL_{2a} or HDL_{3a}, and HDL_{3b} or HDL_{3c} respectively (Otvos JD 1991; Otvos JD 1992). Levels of small HDL particles (7.3 to 8.2nm or HDL_{3b} and HDL_3) are positively related to coronary artery disease risk (Wilson HM 1990; Cheung MC 1991). VLDL particles

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have a similar level of classification: chylomicrons (diameter >100nm) large VLDL and remnants

(60 to 100nm), intermediate VLDL (40 to 60nm), and small VLDL (30 to 40nm) (Otvos JD 1991)

(Otvos JD 1992). Literature is inconclusive on the particular size to which VLDLs are most

health beneficial. Reports have suggested that smaller, TG-rich lipoproteins are more

atherogeneic (Havel 1990; Hodis HN 1995). However, large VLDL particles (and/or chylomicron

remnants) are precursors to small LDL particles and directly proportional to CAD severity as well

(Freedman DS 1998).

LDL Particle Size and Disease Risk

Rizzo published a review on 33 cross-sectional epidemiologic, 18 prospective

epidemiologic, and 8 clinical intervention trials of which the relationships of LDL particle size

and CHD risk were examined. Of the 33 cross-sectional studies analyzed, 25 established a

significant univariate correlation between small-sized LDL particles and CHD. From the 20

cross-sectional studies that utilized multivariate analysis of small LDL size in relation to CHD, 12

trials showed the correlation was not independent of triglycerides and/or HDL-C levels.

Additionally, 16 of the 18 prospective epidemiologic trials reported a significant univariate

correlation of LDL size or density with CHD risk (Rizzo M 2006). This review of the literature

suggests that smaller LDL-C particles correlate highly with increased risk of CVD.

Methods of Determining LDL Particle Size

Several methods of analysis have been used to characterize subpopulations of

lipoproteins, such as nuclear magnetic resonance spectroscopy (NMR), various types of

electrophoresis, ultracentrifugation, and ion mobility. As mentioned, each method has their own

labeling system of identifying particle sizes, however, methods have been closely correlated for

accuracy (Otvos JD 1992). NMR is a single, rapid way to classify all lipoprotein particles based

on size and density. NMR analysis determines particle size within a given range based on the

intensity of a signal given from the particle, which is proportional to its bulk lipid mass concentration given in moles of particles per liter (Otvos JD 1991). With NMR, intermediate-density lipoproteins (IDLs) are included in the quantification of large LDL. This method is accurate and complete and therefore, was the method chosen for the following study.

Using nondenaturing polyacrylamide gradient gel electrophoresis (PAGGE) LDL cholesterol can be separated into seven density subfractions with particle diameters ranging between 22 and 27.2 nm (Krauss RM 1982; Nicholas AV 1986). Subclasses are broken down into the following: LDL-I (27.2-28.5 nm), LDL-IIa (26.5-27.2 nm), LDL-IIb (25.6-26.5 nm), LDL-IIIa (24.7-25.6 nm), LDL-IIIb (24.2-24.7 nm), LDL-IVa (23.3-24.2 nm) and LDL-IVb (22.0-23.3 nm) based on mean peak particle diameter (PPD), sometimes measured in angstroms (Å). Furthermore, particles are divided into phenotype A (large, buoyant LDL particles with diameters >25.5nm or 262 Å or greater) or phenotype B (small, dense particles <25.5nm or 257 Å or less) (St-Pierre AC 2001).

St-Pierre found that high relative and absolute levels of LDL with a diameter less than 25.5nm are associated with a four to six fold increase in the 5-year relative risk of developing CVD (St-Pierre AC 2001). The same researchers also found that high relative and absolute levels of LDL with a diameter of less than 25.5nm are associated with a four to six-fold increase in the 5-year relative risk of CVD including TG and LDL cholesterol levels as well as LDL-PPD (St-Pierre AC 2001). This suggests the concentration and type of cholesterol circulating is important for health, not just total cholesterol numbers.

Density gradient ultracentrifugation (DGU) through vertical auto profile (VAP) technology typically classifies 6 distinct bands of the LDL-C particle based on densities: LDL-1 being the most buoyant and LDL-6 being the most dense (Krauss RM 1982; Chung BH 1986). LDL-1 and LDL-2 are closely linked to pattern A particles, whereas LDL-3 and LDL-4 mainly comprise pattern B particles (Chung BH 1986).

A new method for determining lipoprotein subfractions is through high-resolution ion mobility (Berneis KK 2002). This method uses an ion separation or particle detector system, which separates and counts particles of all sizes based on laminar airflow in an electric field. The major subfraction categories are the same as described by Berneis and Krauss (Berneis KK 2002) and therefore easily comparable. The lipoprotein sizing of each method is summarized in Figure-2.

	Smaller Lipoprotein Particles				**Larger Lipoprotein Particles**	
Nuclear Magnetic Resonance (NMR) †						
LDL Particles		Pattern B			Pattern A	
		L1		L2	L3	
HDL Particles	H1	H2		H3	H4	H5
VLDL Particles		V1	V2	V3 V4	V5	V6
Gradient Gel Electrophoresis* (PAGGE)						
LDL Particles	Pattern B		Intermediate		Pattern A	
		IVb IVa IIIb		IIIa	IIb	IIa	I
HDL Particles		3c		3b	3a	2a	2b
VLDL Particles	Not measured by this method					
Density-gradient Ultracentrifugation (DGU) ‡						
LDL Particles		Pattern B		Pattern A/B		Pattern A
		LDL 4		LDL3	LDL2	LDL1
HDL Particles		HDL 3 (d,c,b,a)		HDL2 (a,b,c)		
VLDL Particles	VLDL 3b		VLDL 3a			VLDL 1+2

Figure 2. Lipoprotein subclasses determined by different commercial methods. Modified from (Bayes HE 2003) *As performed by Berkeley HeartLab Inc.; †as performed by LipoScience; ‡as performed by Atherotech.

Currently, medications have been used as a primary source of managing hypercholesterolemia in otherwise healthy adults. With knowledge of how food additives may safely combat hypercholesterolemia, phytosterols have been a popular focus of recent research.

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Many studies have been conducted using phytosterols in different doses suspended in a fat-emulsion, specifically looking at LDL-C lowering effects. Little emphasis has been put on investigating phytosterols in a non-fat matrix, specifically its effects beyond LDL cholesterol concentration such as qualitative features of lipoproteins and inflammatory markers. The primary aim of this study was to examine the effects two plant sterol preparations, a free 'unaided' plant sterol in skim milk and a triglyceride recrystallized phytosterol (TRP) in a fat-free milk, on circulating markers of lipoprotein particle size and distribution, inflammatory markers, and fat soluble vitamin levels.

Methods

Screening

The University of Connecticut Institutional Review Board approved the study protocol. Subjects were recruited through email list-serves, word of mouth, and posted flyers around the local area. Subjects initially completed screening questionnaires for background information on their medical, exercise and diet history. Women also completed a menstrual history questionnaire regarding information about hormonal supplements and symptoms of menopause to ensure they were postmenopausal. A fasting blood sample was obtained for determination of cholesterol levels. Subjects with blood cholesterol levels between 100 and 129mg/dL were enrolled. Subjects with blood cholesterol between 130 and 215mg/dL were required to obtain written consent from their primary care physician for permission to enroll in this study. Forty-four moderately hyperlipidemic men and post-menopausal women ages 35-70 years old volunteered for screening, which consisted of a fasted lipid panel and complete metabolic panel after reading and signing a written informed consent for participation. Thirty subjects were enrolled secondary to a qualifying LDL-Cholesterol ranging between 100-215mg/dL. Exclusion criteria included individuals who were lactose intolerant, had a medical history of Type 1 or Type 2 Diabetes Mellitus, kidney, liver or any other metabolic or endocrine disorder, or currently using tobacco products. Subjects who took supplements known to affect serum lipid levels (i.e. anabolic steroids, fish oil, niacin, psyllium fiber) or inflammation (i.e. aspirin) were asked to discontinue use to allow for washout of any metabolic effects or, in some cases if used chronically, they were to maintain their habitual use. Subjects needed to be weight stable for the last 3 months, not taking any anti-inflammatory medications, not highly trained or currently exceptionally active, not using tobacco products, and not taking any other lipid or cholesterol medications. Subjects completed questionnaires regarding habitual exercise and nutrition prior to starting the study and asked to keep aspects of current diet and exercise regimen consistent.

Study Design

This was a 12-week intervention study consisting of three sequential 4-week phases during which time all subjects consumed 16 oz/day of bovine milk. This was an open-label trial study where researchers and subjects were aware of milk they were consuming each day. During Phase 1 all subjects consumed 2% milk with no plant sterol, which served as the control period. During Phase 2 all subjects consumed skim milk with 2 g/day of unaided phytosterols, and during Phase 3 they consumed 2 g/day of TRP in fat-free milk. Testing occurred before and after each phase and included fasting blood draws for biochemical analysis (Figure 3).

Milk Supplementation

All milk was provided to subjects. The 2% milk was purchased from Mountain Dairy (Storrs, CT). The milk with added phytosterols for Phase 2 (skim milk) and 3 (fat-free milk) was provided GFA Brands Inc. (Paramus, New Jersey). The PS in Phase 3 were added as described by Patent US 6,638,547 B2 (Perlman D. 2003). Non-esterified phytosterols were added 'unaided' were found to have the surprising property of decreasing the oxidation of fat in the emulsion even when heated by scavenging for free radicals and peroxidases commonly found in rancid fat. The phytosterols were heated to ~230°F and then again to 285°F in soybean oil and then cooled, forming triglyceride recrystallized phytosterol particles in the fat molecule of the milk. This method adds stability to the product.   The final nutrient composition of the milk and plant sterol preparations is provided in Table 1, determined by NutritionistPro™.

Table 1. Milk composition per 8oz serving characteristics supplied to subjects in the three phases*.

	Phase 1 (wk 1-4)	Phase 2 (wk 5-8)	Phase 3 (wk 9-12)
	2% Milk (244.0 gm)	**2 g UP in Skim Milk** (245.0 gm)	**2 g TRP in Fat-Free** (245.0 gm)
Kilocalories	122	83.3	83.3
Protein (g)	8.052	8.257	8.257
Carbohydrate (g)	11.712	12.152	12.152
Fat (g)	4.831	0.196	0.196
Cholesterol (mg)	19.52	4.9	4.9
Saturated Fat (g)	3.067	0.137	0.137

*Subjects drank each supplement twice daily

Subjects were instructed to consume 8oz of milk at 2 different meal times (i.e. breakfast and dinner) or with food, and record times on a log. Subjects were asked to visit the Human Performance Lab at the University of Connecticut weekly to get weighed on a digital scale (OHAUS Corp., Fordham Park, NJ). Subjects answered questions regarding diet and exercise status and changes. Subjects were questioned about any over-the-counter medications taken in the last week, received their new milk supply, and were verbally challenged as to when the milk was consumed. Each week, a new milk log was given to subjects to record the date and time of each supplement taken. Completed logs were collected from subjects each week. Twenty subjects successfully completed the 12-week intervention and ten withdrew. Those who did not complete, the reasons for attrition included time commitment or initiation of medication that would affect cholesterol variables.

Diet Records and Body Composition

Subjects were asked to follow their habitual diet and maintain body weight throughout the 12-week intervention. They were given specific instructions for completing a detailed 3-day diet record and 1-day diet recall in each phase. Diets were scrubbed with subjects at visits and then analyzed by Registered Dietitians for macronutrient composition using nutrition analysis software of NutritionistPro[TM] (Axxya Systems, Stafford, TX).

Body weight was measured weekly and recorded to the nearest 0.1 kg on a digital scale (OHAUS Corp., Fordham Park, NJ) for each subject. Height was recorded at the baseline via tape measurer and recorded to the nearest 0.1cm. Body composition was measured by dual-energy X-ray absorptiometry (DEXA) (Prodigy, Lunar Corporation, Madison, WI) at baseline, and after each 4-week phase and analyzed by the same technician. All females were given a pregnancy test (Quidel Corporation, San Diego, CA) through urine sample prior to each DEXA scan for safety purposes.

Blood Draws and Analysis
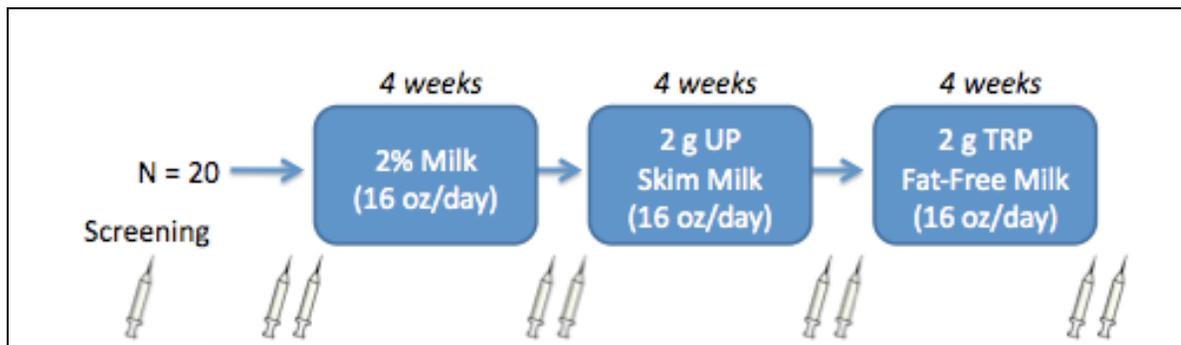
Figure 3. Experimental timeline. Subjects drank 16oz of prescribed milk daily through the 12-week study with duplicate cholesterol blood draws taken every 4 weeks.

Approximately 46mL of blood was obtained (in total) from an anticubital vein into serum, EDTA, serum separator, and sodium citrate tubes by a trained phlebotomist after subjects rested quietly for 10min in the supine position. All blood draws were performed after a 12-hour fast and 24 hour abstinence from intense exercise, alcohol consumption and over-the counter medications. A third blood draw of 10mL in a serum separator tube was taken if duplicate LDL values varied by more than 15%. Whole blood was collected, centrifuged at 1529 x g for 15 minutes at 4°C, and stored in -80°C freezer until analysis. Total cholesterol (TC), HDL-C, TAG, and calculated LDL-C concentrations were determined by Quest Diagnostics (Wallingford, CT). The liquid glucose hexokinase assay was performed on plasma samples, as reported by Folin and

Slein (Folin O 1919; Slein M.W. 1965). First, 3µL glucose standard (Thermo Scientific, Rockford, Illinois, L7517-STD) or vortexed plasma was added to a COSTAR 96-well plate. Glucose hexokinase reagent (300 µL, Thermo Scientific, L7517) was added to the 96-well plate and incubated at 37°C for 3 minutes. The absorbance was then read at 340 nm on a Molecular Devices VERSAmax tunable microplate reader. Insulin was analyzed by ELISA (ALPCO, Salem NH), with sensitivity of 5umol/L, intraassay coefficient of variation (CV) of 7.8% and interassay CV of 11.2%. The assay absorbance was read at 450 nm on a Molecular Devices VERSAmax tunable microplate reader.

Lipoprotein particle size was determined using NMR (LipoScience, Raleigh, NC). This method has best been described by Otvos and colleagues (Otvos JD 1992), and consists of three steps: (1) acquisition of 250-MHz proton NMR spectra of the plasma specimens (0.5 mL, stored at 4°C for up to 5 days) at 45°C , with a Bruker WM-250 spectrometer; (2) deconvolution of the lipid methyl group signal envelope appearing in these spectra at ~0.8ppm, yielding the derived signal amplitudes broadcast by 18 modeled lipoprotein subclasses; and (3) conversion of these signal amplitudes to lipoprotein subclass concentrations by using experimentally determined standards to their chemically measured cholesterol and TG concentrations (Freedman DS 1998).

High Sensitivity C-reactive protein (hs-CRP) was determined by Cobas® (Roche). Plasma for the determination of the acute-phase reactant hs-CRP was mixed with diluents to provide optimum pH and ionic strength for the formation of antigen-antibody complexes. The mixture was then added to a suspension of latex-beads coated with specific antibody to human CRP and the degree of light scattering determined on a Dade-Behring Model 2400 nephelometer (Marburg, Germany). The detection limit of the method is 0.15 mg/L, and the method is linear up to concentrations of 200 mg/L. Interleukin-6 (IL-6), Interleukin-8 (IL-8), monocyte chemotactic protein-1(MCP-1), tumor necrosis factor-alpha (TNF-a), and vascular endothelial growth factor (VEGF) were measured by Luminex® IS 200 system (Luminex, Austin, TX) using xMAP®

technology (Vignali 2000; Zhang JZ 2008) with corresponding antibodies from Millipore Corporation (Billerica, MA, USA) MILLIPEX™ MAP kit from EDTA plasma according to manufacturer's instructions.   Data was analyzed using Beadview software v1.0 (Upstate Cell Signaling Solutions, Temecula, CA). Standard curves for known concentrations of recombinant human cytokines were used to convert median fluorescence intensities (MFI) to cytokine concentrations in pg/ml. IL-6 had a sensitivity of 0.3pg/mL and CV of 7.9% and 11.2%. IL-8 had a sensitivity of 0.2 pg/mL and CV of 6.8% and 11.6%. MCP-1 had a sensitivity of 0.9pg/mL and CV of 5.8% and 11.2%. TNF-a had a sensitivity of 0.1 and CV of 8.5% and 12.9%. VEGF had a sensitivity of 5.9pg/mL and CV of 4.8% and 6.9%.

Vitamin E (as alpha- and gamma-tocopherol) was measured by High Performance Liquid Chromatography (HPLC)-Coularray (ESA INC., Chelmsford, MA). Plasma samples for HPLC analysis were prepared in a room that was protected from direct light as previously described (Clark RM 2006). 200 µl of plasma was mixed with 200 µl of ethanol containing 0.01% BHT and 50 µl of 0.48 µM β-Apo-8'-carotenal (internal standard). The mixture was vortexed for 30 s and then extracted twice with 2 mL of hexane containing 0.01% BHT. Samples were centrifuged at 1000 x g for 5 min at 4 ℃. The hexane layers were combined and evaporated under N_2 gas. The residues were re-constituted in 100 µl of mixture of methanol and ethyl acetate (v/v: 50/50). Carotenoids (lutein, zeaxanthin, β-carotene and lycopene) were analyzed by HPLC-UV (Shimadzu Scientific, Marlborough, MA) as described with minor modifications (Kean EG 2008). The separation of carotenoids was achieved using a YMC carotenoid reverse phase (2.0 x 250 nm, 3µ) polymeric C30 column and a gradient method. Mobile phase A consisted of 1M ammonium acetate in 98% methanol and ethyl acetate was used as mobile phase B. The following gradient profile was performed: 0-10 min, linear gradient from 20-40% B; 10-16 min, linear gradient from 40-99% B; 16-22 min, 99%B; 22-23 min, 99-20%B; 23-30 min, 20%B. A total flow rate of 0.32 ml/min was utilized and 5 µl of sample was injected. Both internal standard and carotenoids were detected at 450 nm.

Statistical Analysis

Dietary composition and body composition results were analyzed using a Phase x Variable repeated measures analysis of variance (ANOVA). NMR lipoprotein results were analyzed using a one-way ANOVA for phases 1, 2, and 3. Fat-soluble vitamins, alone and normalized to cholesterol in mmol/L and inflammation markers were analyzed using a one-way ANOVA. Confidence intervals (95%) and effect size using Cohen's d were calculated on selected values. Significance was set at $p \leq 0.05$ for all results. All table values represent average ± standard deviation unless otherwise stated.

Results

Subjects

The descriptive characteristics of the 20 subjects (13 males, 7 females) are shown in Table 2. On average subjects were overweight with elevated serum total cholesterol and LDL-C but normal HDL-C, triglyceride, glucose and insulin levels. The average subject was enrolled for 85 ± 3.4 days. Compliance to the milk supplementation was 98% based on records turned in.

Table 2. Descriptive characteristics of the 20 completed subjects; 13 males, 7 females.

Subject Characteristics	Mean ± Standard Deviation n=20
Age (yr)	55.0 ± 6.1
Height (cm)	169 ± 10
Weight (kg)	77.9 ± 16.9
Waist circumference (cm)	87.7 ± 11.6
BMI	27.3 ± 5.9
Total Cholesterol (mg/dL)	223.6 ± 31.1
LDL-C (mg/dL)	138.4 ± 22.9
HDL-C (mg/dL)	63.9 ± 21.1
Triglycerides (mg/dL)	106.9 ± 67.7

Mean daily energy and macronutrient intakes are summarized in Table 3. Dietary intake did not change significantly (p >0.05) throughout the intervention in the macronutrient categories or selected compositions of saturated fat, cholesterol, and fiber, represented in Table 3.

Changes in body composition are presented in Table 4. Body composition did not change significantly (p>0.05) throughout the intervention. Waist circumference increased on average 0.5% from Phase 1 to Phase 3, but was not significant.

Table 3. Diet record and recall averages during each phase for the 20 completed subjects. Averages do not include the twice-daily 8oz milk supplements. Statistical analysis found no significant differences (p >0.05) across phases for subjects

Averages for n=20	Phase 1 (wk 1-4)	Phase 2 (wk 5-8)	Phase 3 (wk 9-12)		
	2% Milk	**2 g UP in Skim Milk**	**2 g TRP in Fat-Free Milk**	**Δ (P3-P1) Absolute**	**Δ (P3–P1) Percent**
Kilocalories	1761 ± 639	1914 ± 814	1916 ± 664	155	8.8%
Protein (g)	81 ± 30	81 ± 28	86 ± 27	5	6.1%
Carbohydrate (g)	190 ± 62	206 ± 99	209 ±98	19	10%
Fat (g)	69 ± 36	76 ± 39	77 ± 28	8	11%
Cholesterol (mg)	308 ±229	280 ±191	329 ± 169	21	6.8%
Saturated Fat (g)	25.1 ± 15.5	26.3 ± 16.1	25.7 ± 9.4	0.6	2%
Fiber (g)	14.8 ± 6.4	17.5 ± 7.5	18.2 ±6.0	3.4	22%

Table 4. Dual-Energy X-Ray Absorptiometry results from the 20 completed subjects. Statistical analysis found no significant differences (p >0.05) across phases for subjects.

Averages for n=20	Phase 1 (wk 1-4)	Phase 2 (wk 5-8)	Phase 3 (wk 9-12)		
	2% Milk	**2 g UP in Skim Milk**	**2 g TRP in Fat-Free Milk**	**Δ (P3–P1) Absolute**	**Δ (P3–P1) Percent**
Body Mass (kg)	78.2 ± 17.1	78.2 ± 17.0	78.1 ± 17.0	-0.1	0.00%
Fat Mass (kg)	24.7 ± 10.5	24.6 ± 10.7	24.9 ± 10.9	0.2	0.00%
Lean Body Mass (kg)	50.7 ± 11.6	50.8 ± 11.7	50.4 ± 11.5	-0.3	0.00%
% Body Fat	31.0 ± 10.0%	30.9 ± 10.9%	31.2 ± 10.4%	0.2%	0.00%
BMC (g)	2831 ± 714	2830 ± 708	2835 ± 724	4	0.00%

Table 5. Summary of NMR lipoprotein data analysis results

	Phase 1 (wk 1-4)	Phase 2 (wk 5- 8)	Phase 3 (wk 9-12)				
	2% Milk	2 g UP in Skim Milk	2g TRP in Fat-Free Milk	Avg Δ (P2-P1)*	Avg Δ (P3-P1)*	P-Value^a	Cohen's D
VLDL & CMs	69.7 ± 45.2	63.3 ± 42.0	60.0 ± 37.3	-9.2%	-13.9%	0.358	
Large VLDL & CMs	1.8 ± 3.2	3.7 ± 5.9	2.9 ± 5.0	103.4%	60.1%	0.109	
Medium VLDL	21.7 ± 21.1	25.7 ± 23.7	19.4 ± 17.3	18.2%	-10.5%	0.227	
Small VLDL	46.1 ± 26.6	33.9 ± 17.3^b	37.7 ± 18.9	-26.5% (1.6-18.2)	-18.4% (-2.7-11.5)	0.026	0.23
Total LDL	1372.3 ± 366.6	1305.8 ± 453.0	1251.2 ± 408.3^b	-4.8% (-14.0-186.7)	-8.8% (86-256)	0.022	0.68
Total IDL	55.6 ± 56.5	41.8 ± 56.8	48.1 ± 62.8	-24.7%	-13.4%	0.502	
Large LDL	647.4 ± 354.7	554.5 ± 264.9^b	551.8 ± 287.6^b	-14.3% (12-154)	-14.8% (20-162)	0.008	0.35
Small LDL	669.4 ± 570.3	709.4 ± 557.7	651.4 ± 569.7	6.0%	-2.7%	0.607	
Medium Small LDL	141.2 ± 126.3	148.1 ± 123.3	140.3 ± 127.0	4.9%	-0.7%	0.744	
Very Small LDL	528.2 ± 446.3	561.3 ± 435.6	511.1 ± 443.7	6.3%	-3.2%	0.586	
Total HDL Particles	35.2 ± 5.95	34.8 ± 5.2	34.2 ± 4.9	-1.2%	-3.0%	0.407	
Large HDL	10.4 ± 5.4	9.5 ± 5.2	9.6 ± 4.7	-8.6% (-0.9-1.8)	-8.1% (0.02-2.6)	0.094	0.27
Medium HDL	3.7 ± 5.2	3.6 ± 3.2	5.0 ± 4.3	-2.1%	35.5%	0.226	
Small HDL	21.1 ± 4.9	21.6 ± 3.7	19.6 ± 4.0	2.6%	-7.2%	0.191	
VLDL size	48.1 ± 6.7	49.7 ± 6.70	51.3 ± 10.7	3.3%	6.6%	0.270	
LDL size	21.4 ± 1.0	21.3 ± 0.9	21.4 ± 0.9	-0.4%	-0.2%	0.546	
HDL size	9.2 ± 0.5	9.2 ± 0.5	9.2 ± 0.5	-0.9%	-0.6%	0.191	
Total TGs	114.0 ± 59.3	124.0 ± 78.9	113.2 ± 76.0	8.8%	-0.7%	0.373	
Total VLDL, TGs, CMs	69.2 ± 56.8	83.4 ± 77.2	72.7 ± 70.0	20.5%	5.0%	0.266	
HDL-C total	61.9 ± 20.5	59.0 ± 17.3^b	59.0 ± 17.3^b	-4.6% (0.26-6.1)	-4.5% (-0.3-6.2)	0.016	0.15

^{a}1-Way ANOVA using Phase 1, 2 and 3. ^{b}P<0.05 from corresponding 2% Milk value. ^{c}P<0.05 from corresponding Phase 2 value. *Numbers in parenthesis represent 95% Confidence Intervals. CMs (Chylomicrons); TGs (Triglycerides).

Table 6. Serum glucose, insulin, and inflammatory marker results.

	Phase 1 (wk 1- 4)	Phase 2 (wk 5-8)	Phase 3 (wk 9-12)				
	2% Milk	2g UP in Skim Milk	2g TRP in Fat-Free Milk	Avg Δ (P2-P1)	Avg Δ (P3-P1)	P-Value[a]	Cohen's D
Glucose (mg/dL)	95.2 ± 8.7	95.4 ± 9.9	95.2 ± 8.7	0.2%	0.0%	0.982	
Insulin (pmol/L)	86.0 ± 39.7	87.0 ± 37.9	85.2 ± 29.6	1.2%	-1.0%	0.841	
IL-6 (pg/mL)	9.2 ± 8.0	8.6 ± 7.4	9.0 ± 8.5	-5.9%	-2.0%	0.772	
IL-8 (pg/mL)	22.9 ± 17.3	22.9 ± 19.2	20.3 ± 16.3[b]	0.0% (-2.4-2.4)	-11.2% (-1.0-6.2)	0.057	0.15
MCP-1 (pg/mL)	590 ± 309	575 ± 296	477 ± 259[b]	-2.6% (-72-103)	-19.1% (16-208)	0.027	0.38
TNF-a (pg/mL)	18.2 ± 13.6	18.2 ± 14.0	18.5 ± 16.5	-0.1%	1.7%	0.935	
VEGF (pg/mL)	352 ± 263	327 ± 251	292 ± 254	-7.2%	-17.0%	0.315	
CRP (mg/L)	0.20 ± 0.14	0.19 ± 0.13	0.16 ± 0.11	-3.7%	-21.4%	0.303	

[a]1-Way ANOVA using Phase 1, 2 and 3. [b]P<0.05 from corresponding 2% Milk value. [c]P<0.05 from corresponding Phase 2 value. *Numbers in parenthesis represent 95% Confidence Intervals. IL-6 (Interleukin-6); IL-8 (Interleukin-8); MCP-1 (Monocyte Chemoattractant Protein-1); TNF-a (Tumor Necrosis Factor-alpha); VEGF (Vascular Endothelial Growth Factor); CRP (C-Reactive Protein).

Table 7. Serum fat-soluble vitamin data. Values are shown in absolute numbers and normalized to cholesterol in mmol/L or nmol/L, respectively.

	Phase 1 (wk 1- 4)	Phase 2 (wk 5- 8)	Phase 3 (wk 9-12)		
	2% Milk	2g UP in Skim Milk *	2g TRP in Fat- Free Milk *	P-Value[a]	Cohen's D
Alpha Tocopherol (umol/L)	30.4 ± 9.2	28.0 ± 7.4^b (467-4316)	28.0 ± 8.2^{bc} (676-4129)	0.009	0.27
Alpha Tocopherol (normalized)	0.005 ± 0.001	0.005 ± 0.001	0.005 ± 0.001	0.764	
Gamma Tocopherol (umol/L)	1.39 ± 0.74	1.48 ± 0.73	1.37 ± 0.61	0.895	
Gamma Tocopherol (normalized)	0.000 ± 0.00	0.000 ± 0.00^b	0.00 ± 0.00	0.447	
Lutein (nmol/L)	483 ± 210	421 ± 157^b (22-102)	403 ± 153^{bc} (33-126)	0.002	0.43
Lutein (normalized)	0.000 ± 0.00	0.000 ± 0.00	0.00 ± 0.00	0.083	
Zeaxanthine (nmol/L)	68 ± 29	56 ± 29^b (-1.5-24.4)	55 ± 24^b (-0.79-25.5)	0.049	0.46
Zeaxanthine (normalized)	0.000 ± 0.00	0.000 ± 0.00	0.00 ± 0.00	0.999	
Beta-Carotene (nmol/L)	1039 ± 514	845 ± 452^b (55-330)	727 ± 365^{bc} (198-426)	0.000	0.70
Beta-Carotene (normalized)	0.000 ± 0.00	0.000 ± 0.00^b (-2.3*10-6-4.3*10-6)	0.000 ± 0.00^{bc} (3.2*10-6-5.2*10-6)	0.030	0.39
Lycopene (nmol/L)	779 ± 304	676 ± 253^b (-3.8-208)	668 ± 268^b (7.4-214)	0.037	0.38
Lycopene (normalized)	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00	0.186	

*[a]1-Way ANOVA using Phase 1, 2 and 3. [b]P<0.05 from corresponding 2% Milk value. [c]P<0.05 from corresponding Phase 2 value. * Numbers in parenthesis represent 95% Confidence Intervals.*

TRP supplementation (Phase 3) resulted in a significant 13% reduction of LDL-C, a significant 10% reduction in total cholesterol, and a significant 6% reduction in total cholesterol:HDL-C ratio (results not shown) as compared to Phase 1. Results of the lipoprotein particle size data analyzed through nuclear magnetic resonance showed significant decreases in total LDL particle concentration with the 'aided' triglyceride recrystallized phytosterols from

Phase 3 verses the 'unaided' phytosterol supplementation in Phase 2 (p< 0.05).   Within that LDL

reduction, large LDL particles significantly decreased in both Phase 2 and Phase 3 from the Phase

1 value (p<0.05). In addition, HDL total cholesterol decreased in both Phase 2 'unaided' and

Phase 3 'aided' phytosterols to Phase 1, 2% milk (p<0.05).

Absolute values of alpha tocopherol, lutein, beta-carotene, and lycopene were

significantly decreased by phytosterol supplementation (p <0.05), however, when normalized to

individualized total cholesterol values, only beta-carotene was found to significantly decrease

(Table 7.). Results of the glucose assay and insulin ELISA (Table 7.) show no significant

changes over the 12-week PS supplementation. Inflammatory markers of IL-6, TNF-a, VEGF,

and CRP show no significant changes over the 12 week study, however, IL-8 and MCP-1 were

both significantly decreased in Phase 3 compared to Phase 1 (p <0.05).

Discussion

A patient's LDL cholesterol level is only one of many risk factors used to assess CVD

risk.   ATPIII guidelines do not recognize lipoprotein particle size as a method of monitoring a

person's actual risk level however considerable evidence has linked particle size with risk for

chronic disease. Phytosterols are becoming more recognized as a treatment option to decrease

LDL cholesterol concentration in the blood; however, it remains unclear if phytosterols affect

lipoprotein particle size and other established risk factors such as inflammatory markers. In light

of the controversial literature, we analyzed the effects of lipoprotein particle size after 'unaided'

PS and 'aided' triglyceride recrystallized PS (TRP) supplementation. We show for the first time

that in addition to decreasing LDL-C, TRP incorporated into fat-free milk, was effective at

lowering serum LDL particle concentration, as well as IL-8 and MCP-1 concentrations compared

to an unaided phytosterol.

It is widely accepted that body composition, diet, and exercise may influence cholesterol makeup. In order to eliminate the effects of changes in diet or body composition on lipoprotein changes, we encouraged a similar dietary intake and physical activity pattern throughout this study. Since these confounding variables remained stable the results are most likely due to milk and PS supplementation.

As expected, we found a decrease in LDL cholesterol concentration post supplementation with the 'aided' phystosterols. However, research shows LDL cholesterol levels under represent the number of small LDL or cholesterol-poor particles (Otvos JD 2002; Cromwell WC 2004). Cromwell and colleagues support the theory that individuals with low LDL concentrations also have cholesterol-poor particles . In addition, these researchers analyzed prediction of future CVD events by LDL-C and LDL particle number through NMR and found LDL particle number to be the stronger predictor. These findings suggest that simply having low LDL levels can create an inconsistency between LDL-C and LDL particle number, which can lead to a misconception of actual CVD risk (Cromwell WC 2007). Our findings of a significant decrease of Large LDL particles with PS supplementation and a significant decrease of total LDL particles with TRP supplementation suggests the patented TRP method may be a more beneficial PS choice.

Studies have found with changes in triglyceride levels are the cause of cholesterol composition variability in LDL particles (Otvos JD 2002; Cromwell WC 2004; Freedman DS 2004). Berneis et al. found when triglycerides are elevated, small LDL particles predominate (Berneis KK 2002) causing an increase in CVD risk. Small LDL particles are considered a greater risk because they carry less cholesterol than larger particles because of their triglyceride rich consistency, mainly because of the volume of their lipid core. Parallel to these findings, our data shows a progressive reduction in large LDL particles at triglyceride levels averaging >100mg/dL with both 'unaided' and 'aided' TRP PS supplementation.

Secondary data, such as inflammation markers may pose as an important area of research for PS supplementation since PS have been shown to have anti-inflammatory effects (Devaraj S 2006), Our results found TRP supplementation a significant decrease in monocyte chemoattractant protein-1 (MCP-1), a chemokine that promotes monocyte attraction to the arterial intima and thus increases risk of CVD.   In addition, IL-8 a primary neutrophil chemoattractant expressed by macrophagic foam cells, was also found to decrease with TRP. In the intima, monocytes differentiate into macrophages, which accumulate cholesterol esters and eventually form lipid-laden foam cells. Foam cells are seen as a lipid imbalance (Linton MF 2003), and since our results found an improving cholesterol balance and a decrease in MCP-1 and IL-8, one may speculate may suggest phytosterols may impact MCP-1 and IL-8 receptors (CCR and CXCR2). This would result in a decrease of foam cell production or oxidized LDL-C formation, causing a decrease atherosclerosis development.

Notably, our findings of a significant decrease in fat-soluble vitamins of Vitamin E: alpha-tocopherol, and Vitamin A carotenoids: Lutein, beta-carotene, and lycopene (p <0.05) occurred with both PS types. When normalized to the decreases in cholesterol, beta-carotene was the only carotenoid found to have a significant decrease (p<0.05), however, levels still remain within normal blood values. Concurrent with the literature and suggesting this may be the only side effect of PS supplementation, the decreases in beta-carotene are not associated with any adverse health outcomes. Additionally, this can be prevented by adding sufficient fruit and vegetables to the diet (Noakes M 2002). This can be explained through the decrease in cholesterol absorption at the intestines, which is consistent with the literature.

Overall, it can be concluded there are many ways to assess cardiovascular disease risk. Simply by looking at absolute cholesterol numbers, one may evaluate risk inaccurately. Particle size is becoming a common trend for risk evaluation and phytosterols are increasingly popular as a safe, alternative treatment to medication. Phytosterols have proven to decrease LDL cholesterol in research, but results remain inconclusive on their actions of lipoprotein particle size. Our

findings, similar to some research, found a decrease in LDL particle number, in particular large LDL particle number, but no change in particle size with 'aided' TRP supplementation in fat-free milk. In addition, TRP showed added benefits of attenuating atherogenesis through the significant decreases in IL-8 and MCP-1 cytokines.

This study observed limitations that should be accounted for when considering future research with phytosterol supplementation. For example, subjects experienced an order effect of milk supplementation in this study, where all subjects enrolled in phase 1, 2, then 3, respectively, with no wash out periods in-between. Milk fat content for each phase was not controlled and therefore it is difficult to compare the PS medium by phases over time. Additionally, subjects reported their history and intake frequency of foods with phystosterols, however, this intake was not calculated, analyzed, or accounted for statistically. For future studies determining the effects of phytosterols on lipoproteins, analyzing the exact amount of PS in the blood before, during, and after the phases may prove beneficial to understanding the mechanism of action. Controlling diet or providing an exact diet, and controlling timing of blood draws may help to explain cholesterol blocking action of PS. In addition, measuring oxidized LDL or foam cell production may help in accessing PS impact on CVD risk.

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