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A COMPREHENSIVE DOSE-RESPONSE STUDY OF THE EFFECTS OF PISTACHIOS ON CARDIOVASCULAR DISEASE RISK FACTORS: A TRANSLATIONAL RESEARCH APPROACH INTEGRATING CLINICAL NUTRITION AND MOLECULAR BIOLOGY

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by

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ABSTRACT

Nut consumption reduces risk for cardiovascular disease (CVD). Few studies have evaluated the effects of pistachios on CVD risk factors and they have not evaluated dose-response relationships or lipid-lowering mechanisms. Nutrition studies with a translational research approach integrate clinical nutrition and molecular biology, allowing for the investigation of clinical responses and underlying cellular mechanisms. The present study utilized a translational research approach to comprehensively evaluate the effects of pistachios on CVD. We employed a randomized crossover controlled-feeding study to evaluate the effects of two doses of pistachios, added to a lower-fat diet, on lipids and lipoproteins, apolipoprotein-defined lipoprotein subclasses, and plasma fatty acids. To investigate mechanisms of action, we measured serum cholesteryl ester transfer protein (CETP), indices of plasma stearoyl-CoA desaturase activity (SCD), and gene expression in isolated peripheral blood mononuclear cells (PBMCs). Total cholesterol (TC), LDL-C, non-HDL-C, apoB, and apoB/apoA-I decreased after both pistachio diets; and triacylglycerol and plasma SCD activity decreased after the 3.0 ounce pistachio diet ($P < 0.05$). Pistachios elicited a dose-dependent lowering of TC/HDL-C, LDL-C/HDL-C, and non-HDL-C/HDL-C ($P < 0.01$). We evaluated the effects of pistachios on expression of genes related to inflammation and lipid metabolism (TNFα, IL-1β, IL-6, ICAM, VCAM, CETP, and LCAT) in PBMCs. Furthermore, we investigated the relationship between diet-induced change in CETP expression and change in serum CETP and plasma lipids/lipoproteins. The pistachio-rich diets significantly decreased IL-1β expression compared to baseline ($P < 0.05$). Change in CETP expression in PBMCs predicted change in LDL-C, NONHDL-C, TC/HDL-C, and NONHDL-C/HDL-C in individuals who were diet-responsive with regards to serum CETP. In conclusion, this study demonstrates that pistachios elicit beneficial effects on traditional and emerging CVD risk factors at the protein level in serum/plasma and the transcription level in PBMCs.

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Chapter 1

Introduction

Cardiovascular disease (CVD) is the leading cause of death in the United States. Lipids and lipoproteins play a key role in modulating risk of CVD. It is established that elevated total cholesterol (TC), LDL-cholesterol (LDL-C), and triglycerides (TG) increase CVD risk, while elevated levels of HDL-C exert a cardioprotective effect (1). Traditionally, LDL-C has been the primary target for CVD risk reduction. More recently, other emerging risk factors also have been identified, such as apolipoproteins, lipoprotein (a), C-reactive protein (CRP), homocysteine, fibrinogen and endothelial dysfunction (2). An abundance of data from epidemiologic and randomized controlled trials have demonstrated the effects of diet and individual nutrients on CVD risk factors, resulting in evidence-based dietary recommendations to reduce CVD risk. Identification of dietary factors that modify CVD risk factors is critical for determining optimal dietary interventions to maximize CVD risk reduction on an individual basis. There are numerous mechanisms by which diet can influence risk of CVD, including modification of lipid levels, blood pressure (BP), endothelial function, inflammation, oxidative stress, and insulin sensitivity (3).

Numerous nutrients and whole foods affect CVD risk factors, including saturated fatty acids (SFA), *trans* fatty acids (TFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), dietary cholesterol, stanols and sterols, fiber, vitamins, and minerals, as well as whole grains, fruits, vegetables, dairy, fish, and nuts. Current dietary recommendations by the American Heart Association (AHA) to achieve a desirable blood cholesterol and lipoprotein profile include limiting the intake of foods that are high in SFA and cholesterol, and substituting grains and unsaturated fatty acids from vegetables, fish, legumes, and nuts (4).

The macro- and micro-nutrient composition of nuts makes them an ideal constituent of a healthy dietary pattern. Nuts are a complex plant food matrix providing a unique nutrient package rich in unsaturated fatty acids, fiber, vitamins, minerals, phytosterols, antioxidants, and other bioactive components. Additionally, they are low in SFA and do not contain TFA or dietary cholesterol (5). Due to the consistent data indicating the beneficial effects of nut consumption, nuts have been recommended as part of an optimal diet for the prevention of coronary heart disease (CHD) (4, 6). In 2003, the Food and Drug Administration issued a qualified health claim for nuts due to the association of nut consumption with reduced risk of heart disease, stating that "scientific evidence suggests but does not prove that eating 1.5 ounces per day of most nuts, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease" (7).

Numerous large epidemiologic studies have demonstrated the beneficial effects of nuts on CHD risk. In addition, clinical studies have made evident the lipid-lowering effects of nuts. While these studies were integral in establishing the cardioprotective effects of nuts, most were not designed to investigate mechanisms of action. Furthermore, most studies have been done with almonds and walnuts, while few studies have evaluated the effects of other nuts, such as pistachios. A translational research approach is essential in order to study the lipid and lipoprotein response of pistachio consumption, as well as mechanisms of action. Translational research combines clinical research with basic research, spanning the spectrum from "bedside" to "bench side". Human nutrition studies with a translational research design integrate clinical nutrition and molecular biology, allowing for the investigation of clinical responses and underlying cellular mechanisms. As shown in Figure **1-1**, a translational research approach was utilized in the present study to comprehensively examine the cardiovascular effects and potential mechanisms of pistachios as part of a healthy diet. The effects of pistachio consumption were measured at the protein level in serum and plasma, as well as the mRNA level in isolated peripheral blood mononuclear cells.

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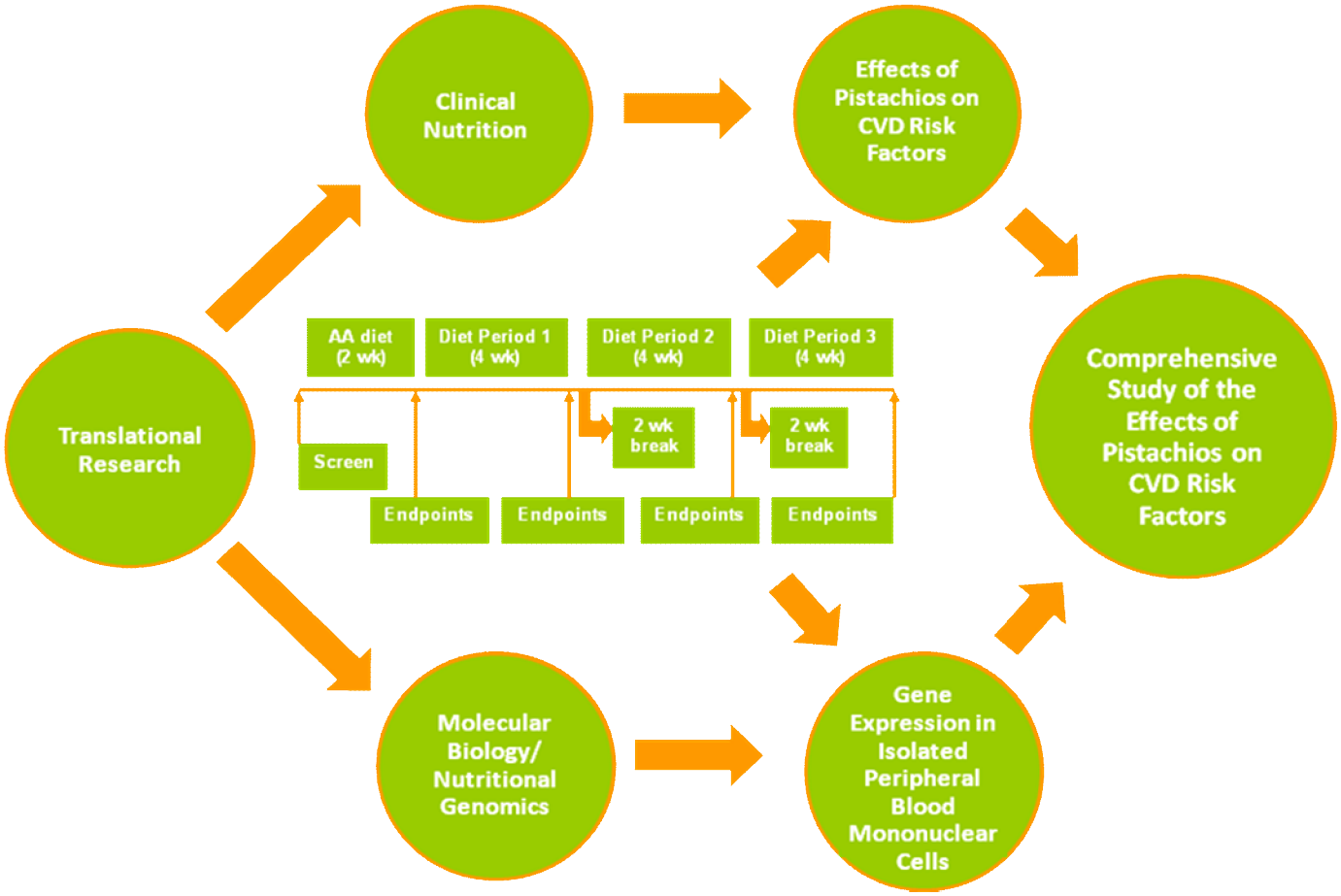


Figure **1-1**: Translational research study design: Comprehensive study of the effects of pistachios on CVD risk factors. Diet periods include: control diet (lower-fat diet with no pistachios), 1.5 oz/d pistachio diet, 3.0 oz/d pistachio diet. AA = average American.

Chapter 2

Literature Review

There is an extensive scientific database demonstrating the cardiovascular effects of nuts as a complex food matrix, as well as the cardiovascular effects of their individual components. This database includes evidence from both epidemiologic studies and clinical trials.

Epidemiologic studies evaluating nut intake and CHD

Large prospective epidemiologic studies, including the Adventist Health Study, Iowa Women's Health Study, Nurses' Health Study, Physicians' Health Study, and Cholesterol and Recurrent Events Study, have demonstrated that increased nut intake is associated with reduced risk of CHD in healthy populations (1-4) and hypercholesterolemic individuals who have experienced a myocardial infarction (MI) (5). The results of epidemiologic studies on primary prevention consistently demonstrate a cardioprotective effect associated with increased nut consumption. The effects of nut consumption on CHD risk are reviewed below, although studies have evaluated multiple outcomes, including non-fatal MI, fatal CHD, and sudden cardiac death. As shown in Figure **2-1**, combined data from prospective cohort studies suggest that there is a 37% reduction in risk for CHD in those consuming nuts frequently (≥ 4 times/week) compared to those who never or rarely consume nuts (RR = 0.63, 95% CI: 0.51 to 0.83) (6). Regression analysis indicates that for each serving of nuts consumed per week, there is an average reduction in risk of CHD death of 8.3%, suggesting a dose-response effect. Thus, the cardioprotective effect of nuts also is observed at lower levels of intake. In addition to beneficial effects on primary prevention of CHD, there was a 25% lower risk of CHD in post-MI individuals who consumed

more than 1 serving/week of nuts versus those who rarely ate nuts or consumed less than 1 serving/month (RR = 0.75, 95% CI: 0.49 to 1.15) (5). In the Physicians' Health Study, individuals who consumed nuts 2 or more times per week had a 47% reduction in risk for sudden cardiac death compared to those who rarely or never consumed nuts (RR = 0.53, 95% CI: 0.30 to 0.92) (4). Collectively, epidemiologic studies demonstrate consistently an independent beneficial effect of nut consumption on risk of CHD across many population groups.

Clinical studies evaluating the effects of nut consumption on lipids and lipoproteins

Numerous free-living and highly-controlled studies have evaluated the effects of specific nuts on lipids and lipoproteins. While the lipid-lowering effects of almonds (7-12) and walnuts (8, 13-17) have been studied most extensively, studies also have demonstrated effects of other nuts, such as pecans (18, 19), macadamia nuts (20), hazelnuts (21, 22), pistachios (23-25), and legume peanuts (26, 27). A systematic review by Mukuddem-Petersen *et al.* demonstrates that compared to control diets without nuts, consumption of almonds (50-100 g/day), walnuts (40-84 g/day), peanuts (35-68 g/day), and pecans (72 g/day), decrease TC by 2-16% and LDL-C by 2-19% (28). It was concluded that the consumption of a heart-healthy moderate-fat diet (~35% of energy from fat) including 50-100 g of nuts at least 5 times/week may significantly decrease TC and LDL-C in individuals with normal and elevated cholesterol. Further research is needed to assess the effects of nuts on TG and HDL-C. The lipid-lowering effects of nuts have been primarily attributed to their fatty acid profile. Specifically, nuts are low in SFA and rich sources of MUFA and PUFA. In the review by Mukuddem-Petersen *et al.*, 10 out of 17 of the controlled feeding studies reported greater observed decreases in LDL-C on the experimental diets than would be expected by predictive equations (29). A previous analysis of controlled feeding studies with nuts indicates that the cholesterol-lowering response is approximately 25% greater than what would be expected

from predictive equations, as shown in Figure **2-2** (30). The difference in the predicted and observed effects on lipids and lipoproteins suggests that other constituents of nuts, such as fiber and phytosterols, may provide additional benefits, resulting in a synergistic effect on lipids and lipoproteins. Additionally, other components of nuts, such as antioxidants, minerals, and phenolic compounds may contribute to the overall cardioprotective effect of nuts. Table **2-1** presents the nutrients contained in various nuts that may be contributing to their beneficial effects on lipids and lipoproteins (29). These nutrients beneficially affect lipid and lipoprotein risk factors in many ways, including reducing cholesterol absorption and thus decreasing LDL-C concentrations, as well as protecting against LDL oxidation. The effects of the constituents of nuts on CVD risk factors are reviewed below, including dietary fatty acids, fiber, phytosterols, antioxidants, vitamins, and minerals.

The effects of fatty acids in nuts on CVD risk

The fatty acid profile of nuts consists of high amounts of MUFA and PUFA and low amounts of SFA. Although approximately 79% of the energy in nuts is from fat, on average, ~91% of energy from fat is from MUFA and PUFA. MUFA is the predominant fat in most nuts constituting ~62% of energy from fat (30). The effects of dietary fat on CVD have been studied extensively. Lifestyle changes in diet have been shown to have significant long-term benefits (31). Overall, randomized clinical trials indicate that replacement of SFA or TFA with MUFA or n-6 PUFA from vegetable oils significantly decreases LDL-C and the ratio of LDL-C/HDL-C (32). Replacement of SFA with carbohydrate (CHO) results in similar decreases in LDL-C and HDL-C, thus the LDL-C/HDL-C ratio is not improved. In addition, replacement of SFA with CHO results in an increase in TG unless fiber is increased. Marine-derived n-3 fatty acids significantly reduce TG and suppress cardiac arrhythmias, with little change in LDL-C or HDL-C

(32). Figure **2-3** demonstrates the predicted changes in serum lipids and lipoproteins when 1% of energy as CHO is replaced by SFA, MUFA, and PUFA (33).

Monounsaturated fatty acids

In the early 1990s, epidemiologic studies reported an increased risk of CHD with higher intake of MUFA (34, 35); however, these studies did not adjust for other classes of fatty acids. In the typical American diet, the major sources of MUFA are beef and dairy fat, which are sources of SFA and TFA, thus adjustment for these fatty acid classes is essential. In the Nurses' Health Study, higher intake of MUFA was associated with a decreased risk of CHD, after adjusting for other fatty acid classes (36). Increases of 5% of energy intake from MUFA (compared with equivalent energy intake from CHO) were associated with a 19% reduction in risk of CHD (RR = 0.81, 95% CI: 0.65 to 1.00; P = 0.05).

Clinical studies consistently demonstrate a TC and LDL-C lowering effect of MUFA (37-42), despite early predictive equations suggesting that MUFA had no effect on lipids and lipoproteins (43, 44). More recent predictive equations indicate that MUFA significantly decrease TC and LDL-C, and increase HDL-C, especially when dietary SFA is low (45). A meta-analysis comparing the effects of high-MUFA diets (22-33% of total energy) versus high-CHO diets (49-60% of total energy) demonstrates that consumption of high-MUFA diets results in slight decreases in TC (-3%), and moderate decreases in VLDL-cholesterol (VLDL-C; -22%) and TG (-19%) (46). In this analysis, the effects of high-MUFA diets on HDL-C were inconsistent, with either slight increases or no changes reported. Overall, there was a 4% increase in HDL-C and no overall effect on LDL-C.

Polyunsaturated fatty acids

N-6 fatty acids

In the 1950s, multiple studies demonstrated that consumption of polyunsaturated vegetable oils significantly decrease serum cholesterol when substituted for SFA (43, 47-49). Four large randomized clinical trials evaluated the effects of PUFA (primarily n-6 PUFA, in the context of a low-SFA diet) on CHD, as reviewed by Sacks & Katan (32). These studies were low in SFA. Three of the four studies reported significant decreases in CVD events. In a study in 846 patients, intake of PUFA (17% of energy) significantly decreased (-34%, $P \leq 0.05$) the combined endpoint of MI, sudden death, or stroke, compared to the control group (5.5% energy from PUFA) (50). In the Oslo Diet-Heart Study, replacement of SFA with PUFA (20% energy from PUFA) in 412 men with previous MI led to a 25% decrease in recurrent MI or sudden death ($P \leq 0.05$) (51). The Finnish Mental Hospital Study was a crossover study in which SFA was replaced with PUFA (14% energy from PUFA) in 676 patients for 6 years. Compared to the control diet (4% energy from PUFA), there was a 43% reduction in the coronary end points of CHD death or MI ($P \leq 0.05$). Overall, these studies demonstrate that intake of high levels of PUFA ranging from 13-21% of energy significantly decrease TC and CHD events by 13-15% and 25-43%, respectively (50-52). A meta-analysis of 27 controlled trials published between 1970 and 1991 confirmed that PUFA was the most potent LDL-C-lowering fatty acid (33). Predictive equations generated from clinical studies indicate that a 1% increase in PUFA decreases TC by 0.024 mmol/l (0.924 mg/dl), and that the cholesterol-lowering response of PUFA is approximately half the cholesterol-raising effect of SFA (53, 54). More recent predictive equations with specific fatty acids indicate that linoleic acid (LA) is the strongest TC- and LDL-C-lowering fatty acid (45). Compared with oleic acid (OA), LA decreases TC and LDL-C, and increases HDL-C (55).

Mechanistically, studies indicate that the cholesterol-lowering effect of n-6 fatty acids is due, in part, to redistribution of cholesterol between plasma and tissue pools (56), and up-regulation of the LDL receptor (57). These data suggest an independent and positive effect of n-6 fatty acids on the regulation of LDL receptor expression. Although cholesterol synthesis is increased, it does not appear to be a major mechanism by which n-6 fatty acids lower plasma LDL-C (56, 58). N-6 fatty acids increase the expression of liver X receptor-alpha (LXRα) leading to increased expression of cholesterol 7α-hydroxylase, the rate-limiting enzyme involved in the conversion of cholesterol to bile acids, and elimination of excess cholesterol. In addition, n-6 fatty acids also inhibit the expression of sterol regulatory element binding protein 1 (SREBP-1), which plays a major role in lipogenesis and cholesterol metabolism (59). The suppression of SREBP-1 prevents the synthesis of unsaturated fatty acids, which may be an indirect mechanism by which LDL receptor expression is increased (60).

N-3 fatty acids

In the 1970s and 1980s, the benefits of n-3 fatty acids on CVD were noted when Inuit populations in Greenland, Northern Canada, and Alaska exhibited significantly lower CVD mortality than would be expected, despite high intake of dietary fat (61-63). The intake of long-chain n-3 fatty acids in these populations was high (5-15 g/day) due to frequent consumption of seal meat and whale blubber. Numerous subsequent studies have demonstrated the cardioprotective effects of fish consumption and intake of α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA).

Epidemiologic studies in the U.S. demonstrate that ALA intake of 0.53 to 2.8 g/day is associated with reduced risk of CVD events (64), fatal ischemic heart disease (IHD) (65), and all-cause mortality (66). Furthermore, the Health Professionals Follow-Up Study reported that a 1%

increase in ALA intake was associated with a 40% lower risk of MI after adjustment for total fat intake ($P < 0.01$) (67). In the Lyon Diet Heart study, a secondary prevention study in post-MI patients, ALA intake of 2.0 g/day (~0.8% of calories) significantly decreased the risk of recurrent coronary events ($P < 0.05$) (68).

ALA has anti-inflammatory effects in some randomized controlled studies. In male dislipidemic individuals, dietary supplementation with ALA, but not LA, significantly decreased CRP, serum amyloid A, and interleukin-6 (IL-6) by 38%, 23%, and 11%, respectively ($P \leq 0.01$) (69). In a study in hypercholesterolemic individuals, compared with an average American diet (7.7% of energy from LA, 0.8% from ALA), a diet high in ALA (6.5% of energy from ALA, 12.6% from LA) decreased CRP, intercellular adhesion molecule-1 (ICAM), vascular cell adhesion molecule-1 (VCAM), and E-selectin ($P < 0.01$ for all). While the cardiovascular epidemiology has been strongly supportive of a benefit of ALA, there are findings from similar population studies in which higher intake of ALA was linked to an increased risk of prostate cancer (70). However, in other studies, no association was found between ALA intake and prostate cancer (71-73).

EPA and DHA have been shown to significantly decrease plasma TG (74), inflammation (75), systolic and diastolic BP (76), and platelet aggregation (77, 78), as well as improve vascular reactivity (79, 80). Due to the numerous beneficial effects of marine-derived n-3 fatty acids reported in the literature, the AHA recommends consumption of 2 servings/week of oily fish and intake of 1 g/d of EPA plus DHA from oily fish or supplements for individuals with CHD (81). Epidemiologic studies have demonstrated a cardioprotective effect of fish consumption (66, 82-85). Intake of EPA and DHA that conferred the lowest risk ranged from 246-919 mg/d. Studies have shown that even in populations with high median intake of EPA plus DHA (900 mg/d), individuals with relatively higher intake have reduced risk for MI, suggesting that higher intakes of n-3 fatty acids elicit even greater reductions in CHD risk (86). Overall, epidemiologic evidence

demonstrates that consumption of as little as 1 serving of fish per month is associated with a reduced risk for CVD.

Two secondary prevention trials have demonstrated beneficial effects of EPA and DHA and fish consumption on recurrent coronary events. In the Diet and Reinfarction Trial (DART) study, there was a 29% reduction in all-cause mortality over a 2-year period in male MI survivors taking fish oil capsules (900 mg/d of EPA and DHA) or 200-400 g of fatty fish per week, providing an additional 500-800 mg/d of n-3 fatty acids (87). In the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI) Prevention Study, subjects with pre-existing CHD randomized to the EPA + DHA supplement group (850 mg/d of n-3 fatty acid ethyl esters) with and without 300 mg/d of vitamin E experienced a 15% reduction in the primary endpoint of death, nonfatal MI, and nonfatal stroke ($P < 0.02$) (88). Furthermore, all-cause mortality was reduced by 20% percent ($P = 0.01$) and sudden death was reduced by 45% ($P < 0.001$), compared with the control group (vitamin E provided no benefit). In the Japan EPA Lipid Intervention Study (JELIS), a randomized controlled study in Japanese individuals of varying health status, long-term use of highly purified EPA in addition to a statin (versus statin alone) reduced risk for major coronary events by 19% (89). A recent meta-analysis of randomized controlled trials in patients with CHD demonstrates a cardioprotective effect of n-3 fatty acids in studies of dietary intake and supplements (0.3-0.6 g/day EPA, 0.6-3.7 g/day DHA) (90). The effects of dietary and non-dietary interventions of n-3 fatty acids were not significantly different for any endpoints.

Despite the evidence supporting a cardioprotective effect of long-chain n-3 fatty acids from the DART, GISSI, and JELIS trials, the effects of EPA and DHA in patients with implantable cardioverter defibrillators (ICDs) remain unclear. In a study in patients with ICDs and a recent episode of sustained ventricular tachycardia (VT) or ventricular fibrillation (VF), Raitt *et al.* found no significant difference in total mortality between the fish oil group (1.8 g/day of EPA/DHA) and the control group. However, there was a shorter time to the first episode of

ICD therapy for VT/VF and an increase in recurrent VT/VF events in patients who received fish oil ($P < 0.001$) (91). In contrast, in a study by Leaf *et al.*, there was a trend in post-MI patients toward a prolonged time to first VT or VF or death from any cause in the fish oil group (2.6 g/day of EPA/DHA; $P = 0.06$) (92). In a recent study by Brouwer *et al.* in patients with ICDs and prior documented malignant VT or VF, there was no significant difference in the combined outcome of VT/VF or death between those who received fish oil (~1 g/day of EPA/DHA) and those who received placebo (93). The discrepancy in these results may be attributed to the populations studied. The results from these studies suggest that fish oil may prevent ventricular arrhythmia in patients with recent MI, with limited amount of scar tissue. Further research is needed before dietary recommendations can be made for patients with ICDs.

Clinical trials consistently have shown that EPA and DHA significantly reduce plasma TG. In a review of 36 human studies, consumption of 3-4 g/d of EPA and DHA resulted in a 25% and 34% decrease in TG in normolipemic (TG < 2.0 mmol/L) and hypertriglyceridemic patients (TG ≥ 2.0 mmol/L), respectively (74). However, LDL-C was increased by 4.5% and 10.8% in normolipemic and normotriglyceridemic patients, respectively (TC and HDL-C were unchanged) (74). In general, the characteristic TG-lowering effect of marine n-3 fatty acids has not been seen at physiologically relevant concentrations of plant sources of n-3 fatty acids. When reported, a TG-lowering effect was found only at very high levels (38 g) of ALA intake (74).

The mechanisms of the potent TG-lowering effect of marine-derived n-3 fatty acids have been investigated in animal and i*n vitro* studies. Marine-derived n-3 fatty acids simultaneously decrease expression of genes involved in lipid synthesis and increase expression of genes involved in fatty acid oxidation. Specifically, EPA and DHA suppress hepatic lipogenesis and VLDL secretion through inhibition of SREBP-1, and increase fatty oxidation in the liver and skeletal muscle via activation of peroxisome proliferator-activated receptor alpha (PPARα). In

addition, EPA and DHA decrease hepatocyte nuclear factor-4α (HNF-4α, promotes the conversion of glucose to glycogen) (94). Overall, this leads to a transition from TG storage to oxidation, ultimately reducing substrate for VLDL synthesis. N-3 fatty acids also accelerate chylomicron clearance and enhance the conversion of VLDL apoB to LDL apoB. N-3 fatty acids increase lipoprotein lipase (LPL), which increases hydrolysis of the TG component of chylomicron and VLDL particles, allowing the removal of the remaining lipoprotein remnants from circulation by the liver (60). Collectively, the effects of EPA and DHA on gene expression support a potential mechanism by which EPA and DHA elicit their TG-lowering effects.

Saturated fatty acids

In the 1970s, the Seven Countries Study reported a significant association between total SFA intake and TC among different populations (95). Subsequent epidemiologic studies also found positive correlations between SFA intake and TC, and SFA intake and incidence of CHD (34, 36, 96). In a more recent analysis of the Seven Countries Study data, associations were reported between individual SFA and TC, and between SFA and CHD mortality. Intake of lauric acid and myristic acid were most strongly associated with TC ($r = 0.84$ and 0.81, respectively) (97). Results from clinical studies support the associations of SFA and TC reported in epidemiologic studies. Numerous meta-analyses of controlled feeding studies evaluating the effects of dietary fatty acids on serum cholesterol have led to the development of predictive equations that estimate how changes in fatty acid classes affect blood cholesterol (33, 44, 45, 98, 99). Regression analyses have demonstrated that for every 1% increase in energy from SFA, LDL-C increases by approximately 0.033-0.045 mmol/L (1.287-1.755 mg/dl) (33, 99, 100). In addition to raising TC and LDL-C, SFA also has been shown to increase HDL-C. For every 1% increase in SFA, HDL-C increases by 0.011-0.013 mmol/L (0.429-0.507 mg/dl) (33, 99, 100).

Furthermore, several equations have been generated to predict the effects of SFA, MUFA, PUFA, as well as individual fatty acids, on TC, LDL-C, and HDL-C (33, 45, 101). Stearic acid has been shown to have no effect on TC, LDL-C, and HDL-C, while myristic acid is more hypercholesterolemic than lauric acid and palmitic acid (45, 101). In a meta-analysis of 60 controlled trials, lauric acid had the greatest LDL-C-raising effect relative to carbohydrate; however, it decreased the ratio of TC/HDL-C due to an increase in HDL-C (102). Myristic and palmitic acids had little effect on the TC/HDL-C ratio due to comparable increases in both TC and HDL-C, whereas stearic acid reduced TC/HDL-C due to slight increases in HDL-C. Collectively, these studies demonstrate that individual SFA differentially affect lipids and lipoproteins (55).

The effects of other components of nuts on CVD risk

Dietary fiber

Dietary fiber includes insoluble fiber (celluloses, lignans, and hemicelluloses) and soluble fiber (pectins, gums, mucilages, and beta-glucans) (103). Although fruits, vegetables, and cereals are the main sources of dietary fiber, nuts contribute a substantial amount of fiber (5-10 g/100 g) (103). Most of the fiber contained in nuts is insoluble; however, nuts do contain some soluble fiber (average soluble fiber fraction = 3%) (104). The cardioprotective effects of dietary fiber have been studied extensively. Epidemiologic studies have found an inverse relationship between intake of total dietary fiber and incidence of CHD (1, 105-110). In the Nurses' Health Study, an increase in dietary fiber of 10 g/day was associated with a 19% decrease in CHD risk (110). Numerous epidemiologic studies have found stronger associations with insoluble cereal fiber compared to soluble fiber from fruits and vegetables (103); however, intervention studies

demonstrate the beneficial effects of viscous soluble fiber (111, 112). A meta-analysis of 20 studies indicates that the reduction in TC per gram intake of viscous fiber (from oat sources) ranges from 0.1-2.5% (113). Greater reductions were seen in hypercholesterolemic individuals (TC ≥ 5.9 mmol/L), particularly when viscous fiber intake was ≥ 3 g/d. Intake of soluble fiber also has been shown to prevent the increase in TG typically associated with low-fat high-CHO diets (114). In the Dietary Approaches to Stop Hypertension (DASH) study, the DASH diet (58% calories from CHO, 27% from total fat), which provided 30 g/d of fiber, resulted in significantly lower TC and LDL-C ($P < 0.0001$) without change in TG, compared to subjects fed a control diet (50% of calories from CHO, 37% from total fat) that provided 11 g/d of fiber (115). In individuals with type 2 diabetes, consumption of a high-fiber diet (30% from total fat, 55% of calories from CHO; 50 g total fiber, 25 g soluble fiber) resulted in a 10% decrease in TG compared to those consuming a moderate fiber diet (30% from total fat, 55% CHO; 24 g total fiber, 8 g soluble fiber) ($P < 0.02$). The higher fiber diet also decreased TC and LDL-C by 6.7% and 12.5%, respectively (116).

Plant stanols and sterols

Plant stanols and sterols are compounds structurally similar to cholesterol that are found in nuts, seeds, vegetable oils, cereals, and beans (117). Compared to the intestinal absorption of cholesterol, which ranges from 35-70%, absorption of plant stanols and sterols is very low, ranging from 0.02-0.3% and 0.4-3.5%, respectively (118). The typical intake of stanols and sterols ranges from 150-400 mg/day (119). The average amount of total phytosterols in nuts is 167 mg per 100 g edible portion. Table **2-2** compares the phytosterol content of commonly consumed nuts.

A meta-analysis of 41 trials demonstrates that intake of 2 g/day of stanols or sterols reduces LDL-C by 10%, with little or no change in HDL-C or TG (119). Decreases in apoB were similar to those of LDL-C, suggesting that stanols and sterols decrease LDL by reducing the number of particles as opposed to affecting size or composition (120). The effects of stanols and sterols are additive to the effects of a healthy diet or cholesterol-lowering drugs. Incorporation of 2.3 g/day of stanol esters into a Step-I diet was shown to double the LDL-C-lowering effect (23%) expected of a Step-I diet alone (121). Studies have demonstrated that addition of plant stanols and sterols to the diets of individuals receiving statins results in a further lowering of LDL-C by 10-20% (122-125). Based on the results of clinical studies, the Third Adult Treatment Panel of the National Cholesterol Education Program recommends 2 g of plant stanols/sterols per day as a diet option to increase LDL-C lowering (126).

Mechanistically, plant stanols and sterols have been shown to interfere with uptake of both dietary and biliary cholesterol from the intestinal tract in humans (127). Since plant stanols and sterols are more hydrophobic than cholesterol, their LDL-C-lowering effects may be due, in part, to displacement of cholesterol from mixed micelles, leading to a decrease in cholesterol concentrations in micelles and a decrease in cholesterol absorption (128). Another potential mechanism is a decrease in the rate of esterification of cholesterol in the enterocyte, leading to reduced cholesterol concentrations in chylomicrons. Plant stanols also have been shown to increase expression of LDL receptor in peripheral blood mononuclear cells (PBMCs); and increase the expression of ATP-binding cassette transporters in intestinal cells, which pump phytosterols from the enterocytes into the lumen of the intestine, thus contributing to reduced cholesterol absorption (129-131).

Antioxidants

Oxidative stress plays an integral role in the pathophysiology of many diseases, including inflammatory diseases, hypertension, CVD, stroke, cancer, AIDS, Alzheimer's disease, and Parkinson's disease (132, 133). A complex endogenous defense system has evolved to counteract oxidative damage that occurs during normal cellular reactions (134). Dietary components also may contribute to antioxidant defense. Dietary plants contain vitamins and minerals (vitamin C, vitamin E, and selenium), multiple carotenoids (α-carotene, β-carotene, lycopene, lutein, and zeaxanthin), and phenolic compounds (flavonoids and resveratrol, reviewed in bioactives section below), all of which function as antioxidants (134-136).

As reviewed by Stanner *et al*. (137), epidemiologic studies generally indicate that intake of antioxidant-rich foods is associated with a reduced risk for diseases related to oxidative stress. In a recent analysis of the Zutphen Elderly Study (n = 559 men, mean age = 72), dietary intakes of α-carotene and β-carotene were inversely associated with CVD mortality after multivariate adjustment (RR = 0.80, 95% CI: 0.66 to 0.99; RR = 0.80, 95% CI: 0.66 to 0.97, respectively) (138). There was no relationship with CVD mortality and other carotenoids, tocopherols, or vitamin C. Results from randomized controlled trials have been unable to confirm the results of epidemiologic studies (139-143). Furthermore, many recent meta-analyses indicate that there is no beneficial effect of supplementation with α-tocopherol, β-carotene, and ascorbic acid on prevention of CVD (endpoints included: nonfatal MI, nonfatal stroke, cardiovascular mortality, all-cause mortality, and fatal MI) (144-146). Some clinical trials have found a beneficial effect of supplementation with selenium on risk of cancer incidence and mortality of numerous types of cancer (lung, liver, prostate, and colorectal) (147). While there has been a focus on α-tocopherol, β-carotene, and ascorbic acid, these antioxidants represent only a fraction of the number of antioxidants in most dietary plants. It is possible that supplementation studies with other

antioxidants would confer greater benefits. Additionally, it is possible that different antioxidants act synergistically with one another, as seen in *in vitro* studies (148). In a study with almonds, flavonoids were shown to act together with vitamins C and E, as well as other bioactive plant compounds, to increase resistance of LDL to oxidation (149). Thus, consumption of foods that are rich in a variety of antioxidants is optimal, due to the possibility that other antioxidants present in foods may provide a protective effect and the potential synergistic effect of various antioxidants. Total antioxidant content (TAC) of nuts was measured by the ferric reducing ability of plasma (FRAP) assay, which measures the reduction of Fe^{3+} to Fe^{2+} in the presence of antioxidants. TAC of nuts ranged from 0.09-23.07 mmol/100 g, with walnuts containing the highest amount, followed by pecans, chestnuts, peanuts, and pistachios (150). To put this into context, TAC of other antioxidant-rich foods (cloves, cinnamon, oregano, berries, grapes, orange, chocolate, wine, coffee, and tea) ranged from 0.8-465.3 mmol/100g . Several *in vitro* studies have found that antioxidants in the extracts from walnuts and almonds inhibit LDL-oxidation (149, 151). Hazelnut oil has been found to decrease lipid peroxides (152). Intervention studies in humans have been inconsistent, with some studies reporting a beneficial effect of walnuts on LDL oxidation (14, 16), whereas others have found no effect of walnuts (15) and almonds (12).

Folate

Folate deficiency is associated with diseases such as megaloblastic anemia and neural tube defects in infants. Low folate status also is associated with elevated plasma concentrations of homocysteine, a risk factor for CVD. Folate is the most important dietary determinant of plasma homocysteine levels due to methylation of homocysteine to methionine (153, 154). Supplementation of folic acid along with vitamins B12 and B6 results in a significant dose-dependent reduction in homocysteine (155). A recent meta-analysis of 30 observational studies

demonstrates that a 25% reduction in plasma homocysteine concentration is associated with decreases in risk of IHD and stroke of 11-16% and 19-22%, respectively (156). However, in a trial in individuals who recently had an acute MI (n = 3749), folic acid + vitamin B12 significantly decreased homocysteine by 27%, but did not reduce the risk of recurrent CVD (composite of recurrent MI, stroke, and sudden cardiac death) (157).

Few studies have evaluated the effect of nut consumption on plasma homocysteine concentration. Two studies in hypercholesterolemic individuals with normal homocysteine concentrations, one with almonds (73 g/day) (10) and one with walnuts (40-65 g/day) (16), reported no effect on plasma homocysteine concentration. However, in another study, incorporation of mixed nuts as part of a folate-rich diet (500 μg of folate) in individuals with hyperhomocysteinemia and coronary artery disease (CAD) resulted in a significant decrease in homocysteine concentration of 8.6% (95% CI: -15.9 to -1.2). This decrease was similar to that from supplementation of synthetic folic acid capsules (500 μg of folate) (8%, 95% CI: -13.3 to –2.7) (158).

Minerals

Nuts contain relatively high amounts of calcium, magnesium, and potassium, and low amounts of sodium. Thus, the mineral profile of nuts matches that of the DASH diet (a diet higher in fruit, vegetables, nuts, whole grains, and low-fat dairy, and lower in red meats), which is a low-sodium high-potassium diet that is also high in calcium and magnesium. The DASH diet has been shown to be effective in lowering BP (159, 160) and serum homocysteine (161). Thus, the mineral profile in nuts could potentially contribute to the cardioprotective effects associated with increased nut consumption.

Calcium

Nuts are one of the few foods that are rich in calcium, among milk and dairy products, legumes, spinach, and sardines (162). Epidemiologic data demonstrate an inverse association with calcium intake and CVD. In the Iowa Women's Health Study, CVD mortality was reduced by 33% when comparing the calcium intakes of women in the highest (> 1425 mg/day) versus lowest (< 696 mg/day) quartiles (RR = 0.67; 95% CI: 0.47 to 0.94) (163). Long-term supplementation with calcium recently has been shown to beneficially affect lipids and lipoproteins, which is consistent with previous clinical studies of shorter duration (164, 165). In a randomized controlled trial in postmenopausal women, calcium supplementation (1 g/day) for 1 year led to a significant increase in HDL-C (-7%, $P < 0.01$) and a non-significant decrease in LDL-C (6%), resulting in a significant increase in the ratio of HDL-C/LDL-C ($P < 0.001$) (166). The authors conclude that the effect of calcium on serum cholesterol concentration may be due to interference with lipid absorption as a result of binding to fatty acids and bile acids in the gut (as previously shown in animal studies) (167). Additional long-term randomized controlled trials in different populations are needed.

The epidemiologic data evaluating the effects of calcium intake and BP are mixed. Results from epidemiologic studies (especially studies in populations with low calcium intake) in the 1990s demonstrate an inverse association between calcium intake and BP, whereas results from other studies are mixed (168-171). In a recent meta-analysis of randomized controlled trials in individuals with raised BP (systolic BP ≥ 140 mmHg, diastolic BP ≥ 85 mmHg), oral calcium supplementation for ≥ 8 weeks significantly decreased systolic BP (-2.5 mmHg, 95% CI: -4.5 to -0.6) (172). There were no significant changes in DBP. Heterogeneity between trials could not be explained by baseline BP or dose of calcium, but it was reduced when poor quality trials were

excluded. Thus, the authors conclude that additional double-blind placebo controlled trials of longer duration and with larger sample sizes are warranted.

Magnesium

Nuts contain significantly more magnesium (~120-300 mg/100 g) than any other common plant food, including fruits and vegetables (162). Magnesium acts as a cofactor for numerous reactions involved in energy metabolism, glucose utilization, and protein and fatty acid synthesis (173). Magnesium deficiency has been associated with numerous conditions, including osteoporosis, hypertension, CHD, congestive heart failure, arrhythmia, and diabetes (174, 175). Epidemiologic studies have demonstrated an inverse association between magnesium intake and risk for diabetes (176, 177), which may explain, in part, the association of frequent nut consumption with reduced incidence of diabetes (178). In the Womens' Health Study, intake of magnesium in women who had no history of CVD or cancer was not associated with total CVD, CHD, non-fatal MI, CVD death, or stroke (179). However, magnesium intake was inversely associated with risk for developing hypertension when comparing women in the highest quintile versus lowest quintile (434 mg/day vs. 256 mg/day, P for trend = 0.03) (180).

Results from epidemiologic studies evaluating the effects of magnesium on BP are inconsistent (181, 182). In a recent review of 12 randomized controlled trials of magnesium supplementation (follow-up of 8 to 26 weeks), there was no significant effect of magnesium supplementation on systolic BP; however, there was a significant decrease in diastolic BP (-2.2 mmHg, 95% CI: -3.4 to -0.9). Similar results were found when poor quality trials were excluded. Heterogeneity between trials could not be explained by baseline BP or dose of magnesium. The authors conclude that more double-blind placebo controlled trials of longer duration and better quality are needed to assess the effect of magnesium on blood pressure (183).

Sodium

Clinical, epidemiological, and animal studies demonstrate that on average, BP increases as intake of dietary salt increases (160). Studies in hypertensive individuals (systolic BP ≥ 140 mmHg, diastolic BP ≥ 90 mmHg) demonstrate consistent reductions in BP with reduced salt intake; however, the reductions in normotensive (systolic BP ≤ 120 mmHg, diastolic BP ≤ 80 mmHg) individuals have not been as consistent. While several meta-analyses have been conducted (184-187), some have shown an effect of salt reduction on BP in normotensive individuals, while others have not (185, 187). However, on average, the median duration of salt reduction in the latter studies was 8 days (187) and 14 days (185). A recent meta-analysis of 28 randomized trials assessed the effects of modest salt reduction for 4 or more weeks on BP in hypertensive (n = 734) and normotensive (n = 2220) individuals (188). In this analysis, the median reduction in urinary sodium was 4.6 g/day and 4.4 g/day in hypertensive and normotensive individuals, respectively. The change in systolic and diastolic BP in hypertensive individuals was -4.96 ± 0.40 mmHg (mean ± SEM; 95% CI: -5.75 to -4.17 mmHg, $P < 0.001$) and -2.73 ± 0.24 mmHg (95% CI: -3.21 to -2.25 mmHg, $P < 0.001$), respectively. In normotensive individuals, the change in systolic and diastolic BP was -2.03 ± 0.27 mmHg (95% CI: -2.56 to -1.50 mmHg, $P < 0.001$) and -0.97 ± 0.21 mmHg (95% CI: -1.39 to -0.55 mmHg, $P < 0.001$), respectively. Furthermore, linear regression analyses demonstrate a dose-response relationship between change in urinary sodium and BP (188).

Highly-controlled clinical trials also have shown a dose-dependent effect of salt intake on BP (189-191). In the DASH trial, the largest of these highly-controlled trials, individuals on the control diet (average American diet) and DASH diet (rich in fruits, vegetables, and low-fat dairy products) were randomized to eat foods with high, intermediate, and low levels of sodium for 30 days (191). The DASH diet was associated with a significantly lower systolic BP at each sodium

level compared to the control diet ($P < 0.001$). Compared to the high sodium phase of the control diet, the low sodium phase of the DASH diet resulted in significant decreases in systolic and diastolic BP of 8.9 mmHg (95% CI: -6.7 to –11.1, $P < 0.001$) and 4.5 mmHg (95% CI: –3.1 to -5.9, $P < 0.001$), respectively. Furthermore, mean systolic BP was reduced by 7.1 mmHg in normotensive individuals and 11.5 mmHg in hypertensive individuals, when comparing the low sodium-DASH and high-sodium control groups (191).

Heterogeneity in individual BP responses to change in salt intake can be attributed, in part, to genetic factors (189, 192-195), and intake of other dietary factors such as potassium. Evidence suggests that individuals' BP response to change in salt intake falls on a continuum, as opposed to simply being salt-sensitive or not (160). Collectively, results from meta-analyses of tightly-controlled clinical trials and studies with modest sodium reductions of longer duration suggest that sodium reduction has a beneficial effect on BP.

Potassium

In a large study evaluating the effects of electrolytes on BP, 24-hour urinary potassium excretion, as a marker for potassium intake, was found to be an independent determinant of BP (196). An increase in potassium intake of 30-45 mmol (1.2-17.6 g) was associated with an average reduction in systolic BP of 2-3 mmHg. Numerous meta-analyses of clinical trials suggest an inverse association between potassium intake and BP in individuals with normal and elevated BP (197-199). Some studies have shown that a high intake of potassium reduces the BP response to increases in salt intake (200, 201). Similarly, studies have shown that increased intake of potassium results in greater reductions in BP when salt intake is high; and reduced intake of salt results in greater reductions in BP when potassium intake is low (160). In contrast, some studies have found an inverse association of potassium and BP independent of sodium intake (196). In

the DASH trial, increases in potassium intake from 37 to 71 mmol/day resulted in a significant decrease in BP regardless of low sodium intake (130 mmol/day). In this study, there was an additive effect of increasing potassium intake and reducing sodium intake (191). A study in mildly hypertensive individuals (diastolic BP between 90-100 mmHg) assessed the effects of 3 diets varying in amounts of potassium and sodium on BP (202). The diets included a high-potassium diet (≥ 100 mmol/day), a reduced-sodium diet (50-75 mmol/day), and a high-potassium low-sodium diet (≥ 100 mmol/day of potassium, 50-75 mmol/day of sodium). Although there were significant decreases in BP between the control diet and all 3 experimental diets ($P < 0.005$), there were no significant differences in BP responses between the treatment diets. This study suggests that increasing intake of potassium decreases systolic and diastolic BP to the same extent as reducing salt intake (8.9 ± 1.0 and 5.8 ± 0.6 vs. 7.7 ± 1.1 and 4.7 ± 0.7, respectively). Furthermore, a combination of reduced salt intake and increased potassium intake did not result in an additive effect on BP (7.9 ± 0.9 and 4.2 ± 0.7, respectively) (202).

In contrast to the studies reporting a beneficial effect of potassium on BP, a recent systematic review found no significant effect of potassium supplementation on BP (203). This was attributed to small sample size in the 2 high-quality trials, short duration of follow-up, and unexplained heterogeneity between trials. Due to the discrepancies, additional highly-controlled randomized trials of longer duration and larger sample size are needed to further investigate the effectiveness of potassium supplementation in reducing BP.

Novel bioactive compounds in nuts

The literature clearly demonstrates that there is a cardioprotective effect of nuts beyond what would be expected based solely on fatty acid profile. Nuts contain many bioactive compounds, including polyphenols, such as flavonoids and resveratrol, phytosterols, squalene,

selenium, and isothiocyanates (151, 204-212). These compounds are contained in small amounts in plant products and have been shown to have protective effects on CVD and cancer. Evidence suggests that in addition to antioxidant activity (213), phenolic compounds also have anti-thrombotic effects, such as reducing platelet aggregation, synthesis of prothrombotic and proinflammatory mediators, and expression of adhesion molecules (214). Multiple epidemiologic studies have found an inverse association between flavonoid intake and risk of CVD (215-219). However, results from a recent study do not support the presence of a protective effect of higher intake of phytoesterogens in low doses (2.2 mg/day) on CVD risk (220). In the Zutphen Elderly Study, high flavonoid intake (30 mg/day) was associated with a 58% reduction in CHD mortality (95% CI: 0.20-0.88) compared with individuals with low intake (< 19 mg/day) (215). Resveratrol is a polyphenol contained in peanuts and pistachios that may decrease CVD risk by multiple mechanisms, including inhibiting LDL oxidative susceptibility *in vitro* (221), platelet aggregation and eicosanoid synthesis (222), and tissue factor protein, which is involved in thrombus formation (223). Resveratrol also has been shown to have chemopreventive activity in mice (224). Squalene, a phytosterol precursor with antioxidant activity, recently has been detected in walnuts, almonds, peanuts, hazelnuts, and macadamia nuts (206). Other bioactive components in nuts include: ellagitannins (polyphenols containing ellagic acid) in walnuts (225, 226); flavonoids and proanthocyanidins in peanuts (211, 227), almonds (149), and pistachios (228); and resveratrol in peanuts and pistachios (212, 228). Further research is needed to identify and quantify the amounts of all bioactive compounds in specific nuts in order to increase our understanding of these bioactive compounds.

Review of clinical studies with pistachios

The beneficial effects of nuts on CHD risk are well documented. Most of the studies have used almonds and walnuts, while few studies have investigated the effects of pistachios. Pistachios are a good source of MUFA and PUFA, and numerous antioxidants including gamma-tocopherol, beta-carotene, lutein, selenium, flavonoids, and phytoestrogens, such as isoflavones and resveratrol (212, 229). In addition, pistachios are the richest sources of phytosterols of all nuts (230). Table **2-3** compares the nutrient profile of pistachios to other commonly consumed nuts. There have been 3 free-living studies that have evaluated the effects of pistachios on lipids and lipoproteins. These studies are summarized in Table **2-4**. Edwards *et al.*(23) first evaluated the effects of pistachios (20% of energy, ~2.4 oz/d) in 10 moderately hypercholesterolemic individuals (TC > 210 mg/dl) in a randomized crossover design study. Pistachios were consumed for 3 weeks and substituted for high fat snacks (or kcals). There were no significant changes in body weight. Diets were analyzed by 1-day food records. Compared to control (participants' regular diets), there were decreases in TC and the ratios of TC/HDL-C and LDL-C/HDL-C ($P < 0.05$), although there were no significant changes in LDL-C, HDL-C, and TG. There were no significant changes in body weight. A recent free-living parallel design study by Kocyigit *et al.* (24) evaluated the effects of consumption of pistachios as 20% of energy (~2.5 oz/d) for 3 weeks in 44 healthy individuals (n = 22 for intervention). There were no significant changes in BMI. Diets were assessed by food records and it was strongly suggested to eat pistachios after dinner. There were significant decreases in TC (-12%), TC/HDL-C (-20%), and LDL-C/HDL-C (-13%), and significant increases in HDL-C (27%) ($P < 0.05$); decreases in TG and LDL-C were not significant. In a free-living crossover design study, Sheridan *et al.* (25) evaluated the effects of pistachios as 15% energy (~2.5 oz/d), substituted for normally consumed high fat snacks (or fat kcals), in 15 moderately hypercholesterolemic individuals (TC > 210 mg/dl) for 4 weeks. There

were no significant changes in BMI. Diets were analyzed by 1-day food records. There were significant decreases in TC/HDL-C (-9%), LDL-C/HDL-C (-14%), and apoB/apoA-I (-13%), and significant increases in HDL-C (6%) ($P < 0.05$); however, there were no significant changes in TC, LDL-C, apoB, and TG. Although these 3 free-living studies with pistachios have demonstrated significant effects on lipoprotein ratios, there were no significant decreases in LDL-C and TG. The lack of effect on LDL-C and TG may be due, in part, to smaller sample size (23, 25), parallel design (24), and free-living conditions and assessment of diet by food records (23-25), which contribute to reduced statistical power and increased variability. Furthermore, these studies have not investigated a dose-effect of pistachios. Due to the limitations of previous studies with pistachios, a dose-response controlled-feeding study with a crossover design is warranted.

Mechanisms of the lipid-lowering effects of nuts

Contribution of fatty acids

Most clinical studies with various nuts have attributed their lipid-lowering effects to fatty acids, specifically the exchanging of saturated fat sources in the diet for unsaturated fats provided by nuts (8-20, 22-24, 231-235). The fatty acids in nuts are an important contributor to their cardioprotective effects. However, it is clear that the observed effects from these studies is much greater than what would be expected based only on their constituent fatty acids (29, 30). Two studies have demonstrated that consumption of fat isolated from almonds as oil (12) or butter (234) results in a similar lipid-lowering effect reported in studies with consumption of almonds as a whole nut. These studies imply that other bioactive components in nuts contributing to their beneficial effects on lipids and lipoproteins are contained in the lipid fraction. Phytosterols are a likely component in the lipid fraction contributing to the added beneficial effect (236). This is

particularly likely with pistachios, which contain the second highest amount of phytosterols of all commonly consumed snack foods, and the highest amount out of all nuts (279 mg/100 g) (230). In addition to phytosterols, pistachios are rich in MUFA and PUFA, particularly OA and LA (**Table 2-3**). Thus, it is likely that OA, LA, and phytosterols are major contributors of the lipid-lowering effects of pistachios.

Potential mechanisms of action

One probable mechanism by which pistachios elicit their lipid-lowering effects is via peroxisome proliferator-activated receptors (PPARs). PPARs are nuclear receptor proteins that directly regulate gene expression when activated by ligands (237-239). Both OA and LA are natural ligands of PPARα (237, 238). PPARα is expressed in many tissues including liver, kidney, heart, and skeletal muscle, and vascular cells such as endothelial cells, vascular smooth muscle cells, and monocytes/macrophages (237). Activation of PPARα stimulates the expression of genes involved in fatty acid and lipoprotein metabolism (240, 241). In particular, PPARα activators decrease plasma TG concentration by decreasing fatty acid synthesis and increasing lypolysis of VLDL via increased expression of LPL and decreased expression of apoCIII (240, 241). In addition, PPARα activators increase plasma concentrations of HDL-C via increased expression of apoA-I and apoA-II, and increase reverse cholesterol transport via increased vascular cell expression of the HDL receptors (ATP-binding cassette transporter-I and scavenger receptor class B type I, SRB-I) (240, 241). More recently, consumption of oxidized phytosterols in rats, specifically the oxidized derivative of campesterol, 5-campestenone, has been shown to activate PPARα in rats (accompanied by decreases in TG) (242). As reviewed by Hovenkamp *et al.* (243), a recent meta-analysis and placebo-controlled intervention trial demonstrate a TG-lowering effect of plant stanol consumption. More research is needed to further investigate this

effect. In summary, pistachios may elicit their beneficial effects on lipids and lipoproteins, in part, due to activation of PPARα by OA, LA, and phytosterols.

Cholesteryl ester transfer protein (CETP) is another protein that may play a role in the lipid-lowering effects of pistachios. Both unsaturated fatty acids and phytosterols regulate CETP, a hydrophobic glycoprotein that is secreted from the liver and circulates in plasma bound mainly to HDL (244). CETP mediates the transfer of cholesteryl esters (CE) and TG between HDL, VLDL, and LDL, resulting in a net mass transfer of CE from antiatherogenic HDL particles to proatherogenic VLDL and LDL particles. As depicted in Figure **2-4,** CETP plays a critical role in reverse cholesterol transport. The issue of CETP inhibition has recently come to light due to the development of CETP inhibitor drugs and more notably, the termination of a large clinical trial of the CETP inhibitor torcetrapib due to increased deaths (245). While numerous cellular, animal, and human studies recently have investigated the effects of complete inhibition of CETP through pharmacological methods, few highly-controlled studies have evaluated the effects of partial inhibition of CETP through diet.

The concept of CETP inhibition as a method to increase HDL-C and decrease LDL-C originated from studies in Japanese populations with a genetic CETP deficiency. Individuals who were heterozygotes for this allele had significantly lower CETP (-40%) and higher HDL-C (30%) with no change in LDL-C, while individuals who were homozygotes for CETP deficiency had higher HDL-C (≥ 100%) and lower LDL-C (-40%), compared with family members without a genetic defect (246-249). Despite the beneficial lipid profile observed with CETP deficiency, the possible atherogenic role of CETP is controversial. CETP may be antiatherogenic in that it increases the rate of reverse cholesterol transport by transporting CE from peripheral tissues to LDL and VLDL, which are then brought to the liver for removal. In contrast, it may be proatherogenic in that it transports CE from HDL, which is protective, to VLDL and LDL, which are atherogenic (250). A decrease in CETP also may decrease oxidation of LDL. In a study in

postmenopausal women (n = 199), there was a direct correlation ($r = 0.3$, $P < 0.05$) between plasma CETP activity and LDL oxidation susceptibility (251). A possible explanation is that increased CETP activity results in TG-rich LDL particles, which are hydrolyzed by LPL, leading to the formation of small, dense LDL particles that are more susceptible to oxidation. Additionally, CETP inhibition may increase antioxidant enzymes associated with HDL, leading to a decrease in LDL oxidation (252).

Studies have indicated an increased (253) and decreased risk (254) of CAD in individuals with CETP deficiency (despite increased HDL-C). Recent studies have suggested that CETP may have varying effects in different sub-populations. Specifically, higher CETP may be beneficial in populations with lower TG concentrations (255, 256). In a nested case-control study conducted in the prospective PREVEND study (n = 111 men), CVD risk tended to be lower with higher plasma CETP after adjustment for age and lipids (hazard ratio = 0.84, 95% CI: 0.69 to 1.03; $P = 0.10$) (255). Plasma CETP was significantly lower in cases (individuals who developed a cardiovascular event during follow-up) than in controls (those who remained free of CVD) with TG $\leq$ 1.38 mmol/l (median value, $P = 0.05$). This was not observed in men with TG > 1.38 mmol/l. The age-adjusted hazard ratio for CVD was 0.46 (95% CI: 0.24 to 0.90) in men with TG $\leq$ 1.38 mmol/l and CETP > 2.26 mg/l (median value), when compared with men with similar TG and CETP $\leq$ 2.26 mg/l. The hazard ratio for CVD was similar in both CETP categories in men with higher TG. Thus, this study demonstrates that higher CETP may be beneficial in men with lower TG (255). In a nested case-control study of the EPIC-Norfolk cohort study, risk of CAD increased with increasing CETP quintiles ($P < 0.05$). In individuals with TG < 1.7 mmol/l (median value), there was no relationship between CETP concentration and CAD risk, while this relationship was strong in individuals with higher TG ($P < 0.05$). Thus, higher CETP was associated with increased risk of future CAD, but only in individuals with higher TG (256). These

2 studies suggest that the effect of CETP depends on metabolic context, particularly TG concentration, and may help explain the discrepant results with CHD risk (256-260).

Due to the beneficial effects on the lipid profile as a result of CETP deficiency (252), 2 potent CETP inhibitor drugs, torcetrapib and JTT-705, were developed as HDL-raising agents (261-263). The beneficial effects of these pharmacological inhibitors were observed in rabbits, where a 4-fold increase in HDL-C was seen with no changes in VLDL-C and LDL-C, leading to a 60% reduction in atherosclerosis (264). In clinical trials, the CETP inhibitors effectively raised HDL-C and lowered LDL-C when added to atorvastatin (265); however, the CETP inhibitor, torcetrapib, led to increased deaths in the experimental group (torcetrapib/atorvastatin) versus control group (atorvastatin), and the trial was terminated. The adverse effects of torcetrapib may be due to off-target effects of that particular drug since they were found to be due to a mechanism that is thought to be unrelated to CETP inhibition (264, 266). It is evident that further research on CETP and CETP inhibition is warranted. Some studies suggest that complete CETP inhibition may have undesirable consequences on reverse cholesterol transport and that partial CETP inhibition ($\leq$ 50%) may be a more beneficial therapeutic option (267). When CETP was extensively inhibited by monoclonal antibody, the delivery of newly synthesized cholesteryl esters to VLDL was completely blocked. In contrast, partial CETP inhibition by monoclonal antibody decreased CE flux to VLDL by less than 20%. This suggests that partial inhibition of CETP may limit suppression of transfers between HDL and VLDL, thus maintaining reverse cholesterol transport.

Dietary factors can influence CETP activity. Studies in animals (268-273) and humans (274-276) indicate that high-fat high-cholesterol diets are associated with an increase in the transfer of CEs from HDL to LDL and VLDL. Studies in rabbits demonstrate that CETP mRNA levels are significantly increased following a high cholesterol diet (269, 277). In hamsters, an OA-enriched diet resulted in a decrease in plasma CETP activity, whereas a high palmitic diet

increased activity (272). Addition of either OA or LA to a high-cholesterol diet reduced the cholesterol-induced increases in TC and LDL-C in hamsters; however, LA reduced HDL-C, whereas OA did not (278). Furthermore, OA inhibited the increase in CETP activity induced by dietary cholesterol, and increased cholesterol 7α-hydroxylase activity, whereas LA did not. Results from this study suggest that mechanistically, OA may elicit beneficial effects on lipids and lipoproteins via decreases in CETP and increases in bile acid synthesis. In a study in monkeys, replacing dietary palmitic acid with elaidic acid resulted in a decrease in HDL-C and increase in CETP activity ($P < 0.05$) (279). Studies in transgenic mice expressing human CETP demonstrate that addition of cholesterol to a low-fat MUFA diet increases activity and mRNA expression of CETP (280). In contrast, addition of cholesterol to a high-fat MUFA diet decreases activity and mRNA expression of CETP. This study indicates that fatty acids interfere with the cholesterol-induced regulation of CETP.

Studies in humans demonstrate that intake of SFA (281-283) and TFA (284, 285) increase CETP concentration. Intake of PUFA has no effect (282, 286, 287) or decreases CETP (marine-derived n-3 fatty acids) (288), and intake of MUFA decreases CETP (282, 285, 286, 289, 290). A randomized controlled-feeding study in 41 healthy normolipidemic men evaluated the effects of 3 different diets (4 weeks each) varying in fatty acid profile on CETP activity (289). Compared to a SFA-rich diet (38% total fat: 20% SFA, 12% MUFA, 6% PUFA; 15% protein, 47% carbohydrate), a Step-I diet (28% energy total fat: 10% SFA, 12% MUFA, 6% PUFA; 15% protein, 57% carbohydrate) and MUFA-rich diet (38% energy total fat: 10% SFA, 22% MUFA, 6% PUFA; 15% protein, 47% carbohydrate) significantly decreased TC, LDL-C, apoB, and CETP ($P < 0.001$). HDL-C was significantly higher after the MUFA diet compared to the Step-I diet ($P < 0.05$). Furthermore, there were significant positive correlations between plasma CETP concentration and TC and LDL-C ($r = 0.39$ and $r = 0.45$, respectively; $P < 0.0001$ for both), as well as change in CETP concentration and change in TC and LDL-C ($r = 0.45$ and $r = 0.46$,

respectively; $P < 0.0001$ for both). Other studies also have found correlations between change in CETP activity and change in TC, LDL-C, and non-HDL-C (282). In a recent free-living study in women with type 2 diabetes, there was an inverse correlation between CETP activity and MUFA content of plasma phospholipids, as an indicator of dietary intake ($r = -0.395$, $P < 0.05$) (286).

In addition to fatty acids, plant stanols and sterols may decrease CETP. In a study in a Japanese population, intake of 2 g/day of plant stanol ester-containing spread for 4 weeks significantly reduced CETP mass by 6% (291). This was accompanied by a significant decrease in LDL-C (9.6%), apoB (8.3%), and oxidized-LDL (20%). A double-blind crossover study in hypercholesterolemic individuals ($n = 60$) demonstrates that CETP single nucleotide polymorphisms may influence lipid and CETP response to plant sterol consumption (292). Consumption of plant sterol ester margarine (1.6 g/d phytosterols) for 4 weeks significantly decreased CETP concentrations ($P < 0.05$). TC and LDL-C were decreased by 10% and 12%, respectively. The relationships of I405V CETP polymorphisms with lipid and CETP response to dietary plant sterol ester were examined and significant reductions in TC occurred only in individuals who were heterozygotes (IV genotype) or homozygotes for the I allele (II genotype) (-4.2 and -7.2%, respectively), and significant reductions in LDL-C occurred only in the II genotype (-9.5%). Furthermore, concentrations of CETP only were decreased in the II genotype (292). The results of this study suggest that response of plasma lipids to plant sterol consumption is related to the I405V CETP polymorphism.

The plasma activity of CETP could potentially be modified by diet due to regulation at the transcriptional or translational level, or due to change in substrate concentration in the plasma (293, 294). Since many conditions related to hypercholesterolemia also increase CETP concentration, plasma cholesterol concentration could potentially regulate activity of CETP. However, studies in transgenic mice support a direct regulatory effect of cholesterol on CETP gene expression. After being fed a high-fat high-cholesterol diet, there was an increase in plasma

CETP concentration and activity (295). Furthermore, transgenic mice expressing cynomolgus monkey CETP had greater diet-induced change in lipids compared to controls (296). In a study in rabbits fed a cholesterol-rich diet, experimental inhibition of CETP activity by antisense oligodeoxynucleotides led to lower plasma TC and higher HDL-C, when compared to rabbits without inhibition (297). Studies in cell culture and transgenic mice show that the CETP gene is directly activated by LXRα and LXRβ (280, 298, 299). Transgenic mice expressing human CETP demonstrate that addition of cholesterol to a low-fat MUFA diet increases CETP activity and mRNA expression (280). In contrast, addition of cholesterol to the high-fat MUFA diet decreases CETP activity and mRNA expression. Thus, at lower-fat levels it appears that the effect of cholesterol overrides the effect of fatty acids, whereas at higher-fat levels fatty acids interfere with cholesterol to regulate CETP (280). In this same study, the high-fat MUFA diet significantly reduced LXRα expression (addition of cholesterol could not rescue the inhibition). These findings suggest that inhibition of LXRα by the high-fat MUFA diet, with or without the presence of cholesterol, may explain the lack of stimulation of CETP. The high-fat MUFA diet also increased expression of PPARα. LXRα and PPARα interact *in vitro* (300-304). PPARα inhibits the binding of LXRα/RXRα to LXR response element, while LXRα inhibits the binding of PPARα/RXRα to PPRE. It is unclear whether the effects of fatty acids and cholesterol on CETP are due to the indirect regulation of LXRα or to the direct interaction of LXR/RXR/PPAR with the CETP promoter. Further research is needed to explore the effect of fatty acids on the regulation of CETP. *In vitro* studies in HepG2 cells show that inhibition of CETP expression by CETP antisense oligodeoxynucleotides has dual effects on HDL metabolism. CETP inhibition decreases the expression and protein levels of SR-BI, an HDL receptor, by 50% (305). In addition, CETP inhibition increases apoA-I expression and secretion by 30 and 92%, respectively. The results of this study suggest that CETP inhibition suppresses catabolism of HDL-CE due, in part, to inhibition of SR-BI, and increases the formation of HDL via increased apoA-I secretion (305). In

another study in HepG2 cells, fatty acids were shown to regulate mRNA levels of CETP (306). Compared to control (1.25% bovine serum albumin), there were significant decreases in CETP mRNA levels after treatment with LA, ALA, gamma-linolenic acid, AA, EPA, and DHA (306). Decreases in CETP mRNA levels were correlated with increases in the degree of unsaturation of the fatty acids.

Taken together, *in vitro*, animal, and human studies demonstrate that CETP plays a key role in lipoprotein metabolism and that dietary fatty acids differentially regulate protein concentrations and mRNA levels of CETP. In addition, recent studies suggest that phytosterols decrease CETP. Clinical studies investigating the effects of diet on CETP have used various oils in test diets to alter fatty acid profiles. While there are many studies that have evaluated the effects of different nuts on lipids and lipoproteins, to our knowledge, there are currently no studies that have examined their effects on CETP.

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Table **2-1**: Nutrients for the reduction of lipids and lipoproteins (per 100 g nuts[1]).

Nut	SF (g)	MF (g)	PF (g)	LA (g)	ALA (g)	Cholest (mg)	Fiber (g)	α-tocopherols (mg)	Total Phytost (mg)	ß-Sitost (mg)
Walnuts	6	9	47	38	9	0	7	0·70	72	64
Almonds	4	32	12	12	0	0	12	25·87	120	111
Macadamias	12	59	1·5	1·30	0·20	0	8	0·57	114	107
Pecans	6	41	22	21	1	0	10	1·4	102	89
Pistachios	6	24	14	13·64	0·26	0	10	1·93	214	199
Hazelnuts	4	46	8	7·83	0·09	0	10	15·03	96	89
Brazils	16	23	24	23·81	0·06	0	5	n/a	n/a	n/a
Cashews	9	27	8	7·66	0·16	0	3	n/a	158	n/a
Pine nuts	8	19	21	21	1	0	4	n/a	141	n/a

[1]Source: USDA National Nutrient Database for Standard Reference. All of the nuts are unsalted; almonds, brazil nuts, hazelnuts, pecans, pine nuts, and walnuts are unroasted; cashews, macadamias, and pistachios are dry roasted; SF = saturated fatty acids, MF= monounsaturated fatty acids, PF = polyunsaturated fatty acids, LA = Linoleic Acid, ALA = Alpha-linolenic acid, Cholest = Cholesterol, Total Phytost = Total Phytosterol, β-Sitost = β-Sitosterol.

Table **2-2**: Phytosterol content of nuts in mg/100 g edible portion.

	β-sitosterol		Campesterol		Stigmasterol		5-avenasterol	Total phytosterols	
Almonds	111[a]	143[b]	5	5	4	5	20[b]	120	199
Brazil nuts	NR	66	NR	2	NR	6	14	NR	95
Cashews	NR	113	NR	9	NR	1	14	158	150
Hazelnuts	89	102	6	7	1	2	3	96	121
Macadamias	108	144	8	10	0	ND	13	116	187
Peanuts	NR	77	NR	13	NR	12	18	NR	137
Pecans	89	117	5	6	3	3	15	102	157
Pine nuts	NR	132	NR	20	NR	1	40	141	236
Pistachios	198	210	10	10	5	2	26	214	279
Walnuts	64	89	7	5	1	ND	7	72	113

Source: [a]USDA Nutrient Database for Standard Reference, [b]Phillips *et al*., 2005. ND = less than the limit of detection, NR = not reported.

Table **2-3**: Nutrients in 1 oz. of tree nuts and peanuts.

Nutrient	Units	Almonds	Cashews	Macadamias	Peanuts	Pecans	Pistachios	Walnuts
	# of kernels/oz	24	18	10-12	28	20 halves	49	14 halves
Calories	kcal	160	160	200	170	200	160	190
Protein	g*	6	4	2	7	3	6	4
Total Fat	g	14	13	22	14	20	13	18
Saturated Fat	g	1	3	3	2	2	1.5	1.5
Monounsaturated Fat	g	9	8	17	7	12	7	2.5
Polyunsaturated Fat	g	3	2	0.5	5	6	4	13
Linoleic acid (18:2)	g	3	2.17	0.37	4.44	5.85	3.87	10.78
Linolenic acid (18:3)	g	0	0.05	0.06	0	0.28	0.07	2.57
Cholesterol	mg**	0	0	0	0	0	0	0
Carbohydrate	g	6	9	4	6	4	8	4
Fiber	g	3	1	2	2	3	3	2
Calcium	%DV***	6	0	2	2	2	4	2
Iron	%DV	6	10	4	4	4	6	4
Magnesium	%DV	20	20	8	10	8	8	10
Phosphorus	%DV	15	15	6	10	8	15	10
Potassium	%DV	6	4	2	6	4	9	4
Sodium	mg	0	5	1	2	0	3	1
Zinc	%DV	6	10	2	6	8	4	6
Copper	%DV	15	30	8	10	15	20	20
Manganese	%DV	35	12	45	30	60	18	50
Selenium	%DV	trace	4	trace	2	2	4	2
Vitamin C	%DV	0	0	0	0	0	trace	0
Thiamin	%DV	4	4	15	8	10	15	6
Riboflavin	%DV	15	4	2	2	2	2	2
Niacin	%DV	6	2	4	20	2	2	2
Pantothenic acid	%DV	0	4	2	4	2	0	2
Vitamin B6	%DV	2	4	6	4	2	20	8
Folate	%DV	2	4	0	10	2	4	6
Vitamin B12	%DV	0	0	0	0	0	0	0
Vitamin A	%DV	trace	0	0	0	trace	6	trace
Vitamin K	mcg****	0	9.84	n/a	0	0.99	3.74	0.76
Vitamin E	%DV	35	0	0	10	6	2	trace
Tocopherol, alpha	mg	7.33	0.26	0.16	1.96	0.4	0.55	0.2
Tocopherol, beta	mg	0.12	n/a	0	n/a	0.11	0.04	0.04
Tocopherol, gamma	mg	0.25	n/a	0	n/a	6.93	6.36	5.9
Tocopherol, delta	mg	0.07	n/a	0	n/a	0.13	0.21	0.54
Total Phytosterols	mg	34	45	32	n/a	29	61	20
Stigmasterol	mg	1	n/a	0	n/a	1	1	0
Campesterol	mg	1	n/a	2	n/a	1	3	2
Beta-sitosterol	mg	31	n/a	30	n/a	25	56	18
Carotene, beta	mcg	1	0	0	0	8	45	3
Carotene, alpha	mcg	0	0	0	0	0	0	0
Cryptoxanthin, beta	mcg	0	0	n/a	0	3	0	0
Lutein + zeaxanthin	mcg	0	7	n/a	0	5	342	3

Source: USDA National Nutrient Database for Standard Reference; *g = gram, **mg = milligram, ***%DV = percent Daily Value, ****mcg = microgram.

Table **2-4**: Clinical studies evaluating the effects of pistachios on lipids and lipoproteins.

Ref.	**Intervention**	**Control**	**Duration, design**	**Sample size, characteristics**	**Pretreatment lipid levels**	**Net effect on lipids**	**Unique features**
Edwards *et al.*, 1999	20% kcal from pistachios (~2.4 oz/d)	Regular diet	3 wk, free-living, RCT	10 moderately hypercholesterolemic (TC > 210 mg/dl)	TC = 243 mg/dl LDL-C = 180 HDL-C = 50 TG = 113 TC/HDL-C = 4.8 LDL-C/HDL-C = 3.2	TC ↓9 NS (median Δ) LDL-C ↓ 11 NS HDL-C ↑ 4 NS TG ↓ 6 NS TC/HDL-C ↓ 0.7 LDL-C/HDL-C ↓ 0.3	Substitute pistachios for high fat snacks (or fat kcals); 1 day food records; No significant changes in body weight (data not shown)
Kocyigit *et al.*, 2006	20% kcal from pistachios (~2.3-2.6 oz/d)	Regular diet	3 wk, free-living, parallel	44 healthy individuals (n = 22 for intervention)	TC = 4.08 ± .69 mmol/L (158 mg/dl) LDL-C = 1.94 ± .43 (75 mg/dl) HDL-C = 1.01 ± .28 (39 mg/dl) TG = 1.18 ± .59 (105 mg/dl) TC/HDL-C = 3.98 ± 1.41 LDL/HDL = 1.8 ± .51	TC ↓ 12% LDL-C ↓ 3% NS HDL ↑ 27% TG ↓ 9% NS TC/HDL-C ↓ 20% LDL-C/HDL-C ↓ 13%	Strongly suggested to eat pistachios after dinner; No nuts, nut butters or nut oils on regular diet; Food records; No significant changes in BMI
Sheridan *et al.*, 2007	15% kcal from pistachios (~2-3 oz/d)	Regular diet	4 wk, free-living, RCT	15 moderately hypercholesterolemic (TC > 210 mg/dl)	TC = 246 ± 5.8 mg/dl LDL-C = 164 ± 6.9 HDL-C = 55 ± 3.5 TC/HDL-C = 4.7 ± .29 LDL-C/HDL-C = 3.1 ± .25 apoB = 132 ± 5.3 apoB/A = 1.0 ± .07	TC ↓ 4% NS LDL-C ↓ 10% NS HDL-C ↑ 6% apoB ↓ 13% NS TC/HDL-C ↓ 9% LDL-C/HDL-C ↓ 14% apoB/apoA ↓ 13%	Substitute pistachios for normally consumed high fat snacks (or fat kcals); 1 day food records; No significant changes in BMI

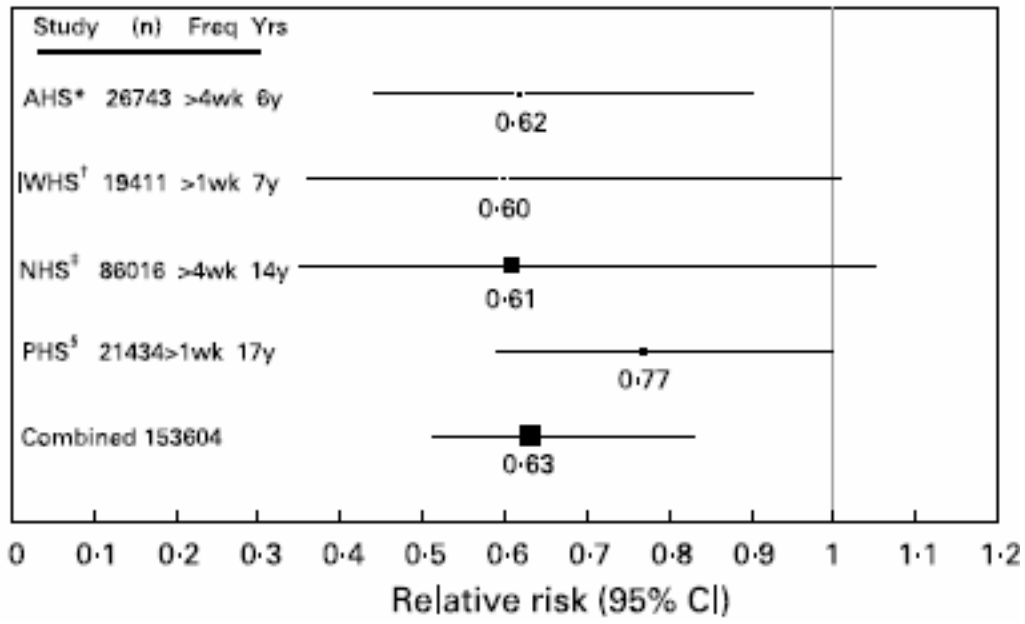


Figure **2-1**: Prospective cohort studies of nut consumption and CHD. Source: Kelly *et al*., 2006. *Adventist Health Study, †Iowa Women's Health Study, ‡Nurses' Health Study, §Physicians' Health Study. Size of marker represents number of subjects. Relative risk and 95% CI derived from the comparison of incidence rates between the highest and lowest frequency of nut consumption and similarly adjusted for multiple risk factors.

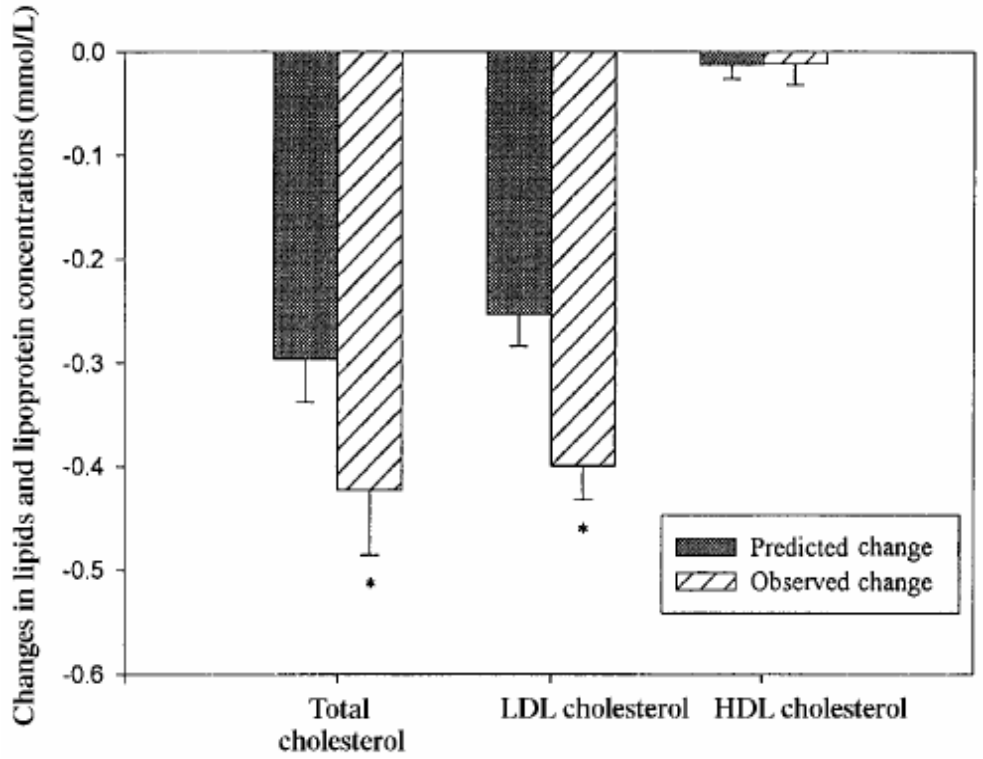


Figure **2-2**: Comparison of observed changes (mean ± SEM) in concentrations of total cholesterol, LDL-cholesterol, and HDL-cholesterol. Source: Kris-Etherton *et al.*, 1999. Predictive equations for plasma cholesterol of Hegsted *et al*, 1993 and Mensink & Katan, 1992 were used (data are means of the results from both equations). *Observed change was significantly different from the predicted change ($P < 0.05$).

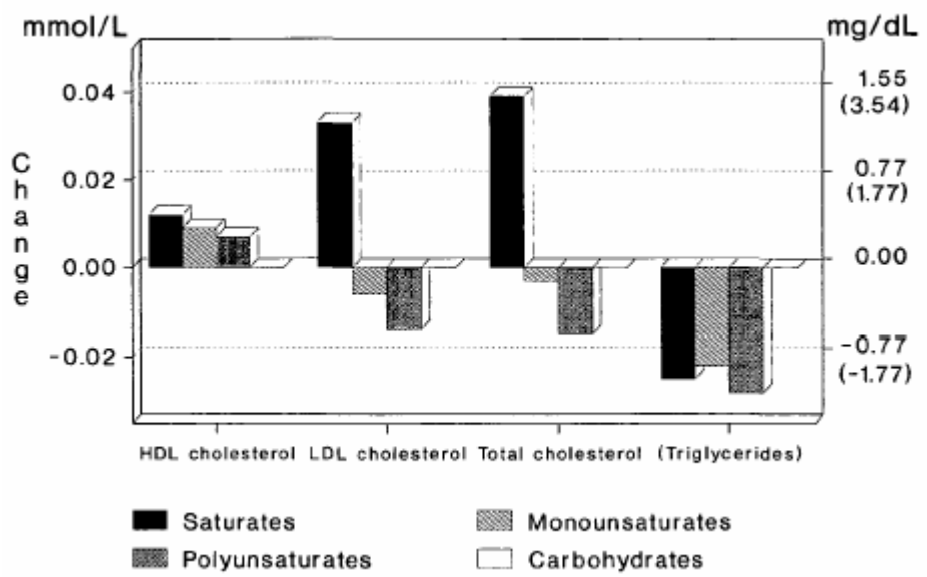


Figure **2-3**: Predicted changes in serum lipids and lipoproteins when 1% of energy as carbohydrate is replaced by saturates, monounsaturates, and polyunsaturates. Values in parentheses represent predicted changes in TG in mg/dl. Source: Mensink & Katan, 1992.

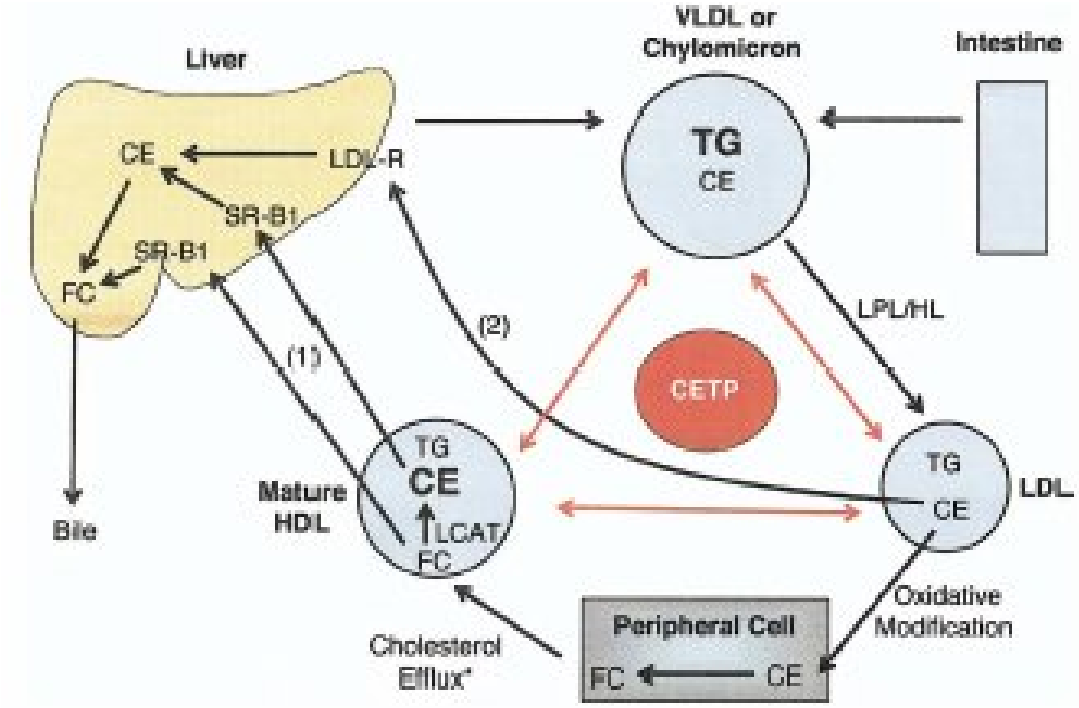


Figure **2-4**: Role of plasma CETP in reverse cholesterol transport. Source: Barter & Kastelein, 2006. CE = cholesteryl esters, FC = free cholesterol (unesterified form), LDL-R = LDL-receptor, LCAT = lecithin:cholesterol acyltransferase, LPL = lipoprotein lipase, HPL = hepatic lipase, SR-B1 = scavenger receptor-B1.

Chapter 3

Integration of Molecular Biology and Nutrition: The Role of Nutritional Genomics in Optimizing Dietary Guidance in Lipids

Abstract

The evolution of the field of nutrition has largely been due to advancements in the techniques of molecular biology. Since the completion of the Human Genome Project, the fields of nutrigenomics, nutrigenetics, pharmacogenetics, metabolomics, and proteomics have evolved. These fields provide immense potential to enhance the study of genes involved in health and chronic diseases and the interactions of nutrients on these genes. The emerging field of lipidomics will provide further insight on the roles of genetics, disease, drugs, and diet, on the regulation of lipid metabolism. Advances in molecular nutrition will continue to increase our understanding of the heterogeneity of individual lipid response to dietary interventions, allowing for a movement towards a more individualized approach for dietary guidance. The potential impact of molecular biology in clinical nutrition research of the future is inducing a shift in the focus of nutrition research from disease treatment and management to one of disease prevention.

Introduction

Integration of the techniques of molecular biology has transformed the field of nutrition, from the study of classical nutrient deficiencies of the 1950s and 1960s, and biomarkers and chronic disease risk in the latter part of the last century, to the study of how nutrients alter gene transcription/translation and physiological processes. The evolution of molecular nutrition has advanced our understanding of the mechanisms that explain diet/nutrient responses. Nutrients are now understood to be bioactive molecules that can affect risk for chronic diseases and impact quality of life. More recently, the study of genomics has changed the direction of nutrition research from a broad focus on public health and development of dietary guidelines at a population level, to a more targeted evaluation of the effects of genetic variation on dietary responses. This individualized approach incorporates the study of the effects of specific nutrients on gene expression (nutrigenomics) and the study of variations in dietary responses due to genetic predispositions (nutrigenetics). Nutritional genomics is the term used to encompass both nutrigenomics and nutrigenetics (1). As we look back at how the field of nutritional science has advanced due to the integration of molecular biology techniques, it prompts the question, how will molecular nutrition affect clinical nutrition research, health policy, and product development in the future? We will use the evidence base of fatty acid effects on cardiovascular disease (CVD) risk to illustrate how our understanding of nutrition has evolved with the developments of molecular biology, and how advances in nutritional genomics will impact clinical nutrition research and practices in the future.

Impact of molecular biology on nutrition research

Developments in molecular biology techniques in the 1970s expanded our understanding of nutrients as molecules that are essential to growth and development, to bioactive molecules that have a role in health maintenance and disease prevention (2). Advancements in recombinant DNA technology led to strategies that enabled dramatic advances to be made in the study and manipulation of DNA (3). This technology vastly increased our knowledge of genes and their role in the inheritance of disease. Integration of molecular biology techniques in basic nutrition research (Table **3-1**) led to the idea that diet and specific dietary components could alter gene expression and play a role in chronic diseases, such as CVD, diabetes, blood pressure, osteoporosis, and cancer. The discovery that diet could alter gene expression and affect an individual's health status was a novel idea that provided immense potential to advance our approaches to treatments of disease. Also, it has provided an increased understanding of how candidate genes explain diet response and, in turn, variability in diet responsiveness.

The evolution of the study of fatty acids

As our understanding of molecular mechanisms increased, it became clear that fatty acids were important signaling molecules owing to their effects on gene expression. By the early 1990s, dietary fat was recognized to have a significant role in the regulation of metabolic pathways in hepatic, adipose, and non-lipogenic tissues (such as immune, intestinal, pancreatic, cardiac, and the brain) (4). The discovery that fatty acids were ligands for nuclear receptors established a direct role for fatty acids in gene regulation (5). Polyunsaturated fatty acids (PUFA) were shown to exert beneficial effects due to upregulation of genes involved in fatty acid oxidation and downregulation of genes involved in lipid synthesis (6). These effects on gene transcription were

found to be mediated by nuclear receptors [peroxisome proliferator-activated receptors (PPARs), liver X receptor, hepatocyte nuclear factor-4α] and transcription factors (sterol-regulatory element binding protein and nuclear factor-κB) (7). Subsequent studies demonstrated that individual PUFAs, for example linoleic acid (LA), alpha-linolenic acid, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA), have differential effects on the expression of proteins involved in lipogenesis (including fatty acid synthase, stearoyl-CoA desaturase and acetyl-CoA carboxylase), inflammation (interleukin-1β), and adhesion molecules (vascular cellular adhesion molecule-1) (8). More recently, there has been a focus on the metabolites of long-chain fatty acids as precursors to prostaglandins, thromboxanes, leukotrienes, lipoxins, and resolvins, and their role in disease (9). By the end of the 20th century, our understanding of the underlying mechanisms of gene regulation by fatty acids had increased markedly, however, there was still relatively little known about the human genome itself. In 1990, an international collaborative effort to sequence the human genome was launched by the International Human Genome Sequencing Consortium, with the ultimate goal of sequencing the approximately 3 billion base pairs in the human genome (10). This effort would revolutionize nutrition research.

The genomics era

The Human Genome Project (HGP) was completed in 2003. Since its completion, the fields of nutrigenomics, nutrigenetics, pharmacogenetics (study of the effects of genetic variation on response to drugs), metabolomics (changes in cellular compounds in response to bioactive nutrients), and proteomics (the effect of nutrients on the set of proteins produced by a species) have evolved (11). A search for "genomics" articles in PubMed results in over 18,300 articles. Of these articles, approximately 10,000 were published in the last 4 years since the completion of the

HGP. Recognizing the importance of the study of nutrient effects on gene regulation, the Journal of Nutrition implemented a nutrient-gene interaction section in 1999. The American Dietetic Association also has recognized the importance of this emerging field with the recent initiation of a nutritional genomics series in their journal in 2005 to address the issues relating to dietitians. The number of articles in the area of nutrient-gene interactions in the last decade has greatly increased, as shown in Figure **3-1**. In 2002, another international collaborative effort was established – The International HapMap Project. The aim of the project was to determine and catalogue the common patterns of DNA sequence variation in the human genome (12). The goal was to characterize the shared patterns in the portion of the genome in which humans differ from one another, with the idea that certain single nucleotide polymorphisms (SNPs) would be associated with each other. In 2005, the International HapMap Consortium published a database of common variation in the human genome (13). The findings revealed the existence of "recombination hotspots" – areas on a chromosome in which certain sets of alleles reside and are associated (i.e., the correlation of certain SNPs). Thus, a small number of select SNPs in a chromosomal region could be genotyped and provide enough information about other common SNPs in that region (12). The utilization of genomics data from the HGP and HapMap Project provides immense potential to enhance the research of genes involved in health and chronic diseases and the interactions of nutrients on these genes. The ultimate goal of the HGP established by the National Institutes of Health is to be able to sequence the entire genome of an individual for $1,000 by the year 2015 (14). Incorporation of high-throughput experimental methods, such as microarray technology, has induced a shift in nutrition from a reductionist approach to an integrative biological systems approach that is driven by data generation. This approach allows for the discovery of unforeseen interactions between nutrients and molecular pathways. In the future, this could lead to achieving the same understanding of the role of diet and genetics of other diseases and metabolic pathways as is currently known of CVD and lipid

responses, as reviewed below. The translation of this research to practice can result in earlier detection of genetic predispositions to disease, improved diagnosis, and development of new approaches to treatments.

Impact of nutritional genomics on the study of fatty acids and dietary guidance

A major challenge in the study of lipid response to dietary interventions is the heterogeneity of individual responses. In an effort to explain this variance, numerous studies have evaluated the interactions of fatty acids with gene variants and their effects on lipid responses. A recent systematic review of the literature demonstrates that nutrient-gene interactions affecting lipid response to diet occurred with SNPs in the apolipoprotein (Apo) genes - *ApoA-I, ApoA-IV, ApoB,* and *ApoE*, although these effects are not consistent across studies and are sometimes conflicting (15). SNPs in the PPARα gene also have been shown to affect lipid responses to dietary intervention (16). The 162V allele was only associated with higher levels of triglycerides (TG) and apoCIII in individuals with a PUFA intake of less than 6% of energy. In contrast, carriers of the 162V allele had lower apoCIII than 162L homozygotes when PUFA intake was high. When PUFA intake was less than 4% of energy, TG levels of 162V allele carriers were 28% higher than those of 162L homozygotes ($P < 0.01$). However, at higher intake of PUFA (> 8% of energy), TG concentrations of 162V allele carriers were 4% lower than 162L homozygotes. Results were similar for n-6 and n-3 fatty acids (16). The differential effects of n-6 and n-3 fatty acids have been reported on carotid-artery intima-media thickness in individuals with variant 5-lipoxygenase genotypes (17). Intake of LA and AA in carriers of two variant alleles was associated with increased intima-media thickness (P for trend < 0.001; P for trend < 0.05, respectively), while intake of EPA plus DHA was inversely associated with intima–media thickness in carriers of two variant alleles (P for trend < 0.01). These associations were not found

in carriers of the common allele (17). Other gene variants affecting lipid response that have been recently reported include apoCIII, lipoprotein lipase, hepatic lipase, and PPARγ (18). Numerous studies have examined the effects of polymorphisms on lipid response to diet; however, the results have been inconsistent. This could be due to inadequate statistical power (i.e., the incidence of the mutation is very small in the population), inconsistent study design, and studying effects of single gene polymorphisms on lipid response (15). In the future, large well-controlled dietary intervention studies that investigate the effects of SNPs in multiple genes will be required. In addition, identification of SNPs with significant frequencies in the population is needed in order to potentially impact a greater number of individuals.

In the last 25 years, progress has been made in the movement towards a more individualized approach for dietary guidance, as evidenced by recommendations made by the American Heart Association (AHA) and the U.S. Department of Health and Human Services (HHS) and Department of Agriculture (USDA). Recent dietary recommendations have increased in specificity for the general population, as well as for specific subpopulations (19, 20). In addition, the recommendations for macronutrients provided as ranges are the framework for individualized diet guidance. In 1980, the recommendation for fat in the Dietary Guidelines for Americans was to "avoid too much fat, saturated fat, and cholesterol" (21). In the most recent dietary guidelines published in 2005, key recommendations for fat include: consume less than 10% of total calories from saturated fatty acids (SFA), consume less than 300 mg/day of cholesterol, keep consumption of *trans* fat as low as possible; and keep total fat intake between 20-35% of calories, with a majority of fats coming from sources of PUFA and monounsaturated fat (20). The range for total fat indicates a need to individualize a total fat target based, in part, on the best health outcome. In addition, considerations were made for specific population groups, such as lower recommended intake of SFA and dietary cholesterol (< 7% of calories and less than 200 mg/day, respectively) as part of a therapeutic diet for adults with elevated LDL-C.

Furthermore, the USDA replaced the "Food Guide Pyramid" with "MyPyramid", a more personalized approach for implementing the new dietary guidelines and promoting the idea of individual variance (19). These changes in recommendations by the USDA and HHS paralleled the increasing specificity of the dietary recommendations by the AHA. In 2000, the Nutrition Committee of the AHA released a revision statement to their dietary guidelines to include considerations for special populations, including older individuals, children, and individuals with specific medical conditions (22), stating that "the present guidelines have begun to incorporate an awareness of genetic and metabolic heterogeneity in optimizing population-based nutritional guidelines for individuals". The influence of genetics on nutrition requirements and dietary response was included under issues that merit further research. Although we can predict change in lipids and lipoproteins in response to varying dietary fat (type and amount) in groups of individuals, we are not yet able to reliably and consistently predict individual response (23). In order to develop techniques to adapt dietary guidelines for individuals, the identification of more diet responsive gene variants is essential. In addition, we need a better understanding of how multiple gene variants on numerous genes interact to influence dietary responses.

Potential impact of molecular nutrition in the future

Molecular nutrition has the potential to significantly impact clinical research, health policy, product development, and health care in the future, as shown in Table **3-2**. In terms of clinical studies, SNP profiles can be used as selection criteria so that randomized controlled trials can be conducted in populations with specific genetic predispositions. This will potentially help clear up the inconsistencies in the data that currently exist. The potential impact in clinical nutrition research is having the ability to predict individual dietary responses and ultimately shift the focus of nutrition research from disease treatment and management to one of disease

prevention (24). In terms of health policy, a targeted approach to developing dietary recommendations to specific subpopulations will be more efficacious than making general recommendations to populations consisting of individuals with diverse genetic backgrounds. The potential impact on the food industry includes enrichment of foods and beverages and development of functional foods and supplements that are tailored to individuals based on their genetic background. The ultimate goal of incorporating nutritional genomics into practice is to minimize risk for disease and maximize genetic potential on an individual basis (25).

Although the potential future benefits are clear, there are numerous challenges and issues that still need to be resolved before nutritional genomics information can be utilized in these realms. In order to clear up the inconsistencies of current studies, large intervention studies that are specifically designed to study nutrient-gene interactions are required. A major challenge is not only identifying key diet-responsive gene variants, but also understanding the cumulative effects of interactions of different SNPs on multiple genes with multiple dietary/lifestyle factors on chronic diseases, such as CVD, diabetes, obesity, and metabolic syndrome. In addition, the application of nutritional genomics in the context of nutrigenetics and related direct-to-consumer genetic testing introduces complex ethical issues. A common public fear/concern is that the misuse of genetic information could result in genetic discrimination by insurance companies and employers. Recognizing the potential for harm and the value of being proactive in managing both the benefits and risk of nutrigenomics, the University of Toronto's Joint Center for Bioethics worked with an expert panel of nutrition scientists to develop a bioethical framework within which nutrigenomic practices and dietary guidance could unfold in a scientific and socially responsible way (26). Issues such as these must be addressed before use of nutritional genomics information can become a reality in practice. A highly integrative approach, involving collaborations of numerous disciplines, will be necessary in order to fully capitalize on the potential of nutritional genomics technology, with the ultimate goal of preemptive nutrition. As

shown in Figure **3-2**, this approach will involve merging our knowledge of structural and functional aspects of nutrients, such as fatty acids, with ethical and regulatory aspects, in order to translate and disseminate our knowledge to affect public health.

Future Perspective: The emerging field of lipidomics

Lipidomics is the large-scale study of non-water-soluble metabolites (lipids) and can be subdivided into architecture/membrane lipidomics and mediator lipidomics (27, 28). This emerging field is a specialized component of metabolomics (29). Similarly to proteomics, metabolomics, or gene expression profiling, lipomic profiling provides information about a broad range of biomolecules. However, unlike the other "-omic" sciences, lipidomics has not been widely utilized in nutrition studies. This is somewhat surprising as relative lipid concentrations yield a complex picture of the regulation of lipid metabolism resulting from genetics, disease, drugs, and importantly diet. Dysregulation of lipid metabolism is primary to diabetes, obesity, CVD inflammation, and a host of other diseases of public health importance. Lipids might not be the first thought to come to mind with diseases such as autism, Alzheimer's, or cancer, but they are critically important to these diseases and virtually all others. Lipids are central to metabolism and integrated into virtually all metabolic processes at some level. Even in those diseases where lipids are not primary causative agents, maintaining proper lipid metabolism is very important to the success of treatment and for maintaining quality of life.

Nonetheless, the study of lipid profiles is perhaps 10 years behind proteins, genes, and transcripts in terms of our technology and understanding. This brings to mind the question, why has lipidomics not been better integrated into nutrition studies? The main reason is not due to our lack of understanding of lipid metabolic pathways, as this is a strength of lipidomics. The main reason is technical and practical. Key methodologies used in lipidomics research include

electrospray ionization, mass spectrometry (MS), and liquid chromatography tandem mass spectrometry (LC-MS/MS); and these methods for assaying lipids in a biofluid are not standardized. An ideal technique would provide quantitative data for each lipid in a sample. MS, chromatography, and nuclear magnetic resonance (NMR) spectroscopy have been used to varying degrees and with varying success. For example, the National Institutes of Health is funding the LIPID MAPS Consortium to identify and quantify all lipids in specific cells using MS. This approach has the advantage of being conducive to high-throughput studies, but it cannot produce highly quantitative profile data. NMR has strong quantitative capabilities but, as with MS, most lipid metabolites produce similar spectra and cannot always be specifically identified. Chromatography is the only solution providing consistent quantitative and qualitative analytical results. Gas chromatography and corresponding MS methods are well established and useful in probing bioactive lipid mediators. Advances in computer hardware, software, algorithms, chromatography, and identification of bioactive mediators help to establish our appreciation for what is currently termed mediator profiling (27, 28). Advances in the use of LC-MS/MS permit profiling of closely related compounds without requiring derivatization of samples. Moreover, the interface of MS/MS with LC permits profiling of lipids with reduced potential for artifacts. Although these approaches can effectively quantify high-abundance lipids, there is still concern that they are less well suited for low-abundance lipids such as eicosanoids or lipid oxidation products. However, given the advances in nanotechnologies and likely increases in sensitivity of mass spectrometers, there is some hope to overcome this limitation. In fact, some have suggested that it should be possible to develop personalized units to continuously monitor an individual's profile of lipid mediators as well as those involved in resolution of inflammation (30).

Thus, at this time, lipid or mediator profiling is in a similar situation as transcript profiling was a number of years ago. Ten years ago, comprehensive gene expression studies were laborious and were being performed in individual laboratories. As the microarray methods

became more routine and the equipment was made available in core or shared laboratories, the inertia for these studies was overcome. Now it is almost commonplace to perform clinical nutrition studies including some comprehensive gene expression analysis. It is a similar story for genotyping where experiments that once took months and yielded little data are now high-throughput and high-content. To have the same trajectory, lipidomics requires the same "platform" of facile techniques with high sensitivity, selectivity, and automation. As mentioned above, the pieces are basically in place, and it is only a matter of time and experience before this –omics technology for lipids catches up with its older established brethren.

Executive summary

Impact of molecular biology on nutrition research

- The discovery that diet could alter gene expression and affect an individual's health status was a novel idea that provided immense potential to advance our approaches to treatments of disease.
- The study of diet-gene interactions has provided an increased understanding of how candidate genes explain diet responses and, in turn, variability in diet responsiveness.

The Genomics Era

- Incorporation of high-throughput experimental methods, such as microarray technology, has induced a shift in nutrition from a reductionist approach to an integrative biological systems approach that is driven by data generation.
- The translation of nutritional genomics research to practice can result in earlier detection of genetic predispositions to disease, improved diagnosis, and development of new approaches to treatments.

Future Perspective: The emerging field of lipidomics

- Utilization of lipidomics in future nutrition studies will provide a complex picture of the regulation of lipid metabolism resulting from genetics, disease, drugs, and importantly diet.

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Table **3-1**: Molecular biology techniques that have impacted basic nutrition research.

- Recombinant DNA technology: restriction endonucleases, cloning vectors
- Hybridization techniques: Southern (DNA), Northern (RNA), Western (protein) blots
- Amplification of DNA sequences: polymerase chain reaction (PCR)
- Protein identification: mass spectrometry
- Knockout mice, transgenic mice
- Gene expression profiles: microarray technology

Table **3-2**: Nutritional genomics in clinical nutrition of the future.

- Identify clinical biomarkers that are responsive to diet and indicate health status
- Use SNP profiles as a screening tool for clinical trials; use specific SNPs as inclusion and exclusion criteria
- Understand the cumulative effects of interactions of multiple SNPs, multiple genes, and multiple environmental factors on disease
- Generate dietary recommendations based on individual genetic make-up
- Expand the role of the dietician to include genetics counselor with bioethics training
- Enrich foods with specific bioactive nutrients aimed at individuals with specific genotypes
- Develop functional foods and dietary supplements targeted at specific populations

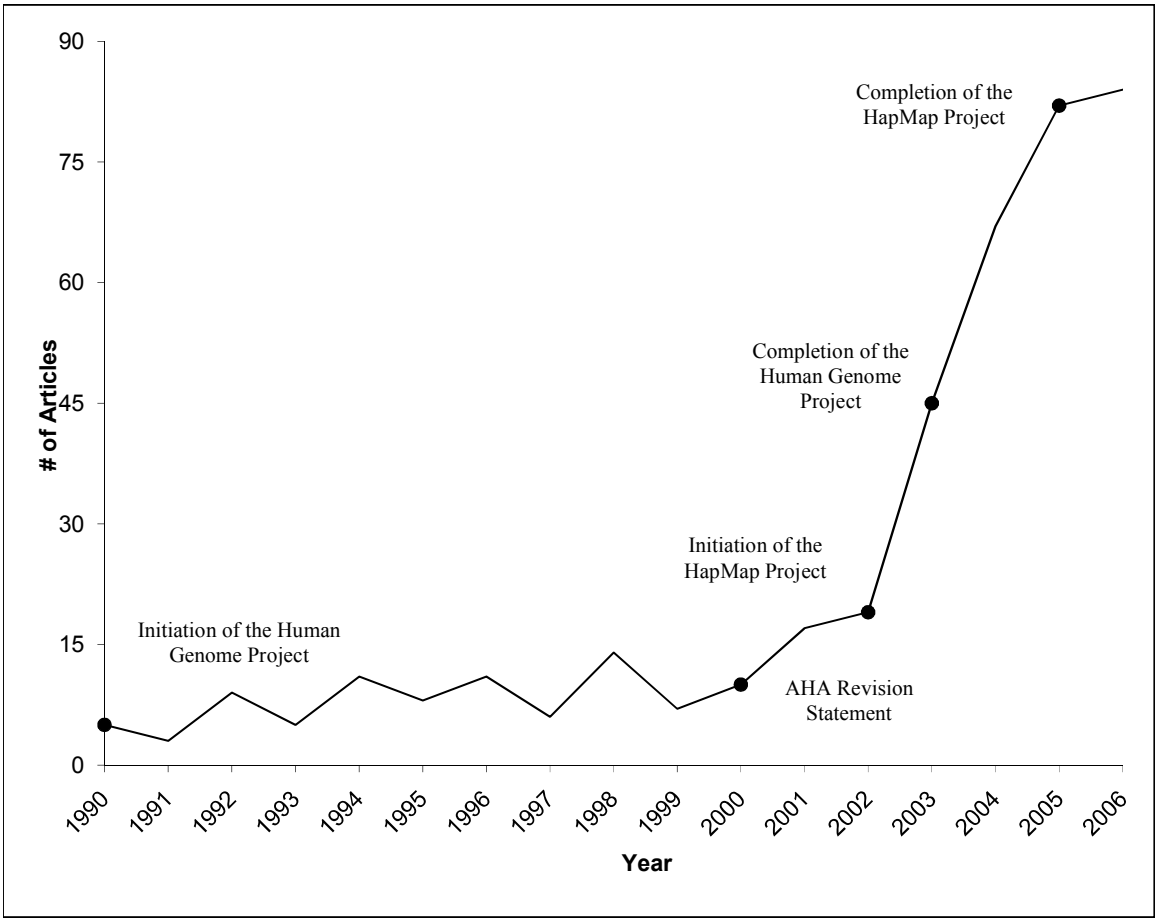


Figure **3-1**: Distribution of nutritional genomics articles in PubMed. Source: www.pubmed.gov.

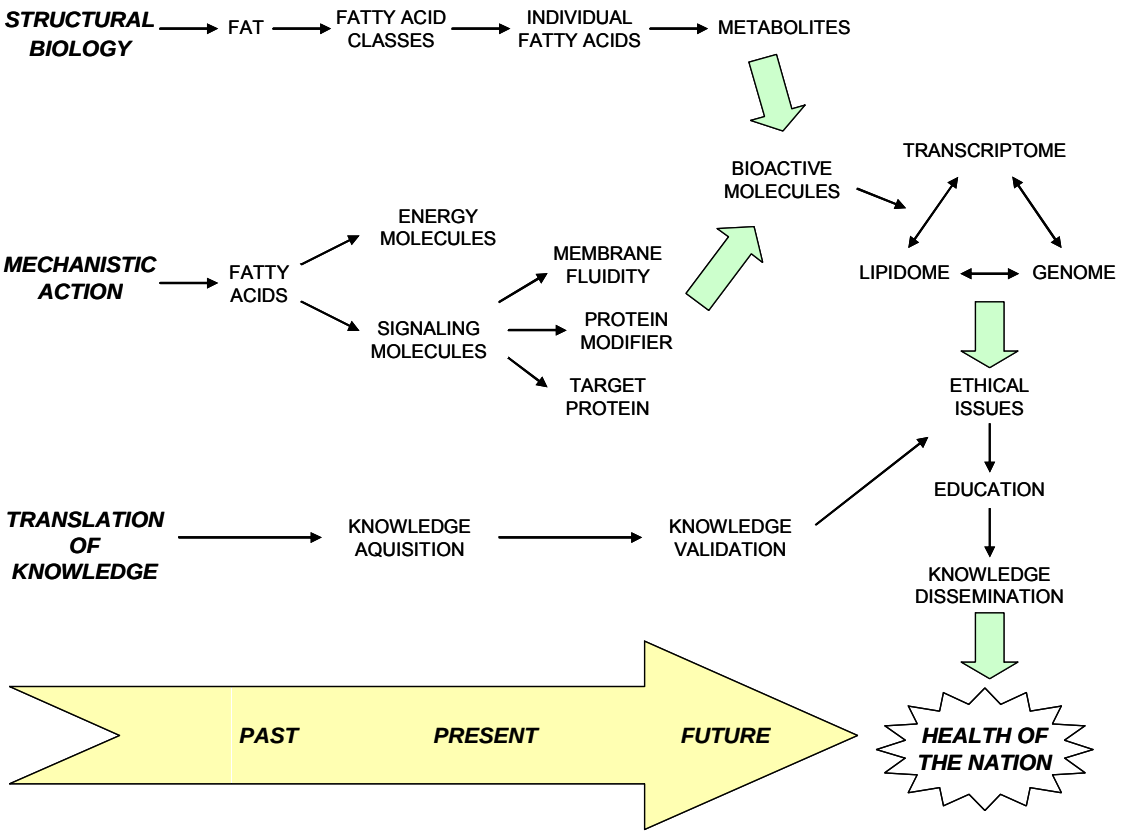


Figure **3-2**: The evolution of the study of fatty acids.

Chapter 4

Effects of Pistachios on Cardiovascular Risk Factors and Potential Mechanisms of Action: A Dose-Response Study

Abstract

Background: Nut consumption lowers cardiovascular disease (CVD) risk. Few studies have examined effects of pistachios on CVD risk factors and they have not evaluated dose-response relationships or lipid-lowering mechanisms. **Objective:** Evaluate the effects of 2 doses of pistachios, added to a lower fat diet, on lipids and lipoproteins, apolipoprotein-defined lipoprotein subclasses, and plasma fatty acids. To investigate mechanisms of action, we measured cholesteryl ester transfer protein (CETP) and indices of plasma stearoyl-CoA desaturase activity (SCD). **Design:** In a randomized, crossover, controlled-feeding study, men (n = 10) and women (n = 18) with LDL cholesterol (LDL-C) ≥ 2.86 mmol/l consumed 3 isoenergetic diets (4 weeks each). Baseline measures were assessed after 2 weeks on a typical western diet. Experimental diets included: lower fat control diet with no pistachios [25% total fat; 8% saturated fat (SFA), 9% monounsaturated fat (MUFA), 5% polyunsaturated fat (PUFA)]; 1.5 ounce pistachio diet [(10% total energy from pistachios; 30% total fat; 8% SFA, 12% MUFA, 6% PUFA); and 3.0 ounce pistachio diet (20% total energy from pistachios; 34% total fat; 8% SFA, 15% MUFA, 8% PUFA). **Results:** Total cholesterol (TC), LDL-C, non-HDL-C, apoB, and apoB/apoA decreased after both pistachio diets ($P < 0.05$ versus control diet). Triacylglycerol and plasma SCD activity decreased after the 3.0 ounce pistachio diet ($P < 0.01$). Pistachios elicited a dose-dependent lowering of TC/HDL-C, LDL-C/HDL-C, and non-HDL-C/HDL-C ($P < 0.01$). **Conclusions:**

Including pistachios in a healthy diet beneficially affects CVD risk factors in a dose-dependent manner, which may reflect effects on SCD and CETP.

Introduction

Epidemiologic and clinical studies have established cardioprotective effects of tree nuts and the legume peanuts (1-4). Almonds and walnuts have been studied most extensively; less is known about effects of other nuts, including pistachios. Pistachios have a unique nutrient and fatty acid profile. They are a good source of unsaturated fatty acids and numerous antioxidants including gamma-tocopherol, beta-carotene, lutein, selenium, flavonoids, and phytoestrogens, such as isoflavones and resveratrol (5, 6). Compared to other popular nuts and seeds, pistachios are one of the richest sources of phytosterols, a dietary factor that reduces LDL-cholesterol (LDL-C) (7). Previous studies have shown beneficial effects of pistachios (15-20% of energy) on lipids and lipoproteins, including reductions in TC/HDL-C, LDL-C/HDL-C, and apoB/apoA-I, and increases in HDL-C; however, significant reductions in LDL-C, VLDL-C, apoA-I, and apoB have not been reported (8-10). Although the fatty acid composition of pistachios has been proposed as the primary mechanism for lipid-lowering (9), previous studies have not reported plasma fatty acid profiles and have not evaluated other potential mechanisms of action. We hypothesized that pistachios would beneficially affect multiple cardiovascular disease (CVD) risk factors including lipids and lipoproteins, apolipoproteins, plasma fatty acids, cholesteryl ester transfer protein (CETP), and stearoyl-CoA desaturase (SCD).

Many studies have assessed effects of nutrients and foods on concentrations of apoA-I and apoB, which have antiatherogenic and proatherogenic effects, respectively. However, individual lipid particles may include several apolipoproteins, and recent work suggests that these unique apolipoprotein profiles may determine their relative atherogenicity and clearance rate (11). Since diet, specifically unsaturated fatty acids, has been shown to affect apolipoprotein profiles (11, 12), this was evaluated in the current study.

The present study comprehensively evaluated the effects of heart healthy diets that included 2 doses of pistachios on CVD risk using multiple risk factors, including lipids, lipoproteins, apolipoproteins, and plasma fatty acids. We also assessed whether the experimental diets affected two metabolic pathways that are essential for lipid metabolism. CETP is a plasma protein that plays a key role in reverse cholesterol transport by transferring cholesteryl esters from HDL particles to LDL and VLDL particles in exchange for triacylglycerols (TG) (13). SCD is the rate-limiting enzyme that catalyzes the synthesis of monounsaturated fatty acids (MUFA, 18:1 and 16:1) from saturated fatty acids (SFA, 18:0 and 16:0) and plays an important role in cholesterol, TG, and lipoprotein metabolism (14). Both CETP and SCD have been shown to be regulated by fatty acids (14, 15) and are important potential mechanisms to explain how pistachios may affect lipids and lipoproteins (Figure **4-1**). To our knowledge, this is the first randomized controlled-feeding study to assess potential mechanisms that may account for the lipid and lipoprotein responses to varying doses of pistachios.

Subjects and methods

Subjects and study design

Twenty-eight men (n = 10) and women (n = 18) with elevated LDL-C (≥ 2.86 mmol/l) completed the study. One subject dropped out due to inability to comply with study protocol. Inclusion criteria were as follows: TG < 3.94 mmol/L, blood pressure < 160/90 mmHg, body mass index (BMI) between 21-35 kg/m^2, and fasting blood glucose levels ≤ 6.93 mmol/l. All participants were otherwise in good health and nonsmokers. Exclusion criteria included: inability to comply with study protocol, use of blood pressure or cholesterol/lipid-lowering medication or substances (psyllium, fish oil, soy lecithin, phytoestrogens), being pregnant or wishing to become

pregnant 6 months before or during the study, lactating 6 weeks before or during the study, having weight loss ≥ 10% body weight 6 months before the study, following vegetarian or weight-loss diets, or having any of the following conditions: previous stroke, diabetes, liver disease, kidney disease, and autoimmune diseases. The Institutional Review Board at the Pennsylvania State University approved the experimental protocol and all subjects provided written informed consent.

The study employed a 4-period randomized crossover controlled-feeding design. A 2-week run-in period preceded the first test diet in order to establish a baseline on a typical American diet. Subjects were then randomized to 3 treatment diets for 4 weeks. Short compliance breaks (average of 2 weeks) separated the diet periods. Study personnel who measured outcome variables were blinded to diet assignments.

Diets

The nutrient composition of the diets is shown in Table **4-1**. Total energy was held constant throughout the 4 feeding periods. An average intake of 2,500 kcal/d was required to maintain weight. The run-in diet was a typical American diet and the control diet was designed as a Step-I diet, both with no pistachios. For the pistachio diets, pistachio intake was calculated as 10% and 20% of total energy and doses ranged from 32-63 g/d and 63-126 g/d, respectively, depending on calorie level assignment. Pistachio diets are referred to by the median amount of pistachios consumed per day (1.5 and 3.0 ounces). Thus, while on the pistachio diets, participants consumed approximately 1-2 servings of nuts per day, according to the FDA definition of a serving. As expected, the pistachio diets were higher in protein and unsaturated fats and lower in carbohydrate. The control and pistachio diets were matched for SFA and cholesterol. All meals and snacks were prepared at the Metabolic Diet Study Center at the Pennsylvania State

University. Blood was drawn on two consecutive days at the end of each diet period. Participants ate 1 meal per day, Monday through Friday, in the center, and had their other meals prepared and packed for offsite consumption. Adherence with the experimental diets was checked daily using compliance questionnaires. In addition, subjects were weighed daily. Pistachios were consumed as a snack (≈50% of dose; roasted and salted) and were incorporated into various recipes (≈50% of dose; roasted and unsalted).

Endpoint assays

Serum and plasma samples were taken from fasting subjects at the end of each diet period. Samples were stored at -80^{o} C until the end of the study so they could be assayed at the same time.

Lipids, lipoproteins, apolipoproteins and CETP

TC and TG were determined by enzymatic procedures with commercially available kits (TC: CHOP/PAP, Boeringer, Mannheim, FRG; TG and free glycerol: Abbott Laboratories, Diagnostic Division, Irving, TX). HDL-C was estimated according to the modified heparin-manganese precipitation procedure of Warnick and Albers (16). LDL-C levels were calculated by Friedewald's equation: LDL-C = TC – (HDL-C + TG/5) (17). Apolipoprotein analyses were carried out in the laboratory of Petar Alaupovic, as previously described (12). Serum CETP concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Wako diagnostics; Richmond, VA) and carried out in the Pennsylvania State University General Clinical Research Center (GCRC) Cytokine Core Laboratory.

Insulin and glucose

Insulin was measured by radioimmunoassay using ^{125}I-labeled human insulin and a human insulin antiserum (18). Glucose was determined by an immobilized enzyme biosensor using the YSI 2300 STAT Plus Glucose & Lactate Analyzer (Yellow Springs Instruments, Yellow Springs, OH) (19). Insulin and glucose were measured at Hershey Medical Center (in Dr. Laurence Demers' laboratory).

Plasma fatty acids

Plasma fatty acids were measured at the University of Guelph (in Dr. Bruce Holub's laboratory). Liquid/liquid solvent extraction was performed and the lower chloroform phase was removed and dried under nitrogen. The dried residue was methylated and fatty acid methyl esters were extracted in hexane and injected into a Varian Gas Chromatograph where the fatty acids were separated on a 60M DB-23 capillary column. The fatty acids were quantified using an internal standard method and are reported as mol %.

Statistical analyses

All statistical analyses were performed using SAS (Statistical Analyses System, Version 9.1, Cary, NC). The natural logarithmic transformation was used on the variable BMI and analysis was performed on transformed values. Means are reported as least squares means ± SEM. The mixed models procedure (PROC MIXED) in SAS was used to test the effects of diet, order, and their interactive effects on each outcome variable. Tukey-Kramer adjusted P-values were used to determine whether the differences in the outcome variables were significant (20,

21). Change scores for each variable were calculated by subtracting the value at the end of the run-in period (the pretreatment baseline) from the end of the treatment value. Within-subject correlations were used to test associations between clinical variables (lipids, lipoproteins, and apolipoproteins) and mechanistic variables (CETP and SCD activity). PROC GLM was used to test whether the slopes of the regression lines were equal across the three diets. When that requirement was met, correlations are reported as pooled values, collapsing across the treatments and taking into account the fact that repeated measurements from each subject are not independent.

Results

Subject characteristics at baseline

Baseline characteristics of the participants are presented in Table **4-2**. Diets were designed to be isocaloric, and there were no significant differences in pre- and post-treatment means for body weight (76.6 ± 2.5 vs.76.1 ± 2.5 kg; $P > 0.05$) and BMI (26.7 ± 0.7 vs. 26.6 ± 0.7 kg/m^2; $P > 0.05$). In addition, there were no significant differences in body weight or BMI when comparing the control diet to the pistachio diets ($P > 0.05$).

Effects of experimental diets on lipids, lipoproteins and apolipoproteins

Compared to the control diet, TC, LDL-C, and non-HDL-C were significantly lower on the 1.5 ounce and 3.0 ounce pistachio diets ($P < 0.001$; see Table **4-3**). The 1.5 ounce and 3.0 ounce pistachio diets decreased LDL-C by 9% and 12%, respectively, as shown in Figure **4-2**. TG ($P < 0.01$), TC/HDL-C ($P < 0.001$), and LDL-C/HDL-C ($P < 0.001$) were significantly lower on

the 3.0 ounce pistachio diet than the control diet. Furthermore, there was a significant difference between the 1.5 ounce and 3.0 ounce pistachio diets for the ratios of TC/HDL-C, LDL-C/HDL-C, and non-HDL-C/HDL-C ($P < 0.05$ for all), indicating a dose-dependent effect. There were no significant differences in HDL-C when comparing the pistachio diets versus the control diet. However, HDL-C was significantly higher in women following the 3.0 ounce pistachio diet compared to the control diet (1.60 ± 0.1 vs. 1.50 ± 0.1 mmol/l, $P < 0.05$).

Compared to the control diet, both pistachio diets significantly reduced apoB and the apoB/apoA-I ratio ($P < 0.01$; see Table **4-4**). Among individual apoB-containing lipoprotein subclasses, the levels of cholesterol-rich LpB were significantly reduced on the 3.0 ounce pistachio diet when compared to the control diet ($P < 0.01$). There was a significant effect of diet on Lp-B:E+Lp-B:C:E, with a trend for significantly lower concentrations on the 3.0 ounce pistachio diet versus the control diet ($P < 0.1$). There were no significant changes in apoCIII.

Plasma fatty acid composition

Table **4-5** presents the composition of plasma fatty acids after each diet. Compared to the control diet, the pistachio diets resulted in significantly lower plasma concentrations of SFA, n-3 PUFA, ALA, EPA, DHA, and n-3/n-6, and significantly higher LA/ALA ($P < 0.05$). In addition, the 3.0 pistachio diet elicited significantly higher total PUFA, n-6 PUFA, and LA relative to the control diet ($P < 0.01$). When comparing the two pistachio diets, the 3.0 ounce pistachio diet resulted in significantly lower SFA, EPA, and n-3/n-6, and significantly higher n-6 PUFA, LA, and LA/ALA ($P \leq 0.05$).

Effects of experimental diets on SCD activity and CETP concentration

Compared to the control diet, there was a significant reduction in the ratio of 16:1/16:0, indicative of plasma SCD activity, after the 3.0 ounce pistachio diet ($P < 0.001$). The diets had no impact on 18:1/18:0. Overall, change in 16:1/16:0 was positively correlated with change in TC ($r = 0.3$, $P < 0.05$), VLDL-C ($r = 0.3$, $P < 0.05$), TG ($r = 0.3$, $P < 0.05$), NONHDL-C ($r = 0.3$, $P < 0.05$), and apoB ($r = 0.3$, $P < 0.05$). Change in 18:1/18:0 was correlated with change in TG ($r = 0.3$, $P < 0.05$) and VLDL-C ($r = 0.3$, $P < 0.05$). Following the 1.5 and 3.0 ounce pistachio diets, respectively, change in 18:1/18:0 was significantly correlated ($P < 0.05$) with concentrations of TC/HDL-C ($r = 0.4$, $r = 0.5$), LDL-C/HDL-C ($r = 0.4$, $r = 0.4$), and NONHDL-C/HDL-C ($r = 0.5$, $r = 0.4$), whereas there was no relationship observed after the control diet. After the 3.0 ounce pistachio diet, change in 18:1/18:0 also was significantly correlated with TG ($r = 0.6$, $P < 0.01$).

In the group as a whole, CETP concentrations were not significantly different among the experimental diets (Table **4-3)**. We also examined the relationships between CETP and lipids and lipoproteins irrespective of diet. Individuals with lower baseline TG (< 1.08 mmol/l median) had significantly higher concentrations of CETP (Figure **4-3**). Furthermore, individuals with higher baseline CETP (≥ 1.3 mg/l median) had significantly lower VLDL-C and TG (Figure **4-4**) and apoCIII (not shown).

Discussion

This study is the first to report significant reductions in LDL-C following consumption of pistachios. In the context of a heart-healthy diet, we showed that adding as little as 1.5 ounces of pistachios per day (equivalent to ~1 serving, or 10% of total energy) lowered LDL-C by 9%. LDL response to the larger dose of pistachios was similar in magnitude (-12%). Recent clinical trials

with statins suggest that this would result in a ~9-12% reduction in coronary heart disease risk (22). Differences in the lipoprotein ratios between the 1.5 ounce and 3.0 ounce diets indicate a dose-dependent response, with greater improvements being observed with increasing intake of pistachios. Prospective studies suggest that TC/HDL-C and LDL-C/HDL-C ratios are more powerful predictors of CVD risk than LDL-C alone (23-25).

Previous studies using similar doses of pistachios (15-20% of total energy) have not found significant changes in LDL-C, VLDL-C, TG, apoA-I, or apoB (8-10), perhaps because they relied on participants to consume pistachios (and replace other foods) under free-living conditions, and assessed intake by diet records. Although one study (n=15) found a 10% reduction in LDL-C, this change was not significant (10). The significant results reported in the present study, in contrast to previous studies, are likely due to adequate sample size and the highly-controlled study design, which allows for increased statistical power and reduced variability in lipid and apolipoprotein values. Controlled-feeding studies are important for studying energy dense foods such as nuts because total calorie intake can be held constant while fatty acid profile of the diets is varied systematically. Our approach was to exchange a fixed percentage of total energy (10% and 20% of total calories) for pistachios, while keeping saturated fat, cholesterol, and kcal constant between the control and experimental diets. This approach also allowed us to customize calorie levels so that body weight was carefully controlled.

The clinical studies that have been conducted with nuts reported LDL-C lowering effects that are greater than predicted on the basis of the fatty acid profile of the test diets (designed with appreciable amounts of nuts/nut oils) (26, 27). Using equations based on fatty acid composition of the diets to predict LDL-C response to the lower dose of pistachios demonstrated that the LDL-C reduction that we observed was 7x greater than the expected value (28). Consequently, the lipid and lipoprotein responses we report not only reflect the fatty acid profile of the diet (which could be mimicked by other fats and oils), but also are the result of other bioactive components (such as

phytosterols and fiber, among others) in pistachios. Irrespective of the mechanism(s), it is obvious that pistachios, and specifically the "package" of nutrients/bioactive factors they bring to the diet, account for the marked beneficial effects on lipids and lipoproteins.

It was surprising that the lower-fat control diet did not decrease TC or LDL-C as designed. The control diet was lower in PUFA and higher in carbohydrates compared to the run-in and pistachio diets, and this may partially explain the lack of LDL-C response. As has been reported in several previous clinical studies [reviewed in (29)], including our own (30), TG concentrations increased during the Step-I diet. Increasing total fat by adding pistachios prevented this potentially adverse response. The TG-lowering effect of the 3.0 ounce pistachio diet compared to the control diet is consistent with the significant reduction observed in non-HDL-C after the 3.0 ounce pistachio diet.

Differentiating between apolipoprotein-defined lipoprotein subclasses provides a new classification system for plasma lipoproteins based on apolipoprotein composition rather than size and density. This classification allows for a better understanding of the atherogenic and metabolic status of individuals. The reduction in apoB on the 3.0 ounce pistachio diet reflects the significant decrease in the atherogenic cholesterol-rich lipoprotein B subclass. Recent evidence suggests that apoB concentrations and the ratio of apoB/apoA-I may be a better predictor than LDL-cholesterol as an index of CVD risk (25, 31, 32). A recent study on the acute effects of individual fatty acids on apoB–containing lipoproteins demonstrates that unsaturated fatty acids differentially affect apoB-containing lipoprotein subclasses (12). Our study is one of the first to test the chronic effects of diet on apolipoprotein-defined lipoprotein subclasses and indicates that pistachio consumption in the context of a heart-healthy diet may reduce concentrations of atherogenic apoB, lipoprotein B, and Lp-B:E+Lp-B:C:E (which includes all lipoprotein classes except HDL).

Our clinical study with pistachios is unique because we examined potential underlying mechanisms that might explain their cholesterol-lowering effects. SCD plays an important role in

cholesterol, TG, and lipoprotein metabolism by catalyzing the synthesis of MUFA, mainly 18:1 and 16:1, from SFA. The ratio of SFA to MUFA in plasma reflects membrane phospholipid composition and increases in this ratio have been implicated in diseases such as CVD, obesity, and diabetes (14). Hepatic SCD1 gene expression is increased by numerous dietary factors including high-carbohydrate diets, glucose, and cholesterol, and it is decreased by PUFA (14). Our results show that consuming a nut that contains high levels of unsaturated fats resulted in a significantly lower ratio of 16:1/16:0. The direct correlations between change in SCD activity and lipids and lipoproteins suggest that SCD activity contributes to the effects we observed. The increase in the ratio of 18:1/18:0 on the pistachio diets compared to the average American run-in diet could be reflective of their higher oleic acid content. Thus, the 16:1/16:0 ratio may be a better marker of SCD activity in the context of a MUFA-rich diet since 16:1 is not found abundantly in the diet.

CETP mediates the transfer of cholesterol esters from HDL to VLDL and LDL in exchange for TG. Although CETP has been studied in great detail in animal and *in vitro* studies, the clinical significance of CETP is controversial. CETP may be anti-atherogenic in that it increases the rate of reverse cholesterol transport, but it may be proatherogenic in that it transports CE from HDL, which is protective, to VLDL and LDL, which are atherogenic (33). Studies in humans have shown that intake of SFA (34-36) and TFA (37, 38) increases CETP, while intake of MUFA decreases (39) and intake of PUFA decreases (40) or has no effect on CETP (15, 35, 41). The lack of a significant effect of diet on CETP in the present study may be due to similar plasma MUFA composition of the three experimental diets and also to the relatively small sample size of the study. Following the 3.0 ounce pistachio diet, there were significant decreases in TG, LDL-C, and VLDL-C, all of which are substrates for CETP; thus, CETP activity may have been reduced following the pistachio diets even though CETP mass was unchanged.

We found that individuals with relatively lower baseline TG (< median value) had significantly higher concentrations of CETP throughout the study compared with individuals with higher baseline TG (≥ median value) and that higher CETP in this sub-population may be beneficial since individuals with higher baseline CETP concentrations (≥ median value) had significantly lower TG and VLDL-C (Figures **4-3** and **4-4**). Our results suggest that TG concentration modifies CETP even in a population with relatively low baseline TG, and that CETP concentration affects lipid and apolipoprotein risk factors (TG, VLDL-C, and apoCIII). The potentially beneficial effect of higher concentrations of CETP in individuals with relatively lower TG found in the present study is consistent with recent studies that have found reduced incident CVD with higher CETP in men with lower TG (42), and a positive association between CETP and CHD events that was restricted to individuals with higher TG (43). Further research is needed to determine how CETP-lowering due to diet affects cardiovascular risk status in different population groups.

Our results extend those of previous nut studies, which have shown LDL-C lowering effects of nuts incorporated into various dietary patterns, by clarifying mechanisms to explain the lipid- and lipoprotein-lowering effects of pistachios. We present evidence that CETP and SCD activity may be modulating the lipid-lowering effect of the pistachio diets. The involvement of multiple mechanisms for CVD risk indicates there are numerous "targets" that affect multiple CVD risk factors. Beyond this, there is evidence based on changes in the SCD ratio that the effects of nut consumption extend beyond CVD. Thus, it will be important to further understand the underlying biological mechanisms that mediate the array of health benefits observed (as well as those that might be predicted) due to nut consumption.

In summary, our study demonstrates a dose-response effect of pistachio consumption within the context of a healthy dietary pattern on CVD risk factors. Importantly, these effects were observed with a low dose of pistachios (~1.5 oz/d), which can be easily incorporated into a

healthy dietary pattern. Due to the very large and convincing evidence base demonstrating multiple health benefits of nut consumption, and now including pistachios, heightened nutrition education efforts are needed to convey this health message to the public. Nutrition educators play a key role in explaining to consumers how nuts, like pistachios, can be incorporated into healthy daily diets. Tools to present this information include MyPyramid, the *2005 Dietary Guidelines for Americans*, and the qualified health claim for nuts (44-46).

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Table **4-1**: Nutrient composition of test diets[1,2].

Diet	Baseline	Control	1.5 oz Pistachio	3.0 oz Pistachio
Calories	2102	2100	2100	2100
Pro (g)	86.7 (16.5)	81.1 (15.4)	87.6 (16.7)	88.6 (16.9)
CHO (g)	261.8 (49.8)	329.5 (62.7)	302.7 (57.6)	281.1 (53.5)
Fat (g)	82.1 (35.1)	59.2 (25.4)	69.0 (29.6)	80.0 (34.3)
Chol (mg)	291.9	288.2	292.7	286.4
SFA (g)	26.2 (11.2)	18.1 (7.8)	18.0 (7.7)	18.0 (7.7)
MUFA (g)	30.6 (13.1)	21.1 (9.1)	28 (12.0)	35.8 (15.3)
PUFA (g)	18.7 (8.0)	10.6 (4.5)	13.5 (5.8)	18.0 (7.7)
LA (g)	4.8 (2.1)	6.0 (2.6)	9.9 (4.2)	14.8 (6.3)
ALA (g)	0.4 (0.2)	1.0 (0.4)	0.6 (0.3)	0.9 (0.4)
Fiber (g)	21.4	32.8	33.3	35.9
Sodium (mg)	3166	3207	2805	2646
Potassium (mg)	2514	2819	2995	3164
Vitamin A (RE)	1813	1943	1985	2048
Vitamin C (mg)	111.8	150.0	160.0	136.3
Calcium (mg)	846.2	854.7	887.3	746.7
Iron (mg)	15.6	20.2	20.6	20.5
Vitamin D (IU)	171.9	182.7	178.7	168.1
Vitamin E (mg)	7.12	6.8	7.2	8.4
Alpha-tocopherol (mg)	4.2	6.1	4.0	4.7
Gamma-tocopherol (mg)	0.9	1.1	9.8	17.2
Folate (mcg)	505.4	751.2	716.3	683.3
Selenium (mcg)	118.8	121.0	111.7	108.0
Lutein (mcg)	2924	2481	2502	2928

[1] Gamma-tocopherol values were approximated from the USDA Nutrient Database (235); all other values were determined by Nutritionist Pro approximations; % kcal values are presented in parentheses; [2]Pro = protein, CHO = carbohydrate, Chol = cholesterol, SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, LA = linoleic acid, ALA = alpha linolenic acid, Lutein = lutein + zeaxanthin.

Table **4-2**: Baseline characteristics of subjects (n = 28).

Characteristic	**M = 10, F= 18**	**Range**
Age (yr)	48 ± 1.5	35-61
BMI (kg/m2)[2]	26.8 ± 0.7	21-34
TC (mmol/l)	5.5 ± 0.1	4.1-6.8
LDL-C (mmol/l)	3.5 ± 0.1	2.3-4.9
HDL-C (mmol/l)	1.5 ± 0.1	1.0-2.5
TG (mmol/l)	1.2 ± 0.1	0.7-1.9
SBP (mmHg)	111.9 ± 2.1	96-141
DBP (mmHg)	69.5 ± 1.1	59-80
Serum Glucose (mmol/l)	5.1 ± 0.1	4.4-6.0
Serum Insulin (μU/ml)	9.27 ± 0.8	1-23

[1]Baseline values are the measurements taken at the end of the 2-week run-in period. Values are expressed as mean ± standard error; SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure; Values are expressed as mean ± standard error; SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure; [2]There were no significant differences in changes in BMI from baseline among the diets.

Table **4-3**: Effects of treatment diets on lipids, lipoproteins, CETP and body weight[1,2].

Diet	Baseline	Control	1.5 oz Pistachio	3.0 oz Pistachio
TC (mmol/l)	5.5 ± 0.1	5.5 ± 0.1	5.1 ± 0.1§º	5.0 ± 0.1§º
LDL-C (mmol/l)	3.5 ± 0.1	3.5 ± 0.05	3.1 ± 0.1§º	3.0 ± 0.1§º
VLDL-C (mmol/l)	0.5 ± 0.04	0.7 ± 0.02º	0.6 ± 0.04	0.6 ± 0.04††
HDL-C (mmol/l)	1.5 ± 0.1	1.4 ± 0.03	1.4 ± 0.1*^	1.5 ± 0.1
TG (mmol/l)	1.2 ± 0.1	1.4 ± 0.1º	1.3 ± 0.1	1.2 ± 0.1††
NONHDL-C (mmol/l)	4.0 ± 0.1	4.0 ± 0.1	3.6 ± 0.1§#	3.5 ± 0.1§º
TC/LDL-C	1.61 ± 0.04	1.60 ± 0.04	1.66 ± 0.04†^	1.68 ± 0.04††#
TC/HDL-C	3.85 ± 0.19	3.98 ± 0.19	3.78 ± 0.19**	3.50 ± 0.19§º
LDL-C/HDL-C	2.48 ± 0.2	2.55 ± 0.2	2.38 ± 0.2*	2.15 ± 0.2§º
NONHDL-C/HDL-C	2.85 ± 0.2	2.98 ± 0.2	2.78 ± 0.2**	2.50 ± 0.2§º
CETP (mg/l)	1.31 ± 0.1	1.32 ± 0.1	1.34 ± 0.1	1.24 ± 0.1
Body weight (kg)	76.8 ± 2.6	75.6 ± 3.4	75.3 ± 3.4	75.5 ± 3.4

[1] Values are expressed as mean ± SEM; [2]Significant effect of diet ($P<0.05$) for all lipid and lipoproteins; ^Compared to baseline: $P<0.05$, #$P<0.01$, º$P<0.001$; †Compared to control: $P<0.05$, ††$P<0.01$, §$P<0.001$; *Significant difference between 1.5 oz Pistachio and 3.0 oz Pistachio: $P<0.05$, **$P<0.01$.

Table **4-4**: Effects of treatment diets on apolipoproteins[1,2].

Diet	Baseline	Control	1.5 oz Pistachio	3.0 oz Pistachio
Total apoA-I	136.4 ± 2.3	135.6 ± 2.3	135.6 ± 2.3	137.0 ± 2.3
LpA-I	34.0 ± 0.79	33.6 ± 0.79	33.4 ± 0.79	33.9 ± 0.79
Lp-A-I:AII	102.5 ± 1.7	102.1 ± 1.7	102.2 ± 1.7	103.1 ± 1.7
Total apoB	98.9 ± 2.0	100.5 ± 2.0	95.8 ± 2.0†	94.9 ± 2.0*††
Lp-B	58.6 ± 0.9	59.6 ± 0.9	58.0 ± 0.9	57.5 ± 0.9††
Lp-B:C	10.1 ± 0.6	10.2 ± 0.6	10.0 ± 0.6	9.3 ± 0.6
Lp-B:E+Lp-B:C:E	15.2 ± 0.8	15.5 ± 0.8	13.9 ± 0.8	13.6 ± 0.8
Lp-A-II:B	15.0 ± 0.8	15.1± 0.8	13.8± 0.8	14.5 ± 0.8
apoB/apoA	0.7 ± 0.02	0.8 ± 0.02	0.7 ± 0.02††	0.7 ± 0.02*††
Total apoCIII	11.8 ± 0.5	12.2 ± 0.5	11.3 ± 0.5	11.6 ± 0.5
apoCIIIR	2.3 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	2.0 ± 0.1
apoCIIIHS	8.2 ± 0.5	8.1 ± 0.5	7.3 ± 0.5	7.8 ± 0.5
apoCIIIHP	3.7 ± 0.2	4.1 ± 0.2	3.9 ± 0.2	3.9 ± 0.2

[1]Values (mg/dl) are expressed as mean ± SEM; [2]LP=lipoprotein; Total apoA-I, particles containing LpA-I alone or in combination with other apolipoproteins: LpA-I, lipoprotein particle containing apolipoprotein A-I; LpA-I:A-II, lipoprotein particle containing apolipoproteins A-I and A-II; Total Apo B, particles containing Lp-B alone or in combination with other apolipoproteins: Lp-B, lipoprotein particle containing apolipoprotein B; Lp-B:C, lipoprotein particle containing apolipoproteins B and C; LpA-II:B, lipoprotein particle containing apolipoproteins A-II and B; Lp-B:E and Lp-B:C:E, lipoprotein particle containing apolipoproteins B:E or B:C:E; Total ApoCIII, particles containing LP-CIII alone or in combination with other apolipoproteins: apoCIIIR, ratio of apoCIII-HS/apoCIII-HP; apoCIIIHS, apoCIII bound to apoA-containing lipoproteins (HDL); apoCIIIHP, apoCIII bound to apoB-containing lipoproteins (VLDL+LDL+IDL); *Significantly different from baseline($P<0.05$). †Compared to control: $P<0.05$, ††$P<0.01$.

Table **4-5**: Effects of treatment diets on plasma fatty acids[1].

Fatty Acid	Baseline	Control	1.5 oz Pistachio	3.0 oz Pistachio
SFA	29.02 ± 0.34	29.80 ± 0.34*	28.89 ± 0.34††^	28.17 ± 0.34**§
MUFA	22.37 ± 0.48	24.22 ± 0.48***	24.45 ± 0.48***	24.09 ± 0.48***
18:1 (OA)	20.03 ± 0.43	21.36 ± 0.43**	21.93 ± 0.43***	21.80 ± 0.43***
PUFA	48.61 ± 0.70	45.97 ± 0.70***	46.67 ± 0.71**	47.74 ± 0.70††
n-6 PUFA	45.11 ± 0.68	42.28 ± 0.68***	43.38 ± 0.68**^	44.66 ± 0.68§
18:2 (LA)	33.0 ± 0.69	30.31 ± 0.69***	31.11 ± 0.69***^	32.64 ± 0.69§
18:3γ (GLA)	0.47 ± 0.04	0.57 ± 0.04**	0.54 ± 0.04	0.48 ± 0.04†
20:4 (AA)	9.44 ± 0.25	9.14 ± 0.25	9.41 ± 0.25	9.41 ± 0.25
n-3 PUFA	3.50 ± 0.09	3.69 ± 0.09	3.29 ± 0.09§	3.08 ± 0.09***§
18:3 (ALA)	0.67 ± 0.03	0.77 ± 0.03***	0.63 ± 0.03§	0.58 ± 0.03**§
20:5 (EPA)	0.51 ± 0.03	0.61 ± 0.03**	0.52 ± 0.03††^	0.44 ± 0.03§
22:5 (DPA)	0.55 ± 0.02	0.57 ± 0.02	0.53 ± 0.02	0.50 ± 0.02†
22:6 (DHA)	1.69 ± 0.07	1.64 ± 0.07	1.55 ± 0.07*	1.50 ± 0.07**†
LA/ALA	51.80 ± 2.24	41.23 ± 2.24***	51.73 ± 2.27§^	58.74 ± 2.24*§
n-3/n-6	0.078 ± 0.002	0.088 ± 0.002***	0.076 ± 0.002§^	0.069 ± 0.002***§
16:1/16:0	0.094 ± 0.01	0.115 ± 0.01***	0.103 ± 0.01	0.094 ± 0.01§
18:1/18:0	2.97 ± 0.08	3.25 ± 0.08**	3.36 ± 0.08***	3.40 ± 0.08***

[1]Values (mol %) are expressed as mean ± SEM; OA = oleic acid, LA = linoleic acid, GLA = gamma-linolenic acid, AA = arachidonic acid, EPA = eicosapentaenoic acid, DPA = docosapentaenoic acid, DHA = docosahexaenoic acid; *Compared to Baseline: $P<0.05$, **$P<0.01$, ***$P<0.001$. †Compared to control: $P<0.05$, ††$P<0.01$, §$P<0.001$. ^Significant difference between 1.5 oz Pistachio and 3.0 oz Pistachio ($P \leq 0.05$).

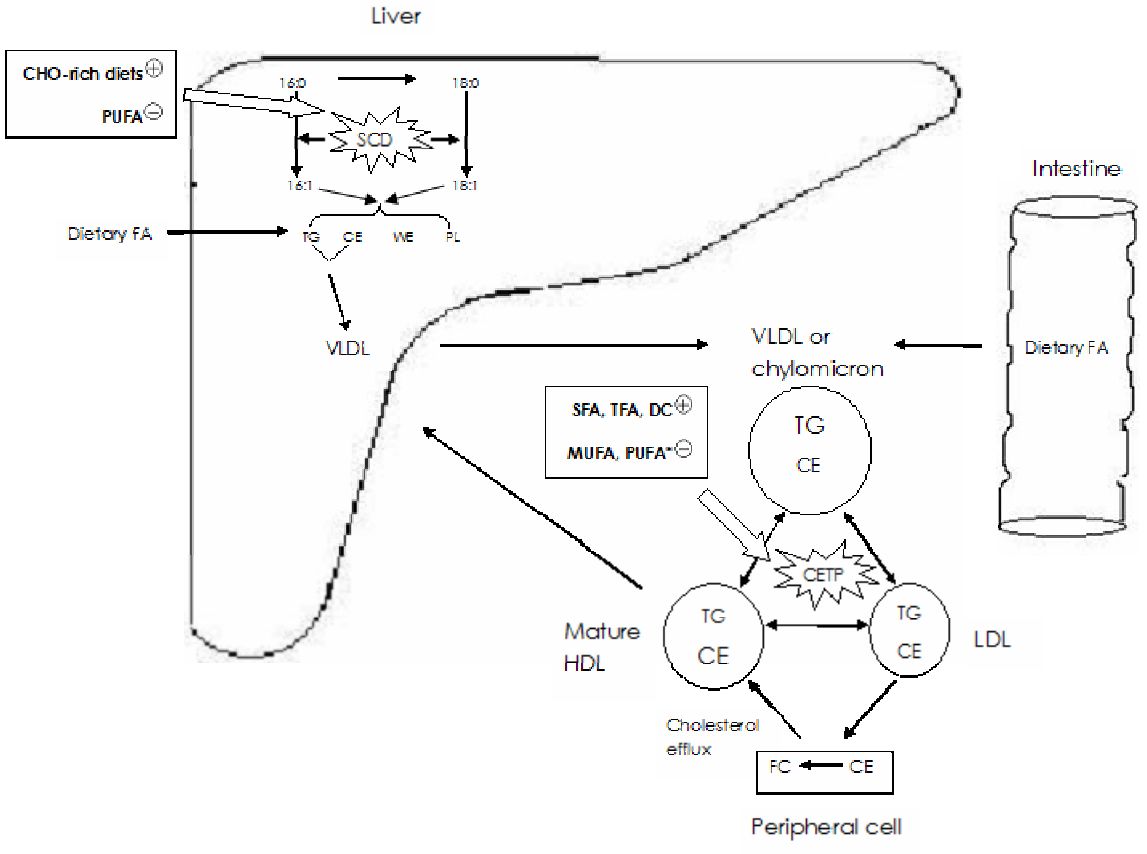


Figure **4-1**: Roles of SCD and CETP in lipid metabolism. SCD is the rate-limiting enzyme catalyzing the synthesis of 16:1 and 18:1, the predominant MUFA of triglycerides, cholesterol esters, wax esters, and phospholipids, from 16:0 and 18:0. CETP mediates the transfer of CE from HDL to VLDL and LDL in exchange for TG from VLDL and LDL to HDL. Thick open arrows denote regulation of dietary factors on SCD and CETP, some of which are investigated in the present study. CHO = carbohydrate, SFA = saturated fatty acids, TFA = trans fatty acids, DC = dietary cholesterol, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, FA = fatty acid, TG = triacylglycerol, CE = cholesterol esters, WE = wax esters, PL = phospholipids, FC = free cholesterol. *Intake of PUFA has been found to decrease CETP or have no effect. Role of CETP in lipoprotein metabolism was modified from Barter & Kastelein, 2006.

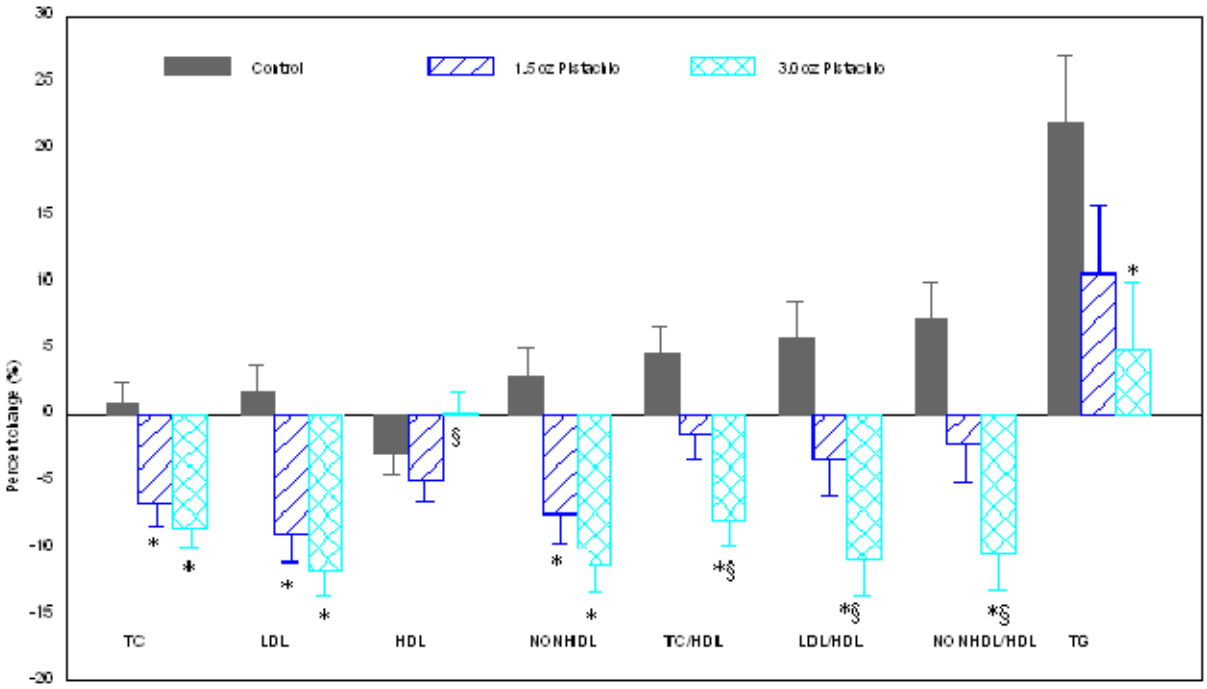


Figure **4-2**: Percent changes (± SEM) in lipids and lipoproteins from baseline. *Significant difference from control diet ($P < 0.05$). §Significant difference between 1.5 oz Pistachio and 3.0 oz Pistachio ($P < 0.05$).

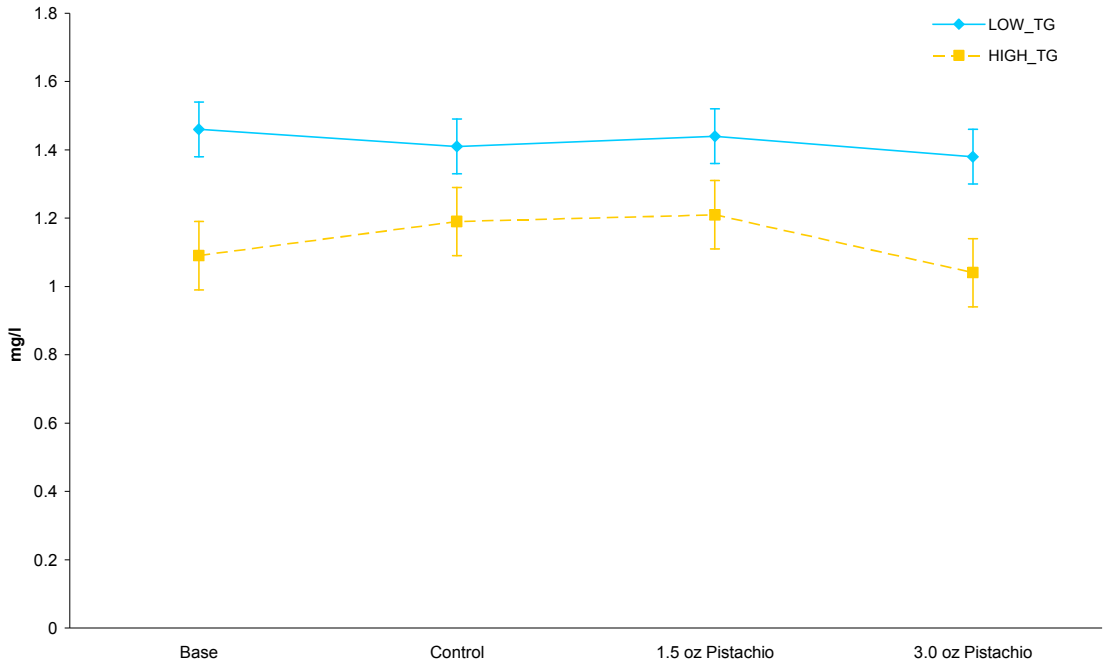


Figure **4-3**: Comparison of CETP in individuals with relatively low and high TG. LOW_TG = TG < 2.5 mmol/l, HIGH_TG = TG ≥ 2.5 mmol/l. Individuals with lower TG have significantly higher concentrations of CETP (significant effect of group, $P < 0.05$).

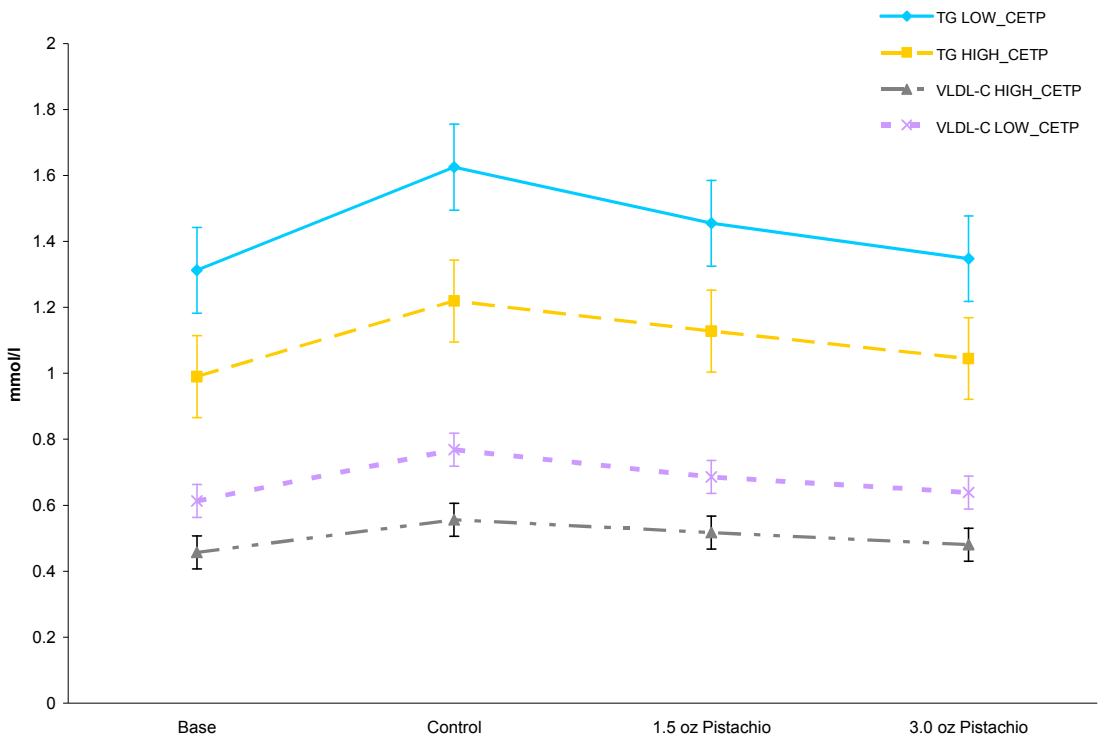


Figure **4-4**: Comparison of TG and VLDL-C in individuals with relatively low and high CETP. LOW_CETP = CETP < 1.3 mg/l, HIGH_CETP = CETP ≥ 1.3 mg/l. Individuals with higher CETP have significantly lower TG and VLDL-C (significant effect of group, $P < 0.05$).

Chapter 5

Pistachio-rich diets affect expression of lipid and inflammatory genes in peripheral blood mononuclear cells

Abstract

Background: Peripheral blood mononuclear cells (PBMCs) have been proposed as a model to study cardiovascular biological systems. Data are lacking on the effects of nutritional interventions that manipulate dietary fat on PBMC gene expression. Furthermore, little is known about the relationship between PBMC gene expression and serum/plasma concentrations of CVD risk factors. **Objective:** To evaluate the effects of pistachio-rich diets on inflammation-related gene expression (TNFα, IL-1β, IL-6, ICAM, and VCAM), and expression of lipid transfer proteins (CETP and LCAT), in PBMCs. In addition, we investigated the relationship between diet-induced change in CETP expression and change in serum CETP and plasma lipids and lipoproteins. **Design:** In a randomized crossover controlled-feeding study, individuals (n = 28) with LDL-cholesterol (LDL-C) ≥ 110 mg/dl consumed 3 isoenergetic diets for 4 weeks each. Baseline measures were assessed after a 2-week run-in diet consisting of a typical western diet. Experimental diets included a Step-I diet with no pistachios (25% total fat), a 1.5 ounce pistachio diet (10% total energy from pistachios, 30% total fat), and a 3.0 ounce pistachio diet (20% total energy from pistachios, 34% total fat). Plasma lipids and lipoproteins, serum CETP, and PBMC gene expression were measured at the end of the 2-week run-in diet and each 4-week experimental diet. **Results:** The pistachio-rich diets significantly reduced IL-1β expression compared to baseline (-15%, $P < 0.05$). Change in CETP expression in PBMCs predicted change in LDL-C, NONHDL-C, and ratios of total cholesterol (TC) to HDL-C (TC/HDL-C), and NONHDL-C/HDL-C in individuals who were diet-responsive with respect to serum CETP.

Conclusions: Consumption of pistachio-rich diets beneficially affects inflammation-related gene expression in PBMCs. Diet-induced change in CETP expression is related to change in plasma lipid risk factors in diet responders (based on serum CETP). Further research is needed to evaluate the effects of diet on expression of other lipid metabolism genes and their relationship to plasma lipid and lipoprotein diet response.

Introduction

The cardioprotective effects of nut consumption are well-documented (1, 2). More recently, we have shown that pistachios beneficially affect plasma lipids, lipoproteins, and apolipoproteins (Gebauer *et al.*, submitted). While the effects of nuts on lipid and inflammatory risk factors in serum/plasma have been established, their effects on the expression of genes related to lipid metabolism and inflammation are unknown.

Since slight alterations in their micro-environments can induce change in expression, genes can act as sensitive measures of change in their macro- and micro-environments. Circulating peripheral blood mononuclear cells (PBMCs) come into contact with every cell in the human body, thus changes within cells and tissues throughout the body may elicit changes in PBMC gene expression (3). Studies have demonstrated that gene expression patterns of PBMCs are distinctive between individuals (4) and that change in expression profiles are characteristic of numerous diseases, such as hypertension, cancer, and arthritis (5-10), and environmental stresses, such as exercise, smoking, and exposure to arsenic and hexachlorobenzene (11-13). In a recent study comparing gene expression in PBMCs to that of 9 tissue types (brain, colon, heart, kidney, liver, lung, prostate, spleen, and stomach), PBMCs expressed approximately 80% of the genes in any of the 9 tissues (14). In another recent study, 166 out of 182 candidate CVD genes were expressed in PBMCs of healthy individuals, including almost all genes involved in lipid metabolism (3). Due to these characteristics, PBMCs have been proposed as a model for researching cardiovascular biology systems (3).

Pistachios are low in saturated fat and rich in unsaturated fats (particularly oleic acid and linoleic acid), antioxidants, and phytosterols (15, 16). We have shown that consumption of pistachio-rich diets leads to significant decreases in plasma lipid risk factors, and increases in

serum concentrations of γ-tocopherol, lutein, and β-carotene (Gebauer *et al.*, submitted; Kay *et al.*, in progress); however, serum assays of inflammatory markers (IL-1β, IL-6, TNF, and CRP) were not sensitive enough to detect change due to diet. It is unknown whether consumption of pistachio-rich diets induces change in PBMC expression of genes related to inflammation and lipid metabolism, which would further our understanding of the biological effects of pistachios and their underlying mechanisms. The present study evaluated the effects of pistachio-rich diets on mRNA expression of genes involved in inflammation and lipid metabolism, and investigated the relationship between diet-induced change in CETP expression and change in serum/plasma lipid risk factors. In animal and *in vitro* studies, high MUFA diets inhibit CETP expression (17); however, studies in humans are lacking. Furthermore, we assessed the utility of PBMC gene expression as a tool for evaluating the effects of dietary interventions and explaining the variance in plasma lipid and lipoprotein response to diet.

Subjects and methods

Subjects and study design

We recruited 29 individuals (M = 10, F = 19) with elevated LDL-cholesterol ≥ 110 mg/dl. One subject dropped out due to inability to comply with study protocol. PBMCs were isolated for 22 individuals (M = 8, F = 14). Inclusion criteria were as follows: triglycerides (TG) < 350 mg/dl, BP < 160/90 mmHg, body mass index (BMI) between 21-35 kg/m^2, and fasting blood glucose levels ≤ 126 mg/dl. All participants were otherwise in good health and nonsmokers. Exclusion criteria included: inability to comply with study protocol, use of blood pressure or cholesterol/lipid-lowering medication or substances (psyllium, fish oil, soy lecithin,

phytoestrogens), being pregnant or wishing to become pregnant 6 months before or during the study, lactating 6 weeks before or during the study, having weight loss ≥ 10% body weight 6 months before the study, following vegetarian or weight-loss diets, or having any of the following conditions: stroke, diabetes, liver disease, kidney disease, and autoimmune diseases. The Institutional Review Board at the Pennsylvania State University approved the experimental protocol and all subjects provided written informed consent.

The study employed a 4-period, randomized crossover controlled-feeding design. A 2-week run-in diet preceded the first experimental diet in order to establish a baseline on a typical western diet. Subjects were then randomized to 3 treatment diets for 4 weeks each, separated by short compliance breaks (average of 2 weeks). Study personnel who measured outcome variables were blinded to diet assignments.

Diets

The nutrient composition of the diets is shown in Table **5-1**. Total energy was held constant throughout the diet periods in order to maintain weight. The 2-week run-in/baseline diet was designed as a typical western diet, higher in total fat and saturated fat. The Step-I diet was designed as a lower-fat diet without pistachios. The pistachio diets were designed as having pistachio intake of 10% and 20% of total energy, with doses ranging from 32-63 g/d and 63-126 g/d, respectively. Pistachio diets are referred to by the median amount of pistachios consumed per day (1.5 and 3.0 ounces). Thus, participants consumed approximately 1-2 servings of nuts per day (while consuming the pistachio diets), according to the FDA definition of a serving. Compared to the baseline diet, the pistachio diets were higher in protein and unsaturated fats and lower in carbohydrate and saturated fat. The experimental diets (Step-I and pistachio diets) were matched for SFA and cholesterol. All meals and snacks were prepared at the Metabolic Diet Study Center

at the Pennsylvania State University. Blood was drawn on 2 consecutive days at the end of each diet period. Throughout the week, participants ate 1 meal per day in the center, and had their other meals prepared and packed for offsite consumption. Adherence with the experimental diets was checked using daily compliance questionnaires. In addition, subjects were weighed daily to ensure that they were not gaining or losing weight. Approximately half of the dose of pistachios was consumed as a snack (roasted and salted) and half of the dose was incorporated into various recipes (roasted and unsalted).

Endpoint assays

Fasting blood draws were performed at the end of each 4-week diet period and at the end of the 2-week baseline diet. Serum and plasma samples were stored at -80^{o}C until the end of the study.

Lipids, lipoproteins and serum CETP

Total cholesterol (TC) and TG were determined by enzymatic procedures with commercially available kits (TC: CHOP/PAP, Boeringer, Mannheim, FRG; TG and free glycerol: Abbott Laboratories, Diagnostic Division; Irving, TX). HDL-C was estimated according to the modified heparin-manganese precipitation procedure of Warnick and Albers (18). LDL-C was calculated by Friedewald's equation: LDL-C = TC – (HDL-C + TG/5) (19). Serum CETP concentration was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Wako diagnostics; Richmond, VA).

RNA isolation, reverse transcription and real time PCR

PBMCs were isolated using the Lymphoprep™ tube (Axis-Shield; Norton, MA), a sterile plastic tube pre-filled with Lymphoprep™ (Ficoll-Isopaque), according to the manufacturer's instructions. Isolated cells were resuspended in RNAeasy RNA Stabilization Reagent (Qiagen, Valencia, CA) and stored at -80^{o}C for RNA extraction. Cells were lysed and harvested using TriReagent (Sigma; St. Louis, MO). The integrity of the RNA was confirmed using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). High capacity cDNA Archive kit (Applied Biosystems; Foster City, CA) was used for reverse transcription. cDNA was amplified by SYBR Green PCR Master Mix (Applied Biosystems; Foster City, CA) and detected by ABI 7000 Sequence Detection System (Applied Biosystems; Foster City, CA). Primer sequences used in real time PCR are presented in Table **5-2**.

Statistical analyses

All statistical analyses were performed using SAS (Statistical Analyses System, Version 9.1.3, Cary, NC). Normality was assessed by the Shapiro-Wilk test using the residuals. A test statistic > 0.9 indicated normal distribution. Logarithmic or other appropriate transformations were used to achieve normality for non-normally distributed variables (expression of CETP, TNFα, IL-1β, IL-6, VCAM, and ICAM, and BMI). Means are reported as unadjusted means ± SEM. Typical transformations did not achieve normality for LCAT, thus non-parametric analyses were used (PROC NPAR1). The mixed models procedure (PROC MIXED) was used to test the effects of diet, order, and their interactive effects on each outcome variable. Analyses were performed on transformed values. Tukey-Kramer adjusted P-values were used to determine whether the differences in the outcome variables were significant. Change scores for each variable were calculated by subtracting the value at the end of the 2-week run-in diet (baseline)

from the value at the end of each 4-week diet period. Pearson correlations (PROC CORR) were used to test associations between change in CETP expression and change in serum/plasma concentrations of lipids, lipoproteins, and serum CETP following the 3.0 ounce pistachio diet. In individuals with gene expression data at baseline and following the 3.0 ounce pistachio diets (n = 17; M = 6, F = 11), serum CETP increasers and decreasers were classified based on a change in serum CETP of ≥ ±5% (mean change across experimental diets) following the 3.0 ounce pistachio diet. PROC GLM was used to compare groups (individuals with increases, decreases, or no change in serum CETP following the 3.0 ounce pistachio diet). Linear regression analysis (PROC REG) was used to examine the strength of the relation between change in serum CETP and change in CETP expression. The model included change in serum concentration as the dependent variable and change in expression as the independent variable. Regression analysis also was used to examine the strength of the relation of change in serum CETP and CETP expression with change in lipids and lipoproteins. The model included change in lipids/lipoproteins as the dependent variable and change in serum CETP or CETP expression as the independent variable. An independent variable was considered to significantly contribute to the variance in the dependent variable when Pr > F was ≤ 0.05. The R^2 value is reported when this condition was met.

Results

Subject characteristics at baseline

Baseline characteristics of all participants (n = 28) are presented in Table **5-3**. As reported previously (Gebauer *et al.*, submitted), body weight was maintained throughout the study.

Effects of treatment diets on inflammation-related gene expression in PBMCs

There was a significant effect of diet overall on IL-1β expression, with lower levels on both pistachio diets and the Step-I diet, compared to baseline ($P < 0.05$); however, individual comparisons between the 1.5 ounce and 3.0 ounce pistachio diet with baseline were not significant. When the pistachio diets were pooled, IL-1β expression was significantly decreased by 15% from baseline ($P < 0.05$, Figure **5-1**). All of the other genes measured were expressed in PBMCs; however, changes were not significantly different across the treatment diets.

Variation in serum CETP response to diet

There was no overall effect of diet on serum CETP; however, there was considerable variation in individual responses. As shown in Figure **5-2**, consumption of the 3.0 ounce pistachio diet increased serum CETP in some individuals, and decreased or did not change in other individuals. Figure **5-3** compares the change in serum CETP from baseline following the 3.0 ounce pistachio diet in these 3 groups (individuals with increases, decreases, or no change). As shown in Figure **5-4**, individuals with increases in serum CETP following the 3.0 oz. pistachio diet had significant decreases in TC, LDL-C, NONHDL-C, TC/HDL-C, and NONHDL-C/HDL-C ($P \leq 0.05$), and no change in HDL-C, VLDL-C, and TG. In contrast, individuals who had decreases in serum CETP had significant increases in VLDL-C and TG, and no change in NONHDL-C, TC/HDL-C, LDL-C/HDL-C, and NONHDL-C/HDL-C. VLDL-C and TG were significantly higher in individuals with decreases in serum CETP ($P < 0.05$), with significant increases from baseline of 27% and 26%, respectively. Baseline characteristics (age, BMI, serum CETP, lipids, and lipoproteins) were not significantly different between the groups (Table **5-3**). As shown in Figure **5-5**, change in CETP mRNA expression in PBMCs did not explain the differences in serum CETP response to the 3.0 ounce pistachio diet. Regression analyses

confirmed that change in CETP expression did not correlate with change in serum CETP ($P > 0.05$). In diet responders (based on increases or decreases in serum CETP after the 3.0 ounce pistachio diet), change in CETP mRNA expression predicted change in LDL-C ($R^2 = 0.4$), NONHDL-C ($R^2 = 0.4$), TC/HDL-C ($R^2 = 0.4$), and NONHDL-C/HDL-C ($R^2 = 0.4$), whereas serum CETP did not. Correlation analysis confirmed these relationships ($r = 0.6$, $P \leq 0.05$ for all). In non-responders, relationships did not exist with CETP mRNA expression and plasma lipids and lipoproteins.

Discussion

We have shown that in addition to beneficial effects on CVD risk factors in serum/plasma (Gebauer *et al.*, submitted), consumption of the pistachio-rich diets significantly reduced IL-1β mRNA expression compared to baseline. We employed a randomized crossover controlled-feeding study to comprehensively evaluate the effects of pistachio-rich diets on CVD risk factors, including effects at the protein level in serum/plasma and the transcription level in PBMCs. Other data from our group demonstrate that pistachio oil reduces expression of inflammation-related genes in HepG2 cells via activation of PPARα (Zhang *et al.*, in progress), a nuclear receptor activated by unsaturated fatty acids and involved in fatty acid metabolism. PPARα exhibits anti-inflammatory properties (20-22). A recent study demonstrated that PPARα is functional in PBMCs, and that changes induced by free fatty acids are likely regulated by PPARα (23). Due to the fatty acid profile of the pistachio-rich diets, specifically the relatively high amounts of unsaturated fatty acids, the decrease in IL-1β expression may be due, in part, to activation of PPARα. The Step-I diet decreased IL-1β expression similarly when compared to the pistachio diets, suggesting that components of the Step-I diet are contributing to the anti-inflammatory effect. Compared to the pistachio diets, consumption of the Step-I diet resulted in significantly

higher plasma concentrations of n-3 fatty acids (ALA, EPA, DPA, and DHA), which exhibit anti-inflammatory effects. Although the higher plasma concentration of n-3 fatty acids following the Step-I diet was unexpected since pistachios contain n-3 fatty acids, it may be due to meals that included tuna fish, which were provided during the Step-I diet period and not the pistachio diets.

We found substantial individual variation in serum CETP response to diet, with increases observed in some individuals, and decreases or no change in others. CETP is predominantly produced in the liver and circulates in plasma bound mainly to HDL (24). The clinical significance of CETP is controversial, as recently reviewed by Dullaart *et al.* (25). It plays a key role in reverse cholesterol transport, the process of cholesterol removal from cells. However, inhibition of CETP leads to significant increases in HDL-C and decreases in LDL-C. In our study, sub-group analysis revealed that differences in groups of individuals who had increases, decreases, or no change in serum CETP following the 3.0 ounce pistachio diet were not explained by baseline characteristics (age, BMI, serum CETP, lipids, and lipoproteins). Furthermore, these differences were not explained by CETP mRNA expression in PBMCs. The underlying biological differences between responders and non-responders are unclear. Further research is needed to determine the occurrences of single nucleotide polymorphisms in different genes in the study population (i.e., PPARα, PPARγ, and CETP) and whether these allelic differences provide insight into the variance in diet response.

Individuals who had decreases in serum CETP following the 3.0 ounce pistachio diet had significant increases in TG and VLDL-C, compared to those who had increases or no change in serum CETP. To our knowledge, this is the first controlled-feeding study to demonstrate that diet-induced decreases in serum CETP in individuals with low TG may have adverse effects on lipids and lipoproteins. Recent studies have shown that the effects of CETP may be dependent on metabolic context. In particular, higher CETP may be beneficial in populations with lower TG [< 1.7 mmol/l (26), <1.38 mmol/l (27)]. The mean baseline TG in our study was 1.2 mmol/l. Thus,

our findings support those data suggesting varying effects of CETP in different populations. In individuals who were diet responsive with respect to serum CETP (increases or decreases), change in CETP expression explained approximately 40% of the variance in lipids and lipoproteins, including LDL-C, NONHDL-C, TC/HDL-C, and NONHDL-C/HDL-C. This relationship was not present in individuals who were non-responsive to diet.

In the present study, we have shown that genes related to inflammation and lipid metabolism are significantly expressed in PBMCs. PBMCs have been proposed as a model for studying biological pathways of CVD because of their unique characteristics. In particular, they continuously circulate throughout the body, coming in contact with all cells; they express a large number of genes; and they are responsive to subtle changes in their micro-environment. Thus, PBMC gene expression profiles, acting as sensors, can be used to investigate the underlying molecular mechanisms of nutrients. In addition, sizeable individual variation exists in PBMC gene expression, thus they may be helpful in understanding the differences in individual response to dietary interventions. A recent study demonstrates that gene expression in PBMCs can be used as a marker of hepatic cholesterol metabolism and can serve as an index for liver to assess the effects of diet and drugs on lipid metabolism (28). Current studies have shown changes in PBMC expression with weight loss (29, 30), *in vitro* zinc treatment (31), and supplementation with plant stanols (32), and psyllium (33). Intervention studies evaluating the effects of dietary fatty acids on PBMC gene expression profiles are lacking; however, a recent study in healthy men (n = 4) found that elevations in free fatty acids during fasting induced significant changes in PBMC gene expression, particularly target genes of PPARα (23). Our study demonstrates that manipulations of type and amount of dietary fat also affect PBMC gene expression. Studies with PBMCs have shown that while there is considerable variation in baseline expression and change in expression between subjects (4, 23, 34, 35), with-in subject variability in PBMC gene expression is small (4,

34, 35). Thus, studies with a crossover design, like the present study, are ideal for studying the effects of dietary interventions on PBMC gene expression.

In summary, we have shown that pistachio-rich diets significantly decrease IL-1β expression in PBMCs. Overall, we have demonstrated that dietary manipulations induce change in lipid and inflammation-related gene expression patterns in PBMCs, and that these alterations in gene expression profiles may help explain change in CVD risk factors in serum/plasma. Specifically, we found that change in CETP mRNA in PBMCs predicted change in lipids and lipoproteins in diet responders (based on serum CETP). Thus, PBMC gene expression may be a useful tool to evaluate molecular mechanisms of nutrients and further our understanding of the heterogeneity of response to dietary interventions, ultimately leading to a more individualized approach for dietary guidance.

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Table **5-1**: Nutrient composition of test diets[1].

Diet	Baseline	Control	1.5 oz Pistachio	3.0 oz Pistachio
Calories	2102	2100	2100	2100
Pro (g)	86.7 (16.5)	81.1 (15.4)	87.6 (16.7)	88.6 (16.9)
CHO (g)	261.8 (49.8)	329.5 (62.7)	302.7 (57.6)	281.1 (53.5)
Fat (g)	82.1 (35.1)	59.2 (25.4)	69.0 (29.6)	80.0 (34.3)
Chol (mg)	291.9	288.2	292.7	286.4
SFA (g)	26.2 (11.2)	18.1 (7.8)	18.0 (7.7)	18.0 (7.7)
MUFA (g)	30.6 (13.1)	21.1 (9.1)	28 (12.0)	35.8 (15.3)
PUFA (g)	18.7 (8.0)	10.6 (4.5)	13.5 (5.8)	18.0 (7.7)
LA (g)	4.8 (2.1)	6.0 (2.6)	9.9 (4.2)	14.8 (6.3)
ALA (g)	0.4 (0.2)	1.0 (0.4)	0.6 (0.3)	0.9 (0.4)
Fiber (g)	21.4	32.8	33.3	35.9
Sodium (mg)	3166	3207	2805	2646
Potassium (mg)	2514	2819	2995	3164
Vitamin A (RE)	1813	1943	1985	2048
Vitamin C (mg)	111.8	150.0	160.0	136.3
Calcium (mg)	846.2	854.7	887.3	746.7
Iron (mg)	15.6	20.2	20.6	20.5
Vitamin D (IU)	171.9	182.7	178.7	168.1
Vitamin E (mg)	7.12	6.8	7.2	8.4
α-toco (mg)	4.2	6.1	4.0	4.7
γ-toco[2] (mg)	0.9	1.1	9.8	17.2
Folate (mcg)	505.4	751.2	716.3	683.3
Selenium (mcg)	118.8	121.0	111.7	108.0
Lutein (mcg)	2924	2481	2502	2928

[1]Percent (%) kcals are presented in parentheses. Pro = protein, CHO = carbohydrate, Chol = cholesterol, SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, LA = linoleic acid, ALA = alpha-linolenic acid, α-toco = alpha-tocopherol, γ-toco = gamma-tocopherol, Lutein = lutein + zeaxanthin.
[2]Gamma-tocopherol values were approximated from the USDA Nutrient Database; all other values were determined by Nutritionist Pro approximations.

Table **5-2**. Primers used in real-time PCR[1].

	Forward	Reverse
IL-1β	CACGGCCACATTTGGTTCTAA	CAGAATGTGGGAGCGAATGAC
IL-6	GCCACTCACCTCTTCAGAACG	CCGTCGAGGATGTACCGAATT
TNFα	ATCAATCGGCCCGACTATCTC	TGGATGTTCGTCCTCCTCACA
ICAM-1	ACTCAGCGGTCATGTCTGGAC	GGCATAGCTTGGGCATATTCC
VCAM-1	AGTGGTGGCCTCGTGAATG	CACGCTAGGAACCTTGCAGC
CETP	CGCATGCTGTACTTCTGGTTCT	TCTCCAGCACTGCCTTGAACT
LCAT	CCGTGACTTCCAACGCTTCTT	TGACTGCAGCCACATGTACCA

[1]IL-1β = interleukin-1-beta, IL-6 = interleukin-6, TNFα = tumor necrosis factor-alpha, ICAM-1 = intracellular adhesion molecule-1, VCAM-1 = vascular cell adhesion molecule-1, CETP = cholesteryl ester transfer protein, LCAT = lecithin:cholesterol acyltransferase.

Table **5-3**: Baseline characteristics of subjects[1].

Characteristic	All (n = 28)	Serum increasers (n= 5)	Serum decreasers (n = 6)	Serum non-responders (n = 6)
Age (yr)	48 ± 1.5	44 ± 3.4	46 ± 3.1	53 ± 3.1
BMI (kg/m2)	26.8 ± 0.7	26.8 ± 1.9	27.0 ± 1.7	27.4 ± 1.7
TC (mg/dl)	210.8 ± 4.7	211.4 ± 13.5	196.5 ± 12.3	211.6 ± 12.3
LDL-C (mg/dl)	133.6 ± 4.3	132.3 ± 14.7	130.7 ± 13.5	135.6 ± 13.5
HDL-C (mg/dl)	57.9 ± 2.9	56.5 ± 5.4	48.7 ± 4.9	52.3 ±4.9
TG (mg/dl)	103.1 ± 7.6	128.0 ± 17.5	91.5 ± 16.0	110.4 ± 16.0
CETP (mg/l)	1.3 ± 0.1	1.1 ± 0.2	1.3 ± 0.2	1.4 ± 0.2

[1]Values are expressed as mean ± SEM. Serum CETP increasers and decreasers were classified based on a change in serum CETP of ≥ ±5% (mean change across experimental diets). Baseline characteristics were not significantly different between groups.

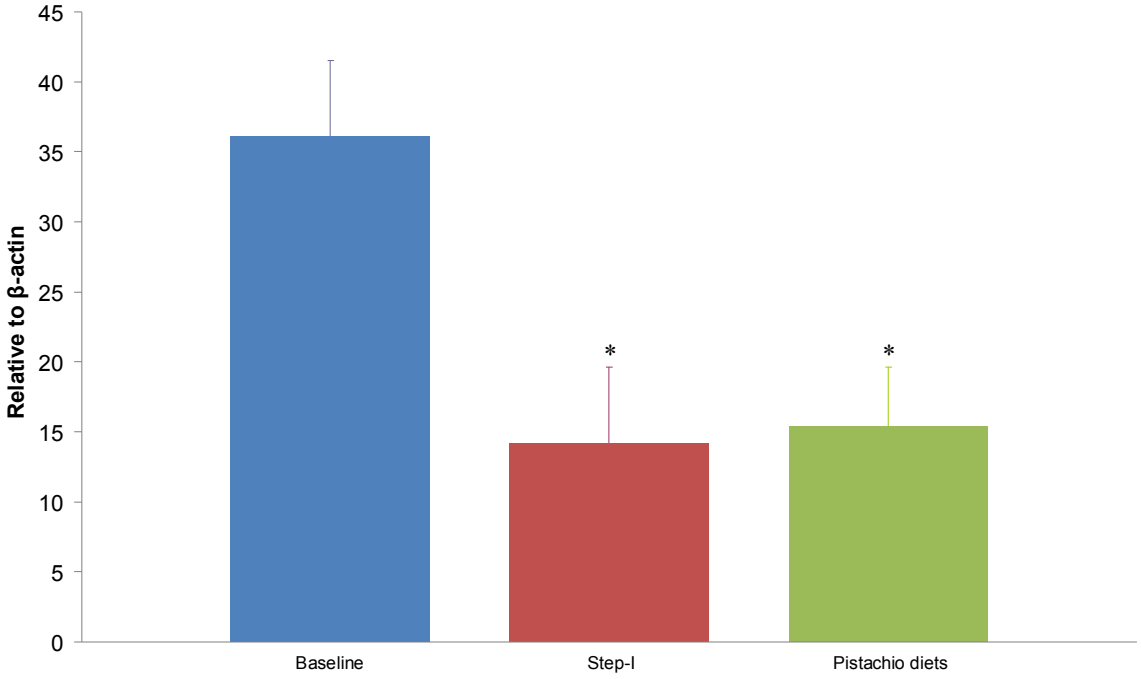


Figure **5-1**: Effects of diet on mRNA expression of IL-1β. *Significantly different from baseline ($P < 0.05$).

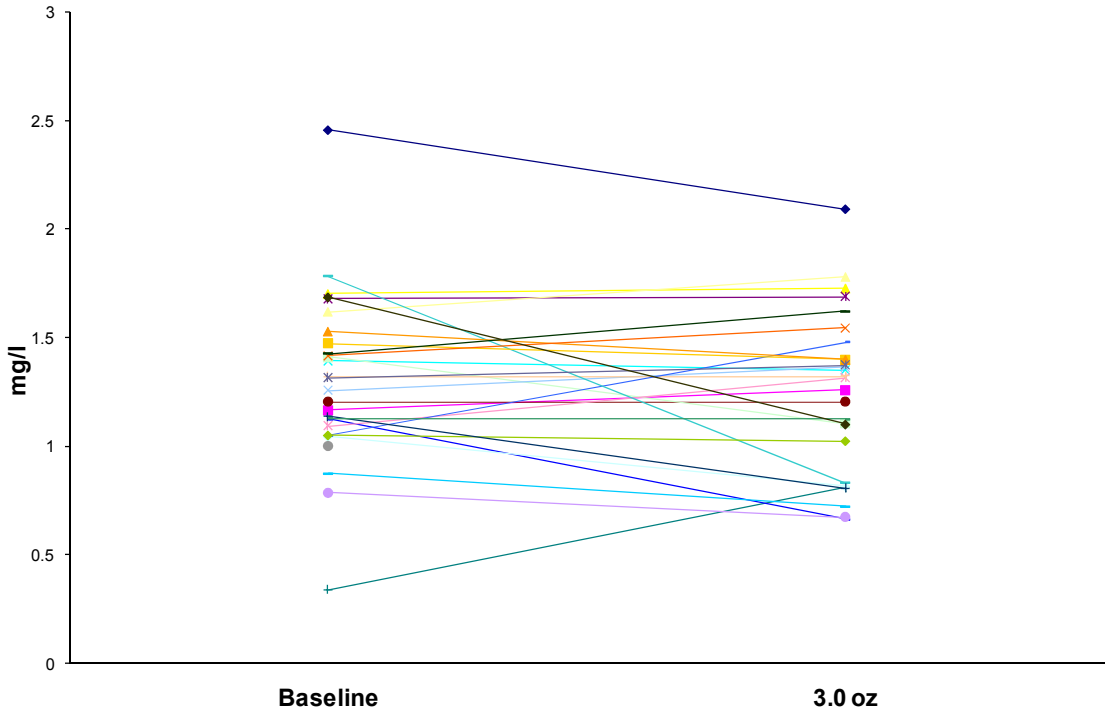


Figure **5-2:** Individual serum CETP responses (mg/l) after consumption of 3.0 oz pistachio diet (n = 28). No significant effect of diet.

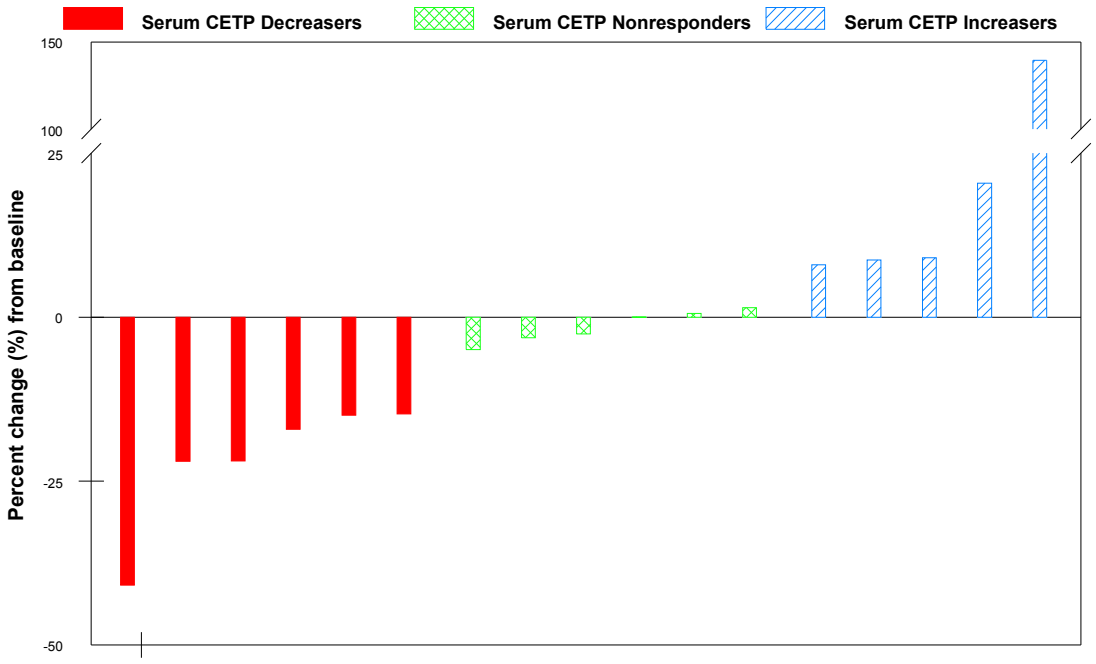


Figure **5-3**: Change in serum CETP from baseline after consumption of the 3.0 oz. pistachio diet. Serum CETP increasers and decreasers were classified based on a change in serum CETP of ≥ ±5% (mean change across experimental diets).

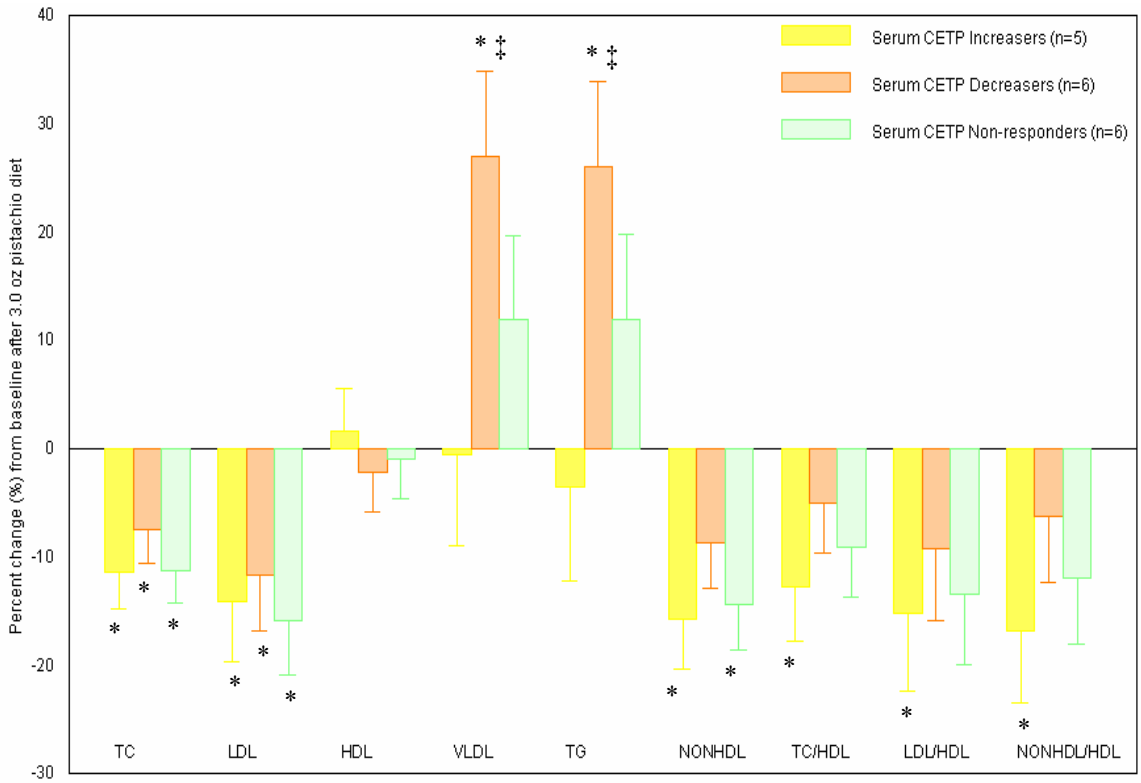


Figure **5-4**: Change in lipids and lipoproteins after consumption of the 3.0 oz. pistachio diet by serum CETP group. Serum CETP increasers and decreasers were classified based on a change in serum CETP of ≥ ±5% (mean change across experimental diets). *Significantly different from baseline ($P \leq 0.05$). ‡Significantly different from serum CETP increasers ($P < 0.05$).

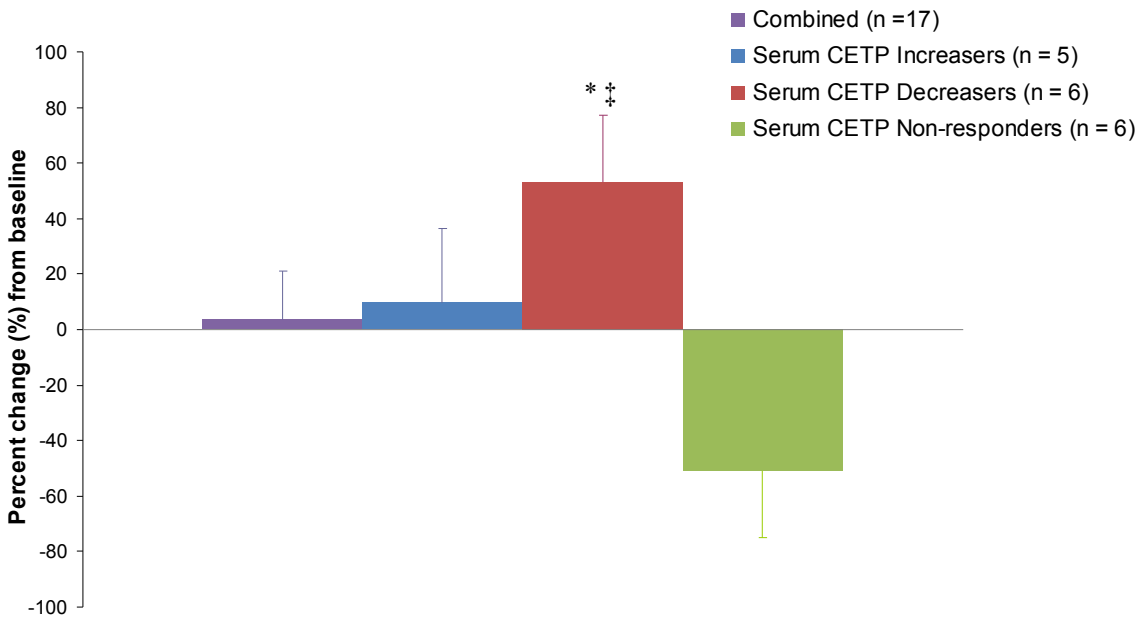


Figure **5-5**: Change in CETP expression in isolated peripheral blood mononuclear cells after 3.0 oz. pistachio diet by serum CETP group. Serum CETP increasers and decreasers were classified based on a change in serum CETP of ≥ ±5% (mean change across experimental diets). *Significantly different from baseline ($P \leq 0.05$). ‡Significantly different from non-responders ($P < 0.05$).

Chapter 6

Summary, Limitations and Future Directions

Summary

Our study clearly demonstrates the beneficial effects of pistachios on numerous cardiovascular endpoints, including lipids and lipoproteins, apolipoprotein-defined lipoproteins, plasma fatty acids, plasma stearoyl-CoA desaturase (SCD) activity, and inflammation-related gene expression in peripheral blood mononuclear cells (PBMCs). Consuming as little as 1 serving of pistachios per day significantly reduces LDL-cholesterol (LDL-C) by 9%. This is the first study with pistachios to report a significant decrease in LDL-C. This reduction is seven times greater than what would be expected based solely on fatty acid composition, suggesting that pistachios deliver a "package" of nutrients and bioactive factors, including unsaturated fats, phytosterols, and fiber, among others, which collectively contribute to their lipid- and lipoprotein-lowering effects. We have shown a dose-dependent effect of pistachios on lipoprotein ratios, with significant decreases of TC/HDL-C, LDL-C/HDL-C, and NONHDL-C/HDL-C, indicating that the lipoprotein-lowering effects of the diets are due to pistachios. The 3.0 ounce pistachio diet also significantly decreased TG compared to the Step-I diet. In addition to beneficial effects on lipids and lipoproteins, pistachios significantly decreased atherogenic apolipoprotein-defined lipoprotein subclasses, including apoB and lipoprotein B. Our results extend those of previous nut studies by clarifying mechanisms to explain the lipid- and lipoprotein-lowering effects of pistachios. Specifically, we present evidence that CETP and SCD activity may be modulating the lipid-lowering effect of pistachios. We have shown that pistachios not only affect proteins in serum and plasma, but also affect gene expression in PBMCs. The pistachio-rich diets significantly decreased IL-1β expression in PBMCs. To our knowledge, this is the first study to evaluate the

effects of a dietary intervention with nuts on expression of lipid and inflammation-related genes in PBMCs, in addition to serum and plasma risk factors. Overall, we have demonstrated that dietary manipulation induces change in PBMC gene expression, suggesting that change in PBMC gene expression patterns can serve as a useful tool for assessing the molecular mechanisms of nutrients and bioactive factors. We have shown that change in CETP expression predicted change in lipids and lipoproteins in individuals who were diet responsive (based on change in serum CETP). Due to the characteristics of PBMC gene expression profiles (large variation between individuals and small variation within individuals), they may be helpful in furthering our understanding of the heterogeneity in response to dietary interventions. Ultimately, this will help characterize individuals who are responsive to dietary interventions versus those who are not, and will lead to a more individualized approach for dietary guidance.

Our study was the first feeding study to evaluate the effects of pistachios in a highly-controlled setting. The crossover design of the study allowed subjects to serve as their own control. Compared with previous studies, the significant effects on numerous cardiovascular endpoints found in our study may be due, in part, to the highly-controlled study design, allowing for increased statistical power and reduced variability. Thus, our study has shown the efficacy of including pistachios as part of a healthy diet, demonstrating the maximum achievable benefit in a rigorously-controlled setting. A major strength of the study was the integrative approach that was utilized, which allowed us to investigate the effects of pistachios at the protein level in serum and plasma, as well as at the transcription level in isolated PBMCs. This study design can serve as a template for translational research approaches to human nutrition studies in order to comprehensively evaluate the effects of dietary interventions as well as their underlying mechanisms.

Limitations

In our study, blood was not collected before the start of each diet period, thus comparisons were made from the end of each 4-week diet period to the end of the 2-week baseline/run-in diet. However, this allowed for comparisons to a highly-controlled baseline/run-in diet, as opposed to each individual's typical diet at the start of the study. With this study design, it is possible that there could have been differences over time (i.e., the first diet period compared to baseline versus the third diet period compared to baseline); however, we tested for a period effect and there was no significant difference between periods and no interaction of period and treatment, which indicates that there was no carryover effect. A limitation of the study was that although there were decreases in CETP substrates when comparing the pistachio diets to the Step-I diet (LDL-C, VLDL-C, and TG), suggesting a decrease in CETP activity, there was no direct measure of CETP activity, only CETP mass. Earlier studies indicated a high correlation between CETP mass and CETP activity; however, recent studies suggest that they may not be highly correlated. It would be interesting to see if the pistachio diets affect a direct measure of plasma CETP activity and whether activity is correlated with change in lipids and lipoproteins, as well as change in CETP mass. We found that the pistachio-rich diets significantly decreased mRNA expression of IL-1β in PBMCs; however, protein concentrations of inflammatory markers in PBMCs were not measured. Change in IL-1β transcription does not necessarily translate to change in protein levels of IL-1β in PBMCs. Finally, we demonstrate that the lipid- and lipoprotein-lowering effects of pistachios are greater than expected from fatty acids. While we suggest that phytosterols are contributing to these effects, we did not measure plasma concentrations of phytosterols.

Future Directions

Our study has demonstrated the efficacy of including pistachios as part of a healthy diet in a rigorously-controlled setting. Further research is needed to assess the effectiveness of incorporating pistachios in a free-living setting. Other free-living studies with pistachios have been conducted; however, in these studies they strongly suggest that pistachios replace high-fat snacks, or are consumed after dinner. These studies did not report significant decreases in LDL-C. In our study, half of the daily dose of pistachios was given as a snack and half of the dose was incorporated into various recipes (Appendix A). This approach may increase long-term adherence to the pistachio diets due to the increase in variety and ease of incorporating pistachios into the diet.

Free-living studies with pistachios have demonstrated weight maintenance with consumption of pistachios. Future studies are needed to assess whether pistachios can be included as part of a weight-loss diet, particularly as part of a dietary treatment strategy for metabolic syndrome. Due to their nutrient profile, pistachios would fit well as part of a moderate-fat weight-loss diet rich in unsaturated fats. In our study, the 3.0 ounce pistachio diet significantly decreased TG compared to the lower-fat Step-I diet. In addition, although not statistically significant, HDL-C values were higher on the 3.0 ounce pistachio diet compared to the Step-I diet, and were maintained from the baseline diet (typical western diet). These beneficial effects were achieved in a relatively healthy population (other than elevated LDL-C), thus it is likely that even greater benefits would be seen in individuals with metabolic syndrome. A moderate-fat diet with pistachios would likely result in similar weight loss as a lower-fat diet without pistachios; however, a moderate-fat weight-loss diet with pistachios may be more beneficial in terms of lipid endpoints than a lower-fat weight-loss diet, particularly in individuals with elevated TG and low HDL-C.

We found distinct differences in individual serum CETP response to diet that were not explained by baseline characteristics or mRNA expression of CETP in PBMCs. Future research should investigate the occurrence of single nucleotide polymorphisms in the study population to provide insight into the differences between responders versus non-responders. In addition, evaluating the effects of other genes involved in lipid metabolism, such as PPAR responsive genes would further investigate the mechanisms by which pistachios are eliciting their lipid- and lipoprotein-lowering effects. Also, it would be interesting to evaluate the effects of pistachios on mRNA expression of SCD to determine whether expression in PBMCs correlates with plasma indices of SCD activity.

There is a vast scientific database demonstrating the numerous beneficial effects of nuts as part of a healthy diet. Nuts contain low amounts of saturated fat and are rich sources of unsaturated fats, plant sterols, and numerous vitamins, minerals, and bioactive factors. Due to their unique nutrient and fatty acid profile, nuts have been shown to beneficially affect numerous endpoints, including lipids and lipoproteins, inflammatory markers, oxidative and hemodynamic measures, and weight status. Nuts are nutrient dense, but they also are calorie dense. Consequently, nuts have been traditionally thought of as a "fatty food" and for years were avoided when following a heart-healthy or weight-loss diet. Unfortunately, the negative connotation associated with nuts has not been completely eliminated in the general public. Efforts are needed to translate the messages from epidemiologic and clinical studies of the benefits of nuts, as well as implementation strategies, to the general public. It is well known that there is a gap in what can be achieved in a highly-controlled clinical setting versus what is actually possible in a free-living setting. Increasing awareness of the immense health benefits of nuts, as well as translating the message that nuts can be consumed long-term while maintaining weight or even losing weight, will increase adherence to nut-rich diets, and consequently reduce the gap between what is achieved in highly-controlled clinical studies versus free-living settings.

Appendix

Pistachio Recipes

Pistachio Crunch Muffins

2 cups whole wheat flour

¾ cup brown sugar, packed

½ cup old fashioned oatmeal, dry

½ cup pistachio kernels, chopped

1 Tbsp baking powder

1 Tbsp orange peel

½ tsp salt

¾ cup skim milk

½ cup unsweetened applesauce

½ cup canola oil

1 egg (or ¼ cup egg whites)

Mix flour, sugar, oats, pistachios, baking powder, orange peel and salt in bowl, stirring with spoon until well blended. Add milk, applesauce, oil and egg all at once. Stir lightly just to mix. Spoon into 12 greased or paper-lined muffin cups. Spoon topping over. Bake at 400ºF for 18 to 22 minutes or until golden brown, turning pan around after 15 minutes for most even browning. Cool for 5 minutes then remove from pan and cool on wire rack. Makes 12 large muffins.

Pistachio Banana Bread Muffins

2 cups whole wheat flour

¾ tsp baking soda

½ tsp salt

½ cup white granulated sugar

½ cup brown sugar, packed

¼ cup butter

½ cup egg whites

4 medium-sized, ripe bananas

1/3 cup fat free-sour cream

1/8 tsp ground cardamom (or you may substitute 1 tsp vanilla extract)

½ cup pistachio nuts, chopped

Preheat oven to 350ºF. In a medium sized bowl, combine flour, baking soda, and table salt, stirring with a whisk. Set aside. In a large bowl, combine the sugars and the butter and beat with hand mixer until well-blended. Add the egg whites to the sugar/butter mixture and beat well. Add the banana, sour cream, and cardamom and beat until well-blended. Add the flour mixture and beat at a low speed just until moist. Stir in pistachios, making sure that they are incorporated evenly throughout the batter.

Spray muffin tin with non-stick cooking spray. Distribute batter evenly among the 12 muffin tins. Place in oven for ~17 minutes. Muffins are done when a toothpick inserted into the center of each muffin comes out clean. Yield: 12 generous muffins.

Pistachio Pesto

2 cup basil leaves

2 garlic cloves

1 cup pistachio kernels

½ cup olive oil

½ cup Parmesan cheese

1 grind black pepper

To make 1 cup of pesto, place 2 cups of basil leaves, 2 garlic cloves, and 1 cup of pistachio kernels in a food processor. Process until smooth, leaving just a bit of texture. With the food processor's motor still running, slowly sprinkle in ½ cup olive oil through the feed tube. Add ½ cup grated Parmesan cheese and a grind of black pepper. Process to combine. Pesto will keep covered in the refrigerator for up to 3 days. Bring to room temperature before serving.

Pistachio Chicken Salad

3 cups chicken, canned (or boneless, skinless chicken breast, chopped)

1 cup red seedless grapes, sliced

2 tsp scallions, minced

2 Tbsp fat free mayonnaise

2 Tbsp fat-free sour cream

2 Tbsp lemon juice

¼ cup unsalted pistachios, chopped

Combine ingredients in a large bowl and mix thoroughly to evenly distribute. Serve atop your favorite whole grain bread or atop a bed of mixed greens. Makes 6 servings.

California Style Pistachio Salad

Dressing:

1 clove garlic, finely minced

1 tsp Dijon mustard

1 Tbsp balsamic vinegar

6 Tbsp orange juice (~1 orange, freshly squeezed)

Salad:

3 cups mixed greens

8 oz boneless, skinless chicken breast, grilled and sliced

1 tart apple, quartered and sliced

½ cup blue cheese, crumbled

½ cup shelled pistachios

For the dressing, mix together the ingredients with a wire whisk. Let set. Wash and dry the mixed greens and divide equally among 4 salad plates. Divide chicken, apple slices, blue cheese, and pistachios over salad. Drizzle dressing over each. Makes 4 servings.

Pistachio Granola

2/3 cup packed brown sugar

¼ cup apple juice

2 cups regular oats

2/3 cup chopped pistachios

2/3 cup bran cereal

2/3 cup raisins

½ tsp ground cinnamon

¼ tsp salt

Spray a large nonstick skillet with cooking spray. Combine sugar and apple juice in skillet and cook over medium-high heat for 3 minutes or until sugar dissolves, stirring frequently. Stir in oats and remaining ingredients; cook 5 minutes or until granola is lightly browned, stirring frequently. Spread granola mixture on a large cookie sheet lined with aluminum foil (sprayed with cooking spray). Allow to cool slightly and place in convection oven for 3-5 minutes to allow granola to harden. Remove from oven and allow to cool completely. Once cool, store in an airtight container for up to 1 week. Yield: 5 cups.

VITA

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PUBLICATIONS

- **Gebauer SK**, Vanden Heuvel JP, Kris-Etherton PM, Gillies PJ. Integration of Molecular Biology and Nutrition: The role of nutritional genomics in optimizing dietary guidance in lipids. Special Report. Future Lipidology. 2007 Feb; 2(2): 165-171.
- **Gebauer SK**, Psota TL, Kris-Etherton PM. The Diversity of Health Effects of Individual trans Fatty Acid Isomers. Lipids. 2007 Aug 11; [Epub ahead of print].
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ORAL PRESENTATIONS

- "Translational Research: Clinical Nutrition Interventions at Penn State." Penn State University/Wageningen University Nutritional Sciences Seminar: University Park, PA, October 2007.
- "The Cardiovascular Health Benefits of Pistachios." Featured speaker of member luncheon at the American Academy of Nurse Practitioners Conference: Indianapolis, IN, June 2007.
- "Pistachios Pack A Powerful Punch". Produce Marketing Association Trade Show: Houston, TX, October 2007.

POSTER PRESENTATIONS

- **Gebauer SK**, Kay CD, West SG, Alaupovic P, Psota TL, Kris-Etherton PM. Pistachios beneficially affect multiple lipid and apolipoprotein CVD risk factors. Experimental Biology: Washington, DC, April 2007.
- Kay CD, **Gebauer SK**, West SG, Kris-Etherton PM. Pistachios reduce serum oxidized LDL and increase serum antioxidant levels. Experimental Biology: Washington, DC, April 2007.
- **Gebauer SK**, West SG, Alaupovic P, Kris-Etherton PM. Including Pistachios in a Step I Diet Favorably Reduces CVD Risk Factors. Experimental Biology: San Francisco, CA, April 2006.