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THE EFFECTS OF MONOUNSATURATED FATTY ACID-ENRICHED DIETS WITH AND WITHOUT AVOCADOS ON CARDIO-METABOLIC RISK FACTORS

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ABSTRACT

Dietary guidance recommends a healthy dietary pattern that is low in saturated fat (SFA) to lower low-density lipoprotein cholesterol (LDL-C), a primary target for cardiovascular disease (CVD) risk reduction. Using either carbohydrates (CHO) or unsaturated fat to replace saturated fat in an average American diet may achieve a similar reduction in LDL-C, but a high intake of dietary CHO may increase triglycerides (TG), lower high density lipoprotein cholesterol (HDL-C), and increase small, dense LDL, along with promoting insulin resistance thereby increasing the risk of metabolic syndrome and type 2 diabetes. These cardio-metabolic risk factors also are targets for dietary intervention to achieve a greater CVD risk reduction. When SFA is replaced with unsaturated fat, the predominant fatty acid class is monounsaturated fatty acids (MUFA) because the recommended intake of polyunsaturated fatty acids (PUFA) is less than 10% of energy. Overall, the substitution of MUFA instead of CHO (especially refine grains and added sugar) for SFA calories may favorably affect other lipid risk factors of CVD.

The oxidative modification of LDL particles is a key factor in the initial process of atherosclerosis. Dietary antioxidant vitamins, polyphenols, and other bioactive compounds from foods (e.g., fruits and vegetables) have been a focus of nutrition because of their role in improving antioxidant status and lowering LDL oxidation. Identifying foods that can beneficially improve multiple cardio-metabolic risk factors is needed for the primary prevention of CVD.

Avocados are a nutrient-dense source of MUFA, rich in vitamins, minerals, fiber, phytosterols and polyphenols. It is unclear whether avocados affect cardio-metabolic risk factors beyond their fatty acid profile. This dissertation investigated the effects of a MUFA enriched moderate fat diets including one avocado per day on established and novel cardio-metabolic risk factors.

A randomized, cross-over, controlled feeding trial was conducted with 45 healthy overweight/obese participants with baseline LDL-C levels in the 25-90th percentile. After a 2 week run-in average American diet (34% fat, 13% SFA, 11% MUFA, 51% carbohydrate, 16% protein) at baseline, three cholesterol-lowering diets (6-7% SFA) were fed (5 weeks each) with a random sequence: a lower-fat diet (LF: 24% fat, 7% SFA, 11% MUFA, 9% PUFA, 59% carbohydrate, 16-17% protein); and two moderate fat diets matched for macronutrients and fatty acids (34% fat, 6% SFA, 17% MUFA, 9% PUFA, 49% carbohydrate, 16-17% protein): the avocado diet included one fresh Hass avocado (136 g) per day, and the moderate fat diet provided mostly the same foods (as the avocado diet) but used high oleic acid oils and low fat dairy products to match the fatty acid profile of one avocado. All three cholesterol-lowering diets met current dietary recommendations on macronutrient percentage range and the serving amount for each food group. Specifically, the grain product in the lower fat diet contained more than half whole grains, which meets current dietary guidelines.

Compared to baseline, all three diets decreased total cholesterol and LDL-C ($p<0.001$ for all). The LF diet significantly increased TG (20.8mg/dL, $p<0.0001$) and very-low-density lipoprotein cholesterol (VLDL-C, 2.6mg/dL, $p=0.0003$), while the AV and MF diets did not. There was a greater HDL-C decrease on the LF diet versus the AV and MF diets ($p=0.03$ and 0.04, respectively). The reduction in LDL-C and non-HDL cholesterol on the AV diet (-13.5mg/dL, -14.6 mg/dL) was greater ($p=0.04$ and 0.01) than the MF (-8.3 mg/dL, -8.7 mg/dL) and LF (-7.4 mg/dL, -4.8 mg/dL) diets. Furthermore, only the AV diet significantly decreased LDL particle number (LDL-P, -80.1nmol/L, $p=0.0001$), small dense LDL cholesterol (LDL_{3+4}, -4.1 mg/dL, $p=0.04$), and the ratio of LDL/HDL (-6.6%, $p<0.001$). In addition, compared to baseline, only the AV diet decreased oxidized-LDL (-7.0 U/L, -8.8%, $p=0.0004$) while the LF diet (-1.6 U/L $p=0.1$) and the MF diet (-3.2 U/L, $p=0.2$) did not. Oxidized-LDL after

consumption of the AV diet was significantly lower ($p=0.05$ and 0.03) than the MF diet and LF diet. HPLC analysis showed that only the AV diet increased plasma lutein by 68.7% from baseline ($p<0.0001$). The increase in lutein in response to the AV diet was significantly greater than the increase by the MF (21.1%, $p=0.7$) and LF (37.6%, $p=0.1$) diets. Both MF and AV diets significantly increased plasma α-carotene (72.8% and 68.4%, $p<0.01$ for both) and β-carotene (15.4% and 12%, $p<0.05$ for both) compared to baseline. The LF diet did not elicit changes in plasma antioxidant vitamins, except for a decrease in γ-tocopherol (-7.8%, $p=0.03$). Interestingly, the change of oxidized-LDL was significantly correlated with the change in small LDL-P ($r=0.32$, $p=0.0002$) and small, dense LDL-C ($r=0.47$, $p<0.0001$) by not large LDL-P ($r=0.15$, $p=0.09$) or large, buoyant LDL-C ($r=-0.03$, $p=0.8$).

Overall, our results showed that a high MUFA, moderate fat diet elicits a more favorable blood lipid profile compared to a lower fat, high carbohydrate diet. Furthermore, the inclusion of one avocado per day as part of a moderate fat, cholesterol-lowering diet has additional benefits on lowering LDL-C, LDL-P, non-HDL-C, small, dense LDL, and oxidized LDL, and increasing plasma lutein. The change in oxidized LDL by the avocado diet may be due to its effect on lowering small, dense LDL. Our results demonstrate that avocados have beneficial effects on cardio-metabolic risk factors that extend beyond their cardio-protective fatty acid profile. Avocados are a source of many cardio-protective nutrients and further larger, long-term clinical studies are warranted to evaluate their role in primary and secondary CVD prevention.

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ABBREVIATIONS

ASCVD	Atherosclerotic cardiovascular disease
95% CI	95% confidence interval
CHO	Carbohydrates
CETP	Cholestryl ester transferase protein
CRP	C-reactive protein
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DGAC	Dietary Guidelines Advisory Committee
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunoassay
FDA	Food and Drug Administration
HDL-C	High-density lipoprotein cholesterol
HDL-P	High-density lipoprotein particle number
HOMA-IR	Homeostatic model assessment of insulin resistance
HPLC	High-performance liquid chromatography
LDL-C	Low-density lipoprotein cholesterol
LDL-P	Low-density lipoprotein particle number
MUFA	Monounsaturated fatty acids
NMR	Nuclear magnetic resonance spectroscopy
oxLDL	Oxidized LDL
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acids
RCT	Randomized controlled trial
SEM	Standard error of the mean
SBP	Systolic blood pressure
sdLDL	Small, dense low-density lipoprotein
SFA	Saturated fatty acids
TC	Total cholesterol
TG	Triglycerides
TNF-α	Tumor necrosis factor-α
US	United States
USDA	United States Department of Agriculture
VAP	Vertical auto profile ultracentrifugation
VLDL-C	Very-low density lipoprotein cholesterol
VLDL-P	Very-low density lipoprotein particle number

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Chapter 1 Introduction

Cardiovascular disease (CVD) is a class of diseases that involve the heart, blood vessels or both. They include coronary heart disease (CHD), cerebrovascular disease (stroke), hypertension, peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. CVD is a ranked as the leading cause of mortality in both developed and developing countries; the global burden of CVD is still rising (1). The 2013 update on heart disease statistics from the American Heart Association (AHA) indicates that the prevalence of CVD in adults in 2010 was 35.3%, and CVD accounted for 31.9% of all deaths. This equates to 1 of every 3 deaths in the US in 2010 (2). Nutrition affects many CVD risk factors. This dissertation is focused on dietary intervention and atherogenic lipoproteins on risk factors of atherosclerotic CVD (ASCVD) (3, 4).

The initiation of atherosclerosis results from a combination of abnormalities in lipoprotein metabolism, oxidative stress, thrombosis and chronic inflammation (5). Multiple factors are involved in the development of CVD, including non-modifiable risk factors (gene, age, gender, family history), and modifiable risk factors (poor diet, smoking, overweight/obesity, physical inactivity). Several risk factors and intervention targets have been identified, including elevated low-density lipoprotein cholesterol (LDL-C, the primary target), low high-density lipoprotein cholesterol (HDL-C), high blood pressure, smoking, diabetes, and increased C-reactive protein (hs-CRP) (3). These targets are used in diet and lifestyle intervention to reduce risk in patients with established CVD (secondary prevention) and prevent the onset of CVD in people at risk who have not yet established CVD (primary prevention).

Besides the traditional risk factors and intervention targets, there are several emerging risk factors that have received increasing attention in clinical nutrition research, such as triglyceride-rich lipoproteins, lipoprotein particle number and size, lipoprotein subclasses and oxidative modification of lipoprotein particles. Although lowering LDL-C has been the primary target for prevention and treatment of CVD, there is residual cardiovascular risk that remains after LDL-C goals are achieved with lipid-lowering treatments, especially in high-risk patients such as those with type II diabetes mellitus or the metabolic syndrome (6, 7). Metabolic syndrome is the clustering of cardio-metabolic risk factors (excess abdominal adiposity, dyslipidemia, elevated blood pressure, and hyperglycemia) related to abdominal obesity and insulin resistance. It may increase the likelihood of developing CVD within 10 years by two-fold (8). The atherogenic lipoprotein phenotype associated with metabolic syndrome includes high triglycerides (TG), increased very low-density lipoprotein (VLDL), low HDL-C, and increased small, dense LDL particles (sdLDL), the latter of which are more susceptible to in vivo oxidation in the artery wall (9). Oxidation of LDL particles is considered to be a key factor in the physiological process that initiates and accelerates the development of the early atherosclerotic lesion (10). However, atherogenic dyslipidemia is largely under-diagnosed and under-treated in clinical practice. According to the recent AHA/ACC guidelines, non-HDL-C has been treated as a secondary therapeutic target for CVD since the residual risk is mainly due to low HDL-C and TG-rich lipoprotein levels (11). The most recent meta-analysis on randomized trials of statins treatment reported that the relative risk reduction of CHD was more closely related to reductions in apolipoprotein B (apoB) than to reductions in either non-HDL-C or LDL-C (12). ApoB may be a better indicator of residual risk than non-HDL-C because it reflects the number of LDL and TG-rich lipoprotein particles. In this context, the direct measure of atherogenic lipoprotein (LDL and TG-rich lipoproteins) particle numbers, small, dense LDL and oxidized LDL may provide

more accurate evaluation for monitoring the effect of diet intervention on CVD risk than traditional lipid biomarkers. Some of these biomarkers are measured and their inter-relationship is discussed in this dissertation: oxidized-LDL, lipid oxidative biomarkers, plasma antioxidant status, sdLDL, lipoprotein subclasses and particle size, TG, apolipoproteins, non-HDL-C, and lipoprotein remnants.

Study Rationale

A focus of dietary recommendations for cardiovascular disease (CVD) prevention and treatment is reducing saturated fat intake, primarily as a means of lowering LDL-cholesterol concentrations. The 2010 Dietary Guidelines (13) recommend healthy diet patterns that contain less than 10% from saturated fat (SFA) for lowering LDL-C, and for further reductions in LDL-C, less than 7% is recommended (14). The 2013 American Heart Association and American College of Cardiology (AHA/ACC) joint Guidelines on Lifestyle Management to Reduce Cardiovascular Risk also recommends a healthy dietary pattern that provides 5 to 6% of calories from SFA to lower LDL-C. A meta-analysis of 60 controlled trials shows all SFAs except stearic acid increased LDL-C and HDL-C (15). A recent Cochrane review reported that reducing SFA lowered the risk of CVD events by 14% (16). In contrast, a meta-analysis that included 16 prospective cohort studies showed poor relative risk of SFA intake for events of CHD, stroke, and total CVD in participants at 30–89 y of age (17). The evidence that supports a reduction in SFA intake must be evaluated in the context of the replacement by other macronutrients and different food sources. Clinical, epidemiological, and mechanistic studies consistently show that the risk of CHD is reduced when SFAs are replaced with PUFAs (18). However, intakes of PUFA have been limited to ≤ 10% of energy due to their potential adverse effects, including a reduction of HDL-C level and increased susceptibility of LDL to oxidation (19).

Besides PUFA, both carbohydrate (CHO) and monounsaturated fatty acids (MUFA) can be used to replace SFA in the diet to achieve the LDL-C lowering effect. However, a high intake of CHO may cause adverse changes in the lipid/lipoprotein profile and glycemic control (20, 21). Numerous studies have shown that low-SFA, high-CHO diets decreased predicted CVD risk based on the reduction in LDL-C. Some large randomized controlled trials (RCTs) showed non-significant benefit (22) or inverse relationship between high CHO intake and CVD risk (23). Many studies have shown that a diet high in complex CHO, whole grain foods and dietary fiber, and low in SFA, benefits CVD risk (24). It was estimated that every serving (28 g/d) of whole grain consumption was associated with a 5% (95% CI: 2%-7%) lower total morality or a 9% (95% CI: 4%-13%) lower CVD mortality (24). A recent meta-analysis concluded that total grain intake and refined grain intake were not associated with CVD risk (25). Overall, there is insufficient evidence to recommend that replacing SFAs with CHO decrease CHD although there might be a benefit if the carbohydrate includes more whole grain products and has a low glycemic index.

From 1971 to 2012, there has been a decrease in total fat intake (-3.3% of total calories) and SFA intake (-2.1% of total calories) in US adults (**Table 1-1**). At the same time, the average intake of carbohydrates (CHO) has increased by 5.1% of total calories. Also, average total calorie intake has been increased 181 kcals. It may due to the impact of dietary recommendations to limit total fat which resulted in increased consumption of refined CHO and added sugar. Diets high in refined carbohydrates, coupled with the rising incidence of overweight and obesity, may lead to a metabolic state that can favor a worsening of atherogenic dyslipidemia that is characterized by elevated triglycerides, reduced HDL cholesterol, and increased concentrations of small, dense LDL particles (26). Hence, a high intake of refined carbohydrates increases the risk of CHD, independent of known coronary disease risk factors (27). In response to these research

findings, 2010 Dietary Guidelines recommended "make at least half your grains whole" in the MyPlate meal plan (13).

Table 1-1 The trend of macronutrients intake in US adults from 1971 to 2012.

Macronutrients	NHANES 1971-1974[a]	NHANES 2010-2012[b]	Change from 1971 to 2012
Daily energy (kcal)	2010	2191	+ 181 kcal
Total fat (% kcal)	37%	33%	- 4%
SFA (% kcal)	13%	11%	- 2%
CHO (% kcal)	44%	49%	+ 5%
Protein (% kcal)	16.7%	15%	- 1.7%

[a] data source: National Health and Nutrition Examination Survey (NAHNES) I; [b] data source: NAHNES 2010-2012.

Clinical studies have demonstrated convincingly that dietary monounsaturated fatty acids (MUFA) beneficially affect metabolic syndrome risk versus dietary CHO. Substituting SFA with MUFA lowers LDL-C, increases HDL-C, and lowers TC/HDL-C ratio. Replacing 5% calories from SFA with MUFA could potentially reduce CHD risk by 7.5% via reduction in the TC/HDL-C ratio (28). However, there is a lack of randomized controlled trials (RCTs) that directly confirm the casual link. Unlike SFA and PUFA with a recommend limit, MUFA intakes are determined by calculating the difference, i.e., MUFA (% energy) = total fat (% of energy) – SFA (% of energy) – PUFA (% of energy) – trans fat (% energy). Thus, MUFA intakes will range with respect to the CHO and total fat composition of the diet. Current dietary guidelines recommend the intake range of total fat is 20% - 35% of total energy, including MUFA up to 20% (**Table 1-2**). The joint FAO/WHO Expert Consultation on Fats and Fatty Acids in Human Nutrition also recommended that MUFA intakes be 15% - 20% of energy (29). Compared to the current macronutrient intake status in US adults, we need to further decrease SFA intake by 4% of total energy. Since MUFA intake is still below the recommended intake range, and CHO intake could be adjusted within the wide range recommended, using either MUFA or complex

CHO to replace SFA can achieve the recommendation for SFA intake. MUFA, however, may beneficially affect metabolic syndrome risk versus CHO.

Table 1-2 Current macronutrient intake of US adults and the recommended range for each macronutrient.

Macronutrients	NHANES 2010-2012[a]	Recommend Intakes[b]
Total fat (% kcal)	33%	20-35%
SFA (% kcal)	11%	≤ 7%
PUFA (% kcal)	7%	Up to10%
MUFA (% kcal)	12%	Up to 20%
CHO (% kcal)	49%	45% - 65%
Protein (% kcal)	15%	10-35%

[a] Data source: NAHNES 2010-2012

[b] Based on Institute of Medicine's Acceptable Macronutrient Distribution Range (30), Dietary Guidelines 2010 (13), and AHA/ACC Diet and Lifestyle Guidelines (14).

It is clear that the inconsistent results on the effect of CHO on CVD risk are mainly due to CHO food sources with different glycemic index. Studies also suggest that the food sources of dietary fatty acids are important in determining their effects on CVD risk than the total amount of the fatty acids. Epidemiologic studies conducted with western populations have reported a neutral or positive association of dietary MUFA and CHD risk (31, 32), but high-MUFA foods in the Mediterranean diet (olive oil and nuts) have been consistently shown to have cardioprotective benefits in epidemiologic studies (33-35) and in a recent long-term RCT. In 2013, the PREDIMED (Prevención con Dieta Mediterránea) trial reported that a Mediterranean diet (supplemented with either extra-virgin olive oil or nuts) reduced the incidence of major CVD events by approximately 30% after five years intervention in men and women (50 to 80 years of age) who were at high risk for CVD (36). Insufficient evidence exists to judge the effect on CHD risk of replacing SFAs with MUFAs, mainly because the available data on MUFAs are limited and confounded by the food sources of MUFAs (e.g., dairy and meats) in Western dietary

patterns. It is evident that we need to identify the preferable food sources of MUFA in a healthy diet.

Of the several dietary factors implicated in CHD prevention, the evidence is most consistent for fruit and vegetables (37, 38), which may due to their rich content of dietary fiber, micronutrients and beneficial bioactive compounds. "Bioactive compounds" are extra-nutritional constituents that naturally occur in small quantities in foods, mainly in fruits and vegetables (39). Both antioxidant (polyphenols, flavonoids and carotenoids) and non-antioxidant bioactive compounds (phytosterols) have been intensively studied, inspired by many epidemiologic studies that have shown protective effects of plant-based diets on CVD and cancer. The benefit of antioxidant bioactive compounds on CVD risk is to reduce oxidant stress and inflammation. Dietary fiber, vitamins, minerals, and phytochemicals in fruits and vegetables are being studied for their role in chronic disease prevention (40). Mechanistic studies have shown that individual antioxidants can successfully reduce antioxidant stress in vitro. However, clinical trials have failed to justify the routine use of antioxidant supplements for the prevention and treatment of CVD (41, 42). In contrast, current evidence supports recommending consumption of a diet high in food sources of antioxidants instead of antioxidant supplements to reduce risk of CVD (43). Recent studies also suggested that the antioxidant potential of plant foods is a function of the synergistic effects of numerous antioxidant compounds (44), which may explain the "antioxidant paradox" of antioxidant supplements. Recently, low fruit and vegetable consumption has been identified as one of the top risk factors for causing the greatest "loss of health" in the Global Burden of Disease 2010 Study (45). Food-based recommendations are more practical for the general public than is nutrient-based dietary advice. If foods are rich in bioactive compounds have consistent cardio-protective benefits, should dietary recommendations for fatty acids focus on specific food sources of fatty acids that also are high in fiber, vitamins, minerals, and

functional bioactive compounds? However, the evidence linking individual foods or food patterns to CVD risk is limited. Thus, identifying specific foods that are good sources of beneficial nutrients and bioactive compounds for CVD risk reduction will be helpful for updating dietary guidelines.

Nuts and olive oil are major food sources of MUFA in the Mediterranean diet and have been extensively studied on their cardio-protective benefits. Avocados are a unique nutrient-dense fruit source of MUFA, which are rich in vitamins, minerals, fiber, phytosterols and polyphenols that have not been studied extensively. The Hass variety, which is mainly consumed in the United States, is relatively high in MUFA and other fat-soluble vitamins. One Hass avocado (136g, without skin and the seed) contains about 13 g of oleic acid, which is similar to the amount of oleic acid in 1.5 oz. almonds or 2-tablespoon olive oil (46). Based on their fatty acid and nutrient profile, avocados could be classified as "nuts" or "fats" and expected to beneficially affect CVD risk. According to USDA, avocados are classified as a fruit (47), which also is relevant since increasing intake of fruit and vegetables is one of the primary dietary recommendations for chronic disease prevention. Evidence about the beneficial effects of avocados on the lipid/lipoprotein profile is based on relatively few clinical studies. Studies with avocados (0.5 to 2 avocados per day) have reported similar or greater TG, LDL-C and TC-lowering effects while maintaining HDL-C compared to low-fat, cholesterol-lowering diets (48-53). There are several limitations, however, of previous studies that include the presence of diabetes, CVD, and other illnesses in some participants (48-51, 53), weight-loss during the feeding period (53), lack of control of the background diet, and variations in the diet interventions (51, 52, 54, 55), including the length of the feeding period and diet design (49, 52, 55), as well as small sample size (48, 50, 52, 53, 55). Thus, it has been challenging to identify a cardio-protective benefit of avocados. Furthermore, in these studies, avocados were used as a source of

MUFA that was substituted for other macronutrients to evaluate only the effect of type and amount of fat on lipids and lipoproteins.

In summary, in order to reduce dietary saturated fat and achieve a cardio-protective diet with an optimal macronutrient intake, there are several paradoxes that need further study:

1) The "CHO paradox": low-SFA, high-CHO diets decreased predicted CVD risk based on the reduction in LDL-C, but high-CHO diets, especially high in refined CHO and simple sugars, may lead to a metabolic state that can favor a worsening of the atherogenic dyslipidemia associated with metabolic syndrome. Current Dietary Guidelines recommend increasing complex CHO intake and the proportion of whole grains in the diet. However, it is still unclear if a lower fat diet using mainly complex CHO and whole grains to replace SFA can offset the effect of high-CHO diet on TG and HDL-C. Also, it remains unclear if the complex CHO induced hypertriglyceridemia is as atherogenic as the high-fat Western diet induced dyslipidemia.

2) The "MUFA paradox": epidemiologic studies conducted with western populations have reported a neutral or positive association of dietary MUFA and CHD risk, but high-MUFA foods in the Mediterranean diet (olive oil and nuts) have been consistently shown to have cardioprotective benefits in observational and interventional studies. The inconsistent results may be due to the available data on MUFA that are confounded by the food sources of MUFA (e.g., dairy and meats) in Western dietary patterns. However, it is still unclear if the cardioprotective effects of high-MUFA foods in the Mediterranean diet are contributed by other nutrients beyond fatty acids in these foods. Few studies have studied the benefits from the bioactive compounds beyond fatty acids content in nutrient-dense MUFA foods, including nuts, olive oil, and avocados.

3) The "antioxidant paradox": individual antioxidants can reduce antioxidant stress in vitro, but antioxidant supplementation has been failed to lower CVD risk in large clinical trials,

even with lowered oxidative stress biomarkers. In contrast, plant foods that are high in antioxidants, such as fruits and vegetables consistently have benefits on CVD prevention. However, studies are lacking to confirm the antioxidant effects from bioactive compounds in plant foods.

In order to investigate these research questions, the current project used avocados as the key food component in a heart-healthy diet to: 1) evaluate if a moderate-fat, high MUFA diet including one avocado a day has additional benefits than a macronutrient-matched diet without avocados; 2) to compare the benefits on CVD risk factors of a moderate-fat diet using MUFA to replace SFA versus a lower-fat diet meeting current Dietary Guidelines (especially recommendations for complex CHO and whole grain products).

Objectives and Hypotheses

The purpose of this dissertation is to investigate the cardio-protective benefits of a MUFA enriched, moderate fat diet on cardio-metabolic risk factors compared to a lower-fat, high complex CHO diet. The results will provide more guidance on the implementation of current dietary guidelines by achieving an optimal macronutrient composition. Specifically, this dissertation evaluated the additional benefits of avocados by comparing the replacement of dietary SFA with CHO versus MUFA from different food sources: one avocado per day or high oleic acid oils/low fat dairy products. This dissertation will address the current controversies on the residual risk of CVD after LDL-C reduction, traditional lipid biomarkers versus advanced lipid testing, the optimal macronutrient profile, and food sources of MUFA.

1. Primary objectives: to determine if a moderate fat diet (using MUFA to substitute for ~6% SFA of total energy in average American diet) including one avocado a day improves risk

factors of and CVD and metabolic syndrome more than a isocaloric lower-fat diet (using complex CHO to substitute for ~6% SFA, with emphasis on whole grains).

Hypotheses: both diets will elicit a LDL-C lowering effect compared to a high-SFA American diet. The lower-fat diet will increase TG, lower HDL-C compared with the average American diet, but the moderate fat diet will not affect TG or HDL-C levels. Furthermore, the moderate fat diet including one avocado a day will increase LDL particle size and insulin sensitivity, and decrease small dense LDL compared with the lower fat diet.

2. Secondary objectives: to determine if a moderate fat diet using one avocado a day to provide the main source of MUFA (~6% of total energy) to substitute for SFA has additional cholesterol-lowering and antioxidant effect compared with an isocaloric moderate diet using vegetable oils to provide the main source of MUFA to substitute for ~6% SFA in the average American diet.

   Hypotheses: the moderate fat diet including one avocado a day will elicit a greater LDL-C reduction than the moderate diet without avocados. Also, consuming one avocado a day will significantly lower oxidation of LDL particles and increase plasma antioxidants.

3. Exploratory objectives: to explore the relationship between the change in lipid oxidative markers, plasma antioxidant, and lipoprotein particle subclasses in response to dietary changes.

Chapter 2 Literature Review

The Literature Review is organized as follows: 1) current dietary guidelines and evidence on the effects of dietary MUFA in CVD prevention; 2) mechanisms of the potential cardioprotective effect of avocados beyond MUFA.

Dietary MUFA and Cardio-Metabolic Risk

The benefits and controversies of dietary MUFA on cardio-metabolic risk

Cumulative evidence from both epidemiological and clinical studies has shown that MUFA beneficially affect multiple CVD risk factors. In the Seven Countries Study, Keys reported that a low prevalence of coronary heart disease was associated with the Mediterranean diet, which sparked interest in subsequent studies that were conducted on high-monounsaturated diets (56). A later randomized diet intervention trial in cardiac patients confirmed the cardio-protective benefit by showing a significant reduction in total mortality associated with Mediterranean diet (34). More specifically, evidence from prospective cohort studies suggests that a diet high in MUFA is associated with a 20% reduced risk of CHD events (57). Within the context of the Mediterranean Diet, dietary MUFA have been associated with a decrease in CHD mortality in prospective studies (33-35). In contrast, epidemiologic studies conducted with Western populations have reported a neutral or positive association of dietary MUFA and CHD risk (31, 32). This inconsistency may reflect the major food sources of MUFA in the United States, many of which coexist with SFA and added sugar (**Table2-1**) (13), while the major food sources of MUFA in the Mediterranean diet are plant based, and provide many micronutrients and bioactive compounds.

Table 2-1 Food sources of oleic acid by percent contribution to intake based on NHANES 2005-2006.

Food item	Contribution to intake %	Cumulative contribution %
Grain-based desserts	8.9	8.9
Chicken and chicken mixed dishes	7.6	16.6
Sausage, franks, bacon, and ribs	5.9	22.5
Nuts/seeds and nut/seed mixed dishes	5.5	27.9
Pizza	5.4	33.3
Fried white potatoes	4.9	38.2
Mexican mixed dishes	4.6	42.8
Burgers	4.1	46.9
Beef and beef mixed dishes	3.9	50.8
Eggs and egg mixed dishes	3.5	54.3
Regular cheese	3.3	57.5
Potato/corn/other chips	3.2	60.7
Pasta and pasta dishes	3.1	63.8
Salad dressing	2.6	66.4
Dairy desserts	2.3	68.7
Yeast breads	2.2	70.9

Current dietary recommendations advise reducing the intake of SFA to reduce CHD risk. Reductions in SFA intake to < 10% of energy require changes in the typical Western dietary pattern, including increases in CHOs, PUFAs or MUFAs (which would be substituted for SFA calories). The DGAC 2010 Report states that a 5% energy replacement of SFA with MUFA can decrease the risk of CVD and type 2 diabetes (13). Clinical evidence suggests that high-MUFA, low-SFA diets are equally as effective as low-fat, low-SFA, and high-CHO diets in decreasing

total and LDL-C, and that they have the added benefits of lowering TG and maintaining concentrations of HDL-C (21).

Other cardio-protective effects of high MUFA diets have been studied. Partial substitution of CHO with MUFA in DASH diet can further lower blood pressure and reduce estimated CVD risk (20). Adding MUFA to the Portfolio diet also achieved further reductions in the ratio of total to HDL cholesterol and reductions in C-reactive protein (58). Several studies have shown that MUFA-rich olive oil and sunflower oil favor increased LDL particle size (59, 60), although the results varied in some studies (61, 62). Cross sectional studies have demonstrated an association between reduced LDL particle size and consumption of a low-fat, high carbohydrate diet (63). Recently, RCT studies of the Mediterranean diet found small dense LDL (sdLDL) particles were decreased in patients with metabolic syndrome or at high risk of CVD after consumption of Mediterranean diet for 12 weeks, 5 weeks, or 1 year (64-66). However, it is unclear which components of the Mediterranean diet elicit the effect.

Consistent with the evidence of MUFA's effect in increasing LDL particle size, MUFA-rich diets have been shown to reduce susceptibility of LDL particles to oxidation compared to average American diet, a Step-1 diet, and a low-fat diet in healthy subjects (62, 67-69). Moreover, studies have found a higher resistance of LDL particles to oxidation after the consumption of a MUFA-rich diet compared with a PUFA-rich diet (59, 70-76).

Studies also have shown that oleic acid lowers cholesteryl ester transfer protein (CETP) concentration (77), and clinical and animal studies strongly support the therapeutic potential of CETP inhibition in increasing reverse cholesterol transport, a very important function of HDL (78-80). MUFA also plays an important role in glycemic control. SFA have been shown to impair insulin sensitivity (81) and replacing dietary SFA with MUFA has shown improvements in insulin sensitivity and glycemic response in individuals having insulin resistance (82-85). Recent study reported that MUFA has a direct action on pancreatic β-cell function and lower insulin

resistance in health men (85). As a replacement for dietary SFA, high MUFA diets have shown improvements in glycemic control, as well as lipoprotein profiles, as compared to high CHO diets for patients with diabetes (86-88). In contrast, clinical studies with healthy subjects did not show differences between high MUFA and high CHO diets on glucose and insulin homeostasis (89-91). However, due to unfavorable metabolic effects associated with high CHO diets, such as increasing TG and decreasing HDL-C, high MUFA diets are beneficial for lowering the risk of diabetes (88, 92). The recent PREDIMED study also showed that a high MUFA, Mediterranean diet improved glucose, insulin, and HOMA score; increased HDL-C and lowered LDL/TG/TC; lowered the prevalence of metabolic syndrome; and lowered the cumulative incidence of diabetes (93). A recent study also reported an enhancement in HDL cholesterol efflux capacity and HDL anti-oxidative status by olive oil. However, the study concluded that the polyphenols in raw olive oil contributed to the benefit (94). It is still unclear if MUFA can elicit the beneficial effects without the existence of bioactive compounds in nuts and olive oil.

In conclusion, both epidemiological and clinical studies have shown a cardio-protective role of MUFA on multiple CVD risk factors. However, there are still several potential benefits of MUFA that are unclear. Further studies are needed to investigate the mechanisms by which MUFA affects CVD and resolve the discrepant results. The Dietary Guidelines 2010 and the 2013 AHA/ACC Lifestyle Guideline all have issued specific food-based recommendations for the intake of dietary MUFA. Specifically, the cardio-protective effect of MUFA needs to be clarified for Western populations that have been on very different dietary patterns than the Mediterranean diet. In a Western diet, MUFA coexist with SFA in many foods, especially in animal and dairy products, while in Mediterranean diet the main source of MUFA is from olive oil and nuts. Specific food-based recommendations and more clinical studies on food sources of MUFA are needed.

The Cardio-protective Effects of Avocados: MUFA and Beyond

The nutrient profile of avocados

Avocados (*Persea americana*) originated in Mexico or Central and South America. The Hass variety represents the majority of avocados consumed in the United States. They are high in MUFA and other fat-soluble vitamins. Avocados have a unique combination of a heart healthy fat profile rich in unsaturated fat, low in saturated fat, and no cholesterol, and include numerous vitamins, minerals and phytochemicals. One Hass avocado (136g, without skin and the seed) contains about 13 g of oleic acid, which is similar to the amount of oleic acid in 1.5 oz. of almonds or 2 tablespoons of olive oil. Furthermore, avocados have a lower energy density than nuts and oils (**Table 2-2**). One whole avocado has a similar nutrient profile as 1.5 ounces of nuts, which has a Heart Heath Claim from the Food and Drug Administration (FDA) (**Table 2-3**) (47). Like almonds and pistachios, avocados are high in MUFA and low in PUFA, which differentiates them from walnuts (**Table 2-3**). Also, avocados have a similar amount of lutein as pistachios, and similar total phenolics content as almonds. Specifically, one avocado contains higher amounts of dietary fiber and potassium than 1.5 oz. almonds, pistachios or walnuts (**Table 2-3**). Being categorized a fruit but high in fatty acids, avocados are also a good source of dietary fat-soluble vitamins. One serving of avocado (1/2 cup) contains similar amount of β-carotene, α-tocopherol, and lutein compared with orange, strawberries, and blueberries (**Table 2-4**). One serving of mango (1/2 cup) contains more β-carotene and less lutein compared to 1 serving of avocado (**Table 2-4**). In contrast to the traditional assumption that avocado is a high fat, high calorie food, 1 serving of avocado contains similar calories as 1 serving of orange, strawberries, blueberries, and mango.

Table 2-2 Energy density of plant sources of MUFA - avocados, nuts and high-oleic acid oils.

Dietary MUFA Source (Plant)	Energy Density (kcal/g)
Avocados	1.67
Nuts/Peanuts	
Almonds	5.75
Pistachios	5.62
Walnuts	6.54
Peanuts	5.67
Macadamia	7.18
Oils	
Olive, canola, safflower, sunflower oils	8.84

Table 2-3 Major nutrient content of one Hass avocado (136g) compared to selected nuts (1.5 oz.).

Nutrient	Hass Avocado (1 fruit, 136g)	Almonds (1.5 oz.)	Pistachios (1.5 oz.)	Walnuts (1.5 oz.)
Calories (kcal)	230	240	240	285
Protein (g)	3	9	9	6
Total fat (g)	21	21	19.5	27
SFA (g)	3	1.5	2.3	2.3
MUFA (g)	13	13.5	10.5	4
PUFA (g)	2.5	4.5	6	19.5
Cholesterol	0	0	0	0
Dietary fiber (g)	9	6	4.5	3
Potassium (mg)	690	300	450	190
Alpha tocopherol (mg)	2.7	10.5	0.9	0.3
Gamma, tocopherol (mg)	0.4	0.3	9	9
Lutein + zeaxanthin (mcg)	369	0	510	4.5
Total phenolics (mg)	260	180	710	670

Table 2-4 Major carotenoids and tocopherols in one serving of Hass avocado and other common fruits.

	Hass Avocado (1/2 cup)	Orange (1 small)	Strawberries (1/2 cup)	Blueberries (1/2 cup)	Mango (1/2 cup)
Calories	50	45	30	40	50
Beta carotene (mcg)	19	68	6	24	528
Alpha tocopherol (mg)	0.6	0.2	0.2	0.4	0.7
Lutein + zeaxanthin (mcg)	81	124	22	59	19

Potential cardio-protective benefits of avocados: beyond MUFA

Besides having a heart-healthy dietary fatty acid profile, avocados also contain several other beneficial bioactive compounds, including fiber, phytosterols, antioxidants, vitamins, minerals, polyphenols (**Table 2-3**). Although the quantity of every nutrient in one avocado is lower than amounts studied in previous supplement trials or epidemiological studies, there might be potential cumulative and synergetic effects of all of the bioactive compounds in avocados that can elicit beneficial effects on multiple cardio-metabolic risk factors.

Potential additional LDL-C lowering effect: phytosterols and fiber

Avocados are a rich source of plant sterols. The phytosterols in avocados include β-sitosterol, campesterol, Δ-5-avenasterol, and Δ-7-avenasterol, the most abundant of which is β-sitosterol (103mg/per unit). Avocados are the richest known fruit source of β-sitosterol (95). A meta-analysis of RCTs has shown that intake of 1.5g/day to 2.5g/day plant sterols/stanols can

decrease LDL-C by 0.25 - 0.42mmol/L. In eight studies using <1.5g of phytosterols, the average LDL-C reduction was 0.25mmol/L (96).

Avocados are a source of plant fiber; 100g of avocado provide 2.11g soluble fiber and 2.7g insoluble fiber (97). One Hass avocado provides about 3 grams of soluble fiber. Jenkins et al. has shown that 5-10 grams of soluble fiber per day reduces LDL-C by approximately 5 percent (98). A soluble fiber intake of 10–25 g/d is recommended by the National Cholesterol Education Program's Adult Treatment Panel III as an additional diet option to decrease LDL-C (99).

Potential effect on glycemic control: seven-carbon sugar and soluble fiber

Avocados contain a unique seven-carbon (C7) sugar, D-mannoheptulose, and its reduced form perseitol. The level of D-mannoheptulose is about 30 mg/g in ripe Hass Avocados. Research on D-mannoheptulose found that it plays a role in regulating blood sugar by improving insulin sensitivity (100-103) and assisting with weight loss (104, 105).

Dietary fiber has been reported to improve the postprandial glycemic response by slowing the digestion and absorption of food, and by regulating several metabolic hormones (106). However, studies evaluating dietary fiber and diabetes in prospective and case-control studies have been mixed (107-109). It may be because dietary fiber is mostly provided by grains in the diets, and the results were influenced by the type of grains used in those studies and the unique nutrient content of different whole grain products. In contrast, a Mediterranean dietary pattern has been shown consistently to be effective in improving insulin sensitivity and is associated with a lower incidence of metabolic syndrome (110). Compared to low-fat, high whole grain diets, Mediterranean-style diets contain more foods that are rich in phytochemicals, antioxidants, and unsaturated fatty acids (e.g., oily fish, nuts, olive oil, red wine, fruits, vegetables, etc.) (111), besides providing whole grains that are rich in dietary fiber. The evidence

suggests it's important to include unsaturated fats in a high whole-grain and fiber-rich diet to improve insulin sensitivity.

Potential antioxidant effects: antioxidants, carotenoids, flavonoids, and polyphenols

Carotenoids are a widely distributed group of naturally occurring lipid-soluble pigments. They provide the red, orange, and yellow colors in fruits and vegetables. Six major carotenoids (lutein, lycopene, zeaxanthin, β-cryptoxanthin, β-carotene, and α-carotene) represent more than 95% of total carotenoids in human plasma (111). Avocados contain the major dietary carotenoids except lycopene (**Table 2-5, Appendix C**). Carotenoids are hypothesized to explain some of the protective effects of fruits and vegetables on risk of cardiovascular disease (112-114). Specifically, increased intake of lutein was protective against the development of atherosclerosis, which has been shown in epidemiologic studies, in vitro cell models, and animal models (115, 116). The suggested mechanism by which carotenoids reduce CVD is by protecting LDL particles from oxidation and by decreasing inflammatory cytokines (117). Also, it has been shown that lutein is primarily transported by HDL-C in the circulation suggesting that lutein may play an important role in HDL antioxidant and anti-inflammatory functions (118). Furthermore, the dietary fat content in avocados increases the bioavailability of carotenoids from other foods. Unlu et al. showed that 150g avocado enhanced the absorption of lycopene and β-carotene in salsa by 4.4 and 2.6-fold, and the absorption of α-carotene, β-carotene, and lutein in salad by 7.2, 15.3, and 5.1-fold, respectively (119).

Avocados are a source of flavonoids and phenolic antioxidants: flavonoids (cyanidin, epicatechin, and epigallocatechin) (120), Coenzyme Q10 (121), Persenone A and B (122). Dietary intake of flavonoids has been associated with reduced risk of CVD in large prospective studies (123, 124). Avocados also contain glutathione, a tripeptide composed of three amino

acids (glutamic acid, cysteine, and glycine) that functions as an antioxidant (125). A study found that avocado's glutathione levels of 8.4 mg per 30 g edible portion, which is higher than several other fruits (126). Avocados are also the highest fruit source of Coenzyme Q10, which can inhibit lipid peroxidation and inhibit the mitochondrial permeability in cardiomyocytes (127). It has been used as a therapeutic drug for CVD patients, especially before cardiac surgery, heart transplantation, and often is prescribed as a supplement for patients who are taking statins (128). Persenone A and B, which are present in avocados, have been shown to inhibit nitric oxide and superoxide generation in vitro (122). These bioactive compounds are summarized in **Table 2-5**. Compared to common fruits, avocados have a moderate antioxidant capacity (**Table 2-6**).

Potential anti-hypertensive effects: potassium

MUFA rich diets were associated with a slight reduction in blood pressure (especially systolic) compared to CHO-rich diets (129). The high potassium content of avocados may also contribute to its role in blood pressure control. A meta-analysis by Whelton et al. concluded that compared with control subjects, was overall pooled estimates of effect of 60 to100 mmol (2340 – 3900mg) daily potassium supplementation on systolic and diastolic blood pressure (BP) were -4.4 and -2.4mm Hg, respectively ($p<0.001$ for both) (130). One avocado contains 690 mg potassium and is one of the richest food sources of potassium. The average consumption of potassium in the U.S. is 3.5g/d, which is lower than the current recommendation of 4.7 g/day for the prevention and control of hypertension (13). Consumption of one avocado per day will help individuals meet potassium recommendation and, as such, improve vascular health and reduce risk of metabolic syndrome. Although avocados do not meet the criteria for a blood pressure lowering claim, the potential additive blood pressure lowering effects of MUFA and potassium would be expected to have an anti-hypertensive effect.

Table 2-5 Major nutrients and bioactive compounds in avocados (beyond fatty acids) with potential cardio-protective benefits.

Nutrients and bioactive compounds	Amount per fruit (136g, without skin and seed)
Viscous fiber	2.9g
β –sitosterol	103mg
Campesterol, stigmasterol, Δ-5-avenasterol, Δ-7-avenasterol	19mg
β -carotene	86mcg
α- carotene	33mcg
Lutein + zeaxanthin	369mcg
Vitamin C	12mg
α-tocopherol	2.68mg
Cyanidin (anthocyanidins)	0.45mg
(-)-Epicatechin	0.5 mg
(-)-Epigallocatechin 3-gallate	0.2 mg
D-mannoheptulose	4.1g
Coenzyme Q_{10}	1.4 mg
Glutathione	38.5mg
Folate	121 mcg
Potassium	690mg
Choline	19.3mg

Table 2-6 Antioxidant and phenolic content of avocados and common fruits.

Fruit	Total Antioxidant Activity (umol TE / g)	Total Antioxidant Activity (umol TE / fruit / Cup)	Total Phenolics (GAE / g)
Blueberries	62-93	9,000 - 13,00	5.3-8.0
Cranberries	95	9,000	7.1
Plums	62-73	41,000 - 48,000	3.7-4.8
Blackberry	53	7,700	6.6
Raspberry	49	6,100	5.0
Strawberries	36	5,900	3.7
Cherries, sweet	34	4,900	3.4
Apples	22-43	1,400-5,900	2.1 -3.5
Avocado, Hass	19	3,300	1.9
Orange, Navel	18	2,500	3.4
Red Grapefruit	15	1,900	2.1
Grapes	11-13	1,800 – 2,000	1.5-1.8
Peaches	19	1,800	1.6
Tangerines	16	1,400	1.9
Apricot	13	1,400	1.3
Mango	10	1,700	2.7
Kiwifruit	9.0	700	2.8
Bananas	9.0	1,000	2.3
Pineapples	8.0	1,200	1.7
Watermelons	1.5	200	0.6

Data are from Wu et al. (131) and adapted from Dreher et al. (46).

Clinical studies of avocados on blood lipids

The cholesterol-lowering effects of avocado were clinically evaluated decades ago. In 1960, Grant reported that the consumption of 0.5 to 1.5 avocados showed positive effects on serum total cholesterol in men (55). This study is not applicable to the general population due to several limitations including: different medical conditions of the subjects, weight-loss during the

feeding period, no control of the diet, and varied length of the feeding periods. Nevertheless, it was the first clinical study that provided preliminary results for further investigation of the cholesterol-lowering effects of avocados. From 1990's to the present, along with the recommendation for a moderate fat diet, several human studies of avocado consumption demonstrated the beneficial effects on blood lipids in a wide variety of diets. **Table 2-7** and **Table 2-8** summarized the design of six avocado consumption intervention trials with 70 men and 146 women with or without diabetes and/or hypercholesterolemia. The experimental diet comparisons are shown in **Table 2-8**.

Four studies compared an avocado enriched, high MUFA (AMF) diet with a low-fat, high complex CHO diet (50-53). Four studies including one weight loss study demonstrated a cholesterol and TG lowering effect of avocados when 300g/d of avocado was substituted for other fat sources (SFA and PUFA) in an isocaloric diet with similar proportions of CHO, protein and fat (i.e., the control diet) (48, 49, 54). Compared to the control diet, the AMF diets elicited a greater LDL-C lowering effect, less decrease in HDL-C and a reduction in TG. The effects are greater in hyperlipidemia subjects (**Table 2-9**, **Figure 2-1**, **and Figure 2-2**). It is of note that two studies reported greater cholesterol lowering effects of the avocado diet than predicted values based on the fatty acid profile in healthy subjects (49, 52), which suggests that there may be additional cholesterol lowering benefits beyond the effect of fatty acid content in avocados (**Figure 2-3**). Predicted values were calculated based on the equations of Mensink et al. (15, 132). In summary, using avocados to replace CHO or other fatty acids in diet can achieve a more heart healthy blood lipid/lipoprotein profile. Also, Carranza-Madrigal et al. proposed a strategy for reducing the high CHO content of a vegetarian diet by incorporating avocados (48).

The six articles also showed several discrepancies due to different diet design, amount of avocado, length of the feeding period, and subjects studied. Alvizouri-Munoz et al. reported no changes in HDL-C and minor hypocholesterolemic effects of avocados, which may due to the

younger age group (18-37 year old) of the study participants (53). An AMF diet also was less effective than the low-fat diet in lowering plasma cholesterol in dyslipidemia II patients in one study (50). In a weight-loss study using 200 g/d of avocados substituted for other fats did not demonstrate a significant difference in cholesterol lowering between the AMF diet and the control diet (54). The result might be influenced by significant weight loss during the study. Further studies are needed to evaluate avocados' cholesterol lowering effect with or without weight loss. Moreover, although previous studies suggested potential additional cholesterol-lowering effects of avocados, well-controlled studies are needed to investigate the potential benefits of avocados beyond MUFA.

Table 2-7 Clinical studies of avocados and blood lipids in healthy subjects and subjects with different clinical conditions.

Study	Subject				Experimental Diet	Study Design	Avocado Amount	Length
	n	Gender	Age	Condition				
(K) Grant et al. (1960)	16	Male	22-72	MI, diabetes, etc	partial substitution of avocado fat for dietary fat	RCT, fixed sequence crossover	0.5 to 1.5 avocados/d	12-87d
(A) Colquhoun et al. (1992)	15	Female	37-58	Healthy	AMF diet vs. LF diet	RCT, crossover	0.5 to 1.5 avocados/d	3 wk
(B) Alvizouri-Munoz et al. (1992)	16	Male 9 Female 7	18-37	Healthy	AMF diet vs. LF diet	RCT, crossover	1-2 avocados/d, contribute 75% dietary fat	4 wk
(H) Lerman-Garber et al. (1994)	12	Female	56	NIDDM	AMF diet vs. LF diet	RCT, crossover	1 avocado/d	4 wk
(D) Carranza-Madrigal et al. (1995) a	8	Male 2 Female 14	58	Dyslipidemia II	AMF diet vs. LF diet	RCT, crossover	1-2 avocados/d, contribute 75% dietary fat	4 wk
(E) Carranza-Madrigal et al. (1995) b	8			Dyslipidemia IV	AMF diet vs. LF diet	RCT, crossover	1-2 avocados/d, contribute 75% dietary fat	4 wk
(F) Carranza-Madrigal et al. (1997)	13	Male 2 Female 11	42-72	Dyslipidemia II	high CHO, vegetarian diet with or without avocado	RCT, crossover	1-2 avocados/d, contribute 75% dietary fat	4 wk
(C) Lopez et al. (1996) a	30	Male 16 Female 14	18-30	Healthy	partial substitution of avocado fat for dietary fat	RCT, parallel	300g/d	1 wk
(G) Lopez et al. (1996) b	22	Male 12 Female 25	35-65	Hyperlipidemia	partial substitution of avocado fat for dietary fat	RCT, parallel	300g/d	1 wk
(I) Lopez et al. (1996) c	15			NIDDM	partial substitution of avocado fat for dietary fat	RCT, parallel	300g/d	1 wk
(J) Pieterse et al. (2005)	61	Male 13 Female 48	21-57	Obese	partial substitution of avocado fat for dietary fat in energy-restricted diet	RCT, parallel	200g/d	6 wk

Table 2-8 Macronutrient components of experimental diets in clinical studies on avocados and blood lipids.

Study	Treatment	% energy CHO		% energy Pro		% energy Fat		% energy from FA (AMF)			% energy from FA (Control)		
		AMF	control	AMF	control	AMF	control	SFA	MUFA	PUFA	SFA	MUFA	PUFA
Colquhoun et al. (1992)	AMF diet vs. LF diet	**42**	**53**	17	21	**37**	**21**	11	**20**	6	7	**8**	6
Alvizouri-Munoz et al. (1992)	AMF diet vs. LF diet	**50**	**60**	20	20	**30**	**20**	N/A	N/A	N/A	N/A	N/A	N/A
Lerman-Garber et al. (1994)	AMF diet vs. LF diet	**40**	**60**	20	20	**40**	**20**	11	**24**	5	6.6	**6.6**	6.6
Carranza-Madrigal et al. (1995)	AMF diet vs. LF diet	**50**	**60**	20	20	**30**	**20**	N/A	N/A	N/A	N/A	N/A	N/A
Carranza-Madrigal et al. (1997)	high-carb, vegetarian diet with or without avocado	**60**	**70**	10	10	**30**	**20**	N/A	N/A	N/A	N/A	N/A	N/A
Lopez et al. (1996)	partial substitution of avocado fat for dietary fat	33	33	14	15	53	52	18	**22**	12	21	**15**	15
Pieterse et al. (2005)	partial substitution of avocado fat for dietary fat	55	55	15	15	30	30	N/A	N/A	N/A	N/A	N/A	N/A

Table 2-9 Comparison of predicted versus observed changes in lipids and lipoproteins in response to an avocado diet.

Study	Experimental Diet	AMF vs. Control							
		Δ TC (mmol/L)		Δ LDL-C(mmol/L)		ΔHDL-C(mmol/L)		ΔTG(mmol/L)	
		Reported	**Predicted**	**Reported**	**Predicted**	**Reported**	**Predicted**	**Reported**	**Predicted**
Colquhoun et al. (1992)	AMF diet vs. LF diet	**-0.2**	**0.07**	**-0.25**	**0.02**	0.19	0.14	**-0.01**	-0.31
Alvizouri-Munoz et al. (1992)	AMF diet vs. LF diet	NS	N/A	NS	N/A	0.17	N/A	**NS**	N/A
Lerman-Garber et al. (1994)	AMF diet vs. LF diet	-0.16	NS	NS	NS	NS	<0.01	**-0.33**	NS
Carranza-Madrigal et al. (1995) a-dislipidemia II patients	AMF diet vs. LF diet	0.28	N/A	0.13	N/A	0.13	N/A	**-0.18**	N/A
Carranza-Madrigal et al. (1995) b-dislipidemia IV patients	AMF diet vs. LF diet	-0.17	N/A	NS	N/A	NS	N/A	**-1.16**	N/A
Carranza-Madrigal et al. (1997)	high-carb, vegetarian diet with or without avocado	NS	N/A	-0.26	N/A	0.16	N/A	**-0.23**	N/A
Lopez LR et al. (1996)a-healthy subjects	partial substitution of avocado fat for dietary fat	**-1.32**	**-0.1**	**-0.85**	**-0.11**	NS	NS	**-0.02**	NS
Lopez LR et al. (1996)b-non-diabetic hypercholesterolamic subjects	partial substitution of avocado fat for dietary fat	**-1.39**	**-0.1**	**1.04**	**-0.11**	NS	NS	**-0.46**	NS
Pieterse, Z et al. (2005)	partial substitution of avocado fat for dietary fat in energy-restricted diet	NS	N/A	NS	N/A	NS	N/A	**NS**	N/A

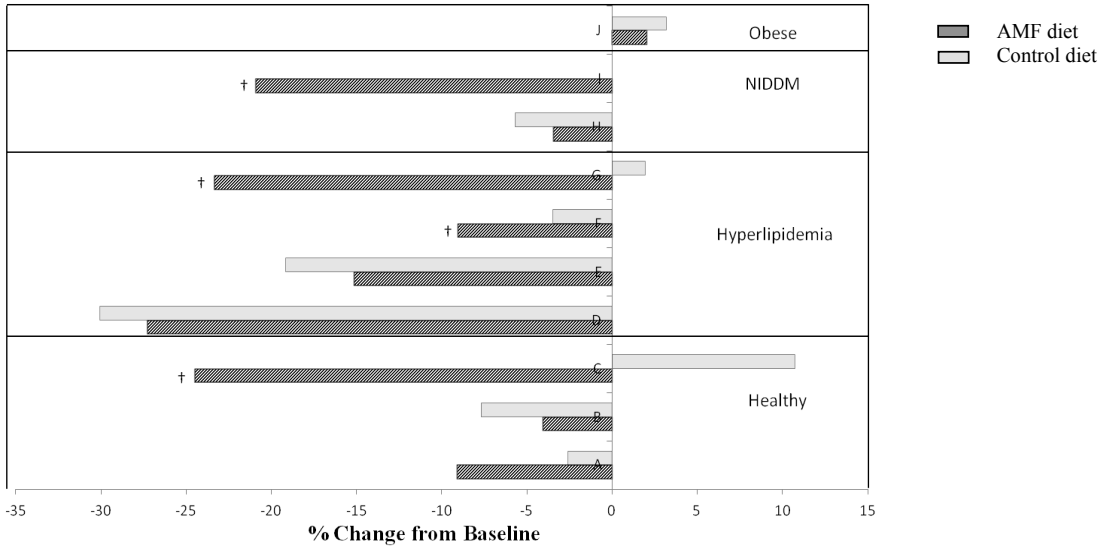


Figure 2-1 Percent change in LDL-C from baseline in avocado diets and control diets in 10 studies.

Each letter represents a study that was described in Table 2-7. † Represents the change in LDL-C by the AMF diet was significantly different from the control diet ($p<0.05$).

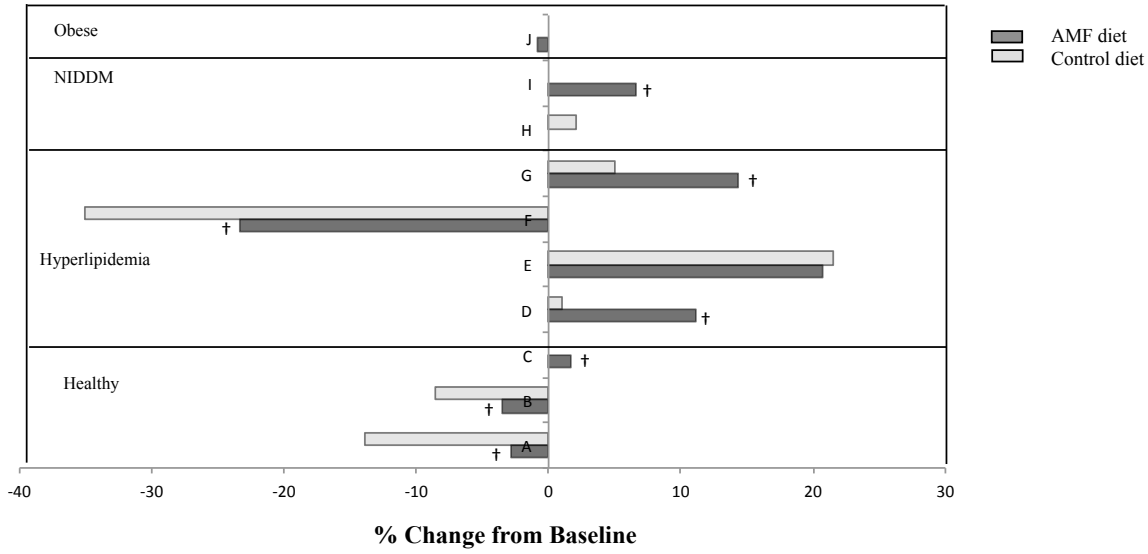


Figure 2-2 Percent change in HDL-C from baseline in avocado diets and control diets in 10 studies.

Each letter represents a study that was described in Table 2-7. † Represents the change in HDL-C by the AMF diet was significantly different from the control diet ($p<0.05$).

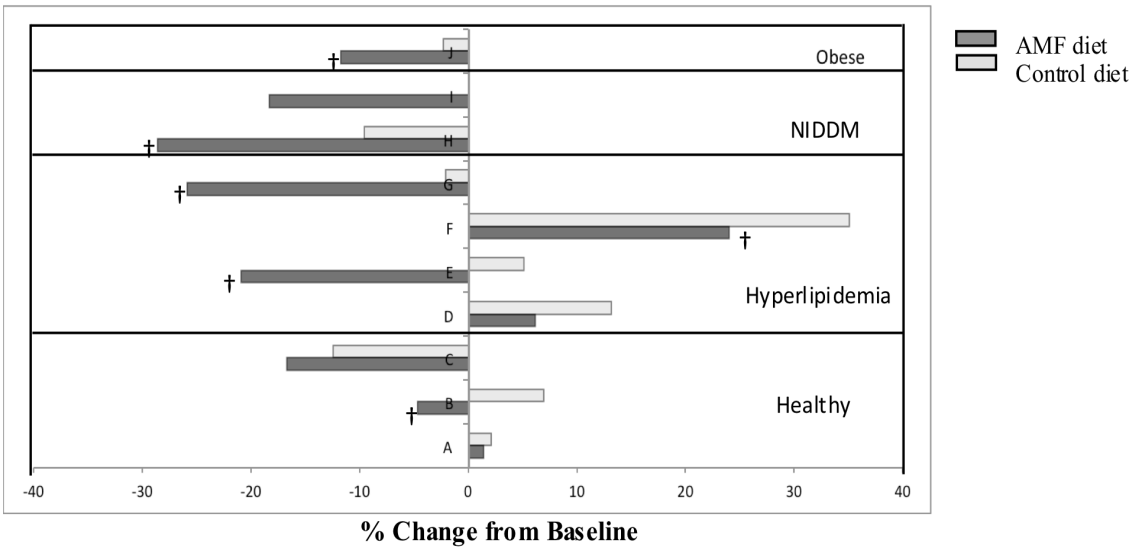


Figure 2-3 Percent change in TG from baseline in avocado diets and control diets in 10 studies.

Each letter represents a study that was described in Table 2-7. † Represents the change in TG by the AMF diet was significantly different from the control diet ($p<0.05$).

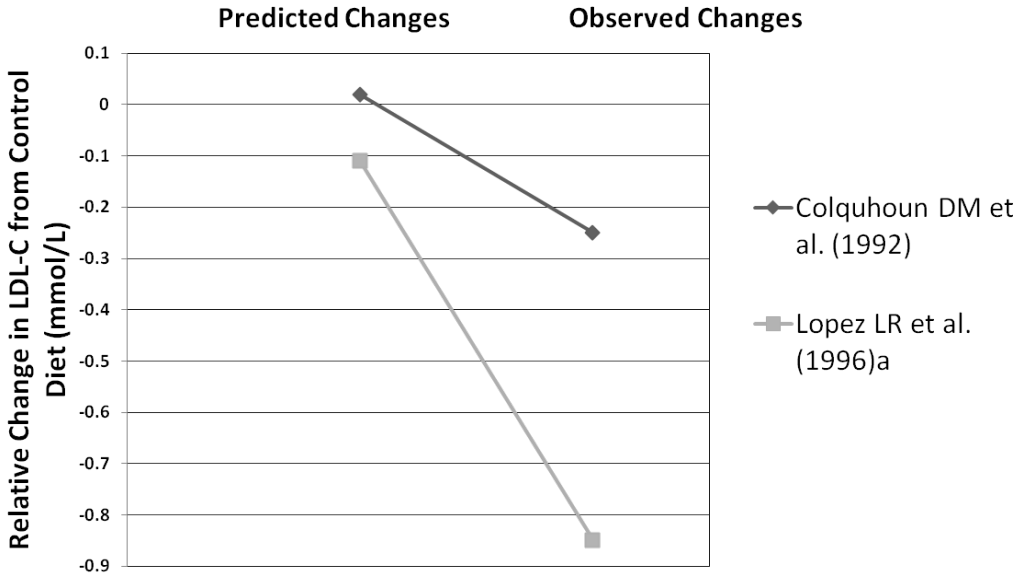


Figure 2-4 Predicted vs. observed changes in LDL-C in two studies using avocados that were substituted isocalorically for other dietary fats in healthy subjects.

Clinical studies of avocados on other cardio-metabolic biomarkers

A study using NHANES 2001-2008 data showed that avocado consumers had significantly higher intakes of vegetables, fruit, diet quality, total fat, monounsaturated and polyunsaturated fats, dietary fiber, vitamins E, magnesium, potassium, and vitamin K, and lower intakes of added sugars compared to avocado non-consumers (133). The odds ratio for metabolic syndrome was 50% lower in avocado consumers vs. non-consumers (133). However, only 347 avocado consumers were identified in 17,567 US adults in this study, the comparison between avocado consumers and non-consumers was not powered and balanced appropriately. This study revealed that frequent consumption of avocados is not common in the United States, based on this study with a relatively small sample size. Adding avocados in the diet could increase the nutritional quality of the diet, not only because avocados are a rich source of many nutrients, but also because avocados can increase the absorption of fat-soluble vitamins, such as carotenoids and lycopenes. Previous studies have demonstrated that avocado consumption can increase the bioavailability of carotenoids from diet (119). Recently, Kopec et al. reported that consumption of one Hass avocado with tomatoes significantly enhanced the efficiency of carotenoid conversion to vitamin A (as measured by retinyl esters) by 4.6 to 12.6-fold (119).

Although frequent consumption of avocado in the United States is not common and clinical studies are lacking, isolated bioactive compounds from avocados have been used for treating diabetes and osteoarthritis. One of the bioactive compounds is the seven-carbon (C7) sugar, D-mannoheptulose, which is used for treating diabetes, due to its effect on β-cell function (95). Another one is avocado/soybean unsaponi (ASU), which contains phytosterol, vitamins and polyphenols from avocado extraction, has been used for the treatment of osteoarthritis due to its potent anti-inflammatory functions (134, 135). However, the anti-oxidant, anti-inflammatory,

and glycemic effects of avocados have not been studied. Although a single dose most likely is not enough, chronic consumption may be of benefit.

Two postprandial studies evaluated effects of avocado consumption on satiety and inflammatory responses using a similar design: adding approximately one-half of an avocado to a meal without matching the nutrient content of the test and control meals. Wien et al. reported that the addition of approximately one-half of a Hass avocado at lunch influenced post-ingestive satiety over a subsequent 3 and 5-hour period in overweight adults (136). However, one-half of an avocado provided an additional 112 kcal to the meal, which may have accounted for the observed increase in satisfaction. Also, no effect on post meal blood glucose or insulin was observed (136). Another study reported reduced activation of the NFκB inflammation pathway in eleven healthy men after consumption of a hamburger meal with the addition of 68g avocado compared to the hamburger meal without any avocado (137). In this study, Li et al. reported a significant vasoconstriction by standard peripheral arterial tonometry (PAT) 2 hours following ingestion of the hamburger, which did not occur when the avocado was consumed with the hamburger (137). IκBα in peripheral blood mononuclear cells (PBMC) was increased at 3 hours (131% *vs.* 58%, $p = 0.03$) when an avocado was consumed with the meat compared to the meat alone, consistent with reduced activation of the NF-kappa B (NFκB) inflammatory pathway. Interleukin -6 (IL-6) increased significantly at 4 hours in the postprandial serum after consumption of the hamburger, but no change was observed when avocado was added (137). However, due to the small sample size and large variance of observed inflammatory markers, this study needs further investigation relative to the anti-inflammatory effects of avocados. There is also an animal study that has reported 5g of avocado in 17.5g chow fed for 5 weeks increased HDL-C associated enzyme, paraoxonase 1(PON1) activity by 33% compared to the control. These studies provide a rationale for larger, longer-term clinical studies to evaluate the effects of avocado consumption on insulin sensitivity and inflammatory responses.

In summary, both epidemiological and clinical studies have shown MUFA's cardioprotective role on multiple CVD risk factors, especially in the context of a Mediterranean diet enriched with nuts and extra virgin olive oil. Although in vitro studies suggested MUFA might affect cardio-metabolic risk factors independently as a functional fatty acid, it's still unclear if MUFA can elicit the beneficial effects without the existence of bioactive compounds in nuts and olive oil.

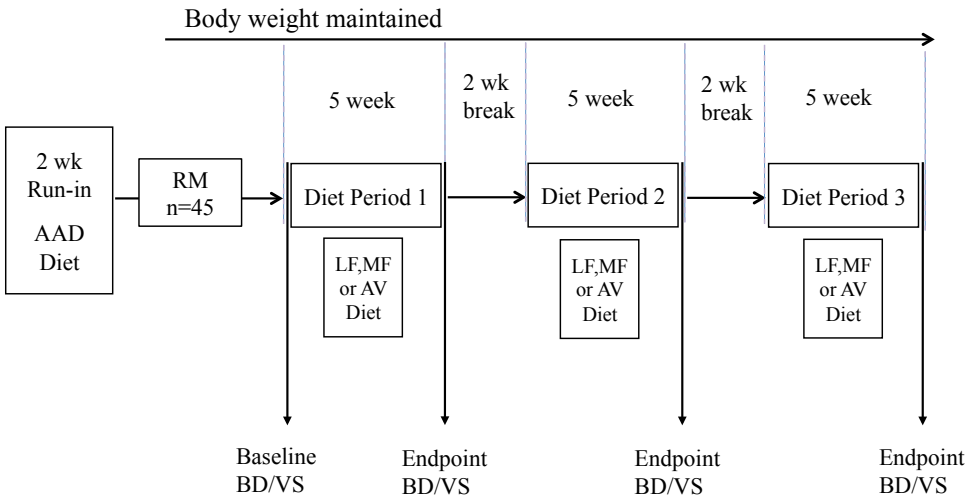
Chapter 3

The AVOCADO Study Protocol: a Randomized, Controlled Clinical Trial

Study Design

A randomized, 3-period crossover study design was implemented. A two-week "run-in" average American diet (AAD) was fed to participants before they were randomly assigned to a treatment sequence of three diet periods (5 week each) with 2-3 week compliance breaks between diet periods. Participants were assigned to random treatment sequences that were generated by balanced permutations. The study design is presented in **Figure 3-1**.

Figure 3-1 Study Design: randomized, crossover controlled feeding.



RM: randomization BD/VS: clinical visit – blood draw and vital signs

We designed three cholesterol-lowering diets: a lower-fat diet (LF:24% fat, 7% SFA, 11% MUFA, 6% PUFA, 59% CHO, 16-17% Pro); and two moderate fat diets matched for macronutrients and fatty acids (34% fat, 6% SFA, 17% MUFA, 9% PUFA, 49% CHO, 16-17% Pro): the avocado diet (AV) included one fresh Hass avocado (136 g) per day, and the moderate

fat diet provided mostly the same foods (as the AV diet) and used high oleic acid oils and low fat dairy products to match the same fatty acid profile as in one Avocado. The MUFA content in AV and MF diets are mainly (>95%) oleic acid (18:1). The LF diet was designed by replacing 6 to 7% of energy from SFA with CHO (from grains that were incorporated in the diet in place of SFA) in the AAD. The nutrient profiles (based on 2100 calories/day) of the experimental diets are presented in **Table 3-1**. For subjects who required more or less than 2100 calories/day, the nutrient composition of the experimental diets was adjusted with fat and fiber containing foods (oils and higher fiber foods) as needed.

AAD diet: The nutrient profiles of the current average American diet are: CHO 51%, protein16%, total fat 34%, SFA12%, MUFA 13%, PUFA7%, cholesterol 336 mg/day (Data source: NHANES, 2005-2006).

Lower-Fat diet (LF): The Lower-Fat diet provided ~24% of calories from fat and met the SFA and cholesterol recommendations of a Step-II diet recommended by the National Heart, Lung, and Blood Association's National Cholesterol Education Program. SFA provided 7% of calories, and cholesterol was < 200 mg/day. The LF diet was designed by replacing 6 to 7% of energy from SFA with CHO (from grains that were incorporated in the diet in place of SFA) in the AAD.

Avocado diet (AV, moderate fat diet with avocado): The AV diet was designed to ensure that all subjects incorporated 1 avocado (~136 g) per day into a moderate fat diet. The AV diet was designed by replacing 6 to 7% of energy from SFA with MUFA and PUFA using one Hass avocado (~136 g fruit pulp, ~13 g MUFA) per day.

Moderate-Fat diet (MF, without avocado): This diet was designed to be the control diet for the AV diet and had an identical fatty acid profile. The MF diet was designed by replacing 6 to 7% of energy from SFA with MUFA using other food sources. To match the macronutrients and fatty acids in the MF and AV diets, and to adjust for the different calorie levels, high oleic

acid oils, low fat cheese, and nuts were used in both diets. About 90% foods in the two diets were identical. Thus, the major difference between the nutrient profiles of the AV and MF diets were the bioactive compounds from one avocado.

Table 3-1 Nutrient profile and food group servings of the study diets.

Nutrient[1]	**AAD**	**LF**	**MF**	**AV**
Total fat*	34	24	34	34
SFA*	13	7	6	6
MUFA*	12	11	17	17
PUFA*	7	6	9	9
Carbohydrate*	51	~ 59	~ 49	~ 49
Protein*	16	16~17	16~17	16~17
Fiber**	17	25	26	35
Cholesterol***	336	<200	<200	<200
Food Groups (servings/day)	**AAD**	**LF**	**MF**	**AV**
Fruits (cups)	0.9	2.3	2.4	3.3
Vegetables (cups)	0.8	1.4	1.7	1.7
Grains (oz.)	7.2	7.6	5.5	5.3
Whole Grains (oz.)	0.5	4.8	2.6	2.6
Meat (oz.)	2.1	1.6	1.6	1.6
Poultry and eggs (oz.)	2.0	2.8	2.8	2.8
Legume, soy, nuts, and peanut butter (oz.)	1.2	0.6	1.4	1.4
Low-fat dairy products (cups)	1.2	2.3	2.3	2.0
Fats and oils (oz.)	0.6	0.33	0.83	0.53

[1]Based on 2100 kcals/day; *% of total calories, ** grams/day, ***mg/day.

Menus (six-day rotating) were developed using the Food Processor SQL software for six calorie levels (1800 to 3600 kcals) in order to meet each participant's energy requirement based on their body weight and level of physical activity. One sample menu is shown in **Table 3-2.** All six-day menus are shown in **Appendix A**. All three diets met current food-based dietary

recommendations except for dairy products in lower calorie levels (1600 to 2100 kcals), which provided approximately two servings per day (**Table 3-1**).

Table 3-2 Sample of one-day LF, MF, and AV diet menus.

	LF	MF	AV
Breakfast	Cereal, farina	Cereal, oatmeal	Cereal, oatmeal
	Blueberries	Blueberries	Blueberries
	Brown sugar	Almonds (sliced)	Almonds (sliced)
	Skim milk	Skim milk	Skim milk
	Orange juice	Orange Juice	Orange Juice
	Low fat yogurt	Nonfat yogurt	Nonfat yogurt
Lunch	Chicken salad	Chicken salad	Chicken salad with half avocado
	(roasted chicken breast, celery, dried cranberries, red bell pepper, onion, canola oil mayonnaise dressing)	(roasted chicken breast, celery, dried cranberries, red bell pepper, onion, canola oil mayonnaise dressing)	(roasted chicken breast, celery, dried cranberries, red bell peppers, onion, fat free mayonnaise dressing, half fresh avocado)
	Peaches (slices)	Crackers	Crackers
	Whole wheat bread	Pears	Pears
Dinner	Pretzels	Carrots	Carrots
	Cereal bar	Cereal bar	Cereal bar
	Turkey taco	Turkey taco	Turkey Taco with half avocado
	(Turkey patty, tortilla chips, taco seasoning, yellow sweet corn, cheddar cheese, unsalted butter, high oleic sunflower oil)	(Turkey patty, tortilla chips, to taco seasoning, yellow sweet corn, cheddar cheese, unsalted butter, high oleic sunflower oil)	(Turkey patty, tortilla chips, taco seasoning, yellow sweet corn, cheddar cheese, unsalted butter, half fresh avocado)
Snack	Apple	Apple	Apple
	English muffin	English muffin	English muffin
	Margarine	Margarine	Margarine
		Jelly	

Subjects and Recruitment

Healthy, overweight men and women (21-70 years old, BMI 25-35 kg/m2) with LDL-C in the 25-90th percentile (NHANES; 105-194 mg/dL for males; 98-190 mg/dL for females) and normal blood pressure (≤140/90 mmHg) or well controlled by blood pressure lowering medication were recruited. All participants were nonsmokers and free of established CVD, stroke, diabetes mellitus, liver disease, kidney disease, inflammatory diseases, thyroid disease (the latter could be controlled by medication), or any other diagnosed diseases that may affect study endpoints. Additional exclusion criteria included: 1) BP ≥140/90 mmHg, BP lowering medications were acceptable if blood pressure was controlled (i.e. ≤140/90 mmHg); 2) A history of myocardial infarction, stroke, diabetes mellitus, liver disease, kidney disease, and thyroid disease (unless controlled on medication); 3) Lactation, pregnancy, or desire to become pregnant during the study; 4) Cholesterol-lowering medication use; 5) Intake of putative cholesterol-lowering supplements (psyllium, fish oil capsules, soy lecithin, niacin, fiber, flax, and phytoestrogens, stanol/sterol supplemented foods); 6) Vegetarianism; 7) Nut allergies (Other food allergies were reviewed on a case-by-case basis); 8) Refusal to discontinue nutritional supplements, herbs or vitamins; and 9) Change of body weight >10% within the 6 months. Volunteers were recruited via the advertisement by State College local newspaper, magazine, flyers, and list-serves on campus. Graduate students or research assistants contacted the volunteers initially with a phone screening. During the phone screening, individuals who did not meet the inclusion criteria (age, BMI, non-smoking status, healthy status, stable body weight, no-food allergies, non-vegetarian, and time commitment) were excluded. In addition, individuals with an eating disorder or vigorous physical activity (>10 hours sports per week), or excessive

alcohol consumption (>10 drinks per week) were excluded as well during the phone screening. After the phone screening, initial qualified participants were scheduled for a clinical screening. Every participant was contacted by study coordinator, review and signed the informed consent. During the screening visit, medical history, diet satisfaction, eating habits, height, weight, vital signs, blood pressure, and fasting blood samples were collected. A CHEM-SCREEN 24 and lipid profile test by Quest Diagnostics were performed; after confirming that all of the biomarkers met the criteria, the participant would be considered qualified and was recruited.

Diet Intervention and Sample Collection

After recruitment, participants were contacted by a study coordinator and the kitchen manager to review menus, discuss food pick up time, schedule each diet period and break period according to the participant's personal schedule. Also, recipes and guidance were given to advise the participant about how to mix certain food ingredients, use weight scales, report weight, complete compliance forms, and report illness or medication use. We used the Harris-Benedict equation(138) (HBE) with a physical activity factor to estimate an individual's basal metabolic rate (BMR) and daily kilocalorie requirements. However, the HBE equation is just an estimate of energy needs based on population data and may not be suitable for each study participant. Thus, we utilized the first two week run-in diet period to further adjust the calorie level until it met the need for each participant to maintain body weight.

During each diet period, all meals (foods and drinks) were provided; participants were required to consume only the foods provided. The run-in diet period allowed participants who discovered they could not comply with the protocol to drop out prior to randomization in order to improve retention during the experimental diets and helped participants achieve a baseline value of the measurements while on a representative, average American diet. Participants came to the

Metabolic Diet Study Center daily during the week to pick up their packed meals for that day; weekend meals were packaged for takeout and picked up on Friday afternoons. Diet compliance, physical activity levels, and medication use were monitored by reviewing daily and weekly monitoring forms. Participants were weighed daily (Monday through Friday) to assess diet compliance and ensure that body weight was maintained. On some holidays (i.e., Thanksgiving, Christmas), subjects were given one free meal with instructions to keep the nutrient components of the meal similar to the planned menu. During compliance breaks, subjects were required to maintain their habitual diet and physical activity practices. Unit foods (muffins, 100 kcal each) that are compositionally identical to the experimental diets were used to adjust calorie levels so that subjects maintained body weight. During the study, caffeine consumption was limited to five (8oz), caffeine-containing beverages/day and alcohol consumption was limited to 2 drinks/week.

At the end of each diet period, after a 12-h overnight fast, subjects came to the Clinical Research Center (CRC) and blood samples were drawn on two consecutive days. If any emergency issues happened during the last week of the diet period, e.g., illness, accidentally alcohol drinking, family emergency or unexpected travel, the endpoint visits were conducted 1-2 days earlier or postponed up to one week. Before the endpoint visit, participants were required to fasting and avoid vigorous physical activity for 12 hours, and refrain from alcohol consumption for 48 hours. During the visit, vital signs were measured and blood samples were collected via veinipuncture. Whole blood samples were centrifuged at 3,200 rpm for 15 min at 4°C, serum and EDTA plasma was separated and aliquoted, and stored at -80°C until the end of the study when all the samples were analyzed at the same time.

Power Calculation

This study was designed to evaluate the effect of a high MUFA diet with one avocado on lipids and lipoproteins, and the control diet was the lower fat diet. Since there have been no studies that evaluated avocado's benefits beyond its MUFA content, we designed a MF diet to match the fatty acid and macronutrient content of the AV diet in order to explore its additional benefits. Consequently, we did not have previous data to power the secondary (exploratory) outcomes – comparison between the MF and the AV diets. Thus, we designed the comparison between AV diet and LF diet as the primary outcome and the comparison between the AV diet and the MF diet as secondary outcome. The sample size was determined by the primary endpoint (LDL-C).

There were two previous studies that had a similar diet design (avocado enriched moderate fat diet versus a low fat diet) and with a similar participant health status (52, 53). However, the two trials were not well controlled, having a small sample size, and their standard deviations (SDs) of the LDL-C change were very different. Power calculations based on these two trials generated either a very large or very small sample size compared to typical controlled feeding studies. Since one avocado has a similar macronutrient content compared to the amount of pistachios used in a pistachio study (139), and the low fat diet and the moderate fat diet including pistachios had similar differences in macronutrients compared to our diet design, we used data from that study to estimate the sample size. In that study, LDL-C of the low fat diet was 3.42±0.11mmol/L (mean ± SEM), and LDL-C on the pistachio diet was 3.08 ± 0.1mmol/L, n=28. The SD of the difference was calculated as $\sqrt{\sigma 1 + \sigma 2}, \sigma = SD^2$. Using the SAS procedure PROCPOWER with PAIREDMEANS statement, a final sample size of 40 was needed to provide 90% power to detect a 10% decrease in LDL-C by the AV diet versus the LF diet with a 2-tailed $\alpha = 0.05$. Considering a 10-15% drop rate, we needed to recruit at least 45 participants.

The Institutional Review Board at The Pennsylvania State University approved the experimental protocol, and all participants signed a written informed consent. The Clinical Trial Registration is: URL: http://www.clinicaltrials.gov. Unique identifier: NCT01235832.

Chapter 4

Effects of MUFA Enriched Moderate Fat Diet with or without One Avocado per Day on Blood Lipids and Lipoproteins, Glycemic Control, and Blood Pressure in Overweight and Obese Adults

Abstract

The current study evaluated the effects of a MUFA enriched, moderate fat diet with or without one avocado per day versus a lower fat, high CHO diet on established CVD risk factors, including blood lipids and lipoproteins, glycemic control, blood pressure, and hsCRP in all subjects from the AVOCADO study. A randomized, crossover, controlled feeding trial was conducted with 45 overweight or obese participants with baseline LDL-C in the 25th to 90th percentile. Three cholesterol-lowering diets (6% to 7% SFA) were fed (5 weeks each): a lower-fat diet (LF: 24% fat); 2 moderate-fat diets (34% fat) provided similar foods and were matched for macronutrients and fatty acids: the avocado diet (AV) included one fresh Hass avocado (136 g) per day, and the moderate-fat diet (MF) mainly used high oleic acid oils to match the fatty acid content of one avocado. Compared to baseline, all three diets decreased total cholesterol (TC) and LDL-C ($p<0.001$ for all), however, the reduction on the AV diet (-16.7 and -13.4 mg/dL) was greater ($p<0.05$) than the MF diet (-10.9 and -8.6 mg/dL) and the LF diet (-8.4 and -7.3 mg/dL). Also, the reduction in non-HDL-C on the AV diet (-14.8 mg/dL) was significantly greater ($p=0.01$) than the MF diet (-8.9 mg/dL). The AV diet also significantly decreased TC/HDL-C and LDL/HDL-C (by -6.2% and -6.6% from baseline, $p<0.001$) whereas the MF and LF diets did not. The LF diet significantly increased TG (20.8mg/dL, $p<0.0001$) and very low-density lipoprotein cholesterol (VLDL-C, 2.6mg/dL, $p=0.0003$), while the AV and MF diets did not. The LF diet also decreased HDL-C greater ($p=0.03$ and 0.04) than the AV and MF diets. In conclusion, a high MUFA, moderate fat diet has benefits on lipids and lipoproteins compared to a

lower fat, high CHO diet. Furthermore, inclusion of one Hass avocado per day as part of a healthy moderate fat diet has beneficial effects beyond its MUFA content on the lipid/lipoprotein profile, especially an additional LDL-C lowering effect.

Introduction

The objective of this study was to evaluate the effects of a MUFA enriched, moderate fat diet with or without one avocado per day on established CVD and metabolic syndrome risk factors. LDL-C is the primary outcome of this study. Numerous mechanistic, animal, and large clinical studies have demonstrated that lowering LDL-C reduces CVD events and mortality (140), and the 2013 AHA/ACA Guideline on the Treatment of Blood Cholesterol to Reduce Atherosclerotic Cardiovascular Disease Risk in Adults continues to emphasize individualized drug therapy for controlling LDL-C in primary prevention of CVD (141). It recommends that individuals without clinical ASCVD or diabetes who are 40 to 75 years of age with LDL-C of 70 to 189 mg/dL and have an estimated 10-year ASCVD risk of 7.5% or higher will be benefit from statin therapy. Also, for adults with primary elevations of LDL-C ≥ 190 mg/dL. Statin therapy is recommended. At the same time, lifestyle modification, especially dietary intervention, can play a major role in CVD prevention and treatment through effects on modifiable risk factors. The dietary approach to lower LDL-C has focused on limiting intake of SFA and trans fat. Multiple clinical trials and epidemiological data have demonstrated that dietary SFA raise LDL-C level by decreasing LDL receptor activity, protein, and mRNA abundance, while unsaturated fatty acids increase these variables (142). Intake of *trans* fatty acids also induces an increase in cholesterol synthesis and LDL-C. In 2013, the FDA made a tentative determination about the ban of any use

of partially hydrogenated oils that contain *trans* fatty acids (143). Cholesterol-raising SFA is still high in western diets and exceeds current recommendations. The 2010 Dietary Guidelines (13) recommend healthy diet patterns that contain less than 10% from saturated fat (SFA) for lowering LDL-C, and for further reductions in LDL-C, less than 7% is recommended (14). The 2013 AHA/ACC Guideline on Lifestyle Management to Reduce Cardiovascular Risk also recommends a healthy dietary pattern that provides 5 to 6% of calories from SFA to lower LDL-C. Compared to the current macronutrient intake status in US adults (**Table 1-2**), we need to further decrease SFA intake by 4% of total energy. Since MUFA intake is still below the recommended intake range, and CHO intake could be adjusted in a wide range (**Table 1-2**), using MUFA or CHO to replace SFA are both options to achieve a healthier diet.

It also has been established that HDL-C is inversely associated with CVD risk and functions as a protective factor (141). HDL-C is included in the assessment criteria for estimating the residual risk of first CVD events after potent statin treatment. Medication and dietary interventions are recommended to lower LDL-C, and increase HDL-C (141). Hence, dietary MUFA has superior effects on lipids versus CHO because a high CHO diet decreases HDL-C, but MUFA maintains HDL-C. Furthermore, high CHO diets (especially refined grains and added sugar) may increase TG enriched apoB lipoproteins and decrease HDL-C, which are associated with increased risk of metabolic syndrome. Metabolic syndrome is related to several cardiovascular risk factors such as insulin resistance, dyslipidemia, hypertension, nonalcoholic fatty liver disease, and prothrombotic and proinflammatory states (144). The combined criteria of metabolic syndrome from medical community are any three of the following: 1) elevated waist circumference (according to population and country-specific definitions); 2) TG ≥ 150 mg/dl; 3) HDL-cholesterol < 40 mg/dl in men and < 50 mg/dl in women; 4) BP 130/85 mmHg or greater; 5) fasting glucose 100 mg/dl or greater (8). It has been suggested that a low-fat, high CHO diet is

associated with the prevalence of metabolic syndrome in different populaitons. However, whole-grain intake, largely attributed to the cereal fiber, is inversely associated with insulin resistance and a lower prevalence of the metabolic syndrome (145). The 2010 Dietary Guidelines recommend 5-8 ounce equivalents of grain product intake in the healthy MyPlate food groups for adults, with an emphasis at least one-half of the grain products from whole grains.

Studies suggest using dietary MUFA to replace SFA and refined CHO promotes healthy blood lipid profiles, mediates blood pressure, improves insulin sensitivity and regulates glucose levels. Moreover, metabolism of dietary MUFA may affect body composition and ameliorating the risk of obesity (146). A meta-analysis of 50 studies on the Mediterranean diet demonstrated a protective role of the Mediterranean diet on components of metabolic syndrome, including waist circumference (−0.42 cm, 95% CI: −0.82 to −0.02), HDL-C (1.17 mg/dl, 95% CI: 0.38 to 1.96), TG (−6.14 mg/dl, 95% CI: −10.35 to −1.93), systolic BP (−2.35 mm Hg, 95% CI: −3.51 to −1.18) and diastolic BP (−1.58 mm Hg, 95% CI: −2.02 to −1.13), and glucose (−3.89 mg/dl, 95% CI: −5.84 to −1.95) (110). The major food sources of dietary MUFA in Mediterranean diet are nuts and olive oil. Nuts are a source of dietary fiber and both nuts and olive oil contain vitamins and minerals and other bioactive compounds that may have additional benefits on lowering the risk of CVD and metabolic syndrome. However, few studies have been differentiated the effects of other nutrients beyond MUFA from these foods. Avocados are a unique nutrient-dense fruit source of MUFA, with lower energy density than nuts and oils, and are rich in vitamins, minerals, fiber, phytosterols and polyphenols that have potential benefits on lowering LDL-C, TG, BP, and enhancing insulin sensitivity. A few clinical trials that used avocados to replace SFA demonstrated a cholesterol-lowering effect of avocados, but in these studies avocados were a source of MUFA that was substituted for other macronutrients to evaluate the effect of the fat content of the test diet on lipids and lipoproteins.

The key questions on the optimal macronutrient subsititution for SFA are: 1) if using MUFA to replace excess SFA is better than using CHO with at least half of the grain products from whole grains; 2) if nutrient-dense food sources of MUFA such as avocados have significant additional benefits on CVD and metabolic syndrome risk beyond MUFA. This study evaluated the effects of dietary MUFA on established lipid risk factors versus a high CHO diet, and investigated if including one avocado per day as a source of MUFA can elicit additional benefits on established risk factors for CVD and metabolic syndrome. We hypothesized that a high-MUFA, moderate fat diet will elicit a similar LDL-C reduction as a lower-fat, high CHO diet, without adverse effects on TG and HDL-C. Secondary, we hypothesized that using one avocado a day to provide the MUFA as a substitute for SFA will have an additional LDL-C lowering effect. Lastly, we explored whether avocados also have additional benefits beyond MUFA on metabolic syndrome risk factors.

Methods

Study Design

The clinical trial design and study protocol of the AVOCADO study has been described in Chapter 3. Briefly, a randomized, 3-period crossover study design was implemented. A two week "run-in" average American diet (AAD: 34% fat, 51% CHO, 17% PRO) was fed to participants before they were randomly assigned to a treatment sequence of three diet periods (5 week each) with a 2-3 week compliance break between diet periods. Participants were assigned to random treatment sequences that were generated by balanced permutations. Three cholesterol-lowering diets (6-7% SFA) were fed (5 weeks each): a lower-fat diet (LF: 24% fat, 59% CHO, 16% PRO); two moderate fat diets (34% fat, 49% CHO, 16% PRO) provided similar foods and

matched for macronutrients and fatty acids: the avocado diet (AV) included one fresh Hass avocado (136 g) per day, and the moderate fat diet (MF) mainly used high oleic acid oils to match the fatty acid content of one avocado. The LF diet was designed by replacing 6 to 7% of energy from SFA with CHO (from grains that were incorporated in the diet in place of SFA) in the AAD. Likewise, the AV and MF diets were designed by replacing 6 to 7% of energy from SFA with MUFA using either one Hass avocado (~136g fruit pulp, ~13g MUFA) per day (for the AV diet) or high oleic acid oils (e.g., sunflower oil and canola oil, for the MF diet) as the main sources of MUFA. To match the macronutrients and fatty acids in the MF and AV diets, and to adjust for the different calorie levels, high oleic acid oils, low fat cheese, and nuts were used in both diets. About 90% of foods in the two diets were identical. Thus, the major difference between the nutrient profiles of the AV and MF diets were due to the bioactive compounds from one avocado. Menus (six-day rotating) were developed using Food Processor SQL software (ESHA Research, Salem, OR) for seven calorie levels (1800 to 3600 kcals) to meet participants' energy requirements. The Harris-Benedict equation with a physical activity factor was used to estimate each participant's basal metabolic rate (BMR) and daily energy requirements. All the menus are shown in Appendix A. Participants were weighed daily (Monday through Friday) to assess diet compliance and ensure that body weight was maintained. Participants were asked to maintain their habitual level of physical activity throughout the study. During the diet periods, participants were required to consume the foods provided only. At the end of each diet period, fasting blood samples were collected on two consecutive days. Serum and EDTA plasma were collected and stored at -80°C until the end of the study.

Laboratory Measurements

Lipids, lipoproteins, and apolipoproteins

Serum concentrations of lipids and lipoproteins were measured by Vertical Auto Profile (VAP test, Atherotech, Inc.), a rapid single vertical spin, density gradient ultracentrifugation technique that directly measures cholesterol concentrations of all lipoprotein classes (LDL, HDL, VLDL, Lp(a), IDL). TG was directly measured by the Architect Plus analyzer. ApoB100, and apoA1 were determined based on the individual LDL pattern for each sample. The within rotor % CV of the VAP method was < 5%; the within day and between day % CV for all variables were <10%(147).

Blood pressure

Blood pressure was measured before the blood draw at one of the two consecutive endpoint visits at the CRC. Subjects were seated and relaxed for 5 minutes before blood pressure was measured. Blood pressure was measured three times by a standard mercury stadiometer (W. A. Baum Co., Copiague) and the average of the 2nd and 3rd repeats was recorded as the final value.

High-sensitivity CRP

Serum high sensitivity C-reactive protein (hsCRP) was determined by a nephelometric method utilizing latex particles coated with (CRP) monoclonal antibodies; the analytical sensitivity was 0.2mg/dL (Quest Diagnostics; assay CV < 8%).

Insulin and Glucose

Plasma fasting insulin was measured by immunoassay; fasting glucose was measured by spectrophotometry (Quest Diagnostics; assay CV was < 8% for both). They can be used to calculate a Homeostasis Assessment Model (HOMA-IR), i.e., a mathematical model that is used evaluate insulin sensitivity (148):

HOMA-IR = fasting insulin (FI in uU/mL) * glucose (FG in mmol/L)/22.5

The HOMA model is used to yield an estimate of insulin sensitivity and β-cell function from fasting plasma insulin and glucose concentrations.

Statistical Analysis

Statistical analyses were performed with SAS software (version 9.2; SAS Institute Inc). Values of outcomes were reported as means ± SEM. A two-sample t test was used to determine significant differences between sexes at baseline for each outcome variable. Change scores were calculated by subtracting baseline values from the endpoint values of each treatment diet period. The mixed models procedure (PROC MIXED) was used to test for effects of diet on the change score of all outcome variables. Diet was entered as a fixed effect; potential carryover effects were assessed by including diet sequence, period and diet-period interaction as a fixed effect in the model; age, BMI and sex were included as covariates to adjust the model; subject was treated as a random effect. The Shaprio-Wilk test of the residuals from the mixed model was used to assess normality. Effects of diet were determined by analysis of the change scores in outcome variables. Tukey post-hoc test was used to adjust for multiple comparisons and determine whether differences between diets were significant. A non-parametric ANOVA model was used to measure endpoints without normal distribution. Age, sex, BMI, diet sequence were also

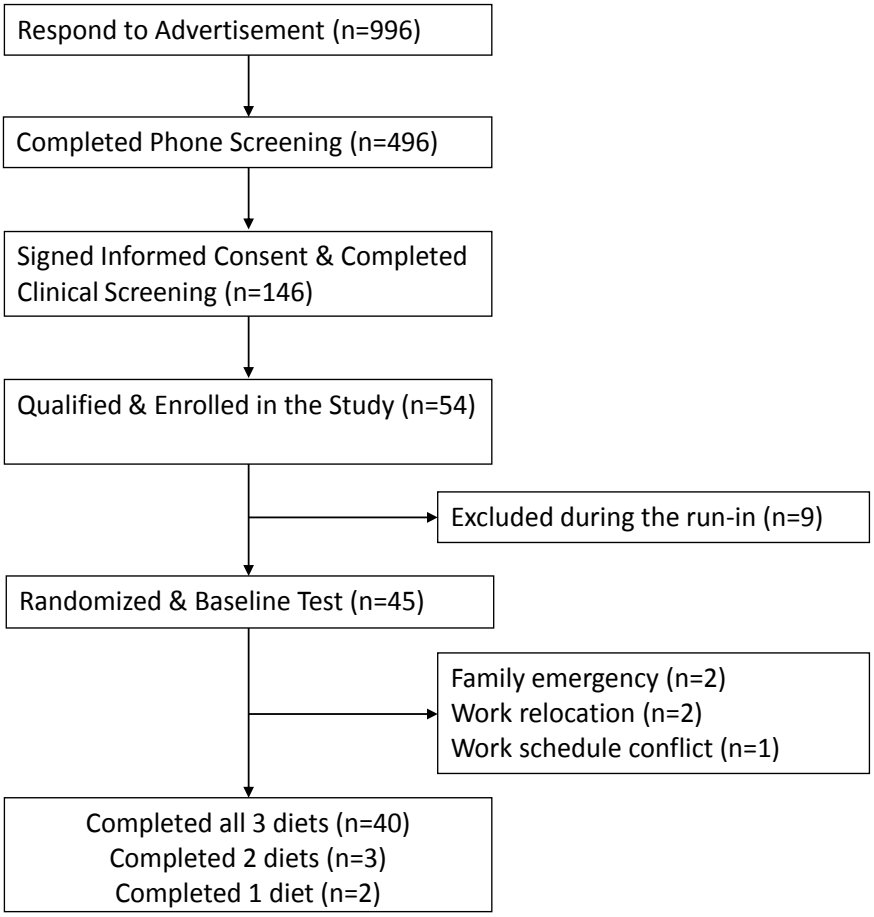
adjusted in non-parametric models. ASCVD 10 year and lifetime risk was calculated using the online ASCVD Risk Estimator tool (http://tools.cardiosource.org/ASCVD-Risk-Estimator/) for every participant at baseline and at the end of each diet. The change in risk score by each diet was estimated as median with 95% CI by the Wilcoxon Signed Rank Test. The ASCVD risk estimate is based on each participant's age, gender, race, smoking status, diabetes status, hypertension treatment, blood pressure, total cholesterol, and HDL-C. Framingham risk score was calculated based on Framingham 10-year risk scoring of developing coronary heart disease (CHD)(149). The Framingham 10-year CHD risk is estimated based on age, gender, smoking status, total cholesterol, HDL-C, and systolic blood pressure.

Results

Participant characteristics

After clinical screening, fifty-four participants were eligible and enrolled in the study. Nine participants were excluded during the two-week run-in diet due to a schedule conflict, personal health issue, or inability to comply. Five participants dropped out during the study: 2 because of a family emergency; 2 because of job relocation; 1 because of a work schedule conflict. These five participants were included in the analysis because 3 of them completed two diets and 2 of them completed one diet. Thus, a total of 45 subjects were included in the final analysis (**Figure 4-1**). All participants successfully maintained body weight during each diet period (within 2 kg weight fluctuation). Based on self-reported monitoring forms, adherence for all participants was 90% as assessed by number of days without skipping meals or consuming non-study foods.

Figure 4-1 Participant recruitment flow.



Baseline subject characteristics are presented in **Table 4-1**. Women were older than men (51 vs 40 yrs, $p<0.05$) and had lower glucose (89 vs 94 mg/dL, $p<0.05$) and higher HDL-C (55.2 vs 44.2 mg/dL, $p<0.05$) compared with men. Nevertheless, no significant interactions of sex and diet treatment by outcome were detected in the final mixed model. Both men and women were at low risk of CHD and metabolic syndrome: 20 participants had less than 1%, and 17 participants had less than 4% 10-year risk of CHD (calculated using Framingham risk score) (149); 13 participants did not have any of the criteria for Metabolic Syndrome, and 30 participants had 1 or 2 metabolic syndrome criteria (based on International Diabetes Federation definition of Metabolic Syndrome) (150) (**Table 4-2**).

Table 4-1 Baseline characteristics of study participants (n=45).

Characteristics	Males (n=27)	Females (n=18)	Combined (n=45)[1]
Age (y)	40 ± 14.1	51 ± 9.1[2]	45 ± 13.3
BMI (kg/m^2)	28.2 ± 2.3	28.2 ± 2.5	18.2 ± 2.4
TC (mg/dL)	192.6 ± 28.4	211.0 ± 35.6	199.9 ± 32.4
LDL-C (mg/dL)	124.8 ± 23.0	133.0 ± 30.3	128.1 ± 26.1
HDL-C (mg/dL)	44.2 ± 10.1	55.3 ± 12.0[2]	48.7 ± 12.1
TG (mg/dL)	113.6 ± 38.8	114.8 ± 41.9	114.0 ± 39.6
Glucose (mg/dL)	94.0 ± 8.9	89.2 ± 5.6[2]	92.2 ± 8.0
Insulin (μIU/mL)[3]	3.0 (1.9, 6.0)	4.0 (2.0, 6.0)	3.5 (1.6, 6.0)
hsCRP (mg/L)[3]	0.8 (0.4, 1.3)	1.1 (0.8, 2.7)	0.9 (0.5, 2.4)
DBP (mmHg)	80.4 ± 7.4	77.2 ± 7.0	79.2 ± 7.4
SBP (mmHg)	118.4 ± 9.8	115.2 ± 11.3	117.2 ± 10.4

[1] Baseline values were obtained before randomization, at the end of two weeks run-in AAD. All values were reported as means ± SEMs, except for insulin and hsCRP. Two-sample T test with SAS was used to detect difference between sexes. Mood's median test with Minitab was used to detect CRP differences between sexes. [2] Values that significantly differed by sex, $p<0.05$. [3]hsCRP and insulin are reported as median with 25% and 75% percentile values.

Table 4-2 Participants' CVD risk and metabolic syndrome criteria at baseline.

Risk Category	Participants at Baseline (n)
Framingham CHD 10-year Risk (%)	
<1	20
1-4	17
5-10	4
10-20	4
[1]ASCVD 10-year Risk (%) (40-69yrs old)	
<5	18
5-10	5
10-20	3
[2]ASCVD Lifetime Risk (%) (20-59yrs old)	
5-10	7
10-30	6
30-50	26
Metabolic Syndrome Risk Factors (n)	
0	13
1-2	30
3-4	2

[1]The ASCVD 10-year risk is only calculated for the 40 to 79 year range; [2]lifetime risk is only calculated for the 20 to 59 year range.

Lipids, lipoproteins, and apolipoproteins

The results for lipids, lipoproteins, and apolipoproteins are presented in **Table 4-3**. The percent change values from baseline are shown in **Figure 4-2**. All three diets significantly decreased total cholesterol (TC) and LDL-C compared to baseline. Compared to the run-in AAD diet, the LF and MF diets reduced TC (LF: -3.9%; MF: -4.7%; $p<0.01$ for both) and LDL-C (LF: -5.3%; MF: -5.9%; $p<0.01$ for both) similarly; while the reduction in TC and LDL-C by the AV diet (-8.1% and -10%; $p<0.0001$ for both) was significantly greater ($p<0.05$) than the LF and MF diets (**Figure 4-2**).

HDL-C was significantly decreased after consumption of the LF diet (-6.2%, $p<0.0001$) and decreased less ($p<0.05$) by the MF diet (-1.8%, $p=0.03$) and AV diet (-1.9%, $p=0.06$) than the LF diet. Also, the LF diet significantly increased TG and VLDL-C by 17.6% and 11%, respectively ($p<0.001$ for both), while the MF and AV diets did not affect TG and VLDL-C. Non-HDL-C was significantly decreased after consumption of the MF and AV diets (-5.3% and -9.5%, $p<0.01$ for both), but was not significantly decreased by the LF diet. Furthermore, the AV diet elicited a greater ($p=0.01$) reduction in non-HDL-C than the MF diet. We observed a small reduction in LP(a) by the LF and MF diets (-0.8mg/dL and -0.5mg/dL, $p<0.05$ for both), but the diet effect in the mixed model was not significant ($p=0.12$).

TC/HDL-C and LDL-C/HDL-C ratios were decreased significantly after consumption of the AV diet (-5.1% and -6.8%, $p<0.01$ for both) but were not significantly affected by the MF and LF diets compared to baseline. Both the TC/HDL-C and LDL-C/HDL-C ratios after consumption of the LF diet were significantly higher than the ratios after consumption of the MF and AV diets ($p<0.05$). The LF diet also significantly increased the TG/HDL-C ratio by 27.2% ($p<0.0001$), whereas the MF and AV diets did not affect TG/HDL-C.

The AV and MF diets significantly decreased apoB-100 from baseline (-7.7% and -4.6%, $P<0.001$ for both), whereas the LF diet did not. There was a trend for a greater ($p=0.06$) reduction in apoB by the AV diet than the MF diet. ApoA1 decreased similarly after consumption of the three diets. The apoB/apoA1 ratio was significantly decreased after consumption of the AV and MF diets (-5.3% and -2.7%, $p=0.0002$ and $p=0.05$) but was not affected by the LF diet.

Table 4-3 Effect of diets on lipids, lipoproteins, apolipoproteins, glucose, insulin, CRP, and blood pressure.

	[1]Baseline (n=45)	LF (n=43)	MF (n=42)	AV (n=43)	Treatment Effect (p)
Cholesterol (mg/dL)					
TC	199.9 ± 4.8	190.7 ± 5.1^{a*}	188.7 ± 4.4^{a*}	182.2 ± 4.3^{b*}	0.005
Non HDL-C	151.2 ± 4.3	145.9 ± 4.6^{a}	141.5 ± 4.0^{a*}	135.3 ± 3.8^{b*}	<.0001
LDL-C	128.1 ± 3.9	120.5 ± 4.2^{a*}	119.0 ± 3.7^{a*}	113.7 ± 3.4^{b*}	0.005
IDL	15.7 ± 0.7	15.8 ± 0.7^{a}	14.7 ± 0.8^{b}	13.7 ± 0.7^{b*}	<.0001
LP(a)	6.6 ± 0.7	6.0 ± 0.7^{a*}	6.3 ± 0.8^{a,b*}	6.4 ± 0.7^{b}	0.02
VLDL	23.3 ± 0.9	25.6 ± 1.3^{a*}	22.6 ± 0.8^{b}	21.7 ± 0.9^{b}	<.0001
HDL-C	48.7 ± 1.8	44.8 ± 1.5^{a*}	47.2 ± 1.5^{b*}	46.9 ± 1.6^{b*}	0.02
TG (mg/dL)	114.0 ± 5.9	134.3 ± 9.8^{a*}	111.1 ± 5.8^{b}	108.6 ± 7.2^{b}	0.0005
Lipoprotein Ratios					
TC:HDL-C	4.29 ± 0.15	4.41 ± 0.16^{a*}	4.15 ± 0.15^{b}	4.00 ± 0.13^{c*}	<.0001
LDL:HDL-C	2.78 ± 0.12	2.79 ± 0.13^{a}	2.64 ± 0.12^{b}	2.52 ± 0.11^{c*}	<.0001
TG:HDL-C	2.54 ± 0.17	3.27 ± 0.30^{a*}	2.53 ± 0.18^{b}	2.46 ± 0.19^{b}	0.0003
Apolipoproteins(mg/L)					
ApoB	101.1 ± 2.8	99.0 ± 2.9^{a}	95.3 ± 2.5^{b*}	92.5 ± 2.5^{b*}	<.0001
ApoA1	144.6 ± 3.0	138.4 ± 2.4*	141.8 ± 2.7*	140.9 ± 2.6*	0.08
ApoB:ApoA1	0.71 ± 0.02	0.72 ± 0.02^{a}	0.68 ± 0.02^{b}	0.67 ± 0.02^{b*}	<.0001
Glucose (mg/dL)	92.2 ± 1.2	91.5 ± 1.0	92.8 ± 1.2	91.0 ± 0.9	0.3
Insulin (µIU/mL)	4.31 ± 0.43	4.30 ± 0.50	4.26 ± 0.43	4.13 ± 0.41	0.8
CRP (mg/L)	1.48 ± 0.23	1.66 ± 0.29	1.43 ± 0.25	1.49 ± 0.23	0.3
HOMA	0.99 ± 0.11	0.98 ± 0.12	0.97 ± 0.10	0.94 ± 0.10	0.6
Blood pressure (mmHG)					
SBP	117.2 ± 1.6	115.0 ± 1.3	115.4 ± 1.5	116.6 ± 1.5	0.6
DBP	79.2 ± 1.1	77.7 ± 1.1	78.4 ± 1.2	78.0 ± 1.1	0.9

[1] Baseline values were obtained before randomization, at the end of two weeks run-in AAD. All values are means ± SEMs. * Significant change compared to baseline AAD, P <0.05. [a/b] Values for diet treatments with different superscript letters are significantly different (Tukey post-hoc test, P<0.05).

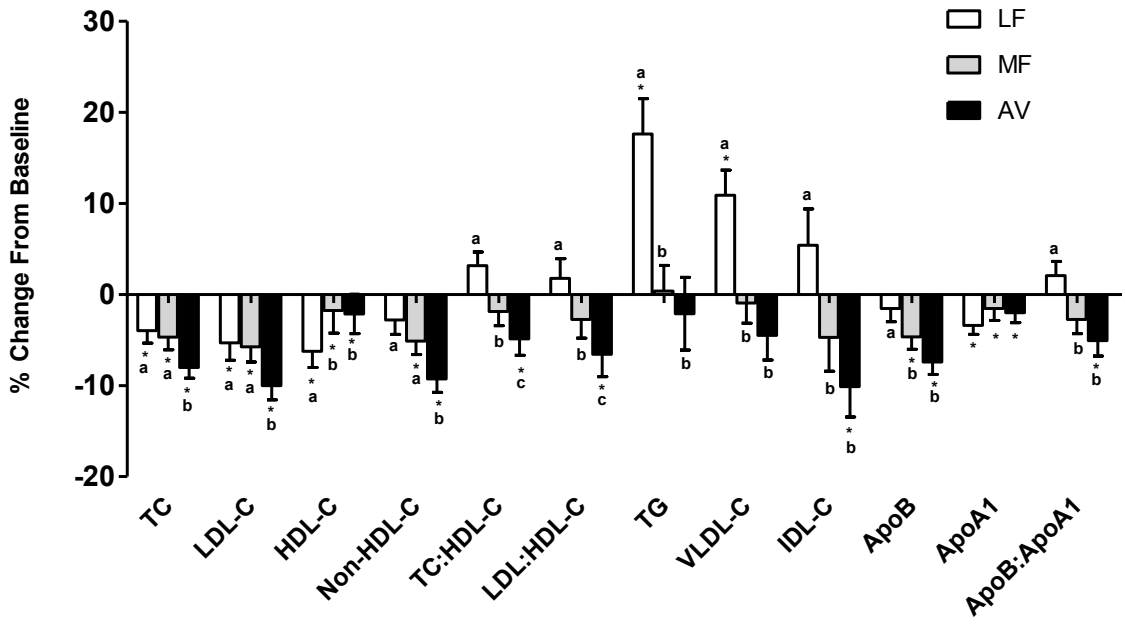


Figure 4-2 Percent change in lipids, lipoproteins, and apolipoproteins (mean ± SEM) from baseline.

*Represents values that are significantly different from the baseline ($p<0.05$). Values with different letters are significantly different (Tukey post-hoc test by SAS, $P<0.05$).

CRP, insulin, glucose, and blood pressure

Fasting CRP, insulin, glucose, systolic and diastolic blood pressure were not affected by any of the three test diets (**Table 4-3**).

CVD risk prediction

The prediction of CHD risk reduction by the Sacks and Katan's model (151) based on the change of LDL-C, HDL-C, and TG showed that the AV diet decreased CHD risk by 9.8% in men and 8.0% in women, which was more effective than the MF diet (**Table 4-4**). The prediction of CVD risk by the 2013 Prevention Guidelines ASCVD Risk Estimator showed significant

improvement on overall 10 year and lifetime risk by the MF and AV diets but not the LF diet (**Table 4-5**).

Table 4-4 Predicted changes in 10-year CHD risk using the Sacks and Katan model.

		LF	MF	AV
Predicted changes in CHD risk[1]	Men	+3.3%	-4.9%	**-9.8%**
	Women	+12.4%	-3.2%	**-8.0%**

[1] CHD prediction was based on a model by Sacks and Katan[127]: Δ LDL: -1mg/dL leads to Δ CHD -1%; Δ HDL: -1 mg/dL leads to Δ CHD + 2% in men and +3% in women; Δ TG: +88 mg/dL leads to Δ CHD: +14% in men and +37% in women.

Table 4-5 Predicted changes in ASCVD risk by three diets.

	[1]Baseline	[2]LF (Δ)	[2]MF (Δ)	[2]AV (Δ)
ASCVD 10-year Risk (%) (40-69yrs old)	2.7 (1.0, 6.3)	-0.05 (CI: -0.35, 0.1) P=0.44	-0.15 (CI: -0.3, 0) P=0.068	-0.15 (CI: -0.45, 0) P= 0.035
ASCVD lifetime Risk (%) (20-59yrs old)	36 (27, 46)	0.0 (CI: -5.0, 0) P=0.2	-5.0 (CI: -6.0, 0) P=0.006	-2.5 (CI: -9.5, 0) P=0.04

[1]Risk at baseline was reported as median with 25% and 75% percentile values. [2]The change in risk from baseline is the estimated median with 95% CI by the Wilcoxon Signed Rank Test. P values are from the test of the median not being equal to 0.

Discussion

To our knowledge, this study is the first randomized controlled feeding trial to evaluate the effects of nutrients and bioactive compounds in avocados beyond their MUFA content. We observed that the AV diet lowered TC, LDL-C, non-HDL-C, TC:HDL-C, and LDL:HDL-C significantly more than the MF diet. Also, there was a trend for the apoB reduction by the AV diet to be greater than the MF diet, which is expected since each atherogenic lipoprotein particle (non-HDL) carries one apoB100 protein. The prediction of CHD risk reduction by the Sacks and Katan's model based on the change of LDL-C, HDL-C, and TG showed that the AV diet decreased CHD risk by 9.8% in men and 8.0% in women, which was more effective in CHD risk reduction than the MF diet. Furthermore, this predicted CHD risk reduction may under-estimate the effect of the AV diet on CHD risk reduction because of the expected benefits of novel risk factors including non-HDL-C, apoB, and lipid/lipoprotein ratios.

Several studies have demonstrated that non-HDL-C and apoB are superior predictors of CVD risk compared to LDL-C (152). Individuals treated with statins who achieve low LDL-C but have high concentrations of non-HDL cholesterol or apoB remain at increased CVD risk (152). In our study, the AV and MF diets significantly decreased non-HDL-C and apoB compared to baseline; while the LF diet did not significantly affect non-HDL or apoB. This may due to an increase in VLDL and IDL by the LF diet. VLDL and IDL have been shown to be independently associated with the prediction, progression, and residual risk of CVD (153). Lipoprotein remnants, including the smaller VLDLs and their catabolic product IDLs, can enter the subendothelial space and increase prothrombotic factors, which contribute to the initial progress of atherosclerosis (154). It has been suggested that TG-enriched VLDL can decrease lipoprotein lipase activity, and increase apoCIII, which may impair the catabolism and clearance of VLDL particles (155). The impaired clearance of TG-enriched VLDL particles may due to an

elevated TG level induced by the high carbohydrate intake on the LF diet. The changes in TG and HDL-C observed herein are consistent with findings in the literature that show a LF diet increases TG and decreases HDL-C compared to a MF diet (88).

The reduction in LDL-C elicited by the AV diet (-10%) is generally in agreement with previous Avocado studies. In two trials conducted in healthy subjects (52, 53), the avocado-enriched diet (0.5-2 avocados per day, 3-4week) decreased LDL-C by 5% and 9% from baseline. Greater reduction in LDL-C was observed in hyperlipidemia subjects. Carranza et al. reported 15% reduction in dyslipidemia IV subjects (n=8) and 27% reduction in dyslipidemia II subjects (n=8) after 4-week consumption of a moderate fat diet including 1-2 avocados per day. We also observed that subjects with baseline LDL-C (≥130mg/dL, n=20) had a greater reduction in LDL-C by the AV diet (-13%, -19mg/dL) than the MF and LF diets. In subjects with normal baseline LDL-C (<130mg/dL, n=25), the MF and LF diets did not decrease LDL-C significantly; whereas the AV diet still elicited a significant reduction in LDL-C (-8%, -9 mg/dL, p=0.0004). Our result indicates that avocados can lower LDL-C effectively in individuals with or without abnormal LDL-C concentrations at baseline.

Some previous avocado studies reported either greater or non-significant reductions in LDL-C. Lopez-Ledesma et al. reported a 24% LDL-C reduction in healthy subjects (n=30) after only one week of hospitalization and consumption of an avocado enriched diet (300g avocado per day) (49). However, all subjects received the same calorie level diet (2000kcal/day) in the study and the body weight was not monitored. Possible weight loss may interfere with the results reported. In contrast, Pieterse et al. found no significant changes in LDL-C by a weight loss diet (200 g avocado per day, 6 week) in obese subjects who had an average 2.3% weight loss (54). In addition, no significant changes in the lipid/lipoprotein profile were observed in both the avocado diet group and the control group that achieved a similar weight loss. However, this study was

conducted in a free-living setting. Garber et al. also reported a non-significant LDL-C reduction (-3%, $p>0.05$) in 12 women with non-insulin-dependent diabetes mellitus (NIDDM) after 4 weeks consumption of an avocado enriched, high MUFA diet (51). In summary, lack of adequate control and a small sample size in these studies may explain why LDL-C did not decrease significantly or was changed with a much greater magnitude in response to an avocado diet.

We did not find any diet effect on CRP, insulin, glucose, and blood pressure. Previous studies have shown that glycemic control, and blood pressure did not differ between the avocado diet and the control diet in diabetic and obese subjects (51, 54). In the present study, both high MUFA diets decreased the TG/HDL-C ratio compared to the LF diet, which demonstrates a benefit of these diets on this marker of insulin sensitivity (156). A study on 449 apparently healthy patients demonstrated that plasma TG/HDL-C ratio ≥ 3.5 provides a simple means of identifying insulin-resistance. The subjects in our study were at low risk of insulin resistance, which was indicated by the baseline TG/HDL-C ratio 2.54 ± 0.17 (means ± SEM). Although the LF diet did not affect insulin or glucose, it significantly increased TG/HDL-C ratio by 27.2% ($p<0.0001$). This finding may due to the increased refined grains and added sugars in some grain products. Although the grain products in the LF diet contained more than 50% whole grains, it still may not overcome the adverse effect on TG/HDL-C. This finding suggests an increased risk for metabolic syndrome, although the net effect of decreased LDL-C and increased TG/HDL-C is unclear. CHO-induced hypertriglyceridemia is among the key unresolved problems in nutrition. It has been clear that simple sugars, refined grains, and fructose-containing sugars can induce hypertriglyceridemia, while dietary fiber in whole grains and vegetables can mitigate the effect. Our study shows that a 10% increase in CHO, even along with an increase in whole grains (+4.3oz), fruits/vegetables (+2 cups) and fiber (+8g) did not offset the adverse effect on TG and HDL-C when substituting SFA with CHO. Although the AV diet contains 9g more fiber (from

one avocado) than the MF diet, it did not lower TG compared to the AAD or the MF diet either. However, participants on the MF and AV diets had TG levels that were significantly lower than on the LF diet. Overall, the results suggest that the diet with an optimal macronutrient composition is more effective than dietary fiber in the control of TG. Hence, a moderate fat diet using dietary MUFA to replace SFA is preferred to achieve LDL-C reduction without elevating TG/HDL-C.

Conclusions

We present novel information that a moderate fat diet low in SFA and high in MUFA that was provided mainly by an avocado per day achieved greater reductions in TC, LDL-C, non-HDL-C, LDL/HDL-C and TC/HDL-C than a high MUFA diet with a similar macronutrient and fatty acid profile in overweight and obese adults. The results of the current study provide convincing evidence that inclusion of a food source of MUFA rich in bioactive compounds confers additional CVD benefits, especially on LDL-C lowering compared with a high MUFA diet without avocados. However, avocados did not show additional benefits beyond MUFA on the risk factors for metabolic syndrome, including TG, HDL-C, blood glucose, and blood pressure.

We also showed that a 10% increase in CHO, even along with a moderate increase in whole grains, fruits/vegetables and fiber did not offset the adverse effect on TG and HDL-C of substituting SFA with CHO, with emphasis on fiber-rich whole grains. A moderate fat diet using

dietary MUFA to replace SFA is preferred to achieve an optimal lipid profile in overweight and obese adults.

This study provides answers to the two key questions we proposed for current dietary guidelines: 1) if using MUFA to dietary replace excess SFA is better than using CHO with at lease one-half of the grain products from whole grains; 2) if a nutrient-rich food source of MUFA such as avocados has significant additional benefits on CVD and metabolic syndrome risk beyond MUFA. First, our results show benefits of increasing MUFA intake to 17% of total energy compared to the current average intake 12% of total energy in US adults. Our results suggest that an optimal macronutrient composition is more effective than dietary fiber in the control of TG and HDL-C, two criteria for metabolic syndrome. Hence, using MUFA to substitute for SFA maybe superior to using CHO within the recommended intake range of macronutrients. Secondly, we demonstrated that including one avocado a day in a moderate fat diet can elicit additional benefits on established CVD risk factors beyond MUFA. Our results will be useful to provide more evidence in the evolution of new dietary guidelines and healthy diet patterns for the primary prevention of CVD.

Chapter 5

Effect of MUFA Enriched Moderate Fat Diet with or without One Avocado per Day on Lipoprotein Particle Number, Size, and Subclasses in Overweight and Obese Adults

Abstract

Recent clinical evidence demonstrates that novel lipid parameters, such as lipoprotein particle number, size and density can improve CVD risk assessment than traditional lipid risk factors. Atherogenic dyslipidemia, which is a key feature of metabolic syndrome, is characterized with a high proportion of small, dense LDL, increased TG, reduced HDL, increased VLDL and remnants, and impaired insulin sensitivity. Well-controlled studies are lacking on the effect of different macronutrient composition and avocado consumption on novel lipid parameters. The current study used two novel advanced lipid-testing methods to measure lipoprotein particle number, size and subclasses in participants in the AVOCADO study. A randomized, crossover, controlled feeding trial was conducted with 45 overweight or obese participants with baseline LDL-C in the 25th to 90th percentile. Three cholesterol-lowering diets (6% to 7% SFA) were fed (5 weeks each): a lower-fat diet (LF: 24% fat); 2 moderate-fat diets (34% fat) provided similar foods and were matched for macronutrients and fatty acids: the avocado diet (AV) included one fresh Hass avocado (136 g) per day, and the moderate-fat diet (MF) mainly used high oleic acid oils to match the fatty acid content of one avocado.

Compared to baseline, the large, buoyant LDL cholesterol and particle number ($LDL_{1+2,}$ large LDL-P) were decreased similarly by all three diets, while only the AV diet significantly decreased LDL particle number (LDL-P, -80.1nmol/L, p=0.0001), small dense LDL cholesterol

(LDL_{3+4}, -4.1 mg/dL, p=0.04) and lipoprotein remnants ($VLDL_3$+IDL, -2.6 mg/dL, p= 0.004) from baseline. The LF diet significantly increased small LDL-P (104.8 nmol/L, p<0.0001) and lipoprotein remnants ($VLDL_3$ + IDL, 1.5 mg/dL, p=0.02). Furthermore, the concentration of small LDL-P and sdLDL-C after consumption of the AV diet was significantly lower (p<0.05) than the MF and LF diet.

In summary, inclusion of one avocado per day as part of a moderate fat, cholesterol-lowering diet has additional benefits beyond dietary fatty acids on lowering total LDL particle number, especially small, dense LDL and lipoprotein remnants. Our results demonstrate that avocados have beneficial effects beyond MUFA on atherogenic lipoprotein subclasses. Also, a lower-fat, high CHO diet may potentially increase the risk of athergenic dyslipidemia associated with metabolic syndrome.

Introduction

Small dense LDL and atherogenic dyslipidemia

Small, dense LDL are linked to atherogenesis: long residence time in plasma, arterial proteoglycan binding, and permeability through the endothelial barrier (157). A high proportion of small, dense LDL, called pattern B, is associated with relative increases in TG and other atherogenic metabolic changes, such as reduced HDL, increased VLDL, and impaired insulin sensitivity (158). Overall, this profile is defined as atherogenic dyslipidemia, which is a key feature of metabolic syndrome. The metabolic pathways of atherogenic dyslipidemia in metabolic syndrome are shown in **Figure 5-1**. Insulin resistance (IR) increases the release of free

fatty acids from the adipocytes, and results in increased plasma free fatty acid levels. An enhanced uptake of free fatty acids by the liver leads to increased TG formation, decreased LDL proteolysis, and higher VLDL production and VLDL apo B-100 secretion into circulation. Once in the bloodstream, the TG in the core of the large TG-enriched VLDL particles exchanges for cholesteryl esters (CE) in the core of LDL via the cholesterol ester transport protein (CETP), producing CE–depleted LDL particles. Since the TG in the core of LDL is more likely to be hydrolyzed by hepatic lipase or lipoprotein lipase, small, dense LDL particles are produced. CETP can also mediate an exchange of CE in HDL particles for the TG in VLDL particles. This TG-enriched HDL and its major protein apoA1 are more likely to be taken up and catabolized by the kidney. These events produce a dyslipidemia pattern characterized by an increased number of small, dense LDL particles, decreased HDL cholesterol, and increased plasma TG levels (159).

It has been shown that the prevalence of LDL phenotype B in both men and premenopausal women is strongly related to the intake of CHO but negatively related to the intake of dietary fat (160). A high intake of CHO (especially refined grains and added sugar), along with low intake of fat, may induce similar atherogenic dyslipidemia as seen in metabolic syndrome. Moreover, some individuals may be more susceptible than others to the adverse effects of high-CHO/low-fat diets, such as the haplotype of ApoA5 gene is strongly associated with increased plasma TG and sdLDL (161). Meanwhile, some individuals may benefit more from high-CHO/low-fat diets on LDL-C reduction. The genetic polymorphisms, mechanisms, and clinical outcomes on CHO-induced hypertriglyceridemia are still unclear relative to identifying individualized dietary interventions.

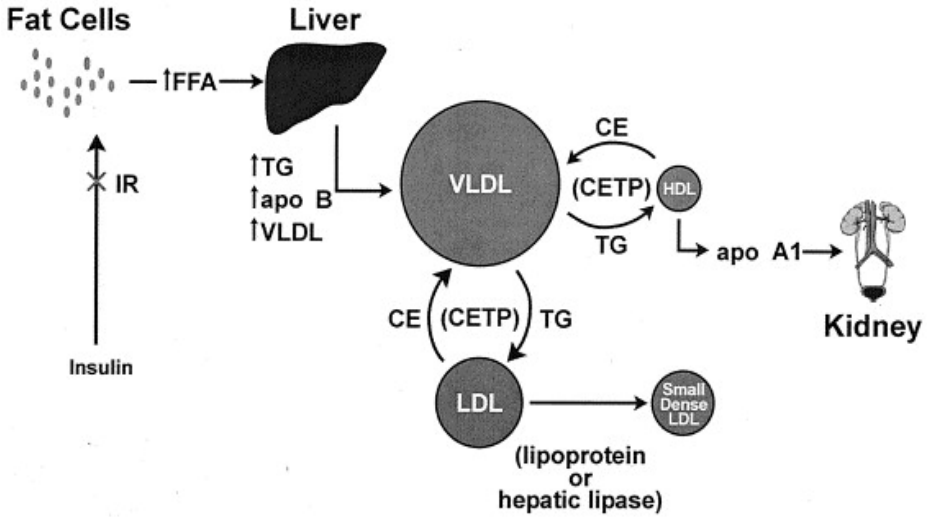


Figure 5-1 Atherogenic dyslipidemia in Metabolic Syndrome.

CE: cholesterol ester; FFA: free fatty acids; IR: insulin resistance. Source: Kwiterovich Jr, 2002 (159)

Advanced lipid testing and clinical application

Advanced lipid testing is recommended to identify patients with substantial residual risk after lowering LDL-C for more aggressive targeting of lifestyle and pharmacologic therapies. It measures the subpopulation of lipoproteins within a lipoprotein class. To date, four methods of advanced lipid testing have been developed commercially and broadly utilized in large clinical trials: nuclear magnetic resonance spectroscopy (NMR) (Liposcience, Inc., Raleigh, North Carolina), vertical rotor ultracentrifugation (VAP) (Atherotech, Birmingham, Alabama), and gradient gel electrophoresis and Ion Mobility (Berkeley Heart Lab, Inc., Berkeley, California). Consistency of the relationships of coronary atherosclerosis progression with measures of small LDL by four independent methods supports their use for managing risk for coronary artery disease (162). NMR and VAP methods are commonly evaluated in large clinical trials. The

NMR lipid test directly measures lipoprotein subclasses particle numbers (**Figure 5-2**), while the VAP test measures the cholesterol content in different lipoprotein subclasses (**Figure 5-3**).

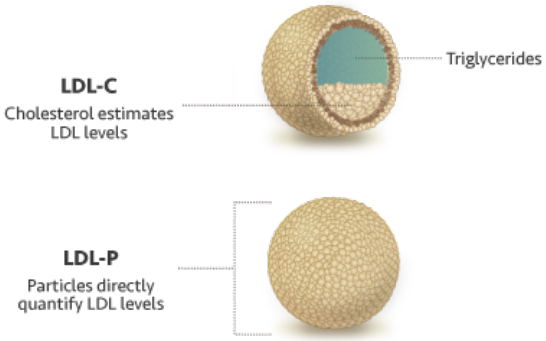


Figure 5-2 Differences in LDL-C and LDL-P measurements.

Recent clinical evidence demonstrates that these novel lipid parameters can improve CVD risk assessment. In a subset of the Framingham Offspring Study (n=531), subjects with a low level of LDL-P (<25th percentile) had a lower CVD incidence (59 events per 1000 person-years) than those with an equivalently low level of LDL-C or non–HDL-C (81 and 74 events per 1000 person-years, respectively) (163). In the Multi-Ethic Study of Atherosclerosis (MESA, n=5598), Otvos et al. reported that for those with discordant levels of LDL-C and LDL-P, only LDL-P was associated with incident CVD (LDL-P HR 1.45, 95% CI: 1.19-1.78; LDL-C HR 1.07, 95% CI: 0.88-1.30) (164). In the Women's Health Study (n=27,533, median follow-up 17.2 years), Mora et al. reported that the CHD risk remained under or overestimated by ≈20-50% for women with discordant LDL-C and LDL-P levels after multivariable adjustment for potentially mediating factors including HDL-C and TG (165). The discordance between LDL-C and LDL-P is due to variation in cholesterol and TG content in LDL particles, which can be assessed by LDL particle size and density (**Figure 5-2**).

Recent large prospective studies have demonstrated that the measurement of sdLDL could be used to identify risk for CHD that may remain undetected using standard lipid measurements only in different populations (166-169). In the Atherosclerosis Risk in Communities (ARIC) study (n=11,419, follow-up 11 years), Hoogeveen et al. found that sdLDL was significantly associated with CHD incidence after adjusting for standard CHD risk factors, even in a subgroup of individuals with an optimal LDL-C level (<100mg/dL) (168). In the MESA study (n=4387,8.5 years of follow up), Tsai et al. reported that elevated sdLDL-C was a risk factor for developing CHD after adjusting for standard CHD risk factors in normoglycemic individuals (169).

There have been some studies that did not show an improved prediction of incident cardiovascular events by markers from advanced lipid testing, as well (170, 171). Although measurements of small LDL may not improve risk assessment beyond conventional lipid measures in all populations, their significant association with disease outcomes, as well as their pathological properties, suggests the potential use of advanced lipid testing in monitoring efficacy of lipid-altering therapy and dietary intervention for reducing CVD risk in individual patients.

Rationale, objectives, and hypotheses of the current study

In Study 1, we reported that a moderate fat diet low in SFA and high in MUFA that was provided mainly by an avocado per day achieved greater reductions in LDL-C and non-HDL-C than a high MUFA diet with a similar macronutrient and fatty acid profile in overweight and obese adults. However, avocados did not show additional benefits beyond MUFA on metabolic syndrome risk factors, including TG, HDL-C, blood glucose, and blood pressure. We also found that a 10% increase in dietary CHO, even along with a moderate increase in whole grains,

fruits/vegetable and fiber did not offset the adverse effect of substituting SFA with CHO on TG and HDL-C.

These results raised two further questions: 1) which lipoprotein subclass of non-HDL-C did avocados affect specifically that was different from the MF diet? 2) Did the LF diet (high in fruits, vegetables, and whole grains) also increase sdLDL level and the risk of atherogenic dyslipidemia, along with its effect on increasing TG/HDL-C? In summary, the primary goal of the current study was to evaluate a MUFA enriched, moderate fat diet including one avocado per day on LDL subclasses, especially small, dense LDL. We hypothesize that the AV diet decreased sdLDL and improved VLDL clearance. Secondly, we explored the relationship between high CHO induced TG and sdLDL production in healthy, overweight and obese adults. We hypothesize that the LF diet increased sdLDL production along with high CHO induced hypertriglyceridemia. Furthermore, we explored the mechanism by which different diet interventions affect lipoprotein metabolism.

Methods

Study Design

The clinical trial design and study protocol of the AVOCADO study have been described in Chapter 3. Briefly, a randomized, 3-period crossover study design was implemented. A two week "run-in" average American diet (AAD: 34% fat, 51% CHO, 17% PRO) was fed to participants before they were randomly assigned to a treatment sequence of three diet periods (5 week each) with a 2-3 week compliance break between diet periods. Participants were assigned to random treatment sequences that were generated by balanced permutations. Three cholesterol-

lowering diets (6-7% SFA) were fed (5 weeks each): a lower-fat diet (LF: 24% fat, 59% CHO, 16% PRO); two moderate fat diets (34% fat, 49% CHO, 16% PRO) provided similar foods and matched for macronutrients and fatty acids: the avocado diet (AV) included one fresh Hass avocado (136 g) per day, and the moderate fat diet (MF) mainly used high oleic acid oils to match the fatty acid content of one avocado. The LF diet was designed by replacing 6 to 7% of energy from SFA with CHO (from grains that were incorporated in the diet in place of SFA) in the AAD. Likewise, the AV and MF diets were designed by replacing 6 to 7% of energy from SFA with MUFA using either one Hass avocado (~136g fruit pulp, ~13g MUFA) per day (for the AV diet) or high oleic acid oils (e.g., sunflower oil and canola oil, for the MF diet) as the main sources of MUFA. To match the macronutrients and fatty acids in the MF and AV diets, and to adjust for the different calorie levels, high oleic acid oils, low fat cheese, and nuts were used in both diets. About 90% of foods in the two diets were identical. Thus, the major difference between the nutrient profiles of the AV and MF diets were due to the bioactive compounds from one avocado. Menus (six-day rotating) were developed using Food Processor SQL software (ESHA Research, Salem, OR) for six-calorie levels (1800 to 3600 kcals) to meet participants' energy requirements. The Harris-Benedict equation with a physical activity factor was used to estimate each participant's basal metabolic rate (BMR) and daily energy requirements. All the menus are shown in Appendix A. Participants were weighed daily (Monday through Friday) to assess diet compliance and ensure that body weight was maintained. Participants were asked to maintain their habitual level of physical activity throughout the study. During the diet periods, participants were required to consume the foods provided only. At the end of each diet period, fasting blood samples were collected on two consecutive days. Serum and EDTA plasma were collected and stored at -80°C until the end of the study.

Laboratory Measurements

Lipoprotein subclasses – cholesterol level

Serum cholesterol concentrations of lipids and lipoproteins (including second-day repeats) were measured using the VAP® (Vertical Auto Profile) test by Atherotech, Inc. The VAP© Test was used as a direct measure of the following lipid and lipoprotein classes and subclasses (**Figure 5-3**): LDL, LDLR, Lp(a), IDL, HDL, HDL2, HDL3, VLDL, VLDL1+2, VLDL3, TC, TG, Non HDL, Remnant Lipoproteins, LDL4, LDL3, LDL2, ApoB100, ApoA1, ApoB100:A1(CV< 8%). During the VAP test, an EDTA plasma sample was diluted and added to a sealed ultracentrifuge tube containing a density-gradient. After the centrifugation, the cholesterol content in the tube was continuously analyzed using the VAP-II analyzer (controlled-dispersion flow analyzer) (147). Finally, a Data Translation analog-to-digital conversion board and the (Data Translation Inc., Marlboro, MA) and software developed in the Data Translation laboratory were used to continuously collect and digitize the absorbance data as the sample is analyzed (147). A cholesterol profile (absorbance curve) was obtained by plotting digitized absorbance units on the Y-coordinate and the relative gradient position (calculated from the sample drain time) on the X coordinate. Then the cholesterol profile was decomposed into curves corresponding to lipoprotein classes using regression models (147).

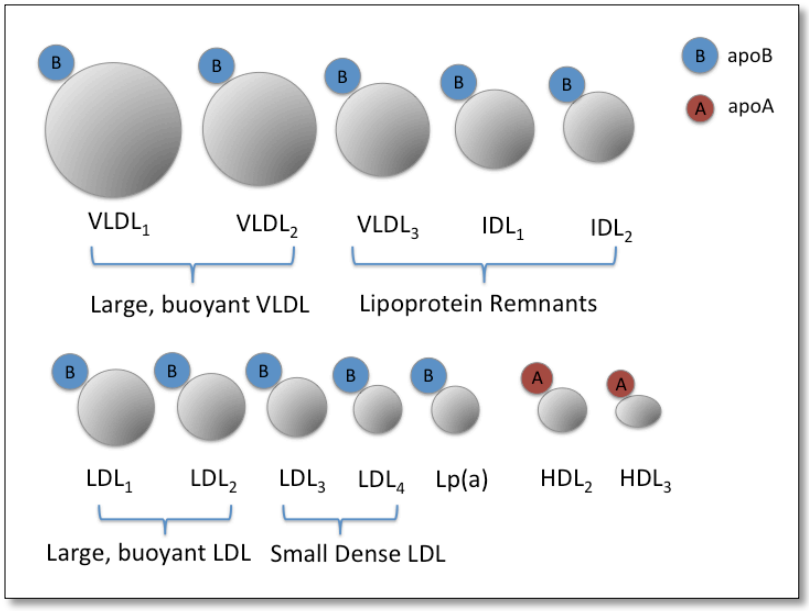


Figure 5-3 VAP lipid test on classification of lipoprotein classes and subclasses.

Lipoprotein subclasses – particle numbers and size

Plasma lipoprotein particle number and size were assessed by a proton nuclear magnetic resonance spectroscopy (NMR LipoProfile III, LipoScience, Raleigh, NC), which measures the particle concentrations of lipoprotein subclasses and average particle size of lipoproteins. Quantification is achieved in a three-step process consisting of measurement of the plasma NMR spectrum followed by computer deconvolution of the spectral data and calculation of the subclass concentrations. A typical plasma NMR spectrum is shown in **Figure 5-4**. The lipoprotein subclasses in the NMR Analyzer are shown in **Figure 5-5**. The spectral region at ~0.8 ppm contains the signals emitted by the methyl group protons of lipids in the lipoprotein particles: phospholipid, cholesterol, cholesterol ester, and triglyceride (172). The amplitude of each lipoprotein particle signal serves as a measure of the concentration of that lipoprotein (172). The lipids in larger particles broadcast signals that are characteristically different in frequency and

shape from the lipid signals emitted by smaller particles. After the NMR measurement, calculations are performed to convert the subclass signal amplitudes to subclass particle number (nmol/L) (172).

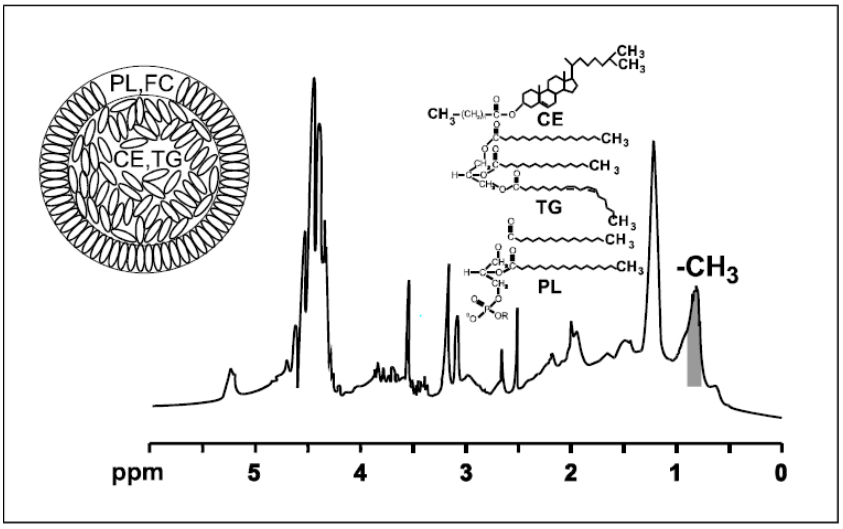


Figure 5-4 A typical NMR spectrum of plasma.

Source: Otvos et al. 2001(172)

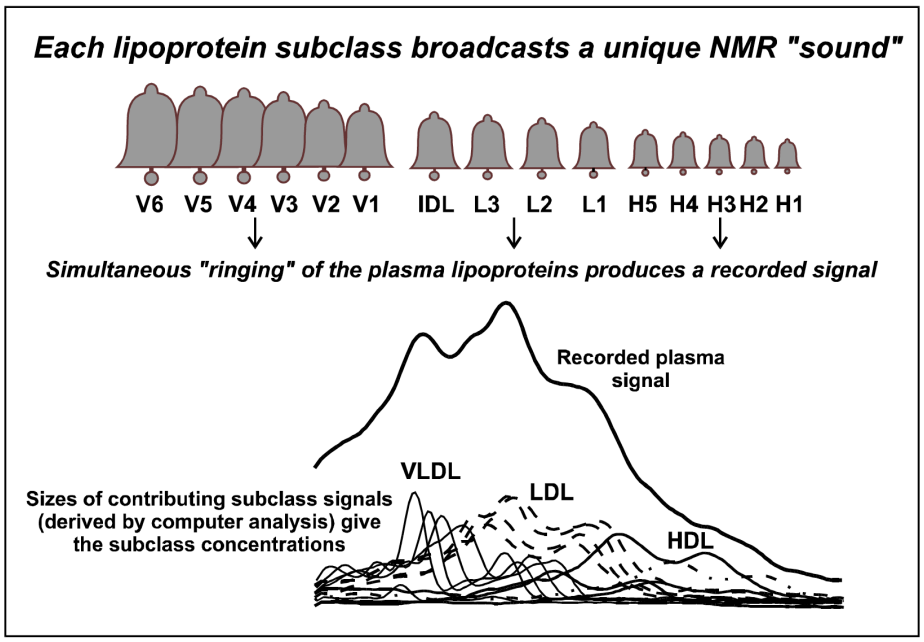


Figure 5-5 Lipoprotein subclasses in the NMR Analyzer.

Source: Otvos et al. 2001 (172)

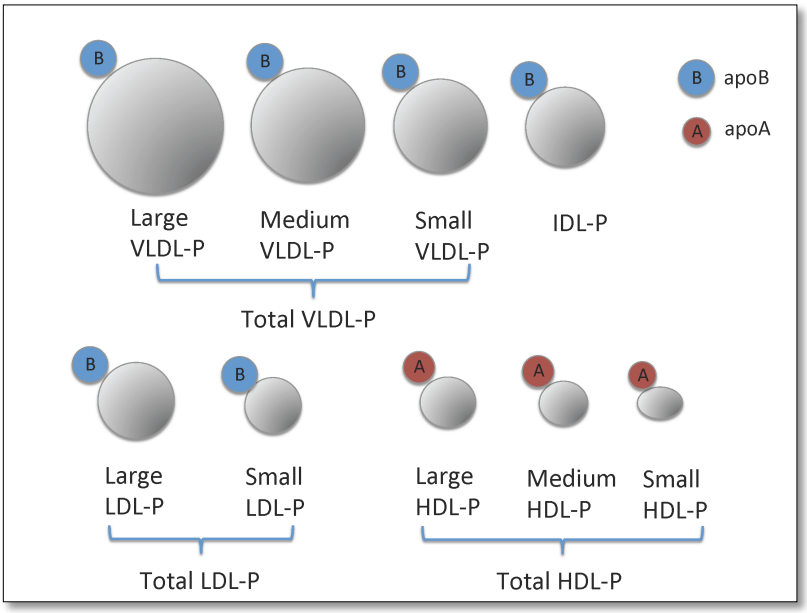


Figure 5-6 NMR lipid test report on classification of lipoprotein classes and subclasses.

Lipid transfer protein activity

We used protein Ex Vivo kits by Roar (Roar Biomedical, Inc) to measure ex vivo activity of cholesteryl ester transfer protein (CETP), plasma protein lecithin:cholesterol acyltransferase (LCAT), and phospholipid transfer protein (PLTP). The Roar ex vivo CETP Activity Assay was performed with all plasma components at near physiological concentration, CETP activity in vitro with the effect of known endogenous inhibitors (apoC1, for example) as occurs in vivo. The assay reagents comprise a donor particle and an acceptor wherein the donor particle contains a substrate for transfer by CETP. The substrate is labeled such that the transfer of the substrate from the donor particle to an acceptor is detected as an indicator of CETP activity. The proprietary substrates enable the detection of CETP-mediated transfer of neutral lipids among the substrates. The CETP transfer activity results in an increase in fluorescence intensity. The Roar LCFC-LCAT Activity Assay is a homogeneous fluorometric assay that is useful for measuring the acyltransferase activity of LCAT by non-enzymatic means. The method uses a reagent

designed for detecting changes in free cholesterol concentration without cholesterol oxidase, peroxidase or the generation of hydrogen peroxide. The Roar PLTP Activity Assay includes proprietary substrates to detect PLTP mediated transfer of fluorescent substrate too. Transfer activity results in increased fluorescent emission intensity from the assay. The inter-assay CV was 3%.

Statistical Analysis

Statistical analyses were performed with SAS (version 9.2; SAS Institute Inc.). The mixed models procedure (PROC MIXED) was used comparing the effects of three diets on the change value (from baseline) of all outcome variables. Potential carryover effects were assessed by including diet sequence, period and diet-period interaction as a fixed effect in the model; age, BMI, sex, diet-sex interaction were included as covariates. The Shapiro-Wilk test was used to assess normality of residuals in the mixed model. Tukey post-hoc test was used to adjust for multiple comparisons of three diets. Correlations between lipoprotein endpoints were determined using Pearson correlation coefficient analysis. Fisher's Z-transformation was used to compare the correlations in different diets.

Results

LDL subclasses

The results of the lipoprotein particle number, size and subclasses are presented in **Table 5-1** and **Table 5-2**. We found discordance between the change in LDL-C and LDL-P on the LF diet. Although LDL-C was significantly reduced, LDL-P did not change (23.8nmol/L, p=0.2)

after the LF diet. The discordance was due to the significant increase in small LDL-P (104.8 nmol/L, p<0.0001) by LF diet. There was a trend for a reduction in LDL-P by the MF diet (-38nmol/L, p=0.07), however, the reduction was significantly (p=0.05) less than the AV diet (-80.1nmol/L, p=0.001). MF failed to significantly decrease LDL-P because it also increased small LDL-P (53.0nmol/L, p=0.01). Similar significant reductions from baseline (p<0.001 for all) in large LDL-P were observed on the LF (-78.4nmol/L), MF (-62.6nmol/L), and AV diets (-60.1nmol/L) (**Figure 5-7**). We observed similar reductions in the cholesterol concentration (**Figure 5-7**) in the large, buoyant LDL particles (LDL_{1+2}) on the LF (-7.1 mg/dL), MF (-7.0 mg/dL), and AV diets (-7.5 mg/dL) compared to baseline (p<0.0001 for all). However, only the AV diet significantly reduced small, dense LDL particles (LDL_3: -4.0 mg/dL, P=0.01; LDL_{3+4}: -4.1mg/dL, P=0.04). The MF diet did not affect LDL_3 and LDL_4, and the LF diet significantly increased LDL_4 from baseline (2.5mg/dL, p=0.03). The frequency distribution of sdLDL (LDL_{3+4}) at baseline and after consumption of 4 diets is shown in **Figure 5-8**. The AAD and LF diet resulted in a similar distribution of sdLDL in 45 subjects, while the MF and AV diets decreased the number of subjects with high sdLDL (>60mg/dL). The MF and AV diets resulted in a similar sdLDL distribution, but the AV diet tended to have fewer subjects with a LDL_{3+4} concentration higher than 40mg/dL compared to the MF diet.

The mean LDL particle size decreased on all three diets: LF (-0.24nm, p<0.0001), MF (-0.21nm, p<0.0001), AV (-0.12nm, p=0.008) (**Table 5-2**). The mean LDL particle size after the AV diet was significantly larger (p=0.03) than the LF diet (**Figure 5-9**). Using the Pearson correlation test, we found that the change in LDL particle size was highly correlated with the change in large LDL-P (r=0.68, p<0.0001); while the change in total LDL-P was highly correlated with the change in small LDL-P (r=0.62, p<0.0001) (**Figure 5-10**). The LDL subclass data from the NMR and VAP profile were generally in agreement with each other: the levels of

LDL_{1+2} and large LDL-P, LDL_{3+4} and small LDL-P were highly correlated at baseline (r=0.7, p<0.0001 for both); their change values on the diets also were modestly correlated (r=0.42 and 0.36, p<0.0001 for both) (**Figure 5-11**).

Table 5-1 Effect of diets on lipoprotein subclasses (cholesterol concentration from VAP test).

	Baseline (n=45)	LF (n=43)	MF (n=42)	AV (n=43)	Treatment Effect (p)
LDL (mg/dL)					
LDL_1	21.3 ± 1.2	19.4 ± 1.1[a]	18.4 ± 0.9[a,b*]	17.9 ± 1.0[b*]	0.03
LDL_2	29.7 ± 2.0	24.7 ± 2.5[*]	25.2 ± 2.1[*]	25.4 ± 2.2[*]	0.6
LDL_3	44.5 ± 2.4	42.2 ± 2.3[a,b]	43.4 ± 1.9[b]	40.0 ± 2.0[a*]	0.01
LDL_4	10.4 ± 1.3	12.6 ± 1.6[a*]	11.2 ± 1.4[a,b]	10.5 ± 1.3[b]	0.04
LDL_{1+2}	51.0 ± 2.8	44.1 ± 3.3[*]	43.6 ± 2.7[*]	43.2 ± 2.9[*]	0.9
LDL_{3+4}	55.0 ± 3.3	54.9 ± 3.5[a]	54.6 ± 3.0[a]	50.5 ± 3.1[b*]	0.01
LDL peak max time (s)	117.0 ± 0.5	115.8 ± 0.6[*]	116.2 ± 0.5[*]	116.4 ± 0.6	0.4
HDL (mg/dL)					
HDL_2	11.6 ± 0.7	10.5 ± 0.6[*]	10.9 ± 0.6[*]	10.8 ± 0.6[*]	0.6
HDL_3	36.9 ± 1.1	34.2 ± 0.9[a*]	36.1 ± 1.0[b]	36.0 ± 1.0[b]	0.01
VLDL (mg/dL)					
$VLDL_{1+2}$	9.9 ± 0.5	11.3 ± 0.8[a*]	9.5 ± 0.5[b]	9.1 ± 0.5[b]	<0.0001
$VLDL_3$	13.3 ± 0.4	14.2 ± 0.5[a*]	13.1 ± 0.5[b]	12.6 ± 0.4[b]	<0.0001
IDL(mg/dL)					
IDL_1	4.3 ± 0.3	4.7 ± 0.3[a*]	4.2 ± 0.3[b]	3.9 ± 0.2[b]	<0.0001
IDL_2	11.4 ± 0.5	11.1 ± 0.5[a]	10.5 ± 0.6[a,b]	9.8 ± 0.5[b*]	0.0005
Remnant Lp (mg/dL)	29.0 ± 1.1	30.0 ± 1.1[a*]	27.8 ± 1.2[b]	26.3 ± 1.0[b*]	<0.0001

All values are means ± SEMs. * Significant change compared to baseline AAD, P <0.05; [a/b] Values in diet treatments with different superscript letters are significantly different (Tukey post-hoc test by SAS, P<0.05).

Table 5-2 Effect of diets on lipoprotein particle size and subclasses (particle number from NMR Lipoprofile).

Lipoprotein Particle	Baseline (n=45)	LF (n=43)	MF (n=42)	AV (n=43)	Treatment Effect (p)
LDL-P, nmol/L					
Total	1145.7 ± 32.5	1168.1 ± 38.3[a]	1099.7 ± 32.3[b]	1054.6 ± 31.4[c*]	<0.0001
Large	476.8 ± 25.9	399.7 ± 28.8[*]	407.0 ± 26.4[*]	408.6 ± 20.2[*]	0.7
Small	557.3 ± 32.5	666.5 ± 41.5[a*]	615.9 ± 34.7[b*]	561.5 ± 31.6[c]	<0.0001
IDL-P, nmol/L	111.6 ± 8.7	102.0 ± 9.3[a]	76.8 ± 6.2[b*]	84.4 ± 7.9[b*]	0.0005
VLDL-P, nmol/L					
Total	62.2 ± 3.9	66.7 ± 4.4[a]	59.6 ± 3.9[a,b]	55.2 ± 4.3[b]	0.02
Large	2.9 ± 0.4	4.5 ± 0.8[a*]	2.5 ± 0.3[b]	2.7 ± 0.4[b]	0.001
Medium	24.2 ± 2.3	31.3 ± 3.1[a*]	24.3 ± 2.5[b]	23.5 ± 3.1[b]	0.02
Small	35.2 ± 2.1	30.9 ± 2.2[*]	32.9 ± 2.0[*]	29.0 ± 2.1[*]	0.1
HDL-P, nmol/L					
Total	30.7 ± 0.7	31.3 ± 0.7	31.4 ± 0.7	31.4 ± 0.6	0.7
Large	5.1 ± 0.4	4.3 ± 0.3[*]	4.6 ± 0.4[*]	4.6 ± 0.3[*]	0.2
Medium	10.3 ± 0.6	12.2 ± 0.8[*]	11.0 ± 0.7[*]	10.9 ± 0.7[*]	0.5
Small	15.3 ± 0.5	14.8 ± 0.6[a]	15.9 ± 0.5[b]	15.9 ± 0.6[a,b]	0.04
Average Particle Size, nm					
LDL	20.87 ± 0.08	20.62 ± 0.09[a*]	20.65 ± 0.08[a,b*]	20.74 ± 0.08[b*]	0.03
VLDL	44.62 ± 0.73	47.18 ± 0.98[a*]	45.04 ± 0.62[b]	45.41 ± 0.68[a,b]	0.02
HDL	9.04 ± 0.06	8.96 ± 0.05[*]	8.94 ± 0.06[*]	8.98 ± 0.06[*]	0.1

All values are means ± SEMs. * Significant change compared to baseline AAD, P <0.05; [a/b] Values in diet treatments with different superscript letters are significantly different (Tukey post-hoc test by SAS, P<0.05).

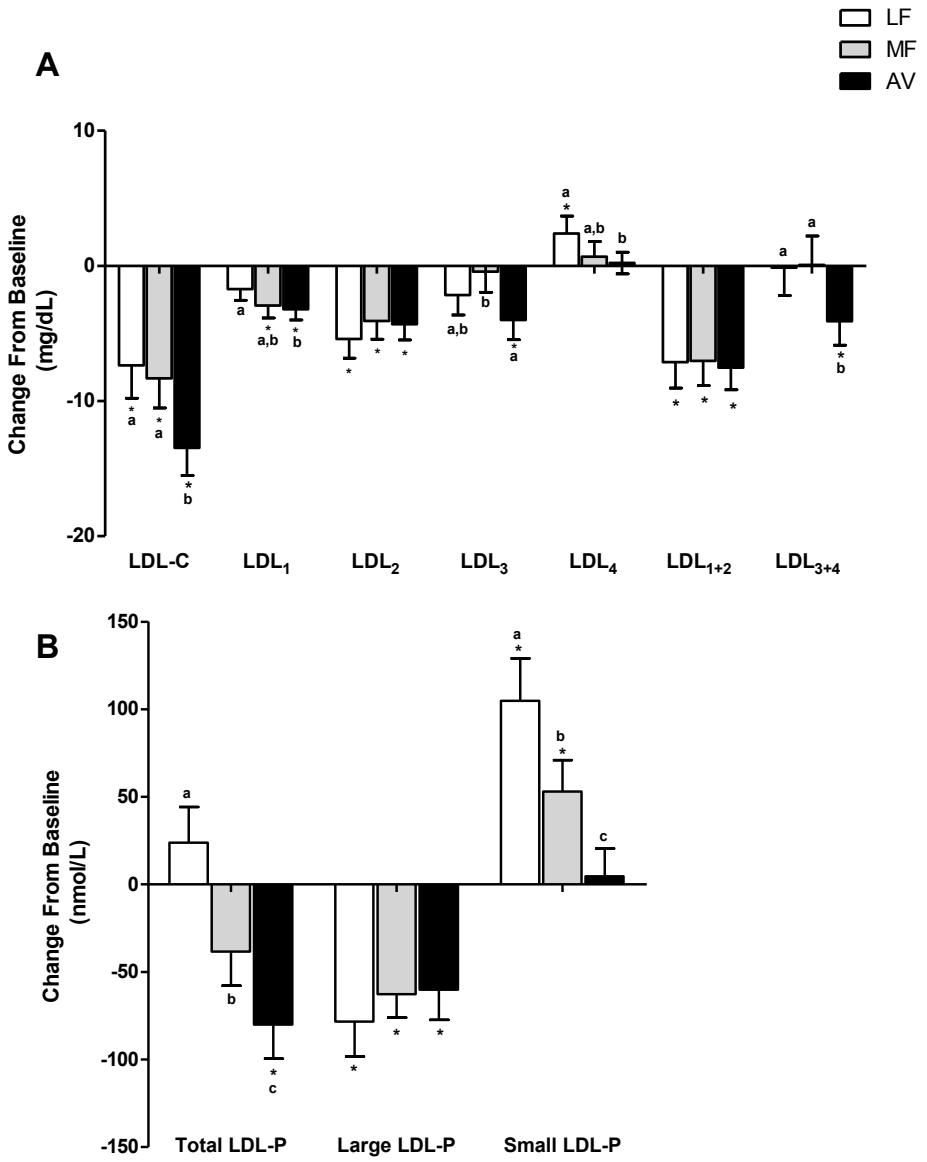


Figure 5-7 Change in LDL subclasses - mean change value (± SEM) from baseline.

A: Change in LDL cholesterol subclasses; **B**: Change in total, large and small LDL particle number. * Represents values that are significantly different from the baseline ($p<0.05$). Values with different letters are significantly different (Tukey post-hoc test by SAS, $P<0.05$).

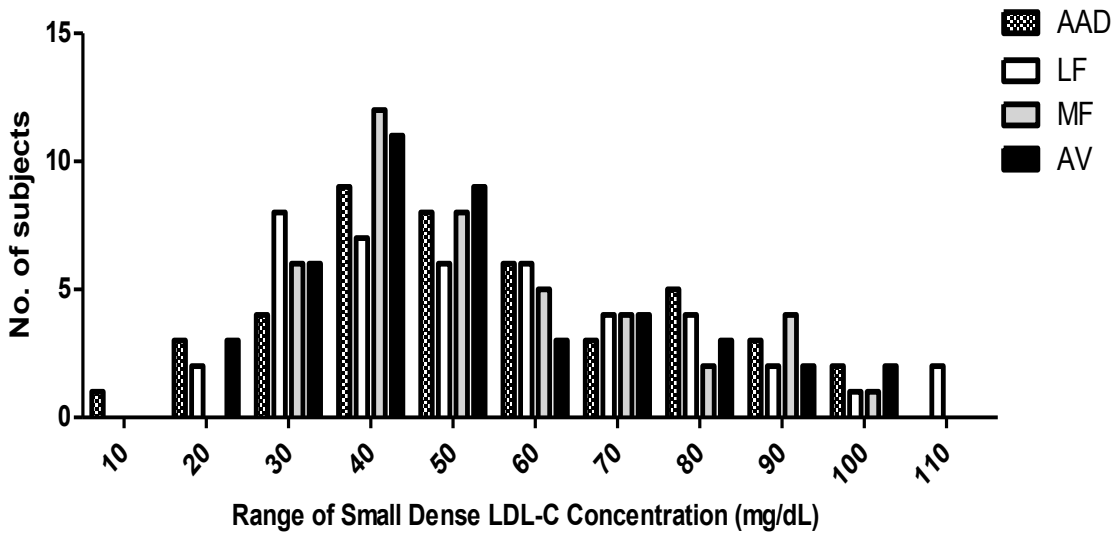


Figure 5-8 Distribution of LDL_{3+4} levels on different diets (histogram of frequency distribution).

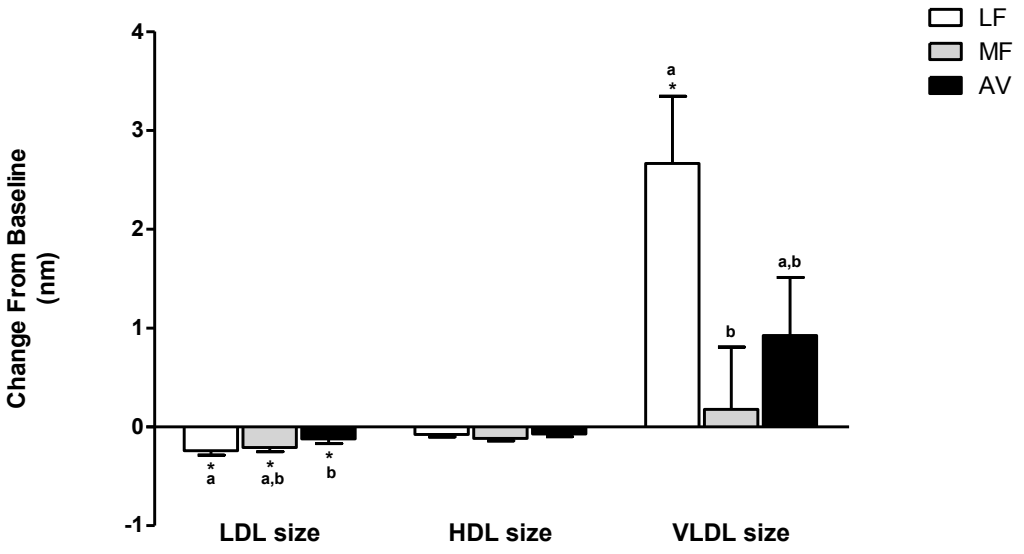


Figure 5-9 Change in average LDL, HDL and VLDL size (± SEM) from baseline.

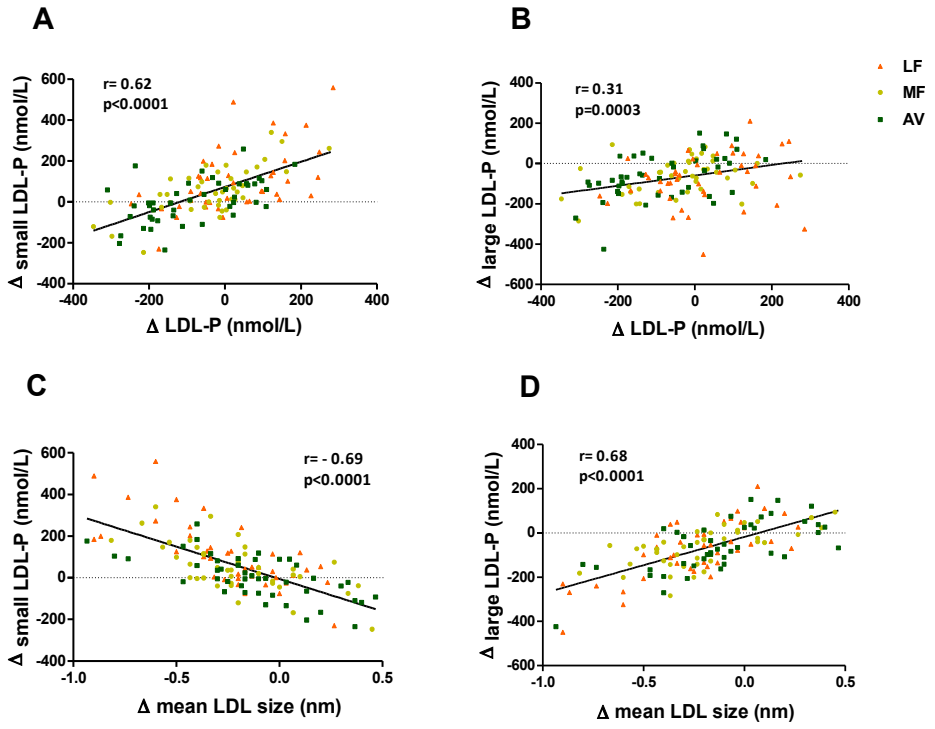


Figure 5-10 Correlation between the change in LDL-P subclasses and LDL particle size.

A. Correlation between the change in small LDL-P and the change in total LDL-P; B. Correlation between the change in large LDL-P and the change in total LDL-P; C. Correlation between the change in small LDL-P and the change in LDL particle size; D. Correlation between the change in large LDL-P and the change in LDL particle size.

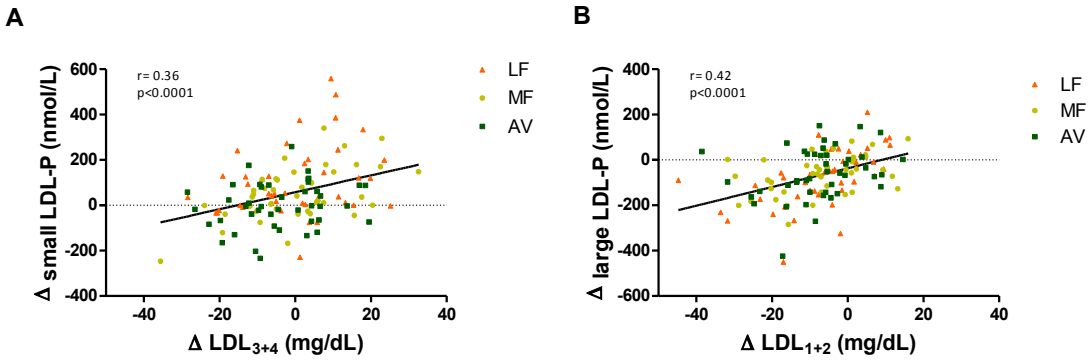


Figure 5-11 Correlation between the change in LDL-P subclasses and LDL-C subclasses.

A. Correlation between the change in small LDL-P and the change in LDL_{3+4}; **B**. Correlation between the change in large LDL-P and the change in LDL_{1+2}.

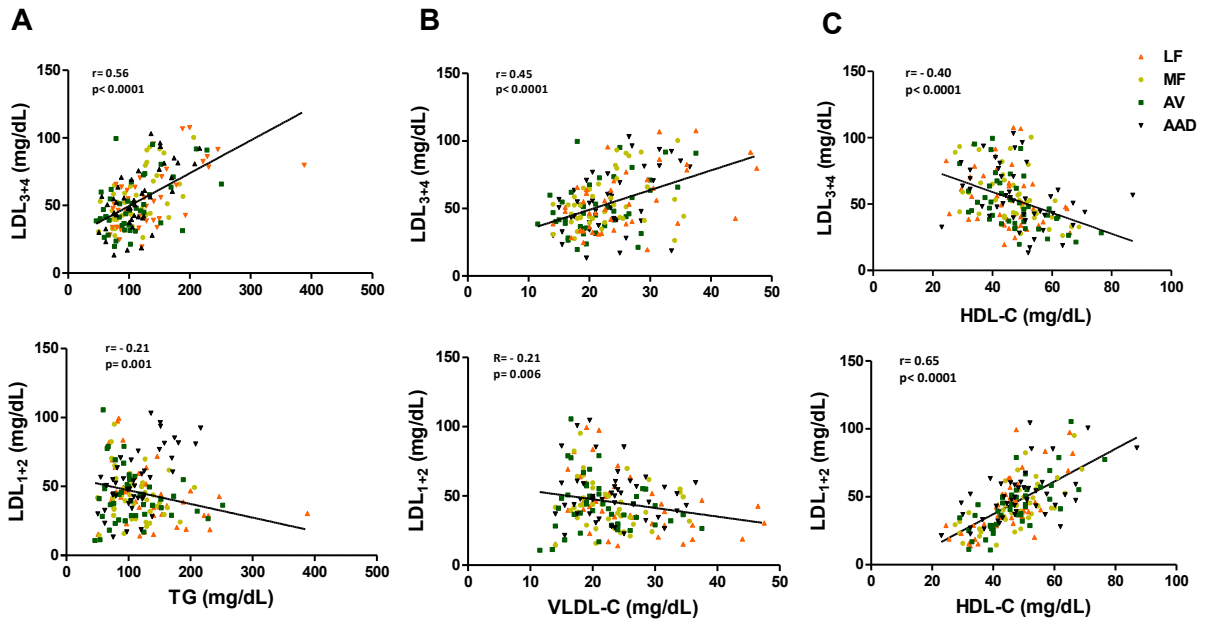


Figure 5-12 Correlations between LDL subclasses and TG, VLDL-C, and HDL-C.

A. Correlation between LDL3+4 and TG, LDL1+2 and TG; **B**. correlation between LDL3+4 and VLDL-C, LDL1+2 and VLDL-C; **C**. correlation between LDL3+4 and HDL-C, LDL1+2 and HDL-C.

VLDL subclasses

The LF diet significantly increased the cholesterol concentration in $VLDL_{1+2}$ (1.5 mg/dL, p=0.0003) and $VLDL_3$ (1.1 mg/dL, p=0.0007), whereas the MF and AV diets had no effect on $VLDL_{1+2}$ (**Table 5-1**, **Figure 5-13**). Also, both $VLDL_{1+2}$ and $VLDL_3$ were significantly higher after consumption of the LF diet compared to the other two diets ($p<0.05$). The concentrations of IDL, IDL_1 and IDL_2 after consumption of the LF diet were significantly higher than the MF and AV diets ($p<0.05$). Lipoprotein remnants ($VLDL_3$ + IDL) were significantly decreased by the AV diet (-2.6 mg/dL, p=0.004) and increased by the LF diet (1.5 mg/dL, p=0.02). The ratio of

apoA1/remnants was significantly decreased on the LF diet (-7.7%, p=0.01) compared to baseline and was significantly lower (p<0.01) than the MF diet and the AV diet.

The results in the change of VLDL subclass particle number were consistent with VAP data except for the significant decrease in small VLDL-P by the LF diet (-5.0 nmol/L, p=0.001); hence, unlike VLDL-C, the increase in total VLDL-P by the LF diet was not significant (4.0 nmol/L, p=0.12) (**Figure 5-13**). The AV but not MF diet significantly decreased small VLDL-P (-5.8 nmol/L, p=0.001), although the comparison between the MF and AV diets was not significant (p=0.2). Similar to $VLDL_{1+2}$, large and medium VLDL-P were significantly increased by the LF diet (1.6 nmol/L and 7.3 nmol/L, p<0.01 for both), while not affected by the MF and AV diets (**Figure 5-13**). The LF diet did not affect IDL-P, whereas the MF and AV diets significantly decreased IDL-P (-28.8 nmol/L and -24.7 nmol/L, p<0.01 for both). The mean VLDL particle size was significantly increased by the LF diet (2.7 nm, p=0.0002) and was significantly larger than the MF diet (p=0.01) but was not different from the AV diet (**Figure 5-9**).

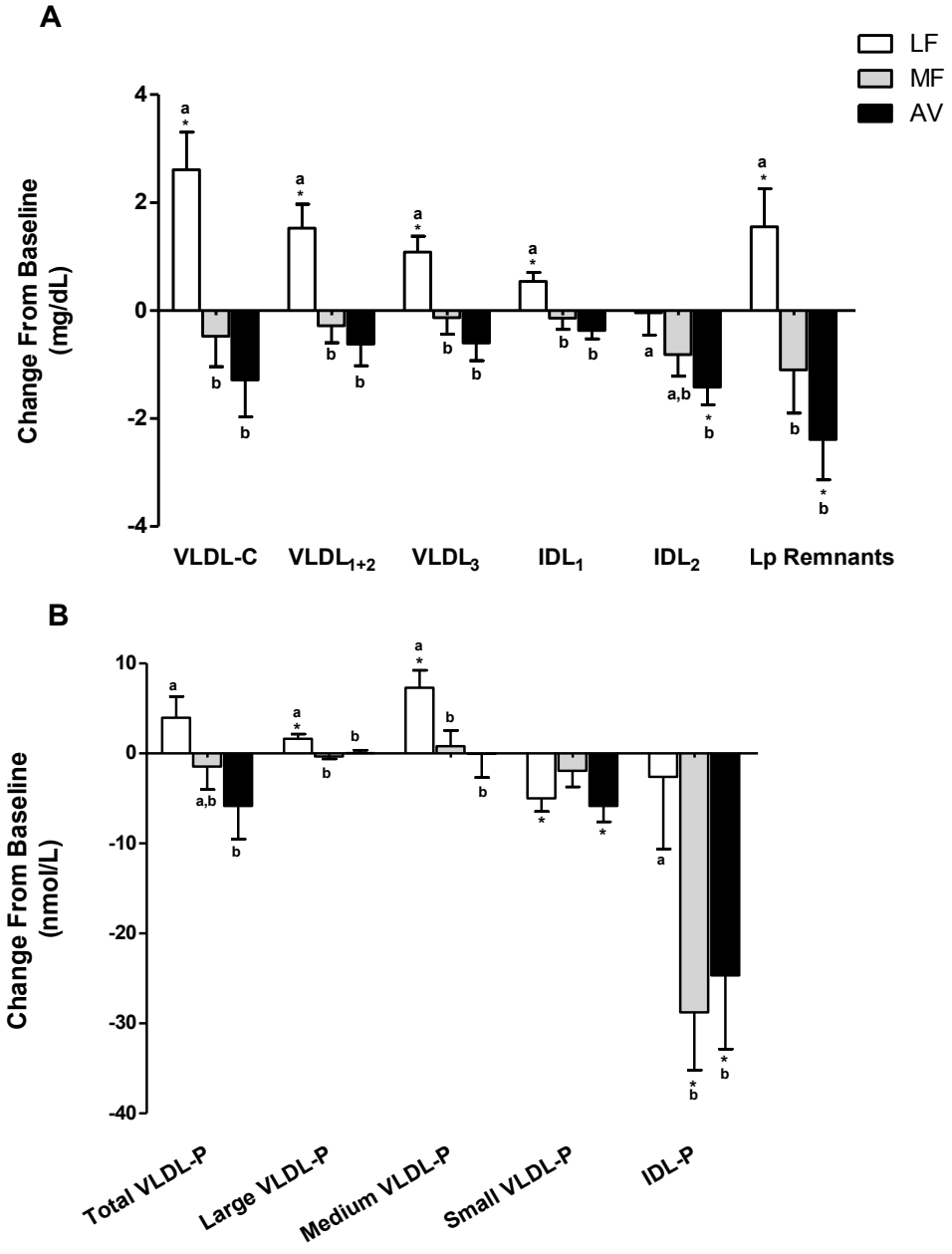


Figure 5-13 Change in VLDL and lipoprotein remnant particles - mean change value (± SEM) from baseline.

A: Change in VLDL and IDL subclasses cholesterol concentration; lipoprotein remnant particles consist of $VLDL_3$ and IDL. **B**: Change in VLDL and IDL subclasses particle number. *values are significantly different from the baseline ($p<0.05$). Values with different letters are significantly different (Tukey post-hoc test by SAS, $P<0.05$).

HDL subclasses

All three diets decreased HDL_2 similarly compared to baseline. However, HDL_3 was significantly decreased after consumption of the LF diet (-2.7 mg/dL, $p<0.0001$) and was not affected by the MF and AV diets (**Table 5-1, Figure 5-14**). Moreover, the concentration of HDL_3 after consumption of the LF diet was significantly lower ($p<0.05$) than the MF and AV diets (**Table 5-1, Figure 5-14**). Unlike HDL-C, total HDL-P was not significantly affected by all three diets. Large HDL-P was decreased similarly by all three diets ($p<0.001$); medium HDL-P was only affected by the LF diet (1.9 nmol/L, $p<0.0001$). There was a trend that small HDL-P after the LF diet was lower than the MF ($p=0.06$) and AV diets ($p=0.08$), which is consistent with the change in HDL_3. The mean HDL particle size was slightly decreased by the LF (-0.07nm), MF (-0.1 nm) and AV (-0.01 nm) diets compared to baseline ($p<0.01$ for all) (**Figure 5-9**).

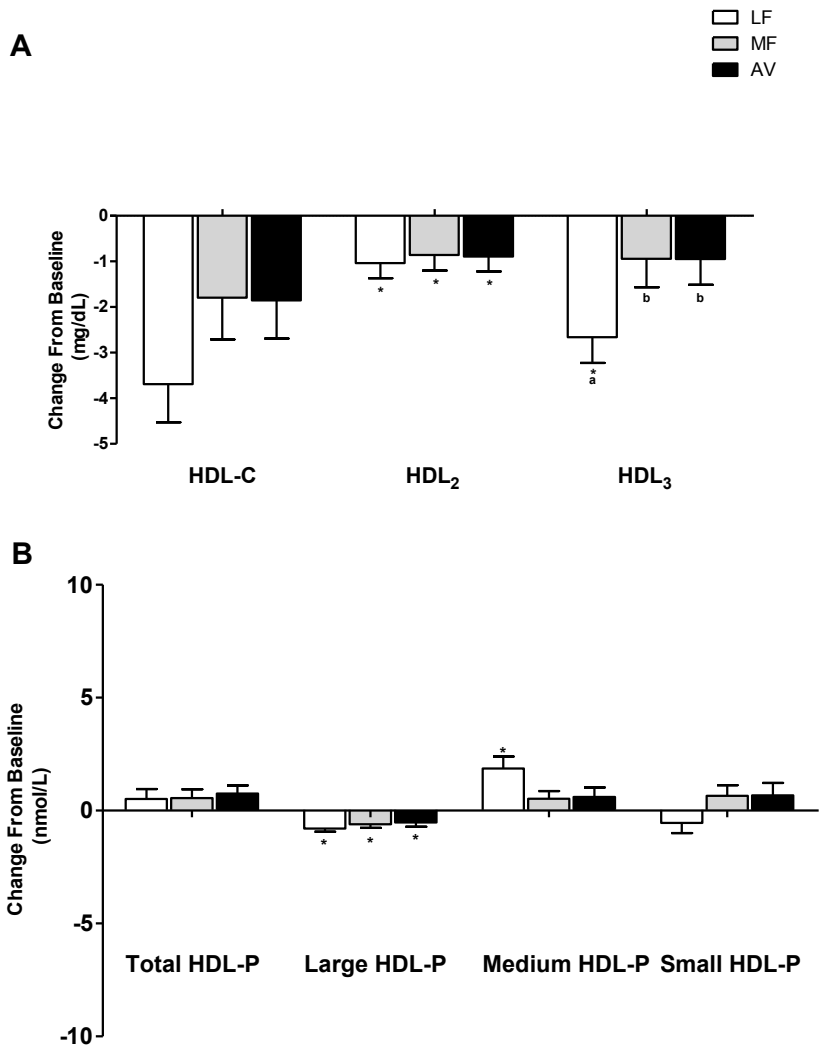


Figure 5-14 Change in HDL subclasses - mean change value (± SEM) from baseline.

A: Change in HDL subclasses cholesterol concentration. **B**: Change in HDL subclasses particle number. *values are significantly different from the baseline ($p<0.05$). Values with different letters are significantly different (Tukey post-hoc test, $P<0.05$).

Activity of lipid transfer proteins

Compared to baseline, the ex vivo CETP activity decreased on both the LF (-3.0 pmoles/µl/hr, p=0.02) and AV diets (-3.2 pmoles/µl/hr, p=0.0007) (**Table 5-3, Figure 5-13**). Only the comparison between AV and MF diet on CETP activity was significant (p=0.02). All three diets did not change PLTP or LCAT activity. We also found the change in CETP activity was significantly correlated with the change in small LDL-P (r=0.49, p= 0.0009) only during the AV diet. At the same time, the change in CETP activity was inversely correlated with the change in large HDL-P (r=-0.48, p=0.001) after the AV diet only (**Table 5-4, Figure 5-14**).

Table 5-3 CETP, PLTP and LCAT activity after consumption of the test diets

Lipid Transfer Protein Activity	Baseline (n=45)	Low Fat Diet (n=43)	Moderate Fat Diet (n=42)	Avocado Diet (n=43)	Treatment Effect (p)
CETP, pmoles/µl/hr	55.3 ± 1.4	52.8 ± 1.5^{*a,b}	54.4 ± 1.2^{*b}	52.1 ± 1.2^{*a,c}	0.02
PLTP, pmoles/µl/hr	20.3 ± 0.6	19.7 ± 0.7	20.0 ± 0.8	20.6 ± 0.7	0.5
LCAT, %change	6.2 ± 0.5	8.5 ± 0.7	8.0 ± 0.6	9.0 ± 0.7	0.5

All values are means ± SEMs. * Significant change compared to baseline AAD, P <0.05; $^{a/b}$ Values in diet treatments with different superscript letters are significantly different (Tukey post-hoc test by SAS, P<0.05).

Table 5-4 Pearson correlation coefficients between change in lipoprotein subclasses and CETP activity by the AV diet

Change value by AV diet from baseline	Δ CETP	Δ PLTP	Δ LCAT
ΔSmall HDL-P	0.03 (P=0.8)	-0.09 (P=0.56)	0.01 (P=0.9)
ΔLarge HDL-P	-0.48 (P=0.001)*	0.12 (P=0.4)	0.07 (P=0.6)
ΔSmall LDL-P	0.49 (P=0.0009)*	-0.08 (P=0.6)	0.05 (P=0.8)
ΔLarge LDL-P	0.09 (P=0.6)	0.09 (P=0.6)	-0.21 (P=0.2)

* Pearson correlation P <0.05.

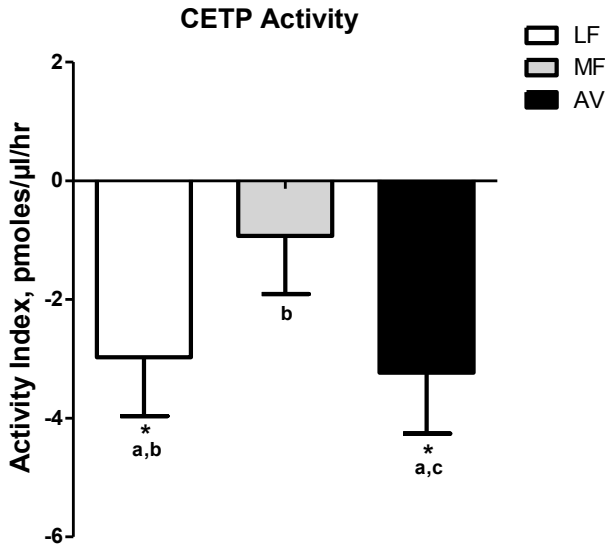


Figure 5-15 The change in CETP activity from baseline by the LF, MF and AV diets.

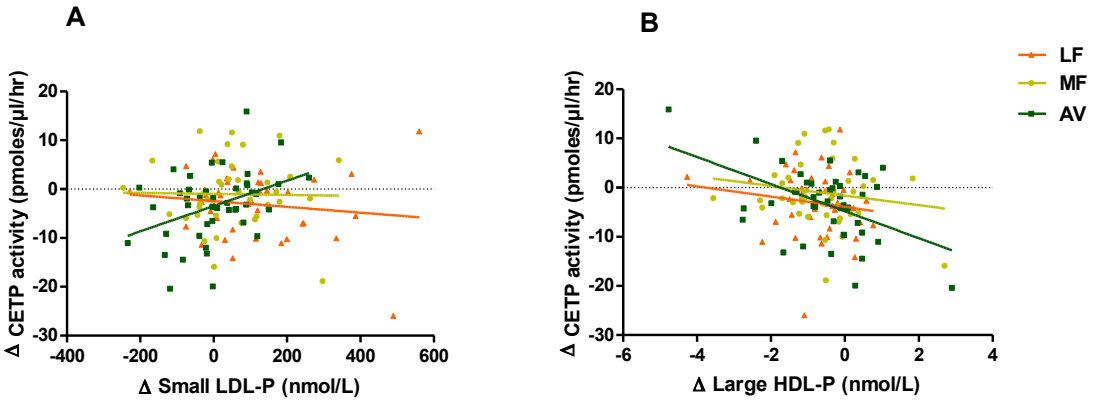


Figure 5-16 Correlations between change in CETP activity and change in LDL and HDL subclasses by three diets.

A: Correlation between change in CETP activity and change in small LDL-P. **B**: Correlation between change in CETP activity and change in large HDL-P.

Discussion

This is the first controlled feeding study using two advanced lipid-testing methods to evaluate lipoprotein subclass change by low fat versus moderate fat diets. We found discordance between change in LDL-C and LDL-P after consumption of the LF and MF diets. Both VAP and the NMR lipid profile showed that the LF, AV and MF diets decreased large, buoyant LDL ($LDL_{1+2,}$ large LDL-P) similarly; however only the AV diet elicited a significant reduction in sdLDL-C, lipoprotein remnants, and also had a lower small LDL-P concentration compared to the MF and LF diet.

The effects of the AV diet on sdLDL may be due to the combined effects of MUFA and other bioactive compounds, especially phytosterols and fiber. Avocados are the richest fruit source of β-sitosterol; one Hass avocado (136g) provides 114 mg of plant sterols, and 100g provides 2.11g soluble fiber and 2.7g insoluble fiber (97). Avocados also contain a unique seven-carbon (C7) ketosugar – mannoheptulose and its polyol form perseitol (about 4g per fruit), which may suppress insulin secretion and promote calorie restriction (102, 103). Sialvera et al. reported that plant sterol supplementation (4g per day for 2 months) decreased sdLDL in metabolic syndrome patients on a western diet (173); Lamarche et al. also reported that including plant sterols (1g per 1000 kcals), viscous fiber (9g per 1000 kcals), soybean protein and almonds in a low-SFA diet for 4 week decreased sdLDL cholesterol (-21%, $p<0.01$) in patients with mildly elevated LDL-C (174). Daily consumption of high-fiber oat cereal providing 14 g dietary fiber/day for 12 week decreased sdLDL-C (-17.3%, $p<0.05$) in overweight men (175). Although the combination of fiber and plant sterols in avocados may contribute to the effect, there may be other bioactive compounds and nutrients that also contribute because of the relatively lower dose of fiber and plant sterols delivered by one avocado compared to other supplementation studies. Dietary MUFA has been associated with lower sdLDL when substituted for SFA, although the

results reported are inconsistent (59, 176, 177). Gill et al. reported that LDL_3 was 25% lower after a high MUFA diet (13.7% energy) versus a low MUFA diet (7.8% energy) in hypercholesterolemic participants (176). Kratz et al. also reported that a high MUFA diet (38.7% energy from fat, 23.2% energy from MUFA) provided by refined olive oil significantly reduced LDL peak particle diameter (-0.36 nm, p=0.012) versus a high SFA diet (16% energy from SFA) (59). However, in another study there was no effect of replacing SFA with MUFA on LDL particle size (177). In the PREDIMED study, nuts but not olive oil supplementation in a Mediterranean diet significantly decreased small LDL-P (65). Nuts also are a good source of MUFA, phytosterols, and viscous fiber. However, the effect of nuts on sdLDL is unclear. Only one study showed that the addition of walnuts to free-living subjects' habitual diets elicited a 13% reduction in the number of sdLDL particles (178). Lamarche et al. showed that a dietary portfolio that incorporated plant sterols, soy protein, viscous fiber and almonds decreased sdLDL by 21%(174). Holligan et al. reported a significant decrease in sdLDL levels after consumption of a moderate fat diet including 2 servings/day of pistachios in healthy adults with high LDL-C (179). One explanation for the inconsistent results is that there might be some synergistic effects of the dietary fiber, plan sterols, MUFA, and other bioactive compounds or nutrients in specific foods and different background diets. Further studies are needed to characterize the bioactive compounds in avocados and investigate the mechanisms by which avocados affect sdLDL production.

The change in LDL particle size and subclasses by the MF and AV diets are different from some Mediterranean diet studies. In the PREDIMED study, the Mediterranean diet supplemented with nuts significantly increased LDL size (0.2 nmol/L [0.1; 0.4]), increased large LDL-P (53.8nmol/L,[18.1; 89.6]), and decreased very small LDL-P (-111nmol/L, [-180; -42]) in a sub-group of subjects (n=156) who were at high CVD risk (65). Richard et al. reported that

consumption of a Mediterranean diet for 5 weeks elicited a reduction in small LDL particles (-11.7%, $p<0.05$), and increased LDL peak particle size (0.18nm, $p<0.05$) compared to an isoenergetic North American control diet (5 weeks) (66). However, in our study, both MF and AV diets significantly decreased large LDL-P and mean LDL particle size. As shown in **Figure 5-10**, the decrease in mean LDL particle size was mostly due to the decrease in large LDL-P. These discrepancies may due to the higher total fat content as well as other beneficial food/nutrient components in the Mediterranean diet. In the PREDMED diet, the Mediterranean diet supplemented with extra virgin oil group did not have the same effects on small LDL-P compared to the nut treatment. Avocados and nuts share similar fatty acid profiles and several bioactive components including fiber and phytosterols, which may contribute to their additional sdLDL lowering effect. Longer term studies are needed to evaluate whether there is a similar "shifting effect" to atherogenic dyslipidemia (i.e., a decrease in small LDL-P, increase in large LDL-P and mean particle size) would occur after long-term consumption of avocados, especially in populations at high risk for metabolic syndrome. However, there is no evidence to suggest that decreasing large LDL-P without increasing small LDL-P or total LDL-P is harmful. Theoretically, the atherogenicity of LDL depends largely on particle number: how many LDL particles enter the artery wall, become modified, and are taken up by macrophage foam cells. sdLDL particles are more easily oxidized, which is consistent with current evidence that total LDL-P and sdLDL are associated with CHD independent of LDL-C concentration. Further analyses are needed to determine if avocados decreased the LDL particle oxidation/modification as well.

The predicted reduction in CHD risk by the AV diet (**Table 4-4 and Table 4-5**) needs further refined by considering changes in LDL-P, sdLDL, and lipoprotein remnants. Clinical studies have found that in individuals with discordant LDL-C and LDL-P levels, the LDL-

attributable atherosclerotic risk is better predicted by LDL-P. Although whether advanced lipid testing should be added to standard CVD risk assessment remains unclear, sdLDL and total LDL-P have potential value in monitoring efficacy of lifestyle intervention on CVD risk (180). In the present study, sdLDL concentration was correlated with an atherogenic lipid/lipoprotein profile while large, buoyant LDL was correlated with a more favorable lipid/lipoprotein profile (**Figure 5-12)**, which is consistent with the characteristics of atherogenic dyslipidemia. Although the AV diet did not reduce TG and increase HDL-C significantly, the reduction of sdLDL by the AV diet may be associated with an overall lower risk of athergenic dyslipidemia, thus, resulting in a decreased risk of metabolic syndrome and CHD.

Several studies have demonstrated that non-HDL-C is also a superior predictor of CVD risk compared to LDL-C due to the atherogenic effect of VLDL and LP remnant particles (181). VLDL and IDL, especially the small, dense Lp remnant particles, have been shown to be independently associated with the prediction, progression, and residual risk of CVD (153, 182). The ratio of apoA1 and lipoprotein remnants also has greater predictive ability for CHD risk than traditional lipid ratios in a recent study (183). In our study, there was a significant correlation between the changes in lipoprotein remnants and changes in TG, which indicates the increased production and impaired clearance of TG-enriched VLDL particles may due to an elevated TG level induced by the high carbohydrate intake on the LF diet. It has been suggested that TG-enriched VLDL decrease lipoprotein lipase activity, and increase apoCIII, which may impair the catabolism and clearance of VLDL particles, resulting in the accumulation of lipoprotein remnants. Consequently, CETP mediated TG enrichment and hepatic lipase mediated lipolysis of apoB-containing particles yields sdLDL and decreases HDL-C (155, 184). Further analyses are needed to explain the mechanism by which avocados modify apoB-containing lipoprotein particle metabolism.

HDL-C was decreased by all three diets in our study, but total HDL-P did not change. The MESA study reported that HDL-P but not HDL-C remained independently associated with cIMT and CHD after adjusting for LDL-P and other possible confounding markers (185). HDL subclass analysis indicated that the HDL-C lowering effect of the LF diet reflected a decrease in HDL_3, which is important for cholesterol efflux and may be up-regulated by MUFA (186, 187). Recent evidence has shown that small, dense HDL_3 particles mediate protection of LDL against oxidation and attenuate apoptosis in endothelial cells (188-190). However, it is still unclear which HDL subfractions may better determine CVD risk (181). Further analyses are needed to determine if HDL functionality, such as cholesterol efflux capacity, is improved after consumption of the AV diet.

The reduction on CETP activity by the AV diet might offer a potential mechanism by which avocados decrease sdLDL since CETP mediated TG enrichment and hepatic lipase mediated lipolysis of apoB-containing particles yields sdLDL and decreases HDL-C. In the present study, CETP activity was positivity associated with sdLDL while negatively associated with large HDL-C. In addition, the correlation existed only for the AV diet. Although studies have shown that oleic oil may lower CETP concentration (191), the MF diet in our study did not affect CETP acitivity. It might be because of the CETP activity reduction by the AV diet was due to other bioactive compounds in avocados, e.g., fiber and phytosterols, rather than the oleic acid. A recent study used the same Roar protein activity kit to measure CETP activity and found CETP activity was directly associated with serum triglycerides and inversely with HDL-C, and was associated with pro-atherogenic reductions in HDL and LDL particle size (191), which agree with our results for the AV diet.

The LF diet also lowered CETP activity, while the MF diet did not. However, the change in CETP by LF diet was not significantly correlated with changes in lipid makers. The reduction

of CETP by the LF diet may be involved in other metabolic pathways. One possible explanation is that because the concentration of TG enrichment VLDL and sdLDL was elevated on LF diet, the CETP had been utilized for TG-CE exchange between lipoproteins in vivo and performed a reduced activity in the ex-vivo assay. The causal relationship between changes in CETP activity and plasma lipid concentrations induced by the diet is not clearly established. One study showed both low-fat and high–monounsaturated fatty acid diets decrease plasma CETP concentrations in young, healthy, normolipemic men (77), which is consistent with our findings. Another possible explanation is that the sdLDL production in response to the LF diet was from the impaired VLDL clearance (in response to high TG) and the conversation of Lp remnants, other than the exchange of TG and CE directly between VLDL and LDL by CETP. Since the LF diet decreased total cholesterol production via reduction of SFA, the cholesterol may alter the intracellular cholesterol-regulatory pool in hepatocytes and therefore decrease CETP secretion in parallel (192). Further studies are needed to investigate the metabolic pathways by which diets affect lipoprotein subclass metabolism.

Our findings on LF diet are in consistent with previous studies. We observed elevated TG by the LF diet, with 10% higher in CHO compared to the baseline AAD, even with higher intake of whole grains, fruits and vegetables. Furthermore, LF diet increased small LDL-P and LP remnant particles. The change on lipid profile by LF diet was associated with higher risk for atherogenic dyslipidemia related to metabolic syndrome. However, metabolic syndrome and atherogenic dyslipidemia in western society are usually associated with high-fat, typical western diet and obesity (7, 193). High-fat western diet induces increased production of VLDL-TG and VLDL particles; whereas a high-CHO diet induced hypertriglyceridemia may have a different metabolic and kinetic basis (194). Hellerstein (195) suggested that the two major macronutrient energy resources (CHO and fat) have independent regulation patterns. Hepatic de novo

lipogenesis may be involved in CHO-induced hypertriglyceridemia, but that the conversion rate of CHO to fat remains low. The major responses to the increased CHO intake are increased glycogen storage and increased whole body CHO oxidation, not increased CHO conversion to fat. Sidossis & Wolfe proposed "the Randle cycle reversed" model that the macronutrient regulatory system appears to match fuel selection to recent CHO intake (196). Associated with the body fuel selection adapted to dietary CHO energy, there is a suppression of adipose lipolysis, increased serum insulin, and reduced percent of lipolytic flux oxidized (196). This model explains why the direct metabolic change to dietary CHO intake relates to plasma lipids more than plasma glucose in our study.

The composition of different types of CHO also determines the metabolic responses to high CHO intake. Simple sugars, especially fructose, have been shown to be a potent stimulator of hepatic lipogenesis (197). Fiber can mitigate the effects of high-CHO diets (198). Therefore, there may be lipogenic or non-lipogenic high CHO diets. Previous studies showed high CHO diets (70%) that were composed predominantly of simple sugars (60%) induced great fasting hypertriglyceridemia and a substantial increase in fractional de novo lipogenesis in the post-absorption state. However, solid food diets composed predominantly of complex CHO also increase fasting TG but not hepatic de novo lipogenesis (199). The LF diet in our study also contained more sugar than the other diets (AAD: 93g, LF: 137g, MF: 109g, AV: 108g), while the increased sugar content was mainly from the added grain products (muffin, granola, bread roll, etc). Although we did not measure hepatic de novo lipogenesis, the LF diet in our study was comprised predominantly of complex CHO, which may increase fasting TG due to the body fuel selection adapted to the increased energy from CHO.

Several studies have compared the kinetics of assembly, production and clearance of elevated VLDL-TG in response to low-fat, high-CHO diets versus the high-fat diets, although with inconsistent results. It is well established that high-fat western diet induces a high-flux state that is characterized by increased production of VLDL-TG and VLDL particles (194). The key question is that whether high CHO intake induced hypertriglyceridemia is due to increased production or reduced clearance of VLDL-TG. It has been suggested that TG-enriched VLDL particles can also be enriched in apoCIII, which inhibits their lipolysis and receptor-mediated clearance, resulting in the greater plasma residence time, accumulation of LP remnants that are further metabolized to small dense LDLs (200). The proposed schema for origins and metabolism of major LDL subclasses described by Krauss is shown in **Figure 5-17.**

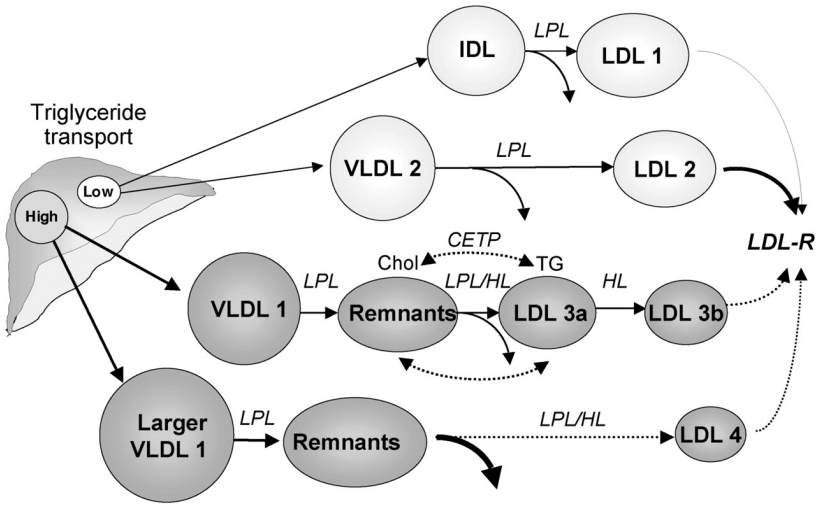


Figure 5-17 Schema for origins and metabolism of major LDL subclasses.
LPL: lipoprotein lipase; HL: hepatic lipase; LDL-R: LDL receptor. Source: Ronald Krauss, 2005 (200), obtained with reprint permission.

Parks et al. (199) studied hepatic production of VLDL-TG and VLDL-apoB kinetics on a high fat diet (35% fat and 50% CHO) versus a low fat diet (15% fat and 68% CHO), with 45% of CHO as simple sugars in both diets on normolipidemic (n=6) and moderately

hypertriglyceridemia (n=5) subjects. They concluded that the major effect of high-CHO diet on the VLDL-TG kinetics was to reduce clearance and prolong half-life without significant effects on production rate. However, the high-fat diet induced a higher production rate of VLDL-TG in subjects with hypertriglyceridemia compared to normolipidemic subjects, but without significant effects on VLDL-TG clearance (199). It appeared that high-CHO diet induced elevated plasma TG due to a reduction on VLDL clearance and not VLDL-TG production. In contrast, Mittendorfer and Sidossis (201) reported different kinetic results of CHO-induced hypertriglyceridemia on 6 normolipidemic subjects. After receiving a very low fat/high CHO diet (10% fat and 75% CHO, 47% simple sugar) or a very high fat/low CHO diet (55% fat and 30% CHO, 40% simple sugar) for 2 weeks, TG production was 65% higher on the high CHO than high fat diet, but with no differences in clearance. This study concluded that the increase in plasma TG on the high-CHO diet was due to over-production and not reduced clearance of VLDL. Compared to the study conducted by Parks et al., Mittendorfer and Sidossis (201) compared more extreme diets and studied normolipdemic subjects with very low baseline TG concentrations (45 mg/dL) in only 2 weeks, which may bias the results.

In summary, high-CHO diets can influence both production and clearance of VLDL-TG, but their potential atherogeneicity may differ from high-fat diet induced hypertriglyceridemia. In our study, LF diet decreased LDL_1 and LDL_2, which may due to larger LDL particles' higher LDL receptor affinity and the increased LDL receptor activity (because of reducing SFA intake). The LF diet increased TG, VLDL particle size, $VLDL_{1+2}$, large and medium VLDL-P; it appears that LF diet increased TG-enriched large VLDL particles as demonstrated in **Figure 5-17**. At the same time, LF diet increased LP remnants (IDL_1 and $VLDL_3$, not smaller IDL_2), LDL4, and small LDL-P. Based on the model proposed by Krauss (**Figure 5-17)**, it is reasonable to conclude that the LF diet increased TG production, yielding large TG-enriched VLDL particles, consequently

reduced VLDL clearance, resulting in larger LP remnants which were further modified to LDL_4 via lipolysis. It also explains why the LF diet lowered CETP activity and did not increase the concentration of LDL_3. However, the in vitro CETP activity test may not be representative of the actual in vivo CETP level and activity. A lipid kinetic study is needed to determine the metabolic pathways that generate sdLDL by the LF diet. Although the generation of TG-enriched large VLDL particles by the LF diet may have a different mechanism versus the high-fat western diet, it appears the LF diet tended to increase atherogenic risk due to the greater plasma residence time of LP remnants and sdLDL.

Conclusions

A high MUFA, moderate fat diet that included one avocado per day achieved greater reductions in sdLDL-C and LDL-P than a similar high MUFA, moderate fat diet without avocados. Thus, inclusion of one avocado per day as part of a moderate fat, cholesterol-lowering diet has additional beneficial effects on lowering LDL-P, especially on more atherogenic small, dense LDL particles. The moderate fat diet include one avocado a day also lowered smaller LP remnants (IDL_2), CETP activity, and LDL_3, suggested that avocado may decrease sdLDL via suppressing CETP, and hence, improving the clearance of VLDL particles. A high MUFA, moderated fat diet without avocados did not lower LDL-P significantly compared to a high-SFA AAD. Our results are the first to demonstrate that avocados have beneficial effects on more atherogenic lipoprotein subclasses, and these effects extend beyond the fatty acids in avocados. Our results demonstrate the importance of selecting nutrient-dense food sources of dietary MUFA to achieve a cardio-protective, moderate fat diet.

We found a lower fat, high complex CHO diet has a discordant effect on LDL-C and LDL-P because it increased sdLDL. Lipoprotein subclass analysis suggest the lower fat diet reduced VLDL clearance, yielded larger LP remnants, and further modified remnants to become small, dense LDL particles. The change in sdLDL concentration was correlated with an atherogenic lipid/lipoprotein profile. In conclusion, our study suggests that a lower fat, high complex CHO diet may increase atherogenic risk in otherwise healthy, overweight and obese individuals. However, further studies are needed to determine the net effect of the lower fat diet on multiple cardio-metabolic risk factors.

Chapter 6

Avocado Consumption Increases Plasma Antioxidants and Lowers Serum Oxidized-LDL in Overweight and Obese Adults

Abstract

Avocados are a nutrient dense source of MUFA that are high in antioxidants. The research presented herein has shown that avocados have an additional LDL-C lowering effect beyond the MUFA content, especially on small, dense LDL particles, which are more susceptible to oxidized in vivo. However, studies about the effect of avocados on oxidative status are lacking.

A randomized, crossover, controlled feeding trial was conducted with 45 overweight or obese participants with baseline LDL-C in the 25th to 90th percentile. Three cholesterol-lowering diets (6% to 7% SFA) were fed (5 weeks each): a lower-fat diet (LF: 24% fat); 2 moderate-fat diets (34% fat) provided similar foods and were matched for macronutrients and fatty acids: the avocado diet (AV) included one fresh Hass avocado (136 g) per day, and the moderate-fat diet (MF) mainly used high oleic acid oils to match the fatty acid content of one avocado. Compared to the baseline, only the AV diet decreased circulating oxidized LDL (oxLDL, -7.0 U/L, -8.8%, $p=0.0004$) while the LF diet (-1.6 U/L $p=0.1$) and the MF diet (-3.2 U/L, $p=0.2$) did not affect oxLDL significantly. Moreover, after consumption of the AV diet, plasma oxLDL was significantly lower ($p=0.05$ and 0.03) than the MF diet and LF diets. HPLC analysis indicated that only the AV diet increased plasma lutein by 68.7% from baseline ($p<0.0001$), and the increase in lutein was significantly greater than the increase observed for the MF (21.1%, $p=0.7$) and LF (37.6%, $p=0.1$) diets. Both MF and AV diets significantly increased plasma α-carotene (72.8% and 68.4%, $p<0.01$ for both) and β-carotene (15.4% and 12%, $p<0.05$

for both) from baseline. The LF diet did not change plasma antioxidant vitamins significantly, except for a decrease in γ-tocopherol (-7.8%, $p=0.03$). All three diets did not affect plasma F2-isoprotane significantly. The change in oxLDL was significantly correlated with the change in small LDL-P ($r=0.32$, $p=0.0002$) and small, dense LDL-C ($r=0.47$, $p<0.0001$) but not with the large LDL-P ($r=0.15$, $p=0.09$) or large, buoyant LDL-C ($r=-0.03$, $p=0.8$).

In conclusion, including one avocado per day into a heart-healthy diet reduced plasma oxLDL and increased lutein concentrations. Thus, these benefits are due to bioactive compounds in avocados. The change in oxLDL induced by the avocado diet was correlated with changes in sdLDL but not large LDL. Importantly, it appears that avocado lutein protects against LDL oxidation, which is of significance because of the cardio-protective benefits this would confer. In addition, a high CHO, lower fat diet meeting the USDA food group recommendations did not increase oxLDL even though it increased sdLDL particles. Our results demonstrate that avocados have beneficial effects beyond MUFA on decreasing the oxidation of atherogenic lipoproteins. Also, an increase in whole grain products, fruit and vegetable consumption in a lower fat, high CHO diet may protect the atherogenic lipoproteins from oxidation.

Introduction

Role of oxidative stress, oxidized-LDL particles in atherogenesis

The current model for the pathogenesis of atherosclerosis is based on several hypotheses, including vascular response to injury, vascular wall retention of LDL, and oxidative modification of LDL. Overall, atherosclerosis is proposed to be an inflammatory disease of arteries. Steinberg et al. (202) proposed the oxidatative modification hypothesis of atherosclerosis in 1989. Native LDL is not atherogenic since cholesterol uptake by LDL receptors is regulated by intracellular cholesterol (203). However, modified forms of LDL, such as acetylated LDL and oxidized LDL, can be taken up by macrophages by the scavenger receptor, leading to substantial cholesterol accumulation and foam cell formation because the scavenger receptor is up-regulated by oxidized LDL (204). During LDL oxidation, the phospholipids, free cholesterol, cholesterol esters, triglycerides, and apolipoprotein B are subject to oxidation. Cholesterol is converted to oxysterols; the polyunsaturated fatty acids in cholesterol esters, phospholipids, and triglycerides are subject to free radical-initiated oxidation and can participate in chemical reactions that amplify the extent of damage (205). As a result, hydroperoxides from cholesterol ester and phospholipid are formed. The breakdown of polyunsaturated fatty acids yields aldehydes and ketones that become conjugated to other lipids or to apoB (206). Also, there is direct oxidative damage to apoB during the process, which decreases the affinity of the LDL receptor for oxidized LDL (207).

LDL particles entering the artery wall can be oxidized by vascular cells (endothelial cells, smooth muscle cells, and macrophages) and oxidizing enzymes that include lipoxygenase and myeloperoxidase in the presence or absence of transition metal ions (208). Some minimally oxidized LDL can be recycled into the circulation and can be detected as oxidized LDL. The

minimally oxidized LDL particle is a direct and indirect chemoattractant for circulating monocytes, and leads to the release of phospholipids that can activate endothelial cells, which express selective adhesion molecules on the surface of arterial endothelial cells. In particular, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) bind monocyte and T lymphocyte, which are leukocytes found in the early atheroma. The leukocytes penetrate into the intima after adhesion to the endothelium by the migratory actions of monocyte chemoattractant protein -1 (MCP-1) and T-cell chemoattractants (209). After residing in the arterial wall, the macrophages express scavenger receptors for oxidized LDL and further differentiate into foam cells. Extensively oxidized LDL can be taken up by macrophages by the scavenger receptors, leading to the formation of foam cells (209). The activated macrophage produces inflammatory cytokines and leads to inflammatory processes and the evolution of a more advanced atherosclerotic lesion. Oxidized LDL also inhibits endothelial cell-dependent arterial relaxation and activates matrix-digesting enzymes that promote plaque instability (210, 211).

Oxidative stress biomarkers and plasma antioxidants in CVD risk assessment

Oxygen free radicals, also termed reactive oxygen species (ROS) are highly reactive and damaging to cells due to the unpaired electrons. An imbalance between ROS and antioxidants in favor of the former is defined as oxidative stress. Oxidative stress has been defined as one of the causative factors in the pathogenesis of CVD, including promoting the oxidation of LDL. Hence, high plasma oxidized LDL concentrations have been recognized as a marker of oxidative stress and CVD risk, and has been the most common oxidative stress marker used in longitudinal studies of CVD. More than 50% of these studies have shown that oxidized LDL was an independent predictor of CVD (212). Specifically, Toshima et al. have demonstrated that plasma

oxidized LDL concentration was a more specific and sensitive marker of CHD risk than TC, TG, HDL-C, LDL-C and TC/HDL-C ratio (213). Thus, adding plasma oxidized LDL to establish CVD risk factors may improve cardiovascular risk assessment in the primary and secondary prevention of CHD.

Biomarkers of oxidative stress represent the redox stress in the microenvironment. Besides oxidized LDL, there are several biomarkers of oxidative stress biomarkers that have been identified, such as lipid peroxidation markers (isoprotanse and malodialdehyde), myeloperoxidase, oxidative protein modifications (nitrotyrosine and S-glutathionylation), and serum antioxidant capacity. F2-isoprostanes, produced from lipid peroxidation of arachidonic acid in cell membranes and LDL particles are currently regarded as the most reliable biomarkers of lipid peroxidation in vivo (214). Oxidation of polyunsaturated fatty acids (PUFAs) in plasma lipoproteins has been proposed to be a critical step in the initial progress of atherosclerosis. Also, oxidized lipids are proatherogenic, in addition to oxidatively modified apoB lipoproteins (215). The generation of isoprostanes from arachidonic acid is primarily through a non-enzymatic process. Isoprostanes are released from the cell membrane into circulation by phospholipases, and can be quantified in tissues, blood and urine. Over the past few years, the measurement of F2-isoprostanes has emerged as one of the most sensitive and reliable biomarkers of lipid peroxidation in vivo and has been evaluated in numerous clinical trials of CHD risk. Several clinical trials demonstrated that F2-isoprostanes are associated with smoking, elevated LDL-C, diabetes, obesity, hypertension, and aging. As a result, F2-isoprostanes are an indicator of oxidative stress and a risk factor for CHD (215).

Plasma antioxidant levels also serve as an indicator of antioxidant status and have been associated with CVD risk in prospective studies. In the Women's Health Study (WHS), women in the upper quartiles compared with the lower half of plasma lycopene levels had a 34% lower risk of CVD (RR: 0.66; 95% CI: 0.47, 0.95) (216). In the Lipid Research Clinics Coronary Primary Prevention Trial and Follow-up Study (LRC-CPPT), men in the highest quartile of serum carotenoids had a 36% lower risk of CHD compared with men in the bottom quartile (RR: 0.64; 95% CI: 0.44, 0.92) after 13 years follow-up (217). In a European prospective study, higher plasma α- and β-carotene concentrations were associated with a lower mortality risk for CVD (RR: 0.83; 95% CI: 0.70, 1.00) after 10 years of follow-up in 1168 elderly men and women (218). Several cross-sectional studies have reported that serum carotenoids levels were inversely associated with inflammatory biomarkers including C-reactive protein (CRP), sICAM-1, and the oxidative stress biomarker F_2-isoprostane, while being positively associated with the antioxidant enzyme SOD (219-221).

Plasma antioxidant concentrations could serve as a measure of the intake of foods rich in antioxidants. Carotenoids are known to increase in plasma or serum in response to dietary interventions (222). Ziebland et al. have shown that an increase in self-reported fruit and vegetable intake increased concentrations of α-carotene, β-carotene, lycopene, β-cryptoxanthin, and ascorbic acid in plasma, and resulted in significant decreases in systolic and diastolic blood pressure (223). In prospective studies, participants with high concentrations of plasma antioxidants had a lower risk of coronary heart disease (223) and stroke (224). However, the clinical significance of the small increase in antioxidant concentrations is unclear.

Dietary factors and oxidative stress

Foods high in antioxidants have been suggested to protect LDL from oxidation. Many studies have evaluated antioxidant vitamin supplementation as well as dietary fatty acids. Supplementation with antioxidant vitamins such as α-tocopherol, vitamin C and β-carotene had been shown to be cardioprotective. However, RCTs using antioxidant vitamins supplements have yielded conflicting results (41, 42). A recent meta-analysis of RCTs of α-tocopherol supplementation found that α-tocopherol (400 IU/day) increased all-cause mortality (225). Also, clinical studies have not convincingly demonstrated that supplementation of α-tocopherol, vitamin C, or β-carotene can protect LDL from oxidation in humans or justify the routine use of antioxidant supplements for the prevention and treatment of CVD (41, 42).

Diet studies on LDL oxidation have shown that a MUFA-rich diet led to a higher resistance of LDL to oxidation compared to PUFA-rich diet, although the underlying mechanisms need further investigation. Omega-6 PUFAs have been shown to promote LDL oxidation, while the effects of omega-3 PUFA fish oil supplements on LDL oxidation remain unclear. Foods rich in polyphenols and antioxidant vitamins such as red wine, soy, nuts, grape juice, cranberry juice, olive oil, strawberries, green tea, cocoa drink, pistachios and almonds have been shown to protect LDL from oxidation (226-229) (230). Fruits and vegetables have been associated with lowered oxidative stress and decreased LDL oxidation (231). The combination these foods including fruits, vegetables, nuts, red wine, olive oil, and fish that contain omega-3 PUFA, are found in the Mediterranean diet that are associated with lower oxidized LDL concentrations (64, 232, 233). These results suggest that a combination of a variety of foods rich in dietary antioxidants is effective for decreasing oxidized LDL. However, it is not clear what all of the specific bioactive compounds are that confer these effects. Identification of specific foods that are high in

antioxidants is important for decreasing susceptibility of the LDL particle to oxidative modification, and thereby reducing CVD risk.

Rationale, objectives, and hypotheses of the current study

Our results (**Chapter 3 and 4**) suggest that the bioactive compounds in avocados beyond their MUFA content can beneficially affect the lipid and lipoprotein profile, especially the small, dense LDL and lipoprotein remnants. Small, dense LDL particles have greater propensity for transport into the sub-endothelial space, increased binding to arterial proteoglycans, and susceptibility to oxidative modification, which play an important role in the pathogenesis of atherosclerosis (208). Previous studies have shown that small, dense LDL particles are more susceptible to in vitro copper-induced oxidative changes (234), especially in patients with atherogenic lipoprotein phenotype, pattern B (235). Furthermore, compared to previous studies on vegetables, fruits, nuts, and Mediterranean diet, avocados are also a significant source of MUFA and are rich in antioxidants and polyphenols, however, their antioxidant effects have not been studied.

We also have shown that a high CHO, lower-fat diet increased small dense LDL particles, despite having LDL-C lowering effect. The traditional "CHO paradox" is based on the LDL-C lowering and TG/HDL-C raising effect of a low fat diet. Our preliminary results further extend the "CHO paradox" because of the discordance between the changes in LDL-C and LDL-P by low fat diet. The increase in TG and small LDL-P and the decrease in HDL-C by the lower fat diet in our study are similar to the metabolic syndrome-associated atherogenic dyslipidemia. However, metabolic syndrome is also associated with a higher concentration of oxLDL, as well as abdominal obesity, hyperglycemia, and hypertriglyceridemia. The key question for the "CHO

paradox" in our study is whether the lower-fat diet induced high sdLDL contributes to a greater LDL oxidation and, hence, induces the initial process of atherosclerosis.

The primary objective of this study is to investigate whether an avocado diet can decrease circulating oxidized LDL and oxidative stress. Furthermore, we evaluated if diet induced changes in sdLDL were associated changes in LDL oxidation. We hypothesized that including one avocado per day in a high MUFA, moderate fat diet will lower oxidized-LDL and overall oxidative stress, and increase plasma antioxidants.

Methods

Study Design

The clinical trial design and study protocol of the AVOCADO study has been described in Chapter 3. Briefly, a randomized, 3-period crossover study design was implemented. A two week "run-in" average American diet (AAD: 34% fat, 51% CHO, 17% PRO) was fed to participants before they were randomly assigned to a treatment sequence of three diet periods (5 week each) with a 2-3 week compliance break between diet periods. Participants were assigned to random treatment sequences that were generated by balanced permutations. Three cholesterol-lowering diets (6-7% SFA) were fed (5 weeks each): a lower-fat diet (LF: 24% fat, 59% CHO, 16% PRO); two moderate fat diets (34% fat, 49% CHO, 16% PRO) provided similar foods and matched for macronutrients and fatty acids: the avocado diet (AV) included one fresh Hass avocado (136 g) per day, and the moderate fat diet (MF) mainly used high oleic acid oils to match the fatty acid content of one avocado. The LF diet was designed by replacing 6 to 7% of energy from SFA with CHO (from grains that were incorporated in the diet in place of SFA) in the AAD.

Likewise, the AV and MF diets were designed by replacing 6 to 7% of energy from SFA with MUFA using either one Hass avocado (~136g fruit pulp, ~13g MUFA) per day (for the AV diet) or high oleic acid oils (e.g., sunflower oil and canola oil, for the MF diet) as the main sources of MUFA. To match the macronutrients and fatty acids in the MF and AV diets, and to adjust for the different calorie levels, high oleic acid oils, low fat cheese, and nuts were used in both diets. About 90% of foods in the two diets were identical. Thus, the major difference between the nutrient profiles of the AV and MF diets were due to the bioactive compounds beyond fatty acids from one avocado. Menus (six-day rotating) were developed using Food Processor SQL software (ESHA Research, Salem, OR) for six calorie levels (1800 to 3600 kcals) to meet participants' energy requirements. The Harris-Benedict equation with a physical activity factor was used to estimate each participant's basal metabolic rate (BMR) and daily energy requirements. All the menus are shown in Appendix A. Participants were weighed daily (Monday through Friday) to assess diet compliance and ensure that body weight was maintained. Participants were asked to maintain their habitual level of physical activity throughout the study. During the diet periods, participants were required to consume the foods provided only. During the clinical visits at baseline and at the end of each diet, fasting blood samples were collected by venipuncture in tubes containing EDTA, and plasma was obtained by centrifugation at 4°C. After separation, plasma was aliquoted to 0.5ml vials and stored at –80°C for up to 18 months. The samples did not undergo any thaw/freeze cycles during storage. Samples were thawed on ice before each assay.

ELISA assay for oxidized-LDL

The Mercodia Oxidized-LDL ELISA kit, a solid phase two-site enzyme immunoassay (Mercodia ab, Sweden) was used for the in vitro quantitative measurement of oxidized LDL-C in

plasma samples. The assay is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the oxidized apoB molecule. The specific murine monoclonal antibody mAb-4E6 was used, which was originally developed by Holvoet et al (236, 237). During incubation, oxidized-LDL in the sample reacts with anti-oxidized LDL antibodies bound to the micro-titration well. After washing, which removes non-reactive plasma components, a peroxidase conjugated anti-human apolipoprotein B antibody recognizes the oxidized LDL bound to the solid phase. After a second incubation and a simple washing step that removes unbound enzyme labeled antibody, the bound conjugate is detected by reaction with 3,3', 5,5'-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint, and then read spectrophotometrically. The detection limit is 0.6 mU/L. Plasma samples were diluted twice to 1/6561 and the final concentration was multiplied by the dilution factor. Assay CV was < 8%.

EIA assay for $F_{2\alpha}$-isoprostane

Plasma $F_{2\alpha}$-isoprostane (8-iso Prostaglandin $F_{2\alpha}$) was measured using Cayman's 8-Isoprostane EIA kit (Cayman Chemical, Ann Arbor, MI). It is a competitive assay that can be used for quantification of $F_{2\alpha}$-isoprostane in plasma, urine, and other sample matrices. The EIA displays an IC50 (50% B/B_0) of approximately 10pg/ml and a detection limit (50% B/B_0) of approximately 2.7 pg/ml. The assay is based on the competition between 8-Isoprostane and an 8-Isoprostane acetylcholinesterase (AChE) conjugate (8-Isoprostane tracer) for a limited number of 8-Isoprostane-specific rabbit antiserum binding sites(238). Because the concentration of the 8-Isoprostane tracer is held constant while the concentration of 8-isoprostane varies, the amount of 8-Isoprostane tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of 8-Isoprostane in the well. This rabbit antiserum 8-Isoprostane complex binds

to the rabbit IgG mouse monoclonal antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of free 8-isoprostane present in the well during the incubation. Assay CV was < 10%.

HPLC methodology for plasma antioxidants

Sample Preparation: 10 µl plasma samples were diluted with 140 µl water and 150 µl ethanol (with 0.1% BHT). Fat-soluble compounds were extracted with two 1 mL aliquots of hexane. After vortexing and centrifugation at 10,000 *g,* the supernatants were removed. The combined supernatants were dried using a Labconco CentripVap concentrator and cold trap system (Kansas City, MO) to remove the organic solvent. The residue was re-dissolved in ethanol spiked with 100 nM trolox (internal standard), vortexed, and filtered through a 0.45 uM PTFE filter prior to injection (VWR International, Radnor PA). Additional plasma samples were spiked with a mixture of commercial standards used to test for degradation during preparation and analysis. Samples were prepared in a relatively dark room with a little indirect light, and brown tubes were used to avoid light. Results of plasma samples spiked with a mixture of commercial standards showed no degradation during sample preparation and analysis.

HPLC Analysis: Tocopherols (α, δ, γ), carotene (α, β), lutein, and retinol were analyzed using an HPLC system consisting of two Shimadzu LC-20AD pumps (Kyoto, Japan), a Shimadzu SIL-20AC refrigerated autosampler (Kyoto, Japan), and an ESA 5500 coulochem electrode array system (CEAS). The potentials of the CEAS were set at 200, 300, 500, and 700 mV. The following commercial standards purchased from Sigma Aldrich (Bellefonte, PA, USA) were used for HPLC analysis: α-, δ-, γ-tocopherol, β-carotene, and retinol. Standard of lutein was purchased from Quality Phytochemicals (East Brunswick, NJ, USA) and standard of α-carotene was purchased from Chromadex (Irvine, CA, USA).

A Supelcosil LC-18 column (150 mm × 4.6 mm, 5 μm; Supelco, Bellefonte, PA, USA) was used for chromatographic separation. The mobile phases consisted of solvent A (20 ammonium acetate buffer, 41.25% acetonitrile and 33.75% ethanol, pH 4.4) and solvent B (20 mM ammonium acetate buffer, 52.25% acetonitrile and 42.75% ethanol, pH 4.4). Mobile phase conditions started at 0% B at 0.6 mL/min with isocratic flow for 2 min. The concentration of B increased to 100% (2-5 min), held at 100% B (5-55 min), and reduced to 0% (55-57 min). Flow rate started at 0.6 mL/min, increased to 1 ml/min (35-36 min), and returned to 0.6 mL/min (60-61 min). The column was re-equilibrated at initial conditions for 3 min prior to next sample introduction. Column temperature was maintained at 35˚C. Injection volume was 50 uL. Compounds were identified by retention time and compared to pure standards (purity ≥ 95%).

HPLC peak areas were calculated using CoulArray software (Thermo Fisher Scientific, Bellefonte, PA, USA). HPLC peak areas were converted to plasma concentration based on external standard curve regression analyses, and the linear regression analysis was carried out using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Statistical Analysis

Statistical analyses were performed with SAS (version 9.2; SAS Institute Inc.). The mixed models procedure (PROC MIXED) was used comparing the effects of the three diets on the change value (from baseline) of all outcome variables. Potential carryover effects were assessed by including diet sequence, period and diet-period interaction as a fixed effect in the model; age, BMI, sex, diet-sex interaction were included as covariates. The Shapiro-Wilk test was used to assess normality of residuals in the mixed model. Tukey post-hoc test was used to adjust for multiple comparisons of three diets. Correlations between plasma antioxidants and lipoprotein endpoints were determined using Pearson correlation coefficient analysis. Fisher's Z-transformation was used to compare the correlations among different diets.

Results

Plasma concentrations of antioxidants

Plasma concentrations of retinol, lutein, α-carotene, β-carotene, α-tocopherol, γ-tocopherol, and δ-tocopherol are shown in **Table 6-1.** The percentage changes of these by the LF, MF and AV diets from baseline are shown in **Figure 6-3**. Treatment effects of diets were only seen for lutein and α-carotene. Compared to baseline, only the AV diet increased plasma lutein significantly (19.6 nmol/L, 68.7%, $p<0.0001$), and the change was significantly greater ($p=0.0005$ and $p=0.008$) than for the LF (1.1 nmol/L, $p=0.7$) and MF (7.0 nmol/L, $p=0.1$) diets. Both MF and AV diets significantly increased α-carotene. The AV diet increased α-carotene by 68.4%

(17.2 nmol/L, p=0.03), and MF diet increased α-carotene by 72.8% (30.2 nmol/L, p<0.001). The LF diet had a trend to increase on α-carotene (25.3%, 7.1 nmol/L, p=0.3). The increase in α-carotene by the MF diet was significantly higher from the LF diet (p=0.009), but was not different from the AV diet (p=0.2). The changes in α-carotene by the AV diet and LF diet were not significantly different from each other either (p=0.3).

Both the MF and AV diets significantly increased β-carotene from baseline, but the changes in β-carotene were not different from the LF diet. The MF diet increased β-carotene by 15.4% (21.4 nmol/L, p=0.01), AV diet increased β-carotene by 12% (16.7nmol/L, p=0.045), while LF diet increased β-carotene with a non-significant trend (11%, 15.0nmol/L, p=0.09).

All three diets did not significantly affect plasma concentration of retinol, α-tocopherol, γ-tocopherol, and δ-tocopherol, except for a slight decrease in γ-tocopherol by the low fat diet (-7.8%, p=0.03). However, there was no treatment difference for γ-tocopherol.

Biomarkers of oxidative stress

Compared to baseline, only the AV diet significantly decreased the plasma concentration of oxidized LDL (-7.1 U/L, p=0.0004). The MF diet (-3.2 U/L, p=0.1) and the LF diet (-1.6 U/L, p=0.2) did not significantly decrease oxidized LDL, although there was a slight trend. Moreover, the reduction in oxidized LDL by the AV diet was significantly greater (p=0.05 and p=0.03) than for the MF and LF diets. All three diets did not significantly affect plasma F2-isoprostane significantly (**Table 6-1, Figure 6-4**).

Correlations between oxidative markers, plasma antioxidants, and lipoproteins

The change in oxidized-LDL was significantly correlated with the change in small LDL-P ($r=0.32$, $p=0.0002$) and small, dense LDL-C ($r=0.47$, $p<0.0001$) but not large LDL-P ($r=0.15$, $p=0.09$) or large, buoyant LDL-C ($r=-0.03$, $p=0.8$). No significant correlations were observed between the change in plasma antioxidants and oxidized LDL (**Figure 6-5**).

Table 6-1 Plasma concentrations of oxidative biomarkers and antioxidants at baseline and after consuming three diets.

Biomarker	[1]Baseline (n=45)	LF (n=43)	MF (n=42)	AV (n=43)	[2]Treatment Effect (p)
Retinol (nmol/L)	204.8 ± 9.2	213.4 ± 9.0	214.5 ± 9.7	200.1 ± 10.0	0.7
Lutein (nmol/L)	33.6 ± 2.7	34.7 ± 2.2[a]	40.6 ± 2.1[a]	53.2 ± 5.2[b*]	0.0004
α-carotene (nmol/L)	39.7 ± 5.0	46.8 ± 6.2[a]	69.9 ± 11.5[b*]	56.9 ± 8.3[a,b*]	0.01
β-carotene (nmol/L)	138.9 ± 9.5	153.8 ± 9.3	160.2 ± 13.2[*]	155.5 ± 11.4[*]	0.8
α-tocopherol (µmol/L)	13.0 ± 0.6	13.7 ± 0.6	13.3 ± 0.7	13.9 ± 0.9	0.8
γ-tocopherol (µmol/L)	2.4 ± 0.2	2.0 ± 0.1	2.0 ± 0.2	2.0 ± 0.3	0.5
δ-tocopherol (µmol/L)	0.3 ± 0.02	0.2 ± 0.01	0.3 ± 0.02	0.3 ± 0.02	0.9
oxidized LDL (U/L)	65.8 ± 2.4	64.3 ± 2.9[a]	61.8 ± 2.2[a]	58.0 ± 2.1[b*]	0.02
Isoprostane (pg/ml)	26.2 ± 1.7	27.1 ± 2.4	25.7 ± 1.5	25.2 ± 1.4	0.6

All values are means ± SEMs. * Significant change compared to baseline AAD, $P<0.05$; [a/b] Values in diet treatments with different superscript letters are significantly different (Tukey post-hoc test by SAS, $P<0.05$).

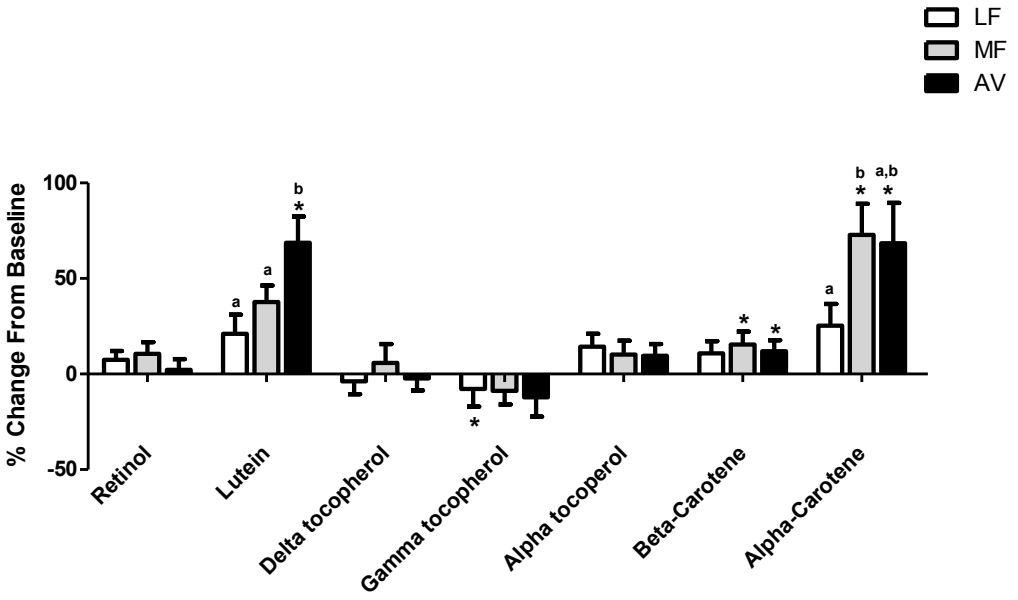


Figure 6-1 The percentage change of plasma antioxidants by LF, MF, and AV diets.

*values are significantly different from the baseline ($p<0.05$). Values with different letters are significantly different (Tukey post-hoc test, $P<0.05$).

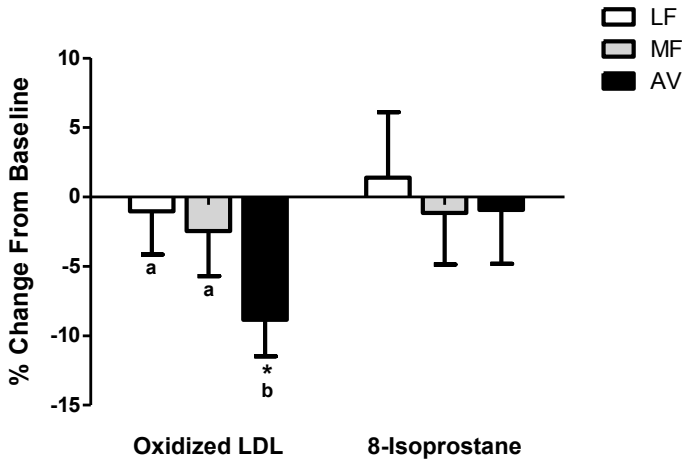


Figure 6-2 The percentage change of plasma oxidative markers by LF, MF, and AV diets.

*values are significantly different from the baseline ($p<0.05$). Values with different letters are significantly different (Tukey post-hoc test, $P<0.05$).

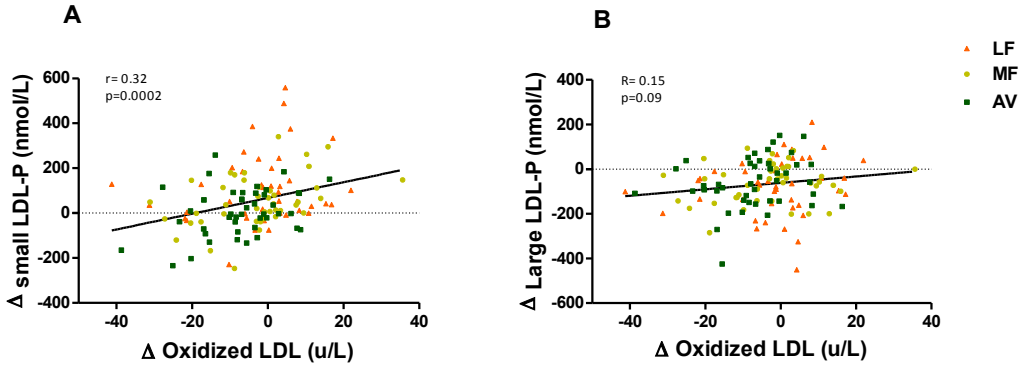


Figure 6-3 The correlation between change in oxLDL and LDL subclasses by LF, MF and AV diets.

A: The correlation between changes in oxLDL and small LDL-P. B: The correlation between changes in oxLDL and large LDL-P.

Discussion

This study is the first randomized controlled feeding trial to our knowledge to evaluate the effects of avocado consumption on biomarkers of oxidation. We found that a high-MUFA moderate fat diet including one avocado per day decreased plasma oxidized LDL significantly after 5 weeks of consumption. Furthermore, we found that the oxidized LDL lowering effect of avocados may due to bioactive compounds beyond fatty acids in avocados, since the moderate fat diet with a matched fatty acid profile did not decrease oxidized LDL. A lower fat, high carbohydrate diet, also higher in fruits, vegetables and whole grains, did not increase oxidized LDL compared to the baseline AAD diet, although it significantly increased small LDL particles.

The change in oxidized LDL was correlated with a change in small, dense LDL but not large, buoyant LDL, especially under the avocado diet. The change in plasma carotenoids did not affect oxidized LDL either. These findings suggest that avocados may decrease oxidized LDL by a mechanism that involves decreasing small dense LDL. This is consistent with the theory that small, dense LDL particles are more susceptible to oxidation. Several factors may influence the

susceptibility of LDL to oxidation, including its size and composition, and the presence of endogenous antioxidants. Small LDL particles are particularly atherogenic as they penetrate the vessel wall more easily than larger LDL particles. Furthermore, small dense lipoprotein particles are more likely to be retained by the extracellular matrix as they have been shown to bind to intimal proteoglycans in vitro (239). LDL particles can contain several antioxidants, such as α-tocopherol (Vitamin E), ubiquinone, and the carotenoids, β-carotene and lycopene. Lower levels of α-tocopherol and ubiquinone have been reported in small dense LDL particles compared with buoyant LDL (240). It has been suggested that surface lipid fluidity and composition may account for the greater susceptibility of small dense LDL to oxidation too (241). Small dense LDL particles that are depleted of free cholesterol have been reported to be less resistant to oxidation (242). Tribble et al. reported that the resistance time to in vitro oxidative challenge of LDL particles was not related to the alpha-tocopherol, beta-carotene, protein, triglycerides or phospholipids content in LDL but was significantly correlated with unesterified cholesterol content ($r = 0.46$; $P < 0.001$) and was inversely associated with cholesterol ester content ($r = 0.28$; $P < 0.05$) (234). This suggests that un-esterified cholesterol in LDL may impart resistance to oxidative modification, possibly by altering properties on the surface monolayer where they reside. This may partially explain the lack of correlation between oxidized LDL and plasma antioxidants in our study.

Based on the change of LDL particles after AV diet, the reduction of oxidized LDL by avocado consumption could be a reflection of a reduction in small, dense LDL particles. However, the result of the LF diet contradicts this hypothesis because although the LF diet significantly increased small LDL-P and total LDL-P, it did not increase oxLDL. oxLDL is also associated with metabolic syndrome; the LF diet decreased HDL-C, increased sdLDL and TG, which is similar to atherogenic dyslipidemia in metabolic syndrome, but it did not increase

oxLDL. The increased intake of whole grains, fruits and vegetables in the LF diet may have had an antioxidant effect and compromised the effect of LF diet on LDL-P and small LDL-P. Fruits and vegetables have been associated with lowered oxidative stress and decreased LDL oxidation (231). In our study, LF and MF diets both are higher in fruits vegetables compared to AAD (**Table 3-1**), but the oxLDL concentration by LF and MF diet did not change significantly. It might due to the short-term intervention of the study as well as the effect on total LDL particles numbers of LF and MF diets.

Different methods have been utilized to assess LDL oxidation. Methodological considerations are important to evaluate the predictive value of LDL oxidation as a biomarker of CVD risk. Most previous studies used the in vitro method measuring the lag time for isolated LDL particles to resist copper (Cu^{2+}) – induced oxidation. Since antioxidants present on the surface of LDL are consumed during an oxidative challenge, a longer lag time reflects a higher resistance of LDL particles to oxidation (243). Immunochemical methods have been developed to determine in vivo circulating oxLDL concentrations in human plasma samples using monoclonal antibodies. The two major antibodies that have been developed to detecting circulating oxLDL are FOH1a/DLH3 and 4E6. The FOH1a/DLH3 recognizes oxidized phosphatidylcholines generated during oxidative modification of LDL and is used in combination with an anti-apoB antibody to identify apoB-containing particles modified by oxidized phosphatidylcholines. The monoclonal antibody 4E6 is directed against a conformational epitope in the apoB-100 moiety of LDL (236). Clinical trials demonstrated that circulating oxLDL is a more sensitive and reliable biomarker of CVD risk compared to the in vitro susceptibility to oxidation of LDL. Although none of these assays are currently approved for routine clinical practice, circulating oxLDL is a useful biomarker for CVD risk prediction in clinical trials.

Circulating oxLDL is elevated in patients with advanced atherosclerosis, such as coronary heart disease and ischemic stroke, several case-control studies have reported high plasma oxLDL concentrations in patients with CHD or elevated risk of CHD compared to healthy individuals (213, 244-246) and also reflects early atherosclerotic changes and metabolic disorders including diabetes, obesity, and metabolic syndrome (193, 213, 247-250). It is hypothesized that the increase of circulating oxLDL in metabolic syndrome and diabetes is due to the occurrence of sdLDL, which is associated with obesity and insulin resistance. In the EPIC (European Prospective Investigation of Cancer)-Norfolk cohort of 45- to 79-year-old apparently healthy men and women followed for ~6 year, After adjusting for age, smoking, diabetes, lipids, and systolic blood pressure, the highest tertiles of oxidized phospholipids on apolipoprotein B-100 particles and Lp(a) were associated with a significantly higher risk of CAD events (odds ratios: 1.67 and 1.64, respectively; $p < 0.001$) compared with the lowest tertiles (251). Toshima et al. have demonstrated that plasma oxLDL concentration was a more specific and sensitive marker of CHD risk than TC, TG, apoB and HDL-C concentrations in patients with established CHD. Also, there was a lack of association of oxLDL with hypertention, hyperlipidemia, smoking, age and gender in that study, suggesting that raised oxLDL levels might be an independent risk factor for CAD (213). Suzuki et al. also reported that circulating oxLDL concentrations were a better marker of CHD in high risk patients than TC, TG, HDL-C, LDL-C, lipoprotein (a) concentrations and TC/HDL-C ratio (246). However, In the Asklepios Study, a Belgian cohort of 2524 apparently healthy middle-aged subjects, elevated oxLDL concentrations were associated with the most CVD risk factors, including age, BMI, waist circumference, blood pressure, serum lipids, smoking, and femoral atherosclerotic plaque (252). In the recent MONICA/KORA Augsburg study (253), the hazard ratio (HR) of CHD for comparing the first and third tertiles of oxLDL was 1.87 (95% CI, 1.33–2.64; $P < 0.001$). Additional adjustments for lipid parameters, inflammatory markers, and markers of endothelial dysfunction attenuated the association (HR, 1.29; 95% CI,

0.88–1.89; P = 0.087) (253). Although circulating oxLDL is a promising biomarker of CVD risk, whether oxLDL is an independent predictor of CAD remains unclear.

α-tocopherol has been found to be the most abundant antioxidant in LDL particles. It has been demonstrated that α-tocopherol can effectively inhibit oxidation of LDL particles in vitro (254). However, Miler et al. performed a meta-analysis of RCTs on the effects of α-tocopherol supplementation found that supplementation of α-tocopherol higher than 400 IU/day may increase all-cause mortality (225). β-carotene supplementation has yielded conflicting results on LDL particle oxidation. The previous controversies on antioxidants (antioxidant paradox) were due to the different measuring methods on LDL oxidation. For example, most clinical trials of α-tocopherol supplementation measured the susceptibility of LDL particles to oxidation in vitro (254). The biological relevance of in vitro LDL oxidation remains uncertain since the in vitro methods used to measure susceptibility of LDL particles to oxidation may not represent the natural oxidation conditions in vivo. Under certain circumstances, α-tocopherol may also act as pro-oxidant molecule when it reacts with lipid radicals and for the α-tocopheroxyl radical, which promotes lipid peroxidation. Only a few studies measured α-tocopherol supplementation on circulating oxLDL concentrations, which has been shown to reflect in vivo oxidation, a marker for CVD (213, 236, 244-246, 253). Two RCTs reported no change in circulating oxLDL concentrations was observed with α-tocopherol supplementation alone or in combination with other antioxidant vitamins (255, 256).

RCTs that have evaluated certain foods using different methods to measure LDL oxidation reported discrepancies too. For example, studies have shown tea consumption had no effect on susceptibility of LDL particles to oxidation (257-259), but other studies reported

consumption of 600ml/day of green tea or tea catechin (500 mg: equivalent to 6 or 7 cups of green tea) significantly decreased circulating oxLDL (260, 261).

The method we used to measure LDL oxidation utilizes the monoclonal antibody mAb-4E6 ELISA assay, which was originally developed by Holvoet et al (236, 237) and has been shown to be of clinical significance in large cohort and RCTs (213, 236, 244-246, 253). In our study, the MF diet did not decrease circulating oxLDL concentration. Some previous studies that reported MUFA protects LDL from oxidation were based on the comparison of MUFA-rich diets versus PUFA-rich diets (226). These results may reflect the presence of more double bonds in PUFA, making them more susceptible to free radical damage (226). Previous studies also reported that high MUFA diets reduced susceptibility of LDL particles to oxidation compared to high CHO/low fat diets in healthy subjects (62, 69). However, the MF diet in our study did not show benefits on lowering oxLDL compared to AAD or LF diets. The inconsistent results may be due to the use of different methods for measuring LDL oxidation.

Foods rich in polyphenols and antioxidant vitamins such as red wine, soy, grape juice, cranberry juice, olive oil, strawberries, green tea, cocoa drink, pistachios and almonds have been shown to protect LDL from oxidation (226-229). The results from clinical trials of the Mediterranean diet suggest a combination of unsaturated fatty acids and dietary antioxidants have beneficial effects on LDL particle oxidation and decreased CVD risk. The major food and nutrient components of Mediterranean diet (e.g., n-3 PUFA, MUFA, tocopherols, carotenoids, ascorbic acid, flavonoids, fruits and vegetables, nuts, olive oil, and red wine) have been shown to reduce LDL oxidation. The consumption of a Mediterranean diet has been associated with decreased circulating oxLDL concentrations in prospective studies (64, 232, 233). Intervention studies also have shown that adherence to the Mediterranean diet pattern lowers LDL oxidization (262-264). A 12-week nutrition intervention promoting the Mediterranean food pattern in healthy

women lead to a 11% reduction in circulating oxLDL concentrations from baseline (262). Pnanagiotakos et al. have shown that individuals who adopted the Mediterranean diet have lower circulating oxLDL concentrations than individuals who adopted a western diet (263). The most recent PREDIMED Study (264) reported that oxLDL decreased ($p<0.05$) in Mediterranean diet groups supplemented with olive oil or mixed nuts. The decrease in the olive oil supplementation group reached significance vs. changes in the control group (low-fat diet) ($P = 0.003$) (264). Studies on olive oil consumption consistently have shown that extra-virgin olive oil with a high phenolic content protects LDL particles from oxidation compared with refined olive oil (265-267).

The lack of effect on oxLDL of the MF diet may be due to the use of high oleic acid vegetable oils that are not rich in polyphenols and antioxidants. Avocados are rich in unsaturated fats (mainly MUFA), antioxidant vitamins, phytosterols, and polyphenols. The nutrient content is similar to nuts, especially pistachios, because they are both high in lutein (**Table 2-3**). The change in oxLDL we observed is consistent with previous studies with nuts. In one study, one serving daily of mixed nuts (mixed raw nuts with skin [15, 7.5 and 7.5 g/d of walnuts, almonds and hazelnuts, respectively]) was associated with lower oxidized LDL concentrations (-7.59 U/L, $p=0.066$) after 12 weeks (230). In a dose-response RCT on almond consumption and CHD risk factors, supplementation of a full dose of almonds (73±3 g/d) but not a half-dose of almonds reduced oxLDL significantly (14.0±3.8%, $P<0.001$) after 1 month (228). Another RCT that evaluated pistachio consumption showed that feeding a low-SFA diet 4 weeks with 1 serving pistachios (32–63 g/d) or with 2 servings pistachios daily (63–126g/d) (oxLDL 43.3±3.02 U/L after consumption) decreased oxLDL significantly ($p<0.05$) compared to a baseline western diet (48.57±3.02 U/L) and the control low-fat diet (51.29±3.02 U/L) (227). In the pistachio study, participants had higher plasma lutein ($P < 0.0001$), α-carotene, and β-carotene ($P < 0.01$) concentrations after consuming the pistachio-enriched diets for 4 weeks than after the baseline

diet. Also, participants showed greater plasma lutein ($P < 0.001$) and γ-tocopherol ($P < 0.05$; 2 servings pistachios/day only) relative to the lower-fat control diet (227). Furthermore, serum lutein and γ -tocopherol were associated with reductions in oxidized-LDL relative to the control diet (227).

Our study also showed a greater increase in plasma lutein in response to the AV diet versus the LF and MF diets. The AV and MF diets significantly increased plasma β-carotene and α-carotene, but only the MF diet achieved significantly affected α-carotene compared to the LF diet. The change in plasma carotenoids and tocopherols reflects a difference in dietary consumption and absorption. The nutrient analysis (by Food Processor SQL) of average daily value of carotenoids and tocopherols in all menus of the four diets is shown in **Table 6-2**. All three cholesterol-lowering diets have higher nutrient-density than the AAD. The AV diet had the highest lutein and zeaxanthin content; the content of α-carotene, β-carotene and α-tocopherol was higher in the AV and MF diets compared to the LF diet. The difference in dietary intake is consistent with the change in plasma antioxidants except for γ-tocopherol. There was a trend for all three diets to decrease plasma γ-tocopherol; the LF diet was significantly different from the AAD. This may due to the increased dietary α-tocopherol intake since plasma and tissue γ-tocopherol are suppressed by α-tocopherol supplementation (268). Also, γ-tocopherol has about 50% of the antioxidant activity and only 10% of the biological activity of α-tocopherol (269).

Studies have demonstrated that the consumption of a carotenoid-containing meal with dietary fat significantly enhances provitamin A carotenoid bioavailability compared with a meal with no added fat (270-272). Avocados are a good source of plant-based dietary fat. The fatty acid profile of Hass avocados is predominantly MUFA (60% oleic, 6% palmitoleic) with some PUFAs (15% linoleic, 2% α-linoleic) and SFAs (16% palmitic, 1% stearic). Studies have shown that avocados and avocado oil can enhance carotenoid absorption from salads and salsa (119). A

recent study reported that the addition of 150g of fresh avocado in a single meal enhanced postprandial absorption of β-carotene and α-carotene from tomato sauce and carrots by 6.6- and 4.8-fold, respectively ($P < 0.0001$ for both) (273). Also, consumption of avocado enhanced the efficiency of carotenoids' conversion to vitamin A (as measured by retinyl esters) by 4.6-fold with tomato sauce ($P < 0.0001$) and 12.6-fold with carrots ($P = 0.0013$) (273). Animal studies have revealed that the chronic consumption of provitamin A carotenoids with higher concentrations of dietary lipids lead to both higher intestinal BCO1 activity and higher hepatic vitamin A stores compared with animals consuming the same meal with less lipid (274, 275). Humans absorb a fraction of intestinally absorbed carotenoids without cleaving them; thus, carotenoids are present transiently in chylomicrons and remnants, as well as LDL and HDL, which also carry carotenoids in the fasted state (276). Besides enhanced enzymatic activity for carotenoid absorption, dietary lipids are necessary for chylomicron synthesis in the enterocyte (276). Plasma retinol levels are normally very stable, with low inter- and intra-individual variations (276). Thus, the high MUFA content of the MF and AV diets may contribute to the increase in plasma carotenoids, too. However, since the LF diet did not have the same amount of dietary carotenoids as the MF diet, it is not possible to conclude that the high MUFA content is the main reason for the increase in plasma carotenoids after consumption of the MF and AV diets. Also, there are limitations in the nutrient database used to calculate nutrients in foods since many factors affect nutrients in foods such as seasonal variation, light, climate, and degree of ripeness, food preparation and processing. Overall, we suggest both the higher unsaturated fat and carotenoid content of the MF and AV diets contributed to the increased plasma carotenoid concentration.

Although our study did not show an association between the decrease in oxLDL and the increase in plasma carotenoids on the AV diet, a decrease in circulating oxLDL on the AV diet

may be the result of both a decrease in sdLDL and an increase in plasma carotenoids, especially lutein. Lutein supplementation in vitro did not show effect on the susceptibility of LDL particles to oxidation in one study (277). Whereas a lutein-rich diet lowered oxLDL in plasma and arota of Guinea pigs after 12 weeks, the lutein-rich diet group presented less focal intimal thickening compared to the control group (116). Epidemiological data from the Los Angeles Atherosclerosis Study and mouse model findings suggested that increasing intake of lutein is protective against the development of early atherosclerosis (115). Lutein and zeaxanthin are largely transported in plasma by HDL (278, 279). HDL can inhibit LDL oxidation and prevent the inhibition of eNOS activity by oxLDL in vivo and the antioxidant properties of HDL may be due to the HDL-associated enzymes, such as paraoxonase. The concentration of HDL_3 after consumption of the AV diet was significantly lower ($p<0.05$) than the LF diet (**Table 5-2**). Recent evidence has shown that small, dense HDL3 particles mediate protection of LDL against oxidation (188-190). Also, avocados are the highest fruit source of Coenzyme Q10, and ubiquinol-10 (reduced form of coenzyme Q10) in LDL acts as an even more powerful antioxidant than α-tocopherol (280). Further studies are needed to determine which bioactive compounds in avocados contribute to the reduction in oxLDL.

Table 6-2 Average content of antioxidant vitamins in the four diets.

Nutrient	AAD	LF	MF	AV
Vit A-IU (IU)	5709	12733	12665	12522
Vit A-RAE (RAE)	752	1105	1031	961
Carotene (RE)	346	969	1051	1061
α-carotene (mcg)	634	1207	1495	1528
β-carotene Eq (mcg)	2078	5813	6305	6370
Retinol (RE)	579	621	506	430
Vit E-IU (IU)	9	13	24	22
Vit E-mg (mg)	6	9	16	15
α-tocopherol (mg)	6	9	16	15
Lutein and Zeaxanthin (mcg)	717	1510	2021	2393
Lycopene (mcg)	159	281	1271	1271

We did not observe a change in plasma F_2-isoprostane. However, this may be partially due to the limitation in the methodology used. The most reliable method for quantifying F_2-isoprostane is stable isotope dilution mass spectrometry. Mass spectrometry coupled to gas chromatography (GC/MS) is generally employed to measure free F_2-isoprostane in plasma and is considered to be the golden standard. Caution must be applied when interpreting the results of F_2-isoprostane immunoassays without adequate validation by GC/MS. Studies comparing the methods have shown very different results depending on the antibodies used, the extent of sample purification and the fluid measured, with some studies finding excellent correlation and other finding poor correlation (215). The correlation between F_2-isoprostane levels obtained using Cayman EIA (used in current study) and GC/MS methods is significant ($r = 0.628$, $P < 0.02$)(281). The Cayman EIA kit we used has shown significant changes on plasma F_2-isoprostane levels in other clinical trials involving statins, smoking cessation, and vitamin E supplementation (215). However, validation by GC/MS at least in a subset of samples will be needed to further validate our results. Also, measuring more biomarkers of oxidative stress, inflammation, and antioxidant capability would be helpful to further understand the effect of avocados on vascular oxidation and inflammation. Such biomarkers include NADPH oxidase, myeloperoxidase, nitrotyrosine, advanced glycation end products (AGEs), TNF-α, IL-1β, IL-6, glutathione, ubiquinol-10, and HDL associated paraoxonase-1, among others.

Conclusions

Including one avocado per day in a heart-healthy diet decreased circulating oxLDL and increased plasma lutein concentrations compared to a typical western diet, a macronutrient and fatty acid-matched moderate fat diet, and a lower-fat, high CHO diet. Based on our findings, we conclude that these benefits are due to bioactive compounds in avocados beyond their fatty acid profile. The decrease in small LDL particles may contribute to the oxLDL lowering effect observed for the avocado diet.

The avocado diet, moderate fat diet and lower-fat diet provided the same amount of fruits and vegetables (except for the fruit portion of one avocado). The dietary intake of carotenoids were increased by the three diets, but only the avocado diet and moderate fat diets increased plasma α-carotene and β-carotene compared to baseline, which is partially due to the higher intake of MUFA enhanced absorption of carotenoids. The changes in plasma carotenoids were not associated with change in oxLDL. Our results suggest that the effect of avocados on lowering small dense LDL is associated with a reduction on circulating oxLDL. However, it is still unclear if plasma carotenoids, or other antioxidants from the avocados that we did not measure, may also contribute to the reduction of oxLDL by avocado consumption.

The increased concentration of small LDL particles by the moderate-fat and lower-fat diets did not induce an increase in plasma oxLDL. The higher intake of fruits and vegetables may protect LDL particles from oxidation. Future studies are needed to determine if the net effect on CVD risk of a lower-fat diet, which is also high in whole grains, fruit and vegetables, is protective or neutral.

In conclusion, it is evident that avocados have a unique nutrient and bioactive profile that plays an important role in reducing LDL oxidation, hence, lowering CVD risk. More long-term

prospective and interventional studies are needed to investigate the effect of avocado consumption on clinical outcomes of CVD and determine the role of avocados in the primary and secondary prevention of CVD.

Chapter 7

Discussion, Limitations, Conclusions and Future Directions

Discussion

The present research has demonstrated that a moderate fat diet including one avocado per day can effectively lower multiple, established and novel cardio-metabolic risk factors, compared to the average American diet and a lower fat, high CHO diet. Although they both lowered the primary target of CVD prevention – LDL-C, the lower fat diet failed to lower non-HDL-C, oxidized LDL, apoB, TC/HDL-C and LDL/HDL-C ratio, whereas it increased TG, sdLDL, VLDL-C, and decreased HDL-C. This study illustrates the benefits of a moderate fat, heart healthy diet that is consistent with current dietary recommendations. The new findings presented herein underscore the benefits of a food source of unsaturated fat that also is a rich source of bioactive compounds. Also of note is that the LF diet met current recommendations for fruits, vegetables, whole grains, etc. and still did not confer the benefits of a moderate fat diet that included an avocado. The results of this dissertation will be helpful for evolving future dietary recommendations.

Although the discordance of LDL-C and LDL-P has been shown in populations at high risk of metabolic syndrome and the LDL pattern B, it is unclear how LDL-C and LDL-P change in response to a dietary intervention. In our study, we first demonstrated that the discordance also existed between the LDL-C and LDL-P in response to the intake of a lower-fat diet. These results have raised questions that require further investigations of lipid metabolism in response to diet intervention. Our results point to the need to consider advanced lipoprotein testing when evaluating the effects of diet on lipids/lipoproteins. However, since our study also showed consistent correlations between the atherogenic dyslipidemia pattern and the sdLDL distribution,

which indicate the traditional lipid measures on TG and non-HDL-C may be adequate to provide evaluation of residual risk of CVD in clinical practice. The novel finding of this study is that when using complex CHO as a substitute for SFA, even within a healthy diet context, there can be an increase in the number of small LDL particles.

This study further explored the "CHO paradox". The LF diet increased TG and HDL-C but decreased LDL-C; however, it did not decrease LDL-P significantly and increased small LDL-P. It also increased remnants, particles that are more easily modified for entry into the artery wall. Although the direct causality of these metabolic changes remains unclear, the increase in sdLDL and LP remnants would predict a higher risk of CVD, which may counterbalance a reduction in LDL-C by replacing SFAs with CHO. Furthermore, lipoprotein subclass analysis suggested the LF diet increased TG production, yielding the largest TG-enriched VLDL particles, consequently reduced VLDL clearance, yielding larger LP remnants, and further modifying to LDL_4 via lipolysis. The Honolulu Heart Study monitored 1156 Japanese-American men for 17 years, reported that while high TG in general was a CVD risk factor, it was specifically the elevation of the remnant fraction of the TG enriched lipoproteins that contributed to CVD risk. Whereas large VLDL particles are inhibited by their size from crossing the endothelial barrier, sdLDL and VLDL remnants can more easily enter the artery wall (282, 283). Although the generation of TG-enriched large VLDL particles by LF diet may have a different mechanism versus the high-fat western diet, it appears the LF diet tended to increase atherogenic risk due to the increased VLDL-TG production and greater plasma residence time of LP remnants and sdLDL.

However, we did not observe any change in circulating oxLDL by the LF diet as expected. One explanation is that we were only able to measure oxidized lipoprotein particles in the circulation rather than in the endothelial cells or within artery wall lesions. Another

explanation is that the increased fruits, vegetables and whole grain consumption in the LF diet protected the small LDL particles and LP remnants from oxidized modification. We are not sure how the LF diet protected sdLDL and LP remnants from oxidation because we did not observe an increase in plasma caroteinoids and tocopherals or a decrease in the lipid peroxidation marker by the LF diet.

The unclear net effect of high-CHO diets also relates to the predisposition of lipoprotein patterns in populations. Subjects started with the more benign LDL pattern A showed less benefit in LDL-C and apoB reduction on a low-fat/high-CHO diet than did subjects who started with the more atherogenic pattern B (284, 285). However, the lower-risk pattern A group represents the majority of the population (70-90%), the potential public health nutrition implications of this model are substantial (194). Krauss (284) summarized an inverse relationship between the percentage of CHO in the diet and the prevalence of LDL subclass pattern B in the population: on 30% fat diets, pattern B has a prevalence of approximately 30% in adult males; on 10% fat diets, pattern B has a prevalence of approximately 60% in men. In our study, the majority of the participants had LDL subclass pattern A (n=28, 62%) at baseline, which represents the distribution of LDL subclass pattern in general western population. Among the participants with pattern A, 3 participants switched to pattern B after the LF diet. Four of them switched to pattern B after the MF diet; one participant switched to pattern B after the AV diet. Among participants who were with pattern B at baseline (n=11, 38%), most of them remained at pattern B after consumption of the three diets. One of them switched to pattern A after the AV diet; five of them switched to pattern A after the MF diet; all of them stayed at pattern B after the LF diet. The trend was not statistically significant. Since our LDL subclass pattern subgroups were not statistically powered and the intervention in our study is too short to see a long-term effect (LDL subclass pattern represents long-term exposure to diet), it was not appropriate to evaluate the

change between LDL subclass patterns in response to different diets in our study. Furthermore, the conversion of pattern A persons to pattern B has unproven implications with respect to CVD risk (194). Also, we observed that participants with pattern B at baseline had a greater reduction in LDL-C on the LF diet, but the increase in TG and decrease in HDL was also greater. The net consequences for CVD risk of the LF diet therefore remain ambiguous. Future studies are needed to determine if the effect on CVD risk of a lower-fat diet, which is also high in whole grains, fruit and vegetables, is protective or neutral.

Our study provides important clinical evidence to interpret the "MUFA paradox". We found that the AV diet but not the MF diet affected several atherogenic lipoproteins, such as the sdLDL and Lp remnants. Also, only the AV diet decreased TC/HDL-C and LDL/HDL-C ratios, and decreased oxidized LDL. At the same time, the MF diet was not as effective as the avocado diet in lowering TC, LDL-C, and non HDL-C. Thus, including one avocado in the diet daily can significantly lower TC, LDL-C, non HDL-C, sdLDL-C, total LDL-P, small LDL-P, oxidized LDL, TC/HDL-C and LDL/HDL-C. This confirmed our hypothesis that avocados have heart healthy benefits beyond MUFA. We also measured the plasma carotenoids and observed a significant increase in plasma lutein solely due to the high lutein content of avocados. Combined with the effect of avocados on lowering sdLDL, oxidized LDL, and Lp remnants, we hypothesize that avocado consumption may affect the inflammatory factors in the initial process of atherosclerosis. We also found that using high-MUFA oils versus complex CHO to replace SFA can lower TG and increase HDL-C, which is consistent with previous RCTs. However, the MF diet had a similar discordant on the change in LDL-C and LDL-P as the LF diet because MF diet also increased small LDL-P. Although the MF diet also increased plasma carotenoids and protected increased small LDL-P from oxidation, long-term intervention studies are still needed to determine its net effect on CVD risk. Our results partially explained the "neutral" effect of

total MUFA consumption on CHD clinical outcomes. Although there is no long-term interventional study on avocado consumption, our results suggest that avocados could be as beneficial as nuts and olive oil in reducing CHD clinical outcomes with long-term consumption.

Our study also provided additional evidence to interpret the "antioxidant paradox" in CVD prevention. In our study, the change in oxidized LDL was correlated with a change in small, dense LDL but not the change in plasma antioxidants in the AV diet. Several factors may influence the susceptibility of LDL to oxidation, including its size and composition, and the presence of endogenous antioxidant compounds, such as α-tocopherol. Both the MF and AV diets increased plasma carotenoids, but MF diet did not lower oxLDL. It appears that the other bioactive compounds (not antioxidants) in avocados that affect LDL particle size contribute more to lowering oxidation of LDL. Circulating oxLDL is elevated in patients with CHD or individuals with early atherosclerotic changes and metabolic disorders including diabetes, obesity, and metabolic syndrome. It is of note that the oxLDL assay that was used in the current study and previous clinical trials is based on detecting conformational changes of apoB protein. The concentration of "circulating oxLDL" we measured represents all modified apoB containing lipoproteins, including VLDL, LP remnants and LDL. Studies suggest that LP remnants are also susceptible to oxidation and involved in the early progression of atherosclerosis (283). Our study demonstrated that a high-MUFA, moderate-fat diet including one avocado a day is effective for improving VLDL and LDL clearance, hence, reducing the oxidation of VLDL remnants and small, dense LDL particles.

We utilized several novel biomarkers of cardio-metabolic risk in our study. The link between LDL-C and the development of CHD is well established and consequently it has been recognized as the primary therapy target for reducing the risk of cardiovascular events (286). However, the contribution of LDL-C to atherosclerosis is complex and individuals at risk of CHD

do not always have elevated levels of LDL-C (287). Two long-term large trials, WOSCOPS (288) and HPS (289) also suggest that statin therapy confers protection of CVD risk and is not due exclusively to lowering of LDL-C. In addition to decreasing LDL-C, it has been shown that statins can also decrease the oxidative susceptibility of LDL particles, and consequently lower the atherogenicity of LDL (290). Atherosclerosis is a complex inflammatory process involving lipoproteins in the arterial wall. Current evidence suggests that controlling inflammation might be as important for CVD prevention as LDL-C reduction. Based on our finding of the relationship between change in sdLDL and oxLDL, the advanced lipid biomarkers such as LDL-P, sdLDL are important measures of atheroschlerosis risk beyond traditional lipid biomarkers. The combination of traditional and novel biomarkers could help better predict the effects of dietary intervention on CHD risk. For example, the populations most likely to experience the greatest increase in TG are most likely to have the greastest LDL-C reduction in response to a high-CHO diet. The implications for cardiovascular risk remain to be clarified. In this study, we used advanced lipid testing and lipoprotein oxidation markers to evaluate the effect of diets on CVD risk; the addition of LDL-P, sdLDL and oxLDL might provide a better prediction in the CVD risk change, and based on our findings, the lower-fat, high CHO diet might have a neutral effect on CVD risk in primary prevention.

Although lipoprotein particle size and number, circulating oxLDL are promising biomarkers of CVD risk, whether they are independent predictor of CAD remains unclear. Furthermore, their diagnostic value in addition to the classical lipid/lipoprotein profile is still controversial. Some large clinical trials have shown a significant relationship between LDL-P, sdLDL, LP remnants, oxLDL and CHD clinical outcomes. However, there is no risk estimator model to predict the change in CVD 10-year and lifetime risk based on changes in these biomarkers. Also, none of them are routinely used in clinical practice. Factors that determine the

clinical utility of a biomarker include the ease and cost of the measurement, its performance characteristics (e.g. sensitivity, specificity, etc.) and evidence for guiding clinical management and improving patient outcome. Although these biomarkers have been identified as novel CVD risk factors, there is a lack of specific therapeutic methods targeting these biomarkers. Their laboratory measurements also are not standardized and are more expensive than traditional lipid/lipoprotein markers. More clinical studies are needed to determine if any of these novel risk factors could be used as effective therapy targets in clinical practice.

Strengths and Limitations

The current study has several strengths. It was a well-controlled clinical trial and we achieved a high level of diet compliance, weight maintenance and had a low dropout rate. The latter is attributable to the run-in diet period, which familiarized participants with the study. The cross-over design eliminated the influence of individual variability and increased statistical power. The diet design allowed us to study three healthy, cholesterol-lowering diets that met current food group recommendations. Unlike previous study designed extreme high fat diets versus low-fat diets, the macronutrient contents of all three diets are within the range of current dietary recommendations. The purpose of our study was to provide clinical evidence to achieve a cardio-protective diet with an optimal macronutrient proportion. Also, we focused on a nutrient-dense food source of MUFA, provided an easy solution in dietary intervention to lower SFA and achieve maximum benefits on CVD prevention. Moreover, our study was designed to differentiate the effects of bioactive compounds in avocados beyond fatty acids. We measured multiple established and novel risk factors for CVD, which provided more information to understand the mechanisms by which avocados affect CVD risk factors. Our study also linked

physical structure modification to the oxidation modification of LDL, which plays a key role in the initial process of atherosclerosis.

The current study also has several limitations. First, in the study design, one avocado per day contributed a different percentage of energy over different calorie levels (7% to 13% over the six calorie levels). We also did not include a dose response group to verify our findings if consuming less than one avocado a day would achieve the same benefits, and if consuming more than one avocado a day would enhance the beneficial effects.

Although we concluded that beneficial effects on CVD risk factors are linked to the bioactive compounds in avocados, we did not identify specific bioactive compounds that conferred this activity, and the underlying mechanisms that elicited the effects. Our study design did not enable us to identify the specific bioactive compounds in avocados. Furthermore, many factors affect polyphenolic compounds in foods such as seasonal variation, light, climate, and degree of ripeness, food preparation and processing. The plasma antioxidant measurements did not consider seasonal change but the study was ongoing through four seasons for 2 years. Participants consumed the avocado diet in different seasons. Seasonal change in certain nutrients in avocados and other fruits and vegetables could be a confounder of our results. However, we did not balance the consumption of diets in four seasons.

The first line of treatment for overweight/obesity is weight loss and physical activity. In our study, weight was maintained so as to not be a confounding variable when studying the effects of avocados on CVD risk factors. However, weight and physical activity have a significant impact on the hypertriglyceridemic response to dietary CHO. It has been demonstrated that weight loss is required for a high-CHO, low-fat diet to decrease LDL-C without worsening TG and HDL. An isocaloric high CHO/low fat diet may not represent a real

life scenario because many studies show that a switch from ad-libitum high-fat to low-fat diets causes weight loss (291). Studies of long-term ad-libitum low-fat/high-CHO diets have demonstrated persistent weight loss associated with no increase in TG (291). Gibney (292) stated, "Given our sedentary lifestyles, high-CHO diets should be recognized as disadvantageous because of their frequent association with elevated TG and low HDL, these effects could be negated by moderate physical activity". It is suggested that the discordance between the international epidemiology data and the potential atherogenic lipid change by low-fat, high-CHO diet is due to the difference in levels of physical activity among populations (26). During our study, we required participants to maintain their usual physical activity level and adjusted calorie levels to maintain body weight. Clearly, weight loss and exercise would elicit beneficial effects on lipids/lipoproteins and CVD risk status. Nonetheless, for individuals who do not lose weight, we have shown that a moderate fat diet high in MUFA, especially from one avocado per day has beneficial effects on lipids/lipoproteins and CVD risk status.

Although we measured several metabolic syndrome risk factors, including HDL-C, TG, glucose, blood pressure, sdLDL and oxLDL, we did not measure an important feature of metabolic syndrome – waist circumference. We did not evaluate if the beneficial changes in cardio-metabolic risk factors were associated with a change in abdominal body fat. Also, since our participants were at low risk of metabolic syndrome at baseline, our findings do not provide any insight about the effects of diet change in individuals with metabolic syndrome. Finally, our participants did not represent the ethnic diversity of the U.S. population.

Future Directions

We have shown that a moderate-fat, high-MUFA, lower-SFA diet including one avocado a day decreased multiple cardio-metabolic risk factors. Since we conducted a short-term intervention study, a longer-term clinical trials as well as observational studies are needed to determine the long-term benefits of avocado consumption. Since our study was only conducted on a small group of otherwise healthy, overweight and obese people with little ethnic diversity, more studies on a diverse population are needed for making specific recommendations for individuals with varying metabolic and genetic backgrounds.

A dose-response study will be important for determining the quantity of avocados that are needed to achieve optimal beneficial effects. Also, it is important to identify the specific bioactive compounds in avocados that account for the sdLDL and oxidized LDL lowering effects. Other possible approaches include using metabolomics to identify the signaling pathways involved and potential key nutrient metabolites; conducting clinical trials with identified candidate bioactive compounds; using certain gene knock-out animal models; and using in vitro approaches to measure the effects of different avocado extracts.

Questions remain about whether the reduction in oxLDL and sdLDL by avocados can effectively lower atherogenesis and achieve clinical significance in CVD prevention and therapy. Long-term interventional studies on population at high risk of CVD are needed to determine the clinical outcome of long-term avocado consumption as well as clinical implications of these novel biomarkers. Also, consumption of avocados under a less-restrict controlled diet is useful to determine their effect on weight loss, clinical and subclinical CVD endpoints in a more real-life environment. Also, since weight loss and physical activity are first line recommendations for

overweight and obesity, further studies are needed to evaluate the benefits of a weight loss diet with avocados and a physical activity intervention.

The CHO-induced hypertriglyceridema is an important nutrition problem. Although studies have shown that increased intake of refined carbohydrates was associated with increased CVD events, the long-term effects of high intake of complex CHO, including whole grains are not conclusive. Our results suggest that a lower-fat diet (10% of total calories less fat than average American diet), high in fruits, vegetables and whole grains, increased VLDL-TG production and VLDL remnants clearance. In contrast, a moderate-fat diet including one avocado per day improved VLDL clearance. These conclusions are based on changes in lipoprotein subclasses and lack of a thorough investigation on mechanisms. First, we need to measure activities of lipoprotein lipase and hepatic lipase to validate our hypotheses; then a lipid kinetic model is needed to determine the metabolic pathways that affect VLDL and LDL clearance by the LF diet and AV diet.

Since we observed that avocados lower LDL oxidation, it will be important to determine if the suppression on LDL oxidation by avocados reduces atherogensis. Biomarkers of oxidation and inflammation that could be studied include NADPH oxidase, myeloperoxidase, nitrotyrosine, advanced glycation end products (AGEs), TNF-α, IL-1β, IL-6, glutathione, and ubiquinol-10, etc. Also, since the LF and AV diets had different effects on HDL subclasses, it is important to determine if the HDL function is affected by the diet. HDL plays a pivotal role in mitigating CVD risk through reverse cholesterol transport by removing excess lipids from the atherosclerotic plaques, contributing to antioxidant defenses, and suppressing pro-inflammatory pathways. HDL particles are critical acceptors of cholesterol from lipid-laden macrophages (cholesterol efflux) and thereby participate in the reduction of pro-inflammatory responses by arterial cholesterol-loaded macrophages. Specifically, the antioxidant capacity of HDL is important to protect LDL

from oxidation. Thus we believe that there is a need to measure HDL macrophage cholesterol efflux capacity using an ex vivo assay (293), and HDL associated paraoxonase-1 activity in plasma samples to evaluate the effect of avocado consumption on HDL function.

Finally, it is important to translate the current research findings into clinical practice and measure the long-term effects of avocado consumption on CVD events in populations at high risk of heart disease, Metabolic Syndrome, and diabetes. Since several biomarkers we measured all serve as measurements of residual risk, such as apoB, TG/HDL-C, sdLDL, a diet intervention study for secondary CHD prevention will have significant clinical implications. Lastly, while we included avocados in a high MUFA, moderate fat diet, it would be interesting to incorporate avocados in other healthy dietary patterns, such as a Mediterranean diet and evaluate avocados compared to other high MUFA foods such as nuts and olive oil. Also, avocados provide MUFA and PUFA as well as several lipid-soluble vitamins that are important for a vegetarian diet. It will be interesting to compare the health effects of avocados alone and together with other nutrient dense, bioactive rich foods in these healthy dietary patterns. The results of these studies will provide important information that will be helpful in identifying new healthy dietary patterns for optimal heart health.

Conclusions

We present novel information that a moderate fat diet low in SFA and high in MUFA that was provided mainly by an avocado per day achieved greater reductions in TC, LDL-C, non-HDL-C, LDL/HDL-C and TC/HDL-C than a high MUFA diet with a similar macronutrient and fatty acid profile in overweight and obese adults. The additional LDL-C and non-HDL-C lowering effects of avocados are from the reduction in small, dense LDL particles and VLDL

remnants, which are atherogenic lipoprotein particles susceptible to oxidation in vivo. The AV diet may decrease small, dense LDL particles by suppressing CETP and improving the clearance of VLDL particles. The AV diet also decreased circulating oxidized LDL and increased plasma lutein concentration compared to the moderate fat diet. Our results suggest that the reduction on circulating oxidized LDL by avocados was associated with decreased small dense LDL particles but not elevated plasma carotenoids. However, it is still unclear if plasma carotenoids, or other antioxidants from the avocados that we did not measure, may also contribute to the reduction of oxidized LDL by avocado consumption.

We found that a 10% increase in CHO, even along with a moderate increase in whole grains, fruits/vegetable and fiber did not offset the adverse effect on TG and HDL-C by replacing SFA with CHO. Also, the lower fat, high complex CHO diet had a discordant effect on LDL-C and LDL-P because it increased small, dense LDL. Lipoprotein subclasses analysis suggest the lower fat diet reduced VLDL clearance, yielded larger LP remnants, and further modified remnants to small, dense LDL particles. The change in small, dense LDL concentration was correlated with a more atherogenic lipid/lipoprotein profile. However, the increased concentration of small LDL particles due to the lower-fat diet did not induce an increase in plasma oxidized LDL. The higher intake of fruits and vegetables may protect small dense LDL particles from oxidation in vivo. Future studies are needed to determine if the effect on CVD risk of a lower-fat diet, which is also high in whole grains, fruit and vegetables, is protective or neutral.

Our results are the first to demonstrate that avocados have beneficial effects on more atherogenic lipoprotein subclasses, and these effects extend beyond the fatty acids in avocados. Furthermore, the decrease in small dense LDL particles may contribute to the reduction on LDL oxidation. Both of the beneficial effects are associated with lowered risk of metabolic syndrome.

However, avocados did not show additional benefits beyond MUFA on the other risk factors of metabolic syndrome, including TG, HDL-C, blood glucose, and blood pressure.

In summary, our study demonstrated that avocados have beneficial effects on cardio-metabolic risk factors that extend beyond their heart-healthy fatty acid profile in generally healthy but overweight and obese adults, and, hence, may contribute to the primary prevention of CVD. A moderate fat diet using a nutrient-dense food source of MUFA to replace SFA is preferred to achieve an optimal lipid profile in overweight and obese adults. Long-term prospective and intervention studies are needed to investigate the effect of avocado consumption on clinical outcomes of CVD and determine the role of avocados in both primary and secondary prevention of CVD.

Appendix A

Six-Day Menus of the Four Study Diets

Menu 1

AAD	LF	AV	MF
Breakfast	**Breakfast**	**Breakfast**	**Breakfast**
Bagel	Skim milk	Skim milk	Skim milk
Cream cheese	OJ	Plain yogurt	Plain yogurt
Apple juice	Granola	Strawberries, fzn	Strawberries, fzn
Jelly	Banana	Granola	Granola
2% milk	English Muffin	Almonds, slivered	Almonds, slivered
	Jelly		
	Butter		
Lunch	**Lunch**	**Lunch**	**Lunch**
Steak, Cheddar, Mushroom Panini	White bread	Whole wheat bread	Whole wheat bread
Potato chips	Turkey	Romaine	Romaine
Chocolate Chip granola bar	Romaine	Turkey	Turkey
Peaches	Mayonnaise	Tomatoes	Tomatoes
	Pretzels	Avocado	Oleic oil Dressing
	RF Provolone cheese	Mayonnaise	Mayonnaise
	Cantaloupe	Pretzels	Pretzels
		Pear	Pear
		Carrots	Carrots
		Vanilla pudding	Vanilla Pudding
			RF Provolone cheese
Dinner	**Dinner**	**Dinner**	**Dinner**
Chicken Fettuccini	Beef Chow Fun	Beef Chow Fun	Beef Chow Fun
Iceberg lettuce	Romaine	Whole wheat dinner roll	Whole wheat dinner roll
Tomatoes	Shredded carrots	Margarine	Margarine
FF Ranch dressing	Whole wheat dinner roll	Green beans, fzn	Green beans, fzn
Biscuit	Margarine	Avocado	
Margarine	AAD Dressing		
Cheddar cheese, shredded	Egg whites (salad)		
Croutons			
Hard boiled egg			
Snack	**Snack**	**Snack**	**Snack**
Almonds	Blueberry yogurt	Celery	Celery
M&M's plain	Graham crackers	Peanut butter	Peanut butter
		Potato chips	Potato chips
			Sour Cream dip

Menu 2

AAD	LF	AV	MF
Breakfast	**Breakfast**	**Breakfast**	**Breakfast**
Blueberry muffin	English Muffin	Banana	Banana
Butter	Ham	English Muffin	English Muffin
2% milk	American cheese	American cheese	American cheese
Granola	Skim milk	Ham	Ham
Apple juice	Apple juice	Skim milk	Skim milk
	Margarine	OJ	OJ
	Cantaloupe		Margarine
Lunch	**Lunch**	**Lunch**	**Lunch**
Mayonnaise	BBQ Chicken Pizza	Pistachios	Pistachios
Hard boiled egg	Carrots	Romaine	Romaine
Dijon mustard	Celery	Tomatoes	Tomatoes
White bread	FF Ranch dressing	Grapes	Grapes
Pretzels	Pear	AAD Dressing	AAD Dressing
Pineapple chunks	Baked potato chips	Margherita Pizza	Margherita Pizza
Baby carrots		Avocado	Oleic oil Dressing
Sour cream dip (for pretzels)			
		Afternoon snack	**Afternoon snack**
		Skim milk	Skim milk
		Whole wheat crackers	Whole wheat crackers
		Peanut butter	Peanut butter
Dinner	**Dinner**	**Dinner**	**Dinner**
Meatloaf w/gravy and whipped potatoes	Sun Dried Tomato Chicken Pesto	Sun Dried Tomato Chicken Pesto	Sun Dried Tomato Chicken Pesto
Green beans, frozen	Green beans, frozen	Broccoli, fzn	Broccoli, fzn
Margarine	Butter	Whole wheat dinner roll	Whole wheat dinner roll
Vanilla pudding	Whole wheat dinner roll	Margarine	Margarine
Dinner roll	Peaches, canned		
Snack	**Snack**	**Snack**	**Snack**
Cheddar cheese, cubed	Vanilla yogurt	Avocado/Guacamole	Cheddar cheese/dip
Cottage cheese	Nutrigrain bar	Tortilla chips	Tortilla chips
Saltines	Granola		

Menu 3

AAD	LF	AV	MF
Breakfast	**Breakfast**	**Breakfast**	**Breakfast**
Pancakes	Cream of Wheat	Oatmeal	Oatmeal
Light syrup	Blueberries, frozen	Blueberries, fzn	Blueberries, fzn
Butter	Brown sugar	Almonds, slivered	Almonds, slivered
2% milk	Skim milk	Skim milk	Skim milk
Scrambled egg	OJ	OJ	OJ
	Yogurt	Blueberry yogurt	Blueberry yogurt
Lunch	**Lunch**	**Lunch**	**Lunch**
Pepperoni Pizza	Chicken Salad	Avocado	Oleic oil Dressing
Peaches	Wheat bread	Chicken salad	Chicken Salad
Rice Krispie treat	Peaches	Saltines	Saltines
Mozzarella string cheese	Pretzels	Pear	Pear
	Peaches, canned	Baby carrots	Baby carrots
		Granola bar	Granola bar
Dinner	**Dinner**	**Dinner**	**Dinner**
Lean Cuisine, Beef Chow Fun	Turkey Taco	Turkey Taco	Turkey Taco
Iceberg lettuce	Tortilla chips	Avocado	Oleic oil Dressing
Croutons	Corn, frozen	RF Cheddar cheese	RF Cheddar cheese
Caesar dressing	RF Cheddar cheese	Corn, fzn	Corn, fzn
Shredded carrots		Tortilla chips	Tortilla chips
M&M's		Butter	
Snack	**Snack**	**Snack**	**Snack**
Bagel	Apple	Apple	Apple
Cream cheese	English muffin	English Muffin	English Muffin
	Margarine	Margarine	Margarine
	Jelly		Jelly

Menu 4

AAD	LF	AV	MF
Breakfast	**Breakfast**	**Breakfast**	**Breakfast**
Bagel	Pancakes	Pancakes	Pancakes
Jelly	Light syrup	Light syrup	Light syrup
Butter	Skim milk	Blueberries, fzn	Blueberries, fzn
Apple juice	Apple juice	Skim milk	Skim milk
2% milk	Egg Beaters	Cottage cheese	Cottage cheese
Pineapple chunks	Margarine	OJ	OJ
			Margarine
Lunch	**Lunch**	**Lunch**	**Lunch**
White bread	Chicken Club Panini	Chicken Club Panini	Chicken Club Panini
Mayonnaise	Celery	Pasta salad	Pasta salad
RF Provolone cheese	Baby carrots	Avocado	Feta cheese
Pretzels	FF Ranch	Carrots	Carrots
Choc chip granola bar	Baked potato chps	Broccoli	Broccoli
Hard boiled egg	Granola bar	Tomatoes	Tomatoes
Turkey		Dressing	Dressing
		Grapes	Grapes
Dinner	**Dinner**	**Dinner**	**Dinner**
Lean Cuisine, Chicken Parm	Chicken Parm	Chicken Parm	Chicken Parm
Broccoli, fzn	Romaine	Romaine	Romaine
American cheese (to melt on broccoli)	Whole wheat dinner roll	Carrots	Carrots
Dinner roll	Butter	Ranch dressing	Ranch dressing
Margarine	Carrots, shredded	Tomatoes	Tomatoes
	Sunflower dressing		Dinner roll
			Margarine
Snack	**Snack**	**Snack**	**Snack**
Almonds	Vanilla yogurt	Avocado	Cantaloupe
Vanilla pudding	Granola	Cantaloupe	Mango
	Pretzels	Mango	Honey Lime Dressing
		Honey Lime Dressing	

Menu 5

AAD	LF	AV	MF
Breakfast	**Breakfast**	**Breakfast**	**Breakfast**
Cracklin' Oat Bran	Bagel	Bagel	Bagel
2% milk	Butter	Avocado	Margarine
Strawberry Poptart	Skim milk	Skim milk	Skim milk
Canned peaches	OJ	OJ	OJ
	Banana	Yogurt	Yogurt
	Jelly	Granola	Granola
Lunch	**Lunch**	**Lunch**	**Lunch**
Lean Cuisine Chicken Club Panini	Whole wheat bread	Mayonnaise	Mayonnaise
Hardboiled egg	Turkey	Whole wheat bread	Whole wheat bread
Pretzels	Mayonnaise	Avocado	RF Provolone cheese
Celery	Romaine	Romaine	Romaine
FF Ranch dressing	Baked potato chips	Bacon	Bacon
M&M's	Peaches, canned	Potato chips	Potato chips
	Vanilla pudding	Turkey	Turkey
		Pear	Pear
Dinner	**Dinner**	**Dinner**	**Dinner**
Lean Cuisine, Lasagna with Meat Sauce	Lasagna with Meat Sauce	Lasagna with Meat Sauce	Lasagna with Meat Sauce
Green beans, fzn	Broccoli, frozen	Whole wheat dinner roll	Whole wheat dinner roll
Biscuit	Whole wheat dinner roll	Butter	Margarine
Butter	Margarine	Broccoli, fzn	Broccoli, fzn
Snack	**Snack**	**Snack**	**Snack**
Chocolate chip granola bar	Wheat pita	Chocolate chip cookie	Chocolate chip cookie
Cottage cheese	Hummus	Almonds	Almonds
Peanuts	Yogurt		

Menu 6

AAD	LF	AV	MF
Breakfast	**Breakfast**	**Breakfast**	**Breakfast**
Scrambled egg	Skim milk	English muffin	English Muffin
Bagel	Apple juice	Peanut butter	Peanut butter
American Cheese	Flour tortilla	Banana	Banana
Apple juice	Egg Beaters	Skim milk	Skim milk
Pineapple, canned	RF Cheddar cheese	OJ	OJ
Butter	Salsa		
Lunch	**Lunch**	**Lunch**	**Lunch**
Veggie burger	Veggie burger	Veggie burger	Veggie burger
Hamburger bun	Hamburger bun	Hamburger bun	Hamburger bun
Mayonnaise	Romaine	Avocado	Oleic oil Dressing
Rice Krispie Treat	FF Mayo	Romaine	Romaine
Pretzels	Pretzels	Carrots	Carrots
RF Provolone cheese	Cantaloupe	Hummus	Ranch dressing
	Nutrigrain bar	Cherry tomatoes	Cherry tomatoes
	RF Provolone cheese	Grapes	Grapes
		Pretzels	Pretzels
Dinner	**Dinner**	**Dinner**	**Dinner**
Lean Cuisine, Salisbury Steak	Apple Cranberry Chicken	Romaine	Romaine
Iceberg lettuce	Whole wheat dinner roll	Avocado	Sunflower dressing
Carrots, shredded	Butter	Cherry tomatoes	Cherry tomatoes
Biscuit	Iceberg	Chicken	Chicken
Butter	Carrots	Ranch dressing	
Ranch	Sunflower dressing	Bacon	Bacon
		Pita chips	Pita chips
		Vanilla pudding	Vanilla pudding
Snack	**Snack**	**Snack**	**Snack**
Strawberry Poptart	Graham crackers	Yogurt	Yogurt
M&M's	Yogurt	Graham crackers	Graham crackers
	Apple		

Appendix B

Recipes and Preparation Guide for the Avocado Meals

Menu 1:

Lunch: turkey sandwich – slice half the avocado and put on top of sandwich

Dinner: Asian stir-fry (Beef Chow Fun) – cube the other half of avocado and mix into stir fry

Menu 2:

Lunch: Margherita pizza – cube half the avocado and put on top of pizza

Snack: Guacamole – will be provided with the fixing's for the guacamole (tomatoes, lime juice, onions, salt/pepper); just requires mixing half the avocado into the rest of the ingredients and enjoy with provided tortilla chips

Menu 3:

Lunch: Chicken salad – cube half the avocado and mix in with chicken salad (which will already be prepared)

Dinner: Turkey Taco – slice or cube half the avocado and mix in with the entrée; could also be mashed/pureed into a more guacamole-type consistency

Menu 4:

Lunch: Pasta salad – cube or slice half the avocado and mix in with the pasta salad (which will already be prepared)

Snack – will receive cantaloupe and mango dressed with a honey-lime yogurt mixture; slice or cube half the avocado and mix in with the other fruits and dressing

Menu 5:

Breakfast: Bagel – slice half the avocado to put on top of bagel; could also be mashed into a spreadable consistency

Lunch: Bacon turkey sandwich– slice half the avocado to put on top of sandwich

Menu 6:

Lunch: Veggie burger – slice half the avocado to put on top of sandwich

Dinner: Chicken Club salad – slice or cube the avocado to put on top of salad

Remember, these are only the original suggestions for how to consume the avocado. Feel free to experiment and be creative – just as long as you figure out a way to eat one whole avocado each day. You can mix half the avocado in with your dinner entrée; eat it with your frozen veggies at dinner, or even eat it plain. The options are limitless.

Appendix C

Nutrient Content of Hass Avocados from the USDA Food Database

Avocados, raw, California (Edible Portion) (47)

Nutrient	Units	Value per 100 g	1.00 X 1 fruit, without skin and seed, 136g	1.00 X 1 NLEA serving, 30g
Proximates				
Water	g	72.33	98.37	21.70
Energy	kcal	167	227	50
Energy	kJ	697	948	209
Protein	g	1.96	2.67	0.59
Total lipid (fat)	g	15.41	20.96	4.62
Ash	g	1.66	2.26	0.50
Carbohydrate, by difference	g	8.64	11.75	2.59
Fiber, total dietary	g	6.8	9.2	2.0
Sugars, total	g	0.30	0.41	0.09
Sucrose	g	0.06	0.08	0.02
Glucose (dextrose)	g	0.08	0.11	0.02
Fructose	g	0.08	0.11	0.02
Lactose	g	0.00	0.00	0.00
Maltose	g	0.00	0.00	0.00
Galactose	g	0.08	0.11	0.02
Starch	g	0.11	0.15	0.03
Minerals				
Calcium, Ca	mg	13	18	4
Iron, Fe	mg	0.61	0.83	0.18
Magnesium, Mg	mg	29	39	9
Phosphorus, P	mg	54	73	16
Potassium, K	mg	507	690	152
Sodium, Na	mg	8	11	2
Zinc, Zn	mg	0.68	0.92	0.20
Copper, Cu	mg	0.170	0.231	0.051
Manganese, Mn	mg	0.149	0.203	0.045

Nutrient	Units	Value per 100 g	1.00 X 1 fruit, without skin and seed, 136g	1.00 X 1 NLEA serving, 30g
Selenium, Se	mcg	0.4	0.5	0.1
Vitamins / Phytochemicals				
Vitamin C, total ascorbic acid	mg	8.8	12.0	2.6
Thiamin	mg	0.075	0.102	0.022
Niacin	mg	1.912	2.600	0.574
Pantothenic acid	mg	1.463	1.990	0.439
Vitamin B-6	mg	0.287	0.390	0.086
Folate, total	mcg	89	121	27
Choline, total	mg	14.2	19.3	4.3
Betaine	mg	0.7	1.0	0.2
Vitamin B-12	mcg	0.00	0.00	0.00
Vitamin A, RAE	mcgRAE	7	10	2
Retinol	mcg	0	0	0
Carotene, beta	mcg	63	86	19
Carotene, alpha	mcg	24	33	7
Cryptoxanthin, beta	mcg	27	37	8
Vitamin A, IU	IU	147	200	44
Lycopene	mcg	0	0	0
Lutein + zeaxanthin	mcg	271	369	81
Vitamin E (alpha-tocopherol)	mg	1.97	2.68	0.59
Tocopherol, beta	mg	0.04	0.05	0.01
Tocopherol, gamma	mg	0.32	0.44	0.10
Tocopherol, delta	mg	0.02	0.03	0.01
Vitamin D (D2 + D3)	mcg	0.0	0.0	0.0
Vitamin D	IU	0	0	0
Vitamin K (phylloquinone)	mcg	21.0	28.6	6.3
Lipids				
Fatty acids, total saturated	g	2.126	2.891	0.638
8:0	g	0.001	0.001	0.000
16:0	g	2.075	2.822	0.623
18:0	g	0.049	0.067	0.015

Nutrient	Units	Value per 100 g	1.00 X 1 fruit, without skin and seed, 136g	1.00 X 1 NLEA serving, 30g
Fatty acids, total monounsaturated	g	9.799	13.327	2.940
16:1	g	0.698	0.949	0.209
17:1	g	0.010	0.014	0.003
18:1	g	9.066	12.330	2.720
20:1	g	0.025	0.034	0.007
Fatty acids, total polyunsaturated	g	1.816	2.470	0.545
18:2	g	1.674	2.277	0.502
18:3	g	0.125	0.170	0.037
18:3 n-3 c,c,c (ALA)	g	0.111	0.151	0.033
18:3 n-6 c,c,c	g	0.015	0.020	0.004
20:3 undifferentiated	g	0.016	0.022	0.005
Cholesterol	mg	0	0	0
Stigmasterol	mg	2	3	1
Campesterol	mg	5	7	2
Beta-sitosterol	mg	76	103	23
Amino acids				
Tryptophan	g	0.025	0.034	0.007
Threonine	g	0.072	0.098	0.022
Isoleucine	g	0.083	0.113	0.025
Leucine	g	0.141	0.192	0.042
Lysine	g	0.129	0.175	0.039
Methionine	g	0.037	0.050	0.011
Cystine	g	0.027	0.037	0.008
Phenylalanine	g	0.228	0.310	0.068
Tyrosine	g	0.048	0.065	0.014
Valine	g	0.105	0.143	0.032
Arginine	g	0.087	0.118	0.026
Histidine	g	0.048	0.065	0.014
Alanine	g	0.106	0.144	0.032
Aspartic acid	g	0.232	0.316	0.070
Glutamic acid	g	0.282	0.384	0.085
Glycine	g	0.102	0.139	0.031

Nutrient	Units	Value per 100 g	1.00 X 1 fruit, without skin and seed, 136g	1.00 X 1 NLEA serving, 30g
Proline	g	0.096	0.131	0.029
Serine	g	0.112	0.152	0.034

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EDUCATION

The Pennsylvania State University Ph.D. in Nutritional Science	2015
California State University, Dietetic Internship	2015
Nanjing Medical University, B.S. Degree in Medicine	2004

PUBLICATIONS

- The effect of a moderate fat diet with and without avocados on lipoprotein particle number, size and subclasses in overweight and obese adults-A Randomized, Controlled Trial. **Li Wang,** Peter L. Bordi, Jennifer A. Fleming, and Penny M. Kris-Etherton. *Journal of American Heart Association;* 2015 Jan 7;4(1). doi: 10.1161/JAHA.114.001355.

- Soy Protein reduces serum cholesterol by both intrinsic and food displacement mechanisms. David JA Jenkins, Arash Mirrahimi, Korbua Srichaikul, Claire E Berryman, **Li Wang**, Amanda Carleton, Shahad Abdulnour, John L. Sievenpiper, Cyril C.W. Kendall, Penny M. Kris- Etherton. *J Nutr;* 2010;140(12):2302S-2312S.

- Atherosclerotic Cardiovascular Disease, *Present Knowledge in Nutrition, 10th edition* (2012). Simone D.Holligan, Claire E. Berryman, **Li Wang**, Michael R. Flock, Kristina A. Harris, Penny M. Kris-Etherton.

- Nutrition, diet quality and cardiovascular health, *Molecular Basis of Nutrition and Aging* (2015). **Li Wang**, Geeta Sikand, and Nathan D. Wong.

HONORS AND AWARDS

- Early Investigator Travel Award at American Heart Association EPI/Lifestyle 2015 Scientific Sessions, 2015
- Clinical Emerging Leader Award Finalist at American Society for Nutrition Scientific Sessions and Annual Meeting, 2014.
- ASN top student travel award at the American Society for Nutrition Scientific Sessions and Annual Meeting, 2013.
- Top award of the Aging and Chronic Disease Prevention RIS group poster competition at American Society for Nutrition Scientific Sessions and Annual Meeting, 2013.
- The 2nd place award of the Young Investigator Competition at National Lipid Association Scientific Sessions and Annual Meeting, 2013.
- Woot-Tsuen Wu Leung Scholarship, College of Health and Human Development, Pennsylvania State University, 2010-2011.