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DIETARY FATS:

A QUANTITATIVE AND QUALITATIVE APPROACH TO REDUCING RISK OF CARDIOVASCULAR DISEASE IN THE FASTING AND FED STATE

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Amy E. Griel

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The thesis of Amy E. Griel was reviewed and approved* by the following:

Penny M. Kris-Etherton
Distinguished Professor of Nutritional Sciences
Thesis Adviser
Chair of Committee

Terryl J. Hartman
Associate Professor of Nutritional Sciences

Keith R. Martin
Assistant Professor of Nutritional Sciences

Sheila G. West
Associate Professor of Biobehavioral Health

David Mauger
Associate Professor of Biostatistics

Michael H. Green
Professor of Nutritional Sciences
Head of the Department of Nutritional Sciences

*Signatures are on file in the Graduate School.

ABSTRACT

Two research studies were conducted in normo- and hypertriglyceridemic individuals to determine the quantitative and qualitative effects of dietary fatty acids in the fasting and fed states, respectively. A randomized controlled feeding study was conducted to evaluate the effects of the chronic consumption of a low-fat versus a moderate-fat diet. The results of the studies conducted demonstrate that chronic intake of dietary patterns at either end of the recommended range of total dietary fat intake (20-35% total kcal) improves levels of serum lipids and lipoproteins. Despite a reduction in fasting triglycerides following the low-fat diet, hypertriglyceridemic individuals had higher levels of atherogenic apolipoproteins following the low-fat diet, compared to the moderate-fat diet. Based on this study, the recommendation to consume a more moderate fat diet must also include a focus on the quality of the fat being consumed. The results of the second study, a randomized crossover design postprandial study indicate that the consumption of different kinds of fatty acids have very different affects on postprandial risk factors for CVD. In particular, the simple heating of a PUFA increased levels of individual trans fatty acid isomers and lipid hydroperoxides, resulting in diverse physiological responses, compared to the same un-heated PUFA. In summary, whereas the implementation of a very healthy low-fat diet may have adverse affects on the apolipoprotein profile of individuals with elevated baseline triglyceride levels, the inclusion of unsaturated fatty acids in a moderate-fat diet, has a significant hypotriglyceridemic effect in individuals, regardless of baseline triglyceride status. In making the recommendation to consume a moderate-fat diet it is important to recognize the effects of individual fatty acids on postprandial risk factors for cardiovascular disease. In particular, the heating of highly unsaturated fatty acids leads to the production of trans fatty acids and lipid oxidation end products. The diverse physiological responses observed thus indicate that a diet rich in mono- and polyunsaturated fatty acids is optimal, when compared to a diet rich in saturated and/or trans fatty acids.

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List of Abbreviations

4-HNE	4-hydroxy nonenal
AA	Acetic acid
AMI	Acute myocardial infarction
AI	Adequate Intake
ATP III	Adult Treatment Panel III
AMDR	Acceptable Macronutrient Distribution Range
ARHQ	Agency for Health Care Policy and Research
ALA	Alpha-linolenic acid
AHA	American Heart Association
ASAP	Antioxidant Supplementation in Atherosclerosis Prevention Study
Apo	Apolipoproteins
AUC	Area under the curve
ARIC	Atherosclerosis Risk in Communities Study
AAD	Average American diet
BP	Blood pressure
BMI	Body mass index
BHT	Butyl-hydroxy-toluene
kcal	Calorie
CHO	Carbohydrate
CVD	Cardiovascular disease
CLAS	Cholesterol Lowering Atherosclerosis Study
CV	Column reservoir volume
CBC	Complete blood count
CI	Confidence interval
CLA	Conjugated linoleic acid
CSFII	Continuing Survey of Food Intakes by Individuals
CAD	Coronary Artery Disease
CHD	Coronary Heart Disease
CRP	C-reactive protein
DBP	Diastolic blood pressure
DASH	Dietary Approaches to Stop Hypertension trial
DELTA	Dietary Effects on Lipoproteins and Thrombogenic Activity Study
DRI	Dietary Reference Intakes
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
ET-1	Endothelin-1
ELISA	Enzyme-Linked Immunosorbent Assay
EIA	Enzyme immunometric assay
EDTA	Ethylenediaminetetraacetic acid
FAMEs	Fatty acid methyl esters
FMD	Flow-mediated vasodilation
GCRC	General Clinical Research Center

HR	Hazard Ratio
HDL-C	High-density lipoprotein cholesterol
hs-CRP	High-sensitivity-CRP
HOMA	Homeostasis Model Assessment
HRT	Hormone replacement therapy
HTG	Hypertriglyceridemic
ICD	Implantable cardioverter defibrillator
IOM	Institutes of Medicine
IL-6	Interleukin-6
IDL	Intermediate-density lipoprotein
IQR	Interquartile range
IHD	Ischemic heart disease
LA	linoleic acid
LPX	Lipid hydroperoxide
LPL	Lipoprotein lipase
Lp	Lipoproteins
LDL	Low-density lipoproteins
LDL-C	Low-density lipoprotein cholesterol
LFD	Low fat (20%) diet
MDA	Malondialdehyde
MeOH	Methanol
MFD	Moderate fat (35%) diet
MUFA	Monounsaturated fatty acid
MI	Myocardial Infarction
NTx	N-telopeptides
NAS	National Academy of Sciences
NCEP	National Cholesterol Program
NHANES	National Health and Nutrition Examination Survey
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NTG	Normotriglyceridemic
OA	Oleic acid
n-6	Omega-6 polyunsaturated fatty acid
n-3	Omega-3 polyunsaturated fatty acid
OmniHeart	Optimal Macronutrient Intake Trial to Prevent Heart Disease Trial
OXPUFA-FL	Oxidized-polyunsaturated test fat load
ORAC	Oxygen radical-absorbing capacity
PA	Palmitic acid
PHFO	Partially hydrogenated fish oil
PUFA	Polyunsaturated fatty acid
P/S	Polyunsaturated fatty acid/saturated fatty acid ratio
PUFA-FL	Polyunsaturated test fat load
PRO	Protein
Q_1	First quartile
Q_3	Third quartile

RDA	Recommended Dietary Allowance
RR	Relative Risk
SFA	Saturated fatty acids
SFA-FL	Saturated test fat load
SDA	Stearadonic acid
SA	Stearic acid
SBP	Systolic blood pressure
TLC	Therapeutic Lifestyle Changes
TC	Total cholesterol
TFA	*trans* fatty acids
Vit E-O•	Tocopheroxyl radical
TAS	Total antioxidant assay
TG	Triglyceride
TAG	Triacylglycerol
US	United States
USDA	U.S. Department of Agriculture
VLDL-C	Very-low density lipoprotein cholesterol
WHI	Women's Health Initiative

Chapter 1
Introduction

Cardiovascular disease (CVD) remains the leading cause of death for both men and women in the United States. In 2002, CVD accounted for 38% of all deaths in the United States; it was estimated that in 2005 700,000 Americans would have a new coronary event and about 500,000 would have a recurrent event(1). CVD claims about as many lives each year as the next 5 leading causes of death combined, including cancer, chronic lower respiratory diseases, accidents, diabetes mellitus, and influenza and pneumonia. Dietary recommendations for the prevention and treatment of CVD have evolved across the decades, driven by the advancement of enhanced research methodologies and techniques. In 1980, the recommendations set forth by the Dietary Guidelines for Americans were to "avoid too much fat, saturated fat, and cholesterol" (2). The most recent Dietary Guidelines for Americans, released in 2005 encourage a healthy eating plan that is "low in saturated fats, *trans* fats, cholesterol, sodium, and added sugars" (3). These recommendations were the first to suggest a range of total dietary fat intake of 20-35% of calories (defined as moderate fat), while still keeping saturated fat to less than 10% of calories and *trans* fats as low as possible. Within these recommendations emphasis is placed on the selection of polyunsaturated (PUFA) and monounsaturated (MUFA) fatty acids from sources such as fish, nuts, and vegetable oils. Rather than focusing on limiting certain foods, this set of dietary recommendations is the first to encourage unsaturated fats within the context of an overall healthy dietary pattern. This evolution in the dietary recommendations directly reflects the expanding knowledge base that has developed over the years with respect to dietary fats and their effect on

CVD and other health outcomes. It is well established that a diet rich in unsaturated fatty acids beneficially impacts risk of CVD, via a reduction in total and low-density lipoprotein cholesterol (LDL-C) and an increase or maintenance of levels of high-density lipoprotein cholesterol (HDL-C).

Just as our understanding of the health effects of dietary fats has increased, so has our knowledge of different risk factors associated with the development and progression of CVD. The Framingham Heart Study played a pivotal role in the identification of traditional risk factors for CVD. The major risk factors that were extensively studied in the Framingham cohort include cigarette smoking, hypertension, serum cholesterol and various lipoprotein fractions, and diabetes mellitus (4). In particular, LDL-C has been considered the primary target of CVD prevention and therapy for decades. Other traditional lipid risk factors include total cholesterol (TC), HDL-C, and triglyceride (TG) levels. The growing data base in the field of lipids and lipoproteins has led researchers to suggest that superior risk prediction may be achieved not only by evaluating specific lipid ratios, such as TC/HDL-C, but also by measuring apolipoproteins (5-10). While these additional lipid parameters have given clinicians and researchers stronger predictive power in determining the incidence of CVD it is no longer considered just a disease of lipid disorders, but is also now considered a disease of inflammation and oxidation.

The oxidative modification of LDL-C is necessary for the uptake and cellular accumulation of cholesterol involved in plaque formation (11). Lipid hydroperoxides, lysophospholipids and other biologically active moieties localize in the lipid fraction of developing atheroma (12). These modified lipids can induce the expression of proinflammatory cytokines and other mediators of inflammation in macrophages and

vascular wall cells (13). C-reactive protein (CRP), an acute-phase marker of inflammation has emerged as the most promising biomarker for inflammation (14) and is now considered an independent predictor for CVD. The impact of oxidative stress however is not simply limited to its effect on LDL-C and inflammation, but extends to other proatherogenic mechanisms. For example, free radical oxygen species can rapidly react with and inactivate nitric oxide (15). Individuals at risk for CVD not only have increased levels of inflammation and oxidative stress, but also have a compromised antioxidant defense system (13,16-18). These processes are related and consequently, a decrease in exogenous antioxidant levels leads to an increase in the production of lipid peroxidation products (lipid hydroperoxides), and an increase in inflammatory markers (such as CRP). Similarly, CRP has been shown to augment the production of the potent endothelium-derived vasoconstrictor, endothelin-1 (19). Endothelin-1 is considered a key mediator in the development of endothelial dysfunction and atherosclerosis (20). The complex interplay of these atherosclerotic processes provides several potential targets for reducing the risk of CVD.

As our knowledge and understanding of dietary fats and their role in the prevention and treatment of CVD has grown, so has the number of remaining research questions. Dietary recommendations are now tailored and focus on the individual patient and specific phenotypes, rather than blanket recommendations for the entire population. We now understand that different population groups have more favorable responses to different amounts of fat in their diet although questions still remain as to what the optimal fatty acid profile is for the prevention of CVD. While recommendations are traditionally made based on fasting biological endpoints following the chronic consumption of a

particular dietary pattern, the fact is that the American population spends a majority of their day in the postprandial state. Because dietary recommendations now emphasize the consumption of a more moderate fat diet, rather than eliminating fat from the diet, it is important to examine the effect of different types of fat on postprandial risk factors for CVD. Delayed clearance of atherogenic lipids is an independent risk factor for coronary heart disease (21). Abnormal transport and metabolism of postprandial triglyceride rich lipoproteins are linked to atherosclerosis in the coronary and carotid arteries, and can be affected not only by different types of dietary fat, but also by baseline triglyceride levels (22). Consequently, gaining a better understanding of how different fat and fatty acid profiles affect risk factors for CVD, both in the acute and chronic physiological state will facilitate revising dietary recommendations for individuals to further decrease risk of CVD.

The purpose of the first study conducted was to test the new range of the 2002 DRI dietary fat recommendation (20-35% total fat) in both normo- and hypertriglyceridemic individuals to determine the optimal macronutrient profile to optimize CVD risk factors. The purpose of the second study was to investigate the effects of consuming different types of fat on postprandial lipids, and markers of inflammation, oxidative stress, antioxidant potential and endothelial health, given our new recommendations to consume a diet rich in unsaturated fatty acids. The results of the present studies indicate that a moderate-fat diet can dramatically reduce the risk of CVD via improvements in the lipid profile. Compared to a low-fat diet (20% of calories), the consumption of a moderate-fat diet (35% of calories) lowered the ratio of TC:HDL-C by 0.47 units, representing an estimated ~25% reduction in risk of MI

(Chapter 3; Study 1). The recommendation to consume a moderate-fat diet must also focus on the quality of the dietary fats in the diet. The results of the second study demonstrate that the consumption of different types of dietary fatty acids have marked differences on postprandial CVD risk factors. In particular, the heating of a PUFA-rich dietary fat has the capacity to generate trans fatty acids, which can then elicit adverse *in vivo* physiological responses. This is important because diets rich in unsaturated fatty acids are recommended for the prevention of CVD. When making this recommendation careful consideration should be taken regarding not only the types of fat chosen, but also the preparation of the dietary fats themselves.

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Chapter 2
Literature Review

The Effects of Dietary Components on Cardiovascular Disease Risk

Total Dietary Fat

Dietary fat is an essential nutrient, serving as an energy supply, and as a carrier for the absorption and transport of fat-soluble vitamins. Fat also is a source of essential fatty acids, antioxidants, and numerous bioactive compounds, serve as building blocks of membranes and play a key role in the regulation of numerous biological functions (1). The current dietary recommendations for the general public of the United States of America, set forth by the Dietary Guidelines Advisory Committee Report states that total fat intake should be 20 to 35% of calories for adults (2). This recommendation is in agreement with the Institute of Medicine's (IOM's) Acceptable Macronutrient Distribution Range (AMDR) of 20-35% of calories from fat, although it is slightly different from the recommendation set forth by the National Cholesterol Program's (NCEP) Adult Treatment Panel III (ATP III) (25-35% of calories). The lower limit of the total fat recommendation is set at 20% of calories from fat because at intakes less than this, serum TG levels increase and HDL-C levels decrease. The target population for the ATP III recommendations is individuals at risk for CHD, therefore the NCEP sets the lower limit for total fat intake to be 25% of calories to minimize this adverse affect on the lipid profile. At an intake of 20% of calories from fat it also is difficult to achieve the recommended intake of several nutrients, including the essential fatty acids and vitamin E. A menu modeling process completed by the U.S. Department of Agriculture's (USDA's) Center for Nutrition Policy and Promotion revealed that vitamin E Recommended Dietary Allowance's (RDA's) were met only at the 3,000 and 3,200

calorie levels when testing dietary patterns consistent with increases in the percentage of calories from fat and increases in the energy content. When testing the Adequate Intakes (AIs) of the essential fatty acids linoleic acid (LA) and α-linolenic acid (ALA), few food patterns met the recommended levels at 20% of calories from fat, most did at 25%, and all models met the AIs for LA and ALA at 30 and 35% of calories from fat. The upper limit of fat intake (35%) is set in order to restrict the saturated fat content of the diet. Because higher intakes of total fat are associated with increased saturated fatty acid intakes, diets that are > 35% of calories from fat generally provide high levels (>10% of calories) of saturated fatty acids. Diets with > 35% of calories from fat also may increase the risk of consuming excess calories, risk of certain cancers (breast and colorectal), and may promote a prothrombotic state. Thus, the fat recommendations are anchored at the lower end to prevent nutrient deficiencies and at the high end to limit consumption of saturated fat.

Numerous clinical studies have been conducted to compare the plasma lipid and lipoprotein responses to different blood cholesterol-lowering diets that vary in the amount of total fat and carbohydrate. Low-fat/high-carbohydrate diets (18-30% of calories total fat) have been compared with higher fat diets (30-40% of calories) that provide similar amounts of SFA (4-12% of calories) and dietary cholesterol (<100-410 mg/day). Consistently, a low-fat, high-carbohydrate diet compared with a higher fat diet (both relatively low in SFA and cholesterol) decreases LDL-C levels to a similar degree (3-15). Low-fat diets, however, decrease the cardioprotective HDL-C. Since HDL-C is proportionately decreased as LDL-C is decreased, the ratio of LDL-C to HDL-C does not change (16). On a moderate-fat diet, however, when unsaturated fatty acids replace SFA,

LDL-C decreases proportionately more than HDL-C thereby decreasing the LDL:HDL-C ratio (17). Low-fat, high-carbohydrate diets also increase fasting TG versus moderate-fat diets, both of which are low in saturated fat. The recent results from the Women's Health Initiative (WHI), a randomized controlled trial of over 48,000 postmenopausal women, reported no benefit of a low-fat diet intervention designed to provide 20% of calories from fat on CVD risk, likely because the intervention group did not attain their target fat goal (i.e., they consumed 29% of calories from fat) (18). Although the intervention was directed at a reduction of total fat a subgroup analysis showed a trend towards a reduction in CHD risk in women without existing CVD at the start of the study who attained the lowest levels of saturated fat (<6.1% calories) [adjusted HR=0.82; 95% CI: (0.67, 0.99); p=0.05] and trans fat (<1.1% calories) [adjusted HR=0.84; 95% CI: (0.69, 1.02); p=0.10] or the highest intake of fruits and vegetables (>6.5 servings/day) [adjusted HR=0.89; 95% CI: (0.74, 1.06); p=0.11]. In some individuals however, a low-fat, high-carbohydrate diet, compared to a higher fat diet, induces atherogenic dyslipidemia (19).

Atherogenic dyslipidemia is characterized by small dense LDL-C particles, high levels of TG and low HDL-C levels (20). In sedentary, overweight or obese populations, in particular, low-fat, high-carbohydrate diets increase the prevalence of this phenotype. This phenotype is associated with increased risk of CHD (21). Viscous fiber may attenuate the hypertriglyceridemic response to dietary carbohydrate (19). Within the range of total fat evaluated in the controlled feeding studies conducted to date, there is a linear dose-response relationship between total fat content of the diet and the changes in HDL-C and TG (22), such that with increasing levels of total dietary fat TG levels decrease and HDL-C levels increase (Figure 1). Weighted least-squares regression

analysis revealed that for every 5% decrease in total fat, HDL-C levels would decrease by 2.2% and TG levels would increase 6%. When recommending the consumption of a moderate fat diet, attention must be placed on the types of fatty acids within this diet.

Types of Dietary Fat

Saturated Fatty Acids

It is well established that dietary saturated fatty acids (SFA) raise total and low-density lipoprotein cholesterol (LDL-C) levels (17,23-25), and thereby increase risk of CVD. The first dietary guidelines for the general public were released by the American Heart Association in 1957 and recommended that polyunsaturated fatty acids (PUFA) replace SFA (26). The recommendation to decrease SFA has been supported for over 40 years based on a robust database from both clinical and epidemiologic studies. The Dietary Guidelines for Americans 2005 recommends that saturated fat consumption be < 10% if calories and that total fat provide 20-35% of calories (1). Specifically, for adults with LDL-C < 130 mg/dL (3.38 mmol/L), saturated fat intake should be < 10% of calories. For adults with elevated LDL-C levels [$\geq$ 130 mg/dL (3.38 mmol/L)], less than 7% of calories should come from SFA. These recommendations are in agreement with those of the NCEP's ATP III recommendations for saturated fat intake. The Dietary Effects on Lipoproteins and Thrombogenic Activity (DELTA) Study was an important study that investigated the effects of decreasing SFA on lipids and lipoproteins (27). The DELTA Study was a multi-center study that compared an average American diet (AAD) (34% calories total fat, 15% calories SFA) with a Step I diet (28.6% calories total fat, 9% calories SFA), and a low-SFA diet (25.3% calories total fat, 6.1% calories SFA) (27). TC was reduced 5% and 9% on the Step I and low-SFA diets, respectively, compared with

the AAD (both P<0.01). LDL-C and HDL-C were reduced similarly by 7% and 11%, respectively, on both the Step I and low-SFA diets versus the AAD (both P<0.01).

The Optimal Macronutrient Intake Trial to Prevent Heart Disease (OmniHeart) Trial was designed to compare the effects of 3 low SFA diets, varying in the macronutrient profile, on serum lipids and blood pressure (28). Participants with pre-hypertension or stage 1 hypertension (n=164) were randomized in this 3-period crossover design controlled feeding study. Each diet period lasted for six weeks, with a two to four week washout period between each of the diet periods. The three diets were 1) a CHO-rich diet (58% CHO, 15% PRO, 27% total fat, 6% SFA, 13% MUFA, 8% PUFA), 2) a diet rich in protein (48% CHO, 25% PRO, 27% total fat, 6% SFA, 13% MUFA, 8% PUFA), and 3) a diet rich in unsaturated fat (48% CHO, 15% PRO, 37% total fat, 6% SFA, 21% MUFA, 10% PUFA).

When compared with baseline, levels of blood pressure, LDL-C and estimated CVD risk were lower on all three diets (28). Comparisons between the three test diets showed beneficial effects of the diets rich in protein and unsaturated fats, above those effects observed for the CHO-rich diet. When compared to the CHO-rich diet, the protein-rich diet decreased mean SBP by 1.4 mmHg (p<0.01) in normotensive individuals and by 3.5 mmHg (p<0.01) in hypertensive individuals. The protein-rich diet also elicited a greater decrease in LDL-C [3.3 mg/dL (0.09 mmol/L); p=0.01], HDL-C [1.3 mg/dL (0.03 mmol/L); p<0.05], and triglycerides [15.7 mg/dL (0.17 mmol/L); p<0.001], compared to the CHO-rich diet. The diet rich in unsaturated fatty acids had a similar BP-lowering effect, decreasing mean SBP by 1.3 mmHg (p<0.01) in normotensives and by 2.9 mmHg (p<0.05) in hypertensives, beyond those effects

observed following the CHO-rich diet. Compared to the CHO-rich diet, the diet rich in unsaturated fatty acids had no significant effect on levels of LDL-C, but significantly increased HDL-C [1.1 mg/dL (0.03 mmol/L); $p<0.05$] and lowered triglycerides [9.6 mg/dL (0.11 mmol/L); $p<0.05$]. There were no differences between the diet effects on mean SBP and DBP when comparing the protein-rich and unsaturated fatty acid-rich diets. The protein-rich diet did however significantly lower levels of total cholesterol [-5.0 mg/dL (-0.13 mmol/L); $p<0.001$], compared to the unsaturated fatty acid rich diet, primarily due to the decrease in HDL-C observed on the protein-rich diet and the corresponding increase in HDL-C on the unsaturated fatty acid-rich diet. Levels of triglycerides also were significantly reduced on both the unsaturated fatty acid diet [-9.3 mg/dL (-0.10 mmol/L)] and the protein-rich diet [-16.4 mg/dL(-0.18 mmol/L)], although more so on the protein rich ($p<0.05$). As a result of their enhanced reduction in total cholesterol and blood pressure levels, diets rich in protein and unsaturated fatty acids similarly reduced the estimated Framingham 10-year CVD risk (-21.0% and –19.6%, respectively), compared with a diet rich in carbohydrates (-16.1%).

Early studies by Keys et al. (29) and Hegsted et al. (30) in the 1960s used regression analysis to evaluate the effect of individual fatty acids on levels of total cholesterol (TC) in humans. Predictive equations estimate that SFA raises TC compared to carbohydrates and MUFA (which both have neutral effects), while PUFA lowers TC. Clinical studies also have demonstrated the LDL-C raising effect of SFA (31,32). In a meta-analysis of 395 dietary experiments, among 129 groups of individuals, Clarke et al. (33) demonstrated a strong positive relationship between SFA intake and levels of TC (Figure 2). Results of regression analysis of clinical studies have determined that for

every 1% increase in energy from SFA, LDL-C levels will increase approximately 1.3-1.7 mg/dL (0.033-0.045 mmol/L) (Figure 3) (17,24,33). In addition to raising TC and LDL-C, SFA also has been shown to increase HDL-C levels. It is estimated that for every 1% increase in SFA, HDL-C will increase by 0.4-0.5 mg/dL (0.011-0.013 mmol/L) (17,24,33).

In addition to equations that incorporate classes of fatty acids, equations have been generated to predict how alterations in individual dietary fatty acids affect TC, LDL-C, and HDL-C. Recent regression analyses have demonstrated that stearic acid (18:0) has no effect on TC, LDL-C, and HDL-C (34), while myristic acid (14:0) is more hypercholesterolemic than lauric acid (12:0) and palmitic acid (16:0) (35). A recent meta-analysis of 60 controlled trials determined the effects of different SFA relative to carbohydrate (CHO) on the TC/HDL-C ratio (36). Although lauric acid was found to increase LDL-C the most, it decreased the ratio of TC/HDL-C due to a greater increase in HDL-C levels relative to TC. Myristic and palmitic acids had little effect on the ratio due to similar increases in both TC and HDL-C. Stearic acid reduced the ratio due to a slight increase in HDL-C.

The Seven Countries Study was a classic epidemiologic study that reported a strong positive correlation between SFA intake and CHD mortality rates, as well as a significant association between total SFA intake and TC (37). Subsequent epidemiologic studies also have found correlations with classes of SFA and TC levels and incidence of CHD (38,39). In a more recent analysis of the Seven Countries Study, strong positive associations were reported between 25-year death rates from CHD and average intake of the four major saturated fatty acids: lauric, myristic, palmitic, and stearic acid ($r>0.8$)

(40). Specifically, intakes of lauric acid (12:0) and myristic acid (14:0) were most strongly associated with TC (r=0.84, r=0.81, respectively). However, a recent epidemiologic study has questioned the relationship between the intake of SFA and risk of CHD.

Mozaffarian and colleagues analyzed data from the Estrogen Replacement and Atherosclerosis trial in a population with a lower total fat intake (25% calories), and found that a greater SFA (10.6 to 16% calories) intake was associated with less progression of coronary atherosclerosis in postmenopausal women, compared to women with a lower intake of SFA (3.5 to 7% calories) (41). The results of this study are in contrast to previous work using the Nurses' Health Study cohort, a population without pre-existing CVD or type 2 diabetes. Hu and colleagues compared four different risk models for energy adjustment when examining the associations between the intake of the four major types of fat (SFA, monounsaturated (MUFA), PUFA, and *trans* fat) (42). In contrast to the results presented by Mozaffarian and colleagues, the Hu study found that SFA and *trans* fats were associated with an increased risk of CHD. Earlier results from the Nurses' Health Study indicated that for every 5% increase in energy intake from SFA, as compared with an equivalent energy intake from carbohydrates, risk of CHD would increase 17% (RR, 1.17; 95% CI, 0.97 to 1.41; p=0.10) (39). More recent work from the Nurses' Health Study has demonstrated that a higher intake of long-chain SFA was associated with an increased risk of CHD [RR=1.14; 95% CI: (0.93, 1.39); p=0.03], whereas intake of short- and medium-chain SFA was not [RR=1.07; 95% CI: (0.89, 1.30); p=0.78] (43). Intake of SFA among adults ages 20 to 74 years decreased from 13% of calories in 1971-1974 to 11% of calories in 1999-2000 (44). Thus, while the

results of the Mozaffarian study bring to light important questions regarding the role of SFA in a heart healthy diet, the "higher" intake of SFA reported in their study is actually close to current intakes in the US population. Collectively, the results from the Nurses' Health Study, in conjunction with those reported by Mozaffarian and colleagues(41), are provocative since they raise important questions about whether different diets should be recommended for different groups of postmenopausal women for the prevention and treatment of CVD.

Trans Fatty Acids

Current dietary guidelines recommend that intake of *trans* fatty acids (TFA) be kept as low as possible ($\leq$ 1% of calories), for all population groups. This recommendation put forth by the 2005 Dietary Guidelines Advisory Committee (2) is supported by a systematic, extensive review of the evidence collected by the Institutes of Medicine (20 controlled trials and 11 epidemiologic studies) (22), and the evidence review conducted by the NCEP ATP III Report Committee (20). Based on CSFII data from 1989-1991 the estimated mean TFA intake for the US population, ages 3 and older was 2.6% of calories (45). Industrial sources of TFA account for 80% of TFA intake, whereas 20% comes from animal sources. Elaidic acid (t-18:1) is the predominant *trans* fatty acid found in some hydrogenated fats and is produced in the deodorization of vegetable oils. These industrial sources of TFA are commonly found in commercially prepared baked products, fried foods, and margarine. Meat and dairy products contain naturally occurring TFA as vaccinic acid and conjugated linoleic acid (CLA). The effects of TFA on TC, LDL-C, and HDL-C have been compared to other fatty acids via the development of blood cholesterol predictive equations. Results from a study by Lichtenstein and colleagues indicates that TFA increase TC [Δ TC = 2.77(Δ SFA) +

2.52(ΔTFA)] and LDL-C [Δ LDL-C = 2.46(Δ SFA) + 2.04(ΔTFA)] less than SFA, but they also lower HDL-C [Δ HDL-C = 0.22(Δ SFA) – 0.23(ΔTFA)] more than SFA (46). In addition, LDL particle size is decreased (47), and lipoprotein(a) is increased (48) (49,50) by TFA.

Several clinical trials have reported an HDL-C-lowering effect of TFA when compared with saturated fat. In a study conducted by Mensink et al. (51), subjects were placed on three diets that were identical in nutrient composition except that 10% of calories were either from oleic acid (MUFA), *trans* isomers of oleic acid, or SFA. The mean HDL-C level was the same on the SFA and oleic acid diets, but was 6.5 mg/dL (0.17 mmol/L) lower on the TFA diet ($p<0.0001$). Likewise, a high TFA diet (9.2% of calories TFA, 12.9% of calories SFA) produced a greater reduction in HDL-C [13.8 mg/dL (0.36 mmol/L)], compared with a high SFA diet (0% of calories TFA, 22.9% of calories SFA) (52). These studies and others (53) indicate that TFA are unfavorable due to their HDL-C lowering effect relative to SFA, and their LDL-C raising effect.

A clinical trial conducted by Judd et al. (54) utilized a randomized crossover design to evaluate the effects of replacing carbohydrates with TFA on LDL-C. Subjects were fed diets providing approximately 15% of calories from protein, 39% from total fat, and 46% from carbohydrate. TC was increased by 5.8% and LDL-C was increased by 10% when TFA replaced 8% of the energy provided by carbohydrate. When 8% of the calories provided by carbohydrate were replaced with a combination of 4% TFA and 4% stearic acid (known to have a neutral effect on plasma lipids), TC was increased by 5.6% and LDL-C was increased by 8.7%. In a review of the TFA studies that have been conducted, a linear dose-dependent relationship was reported between TFA intake and

the LDL:HDL ratio from intakes of 0.5 to 10% of total calories (Figure 4) (55). The magnitude of this effect is greater for TFA than for SFA. A clinical trial conducted by Lichtenstein et al. (46), demonstrated that increases in TFA elicit a dose-response increase in LDL-C. The study also indicated that at levels higher than typically consumed in the diet (2.6% of calories), TFA decrease HDL-C.

Several epidemiologic studies also have found an adverse effect of TFA on blood lipids and lipoproteins. In the Seven Countries Study, the average intake of elaidic acid (TFA) was positively associated with TC levels ($r=0.70$, $p<0.01$) and 25-year mortality rates from CHD ($r=0.78$, $p<0.001$) (40). This association has since then been confirmed by other epidemiologic studies (56,57). Using follow-up data from the Nurses' Health Study, Hu et al. (39) found that compared with equivalent energy from carbohydrate, for every 2% incremental increase in energy from TFA, the relative risk (RR) for CHD was 1.93 (95% CI, 1.43-2.61; $p<0.001$). The RR for TFA was higher than that for 5% of calories from SFA, and 5% from total fat (RR 1.17; 95% CI, 0.97-1.41; $p=0.10$, and RR 1.02; 95% CI, 0.97-1.07; $P=0.55$, respectively). Studies have shown that TFA increase CHD risk by various lipid-mediated mechanisms including raising LDL-C, lowering HDL-C, and raising TG (58).

Beginning in January 2006 the Food and Drug Administration is requiring that all Nutrition Facts labels must disclose the TFA content of food products, just as they now disclose other fats, cholesterol, sugar and protein. This has led the food industry to seek substitutes for TFA in their products and to reformulate many of these products that contain TFA. Food makers, such as Kraft Foods are replacing partially hydrogenated soybean oil with palm oil, an oil rich in SFA. Although palm oil is rich in SFA, it does

not contain any TFA. Palm oil also is cheaper than soybean oil and, because it is a semi-solid product, it provides the pleasing mouth feel needed for cookies, crackers and pastries. Given the detrimental effects of both TFA and SFA on CHD risk the most prudent advice for consumers would be to avoid products containing these fat sources.

Polyunsaturated Fatty Acids

The results of many clinical and epidemiologic studies indicate that the intake of PUFA may confer beneficial effects on CVD mortality. Based on the 2005 Dietary Guidelines for Americans (1) and the Institute of Medicine's (IOM) AMDR for fatty acids (59), current recommendations for the dietary intake of n-6 PUFA is between 5-10% of calories. Recommendations for the intake of α-linolenic acid (n-3) are between 0.6 and 1.2% of calories. Given the biological potency of the longer chain n-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the IOM has recommended that up to 10% of the AMDR for α-linolenic acid can be consumed as EPA and/or DHA (59). This translates into an intake of 0.06-0.12% of calories from EPA and/or DHA. This recommendation can be met by consuming two 4-ounce servings of fatty fish per week. These recommendations for the dietary intake of n-6 and n-3 PUFA are in agreement with the recommendations of the NCEP ATP III Guidelines (20), and many professional organizations including the American Diabetes Association and the American Heart Association. Based on CSFIII 1989-91 data, mean intake of n-6 PUFA for adults is approximately 5-6% of calories (45). The total median intake of n-3 fatty acids for men and women ranges from 1.3-1.8 g/day and 1.0-1.2 g/day, respectively (59). While many studies have shown an association between dietary PUFA and reduced CVD mortality after adjusting for SFA (60); other studies, such as the Seven Countries Study, reported no significant association between PUFA intake and CVD (37,40,61).

Omega-6 (n-6) Polyunsaturated Fatty Acids

Some of the earliest clinical trials evaluated the effects of diets high in PUFA, ranging from 13-21% of energy, on TC and CHD events (62-65). Three of these studies reported a 13-15% decrease in TC, which was accompanied by a 25-43% decrease in CHD events (62-64). Predictive equations have demonstrated that a 1% increase in PUFA results in a reduction of TC by 0.9 mg/dL (0.024 mmol/L) (29,30). The TC lowering effect is approximately half of the cholesterol-raising effect of SFA (29,30). More recent predictive equations developed for individual fatty acids demonstrate that linoleic acid (LA) is the strongest TC and LDL-C lowering fatty acid. Some studies have also shown that LA raises HDL-C when compared with stearic acid (18:0) (66). A study by Mattson and Grundy (31), however, reported an HDL-C lowering effect [-5.0 ± 1.7 mg/dL (-0.13 ± 0.04 mmol/L); $p<0.02$] of PUFA at very high levels (28% of kcals) in normotriglyceridemic individuals. Other studies have reported no significant change in HDL-C with a high PUFA intake (67).

Specific associations with linoleic acid (LA), the predominant n-6 fatty acid, and coronary disease risk also have been inconsistent. A cross-population study in healthy men found an inverse association between n-6 levels in adipose tissue and mortality rate from CAD (68). In contrast, a recent study in an Israeli population consuming PUFA as 10% of total energy did not find an association between LA intake and acute myocardial infarction (AMI) (69). However, there was a positive association between arachidonic acid, the long chain derivative of LA, with AMI ($p=0.004$). Thus, after multivariate adjustment there was no indication of an adverse association between LA and AMI.

A large number of epidemiologic studies also have investigated the association between n-6 PUFA and CVD risk. The results of two population studies (70,71) indicate that PUFA intake is negatively associated with CVD mortality, after adjustment for dietary SFA intake. In addition, several prospective studies (39,72-74), two longitudinal studies (75,76) and one cross-sectional study (77) also have reported a beneficial association of dietary PUFA with CVD morbidity and mortality. Specifically, results from the Nurses' Health Study indicate that as compared with equivalent energy from carbohydrates, the relative risk of CHD for a 5% increment in energy from PUFA was 0.62 (95 % CI, 0.46 to 0.85; $p< 0.01$) (39). A 20-year follow-up report from the Nurses' Health Study demonstrated similar findings, with a multivariate RR of 0.75 (95% CI, 0.60 to 0.92; $p<0.01$) when comparing the highest quintile (7.4% of calories) to the lowest quintile (5.0% of calories) of dietary PUFA (61,78). Conversely, several epidemiologic studies (38,69,79), including the Seven Countries Study (40,80), have found no significant association between dietary PUFA and CVD. In summary, while the epidemiological evidence is mixed, the results of clinical trials demonstrate that the consumption of dietary PUFA, within recommended ranges can beneficially impact CVD risk.

Omega-3 (n-3) Polyunsaturated Fatty Acids

Data from the evidence-based report from the HHS Agency for Health Care Policy and Research (ARHQ) entitled 'Effects of Omega-3 Fatty Acids on Cardiovascular Disease' (81) provide convincing evidence concerning protection against CVD and n-3 fatty acids. This AHRQ report summarizes 22 prospective studies that were conducted in many countries, including the United States, China, Japan, and

countries in the Mediterranean and Northern Europe. A majority of the cohorts that were looked at had several thousand subjects, with most aged 40 years or older. Overall, this report concluded that the evidence from both primary and secondary prevention studies support the hypothesis that the consumption of n-3 fatty acids (EPA, DHA, ALA), fish, and fish oil reduce all-cause mortality, and various CVD outcomes, including sudden death and cardiac death (81).

The proposed mechanisms by which n-3 fatty acids protect against CHD include increased stabilization of atherosclerotic plaques; decreased production of adhesion molecules; eicosanoids; cytokines; and increased endothelial relaxation and vascular compliance (82). In addition, n-3 fatty acids are known to influence the lipid profile. Overall, studies have observed slight increases in LDL-C (5-10%) and HDL-C (1-3%) and substantial decreases in TG levels (25-20%) with marine based n-3 fatty acid supplementation (< 7 g n-3 fatty acids/d) (83). Studies have also tested the hypothesis that n-3 fatty acids will protect against ventricular arrhythmias. In particular, two studies have examined the effects of EPA and DHA in patients with implantable cardioverter defibrillators (ICDs) (84,85). Raitt *et al.* found no overall benefit of n-3 fatty acids on time to the first ICD therapeutic discharge; in a subset of patients who had a history of ventricular tachycardia or fibrillation, the time to first discharge was actually shortened by n-3 supplementation (84). Total deaths, however, tended to be fewer in the n-3 group (4 vs 10; p=0.16). On the other hand, Leaf *et al.* found a beneficial effect of n-3 fatty acids on a combined endpoint of time to discharge or death (85). The latter study provided twice as much n-3 fatty acids (2.6 g of EPA and DHA vs. 1.3 g), included twice as many patients (402 vs 200), and the patients were much less likely to have significant

heart failure. These factors (and probably others) may explain the divergent results of these two studies. Although these results are intriguing, firm dietary recommendations for n-3 PUFA supplementation in patients with ICDs must await the results for other ongoing studies.

The primary effect of marine sources of n-3 fatty acids on the lipid profile is due to their TG-lowering effects. In a review of 44 intervention studies (86), supplementation of 0.5 to 25 g of n-3 fatty acids from fish oils for an average of 6 weeks elicited a substantial decrease in TG levels (10-20%), while LDL-C and HDL-C concentrations did not change. In addition, a study of longer duration (16-wk) found that a low dose of n-3 PUFA from fish oil (1g/d) decreased fasting TG levels by 21% (87). Consumption of 3-4 g/d of EPA and DHA results in a 25% decrease in TG in normotriglyceridemic individuals [TG < ~178 mg/dL (2 mmol/L)] and 34% decrease in TG in hypertriglyceridemic individuals [TG > ~178 mg/dL (2 mmol/L)] (83). The characteristic TG-lowering effect appears specific to marine sources of n-3 fatty acids and is generally not observed with plant sources of n-3 fatty acids. However, a TG-lowering effect has been found at very high levels (38 g) of ALA intake (83).

In addition, marine-derived n-3 fatty acids have been shown to increase HDL-C levels 5-15% in recent supplementation trials (88-92). Likewise, slightly elevated LDL-C levels have been a consistent finding in the n-3 fatty acid supplementation trials (93-96). In a review, LDL-C was increased by 4.5% in normolipemic patients and 10.8% in the hypertriglyceridemic patients consuming 3-4 g/d of EPA and DHA (83). Results of the Agency for Healthcare Research and Quality (AHRQ) Report indicate that compared to placebo, pooled n-3 intakes would be expected to cause a mean increase of 1.53 mg/dL

(95% CI, -0.82, 3.87) in HDL-C and a 5.12 mg/dl (95% CI, -1.02, 11.25) mean increase in LDL-C in type 2 diabetics (97). Several studies suggest that the elevation of LDL-C probably relates to an increase in LDL particle size (94,96).

In the GISSI Prevenzione Study (98), the largest (N=11,324) prospective clinical trial to test the efficacy of n-3 fatty acids for secondary prevention of CHD, subjects were randomized to the EPA + DHA supplement group (850 mg/d of omega-3 fatty acid ethyl esters), with and without 300 mg/d of vitamin E. Individuals in the supplement group compared with the control group experienced a 15% reduction in the primary endpoint of death, nonfatal myocardial infarct, and nonfatal stroke ($p<0.02$). In addition, all-cause mortality and sudden death were reduced by 20% ($p=0.01$) and 45% ($p<0.001$), respectively, compared with the control group, with Vitamin E providing no benefit.

An inverse association between n-3 fatty acids and CAD has been found in numerous epidemiologic studies. In the Seven Countries Study, a nonsignificant negative correlation ($r=-0.28$) was observed between fish consumption and CAD mortality despite large differences in fish consumption among the cohorts. An inverse correlation also was found in the 25-year follow-up of the study with n-3 fatty acid intake and 25-year CAD mortality rates ($r=-0.36$) (40). Epidemiologic studies also have found an association between α-linolenic acid (ALA), specifically, and CHD risk. In the Health Professionals Follow-Up Study, a 1% increase in ALA intake was associated with a 40% lower risk of CHD (73).

Conversion of alpha-linolenic acid (ALA) in vivo

Evidence suggests that EPA and DHA have a much higher biological potency, compared to that of ALA. Dietary ALA is converted to EPA and DHA via a series of

desaturation and elongation steps (Figure 5). Although this conversion of EPA and DHA does occur, it is thought to proceed slowly and is not well understood in humans. The results of a radio-labeled tracer study in 6 healthy women (aged 28 $\pm$ 4 years, BMI 22.4 $\pm$ 2.8 kg/m^2) indicate that the net fractional ALA inter-conversion was 21% EPA, 6% DPA, and 9% DHA (99). This study also suggests differential partitioning of ALA, EPA, and DHA between plasma lipid classes, which may facilitate the targeting of each of these individual fatty acids to specific tissues. In particular, these data suggest that while plasma cholesteryl ester may act as a long-term source of ALA within the circulation, EPA, DPA and DHA are primarily associated with phosphatidylcholine. Previous studies in men have shown that the conversion of ALA to EPA was ~8%, with conversion to DHA ranging from 0-4% (100,101). In a randomized, parallel arm study the long-chain conversion of ALA and LA was evaluated following diets rich in either flaxseed oil or sunflower oil in moderately hyperlipidemic men (n=38) using ^{13}C stable isotopes (102). The results indicated that reducing the dietary ratio of LA/ALA (27.9 to 0.5) downregulates LA conversion to AA and likely upregulates ALA conversion to EPA. The overall conversion rates of LA and ALA, when adjusted for dietary intake, were low and of similar magnitude (0.18% and 0.26%, respectively); conversion to DHA was minimal (<0.01%) (102). In a study designed to estimated the in vivo conversion of ALA following long-term intake, it was determined that nearly 7% of dietary ALA was incorporated into plasma phospholipids following 28 days of a high ALA diet (7% calories LA and 0.4% calories ALA) (103). From this pool, 99.8% was converted to EPA and 1% converted to DPA and subsequently to DHA. The results of the above studies thus suggest that women may possess a greater capacity for ALA conversion,

compared to that of men (~21% and ~8% conversion of ALA to EPA for women and men, respectively).

The fatty acid biosynthesis of ALA (n-3 PUFA) competes with linoleic acid (n-6 PUFA) for the enzymes required for the production of longer-chain fatty acids, prostaglandins and various eicosanoid end-products. The rate-limiting enzyme in these processes, which ultimately determines whether pro- or anti-inflammatory eicosanoids are produced, is the delta-5-desaturase enzyme (Figure 5). The degree of conversion from ALA to EPA and DHA is thus influenced by the amount of n-6 PUFA in the diet. Research indicates that in the presence of a background diet rich in SFA, the conversion of ALA to EPA is 6% and 3.8% for DHA. Conversely, with a background diet rich in n-6 PUFA, conversion is reduced by 40-50% (104). Thus as a result of this competition for enzyme activity, a high intake of n-6 PUFA results in a decrease in the conversion of ALA to EPA and DHA. This has led many researchers to question what the appropriate ratio of n-6:n-3 PUFA should be not only for the optimal conversion of ALA to EPA and DHA, but also for the prevention of chronic disease.

In a recent clinical study a 3-period randomized crossover design was used to evaluate the effect of altering the n-6:n-3 PUFA ratio on risk factors for CVD (105). Twenty-three hypercholesterolemic subjects consumed each diet for 6 weeks: 1) Average American diet [AAD; [34% total fat, 13% SFA, 13% MUFA, 9% PUFA (7.7% LA, 0.8% ALA)]], 2) Linoleic Acid diet [LA; [37% fat, 9% SFA, 12% MUFA, 16% PUFA (12.6% LA, 3.6% ALA)]], and 3) α-Linolenic Acid diet [ALA; [38% fat, 8% SFA, 12% MUFA, 17% PUFA(10.5% LA, 6.5% ALA)]]. The n-6:n-3 ratios of the three experimental diets were 9.0, 3.5 and 1.5, respectively. The results of this study indicated that a diet high in

n-3 PUFA, with a low n-6:n-3 ratio, appears to decrease CVD risk by inhibiting vascular inflammation and endothelial activation beyond its lipid-lowering effects, as indicated by reductions in C-reactive protein, intercellular cell adhesion molecules, vascular cell adhesion molecules and E-selectin (105). The results of an ancillary study (106) conducted to investigate the effects of the above diets on bone health indicate that incorporating walnut products and flaxseed oil into the diet reduces serum (N-telopeptides) NTx, a marker of bone resorption. The reductions in NTx were related to the amount of ALA each diet provided, and were inversely related to the presence of saturated fatty acids in the serum.

The design of this study is unique in that the high ALA diet also had a high level of LA. While in many diet designs the n-6:n-3 ratio is manipulated by decreasing the LA, and increasing the ALA, the design used in the present study was to slightly decrease the level of n-6 and to increase the level of ALA to achieve the desired ratio. The variables that can affect the n-6:n-3 PUFA ratio were reviewed recently and are presented in Table 1 (107). It is apparent that the ratio of n-6:n-3 PUFA alone does not provide sufficient evidence to understand the biological consequences of diets that vary in PUFA content. Rather, it is important to know absolute amounts of all dietary n-6 and n-3 fatty acids to sufficiently evaluate study outcomes and the biological activity of the longer-chain n-6 and n-3 PUFA. The overarching goal should be to collect as much information as possible about the amount of all constituent n-6 and n-3 PUFAs so that the ratio can be calculated and put in an appropriate context.

The evidence from 5 large US epidemiologic studies (108-112) indicates that the average intake of EPA and DHA (estimated from fish consumption) associated with the

lowest risk of coronary events is approximately 496 mg/day. A daily intake of 496 mg/day corresponds to an intake of 3.5 g/week, or two 4-ounce servings of high n-3 fish per week. Given the limited conversion of ALA to EPA and DHA in vivo, it is important that humans consume an adequate amount of EPA and DHA from dietary sources, such as fatty fish, that are rich in these fatty acids. Based on NHANES 1999-2000 data, mean intake of fish is 2.92 ounces/week, with the majority of this intake (63%) as finfish and shellfish, which contain less than 500mg n-3 PUFA per 3-ounce serving (1). While current recommendations are to increase the consumption of fish high in n-3 PUFA, for many individuals who do not eat fish, it may be difficult to implement this guidance. In addition, if all Americans consumed the recommended amount of fish the pressure on the global fish stocks and aquaculture would exceed the supply that is available. An alternative source of EPA is vegetable oils containing stearadonic acid (SDA). In a recent study, vegetable oils containing SDA increased tissue EPA concentrations more than did ALA-containing vegetable oils (113). Consumption of SDA also increased plasma, erythrocyte, platelet and mononuclear cell concentrations of EPA and DPA, but not DHA. An intake of 1g SDA/day would be expected to increase tissue concentration of EPA equivalent to those that were observed in the GISSI Prevention Study (113). Thus use of SDA-containing oils in food manufacturing could provide a wide range of dietary alternatives for increasing tissue concentrations of EPA. Given the limited conversion of ALA to EPA and DHA in vivo and the inability of complete conversion of SDA to DHA, current evidence suggests that dietary intake of sources of EPA and DHA should be strongly recommended in the American diet.

Monounsaturated Fatty Acids

Monounsaturated fatty acids (MUFA) are often used to replace calories from saturated and trans fatty acids, in cholesterol-lowering diets. Dietary MUFA are present in virtually all fat-containing foods and they provide an excellent vehicle to achieve total fat recommendations, within the context of the dietary recommendations for saturated and polyunsaturated fatty acids. Current dietary guidelines recommend that dietary MUFA intake can provide up to 20% of total calories (20). Based on data from CSFII (1994-1996) (22), the third National Health and Nutrition Examination Survey (1988-1994) (114), and the 1987-1988 Nationwide Food Consumption Survey (115), dietary MUFA intake in the United States is approximately 12-14% of total calories. The results of clinical and epidemiologic studies have demonstrated that there is an inverse relationship between dietary MUFA intake and the TC:HDL-C ratio (Figure 6). In addition, when MUFA are substituted for saturated fatty acids in equal amounts, levels of LDL-C decrease and improvements in insulin sensitivity are observed.

Results of a meta-analysis of clinical feeding studies indicate that the regression coefficients for the effects of MUFA on LDL-C and HDL-C are -0.0008 and +0.006, respectively (33). Furthermore, a subsequent meta-analysis conducted by Garg et al. (116) found that diets high in MUFA vs. diets high in carbohydrate reduce fasting TG levels by 19%, decrease VLDL-C by 22%, and moderately increase HDL-C without negatively affecting LDL-C. Grundy and Mattson (10,31) have demonstrated the replacing SFA with MUFA lowers LDL-C levels without lowering HDL-C. Kris-Etherton et al. (66) demonstrated that replacing SFA with MUFA (37% calories total fat, 22% calories MUFA, 47% calories CHO) versus CHO (30% calories total fat, 15% calories MUFA and 54% calories CHO) resulted in comparable decreases in LDL-C

(6.3% and 7.0%, respectively). The blood cholesterol-lowering diet high in CHO and low in fat decreased HDL-C by 7.7% and increased TG by 6.9%, whereas the diet high in MUFA only decreased HDL-C by 4.1% and decreased TG by 4.6% (117).

A review of 18 well-controlled clinical studies compared the effects of substituting either MUFAs or carbohydrate for SFA in the context of a blood cholesterol-lowering diet (118). A series of recent publications have demonstrated that when substituted for carbohydrates, dietary MUFA have a beneficial impact on components of the metabolic syndrome and lipid abnormalities associated with atherogenic dyslipidemia (1). Following an extensive review of the literature, the 2005 Dietary Guidelines Advisory Committee concluded that "compared with a high-carbohydrate diet (>65% of calories from carbohydrate), a diet that provides approximately 20% of total calories from MUFA and 35% from total fat improves glycemic control in individuals with type 2 diabetes mellitus who maintain their body weight. Specifically, such a diet may decrease triglyceride and increase HDL-C concentrations." (119)

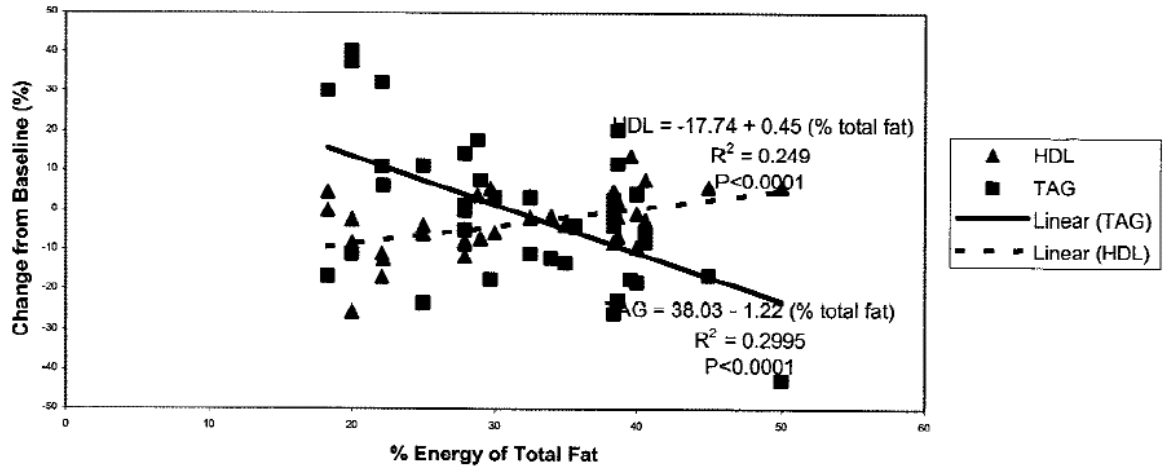
Epidemiologic studies have found inverse associations between MUFA intake and risk of CHD and ischemic heart disease (IHD) after adjusting for SFA and dietary cholesterol (39,70,120). Furthermore, the Seven Countries Study showed that rates of coronary artery disease (CAD) were low despite moderately high total fat intakes when SFA was replaced with MUFA (37). Mortality rate from CHD is lower in Mediterranean populations and they consume a diet that differs in many ways from a Western diet, including widespread use of olive oil, a major source of oleic acid, as their principal source of fat.

Table 2.2: Summary of dietary fat recommendations.

Total Fat	20-35% of calories
Saturated Fatty Acids	< 10% of calories; < 7% of calories for those at risk for cardiovascular disease and diabetes mellitus
Trans Fatty Acids	As low as possible; <1% of calories
n-6 Polyunsaturated Fatty Acids	5-10% of calories
n-3 Polyunsaturated Fatty Acids	0.6 and 1.2% of calories; up to 10% from EPA + DHA (0.06-0.12% of calories)
Monounsaturated Fatty Acids	Up to 20% of calories

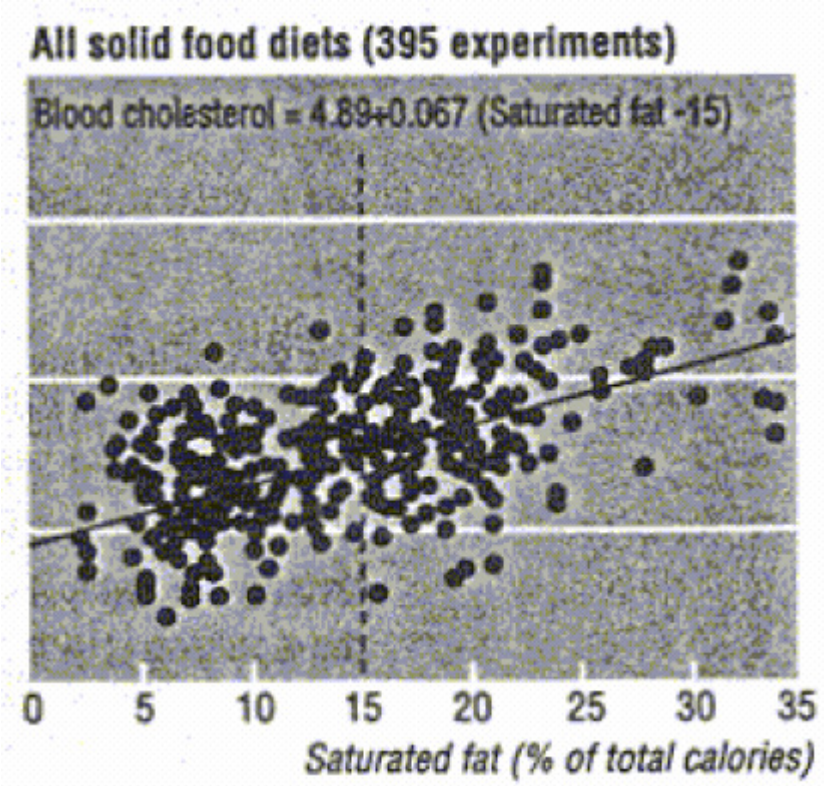
References: IOM/NAS Dietary Reference Intakes for Macronutrients, 2002 (22), NCEP ATPIII Guidelines (20).

Figure 2.1: Relationship between percent of total fat intake and change in triacylglycerol (TAG) and HDL-C (HDL) concentrations.



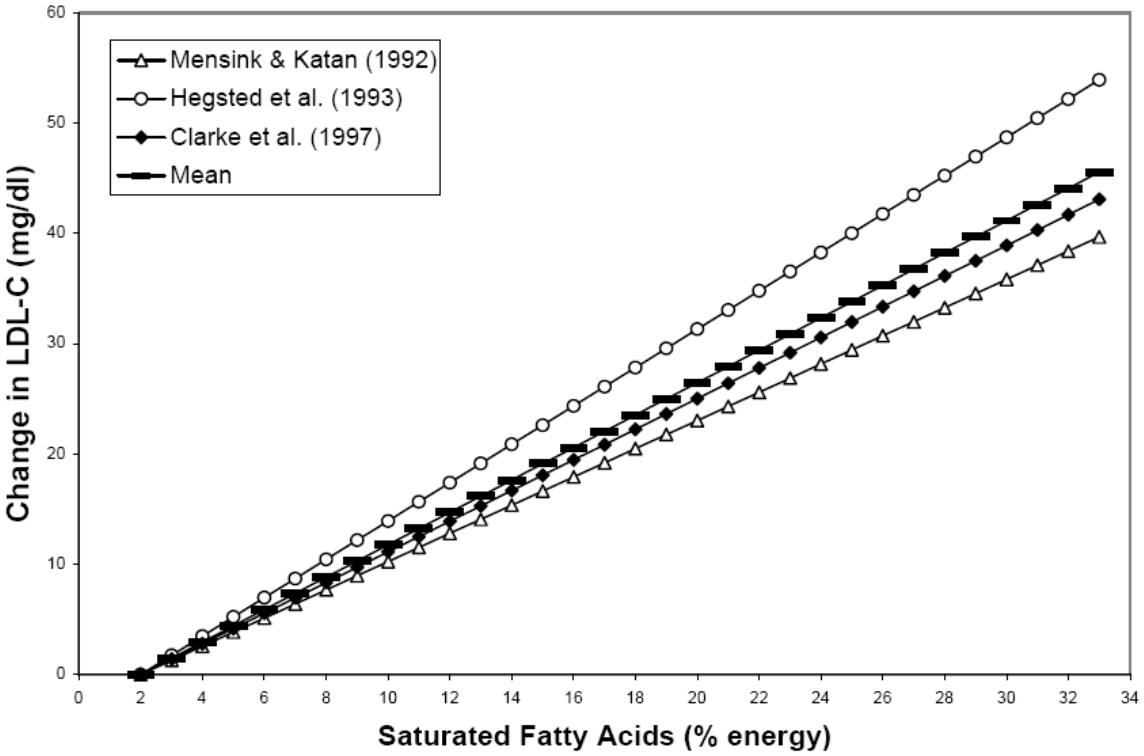
Reference: IOM/NAS Dietary Reference Intakes for Macronutrients, 2002 (22).

Figure 2.2: Relationship between serum total cholesterol concentrations and saturated fatty acid intake.



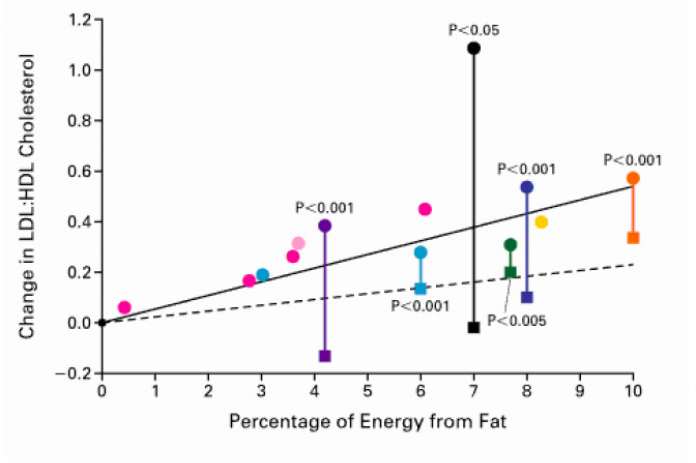
Reference: IOM/NAS Dietary Reference Intakes for Macronutrients, 2002 (22).

Figure 2.3: Calculated Changes in Serum LDL cholesterol concentration in response to percent change dietary saturated fatty acids.



Reference: IOM/NAS Dietary Reference Intakes for Macronutrients, 2002 (22).

Figure 2.4: Change in the LDL:HDL cholesterol concentrations with increasing energy intake from saturated and trans fatty acids.



Solid line represents the best-fit regression for *trans* fatty acids. Dotted line represents the best-fit regression for saturated fatty acids.

Reference: IOM/NAS Dietary Reference Intakes for Macronutrients, 2002 (22); (from Ascherio et al., 1999) (121).

Figure 2.5: Biosynthesis of n-6 and n-3 polyunsaturated long-chain fatty acids.

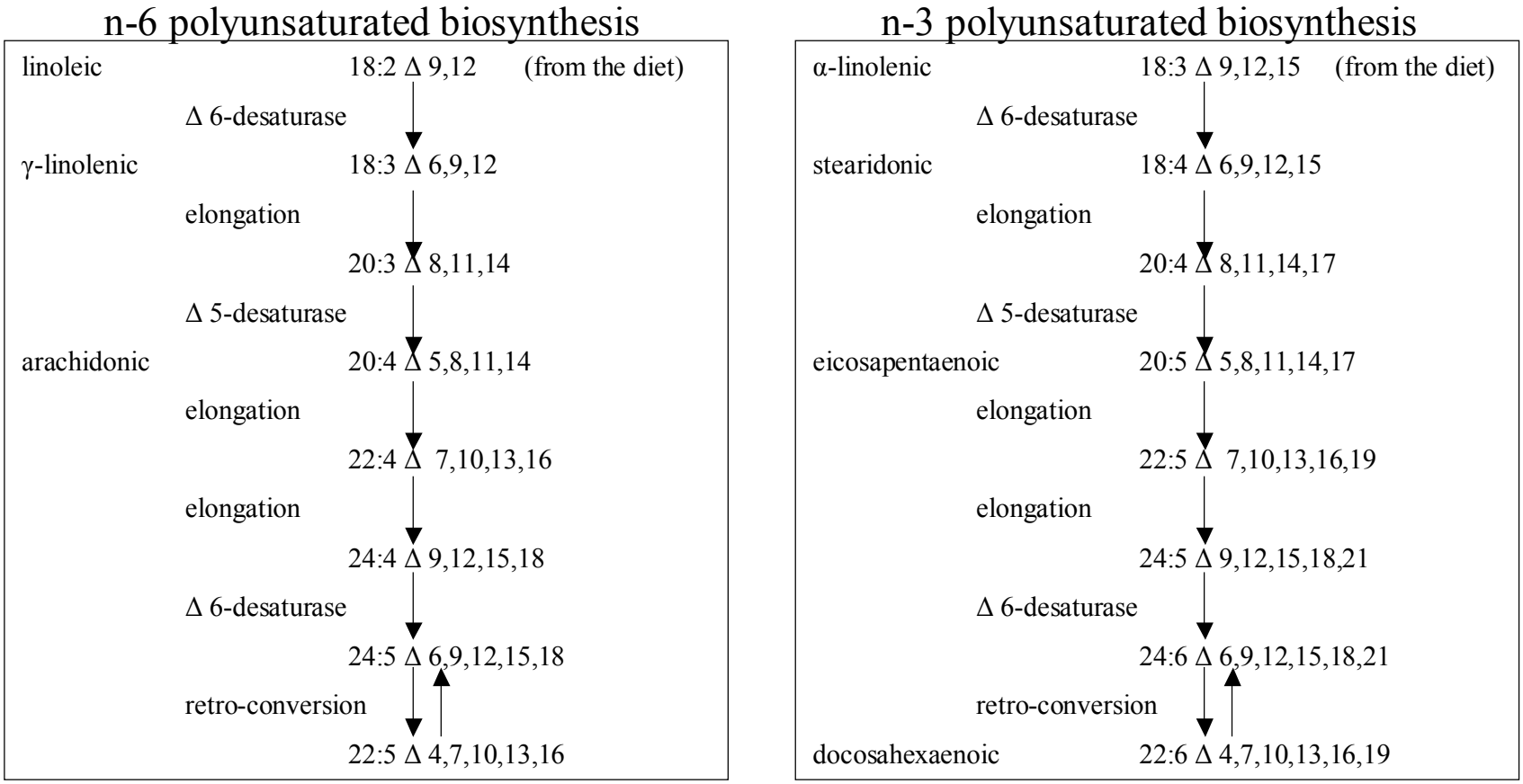


Table 2.1: Multiple strategies for changing the n-6:n-3 ratio.

Strategy	Impact on Ratio[a]
Increase the ratio	
Increase linoleic acid	+ +
Increase arachidonic acid	+
Increase both linoleic acid and arachidonic acid	+ + +
Decrease α-linolenic acid	+
Decrease EPA + DHA	+
Decrease α-linolenic acid and EPA + DHA	+
Increase linoleic acid and decrease α-linolenic acid	+ + + +
Decrease the ratio	
Decrease linoleic acid	+ +
Decrease arachidonic acid	+
Decrease both linoleic acid and arachidonic acid	+ + +
Increase α-linolenic acid	+
Increase EPA + DHA	+
Increase α-linolenic acid and EPA + DHA	+
Decrease linoleic acid and increase α-linolenic acid	+ + + +

[a] Magnitude of impact: + = least impact; + + + + = greatest impact on ratio
From Gebauer et al. [112]

Dietary Fiber

Numerous studies have demonstrated that diets rich in soluble fiber are more effective in lowering blood cholesterol levels than are diets rich in insoluble fiber (122-128). The National Academy of Sciences and the Institutes of Medicine have set the AI for dietary fiber to be 14g per 1,000 calories (22). Based on data from the Third National Health and Nutrition Examination Survey (1988-94), the mean intake of dietary fiber was 18 grams for males and 14 grams for females (129). In the same survey, mean total caloric intake for males was ~2,500 calories and ~1,800 calories for females; mean fiber intake per 1,000 calories is thus estimated to be 7.2 grams and 7.8 grams for males and females, respectively. Americans are thus currently consuming half of the recommended amount of dietary fiber. The IOM recommendation is based on evidence from clinical and epidemiologic studies that have investigated the role of fiber in decreasing the risk for cardiovascular disease. The key soluble fibers are β-glucan (found in oats, barley, and yeast), psyllium (found in husks of blonde psyllium seed), and pectin (found in fruit). Several properties of soluble fiber; including viscosity, bile acid binding capacity, and potential cholesterol synthesis-inhibiting capacity after fermentation in the colon (130,131), contribute to its cholesterol-lowering effect (132).

A high fiber intake prevents or attenuates the hypertriglyceridemic response to a high carbohydrate (CHO) diet, that is often also low in total fat (133). The traditional adoption of a high-carbohydrate, low-fat diet can produce an unfavorable lipid profile by decreasing HDL-C and increasing TG (134). Although the mechanism has not been confirmed, some studies suggest that this hypertriglyceridemic response is the result of reduced VLDL-TG clearance (135), while others attribute it to increased VLDL-TG

secretion because of increased hepatic fatty acid availability resulting from increased influx of fatty acids and decreased hepatic fatty acid oxidation (136,137). Elevated levels of blood TG are considered an independent risk factor for CHD (138,139); a 90.9 mg/dL (1 mmol/L) increase in fasting blood TG is associated with a 76% and 31% increase in CVD risk in women and men, respectively (140). Several studies note that increasing dietary fiber diminishes the adverse effects of a low-fat, high-CHO diet on HDL-C and TG concentrations (122,141-143).

An extensive review of 14 studies (133) found that high-CHO (60% calories CHO), low-fiber (6g/1000 kcal) diets elicited higher fasting serum TG levels by a mean of 53% (95% CI, 34% to 71%), compared to low-CHO (<45% calories CHO), low-fiber diets. The opposite was true for high-CHO, high-fiber (29g/1000 kcal) diets, which modestly lowered TG by 10% (95% CI, -2% to -17%) compared to low-CHO (42% calories CHO), low-fiber (7.5g/1000 kcal) diets. Even modest increases in dietary fiber from 10 to 22 g/1000 calories has been associated with a 10% reduction in fasting TG levels in patients with type 2 diabetes consuming a moderate-CHO diet (55% calories CHO) (144). Garg et al. (145) conducted an innovative study comparing the TG response of two diets matched for fiber content (25 g/d), but varied in levels of carbohydrate in hypertriglyceridemic diabetic individuals (n=8). The high-CHO diet (60% calories CHO) resulted in a 27.5% ($p < 0.002$) increase in TG compared to the low-CHO diet (35% calories CHO).

Emerging evidence suggests that increases in blood TG levels, may contribute to increased concentrations of small, dense LDL particles, which are atherogenic (146). A recent study in 36 overweight men aged 50-75 years found that consumption of two large

servings of oats daily (about 14g/d dietary fiber) substantially decreased small, dense LDL-C (-17.3%) and LDL particle number (-5.0%) compared to the wheat control (+60.4% and +14.2%, respectively) (147). More importantly, although carbohydrate intake increased and total and saturated fat intakes decreased, HDL-C and TG levels remained stable in subjects who consumed the high-fiber oat cereals. Other emerging data suggest that dietary fiber is inversely associated with C- reactive protein (148,149).

In addition to the cardioprotective effects of fiber on TG levels, fiber also beneficially affects total and LDL-cholesterol levels. A meta-analysis of eight studies reported that 10g/day of psyllium reduced TC and LDL-C by 4% and 7%, respectively (150). Another meta-analysis of 67 controlled dietary studies (142) found that for each gram of soluble fiber from oats, psyllium, pectin, or guar gum, TC concentrations decreased by 1.42, 1.10, 2.69, and 1.13 mg/dL (0.037, 0.028, 0.070, and 0.026 mmol/L), respectively. LDL-C decreased by 1.23, 1.11, 1.96, and 1.20 mg/dL (0.032, 0.029, 0.055, and 0.033 mmol/L), respectively, demonstrating that the cholesterol-lowering effects of these soluble fibers are comparable. Beneficial effects of fiber intake also have been observed in healthy populations. In a study of normolipidemic and normotensive subjects (n=53), increased dietary fiber intake (30.5 g/d total fiber and 4.11 g/d soluble fiber) over 3 months significantly reduced LDL-C by 12.8%, while TG and HDL-C did not change (151).

Overall, epidemiologic studies lend convincing support to the hypothesis that individuals with a higher intake of dietary fiber, especially from whole grains (152-158), have a lower risk of CVD than those who consume a low fiber diet. In the Health Professionals Follow-up Study (159) the age-adjusted relative risk for total myocardial

infarction was 0.59 among men with the highest quartile of total dietary fiber intake (median 28 g/day) compared with men with the lowest quartile (median 12.4 g/day). The relative risk for fatal myocardial infarction in the highest quartile was 0.45 compared to the lowest quartile of fiber intake. In the Nurses' Health Study, Wolk et al. (160) reported that an increase of 10g/day dietary fiber was associated with a 20% reduction in CHD risk. Similarly, a recent meta-analysis of ten prospective cohort studies (91,058 men and 245,186 women) found that each 10-g/d increment of total dietary fiber was associated with a 14% reduction in risk of all coronary events and 27% reduction in coronary mortality (161). The importance of whole grains as a source of fiber has been demonstrated in several studies (153,157). A recent meta-analysis of 12 population-based cohort studies found that whole grain foods significantly reduced the risk of CHD by approximately 26% after adjustment for multiple CHD risk factors (162). The inverse association of whole grains was stronger than for cereal fiber, fruits, or vegetables, suggesting that three servings of whole grains per day may be important to cardiovascular health. Several additional studies have suggested that the cardioprotective benefit of regular whole grain consumption may be conferred via favorable effects on risk factors associated with CVD, including hypertension (163-165), type 2 diabetes (164,166-168), and other metabolic risk factors (166,167). Therefore, consistent with the 2005 Dietary Guidelines for Americans, the public should focus on choosing high-fiber sources of carbohydrate, particularly in the context of a low-fat/high-carbohydrate diet to decrease risk of chronic disease.

Antioxidants

One of the primary theories associated with the pathophysiology of cardiovascular disease is based on oxidative stress. Oxidative stress is defined by Sies as "a disturbance in the prooxidant-antioxidant balance in favor of the former" (169). Thus, oxidative stress results when there is an imbalance between the production of various reactive species and the ability of the organism's natural protective mechanisms to cope with these reactive compounds. One potential therapy for the prevention of damage as a result of oxidative stress is the use of dietary antioxidants, including Vitamins E, C and β-Carotene.

Vitamin E is a collective name for molecules that exhibit the biologic activity of α-tocopherol, a fat-soluble vitamin. Vitamin E is found in a number of common foods, such as vegetable oils, nuts, green leafy vegetables, and in cereals that have been fortified with Vitamin E. The Dietary Reference Intake (DRI) for Vitamin E for adults is 15 mg (22 IU) per day, with an upper limit of 1000 mg (1500 IU) per day (170). Because Vitamin E can act as an anticoagulant, an upper limit was established to avoid any risk of bleeding. The primary function of Vitamin E lies within its antioxidant properties.

Vitamin E functions as a powerful antioxidant. It acts in vivo as a chain-breaking antioxidant that prevents the propagation of free radical damage in biologic membranes. In the presence of Vitamin E the superoxide free radical is reduced, forming a tocopheroxyl radical (Vit E-O•). This tocopheroxyl radical (Vit E-O•) then travels from lipid bilayer into the aqueous domain where it reacts with a hydrogen donor, such as Vitamin C, thiols, or glutathione. This results in the oxidation of Vitamin C, or the thiol involved, and the return of Vitamin E to its reduced state. In the absence of Vitamin E,

the superoxide radical may be reduced, however this reduction is different and leads to the production of more superoxides. As shown here in the regeneration of Vitamin E, Vitamin C also acts as a powerful antioxidant. Vitamin C is the most effective and versatile of the water-soluble dietary antioxidants. It readily donates electrons to quench the reactive free radicals and oxidant species, such as the tocopheroxyl radical. Once it has regenerated the Vitamin E, the oxidized Vitamin C can be easily returned to its reduced state by ubiquitous electron donors, such as NADPH.

Despite the results of many clinical studies, epidemiological studies have failed to show a beneficial relationship between vitamin E supplementation and risk of cardiovascular disease. A recent meta-analysis, conducted by Miller et al., actually showed a potential negative effect of high-dose vitamin E supplementation on all-cause mortality (171). Nineteen randomized-controlled trials were selected, representing 135,967 participants and 12,504 all-cause deaths. The vitamin E dosages ranged from 16.5 to 2000 IU/day, with a median dosage of 400 IU/day. Several important points to remember when interpreting the results of this meta-analysis are that many of the trials that tested high dosages of vitamin E involved adults that were already suffering from chronic diseases, most often coronary heart disease. In addition, 10 out of the 19 trials included vitamin E and other nutritional supplements so they are really not testing the affects of vitamin E on its own. Finally, the trials that tested the higher dosages ($\geq$ 400 IU/d) were smaller on average and included a total of 40,950 patients, or 30.1% of the total meta-analysis population.

Results of the meta-analysis showed no effect of vitamin E supplementation on all-cause mortality. The pooled risk difference comparing vitamin E with control was 10 per

10,000 persons; the risk ratio was 1.01 (95% CI: 0.98 – 1.04; $p > 0.2$). The studies were then divided into low-dosage (< 400 IU/d) and high-dosage ($\geq$ 400 IU/d) vitamin E trials. Eight trials tested low-dosage vitamin E supplementation, with a pooled risk difference of –16 per 10,000 persons and a risk ratio of 0.98 (95% CI: 0.96 – 1.01; $p<0.2$). In the eleven high-dosage trials the pooled risk difference was 39 per 10,000 persons, with a risk ratio of 1.04 (95% CI: 1.01 – 1.07; $p=0.035$). This led the researchers to conclude that there may be a potential harmful effect of high-dosage vitamin E supplementation.

In response to these results, a dose-response analysis was performed to determine if all-cause mortality progressively increased as vitamin E dosage increased. The results indicated a cut-point of 150 IU/d, such that dosages above 150 IU/d may incur an increased risk for all-cause mortality, whereas dosages below 150 IU/d are not associated with an increased risk. While these results may indicate that high dosages of vitamin E are associated with increased all-cause mortality risk, it is important to remember that this meta-analysis does have several potential sources of error, as discussed above. Despite these results, many observational and clinical studies have shown beneficial affects of vitamin E supplementation.

One of the primary observational studies that has evaluated the relationship between Vitamins E and C and CVD is the Nurses' Health Study (172). The results of the Nurses' Health Study indicated that the risk of major coronary disease was lowest in women within the highest compared with those within the lowest quintile of reported vitamin E intake after adjustment for age and smoking status (RR 0.66; 95% CI, 0.50 to 0.87). The range of Vitamin E intake in this study, including dietary sources and supplements, was 21.6 to 1,000 IU, with a mean intake of 208 IU. Thus, the lower risk

for CVD that was observed was associated with levels of vitamin E intake that were achievable only by supplementation, with a 43% lower risk for vitamin E supplement users versus nonusers. While these observational data provide an argument for the use of Vitamin E in CVD risk reduction, results from the controlled clinical trials conducted, utilizing a more rigorous design, have yielded mixed results. In the Atherosclerosis Risk in Communities Study (ARIC) dietary intake was assessed using a 66-item semi quantitative food frequency questionnaire in 11,307 males and females, aged 45 to 64 years (173). Results of this study demonstrated that there was an inverse relationship between carotid artery wall thickness and both Vitamin C and α-tocopherol intake. The inverse relationship between arterial wall thickness and α-tocopherol intake was only significant for women. No significant relationships were determined for participants younger than 55 years of age, thus the researchers determined that these potential protective effects might be more prominent in individuals older than 55 years of age.

The Cholesterol Lowering Atherosclerosis Study (CLAS) (174) was a secondary prevention trial, with subjects randomized to receive either colestipol and niacin plus a diet, or a placebo plus diet. The investigators defined supplementary Vitamin E intake as $\geq$ 100 IU/day, and supplementary Vitamin C intake as $\geq$ 250 mg/day. The results indicated that supplementary Vitamin E intake may be effective in reducing the progression of atherosclerosis in subjects not treated with lipid-lowering drugs. There was no effect found for Vitamin E in the group receiving lipid-lowering agents. Several other clinical trials have investigated the effects of dietary antioxidants on atherosclerotic events. While the results are controversial, a majority of these trials have determined that

supplemental Vitamin E at doses ranging from 50-400 mg/day do not provide significant reduction in the risk of atherosclerotic disease (175).

One of the largest studies to test the effects of both Vitamin E and Vitamin C was the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) Study (176). The subjects were 520 smoking and nonsmoking hypercholesterolemic men and postmenopausal women, aged 45-69 years. The study included a randomized, double-blinded 3-year treatment period, followed by an open 3-year treatment period. During the first 3 years of treatment the subjects in the experimental groups received either 136 IU Vitamin E, 250 mg slow-release Vitamin C twice a day or both Vitamin E and C. During the 3-year open treatment period all of the individuals from the treatment groups that remained were placed on the Vitamin E and C combination. Following this six-year treatment period the researchers determined that supplementation with Vitamin E and slow-release Vitamin C does slow down atherosclerotic progression in hypercholesterolemic individuals.

As a result of all this conflicting evidence, scientists are left questioning the health effects of antioxidants, and accordingly, what to recommend both with respect to diet and supplement use. In 1999, based on the available scientific evidence, the American Heart Association (AHA) stated that the most prudent advice for individuals is “to consume a balanced diet with emphasis on antioxidant-rich fruits, vegetables and whole grains" (177). Following the release of the meta-analysis by Miller, et al, the AHA was again prompted to make a recommendation based on the more recent clinical trials (178). In this latest science advisory they concluded, “At this time, the scientific data do not justify the use of antioxidant vitamin supplements for CVD risk reduction. … No consistent data

suggest that consuming micronutrients at levels exceeding those provided by a dietary pattern consistent with AHA Dietary Guidelines will confer additional benefit with regard to CVD risk reduction." (178)

Emerging risk factors for Cardiovascular Disease

Apolipoproteins

To recognize the pivotal role played by apolipoproteins (apo) in CVD, Dr. Peter Alaupovic's group has introduced an alternative classification system of lipoproteins based on apolipoprotein composition rather than density properties as the criterion for their identification and differentiation (179). Based on this classification system, there are two lipoprotein classes, one of which is characterized by the presence of apoA and the other by apoB. The apoA-containing lipoproteins of high density properties consist of three lipoprotein families defined by their apolipoprotein composition as Lp-A-I, Lp-A-I:A-II, and Lp-A-II. The apoB-containing lipoproteins encompass five major lipoprotein families called Lp-B, Lp-B:E, Lp-B:C, Lp-B:C:E and Lp-A-II:B:C:D:E. The Lp-B and Lp-B:E, the major lipoproteins of the LDL density segment, are cholesterol-rich lipoproteins, whereas the other three lipoproteins, present predominantly in VLDL and IDL density segments, are intact or partially delipidized triglyceride-rich lipoproteins . Each of the apoA- and apoB-containing lipoprotein families has a unique apolipoprotein composition and specific metabolic properties.

Alterations in the metabolism of triglyceride-rich lipoproteins are important in the pathogenesis of atherosclerosis (180). Consequently, there is increased interest in learning more about the role of abnormal triglyceride-rich lipoproteins in the atherogenic process. Remnant particles (of chylomicrons and VLDL) have a prolonged residence time in plasma and have been shown to be atherogenic, especially those that contain apoB:apoC-III (181-186). The presence or absence of apoE, apoC-II and apoC-III in triglyceride-rich lipoproteins is an important determinant of their metabolic fate and

atherogenicity. For example, elevated plasma levels of apoC-III are associated with increased levels of VLDL and decreased fractional catabolism in humans (187). Moreover, several studies have demonstrated increased secretion rates of newly synthesized apoC-III into plasma of subjects with hypertriglyceridemia (188-190). Based on available evidence, increased production of apoC-III may be important in the development of hypertriglyceridemia. In a recent study, a quantitative proteomic approach was used to characterize the exchangeable apolipoproteins present in small, dense LDL and large, buoyant LDL subclasses in individuals with the metabolic syndrome and type 2 diabetes (191). Both groups of individuals exhibited subclinical atherosclerosis and the B LDL phenotype, characterized as having small dense LDL particles. The small dense LDL from both groups of patients were enriched in apoC-III and were depleted of apoC-I, apoA-I, and apoE compared with matched healthy controls with the A phenotype. This study provides further evidence for the association of apoC-III with atherogenic dyslipidemia, characterized by hypertriglyceridemia, low levels of HDL-C and small, dense LDL-C particles.

There is little information about the effect of nutrients on the levels of Lp-A-I and particles. Fumeron et al. (192) investigated the effect of a high ratio of polyunsaturated to saturated fatty acids (P/S = 1.2) on Lp-A-I and Lp-A-I:A-II particles in a group of young adult males fed diets high in either butter (P/S = 0.2) or sunflower margarine (P/S = 1.2) for 3 weeks each. Plasma cholesterol, LDL-cholesterol and apoB levels were significantly lower on the latter diet. However, this positive effect was counter-balanced by a decrease in anti-atherogenic HDL_2-cholesterol and Lp-A-I. Delplanque et al. (193) showed that the negative effect of polyunsaturated acids on the levels of Lp-A-I particles

may be avoided by the use of an equal ratio of polyunsaturated to monounsaturated fatty acids; diets high in monounsaturated oleic acid were shown to increase the levels of Lp-A-I:A-II but not those of Lp-A-I particles. To date, the only diet study evaluating the effect of a high fat diet (40% of calories from fat) on the levels of individual apoB-containing lipoprotein families has shown differences in the postprandial concentration and percent composition of these lipoprotein families between normolipidemic and hypertriglyceridemic subjects; the main difference between these two groups of subjects was a greater postprandial increase of atherogenic Lp-B:C particle levels in the latter than in the former subjects. Although based on a small number of subjects, these results suggest that measurement of individual apoA- and apoB-containing lipoproteins provide a new means of characterizing the effect of various nutrients and diets on maintaining normal and correcting abnormal lipid transport processes.

While many believe that levels of specific apolipoproteins provide additional evidence for CVD risk assessment, results of a recent study have disputed this observation. Ridker et al. (194) completed an analysis of the Women's Health Study to directly compare the clinical utility of total cholesterol, low-density lipoprotein cholesterol (LDL-C), HDL-C, non-HDL-C, apolipoproteins A-I and B(100), hs-CRP, and the ratios of total cholesterol to HDL-C, LDL-C to HDL-C, apolipoprotein B(100) to apolipoprotein A-I, and apolipoprotein B(100) to HDL-C as predictors of future cardiovascular events in women. The results of this study indicated that non-HDL-C (HR=2.51, 95% CI, 1.69-3.72) and the TC:HDL-C ratio (HR=3.81 (95% CI, 2.47-5.86) were as good or better than the apolipoprotein fractions in the prediction of future cardiovascular events.

Postprandial Triglycerides (TG)

The delayed clearance of postprandial lipemia is an independent risk factor for coronary heart disease (CHD) (195). Abnormal transport and metabolism of postprandial TG-rich lipoproteins are linked to atherosclerosis in the coronary and carotid arteries (146). At a given amount of dietary fat, postprandial lipids are cleared more slowly in individuals who have a higher baseline TG level. A delayed clearance of these atherogenic TG-rich lipoproteins is thought to create a metabolic milieu that results in the promotion of atherogenesis. Thus, a dietary pattern that reduces fasting and postprandial plasma TG may decrease the accumulation of atherogenic, TG-rich lipoproteins resulting in decreased risk for atherosclerosis.

A number of studies have been conducted to assess postprandial TG clearance with respect to n-3 PUFA. Most studies have been done with fish-derived n-3 PUFA and have shown increased postprandial lipid clearance (196), driven by the potent hypotriglyceridemic effect of n-3 PUFA. A study conducted in physically active males illustrated that the supplementation of 4.0 g of n-3 PUFA/d for 5 weeks decreased postprandial TG area under the curve (AUC) by 42% (197). When a single bout of exercise (1-hour treadmill run) was completed 12 hours prior to the high fat test challenge, the reduction in the postprandial TG AUC elicited by n-3 PUFA supplementation was significantly enhanced (-58%), beyond that of the effects seen with n-3 PUFA supplementation alone.

A study conducted by Finnegan and colleagues (198) compared the effects of α-linolenic acid (ALA) and EPA+DHA on several cardiovascular disease risk factors, including postprandial blood lipid concentrations. The study employed a placebo-

controlled parallel arm design involving 150 moderately hypercholesterolemic individuals. Subjects were randomly assigned to receive one of five interventions: 1) 0.8 g EPA+DHA/d, 2) 1.7 g EPA+DHA/d, 3) 4.5 g ALA/d, 4) 9.5 g ALA/day, and 5) n-6 fatty acid control. A postprandial fat challenge was completed at baseline and following 6 months of treatment. While the decrease in fasting TG levels following the supplementation of 1.7 g/d EPA+DHA (-7.7%) was significantly different ($p<0.05$) from the supplementation of 9.5 g/d ALA (10.9%), there were no treatment differences on the postprandial response to a standard fat load. This lack of effect could possibly be explained by the restricted range of baseline TG levels for the study subjects and the fact that comparisons were made to an n-6 PUFA control, rather than to a standardized SFA fat load.

Very few studies have been conducted to evaluate the effects of different dietary patterns on postprandial TG response. In a recent 2-period randomized crossover design study (199), the effects of a hypoenergetic very-low-carbohydrate (<10% calories as carbohydrate) and a hypoenergetic low-fat diet (<30% calories as total fat) on fasting blood lipids and postprandial lipemia were studied in overweight men. An oral fat tolerance test (86% calories total fat, 11% calories carbohydrate, 3% calories protein) was completed at baseline and at the conclusion of each of the test diet periods. Postprandial TG levels were significantly reduced following the consumption of both test diets, compared to baseline, with the greatest reduction following the very-low-carbohydrate diet (-34%). These results indicate that short-term use of a hypocaloric very-low-carbohydrate diet has beneficial effects on postprandial TG levels. This effect likely is mediated by the reduction in fasting TG levels and a beneficial effect on other

components of the metabolic syndrome, including small dense LDL-C particles, markers of insulin resistance and the TG:HDL-C ratio. In several other studies, similar postprandial TG-lowering was observed in normal weight, normolipidemic men (-29%) following a ketogenic diet (61% calories total fat, 8% calories carbohydrate, 30% calories protein) (200), and women (-31%) following an isoenergetic very-low-carbohydrate diet (60% calories total fat, 10% calories carbohydrate, 30% calories protein) (201), independent of weight loss.

In addition to postprandial TG levels, adverse effects on endothelial function in the postprandial period are also considered to be potential pro-atherogenic mechanism. In a classic study, Vogel et al. (202) assessed the direct effect of postprandial triglyceride-rich lipoproteins on endothelial function. Ten healthy, normocholesterolemic volunteers were studied before and for 6 hours after single isocaloric high- and low-fat meals (900 calorie; 50 and 0 g fat, respectively). Endothelial function was assessed using flow-mediated dilation of the brachial artery. As a result of the high-fat meal serum TG increased from 94 $\pm$ 55 mg/dl (1.03 $\pm$ 0.61 mmol/L) to 147 $\pm$ 80 mg/dl (1.62 $\pm$ 0.88 mmol/L) after 2 hours ($p = 0.05$). Flow-dependent vasoactivity decreased from 21 $\pm$ 5% preprandially to 11 $\pm$ 4%, 11 $\pm$ 6%, and 10 $\pm$ 3% at 2, 3, and 4 hours after the high-fat meal, respectively (all $p < 0.05$ compared with low-fat meal). These results thus demonstrate that a single high-fat meal, rich in SFA, transiently impairs endothelial function and provides a potential process by which high-SFA, high-fat meals create a pro-atherogenic environment in the postprandial period. Results of a recent study indicate that endothelial dysfunction observed after the consumption of a high-fat meal is

associated with augmented oxidative stress manifested by the depletion of serum antioxidant enzymes and increased excretion of oxidative modification products (203).

In summary, a series of pro-atherogenic mechanisms are induced by the consumption of a high-fat meal. In particular, the delayed clearance of TG-rich lipoproteins is accompanied by increased levels of oxidative stress, inflammation and deleterious effects to endothelial health. Supplementation with marine sources of n-3 PUFA elicits a beneficial reduction on postprandial TG levels; this effect likely is mediated by the potent hypotriglyceridemic properties of marine n-3 PUFA in the background diet (204). In addition, the use of a very-low-carbohydrate diet may be an effective means for eliciting a reduction in postprandial TG levels, and may have additional beneficial effects on risk factors associated with the metabolic syndrome. Given the important role of dietary PUFA in reducing risk of CVD, questions remain with regards to the acute effects of ingesting high levels of PUFA and their potential for oxidative modification.

C-reactive protein

Given the expansion in our knowledge of the pathophysiology of cardiovascular disease, we now know that inflammation plays a key role in the local, myocardial, and systemic complications associated with the atherosclerotic process (205). It is estimated that inflammation is the underlying cause of approximately 80% of all sudden cardiac deaths (206). Within the process of plaque formation and plaque rupture the activation of macrophages, T lymphocytes, and smooth muscle cells leads to the release of adhesion molecules, cytokines, chemokines, and growth factors (207). One particular cytokine involved in this process, interleukin-6 (IL-6) acts as a messenger cytokine, stimulating

the release of C-reactive protein (CRP) from hepatocytes into the circulation (208). While IL-6 appears to play a pivotal role in the identification of CVD risk, day-to-day measures of IL-6 are subject to a great deal of variability, whereas levels of CRP are much more stable (209,210). CRP has thus emerged as a very powerful predictor of risk for cardiovascular disease. Clinical cutpoints for levels of CRP, as defined by the American Heart Association and the Center for Disease Control and Prevention are: <1 mg/L (low risk, 1.0-3.0 mg/L (average risk), and >3.0 mg/L (high risk) (211).

Data from the Women's Health Study (212,213) indicate that out of four markers of inflammation (high-sensitivity-CRP, serum amyloid A, IL-6, and soluble-intracellular adhesion molecule-1), hs-CRP was the most significant predictor of risk in univariate analysis. Within the same study hs-CRP was also significantly better than homocysteine, lipoprotein(a), and LDL-C levels in predicting CVD risk. Furthermore, in a recent analysis of the Women's Health Study (194), after adjustment for age, blood pressure, smoking, diabetes, and obesity, hs-CRP added additional prognostic information beyond that obtained with traditional lipid measures. When divided into quintiles of hs-CRP levels, the lipid ratio of TC:HDL-C had the strongest association with CVD risk, with a HR of 3.81 (95% CI, 2.47-5.86; $p<0.001$) for the highest quintile of hs-CRP level, compared to the lowest quintile (194).

Increased levels of CRP are strongly correlated with the prevalence of obesity. In particular, a recent study has shown that mean CRP levels were significantly higher in the presence of central obesity (2.45 vs. 1.24; $p<0.001$) (214). Although the association between CRP and obesity is well documented, the connection between CRP and various dietary components is not well understood. Despite an observational study that reported

a positive correlation between high glycemic load foods and CRP (215), a recent clinical trial has shown that a high intake of sucrose has a modest effect on CRP levels (216). Despite a 151% increase in sucrose intake, resulting in a 1.6 kg weight gain, levels of CRP increased only 6% over a 10-week period. Results of a recent weight loss intervention demonstrated that the dietary carbohydrate/protein ratio had no effect on levels of CRP, but that CRP was positively associated with indices of body fatness (217). In a study designed to test the effectiveness of 4 popular diets: Atkins (carbohydrate restriction), Zone (macronutrient balance), Weight Watchers (calorie restriction), and Ornish (fat restriction), decreased levels of CRP were significantly associated with weight loss, with no significant differences between any of the test diets (218). In weight stable subjects, levels of CRP have been positively associated with intakes of *trans* fatty acids (219), and low intakes of vitamin C (220), and negatively associated with intakes of ALA (105). The results of a study evaluating the association between individual dietary factors and levels of CRP indicate that in women, there are slight negative correlations between intakes of total fat ($r = -0.13$, $p=0.011$), saturated fat ($r = -0.13$, $p=0.011$), monounsaturated fat ($r = -0.13$, $p=0.010$), polyunsaturated fat ($r = -0.14$, $p=0.007$) and n-3 polyunsaturated fat ($r = -0.14$, $p=0.004$) (220) with CRP. While this may be surprising to some, the observed effect may be an example of the lipemic benefits observed on a moderate fat diet, compared to a low fat diet that may induce atherogenic dyslipidemia and the related metabolic consequences. Thus, the specific effects of individual dietary fatty acids and dietary patterns on levels of CRP are not completely understood at this time.

Oxidative stress

With regards to the cardiovascular system, oxidative events trigger biochemical changes in the arterial wall. The basis for these changes is the oxidation of low-density lipoproteins (LDL) in the subendothelial space (221). These oxidized LDL stimulate the expression of adhesion molecules on endothelial cells, are chemotactic, and have the ability to up-regulate cell scavenger receptors (222). The actions of oxidized LDL that lead to a pro-atherogenic environment include the recruitment of monocytes and macrophages from the bloodstream into the subendothelial space and an excessive deposition of lipids in this endothelial space. The retention of the intimal macrophages and the presence of oxidized LDL molecules lead to the formation of macrophage-derived foam cells. These lipid-laden foam cells accumulate and form a lesion. The uncontrolled uptake of lipoproteins and cholesterol only contributes to the growth of the lesion, resulting in plaque formation. In addition to plaque formation, these events also lead to a thickening of the intima and a narrowing of the vessel. The results of this damage often include the formation of a thrombosis and plaque rupture, resulting in an acute myocardial infarction. In the postprandial period these events have to potential to be exaggerated in the presence of delayed clearance of TG-rich lipoproteins and the accumulation of oxidized fatty acids. When considering the extent of lipid peroxidation that may take place, three factors need to be considered: 1) the generation of oxygen free radicals, 2) the presence of lipid substrates, and 3) the activity of antioxidants (223).

Although several trials have tested the effect of antioxidant supplementation on in vitro lipid peroxidation and in vitro oxidative modification of LDL-C (224-230), few studies have evaluated the effects of dietary patterns on these processes. Therefore,

within the Dietary Approaches to Stop Hypertension (DASH) trial, an ancillary study was conducted to test the hypothesis that reduced-fat diets, rich in fruits and vegetables would beneficially affect lipid peroxidation (223). Levels of lipid peroxidation were assessed via breath ethane and malondialdehyde (MDA). While there were significant increases in breath ethane, indicating a decrease in lipid peroxidation across the test diets, there were no significant decreases in levels of MDA. Total antioxidant capacity was determined using the oxygen radical-absorbing capacity (ORAC) assay. Following the consumption of both the combination diet and the fruits and vegetables diet, levels of serum ORAC increased significantly, supporting the hypothesis that diets rich in fruits and vegetables can increase the antioxidant capacity of the serum. The ORAC assay has a high degree of specificity and measures the capacity of an antioxidant to directly quench free radicals (231). The ORAC assay is unique in that it takes free radical action to completion and uses the area-under-the-curve (AUC) technique for quantification, and, thus combines both inhibition percentage and the length on inhibition time of the free radical action by antioxidants into one single number (232).

In addition to assessing the activity of antioxidants, it is also important to quantify the other end of the equation, the degree of lipid peroxidation present (233,234). Lipid peroxidation results in the formation of highly reactive and unstable hydroperoxides of both saturated and unsaturated lipids. Traditionally, lipid peroxidation is quantified by measuring malondialdehyde (MDA) and 4-hydroxy nonenal (4-HNE), the degradation products of polyunsaturated fatty acids (PUFAa) hydroperoxides (235-237). These assays however are non-specific and thus often lead to an overestimation of lipid peroxidation. Given the limitations of the indirect methods, direct measurement of lipid

hydroperoxides is the obvious choice. Consumption of a meal containing oxidized and oxidizable lipids increases levels of plasma lipid hydroperoxides (238). This is then associated with increased susceptibility of LDL to oxidation, due to structural perturbation at the particle surface brought about by lipid oxidation products (239). Since many individuals spend a majority of their day in the postprandial state, and the significant role that oxidation plays in the pathogenesis of CVD, several important questions remain regarding the acute ingestion of pre-oxidized and oxidizable fatty acids.

Endothelial Health

Although once thought of as a protective inert barrier, the vascular endothelium is now regarded as a metabolically active tissue that plays a pivotal role in the development of atherosclerosis (240). The vascular endothelium plays a key role in the local regulation of vascular tone by the release of vasodilator substances, such as nitric oxide (NO) and vasoconstrictor substances including free radicals and endothelin-1 (ET-1) (241). Endothelin-1, originally described as a potent vasoconstrictor (242), is now considered a key mediator in the development of endothelial dysfunction and atherosclerosis (243). Although the degree of endothelial dysfunction within the coronary microvasculature correlates with total serum cholesterol levels (244), the use of biomarkers that are more strongly associated with endothelial health is important in better defining CVD risk.

In a recent study Sainani et al. (245) compared the circulating levels of ET-1 in patients with coronary artery disease (CAD) with those of healthy controls. In addition to significantly higher levels of ET-1 in the CAD patients, compared to the healthy controls, tissue ET-1 was also observed in the smooth muscle cells of the intimal and medial layers

of the aortas of patients diagnosed with CAD. In addition to increased levels in the fasted state, levels of ET-1 are also increased in the postprandial state, in conjunction with a rise in triglycerides and insulin levels. In a study of subjects with the metabolic syndrome, an acute increase in triglycerides, stimulated by an intralipid infusion significantly enhanced levels of ET-1 (246). These increased levels of ET-1 were further increased by the synergistic contribution of high insulin (induced by an insulin bolus combined with a euglycemic clamp) and triglyceride levels. Few studies have focused on dietary interventions to reduce the release of ET-1 in the postprandial period. One such study evaluated the effects of the polyphenols from red wine on the synthesis of ET-1 in cultured bovine aortic endothelial cells (247). The decrease in ET-1 synthesis was a result of suppression in the transcription of the ET-1 gene. In a recent study, pretreatment with vitamin C inhibited the endothelium-dependent and endothelium-independent impairment of vasodilation and the release of IL-6 induced by ET-1 infusion (248). These results therefore indicate that the impairment of vascular function and the stimulated release of IL-6 by ET-1 involves increased levels of oxidative stress. Given the utility of ET-1 as a predictor of endothelial health, questions remain regarding the effect of different types of dietary fat on the postprandial release of ET-1 and the associated pro-atherogenic events.

Although LDL-C has traditionally been the primary target for the prevention of CVD, we now know that the pathophysiology of CVD extends beyond that of lipid disorders alone. CVD is now also considered a disease of inflammation, oxidation and endothelial dysfunction. The identification of emerging risk factors associated with each

of these processes has provided researchers and clinicians with enhanced knowledge regarding the prevention, diagnosis and treatment of CVD.

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Chapter 3
Examining the DRI Total Fat Recommendation in Healthy Individuals and Those at Risk for Cardiovascular Disease

Background

Cardiovascular disease (CVD) is the leading cause of death for both men and women in the US. Almost 2,600 American die of CVD each day, with an average of 1 death every 34 seconds (1). Many of the major risk factors for CVD can be decreased by diet, including elevated total cholesterol (TC), LDL cholesterol (LDL-C) and triglyceride (TG) levels and a low level of HDL cholesterol (HDL-C). Decreasing cholesterol-raising nutrients (i.e., saturated fat, *trans* fat and dietary cholesterol) and modifying the amount of total fat can markedly affect these key modifiable risk factors for CVD. There has been a long-standing focus on levels of low-density lipoprotein cholesterol (LDL-C) as the primary target for reducing CVD risk (2). Elevated levels of LDL-C are not only a major risk factor for CVD (3-6), but a strong body of literature also indicates that decreasing LDL-C decreases CVD risk (2,7-9). Because many individuals with the atherogenic lipid profile do not have increased levels of LDL-C, heightened interest has developed in other coronary risk factors, including features of atherogenic dyslipidemia (10,11).

The atherogenic lipid profile is characterized by high levels of serum triglycerides (TG), low HDL-C levels, and small dense low-density lipoprotein cholesterol (LDL-C) particles (2,12,13). This phenotype is generally prevalent in individuals with insulin resistance/hyperinsulinemia and is also associated with increases in inflammation, endothelial dysfunction, the postprandial accumulation of remnant lipoproteins and a prothrombotic state (14). Each of these physiological states is associated with an

increased risk of cardiovascular disease (CVD), thus the clustering of these disorders represents a significant increase in overall CVD risk (15). Elevated levels of serum TG alone are considered an independent risk factor for CVD (16,17); a 91 mg/dL (1 mmol/L) increase in fasting blood TG is associated with a 76% and 31% increase in CVD risk in women and men, respectively (18). Given the diversity in the risk profile of these two groups of individuals (i.e. isolated high LDL-C vs. atherogenic dyslipidemia), the question then remains: which diet is optimal for the reduction of CVD risk in these metabolically diverse groups? Traditionally, low-fat, low-saturated fat diets have been recommended for reduction of LDL-C levels (19-31). These more traditional low-fat diets however increased the prevalence of atherogenic dyslipidemia, particularly in sedentary, overweight or obese populations, via an increase in TG and a decrease in HDL-C. The new generation of low-fat, higher-carbohydrate diets that emphasize complex carbohydrates and are high in dietary fiber, however have not elicited the adverse affects once seen on levels of TG and HDL-C (32-34).

Dietary recommendations for the prevention and treatment of CVD have evolved across the decades, driven by the results of both clinical and epidemiological research trials. Based on the tendency of traditional low-fat diets to induce atherogenic dyslipidemia, the National Cholesterol Education Program's Adult Treatment Panel III (ATP III) report recommends a diet that is 25-35% of calories from total fat (2). Additional dietary components included in the ATP III Therapeutic Lifestyle Changes (TLC) Diet include a focus on the reduction of saturated fats (<7% of calories) and cholesterol (<200 mg/day), 20-30 g/day of dietary fiber (including 5-10 g/day of viscous fiber), 2 grams per day of plant stanol/sterol esters, weight reduction and physical

activity. The most recent Dietary Reference Intakes (DRIs) for macronutrients, issued by the Food and Nutrition Board of the National Academies in 2002 (35) recommend the following macronutrient distribution ranges: 20-35% of calories for total fat (5-10% for PUFA, 0.6-1.2% of energy for α-linolenic acid, of which up to 10% can come from EPA + DHA), 45-65% of calories for carbohydrate, and 10-35% of calories from protein. The recommendation for dietary fiber is 14 g/1000 calories. The DRI Report advises that saturated fatty acids, *trans* fatty acid and dietary cholesterol intake be as low as possible within the context of a healthy diet. These recommendations are intended for the general public, whereas the guidelines set forth in ATP III are for individuals at risk for CVD. Since then the Dietary Guidelines for American were released in 2005 (36).

Many of the same recommendation that were made in the DRI report have been maintained in the 2005 Dietary Guidelines for Americans. One of the advantages of these dietary recommendations is that the wide ranges allow for great flexibility in diet planning for healthy individuals. These guidelines however may also be implemented in individuals who exhibit different lipid abnormalities. Of note is that the new guidelines focus on the type of fat that is recommended, rather than requiring tight limitations on total fat intake, which has been the focus of dietary guidance for over twenty years. Still, a question that needs to be addressed is what is the ideal dietary macronutrient profile(s) for prevention of chronic disease in individuals who differ metabolically at baseline? Studies are needed that compare different diets that meet the new macronutrient guidelines to determine which macronutrient profile has the greatest impact on markers of coronary risk in individuals exhibiting different lipid disorders.

It is well established that a moderate fat diet that emphasizes unsaturated fat decreases plasma triglyceride levels compared with a lower fat diet. Within the range of total fat (18-40% kcal) evaluated in controlled feeding studies, there is a linear dose-response relationship between decreases in total fat content of the diet and decreases in HDL-C and increases in fasting TG (35). In one study, with normotriglyceridemic subjects, Zhao et al. showed that a diet high in PUFAs (16-17% energy) compared with an Average American Diet (8.5% energy from PUFA) resulted in a lower triglyceride level (TG decrease of ~ 24mg/dL) (37). In another study, Kris-Etherton et al. showed that a cholesterol-lowering diet high in monounsaturated fat (17-21% energy) resulted in significantly lower plasma triglycerides compared with a blood cholesterol-lowering low-fat diet (27).

The effects of altering the total amount of dietary fat in the diet on emerging risk factors for CVD require further investigation. The classification system of lipoproteins based on apolipoprotein composition rather than density properties as the criterion for their identification and differentiation (38) has provided researchers with greater detail about the structure and function of specific lipoproteins. Based on this classification system, there are two lipoprotein classes, one of which is characterized by the presence of apoA and the other by apoB. The apoA-containing lipoproteins of high density properties consist of three lipoprotein families defined by their apolipoprotein composition as Lp-A-I, Lp-A-I:A-II, and Lp-A-II. The apoB-containing lipoproteins include five major lipoprotein families called Lp-B, Lp-B:E, Lp-B:C, Lp-B:C:E and Lp-A-II:B:C:D:E. The Lp-B and Lp-B:E, the major lipoproteins of the LDL density segment, are cholesterol-rich lipoproteins, whereas the other three lipoproteins, present

predominantly in VLDL and IDL density segments, are intact or partially delipidized triglyceride-rich lipoproteins . Each of the apoA- and apoB-containing lipoprotein families has a unique apolipoprotein composition and specific metabolic properties.

Based on the findings of previous studies, the chronic level of total fat intake affects TG levels. Paradoxically, it is the low-fat high-carbohydrate diets that increase baseline serum TG levels via an increase in de novo lipogenesis; moderate-fat diets that are rich in unsaturated fatty acids decrease TG levels in part due to the replacement of carbohydrates with unsaturated fatty acids. The purpose of the present study is to evaluate the extremes of the 2002 DRI dietary fat recommendations (20 vs. 35% of calories) within the context of a healthy diet that meets all other DRI recommendations for both macronutrients and micronutrients. In addition, this study will evaluate the effects of different amounts of total fat (20 vs. 35% of calories) on the lipid and apolipoprotein profile and inflammatory status of individuals with normal and elevated baseline serum TG levels. It is thought that a moderate fat diet (35% of calories) that emphasizes unsaturated fat will decrease fasting plasma triglycerides compared with a lower-fat diet (20% of calories). Concurrently, subjects with hypertriglyceridemia vs. normotriglyceridemia will have a greater triglyceride lowering response when fed a moderate-fat vs. a low-fat diet.

Methods

Subjects

Thirteen individuals (11 males and 2 females) participated in the present study. Six individuals (4 males, 2 females) with normal baseline triglyceride levels (TG < 150 mg/dL) and 7 individuals (7 males, 0 females) with elevated baseline triglyceride levels

(TG >150 mg/dL), as determined by current diagnostic guidelines from the ATP III report were recruited to participate in this study. Subjects were otherwise reasonably healthy men (30-65 years of age) or post-menopausal women with no other major co-morbidities. The eligibility criteria included: BMI < 35 kg/m^2, LDL-cholesterol < 160 mg/dL, systolic blood pressure < 145 mmHg, and diastolic blood pressure < 95 mmHg; not on lipid-lowering or blood pressure medication or other medications known to affect lipid levels. Baseline subject characteristics are shown in Table 3.1. Subjects in the present study were quite representative of the population in the U.S. that is at high risk for cardiovascular disease. The two females that participated were postmenopausal, and had not received hormone replacement therapy (HRT) for the 6 months prior to the start of the study. The Institutional Review Board at the Pennsylvania State University approved the experimental protocol and all subjects provided written informed consent before enrollment in the study.

Study Design

A randomized, double-blind, two-period crossover design was employed (Figure 3.1). Subjects were recruited via advertisements in the local newspaper and fliers distributed across the campus of the Pennsylvania State University. Subjects who met the initial criteria during a phone screen reported to the General Clinical Research Center (GCRC) on the campus of the Pennsylvania State University for further screening. At each screening visit blood was drawn for chemistry and lipid panels. Subjects also completed a medical history form and had their blood pressure and weight measured.

Subjects were randomly assigned to receive one of the two experimental diets during the first four-week period. Throughout the course of the study each subject

consumed every diet during two separate diet periods. Diet periods lasted 4 weeks and were separated by an approximate 2-week compliance break, during which subjects consumed their usual diet.

Subjects consumed either breakfast or dinner at the GCRC on Monday through Friday; lunches and weekend meals were prepared or packed for offsite consumption. Diet compliance was monitored by the staff and by the review of daily and weekly monitoring forms. Subjects' baseline body weights were maintained throughout the course of the study. Subjects were instructed to maintain their usual activities and exercise levels throughout the study.

Experimental Diets

The two experimental diets were designed to meet the two extreme ends of the 2002 DRI recommendations for dietary fat: 1) low fat (20%) diet (LFD), and 2) moderate fat (35%) diet (MFD). In addition, the fatty acid profile was similar in both diets with regards to saturated fatty acids (SFA), polyunsaturated fatty acids (PUFA), α-linolenic acid (ALA), linoleic acid (LA) and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and their recommendations as a percentage of total calories (Table 3.2). MUFA provided the remaining fat in each test diet to achieve either 20% or 35% of calories from total fat. For each of the two experimental diets eight calorie levels (1800 - 3900 kcal) were developed, with a 3-day meal rotation, to maintain subjects' baseline body weight throughout the course of the study.

Several different oils were used to achieve the desired fatty acid composition of each diet. In the LFD, walnut, grapeseed, and flaxseed oil were used. For the MFD, almond, olive, grapeseed, flaxseed, and safflower oils were used to increase the total

amount of fat, while maintaining ratios similar to those in the LFD. To achieve the recommended levels of EPA and DHA in the diets subjects consumed salmon twice a week (once per each three-day meal rotation) in the form of salmon pate. To achieve the recommended fiber intake focus was placed on whole grain, high-fiber sources of carbohydrates. This included the use of a wide variety of fruits, vegetables, whole-wheat bread, whole-wheat noodles, and brown rice. The three day menu cycle for each of the diets is presented in Table 3.3.

Serum Samples

Twelve hour fasting blood samples were taken by venipuncture on two consecutive days at the end of each diet period. Whole blood was centrifuged at 3000 rpm for 15 minutes at -4^{o}C. Serum samples were aliquoted and stored at -80^{o}C until the conclusion of the study when all samples were analyzed together.

Lipids, Glucose and Insulin

Serum total cholesterol (TC) and triglycerides (TG) were quantified using enzymatic assays (CHOP/PAP, Boeringer, Mannheim, FRG, Abbott Laboratories, Diagnostic Division, Irving, TX) conducted at the Core Laboratory of the General Clinical Research Center on the Hershey Medical Center's campus of the Pennsylvania State University (Hershey, PA). High-density lipoprotein cholesterol (HDL-C) was estimated according to the modified heparin-manganese precipitation procedure of Warnick and Albers (38). LDL-C levels were calculated by the Friedewald's equation: LDL-C = TC – (HDL-C + TG/5) (39). Samples were also assayed for levels of fasting glucose and insulin using standard methodology at the Hershey GCRC Core Lab. HOMA Scores were calculated using the updated HOMA model (HOMA2) which takes

into account variations in hepatic and peripheral glucose resistance, increases in the insulin secretion curve for plasma glucose concentrations above 10 mmol/L (180 mg/dL) and the contribution of circulating proinsulin (40). The HOMA2 model estimates steady state beta cell function (HOMA_B), insulin sensitivity (HOMA_S), and insulin resistance (HOMA_R). HOMA_R (which is the inverse of HOMA_S) = (insulin X glucose)/22.5; HOMA_B = 20 X insulin/(glucose - 3.5).

Apolipoproteins

Apolipoproteins (apo) A-I , B , and C-III were determined by electroimmunoassays in the laboratory of Dr. Petar Alaupovic according to methods described previously (41).

C-Reactive Protein

C-reactive protein was measured by high sensitivity competitive Enzyme-Linked Immunosorbent Assay (ELISA) developed in the Cytokine Core Laboratory at the GCRC at Penn State University, University Park Campus.

Assessment of Vascular Reactivity.

The ultrasound protocol closely followed recommendations by Corretti et al and we have shown excellent reproducibility with this protocol . Brachial arterial diameter were measured using an Acuson Aspen™ duplex ultrasound imaging system (Siemens AG, US) with a 10 MHz linear array transducer. Longitudinal, 2-dimensional images of the brachial artery 5 to 10 cm above the elbow of the right arm were stored on SVHS tape during quiet rest (1 min), arterial occlusion via inflation of a cuff on the forearm (distal to the target artery) to 200 mmHg (5 min), and reactive hyperemia (2 min) by a sonographer with extensive training in vascular ultrasound. Frames for analysis were sampled at end diastole and diameters were measured continuously using automated edge-detection

software (Brachial Analyzer, MIA, Iowa City, IA), with manual review of arterial boundaries by a trained technician. FMD was measured as the percent change (Δ%) in arterial diameter from the average diameter under resting conditions to the peak diameter recorded during the deflation period.

Statistical Analysis

All statistical analyses were performed using SAS for WINDOWS, release 9.1 (SAS Institute, Cary, NC). The inter-quartile range (IQR) was used to detect the presence of potential outliers. The PROC UNIVARIATE statement in SAS was used to generate a boxplot and IQR for each of the variables at baseline. Observations that were outside of Q_1-(1.5*IQR) and Q_3+(1.5*IQR) were flagged as potential outliers; there were no outliers outside of the Q_1-(3*IQR) and Q_3+(3*IQR) range. All analyses were then completed without potential outliers to determine their impact. The elimination of potential outliers did not change any of the main effects observed, thus all data reported includes all thirteen individuals.

The Shaprio-Wilk test of the residuals from the mixed model (PROC MIXED) was used to test for the normality of each variable. A W statistic >0.90 indicated that the variable was normally distributed. Non-normally distributed variables were log-transformed to achieve normality. Prior to log transformation any variable with a value <1 was adjusted; the lowest value for the variable of interest was subtracted from one. The difference was then added to each value such that the lowest value was equal to one after adjustment and prior to log transformation. For the mixed models analysis levels of serum TG and CRP were log-transformed as described above. All analyses were performed on transformed values; all means reported represent unadjusted means.

The mixed models procedure (PROC MIXED) was used to test for effects of diet, group, order of diet presentation, period, and the diet*group interaction on the levels of all serum outcome variables. Tukey-Kramer adjusted *P* values < 0.05 were used to determine whether the differences in the outcome variables were significant. All of the p-values and least squares means that are presented were taken from the mixed model including diet, group, order, period and the diet*group interaction.

Results

All thirteen individuals enrolled in the study at baseline completed the study (Table 3.1). There were no statistically significant differences between the age, BMI, TC levels, and LDL-C levels of the NTG and HTG groups. Individuals in the HTG group had significantly lower levels of HDL-C ($p<0.01$) and higher levels of TG ($p<0.001$), compared to the NTG individuals.

Lipid and Apolipoprotein Responses

Blood lipids at baseline and following the consumption of the two experimental diets are listed in Table 3.4. While the LFD elicited a reduction in TG (11.5%), TG reductions were greater ($p<0.01$) following the MFD (30.5%), across both groups. The TG-lowering response on the LFD was driven by the HTG response (-22%); levels of TG were unchanged for the NTG group following the low-fat diet. The NTG and HTG groups both experienced large decreases in TG following the MFD (28% and 35%, respectively), however this change was not statistically significant in either group alone. The MFD increased HDL-C 6.1%, compared to a 2.6% reduction on the LFD ($p<0.05$) across both groups. Individuals in the NTG group had slight decreases in levels of HDL-C following the LFD and maintained baseline levels of HDL-C following the MFD. The

HTG group maintained their baseline levels of HDL-C following the LFD and experienced a slight increase in HDL-C following the MFD. Levels of LDL-C did not statistically differ across diets (LFD: 17% and MFD: 18%), compared to baseline. The NTG group experienced a greater reduction ($p<0.05$) in LDL-C during the MFD (25%) compared with the LFD (18%). The HTG group experienced the greatest reduction in LDL-C following the LFD (16%), compared to the reduction observed following the MFD (11%); this difference was not statistically significant. The ratios of LDL-C:HDL-C and TC:HDL-C were significantly higher in the HTG group, compared to the NTG group ($p<0.05$), across all diets. Compared to the LFD (-0.15), the MFD elicited the greatest reduction in the LDL-C:HDL-C ratio (-0.46; NS). The ratio of TG:HDL-C was also significantly higher in HTG individuals, compared to NTG individuals ($p<0.01$) across all diets. The TG:HDL-C ratio was significantly lower following the MFD, compared to baseline ($p<0.05$) across both groups. In addition there was a group*diet interaction effect for the ratio of TG:HDL-C (see Figure 3.2).

The levels of plasma apolipoproteins in response to the two experimental diets are listed in Table 3.5. There was no diet or group effect on the levels of total apoB-containing lipoproteins. There was a main effect of group and diet and a group*diet interaction effect on the levels of LpB:C ($p<0.01$; Figure 3.3). Levels of LpB:C were 18.3% higher on LFD compared to the MFD ($p<0.01$), across both groups. On the LFD, HTG subjects had significantly higher levels of LpB:C, compared to NTG Subjects ($p<0.01$). In addition, HTG subjects had significantly higher levels of LpB:C on the LFD, compared to the MFD ($p<0.01$). Across both diets, HTG subjects had higher levels of plasma apoC-III and apoC-III-HP (apoC-III associated with apoB) than the NTG

group. Levels of LpB were higher on the LFD, compared to MFD ($p=0.08$) across both groups. The ratio of ApoB/ApoA-1 was significantly higher in HTG, compared to NTG ($p<0.05$), across both diets.

Glucose, Insulin and HOMA Responses

There were no differences in levels of fasting glucose and insulin across diets or across groups. HTG subjects, however, tended to have higher levels of glucose and insulin compared to the NTG subjects (Table 3.6). There was no main effect of group or diet on any of the calculated HOMA Scores (HOMA_B, HOMA_S, HOMA_IR).

C-reactive protein Responses

CRP levels are represented as [median (range)]. CRP levels following the LFD were 1.51 (0.95-2.90) mg/L for the NTG group and 1.50 (1.43-2.85) mg/L for the HTG group. Following the MFD, CRP levels were 1.39 (0.99-1.51) mg/L for the NTG group and 1.86 (1.04-5.50) mg/L for the HTG group. These differences were not statistically significant.

Vascular Reactivity Responses

There were no main effects of diet or group for measures of vascular reactivity. Mean baseline diameter was 4.30 $\pm$ 0.31 mm for the NTG group and 4.57 $\pm$ 0.29 mm for the HTG group. Peak diameter and FMD scores following both diets are listed in Table 3.7.

Discussion

The results of the present study demonstrate that dietary patterns at either end of the recommended range of total dietary fat intake (20-35% total kcal) improve levels of serum lipids and lipoproteins in NTG and HTG individuals. Fasting levels of serum TG

were reduced in response to both a low-fat and a moderate-fat diet. The hypotriglyceridemic affect of the low-fat diet was driven primarily by the reduction in TG achieved in the HTG group. Of note however is that the LFD did not elicit an increase in fasting TG levels in either group; TG levels were maintained in the NTG group. Because the two experimental diets were both low in saturated fat (~5% of calories) and matched for dietary fiber (14g/1000 calories), they both resulted in a similar LDL-C-lowering response, as would be expected. The reduction in HDL-C following the LFD and the increase observed following the MFD also was expected. Of note in the present study is that despite a reduction in levels of serum TG on the LFD, HTG individuals experienced increased levels of the atherogenic apolipoprotein LpB:C, compared to the MFD. Levels of LpB:C remained unchanged across diets in the NTG group. These results indicate that while either a low-fat or moderate-fat diet (both low in saturated fats) may be recommended for individuals with normal fasting TG at baseline. On the contrary, it is evident that a moderate-fat diet, low in saturated fatty acids should be recommended for individuals with elevated fasting TG levels to prevent any potential increase in atherogenic apolipoproteins. These findings are of particular importance in the clinical setting where advanced lipid testing (i.e. apolipoproteins) does not routinely occur. For example, it is possible that while we may think we are improving the lipid profile by decreasing TG with a LFD, it is possible that in individuals with elevated TG we may actually be eliciting a pro-atherogenic apolipoprotein response.

Current dietary recommendations have provided individuals with suggested ranges of macronutrient intakes. Rather than focusing on simply reducing levels of total dietary fat, for the first time a range of dietary fat is recommended. Traditionally, low-

fat, high-carbohydrate diets increase levels of fasting TG and decrease levels of the HDL-C when compared to moderate-fat diets, both of which are low in saturated fat (42). The inclusion of viscous fiber into a meal plan, however, will attenuate the hypertriglyceridemic response to dietary carbohydrate (42). Within the range of total fat (18-40% kcal) evaluated in controlled feeding studies, there is a linear dose-response relationship between total fat content of the diet and the changes in HDL-C and TG (35). Weighted least-squares regression analysis revealed that for every 5% decrease in total fat, HDL-C levels would be expected to decrease by 2.2% and TG levels would be expected to increase 6%. In some individuals, reduced-fat, high-carbohydrate diets may induce atherogenic dyslipidemia (42), which is characterized by small dense LDL-C particles, high TG levels and low HDL-C levels. In sedentary, overweight or obese populations, in particular, reduced-fat, high-carbohydrate diets increase the prevalence of this phenotype, resulting in a higher risk of CVD among these population groups (15). The results of the present study demonstrate that a high fiber, well-balanced low-fat diet (LFD; 20% kcal) can prevent an increase in fasting TG and reduce these levels in individuals with normal and elevated baseline TG levels. In particular, the results illustrate the potent TG-lowering effect of a moderate-fat diet (MFD; 35% kcal), rich in unsaturated fatty acids, in individuals with elevated baseline TG levels. Based on the 15% reduction in total fat between the MFD and LFD, it would be expected that the MFD would have elicited an 18% reduction in fasting TG levels and a 6.6% increase in levels of HDL-C (35). The observed changes in the present were greater than those that would be predicted; compared to the LFD, the MFD reduced levels of fasting TG by 19.5% and increased levels of HDL-C by 7.7%, possibly due to the rich fiber content of the diets.

Consistently, a low-fat, high-carbohydrate diet compared with a moderate fat diet (both relatively low in SFA and cholesterol) decreases LDL-C levels similarly (19-31). Since HDL-C is also proportionately decreased as LDL-C is decreased, the ratio of LDL-C to HDL-C does not change (43). On a moderate-fat diet, however, when unsaturated fatty acids replaces SFA, LDL-C decreases proportionately more than HDL-C thereby decreasing the LDL:HDL-C ratio (44). In the present study levels of LDL-C were decreased similarly across both the LFD (17%) and MFD (18%), compared to baseline. Concurrently, levels of HDL-C were slightly reduced following the LFD (-2.8%) and were increased following the MFD (+4.7%), compared to baseline. As a result the ratio of LDL-C:HDL-C was lower following the MFD (2.79), than it was following the LFD (3.10), compared to baseline (3.25). Epidemiologic evidence suggests that for every 1 unit decrease in the TC/HDL-C ratio, there is a 53% decrease in the risk of myocardial infarction (MI) (45). In the present study the ratio of TC:HDL-C was 0.47 units lower following the MFD, compared to the LFD, representing a ~25% reduction in risk of MI.

The measurement and classification of serum apolipoproteins has provided additional prognostic information for determining risk of CVD. In particular, it has been demonstrated that the three major TG-rich lipoprotein families have different affinities for lipoprotein lipase (LPL) despite almost identical triglyceride and apoC-III contents; thus, the Lp-B:C:E particles have been found to be the most efficient substrate for LPL resulting in faster clearance followed by decreasing efficiency of Lp-B:C and low efficiency of Lp-A-II:B:C:D:E particles (24). Results of a preliminary study have suggested that individual apoB-containing lipoprotein families also have different binding affinities and rates of uptake by human THP1 macrophages. The highest

accumulation of neutral lipids and apoB was caused by Lp-B:C and Lp-B:C:E particles; macrophages incubated with these particles accumulated lipids and apoB by an apparently unsaturable mechanism and were readily transformed into foam cells. In contrast, Lp-A-II:B:C:D:E and especially Lp-B particles were taken up by macrophages at much lower rates via a saturable mechanism. In the present study levels of the pro-atherogenic LpB:C subclass were significantly higher following the LFD, compared to the MFD diet. In addition, there was a diet by group interaction such that HTG individuals had higher levels of LpB:C following the LFD, compared to the MFD; and HTG individuals experienced higher levels, compared to NTG individuals following the LFD. While it appears as though the LFD may have induced higher levels of this atherogenic apolipoprotein, it is possible that the baseline levels were higher than those observed following the LFD. Regardless, the results of the present study do indicate that the MFD has a stronger anti-atherogenic effect on levels of atherogenic apolipoproteins, compared to the LFD.

Although once thought of as simply a disease of lipid disorders, it is now well established that inflammation plays a key role in the local, myocardial, and systemic complications associated with the atherosclerotic process (46). It is estimated that inflammation may be the underlying cause of approximately 80% of all sudden cardiac deaths (47). Epidemiological evidence from the Women's Health Study (48,49) indicates that out of four markers of inflammation [high-sensitivity-C-reactive protein (hs-CRP), serum amyloid A, IL-6, and soluble-intracellular adhesion molecule-1], hs-CRP was the most significant predictor of risk in univariate analysis. Within the same study hs-CRP was also significantly better than homocysteine, lipoprotein(a), and LDL-C levels in

predicting CVD risk. Furthermore, in a recent analysis of the Women's Health Study (50), after adjustment for age, blood pressure, smoking, diabetes, and obesity, hs-CRP added additional prognostic information beyond that obtained with traditional lipid measures. When divided into quintiles of hs-CRP levels, the lipid ratio of TC:HDL-C had the strongest association with CVD risk, with a HR of 3.81 (95% CI, 2.47-5.86; $p<0.001$) for the highest quintile of hs-CRP level, compared to the lowest quintile (50). Clinical cutpoints for levels of CRP, as defined by the American Heart Association and the Center for Disease Control and Prevention are: <1 mg/L (low risk, 1.0-3.0 mg/L (average risk), and >3.0 mg/L (high risk) (51).

Increased levels of CRP are strongly correlated with the prevalence of obesity (52-55). Although the association between CRP and obesity is well documented, the connection between CRP and various dietary components is not well understood. Results of a recent weight loss intervention demonstrated that the dietary carbohydrate/protein ratio had no effect on levels of CRP, but that CRP was positively associated with indices of body fatness (56). In a study designed to test the effectiveness of 4 popular diets: Atkins (carbohydrate restriction), Zone (macronutrient balance), Weight Watchers (calorie restriction), and Ornish (fat restriction), decreased levels of CRP were significantly associated with weight loss, with no significant differences between any of the test diets (57). In weight stable subjects, levels of CRP have been positively associated with intakes of *trans* fatty acids (58), and low intakes of vitamin C (59), and negatively associated with intakes of ALA (37). Given that both the LFD and MFD diets in the present study were well-designed, heart healthy diets, levels of CRP were unchanged across the two diets. Levels of CRP tended to be higher in HTG subjects,

compared to NTG, indicating that these individuals are at an increased risk for CVD. Despite an increase in dietary fat on the MFD, the fatty acid profile of the diet remained consistent with that of the LFD, and of a pattern that is associated with decreased risk of CVD. Because CRP levels were not assessed at baseline, it is not possible to detect from this data whether a reduction in levels of CRP were observed between the two experimental diets and baseline.

A strong body of literature from animal and in vitro studies suggests that endothelial dysfunction is an early event in atherosclerosis (60). One of the most frequently used techniques for measuring endothelium-dependent vasodilation involves the measurement of dynamic changes in arterial diameter using high frequency ultrasound (61). This noninvasive technique has allowed researchers to demonstrate impaired endothelial function in individuals at high risk of coronary disease, long before the clinical manifestations of arterial disease are present. For example, numerous studies have reported impaired FMD in patients with CVD (62) and type-2 diabetes (63). There is strong evidence that lowering cholesterol (64), reducing blood pressure (65), and beginning an exercise program (66) significantly increase FMD.

Studies examining the effects of altering the macronutrient profile of the diet on FMD have produced mixed results. Improvements in FMD (+3.3%) have been observed when comparing a Mediterranean-style diet (38% total fat; <10% SFA, 22% MUFA, 6% PUFA) to a high saturated fat diet (38% total fat; 20% SFA, 12% MUFA, 6% PUFA); no benefit was observed however when a low-fat diet (28% total fat; <10% SFA, 12% MUFA, 6% PUFA) was compared to the high saturated fat diet (67). Similarly, de Roos et al. observed no difference in FMD following either a low-fat diet (26% total fat; 10%

SFA, 8% MUFA, 7% PUFA), or a high MUFA diet (44% total fat; 16% SFA, 19% MUFA, 9% PUFA) (68). Impairments in FMD (-1.8%) were also observed following the consumption of a diet high in trans fatty acids (37% total fat; 0.4% *trans*, 13% SFA, 18% MUFA, 5% PUFA), compared to a diet rich in saturated fat (41% total fat; 9.4% *trans*, 23% SFA, 9% MUFA, 7% PUFA) (69). When compared to a Mediterranean style diet (33% total fat, 8% SFA, 18% MUFA, 4% PUFA), a diet high in PUFA (33% total fat; 5% SFA, 14% MUFA, 12% PUFA) elicited improvements in FMD (+2.3%) (70). In a recent study, a high saturated fat (37% total fat; 19% SFA, 12% MUFA, 4% PUFA) diet caused a ~50% deterioration in FMD compared to diets rich in PUFA (36% total fat; 9% SFA, 10% MUFA, 15% PUFA), MUFA (37% total fat; 8% SFA, 19% MUFA, 7% PUFA) and carbohydrates (18% total fat; 7% SFA, 6% MUFA, 3% PUFA) (71). In general the results of these studies indicate that the chronic consumption of a diet rich in saturated fat will cause impairment in FMD. Thus it is possible that the lack of a diet effect in the present study is due to the fact that both of the diets were very low in saturated fat (5% kcal). Further research is needed to determine if the absolute amount of fat in the diet causes an impairment of FMD, when the fatty acid profile is held constant.

Study Limitations:

A major limitation of the present study is the small sample size of the entire sample and of the two groups evaluated. Although the crossover design maximizes the power of the study by enabling each subject to serve as his/her own control, it is possible that with a greater number of total subjects, and thus subjects within each group significant differences may have existed for levels of CRP, more of the apolipoproteins, and measures associated with insulin resistance. A greater sample size may have also

provided a more diverse sample, and thus elicited responses that would be more representative of the general population. Another limitation of the present study is the lack of baseline measurements for some of the biomarkers (i.e. apolipoproteins and CRP). It is possible that if baseline parameters were available that significant effects of diet would have been observed, as was seen with the observed change in serum lipids from baseline.

Conclusions:

Current dietary recommendations provide suggested ranges for macronutrient intake. This not only allows for flexibility in menu planning but also allows for the individualization in menu planning based on individual characteristics and metabolic tendencies. Although traditional low-fat, high-carbohydrate diets have been shown to increase fasting TG levels and decrease levels of HDL-C these effects were prevented in this study by focusing on the quality of the dietary carbohydrate in the experimental diets. The use of whole wheat bread, a high fiber cereal and various fruits and vegetables provided levels of dietary fiber in accordance with current recommendations (14g per 1000 kcal). In addition, the focus on the quality of the fat in the experimental diets allowed for a fatty acid distribution that met recommendations for dietary SFA, MUFA, PUFA and n-3 PUFA. This was achieved by choosing low-fat and fat-free dairy products, lean cuts of meat, including salmon twice a week and focusing on various oils rich in unsaturated fatty acids, including safflower, olive, grapeseed, flaxseed, walnut and almond oils. Each of the above choices and manipulations of the experimental diets (i.e. choosing high-fiber cereals and low-fat diary products) are strategies that can easily be implemented into the lives of individuals.

Individuals with elevated baseline TG are at an increased risk for CVD. The results of this study indicate that individuals with elevated baseline TG levels should be encouraged to eat a moderate fat diet, rich in unsaturated fatty acids (35% of calories), rather than a lower-fat diet (20% of calories). Individuals with elevated baseline TG experience exaggerated increases in postprandial TG levels. Given that postprandial TG levels are also an independent risk factor for CVD, reductions in fasting TG levels would be expected to greatly reduce risk for CVD. In making this recommendation to consume a moderate-fat diet rich in unsaturated fatty acids it is important to understand the effects of different kinds of fat on postprandial markers of atherogenesis in individuals both with normal and those with elevated baseline TG levels.

Table 3.1: Baseline subject characteristics

	All	Normotriglyceridemic (NTG)	Hypertriglyceridemic (HTG)
N	13 (2F, 11M)	6 (2F, 4M)	7 (0F, 7M)
Age (y)	45.8 ± 2.9[#]	49.5 ± 4.2	42.7 ± 3.9
BMI (kg/m^2)	27.9 ± 0.9	27.4 ± 1.1	28.7 ± 0.8
Total Cholesterol (mg/dL)	208.5 ± 7.8	209.5 ± 10.8	208.5 ± 10.1
LDL-Cholesterol (mg/dL)	130.6 ± 6.7	137.5 ± 6.3	122.8 ± 10.7
HDL-Cholesterol (mg/dL)	42.6 ± 3.1	53.8 ± 6.0	31.4 ± 3.6*
Triglycerides (mg/dL)	176.6 ± 18.2	91.0 ± 10.9	271.7 ± 30.4[¶]

Data reported as least squares mean ± standard error; F = female, M = male

[#]Arithmetic mean ± standard error

*$p<0.01$ when compared with NTG

[¶]$p<0.001$ when compared with NTG

Figure 3.1: Study schematic

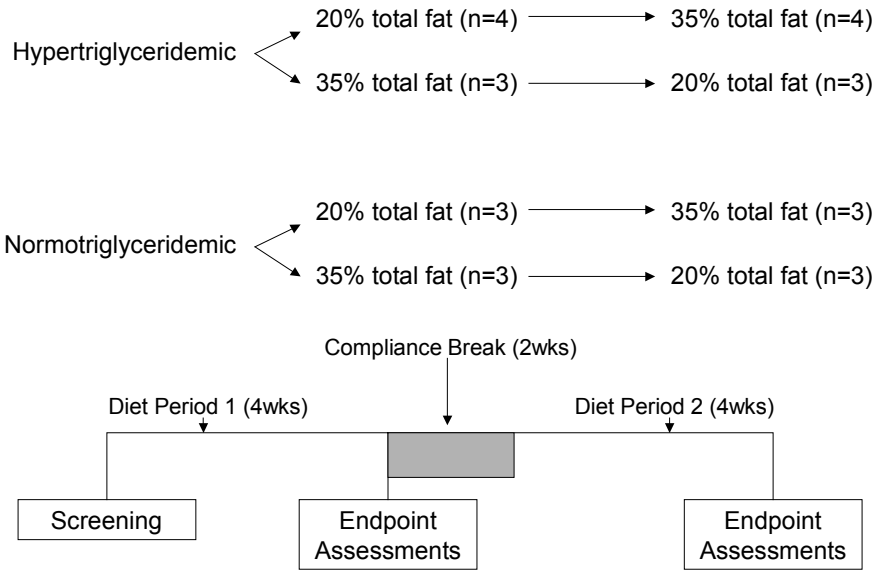


Table 3.2: Macronutrient composition of the two experimental diets. (% total calories)*

Nutrient	Low-Fat Diet (LFD)	Moderate-Fat Diet (MFD)
Total Fat	20	35
SFA	5	5
MUFA	6.5	21.5
PUFA	8.5	8.5
LA	7.5	7.5
ALA	0.9	0.9
EPA + DHA	0.09	0.09
Protein	18	18
Carbohydrate	62	47
Fiber	14 g / 1000 kcal	14 g / 1000 kcal
Cholesterol	<150 mg	<150 mg

*Calculated using Nutritionist Pro; SFA=saturated fatty acids, MUFA=monounsaturated fatty acids, PUFA=polyunsaturated fatty acids, LA=linoleic acid, ALA=α-linolenic acid, EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid

Table 3.3: Menu cycle.

Day 1:

Low-Fat Diet (LFD)		Moderate-Fat Diet (MFD)
Breakfast		***Breakfast***
Cheerios Cereal		Cheerios Cereal
Banana		Banana
Whole Wheat Bread and Jelly Packet		Whole Wheat Bread and Jelly Packet
Orange Juice		Orange Juice
Skim Milk		Skim Milk
Lunch		***Lunch***
Onion Sandwich Roll		Onion Sandwich Roll
Roast Beet		Roast Beet
Dijon Mustard Sauce (with Safflower Oil)		Dijon Mustard Sauce (with Grapeseed Oil)
Baby Carrots and Celery Sticks		Baby Carrots and Celery Sticks
Ranch Dressing (with Walnut Oil)		Ranch Dressing (with Almond Oil)
JELL-O Snack		
Dinner		***Dinner***
Chicken Breast		Chicken Breast
Broccoli		Broccoli
Romaine Lettuce, Tomato, Cucumber, Baby Carrots		Romaine Lettuce, Tomato, Cucumber, Baby Carrots
Catalina Dressing (with Flaxseed Oil)		Catalina Dressing (with Grapeseed and Flaxseed Oil)
Seasoned Brown Rice (with Grapeseed Oil)		Seasoned Brown Rice (with Olive Oil)
Strawberries		Strawberries
Whole Wheat Bread		Whole Wheat Bread
Butter		
Slivered Almonds		
Snack		***Snack***
Lorna Doone Shortbread Cookie		Slivered Almonds

Day 2:

Low-Fat Diet (LFD)		Moderate-Fat Diet (MFD)
Breakfast		***Breakfast***
French Toast		French Toast
French Toast Spread (with Safflower and Grapeseed Oil)		French Toast Spread (with Safflower and Grapeseed Oil)
Skim Milk		Skim Milk
Raspberries		Raspberries
Lunch		***Lunch***
Salmon Pate (with Grapeseed Oil)		Salmon Pate (with Safflower Oil)
Crackers		Crackers
Apple		Apple
Fig Newtons		Fig Newtons
JELL-O Snack		
Dinner		***Dinner***
Roast Beef and Fat Free Gravy and Whole Wheat Noodles (with Flaxseed and Grapeseed Oil)		Roast Beef and Fat Free Gravy and Whole Wheat Noodles (with Flaxseed, Grapeseed and Safflower Oil)
Corn		Corn
Spinach		Spinach
Mushrooms		Mushrooms
Fat Free Italian Dressing		Fat Free Italian Dressing
Whole Wheat Bread		Whole Wheat Bread
Butter		Cooked Egg Whites
Snack		***Snack***
Fruit Cup Snack		Light Fruit Cocktail
Skim Milk		Skim Milk
		All Bran with Extra Fiber

Day 3:

Low-Fat Diet (LFD)		Moderate-Fat Diet (MFD)
Breakfast		***Breakfast***
Non-fat plain yogurt		Non-fat plain yogurt (with Safflower Oil)
Granola (with Safflower Oil)		Granola (with Safflower Oil)
Blueberries		Blueberries
All-Bran Cereal with Extra Fiber		All-Bran Cereal with Extra Fiber
Apple Juice		
Lunch		***Lunch***
Skim Milk		Skim Milk
Deli Ham		Deli Ham
Rye Bread		Rye Bread
Honey Mustard Spread (with Safflower Oil)		Honey Mustard Spread (with Grapeseed Oil)
Pear		Pear
Dinner		***Dinner***
Turkey Taco Meat (with Flaxseed Oil)		Turkey Taco Meat (with Safflower Oil)
Romaine Lettuce		Romaine Lettuce
Tomato		Tomato
Baked Tortilla Chips		Baked Tortilla Chips and Salsa
Black Beans (with Grapeseed Oil)		Black Beans (with Flaxseed and Grapeseed Oil)
Cheddar Cheese (2%)		Cheddar Cheese (2%)
Fat Free Chocolate Snack Pack Pudding		Fat Free Chocolate Snack Pack Pudding
Snack		***Snack***
Raisins		Raisins
Pretzels		Pretzels

Unit Foods

LFD: Cranberry Orange Muffin		MFD: Apple Cinnamon Muffin
Sources of Fat: Walnut, Safflower, Grapeseed Oils and Butter		Sources of Fat: Walnut and Safflower Oils, Butter
Sources of Fiber: Wheat Flour, Whole Grain Wheat Flour, All-Bran with Extra Fiber Cereal		Sources of Fiber: Wheat Flour, Whole Grain Wheat Flour, All-Bran with Extra Fiber Cereal

Table 3.4: Lipid response to the two experimental diets.

	Baseline	LFD	MFD	Main effect of Diet	Main effect of Group	Diet*Group Interaction
TG				<0.05	<0.001	-
All Subjects	177.0 ± 18.6	146.9 ± 18.6	118.3 ± 18.6[#]			
NTG	91.0 ± 27.3	89.3 ± 27.3	65.8 ± 27.3			
HTG	263.0 ± 25.3	204.5 ± 25.3	170.8 ± 25.3			
Total Cholesterol				<0.001	-	<0.05
All Subjects	208.5 ± 7.8	179.3 ± 7.8*	175.1 ± 7.8*			
NTG	209.5 ± 10.8	182.0 ± 10.8[#]	169.7 ± 10.8[¶]			
HTG	208.5 ± 10.1	175.2 ± 10.1[¶]	179.4 ± 10.1[¶]			
LDL-Cholesterol				<0.001	-	-
All Subjects	131.0 ± 6.8	109.0 ± 6.8*	107.2 ± 6.8*			
NTG	137.5 ± 9.9	113.0 ± 9.9	103.0 ± 9.9			
HTG	124.5 ± 9.2	104.9 ± 9.2	111.4 ± 9.2			
HDL-Cholesterol				-	<0.01	-
All Subjects	42.6 ± 3.2	41.4 ± 3.2	44.6 ± 3.2			
NTG	53.8 ± 4.7	51.0 ± 4.7	53.3 ± 4.7			
HTG	31.4 ± 4.4	31.8 ± 4.4	35.8 ± 4.4			
LDL-C:HDL-C				<0.001	<0.01	-
All Subjects	3.25 ± 0.42	3.10 ± 0.54[#]	2.79 ± 0.54*			
NTG	2.62 ± 0.56	2.42 ± 0.67	2.47 ± 0.67			
HTG	3.89 ± 0.64	3.77 ± 0.64	3.11 ± 0.64			
TC:HDL-C				<0.01	<0.05	-
All Subjects	4.72 ± 0.48	5.24 ± 0.60	4.77 ± 0.48[§]			
NTG	3.42 ± 0.64	4.12 ± 0.76	4.11 ± 0.76			
HTG	6.01 ± 0.72	6.36 ± 0.72	5.43 ± 0.72			
TG:HDL-C				-	<0.01	-
All Subjects	5.28 ± 0.71	4.08 ± 0.84	3.24 ± 0.84			
NTG	1.83 ± 0.97	1.81 ± 1.10	1.52 ± 1.10			
HTG	8.72 ± 1.04	6.35 ± 1.04	4.96 ± 1.04			

Data reported as least squares mean ± standard error; *$p<0.001$ when compared to baseline; [¶]$p<0.01$ when compared to baseline; [#]$p<0.05$ when compared to baseline; [§]$p<0.01$ when compared to LFD. LFD = low-fat diet (20%), MFD = moderate-fat diet (35%), TG = triglycerides, NTG = normotriglyceridemic, HTG = hypertriglyceridemic, LDL = low-density lipoproteins, HDL = high-density lipoproteins.

Figure 3.2: TG:HDL-C ratio responses to experimental diets.

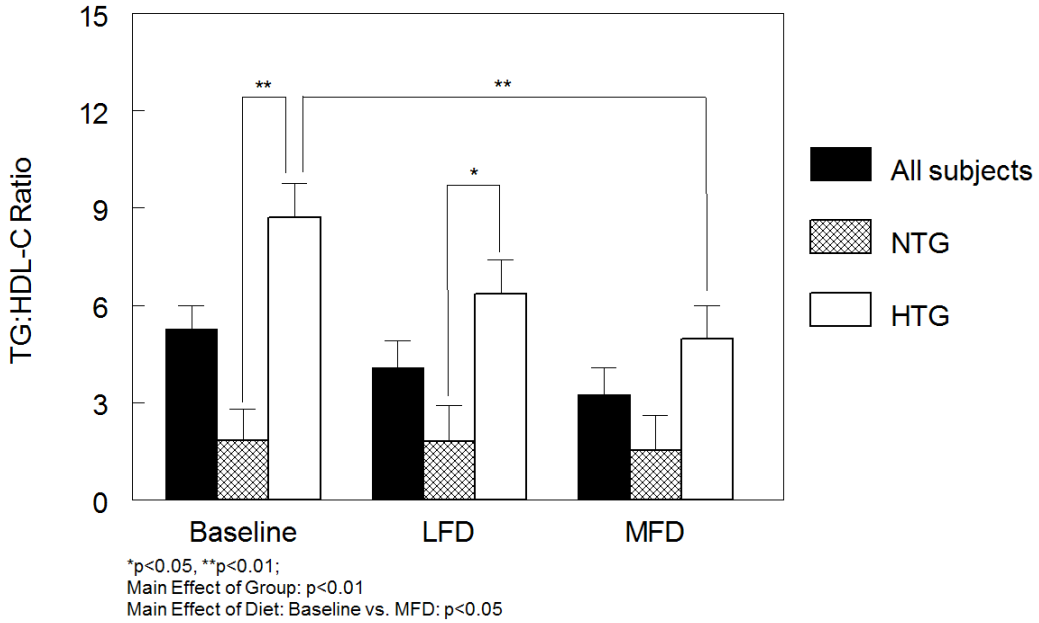
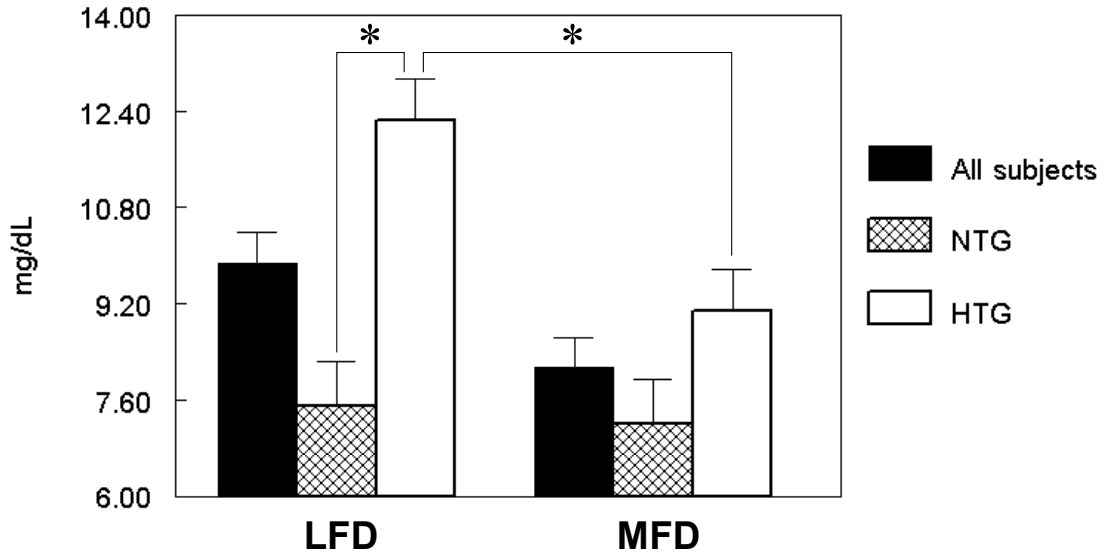


Table 3.5: Plasma apolipoprotein responses to two experimental diets.

	LFD	MFD	Diet Effect	Low TG	High TG	Group Effect	Diet*Group Interaction
ApoC-III	12.46 ± 0.63	11.62 ± 0.63	NS	**10.68 ± 0.78**	**13.4 ± 0.73**	**0.0287**	NS
ApoC-IIIR	1.91 ± 0.16	2.14 ± 0.16	NS	**2.42 ± 0.18**	**1.63 ± 0.17**	**0.0099**	NS
ApoC-IIIHS	7.7 ± 0.52	7.43 ± 0.52	NS	7.37 ± 0.61	7.76 ± 0.57	NS	NS
ApoC-IIIHP	4.25 ± 0.25	3.79 ± 0.25	NS	**3.14 ± 0.26**	**4.9 ± 0.24**	**<0.001**	NS
ApoA-IP	133.86 ± 4.72	135.1 ± 4.72	NS	140.42 ± 6.28	128.54 ± 5.84	NS	NS
LpA-1	31.31 ± 1.20	32.21 ± 1.20	NS	**34.21 ± 1.69**	**29.31 ± 1.58**	**0.0603**	NS
LpA-1A2	102.54 ± 3.75	102.89 ± 3.75	NS	106.22 ± 4.75	99.21 ± 4.42	NS	NS
ApoB Total	98.64 ± 2.31	96.76 ± 2.31	NS	**91.23 ± 3.05**	**104.18 ± 2.84**	**0.0111**	NS
LpB	57.00 ± 1.34	55.46 ± 1.34	0.0754	56.12 ± 1.88	56.34 ± 1.75	NS	0.0722
LpB:C	**9.88 ± 0.50**	**8.15 ± 0.50**	**0.0025**	**7.37 ± 0.66**	**10.67 ± 0.62**	**0.0045**	**0.0079****
LpA2B Total	15.38 ± 1.60	17.18 ± 1.60	NS	**12.12 ± 1.76**	**20.45 ± 1.64**	**0.0061**	NS
LpBE:BCE	16.38 ± 0.66	15.97 ± 0.66	NS	15.63 ± 0.73	16.72 ± 0.68	NS	NS
ApoB/ApoA-1	0.74 ± 0.03	0.73 ± 0.03	NS	**0.66 ± 0.04**	**0.82 ± 0.04**	**0.0226**	NS

**See Figure 3.3.

Figure 3.3: Levels of LpB:C across the two experimental diets.



main effect for diet and group ($p<0.01$); * $p<0.01$ for diet* group interaction

Table 3.6: Levels of glucose and insulin following both of the two experimental diets (least squares mean + s.e.).

	LFD	MFD
Fasting Glucose		
All Subjects	89.2 ± 1.9	89.3 ± 1.9
NTG	87.5 ± 2.7	87.5 ± 2.7
HTG	90.9 ± 2.6	91.1 ± 2.6
Fasting Insulin		
All Subjects	13.4 ± 1.7	13.6 ± 1.7
NTG	11.5 ± 2.5	11.5 ± 2.5
HTG	15.2 ± 2.3	15.7 ± 2.3

No differences are statistically significant.

Table 3.7: Vascular reactivity response to the two experimental diets (l.s. mean + s.e.).

	LFD	MFD	Main effect of Diet	Main effect of Group	Diet*Group Interaction
Peak Diameter (mm)			NS	NS	NS
All Subjects	4.70 ± 0.20	4.78 ± 0.20			
NTG	4.60 ± 0.29	4.57 ± 0.29			
HTG	4.81 ± 0.27	4.98 ± 0.27			
FMD Score (%)			NS	NS	NS
All Subjects	7.48 ± 0.95	7.07 ± 0.95			
NTG	6.88 ± 1.39	6.81 ± 1.39			
HTG	8.07 ± 1.29	7.34 ± 1.29			

NS = not significant

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Chapter 4
Effects of Different Fats on Postprandial Lipid Clearance and Associated Markers of CVD Risk

Background

Postprandial triglyceride level is an emerging risk factor for cardiovascular disease (CVD). Delayed clearance of triglyceride-rich postprandial particles may stimulate a number of pro-atherogenic events that include increases in oxidized fatty acids, oxidized stress, inflammation and endothelial dysfunction (1). As a result of frequent eating episodes, the postprandial state can span as much as 12 to 14 hours each day. It is evident that this alone, as well as perturbations in postprandial lipid metabolism, can play a role in the initiation and progression of atherosclerosis. Studies have shown that both type and amount of dietary fat modulate postprandial lipid clearance and vascular biology (2). In addition, baseline triglyceride level is an important component in postprandial lipid clearance; elevated triglyceride levels result in delayed triglyceride clearance (3). Based on the results of the previous study, compared to a lower-fat diet (20% of calories), a moderate-fat diet (35% of calories) is more effective at reducing levels of fasting triglyceride levels in normo- and hyper-triglyceridemic individuals. Recommendations therefore to consume a moderate-fat diet that is rich in unsaturated fatty acids is warranted for a greater reduction of fasting triglyceride levels.

Oxidative stress plays a key role in the pathogenesis of CVD (4). Another important event that occurs during postprandial lipoprotein clearance is oxidative modification of dietary fatty acids. Unsaturated fatty acids in triglycerides that circulate in postprandial lipoproteins and endogenously synthesized lipoproteins are targets of many oxidative processes that lead to an accumulation of oxidized fatty acids that

promote atherogenesis. In addition, during the postprandial period levels of oxidative stress have the potential to be exaggerated in the presence of delayed clearance of triglyceride-rich lipoproteins and the accumulation of oxidized fatty acids. The degree of lipid peroxidation depends on three factors: 1) the generation of oxygen free radicals, 2) the presence of lipid substrates, and 3) the activity of antioxidants (5). Results from animal research indicate that in diabetic animals, oxidized lipids are absorbed more efficiently (6). Also of importance when discussing the role of oxidative stress in the progression of CVD is the antioxidant status of the individual. For example, individuals with pre-existing atherosclerosis have been found to have significantly lower blood antioxidant status compared to healthy controls (7). Evidence suggests that dietary antioxidants present in red wine (8), grape seed extract (9), blueberries (10) and olive oil (11,12) all have the potential to increase levels of antioxidant capacity in the postprandial state. Little is known however, about the effects of fats varying in their degree of oxidative susceptibility (i.e. monounsaturated vs. polyunsaturated fatty acids) on postprandial levels of oxidative stress and antioxidant capacity. Results of a recent study indicate that oxidative stress manifested by the depletion of serum antioxidant enzymes and increased excretion of oxidative modification products is associated with endothelial dysfunction observed after the consumption of a high-fat meal (13).

In addition to postprandial TG levels and increases in levels of oxidative stress, adverse effects on endothelial function in the postprandial period are also considered to be a potential pro-atherogenic mechanism. In a classic study, Vogel et al. (14) assessed the direct effect of postprandial triglyceride-rich lipoproteins on endothelial function. As a result of the high-fat meal serum TG increased from 94 $\pm$ 55 mg/dl to 147 $\pm$ 80 mg/dl

after 2 hours ($p = 0.05$) and flow-dependent vasoactivity decreased from 21 $\pm$ 5% preprandially to 11 $\pm$ 4%, 11 $\pm$ 6%, and 10 $\pm$ 3% at 2, 3, and 4 hours after the high-fat meal, respectively (all $p < 0.05$ compared with low-fat meal) in 10 healthy, normocholesterolemic individuals. In an acute study designed to evaluate the effects of different fat loads on postprandial triglyceride clearance and flow mediated dilation (FMD) of the brachial artery, subjects with an elevated baseline triglyceride level (>150 mg/dL) had different vascular responses to the test fats compared to normotriglyceridemic subjects (15). As a whole, the entire sample experienced an increase in FMD in response to fat load high in monounsaturated fatty acids (MUFA). In particular, hypertriglyceridemic individuals responded favorably to two different fat loads high in omega-3 fatty acids, one of which was high in α-linolenic acid and the other which was high in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Although the use of FMD is often used in determining the health of the endothelial (1,16), biomarkers are also available for estimating risk of CVD associated with endothelial health and dysfunction.

Although the degree of endothelial dysfunction within the coronary microvasculature correlates with total serum cholesterol levels (17), the use of biomarkers that are more strongly associated with endothelial health can better define CVD risk. Endothelin-1 was originally described as a potent vasoconstrictor (18) and its overproduction is now considered a key mediator in the development of endothelial dysfunction and atherosclerosis (19). Evidence has shown that circulating levels of ET-1 are significantly higher in patients with CAD, compared to the healthy controls; tissue ET-1 was also observed in the smooth muscle cells of the intimal and medial layers of the

aortas of patients diagnosed with CAD (20). In addition to increased levels in the fasted state, levels of ET-1 also are increased in the postprandial state, in conjunction with a rise in triglycerides and insulin levels (21). Few studies have focused on dietary interventions to reduce the release of ET-1 in the postprandial period. One study evaluated the effects of the polyphenols from red wine on the synthesis of ET-1 in cultured bovine aortic endothelial cells (22). Given the utility of ET-1 as a predictor of endothelial health, questions remain regarding the effect of different types of dietary fat on the postprandial release of ET-1 and the associated pro-atherogenic events.

Based on previous findings, chronic intake of total fat (20 vs. 35% of calories) appreciably affects triglyceride levels. While both test diets decreased triglyceride levels, the moderate-fat diet (35% of calories) elicited a greater hypotriglyceridemic response. There are many important questions that remain to be addressed. With the recommendation to consume a moderate fat diet also comes the recommendation to consume a moderate fat diet with a cardioprotective fatty acid profile. Given the fact that postprandial lipids are affected by the type of fat and that individuals with normal and elevated baseline triglyceride levels respond differently to various types of fat in the postprandial period, studies are needed to define the ideal fatty acid profile with respect to postprandial lipid clearance in both normo- and hyper-triglyceridemic individuals.

It is thus suggested that an increase in the consumption of dietary oxidized lipids may play a role in the elevated levels of oxidized lipids in the lipoproteins, with the effects being greater for diabetic animals. While it is known that a fat load high in saturated fat will elicit pro-atherogenic postprandial responses in humans, the postprandial effects of polyunsaturated fatty acids, and pre-oxidized fatty acids in

particular are not well understood. Given that Americans spend a majority of their day in the postprandial state and the significant role of oxidative stress in the pathogenesis of CVD, several important questions remain regarding the acute ingestion of pre-oxidized and oxidizable fatty acids.

The purpose of the proposed pilot study therefore, is to evaluate the effects of polyunsaturated fatty acids (PUFA) and pre-oxidized PUFA, compared to a standardized saturated fatty acid (SFA) fat load in the postprandial period on traditional and emerging risk factors of CVD. Specifically, it is important that we better understand the role that dietary PUFA have in generating oxidized lipid end products, and how these may modulate important atherogenic events, including inflammation and vascular dysfunction. Although biomarkers such as C-reactive protein (CRP) (23) and ET-1 (19) are predictors of CVD risk in the fasted state, little is known about their utility in the postprandial period, specifically in response to different fatty acids. This is particularly important because recommendations for dietary PUFA span a wide range (5-10% of calories), as does the total fat recommendation (20-35% of calories). Collectively, it is important to know whether higher intakes of PUFA, fatty acids that are highly susceptible to oxidation, would induce a greater oxidative stress burden and downstream effects. In addition, with the increase in food eaten away from home, it is possible that individuals are consuming a greater number of pre-oxidized fatty acids principally from deep-fried foods that are prepared with fats and oils that are heated to high temperatures and often reused. In addition, the effects of consuming pre-oxidized PUFA will be compared to the consumption of un-altered PUFA on postprandial risk factors for CVD. It is thought that an acute fat load, rich in saturated fatty acids (SFA) will elicit a greater increase in

postprandial triglyceride (TG) levels, compared to a fat load rich in polyunsaturated fatty acids (PUFA), with a fat load rich in oxidized PUFA eliciting an intermediary response. Due to their increased level of risk and propensity to exhibit delayed clearance of TG-rich remnant lipoproteins, it is also hypothesized that individuals with elevated baseline TG levels will have an exaggerated postprandial TG response and associated increases in postprandial levels of oxidative stress, inflammation, endothelial dysfunction and decreases in antioxidant capacity, compared to individuals with normal baseline TG levels.

Methods

Subjects

Subjects with high (n=4; $\geq$ 150 mg/dL) and low (n=4; < 150 mg/dL) fasting triglyceride levels were recruited for this study. Subjects were otherwise reasonably healthy men or post-menopausal women (30-65 years of age) with no other major co-morbidities. The eligibility criteria used to determine if subjects were eligible are: BMI < 35 kg/m^2, LDL-cholesterol < 160 mg/dL, systolic blood pressure < 145 mmHg, and diastolic blood pressure < 95 mmHg; not on lipid-lowering or blood pressure medication or other medications known to affect lipid levels.

Study Design

After appropriate consent was obtained, subjects reported to the General Clinical Research Center (GCRC) on the University Park campus of The Pennsylvania State University for screening. Each screening visit included height, weight, and blood pressure measurement, a chem 24 profile, lipid panel and complete blood count (CBC). If all of the above eligibility criteria were met, subjects were then stratified into either the

normo- or hyper-triglyceridemic group (with 150 mg/dL as the cut point). Each subject then reported to GCRC for 3 separate test days, with the same test protocol for each visit. Prior to the first test day each subject completed a food record for the 48 hours prior to their test day. The subjects were then asked to match this intake as closely as possible before each of the two remaining test days. After a baseline blood draw, following a 12-hour fast, every subject was given a "milkshake" to drink that included one of the 3 different types of fat. Each milkshake was consumed within 15 minutes. Following the complete consumption of the milkshake a timer was started to ensure accurate timing of subsequent blood draws. Blood was collected at 30 minutes, 1, 2, and 4 hours after the complete ingestion of the milkshake. Each subject repeated this protocol on 2 additional days, with each test day separated by at least one week. Each of the three treatments was presented to the subjects in a randomized, blinded fashion.

Experimental Fat Loads

Each experimental fat load was prepared by the GCRC dietetic staff and delivered to the test area in the GCRC. The test product consisted of a "milkshake" type drink containing the 3 different fat loads (equal to 50g total fat). The protein and carbohydrate source in each of the three different test drinks was identical. The predicted macronutrient composition of the test fat loads is listed in Table 4.1. The predominant fat source in the high saturated fat (SFA-FL) fat load was olive oil and light cream. The oil used for both the high PUFA (PUFA-FL) and high-oxidized PUFA (OXPUFA-FL) fat loads was refined, bleached and deodorized) linoleic safflower oil without added vitamin E. The recipes for each of the test fat loads, containing the amounts of oils used are listed in Table 4.2. The amount of vitamin E/tocopherols in the oil was ~314ppm of α-

tocopherols, and ~21ppm of γ-tocopherols. (California Oils, Richmond, CA). Average tocopherol content of safflower oil is ~234-600 ppm α-tocopherol and ~0-12 ppm γ-tocopherol (24). On the day prior to each of the OXPUFA-FL test days the safflower oil was heated to 165ºF and maintained at that temperature for three hours in a standard deep fat fryer machine. The oil was then kept in a dark refrigerator at 4ºC until use in the preparation of the milkshakes the next morning. The heating of the oil was completed by a single person using the same procedure for each OXPUFA-FL to ensure the production of a standard test product for all subjects.

Serum Samples

Venous blood samples were collected into the appropriate tubes with anti-coagulants when necessary. Serum samples for baseline and postprandial lipids, c-reactive protein and ORAC were recovered by low-speed centrifugation at 3000 rpm for 30 min at 4°C. Plasma samples for endothelin-1 were collected in vacutainer tubes containing EDTA. Prior to centrifugation 50 μL of Aprotinin per 1 mL whole blood was added to each sample; samples were then recovered by low-speed centrifugation at 1,600 rpm for 15 minutes at 4°C. Serum samples for the determination of lipid hydroperoxides and fatty acids were collected in heparin-containing vacutainer tubes. Following the recovery of the serum by low-speed centrifugation at 3000 rpm for 30 min at 4°C, 0.5 μL of 0.5 M butyl-hydroxy-toluene (BHT) solution was added to each 2 mL cryovials to reduce the occurrence of *in vitro* oxidation. All serum samples were then stored at -80°C until the conclusion of the study when all samples were assayed together.

Baseline Lipid Profile and Postprandial Lipids

Serum total cholesterol (TC) and triglycerides (TG) were quantified using enzymatic assays (CHOP/PAP, Boeringer, Mannheim, FRG, Abbott Laboratories, Diagnostic Division, Irving, TX) conducted at the Core Laboratory of the General Clinical Research Center on the Hershey Medical Center's campus of the Pennsylvania State University (Hershey, PA). High-density lipoprotein cholesterol (HDL-C) was estimated according to the modified heparin-manganese precipitation procedure of Warnick and Albers (25). LDL-C levels was calculated by the Friedewald's equation: LDL-C = TC – (HDL-C + TG/5) (26).

Fasting and Postprandial Serum Fatty Acids and Fat Load Fatty Acids

Analysis of serum fatty acids was conducted in the laboratory of Dr. Ken Stark at the University of Waterloo in Waterloo, Ontario, Canada. Samples were extracted with the addition of an internal standard (22:3n-3 methyl ester; Nu-Chek-Prep, Elysian, MN, USA) using a modified Folch procedure and fatty acid methyl esters (FAMEs) were prepared from the total lipid extract by methylation with boron trifluoride in methanol (14% wt/vol; Alltech Assoc., Deerfield, IL, USA) according to the method of Morrison and Smith (27) as modified by Masood et al.(28).

Conventional analyses were performed on a Shimadzu GC-17A Gas Chromatograph equipped with a split/splitless injector in split mode, an AOC-17 automatic liquid sampler, and flame ionization detection. Initial gas chromatography analyses were performed on a DB-FFAP 30 m x 0.25 mm i.d. x 0.25 μm film thickness (J&W Scientific from Agilent Technologies, Palo Alto, CA), nitroterephthalic acid modified, polyethylene glycol, capillary column. In order to resolve cis-trans isomers, a

HP-88 100 m x 0.25 mm i.d. x 0.2 µm film thickness (Agilent Technologies, Palo Alto, CA) cyanopropyl-methylarylpolysiloxane capillary column was used.

Fasting and Postprandial C-Reactive Protein

C-reactive protein was measured by a high sensitivity sandwich Enzyme-Linked Immunosorbent Assay (ELISA) developed in the Cytokine Core Laboratory at the GCRC at Penn State University, University Park Campus.

Fasting and Postprandial Whole ORAC, Slow ORAC and Fast ORAC

Analysis of serum samples for the ORAC assay was conducted at the Genox Corporation. The ORAC assay measures the time-dependent decrease in the fluorescence intensity of the b-PE marker protein, resulting from oxygen radical damage. On a molar basis, the b-PE protein reacts with oxygen radicals over 100 times slower than most biological antioxidants such as thiols, uric acid, bilirubin and ascorbate. However, b-PE is over 60 times more reactive than other non-antioxidant proteins. Therefore, all other active antioxidants are completely oxidized before the b-PE protein gets oxidized, thus facilitating the measurement of the total antioxidant capacity of the sample being tested. Each point on the whole serum ORAC curve reflects the level of antioxidant protection at that time, which is made up of many different antioxidants. Genox Corporation has developed a computer macro which measures the amount of fast acting antioxidants, such as ascorbic acid, phenolic acids etc. that protect the b-PE protein 100% by completely inhibiting free radical propagation. The slow acting antioxidants consist of the tocopherols, polyphenols, flavonoids, and, in addition, certain proteins and lipids, that are found in the blood stream (29).

Fasting and Postprandial Serum Lipid Hydroperoxides & Milkshake Lipid Hydroperoxides

Levels of fasting and postprandial serum lipid hydroperoxides and lipid hydroperoxides present in the milkshakes were determined using a lipid hydroperoxide (LPO) colorimetric assay (Cayman Chemicals, Ann Arbor, Michigan) with brief modifications. Lipid hydroperoxides were first extracted using chloroform. 1 mL serum was extracted into 2 mL chloroform following deproteination. For the milkshakes, 1 mL milkshake was extracted. Extracts were then stored at –80˚C until the assay was performed. The LPO assay was performed according to standard kit procedures using 750µL cholorform serum extract. Each sample was run in triplicate and mean values were used for data analysis. Serum that had been hemolyzed during the blood collection process were run in triplicate twice; once with a triphenylphosphine solution and once without the added solution. Triphenylphosphine reduces hydroperoxides very efficiently and eliminates the color development due to hyroperoxides in the assay. The absorbance after treating the samples with triphenylphosphine was then subtracted from the sample data to correct for any absorbance not due to hydroperoxides. All absorbance values were read at 490 nm using a Biotec ELx ultran microplate reader utilizing KinetiCalc version 4 software (Bio-Tec Instruments Inc., Winooski, VT). The average value for the blank wells was then subtracted from the average absorbance value for each sample and standard. The corrected absorbances of the standards were then plotted to determine a standard curve for each of the test plates that were run. Calculations of the lipid hydroperoxide values were then performed using the equation obtained from the linear regression line of the standard curve.

Fasting and Postprandial Endothelin

Fasting and postprandial levels of endothelin-1 were determined using a standard enzyme immunometric assay (EIA) kit (Assay Designs, Ann Arbor, Michigan) following solid phase extraction of the plasma samples. Briefly, solid phase extraction was performed using 500mg C18 Sep-Pak 6-mL cartridges. Samples were de-proteinated by adding an equal volume (1.5 mL) of 20% acetic acid (AA) to the sample (1.5 mL) before centrifugation at 3000 x g for 10 minutes at 4°C. The columns were equilibrated with one column reservoir volume (CV) of 100% methanol (MeOH), followed by one CV water and one CV 10% MeOH. Following equilibration the de-proteinated supernatant was applied to the Sep-Pak column. The column was then washed with one CV 10% AA, followed by 2 CV ethyl acetate. The sample was eluted by applying 6 mL MeOH/0.05M ammonium bicarbonate (80/20) to the column. The eluant was collected and evaporated to dryness using a centrifugal concentrator under vacuum. All samples were then stored at -80°C until they were reconstituted for use in the EIA kit. Samples were reconstituted with the assay buffer provided with the kit immediately before use; each sample was run in duplicate. Standard kit procedures were followed; absorbance values were read at 450 nm, using a Biotec ELx ultran microplate reader utilizing KinetiCalc version 4 software (Bio-Tec Instruments Inc., Winooski, VT). The average absorbance was then calculated for each sample and standard by subtracting the average absorbance for the blank wells from the average of the duplicate sample/standard wells. Endothelin-1 concentrations were then calculated using the equation obtained from the linear regression line of the standard curve.

Statistical Analysis

All statistical analyses were performed using SAS for WINDOWS, release 9.1 (SAS Institute, Cary, NC). The inter-quartile range (IQR) was used to detect the presence of potential outliers. The PROC UNIVARIATE statement in SAS was used to generate a boxplot and IQR for each of the variables at baseline. Observations that were outside of Q_1-(1.5*IQR) and Q_3+(1.5*IQR) were flagged as potential outliers; there were no outliers outside of the Q_1-(3*IQR) and Q_3+(3*IQR) range. All analyses were then completed without potential outliers to determine their impact. The elimination of potential outliers did not change any of the main effects observed, thus all data reported includes all eight individuals. Due to the postprandial nature of the study extreme values in the postprandial time period were not excluded based on the principle that they may be representing a hypo- or hyper-physiological response.

The Shaprio-Wilk test of the residuals from the mixed model (PROC MIXED) was used to test for the normality of each variable. A W statistic >0.90 indicated that the variable was normally distributed. Non-normally distributed variables were log-transformed to achieve normality. Prior to log transformation any variable with a value <1 was adjusted; the lowest value for the variable of interest was subtracted from one. The difference was then added to each value such that the lowest value was equal to one after adjustment and prior to log transformation. For the analysis of serum levels, serum triglycerides and serum trans fatty acids were log-transformed as described above. All analyses were performed on transformed values; all means reported represent unadjusted means.

Serum fatty acids were analyzed as individual fatty acids and as groups of fatty acids. Each of the individual serum fatty acids were included in their respective fatty acid class (i.e. SFA, MUFA, PUFA, n-6 PUFA, n-3 PUFA, trans fatty acids) for analysis. In addition, trans fatty acid isomers were analyzed individually and as groups based on their structures (i.e. 18:1-trans and 18:2-trans). To explore the effects of fatty acids that are highly susceptible to oxidation on the oxidation and antioxidant measures, the number of double bonds was calculated for each fatty acid. This crude estimate of the number of double bonds was calculated by multiplying the concentration of each fatty acid by the number of double bond for the fatty acid. These calculations from the individual fatty acids were then summed into the following groups: total number of double bonds, one double bond, 2 or more double bonds and trans double bonds.

The mixed models procedure (PROC MIXED) was used to test for effects of treatment, group, time, order of treatment presentation, visit, and their interactions (treatment*time, treatment*group, group*time, treatment*time*group) on the levels of the outcome measurements (i.e. TG, CRP, ET-1, LPX and ORAC values) and serum fatty acids. In addition, change scores, from baseline (1-0, 2-0 and 4-0), were calculated for each of the above variables and serum fatty acids. The mixed models procedure was then used to test for effects of treatment, group, time (change), order of treatment presentation, visit, and their interactions (treatment*time, treatment*group, group*time, treatment*time*group) on the changes observed. Tukey-Kramer adjusted P values < 0.05 were used to determine whether the differences in the outcome variables were significant. All of the p-values and least squares means that are presented were taken

from the mixed model including treatment, group, time and their interactions (treatment*time, treatment*group, group*time, treatment*time*group).

Pearson correlations were performed by both treatment and time to investigate possible relationships between levels of serum fatty acids and each of the outcome variables (i.e. TG, CRP, ET-1, LPX and ORAC values). Similar correlations were run between the changes in fatty acids and the changes in each of the outcome variables. Each of these correlations was then transformed using the Fisher z-transformation. Correlations within each treatment were then averaged to obtain an overall correlation for each treatment. Averaged correlations were then back-transformed with the Fisher z-transformation to obtain overall correlation estimates. The magnitude of the overall transformations was then investigated to determine possible relationships between fatty acids and each of the outcome variables.

Power Analyses

In this study, all subjects consumed each of the fat loads and therefore acted as their own control in comparisons between postprandial challenges. This type of design increases the statistical power to detect differences between diets over the between-subjects design in which each treatment (fat load) is comprised of different individuals. SAS-PC Analyst (Version 8) was used for all power analyses. A two-tailed t-test was used with power set to 0.80 and alpha equal to 0.05.

The data used for power calculations were selected from a postprandial study conducted in ten healthy men (30). This study was designed to test the effects of different types of fat on postprandial lipid and TG responses. The test meals used were 1) rich in SFA, 2) rich in MUFA, and 3) rich in n-6 PUFA. Serum triglycerides increased

rapidly (1 hour) and peaked 2-4 hours following the test meal, with the greatest responses observed following the MUFA and PUFA meals. We estimated the sample size needed to detect a physiologically significant postprandial peak in triglycerides with our postprandial challenges to be 3 subjects, with alpha set to 0.05 and power set to 0.80.

A second postprandial study was used to determine the sample size needed to detect a significant change in postprandial levels of lipid hydroperoxides (31) in response to a typical American breakfast. Serum lipid hydroperoxide levels peaked at 2 hours, increasing 153% from baseline. Using the data from this study, the estimated sample size needed to detect a meaningful change in lipid hydroperoxides would be 11 subjects, with alpha set to 0.05 and power set to 0.80. All of the subjects in this study were normotriglyceridemic at baseline. Because half of our subjects had elevated levels at baseline it is expected that they will have an exaggerated response, compared to the present study. In addition, the use of pre-oxidized fatty acids is expected to elicit a response which would inherently be greater than the observed response in this study.

Results

Baseline Subject Characteristics

All eight subjects enrolled in the study completed the three test days. Baseline subject characteristics are listed in Table 4.3. Subjects ranged in age from 39 to 55 years; body mass index ranged from 19.1 to 32.1 kg/m^2. Subjects in the both the NTG and HTG groups had slightly elevated total (~211 mg/dL) and LDL-cholesterol (~124 mg/dL) levels. Consistent with the design of the study the HTG group had significantly higher

levels of serum triglycerides ($p<0.05$) and lower levels of HDL-cholesterol. There were no other significant differences between the two groups.

Fatty Acid Composition of Test Fat Loads

The calculated versus the assayed fatty acid composition of each of the test fat loads is listed in Table 4.4. Compared to the two PUFA-rich test fat loads, the SFA-FL was higher in SFA (18.4% total fatty acids) and MUFA (70.2% total fatty acids). As predicted, the OXPUFA-FL and PUFA-FL were rich in PUFA (76.6 % and 77.1 % total fatty acids, respectively). The predominant PUFA in these two test loads were n-6 PUFA (76.5 % and 77.0 % total fatty acids, respectively), with trace amounts of n-3 PUFA (0.06 % and 0.09 % total fatty acids, respectively). Levels of assayed trans fatty acids were highest in the SFA-FL (0.63% total fatty acids), lowest in the PUFA-FL (0.27% total fatty acids) and intermediary in the OXPUFA-FL (0.43% total fatty acids).

The concentration of the major groups of fatty acids and the predominant individual fatty acids are listed in Table 4.5. The predominant SFA in the test fat loads were palmitic acid (16:0; PA) and stearic acid (18:0; SA). Oleic acid (18:1n9; OA) was the MUFA fed in the highest concentration and linoleic acid (18:2n6) and α-linolenic acid (18:3n3) were the main n6 and n3 PUFA, respectively, in the test fat loads. The distribution of individual trans fatty acid isomers is presented in Figure 4.1. The trans fatty acid isomers detected in each of the test fat loads were 18:2-trans, trans, 18:2-trans, cis, 18:2-cis, trans, 18:1-trans-11. In addition, the trans fatty acid isomer 18:1-trans-12 also was detected in both the OXPUFA-FL and PUFA-FL test fat loads.

The total number of calculated double bonds was higher in the two PUFA-rich fat loads (~164 double bonds each), compared to the SFA-FL (~90 double bonds). The

majority of the double bonds in the OXPUFA-FL and the PUFA-FL were within fatty acids containing 2 or more double bonds, whereas the SFA-FL contained a greater number of fatty acids with single double bonds.

Comparison of the Fatty Acid Composition of Test Fat Loads following heat treatment

As a result of the heat treatment, the OXPUFA-FL exhibited higher levels of trans fatty acids, compared to the PUFA-FL, especially for 18:2-trans, trans. The increase in each of the measured trans fatty acid isomers is presented in Figure 4.2. The greatest increase observed following heat treatment was in the 18:2-*trans, trans* isomer. In addition there were minimal increases in total MUFA, and very small decreases in total PUFA and n-3 PUFA following heat treatment. There was no change in the calculated estimates of the number double bonds following heat treatment.

Serum Fatty Acid Concentrations

Serum levels of total fatty acids were increased significantly from 0 to 4 hours ($p<0.001$) across all treatments. In general, serum fatty acid profiles reflected the fatty acid composition of the test fat load consumed. In particular, there was a significant increase in MUFA following the SFA-FL, compared to the two PUFA-rich fat loads ($p<0.01$); and a significant increase in total PUFA and n-6 PUFA following the consumption of the two PUFA-rich fat loads compared to the SFA-FL ($p<0.05$). Serum levels of n-3 PUFA were significantly higher following consumption of the SFA-FL, compared to the OXPUFA-FL ($p<0.05$), with the PUFA-FL eliciting an intermediary response. There was a significant treatment*time interaction for serum levels of trans fatty acids; following the OXPUFA-FL there was a significant increase between 0 and 4 hours (Figure 4.3; $p<0.05$). In general, HTG individuals (3805 mg/100mL) had higher

levels of serum fatty acids compared to their NTG counterparts (3176 mg/100mL). In particular, HTG individuals had significantly higher concentrations of serum SFA, MUFA and trans fatty acids, compared to NTG individuals ($p<0.05$) across all fat loads.

Baseline levels of trans fatty acids were not different across all treatments. When analyzing individual trans fatty acid isomers as a percent of total fatty acids, there was a significant increase in the concentration of 18:1-trans-12 ($p<0.001$), 18:1-trans-11 ($p<0.0001$), 18:1-trans-9 ($p<0.05$), 18:1-trans-6 ($p<0.05$), 18:2-trans, trans ($p<0.0001$) and 18:2-cis, trans ($p<0.05$) over time. The change in the levels of 18:1-trans-11 ($p<0.05$), 18:1-trans-6 ($p<0.01$), 18:2-trans, trans ($p<0.01$), 18:2-trans, cis ($p<0.0001$) and 18:2-cis, trans ($p<0.01$) was significantly higher following the OXPUFA-FL, compared to both the SFA-FL and PUFA-FL (Figure 4.4).

There were no differences among the calculated estimate of serum double bonds across treatments (Figures 4.5a-c). Despite the consumption of different numbers of double bonds across treatments, there was no difference in the estimate of double bonds in the serum across treatments (SFA-FL: 4725 double bonds, OXPUFA-FL: 4770 double bonds, PUFA-FL: 4734 double bonds; $p=0.95$). There also was no main effect of treatment for the number of double bonds contained in fatty acids with 2 or more double bonds. There was however, a significant treatment effect for the number of single double bonds, such that the SFA-FL elicited the greatest number of serum single double bonds compared to the two high-PUFA fat loads ($p<0.01$).

Postprandial Triglyceride (TG) Response to Test Fat Loads

There was a significant increase in serum TG levels ($p<0.0001$) following the consumption of each of the three test fat loads. The increase in TG levels was

significantly higher following the SFA-FL, compared to the PUFA-FL ($p<0.01$) (Figure 4.6). The TG response following the OXPUFA-FL was intermediary between the SFA-FL and PUFA-FL (Figure 4.6). There was a significant main effect of group such that HTG individuals had higher TG levels across time compared to NTG individuals ($p<0.05$) (Table 4.6).

Results of the overall Pearson correlation analysis indicated that levels of serum TG were associated with levels of MUFA (SFA-FL: r = 0.74, OXPUFA-FL: r = 0.83, PUFA-FL: r = 0.80) and trans fatty acids (SFA-FL: r = 0.75, OXPUFA-FL: r = 0.84, PUFA-FL: r = 0.82) across each of the three test fat loads. Strong associations also were also present between levels of TG and the 18:1-trans fatty acids isomers (SFA-FL: r = 0.71, OXPUFA-FL: r = 0.87, PUFA-FL: r = 0.89) and the 18:2-trans fatty acid isomers (SFA-FL: r = 0.74, OXPUFA-FL: r = 0.68, PUFA-FL: r = 0.53) across all treatments.

Postprandial Lipid Oxidation and Antioxidant Capacity Following Test Fat Loads

Concentrations of lipid hydroperoxides in each of the test fat loads were: SFA-FL: 5.4 μM, PUFA-FL: 0.7 μM, and OXPUFA-FL: 26.0 μM. Serum lipid hydroperoxide (LPX) levels peaked at 2 hours following the consumption of all three test fat loads (Figure 4.7). The highest peak was observed following the SFA-FL, with the lowest peak following the PUFA-FL. The peak in LPX following the OXPUFA-FL closely resembled the peak following the SFA-FL; there were no significant effect of treatment or time on postprandial levels of LPX. There were no strong associations determined between serum fatty acids or the number of double bonds and levels or changes in postprandial LPX.

There was a significant increase in levels of Whole ORAC ($p<0.0001$), Slow ORAC ($p<0.01$) and Fast ORAC ($p<0.0001$) across all treatments between 0 and 4 hours (Figures 4.8-4.10). There was a significant treatment effect for levels of Whole ORAC and Fast ORAC, such that the SFA-FL produced a greater increase compared to the PUFA-FL ($p<0.05$), with the OXPUFA-FL eliciting an intermediary response. There was a significant main effect of group for levels of Slow ORAC, with NTG individuals having higher levels compared to HTG individuals ($p<0.05$) (Table 4.6).

Strong associations were observed between levels of Whole ORAC and MUFA (SFA-FL: $r = 0.61$, OXPUFA-FL: $r = 0.74$, PUFA-FL: $r = 0.54$) and between Whole ORAC and trans fatty acids (SFA-FL: $r = 0.56$, OXPUFA-FL: $r = 0.78$, PUFA-FL: $r = 0.58$) across all treatments. Levels of Whole, Slow and Fast ORAC also were correlated with the number of double bonds calculated from the serum fatty acids. See Table 4.7 for the correlation matrix. In general, the total number of double bonds, fatty acids with one double bond and trans fatty acid double bonds were positively associated with Whole and Fast ORAC, and negatively associated with Slow ORAC.

Postprandial Inflammatory and Vascular Response to Test Fat Loads

There were no main effects of time, treatment or group for postprandial levels of C-reactive protein (CRP) (Figure 4.11). Two HTG individuals had baseline CRP levels that fell between the [1.5 x (IQR)-3.0 x (IQR)] range when testing for outliers. Separate analyses were completed without these two individuals and the results were unchanged. Data presented for baseline and postprandial CRP therefore reflects all eight individuals.

There was a peak in serum levels of endothelin-1 (ET-1) at 2 hours ($p<0.001$) following the consumption of all three test fat loads (Figure 4.12). There were no main

effects of diet or group on postprandial levels of ET-1. There was a significant group*time interaction such that NTG individuals had significantly higher levels of ET-1 at 2 hours, compared to 4 hours ($p<0.01$) and significantly lower levels at 4 hours compared to 2 hours ($p<0.0001$) (Table 4.6). There were no apparent relationships between levels or changes of serum fatty acids and CRP or ET-1.

Effect of Baseline Triglyceride Status on the Postprandial Responses to Test Fat Loads

There was a trend for a main effect of group ($p<0.10$) in the postprandial LPX responses to the three test fat loads such that NTG individuals had higher levels across time compared to HTG individuals (Table 4.6). There was no main effect of group for postprandial ET-1 levels. In response to the OXPUFA-FL HTG individuals experienced a continued increase in ET-1 across the four-hour time period, whereas for all other treatments both groups experienced a peak at 2 hours and a subsequent decrease in ET-1 levels at 4 hours (Table 4.6). Although HTG individuals tended to have higher levels of CRP across both time and treatment when compared to their NTG counterparts, this difference was not statistically significant (Table 4.6).

Individuals with elevated baseline TG levels tended to have higher levels of Whole and Fast ORAC when compared to individuals with normal baseline TG levels, however this difference was not statistically significant. There was a main effect of group for levels of Slow ORAC, such that NTG individuals had higher levels compared to HTG individuals ($p<0.05$) (Table 4.6). Levels of serum TG also were correlated with levels of Whole ORAC across all treatments (SFA-FL: $r = 0.90$, OXPUFA-FL: $r = 0.73$, PUFA-FL: $r = 0.79$). When postprandial TG levels are included as a covariate in the mixed model, there is a significant main effect of group for Whole ($p<0.05$) and Slow

ORAC ($p<0.0001$) and a trend for a main effect of group for Fast ORAC ($p<0.06$). The inclusion of postprandial TG levels as a covariate ($p<0.0001$) eliminated any main effect of treatment previously observed for postprandial Whole and Fast ORAC responses.

Discussion

Based on the previous study, a moderate-fat diet (35% of calories), compared to a low-fat diet (20% of calories) elicits a greater reduction in fasting TG levels, particularly in individuals with elevated TG levels. The recommendation to consume a moderate fat diet includes a focus on the quality of the fat being consumed. The results of the present study indicate that the consumption of different kinds of fatty acids can have very different effects on postprandial risk factors for CVD. In particular, the simple heating of a PUFA increased levels of individual trans fatty acid isomers, resulting in diverse physiological responses, compared to the same un-heated PUFA. Thus, both type of fat and the method of preparation are important issues to consider.

The heat treatment used in the present study (165ºF for 3 hours on the day prior to consumption) elicited an increase in individual trans fatty isomers, compared to the identical unheated oil. Despite the fact that a greater concentration of trans fatty acids was fed in the SFA-FL, levels of serum trans fatty acids increased significantly only following the OXPUFA-FL, indicating a difference in absorption and/or clearance of natural vs. heat-modified trans fatty acids. Little is known however about the absorption and utilization of individual trans fatty acid isomers. In a study designed to evaluate the incorporation of cis-9, trans-11 linoleic acid and trans-10, cis-12 linoleic acid into plasma and peripheral mononuclear cell lipids of healthy men, both isomers were incorporated in

a dose-dependent manner (32). Each of these trans fatty acid isomers were greater in the test fat loads and in the serum for the OXPUFA-FL compared to the SFA-FL. This suggests a greater increase in serum trans fatty acids following the OXPUFA-FL, compared to the SFA-FL. In addition, total 18:1-trans, as a percentage of total fatty acids, also were higher following consumption of the OXPUFA-FL. The identification of specific absorption and clearance properties of individual trans fatty acid isomers is needed to better identify the direct *in vivo* physiological responses of these individual isomers.

Increases in postprandial TG levels and the delayed clearance of TG-rich postprandial particles may stimulate increases in oxidative stress, inflammation and endothelial dysfunction (1). Serum levels of TG were significantly elevated at 4 hours following each fat load, with the SFA-FL eliciting the highest levels and the PUFA-FL producing the lowest levels in both NTG and HTG individuals. The peak TG response following the OXPUFA-FL was intermediary between the SFA-FL and the PUFA-FL, indicating that the heat modification of the dietary PUFA resulted in a different rate of absorption and/or clearance of dietary TG. Pearson correlation analysis revealed strong associations between levels of both MUFA and trans fatty acids and serum TG across all treatments. In a recent study, the postprandial TG effects of partially hydrogenated fish oil (PHFO), rich in trans unsaturated fatty acids, were compared with those of palm oil and lard in eight healthy males (33). The results indicated that the shorter-chain fatty acids (18:0, 18:1-trans and 16:1-trans) were more readily absorbed 4 hours after the PFHO treatment, compared to the longer-chain fatty acids (C20-22, cis or trans isomers). Thus, despite the fact that the longer-chain fatty acids represented 40% of the total fatty

acids, compared to only 21% for the shorter-chain fatty acids, there was a decrease in the absorption efficiency of the longer-chain fatty acids. Although all of the trans fatty acid isomers fed in the current study were 18 carbons or less it is possible that the individual isomers themselves exhibit different absorption properties based on the location of the trans double bonds.

Baseline TG level is an important component in postprandial lipid clearance; elevated triglyceride levels result in delayed triglyceride clearance (3). Although it was believed that individuals with elevated baseline TG levels would have an exaggerated response to the test fat loads compared to individuals with normal baseline TG levels, the absolute change in TG levels across 4 hours was identical across both groups. It is possible that the small sample size in the present study was not sufficient to detect these differences across the two groups. Evidence suggests that de novo lipogenesis is stimulated after the consumption of a mixed meal (34). In a study of eight healthy men maximal postprandial de novo lipogenesis ranged from 10.3% to 37.5%, peaking at 4.2 hours after the meals (35). It is thus possible that individuals in the present study experienced different rates of postprandial de novo lipogenesis, resulting in similar overall increases in serum TG over the 4-hour period. In addition, although TG levels previously have been shown to peak at 4 hours, it is possible that levels may have continued to increase beyond this timepoint in the present study and thus differences beyond 4 hours were not detected.

The consumption of a meal containing both oxidized and oxidizable lipids results in an increase in the plasma concentration of lipid hydroperoxides. This increase in oxidation products is associated with increased susceptibility of LDL to oxidation, due to

structural perturbations at the particle surface (36). Levels of serum lipid hydroperoxides (LPX) in the present study peaked at 2 hours following the consumption of the test fat loads, with no difference across the three treatments. This is in agreement with a previous study in nine healthy males following the consumption of a high-fat (55% total calories) breakfast meal (31). The variability in peak LPX levels in this study however was great; six subjects had increases ranging from 40 to 112%, one individual showed very little change and two subjects showed large increases (~250%). The variability in the postprandial increases in LPX in the present study also was very diverse across the subjects, ranging from decreases (-5% to -40%) between 0 and 2 hours to increases greater than 600%. This extreme difference may be an artifact of the measurement limitations associated with postprandial LPX in the present study. Serum samples for the determination of LPX were stored at -80ºC in the presence of BHT to reduce the occurrence of *in vitro* oxidation and extracted within 9 months of the end of the study. If the serum samples had been extracted immediately following each test day it is possible that the samples would have been more stable over time and thus more accurate estimates of LPX would have been determined. Regardless, we were still able to detect an increase in serum LPX levels 2 hours after the consumption of each test fat load. Given the high variability in the measurement of postprandial LPX, it is possible that a larger sample size is needed to detect changes in these levels in response to different types of dietary fatty acids, including pre-oxidized fatty acids and fatty acids that are highly susceptible to *in vivo* oxidation.

The ORAC assay measures the capacity of an antioxidant to directly quench free radicals (37). The ORAC assay is unique in that it takes free radical action to completion

and uses the area-under-the-curve (AUC) technique for quantification, and, thus combines both inhibition percentage and the length of inhibition time of the free radical action by antioxidants into one single number (38). In the present study there were significant increases in all measures of ORAC following the consumption of all three test loads. Whole and Fast ORAC levels were significantly higher, indicating a higher level of antioxidant capacity following the SFA-FL, compared to the PUFA-FL; the OXPUFA-FL exhibited an intermediary response. The increases observed in antioxidant capacity following the consumption of essentially an antioxidant-free fat load may be explained by the properties of the ORAC assay. Because the ORAC assay measures the free radical action to completion, this allows for the contribution of all oxidizable substrates within the sample to prevent the oxidation of the target molecule (10), including unsaturated fatty acids.

In the present study strong positive correlations were observed between the levels of MUFA, trans fatty acids, the calculated number of double bonds, fatty acids containing one double bond and trans fatty acid double bonds and levels of Whole and Fast ORAC; similar negative correlations were observed between these calculated double bond estimates and levels of Slow ORAC. The SFA-FL contained the greatest amount of MUFA and thus the greatest number of single double bonds; it is possible that these single double bonds acted to quench the free radicals within the ORAC assay, thus resulting in the highest levels of ORAC at 4 hours. On the contrary, it is possible that the multiple double bonds present in the OXPUFA-FL and PUFA-FL experienced oxidative propagation when exposed to the free radicals in the ORAC assay, resulting in lower levels of total antioxidant capacity. In addition, as previously reported (10), levels of

serum TG were strongly correlated with levels of Whole and Fast ORAC across the three treatments (SFA-FL: r = 0.90, OXPUFA-FL: r = 0.73, PUFA-FL: r = 0.79 and SFA-FL: r = 0.83, OXPUFA-FL: r = 0.60, PUFA-FL: r = 0.71, respectively). When serum TG levels were included as a covariate in the mixed models analysis of the ORAC measures the previously observed treatment effects were no longer significant, indicating that the postprandial nature of the study may interfere with an accurate assessment of antioxidant capacity via the ORAC assay. Similar results were obtained in a postprandial study evaluating the effect of blueberry consumption on serum antioxidant status in healthy men (10). Within this study levels, of ORAC also were significantly higher following the consumption of the standard control high-fat meal and were significantly correlated with levels of serum TG. However, postprandial antioxidant status also was assessed by the total antioxidant assay (TAS), an assay which measures the change in radical concentration over a 10-minute incubation period. No significant increase in antioxidant capacity was observed with the TAS assay following the consumption of the high-fat meal alone.

Whereas the Fast ORAC levels represent the activity of water-soluble antioxidants, or quick-acting antioxidants, levels of Slow ORAC represent the activity of lipid-soluble antioxidants. Levels of Slow ORAC were negatively correlated with the calculated number of double bonds and levels of serum TG following the PUFA-FL, indicating that an increase in fatty acids that were highly susceptible to oxidation was associated with decreases in antioxidant capacity. Thus, levels of lipid-soluble antioxidant capacity may be less sensitive to the acute postprandial state and may more accurately reflect an individual's background antioxidant capacity. Further studies are needed to determine

the effect of background and postprandial indices of antioxidant capacity in the presence of fatty acids that are highly susceptible to oxidation and free radical damage.

In the present study there were no significant effects of treatment or time on postprandial levels of CRP. Previous studies have reported slight decreases (39), no affect (13) and increases (40) in levels of postprandial CRP in response to a high-fat meal. In the study conducted by Carroll et al (40), the addition of either pre-breakfast or pre-supper vitamin supplementation (800 IU vitamin E and 1 g vitamin C) was compared to the postprandial affects observed to the high-fat supper alone. The results indicated that both pre-breakfast and pre-supper vitamin supplementation prevented the meal-induced increase in CRP observed with no vitamin supplementation. Because measures of CRP are variable among individuals it is possible that a larger sample size is needed to detect any acute postprandial affects to different types of dietary fat. In addition, the utility of CRP as an accurate marker of inflammation in the postprandial state may not be warranted due to the high degree of intra-individual variability.

Serum levels of ET-1 increased significantly in the present study 2 hours after the consumption of all three test fat loads. This adverse affect on endothelial function is in agreement with previous studies that have demonstrated postprandial increases in ET-1 (21), significant decreases in vascular compliance, and markedly reduced levels of plasma nitric oxide metabolites in normal-weight young subjects with an insulin resistance phenotype (41) following the consumption of a high-fat meal (50g total fat; 14g SFA). Although there was no main effect of treatment on postprandial levels of ET-1 it is important to note that fat loads rich in both saturated and unsaturated fatty acids elicit an increase in this marker of endothelial dysfunction. In addition, the assessment of

endothelial health in the postprandial state, via flow-mediated vasodilation (FMD), is most often assessed 4 hours after consumption of the high-fat meal (1,15). The results of the present study indicate that there may be perturbations in endothelial function that occurs and is resolved before the traditional 4-hour postprandial timepoint. Further studies that include more frequent measures of FMD in the postprandial state are recommended to determine the optimal timing of the assessment of endothelial health.

It was originally hypothesized that individuals with elevated TG levels would have more adverse postprandial responses compared to individuals with normal baseline TG levels. As discussed earlier, although levels of TG were higher at 4 hours in HTG individuals, compared to NTG individuals, the absolute change in TG over the 4-hour period was not different. Similarly, although levels of CRP tended to be higher in HTG individuals compared to NTG individuals, there was not a significant effect of group. Individuals with elevated baseline TG levels also exhibited higher levels of Whole and Fast ORAC throughout the postprandial period, whereas individuals with normal baseline TG levels had higher levels of Slow ORAC across time. When serum TG were included in the mixed model analysis of ORAC levels there was a significant main effect of group that was not observed for the traditional mixed analysis. This indicates that the higher levels of ORAC observed in the HTG group is likely an artifact of their higher TG levels over time, compared to the NTG group. In addition, these results also indicate that levels of Slow ORAC may not be as sensitive to levels of serum TG; because NTG individuals had higher levels of Slow ORAC, representing lipid-soluble antioxidants, it is possible that they have a high background antioxidant defense system, compared to HTG individuals.

In response to the three test fat loads NTG individuals tended to have a higher peak of both ET-1 and LPX. Although it cannot be determined from the present study this may be a result of difference in the absorption and/or clearance of the dietary fats fed. In response to the OXPUFA-FL, HTG individuals experienced a continued increase in ET-1 across the 4-hour period, whereas ET-1 peaked at 2 hours for all other treatment and group combinations. It is possible that the consumption of heat-modified trans fatty acids present in the OXPUFA-FL have a detrimental effect on endothelial function in individuals with elevated baseline TG levels. Due to the small sample size of this study and of the HTG group, further research is needed to determine the impact of consuming different fatty acids on postprandial ET-1 responses. In a study designed to evaluate the effects of antioxidant supplementation on postprandial oxidative stress and endothelial dysfunction, no difference in peak levels of ET-1 were detected between individuals with type 2 diabetes, individuals with impaired glucose tolerance and healthy controls (42), indicating that baseline health status may not have a significant impact on postprandial levels of ET-1.

Study Limitations:

The small sample size of the present pilot study may be a major limiting factor in determining main effects of treatment and/or group on the measured postprandial risk factors for CVD. When calculating correlations by both treatment and time for the detection of relationships between serum fatty acids and the various outcome measurements the sample size was reduced to eight, thus generalizations to the general public are not possible. It is possible however that with a larger sample size, which is more representative of the general population, that stronger associations and greater

differences may have been observed. Another limitation of the present study is that the fatty acid analysis of the test fat loads was completed on multiple aliquots of one milkshake sample. For the OXPUFA-FL in particular it is possible that the heating process produced different concentrations of the trans fatty acid isomers across different test days. Multiple trials of heating the test fat and preparing multiple OXPUFA-FL milkshake samples for analysis would provide a measure of variability in milkshake trans fatty acid isomer content.

Conclusion

The results of the present study indicate that the consumption of different types of fatty acids elicits adverse effect on postprandial markers of atherosclerosis, lipid oxidation and endothelial dysfunction, regardless of baseline TG status. In addition, the heating of an oil rich in PUFA generates the formation of trans fatty acid isomers and increased levels of lipid hydroperoxides which have diverse physiological responses in the postprandial state. Future studies are needed to better determine the associations between serum fatty acids and individual trans fatty acid isomers in particular on postprandial risk factors for CVD.

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Tables

Table 4.1: Calculated fatty acid composition of test fat loads.

	High Saturated Fat (SFA-FL)	High Oxidized Polyunsaturated Fat (OXPUFA-FL)	High Polyunsaturated Fat (PUFA-FL)
Calories	629 kcal	629 kcal	629 kcal
Protein	15.2 g (9.7% kcal)	15.0 g (9.6% kcal)	15.0 g (9.6% kcal)
Carbohydrate	31.6 g (20.1% kcal)	31.3 g (20.0% kcal)	31.3 g (20.0% kcal)
Total Fat	50.0 g (71.6% kcal)	50.0 g (71.8% kcal)	50.0 g (71.8% kcal)
SFA MUFA PUFA n-6	12.2% kcal 50.4% kcal 5.7% kcal 5.4% kcal	5.1% kcal 10.4% kcal 52.8% kcal 52.7% kcal Subjected to HEAT Treatment	5.1% kcal 10.4% kcal 52.8% kcal 52.7% kcal

Table 4.2: Ingredients in experimental test fat loads

	High Saturated Fat (SFA-FL)	High Oxidized Polyunsaturated Fat (OXPUFA-FL)	High Polyunsaturated Fat (PUFA-FL)
Skim Milk	440g	440g	440g
Light Whipping Cream	9.0g	-	-
Olive Oil	46.4g	-	-
Safflower Oil	-	49.2g*	49.2g
Splenda	10.0g	10.0g	10.0g

*Subjected to heat treatment

Table 4.3: Baseline subject characteristics

	All Subjects (n=8)	NTG Subjects (n=4)	HTG Subjects (n=4)
Age (years)	47.4 ± 6.0	49.8 ± 6.1	45.0 ± 5.7
Body Mass Index (kg/m^2)	26.5 ± 4.3	25.3 ± 4.3	27.8 ± 4.4
Total Cholesterol (mg/dL)	211.0 ± 40.2	191.0 ± 10.9	231.0 ± 50.8
LDL-Cholesterol (mg/dL)	124.1 ± 26.9	119.3 ± 4.6	129.0 ± 40.1
HDL-Cholesterol (mg/dL)	47.4 ± 11.6	53.3 ± 9.2	39.7 ± 11.0
Triglycerides (mg/dL)	163.9 ± 91.7	92.5 ± 28.8*	235.3 ± 72.0*

Data expressed as mean ± standard deviation; *p<0.05

Table 4.4: Calculated vs. assayed fatty acid composition of test fat loads (% of total fatty acids) (means).

	SFA Test Fat Load		**OXPUFA Test Fat Load**		**PUFA Test Fat Load**	
	Predicted	**Assayed**	**Predicted**	**Assayed**	**Predicted**	**Assayed**
SFA	17.1	18.4	7.1	9.6	7.1	9.4
MUFA	70.4	70.2	14.5	13.4	14.5	13.3
PUFA	8.0	10.8	73.8	76.6	73.8	77.1
N6 PUFA	7.5	10.1	73.7	76.5	73.7	77.0
N3 PUFA	-	0.64	-	0.06	-	0.09
Trans	-	0.63	-	0.43	-	0.27

Table 4.5: Fatty acid concentration of test fat loads (mg/100mL) (mean).

	SFA Fat Load	OXPUFA Fat Load	PUFA Fat Load
SFA	18.09	9.48	9.24
MUFA	69.18	13.17	13.07
PUFA	10.63	75.50	75.90
n3 PUFA	0.63	0.08	0.09
n6 PUFA	9.99	75.40	75.76
n6:n3 ratio	15.8	898.5	840.0
trans	0.62	0.43	0.27
16:0	13.35	6.15	6.14
18:0	3.30	2.37	2.35
18:1n9	66.20	12.23	12.18
18:2n6	9.79	75.35	75.72
18:3n3	0.63	0.06	0.09

Table 4.6: Postprandial Responses to Test Fat Loads (least squares means ± standard error)

	SFA-FL	OXPUFA-FL	PUFA-FL	Main effect of Treatment	Main effect of Group	Main effect of Time	Treat*Group Interaction	Group*Time Interaction
Triglycerides (mg/dL)				**<0.01**	**<0.05**	**<0.0001**	**-**	**-**
All Subjects – 0 hour	135.0 ± 17.3	129.8 ± 17.3	122.9 ± 17.3					
1 hour	167.3 ± 17.3	159.8 ± 17.3	134.6 ± 17.3					
2 hour	178.5 ± 17.3	162.4 ± 17.3	153.1 ± 17.3					
4 hour	219.1 ± 17.3	195.3 ± 17.3	158.8 ± 17.3					
NTG – 0 hour	90.8 ± 24.4	86.0 ± 24.4	88.0 ± 24.4					
1 hour	135.3 ± 24.4	130.0 ± 24.4	108.0 ± 24.4					
2 hour	143.5 ± 24.4	136.0 ± 24.4	135.8 ± 24.4					
4 hour	170.5 ± 24.4	153.8 ± 24.4	129.0 ± 24.4					
HTG – 0 hour	179.3 ± 24.4	173.5 ± 24.4	157.8 ± 24.4					
1 hour	199.3 ± 24.4	189.5 ± 24.4	161.3 ± 24.4					
2 hour	213.5 ± 24.4	188.8 ± 24.4	170.5 ± 24.4					
4 hour	267.8 ± 24.4	236.8 ± 24.4	188.5 ± 24.4					
C-reactive protein (mg/L)				**-**	**-**	**-**	**<0.05**	**-**
All Subjects – 0 hour	1.58 ± 0.75	2.64 ± 0.75	1.72 ± 0.75					
1 hour	2.52 ± 0.75	2.15 ± 0.75	1.75 ± 0.75					
2 hour	1.82 ± 0.75	1.65 ± 0.75	1.36 ± 0.75					
4 hour	1.81 ± 0.75	2.42 ± 0.75	1.66 ± 0.75					
NTG – 0 hour	1.37 ± 1.06	1.31 ± 1.06	1.08 ± 1.06					
1 hour	2.24 ± 1.06	1.19 ± 1.06	0.98 ± 1.06					
2 hour	1.62 ± 1.06	0.77 ± 1.06	0.79 ± 1.06					
4 hour	1.43 ± 1.06	1.09 ± 1.06	1.12 ± 1.06					
HTG – 0 hour	1.79 ± 1.06	3.97 ± 1.06	2.36 ± 1.06					
1 hour	2.80 ± 1.06	3.11 ± 1.06	2.53 ± 1.06					
2 hour	2.02 ± 1.06	2.52 ± 1.06	1.92 ± 1.06					
4 hour	2.19 ± 1.06	3.76 ± 1.06	2.19 ± 1.06					

Table 4.6 continued

	SFA-FL	OXPUFA-FL	PUFA-FL	Main effect of Treatment	Main effect of Group	Main effect of Time	Treat*Group Interaction	Group*Time Interaction
Endothelin-1(pg/mL)				**-**	**-**	**<0.001**	**-**	**<0.01**
All Subjects – 0 hour	3.34 ± 1.20	2.91 ± 1.20	4.09 ± 1.20					
2 hour	6.27 ± 1.20	6.32 ± 1.20	6.95 ± 1.20					
4 hour	2.79 ± 1.20	3.93 ± 1.20	3.39 ± 1.20					
NTG – 0 hour	2.81 ± 1.70	3.11 ± 1.70	4.06 ± 1.70					
2 hour	6.02 ± 1.70	8.13 ± 1.70	7.73 ± 1.70					
4 hour	0.82 ± 1.70	0.91 ± 1.70	2.06 ± 1.70					
HTG – 0 hour	3.87 ± 1.70	2.72 ± 1.70	4.13 ± 1.70					
2 hour	6.53 ± 1.70	4.51 ± 1.70	6.17 ± 1.70					
4 hour	4.76 ± 1.70	6.95 ± 1.70	4.73 ± 1.70					
Lipid Hydroperoxides (µM)				**-**	**<0.10**	**<0.12**	**-**	**-**
All Subjects – 0 hour	0.63 ± 0.98	1.27 ± 0.98	1.22 ± 0.98					
2 hour	3.15 ± 0.98	2.92 ± 0.98	2.15 ± 0.98					
4 hour	2.33 ± 0.98	1.94 ± 0.98	1.15 ± 0.98					
NTG – 0 hour	0.73 ± 1.39	2.55 ± 1.39	1.63 ± 1.39					
2 hour	4.51 ± 1.39	2.52 ± 1.39	3.18 ± 1.39					
4 hour	3.22 ± 1.39	3.07 ± 1.39	1.15 ± 1.39					
HTG – 0 hour	0.54 ± 1.39	~0.00 ± 1.39	0.81 ± 1.39					
2 hour	1.78 ± 1.39	3.32 ± 1.39	1.12 ± 1.39					
4 hour	1.43 ± 1.39	0.81 ± 1.39	1.15 ± 1.39					

No significant Treatment*Time or Treatment*Time*Group interactions for any of the above endpoints

Table 4.6 continued

	SFA-FL	OXPUFA-FL	PUFA-FL	Main effect of Treatment	Main effect of Group	Main effect of Time
Whole ORAC (µM)				**<0.05**	**-**	**<0.0001**
All Subjects – 0 hour	3678.4 ± 454.7	3625.3 ± 454.7	3488.0 ± 454.7			
4 hour	5974.8 ± 454.7	5471.4 ± 454.7	4537.6 ± 454.7			
NTG – 0 hour	3106.0 ± 643.0	3014.8 ± 643.0	3033.4 ± 643.0			
4 hour	5387.3 ± 643.0	5426.3 ± 643.0	4335.3 ± 643.0			
HTG – 0 hour	4250.7 ± 643.0	4235.9 ± 643.0	3942.5 ± 643.0			
4 hour	6562.4 ± 643.0	5516.5 ± 643.0	4739.9 ± 643.0			
Slow ORAC (µM)				**-**	**<0.05**	**<0.01**
All Subjects – 0 hour	285.7 ± 18.6	288.1 ± 18.6	282.7 ± 18.6			
4 hour	368.9 ± 18.6	331.2 ± 18.6	328.0 ± 18.6			
NTG – 0 hour	318.4 ± 33.5	345.2 ± 33.5	313.7 ± 33.5			
4 hour	402.7 ± 33.5	390.3 ± 33.5	392.2 ± 33.5			
HTG – 0 hour	253.0 ± 33.5	231.0 ± 33.5	251.7 ± 33.5			
4 hour	335.2 ± 33.5	272.2 ± 33.5	263.8 ± 33.5			
Fast ORAC (µM)				**<0.09**	**-**	**<0.0001**
All Subjects – 0 hour	1953.9 ± 538.3	2159.0 ± 538.3	1820.9 ± 538.3			
4 hour	4545.1 ± 538.3	4100.3 ± 538.3	2990.8 ± 538.3			
NTG – 0 hour	1501.8 ± 761.3	1509.6 ± 761.3	1472.0 ± 761.3			
4 hour	3941.3 ± 761.3	4259.4 ± 761.3	2699.4 ± 761.3			
HTG – 0 hour	2406.0 ± 761.3	2808.4 ± 761.3	2169.8 ± 761.3			
4 hour	5149.0 ± 761.3	3941.2 ± 761.3	3282.1 ± 761.3			

No significant interactions effects for measures of Whole, Slow or Fast ORAC

Table 4.7: Correlations between the calculated number of double bonds in serum and measures of antioxidant capacity.

	Fat Load	Whole ORAC	Slow ORAC	Fast ORAC
Total number of double bonds	SFA-FL	0.25	0.13	0.08
	OXPUFA-FL	0.61	-0.12	0.54
	PUFA-FL	0.35	-0.51	0.33
1 or more double bonds	SFA-FL	0.61	0.13	0.41
	OXPUFA-FL	0.74	-0.31	0.62
	PUFA-FL	0.54	-0.59	0.51
2 or more double bonds	SFA-FL	0.04	0.10	-0.09
	OXPUFA-FL	0.44	0.00	0.40
	PUFA-FL	0.22	-0.35	0.22
trans double bonds	SFA-FL	0.57	-0.21	0.41
	OXPUFA-FL	0.78	-0.18	0.65
	PUFA-FL	0.58	-0.51	0.53

Figures

Figure 4.1. *trans* fatty acid isomer concentration of test fat loads

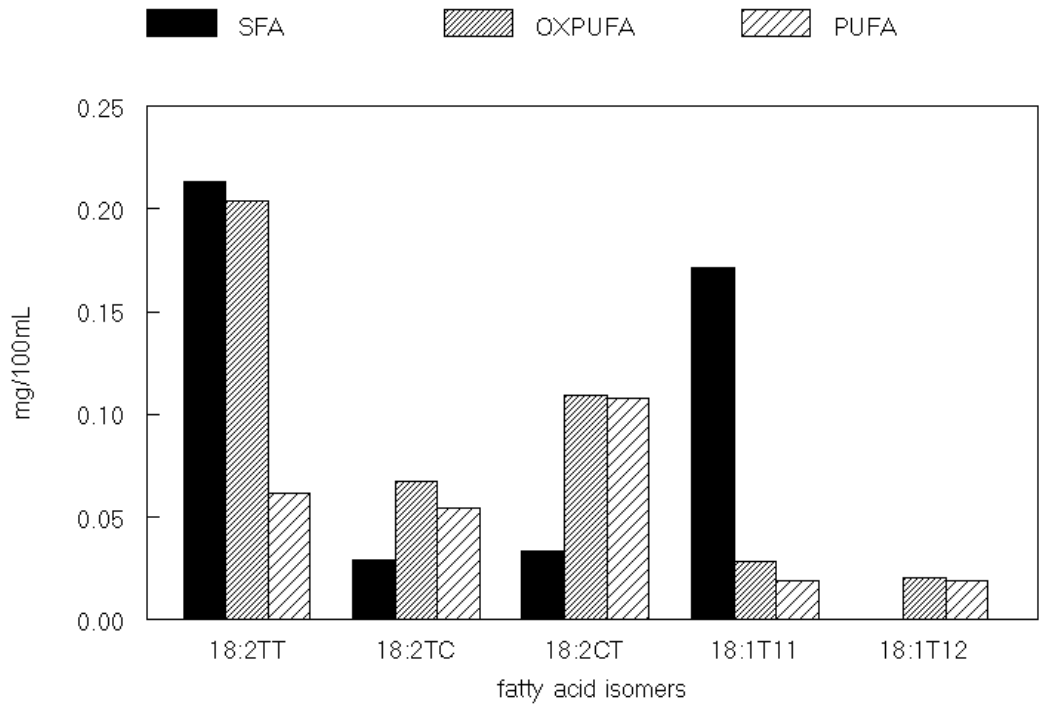
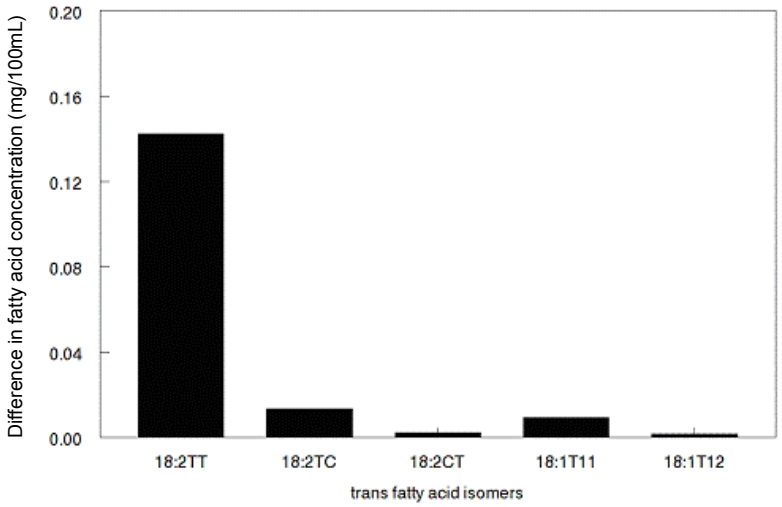


Figure 4.2: Increase in trans fatty acid isomers following heat treatment.



Fatty acid concentration calculated as OXPUFA-FL-PUFA-FL for each individual isomer.

Figure 4.3: Serum *trans* fatty acid concentrations in response to test fat loads.

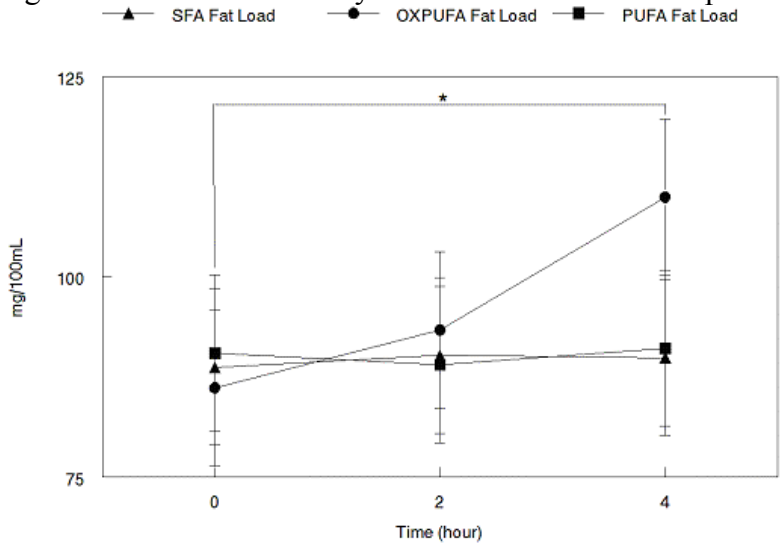
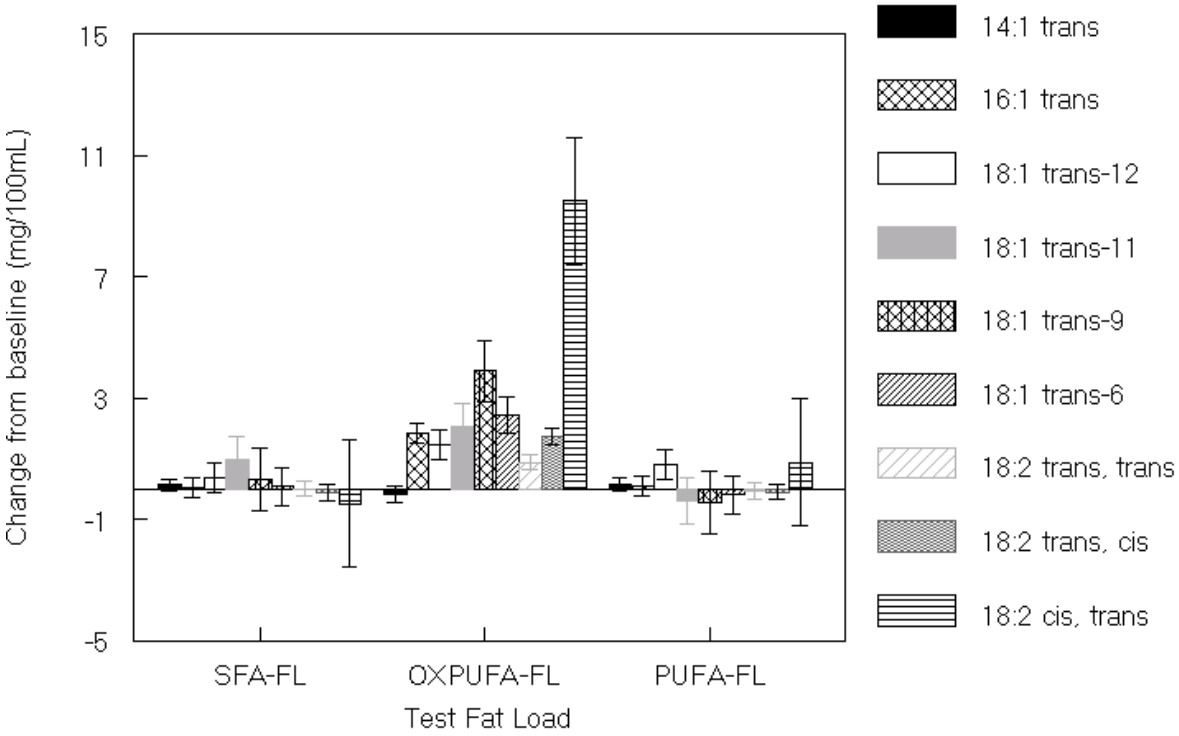


Figure 4.4: Absolute change in individual *trans* fatty acid isomers (0 to 4 hours)



Changes in 18:1-trans-11 ($p<0.05$), 18:1-trans-6 ($p<0.01$), 18:2-trans, trans ($p<0.01$), 18:2-trans, cis ($p<0.0001$) and 18:2-cis, trans ($p<0.01$) are significantly greater following the OXPUFA-FL, compared to both the SFA-FL and PUFA-FL

Figures 4.5a-c: Calculated number of double bonds in serum fatty acids in response to the consumption of the test fat loads.

Figure 4.5a: Response Following SFA-FL

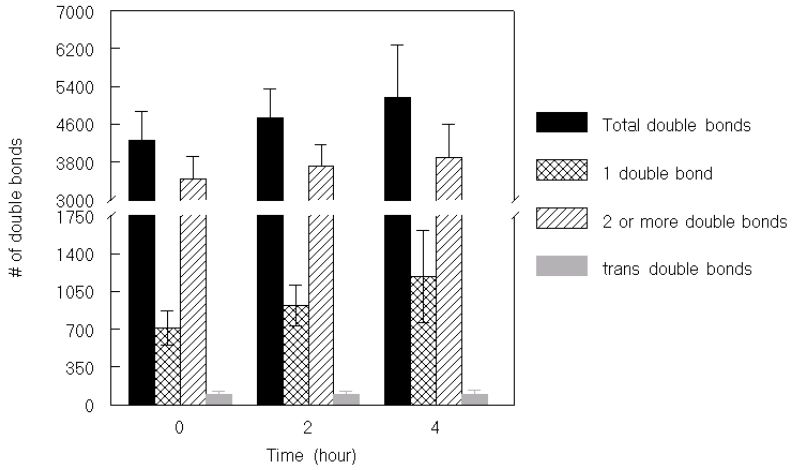


Figure 4.5b: Response Following OXPUFA-FL

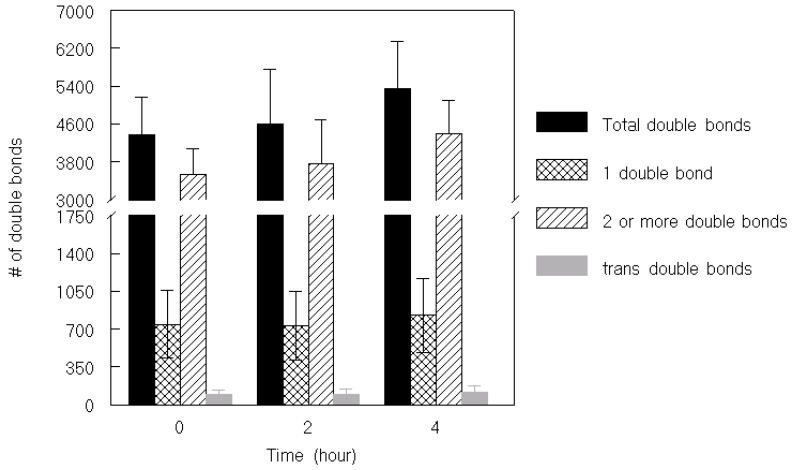


Figure 4.5c: Response Following PUFA-FL

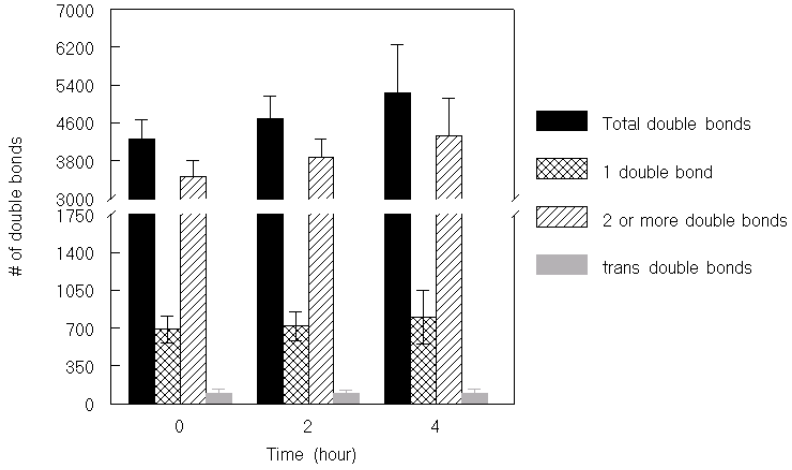
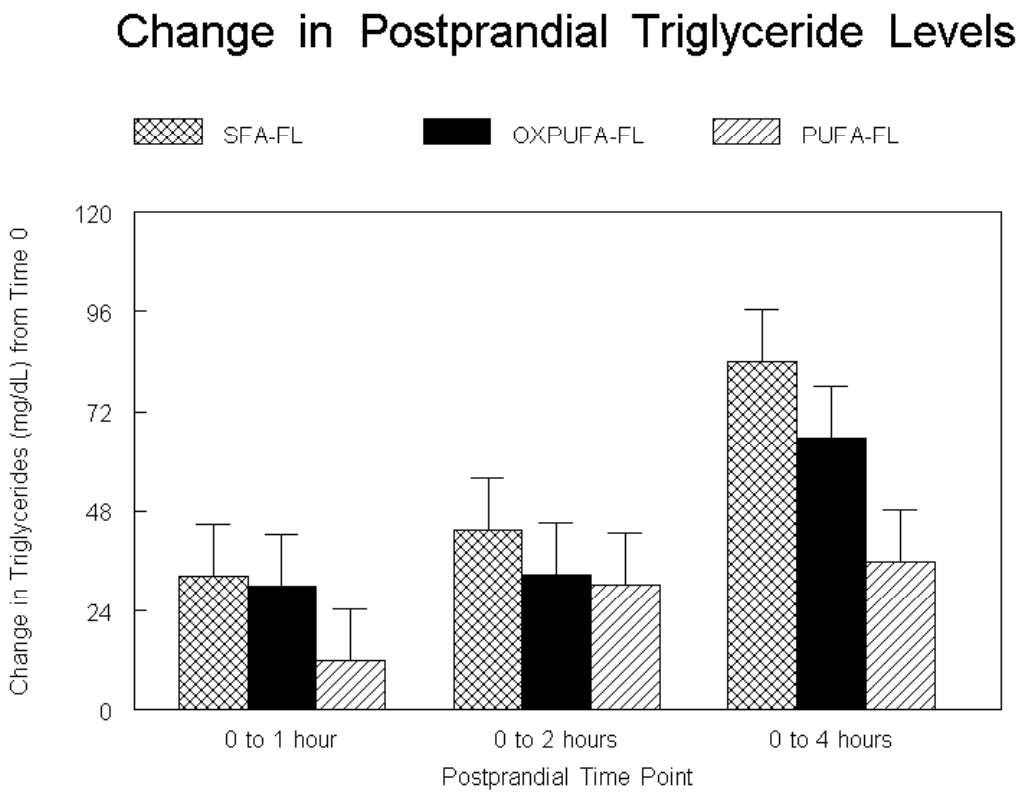
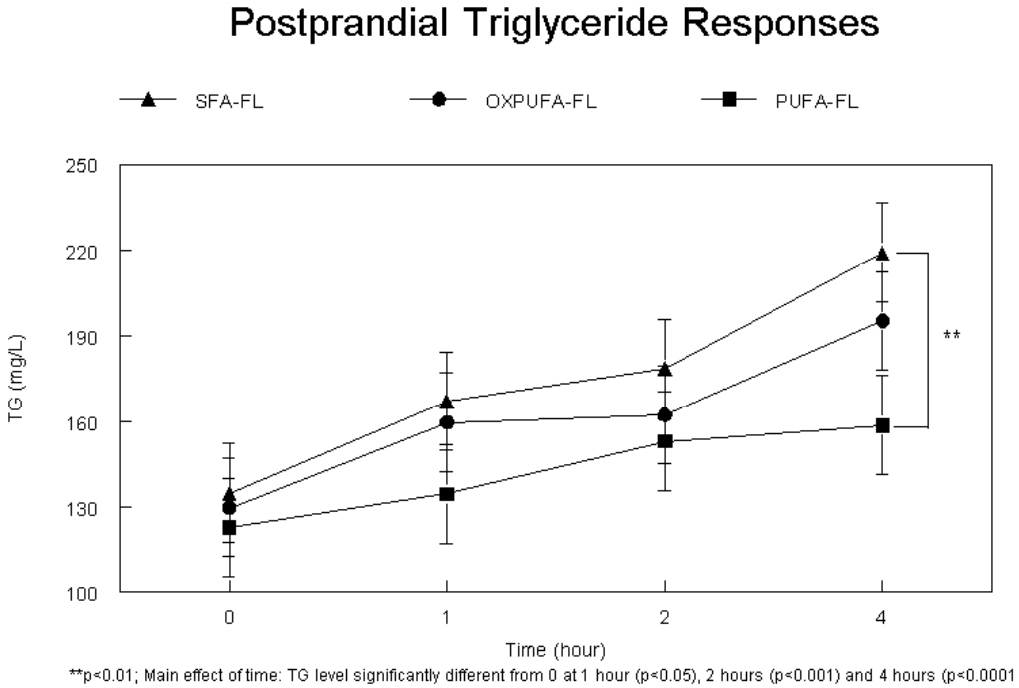


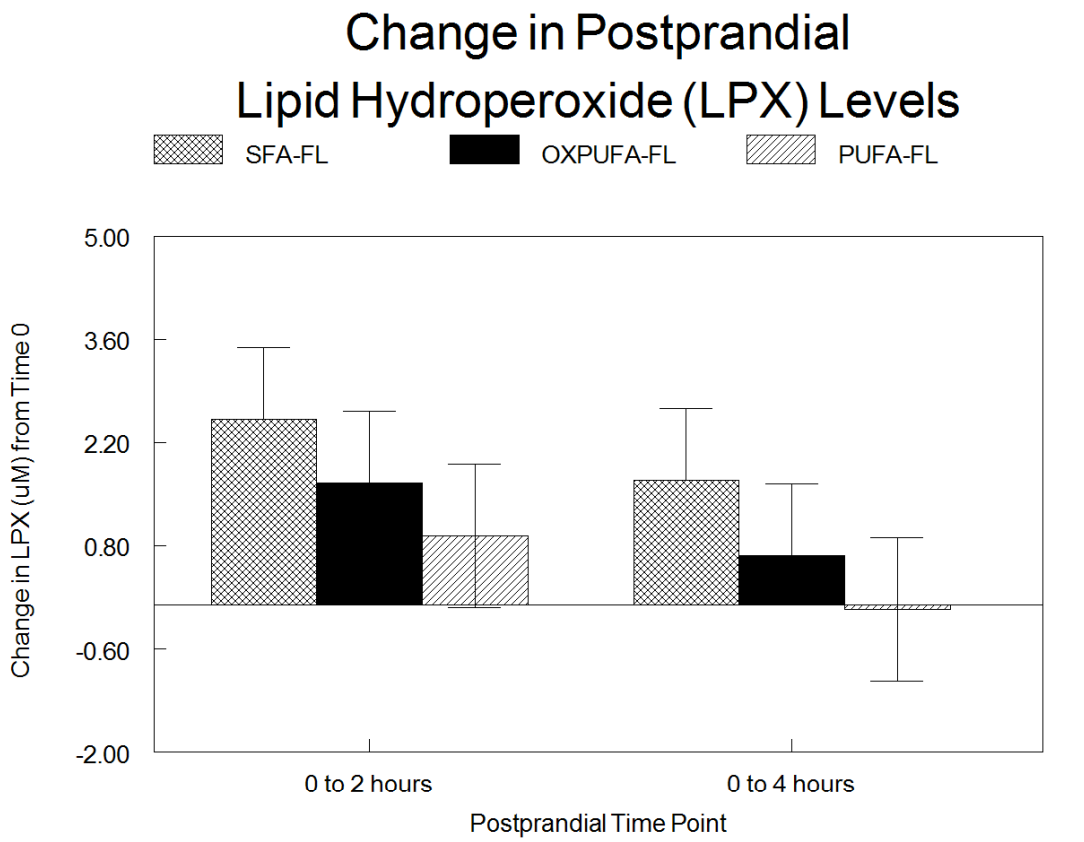
Figure 4.6: Triglyceride response to test fat loads





**p<0.01; Main effect of time: TG level significantly different from 0 at 1 hour (p<0.05), 2 hours (p<0.001) and 4 hours (p<0.0001

Figure 4.7: Lipid hydroperoxide responses to test fat loads



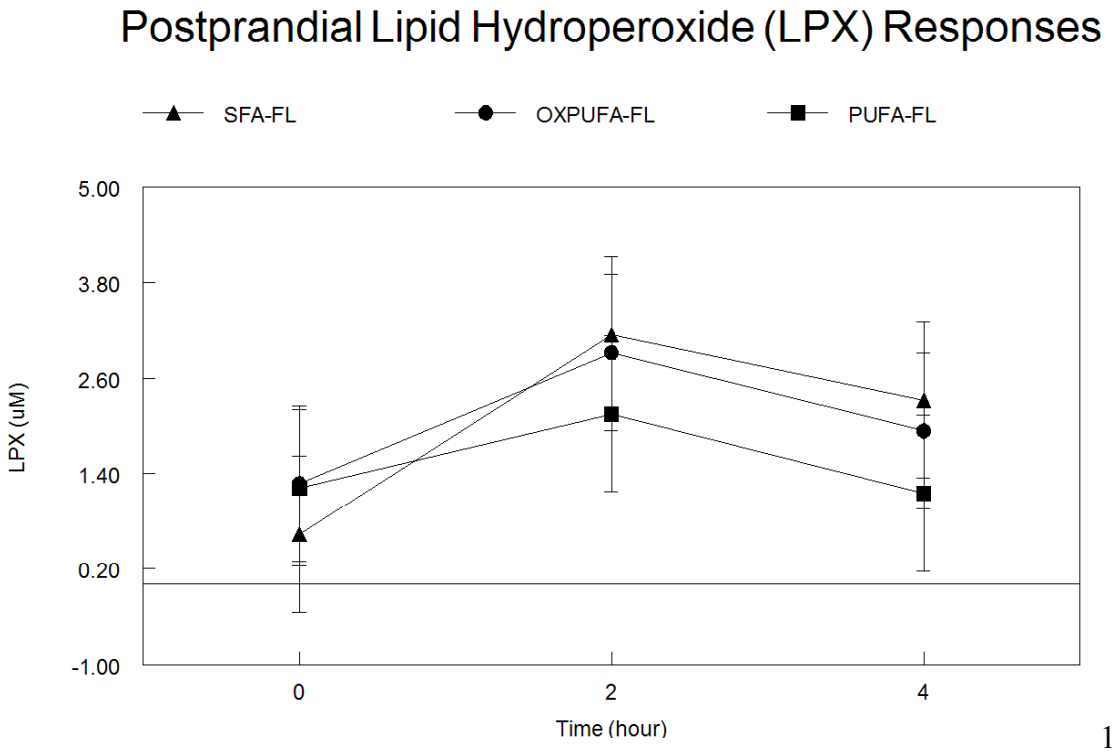
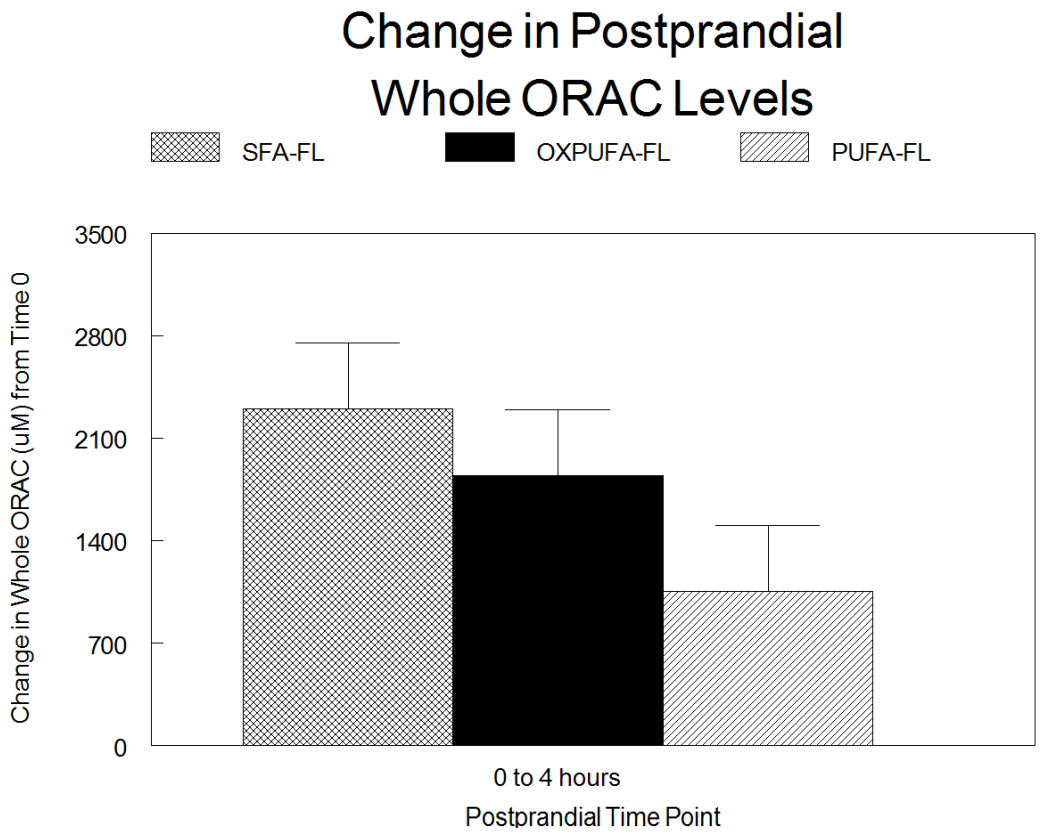
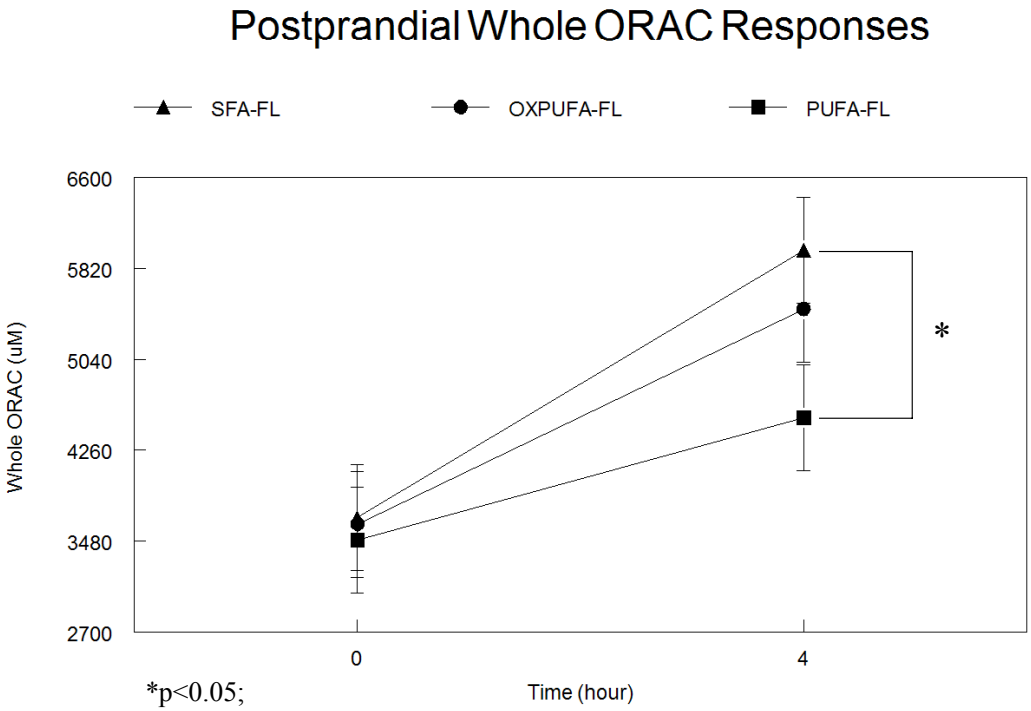


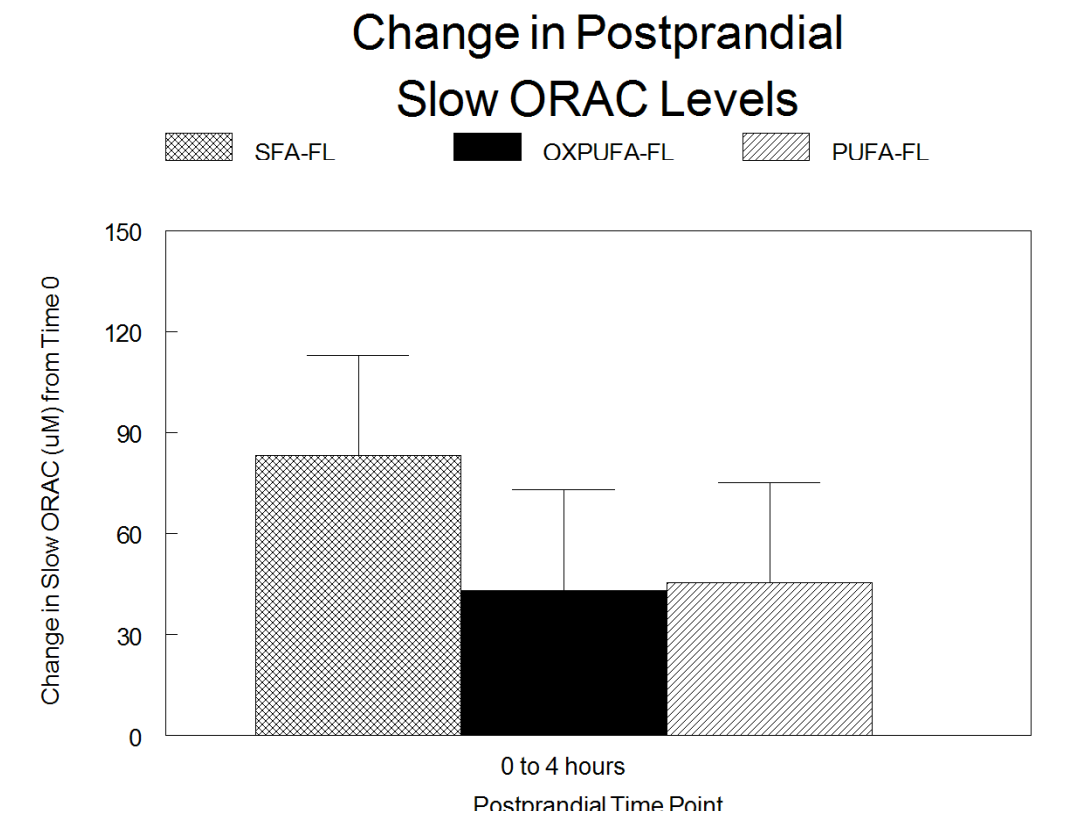
Figure 4.8: Whole ORAC responses to test fat loads

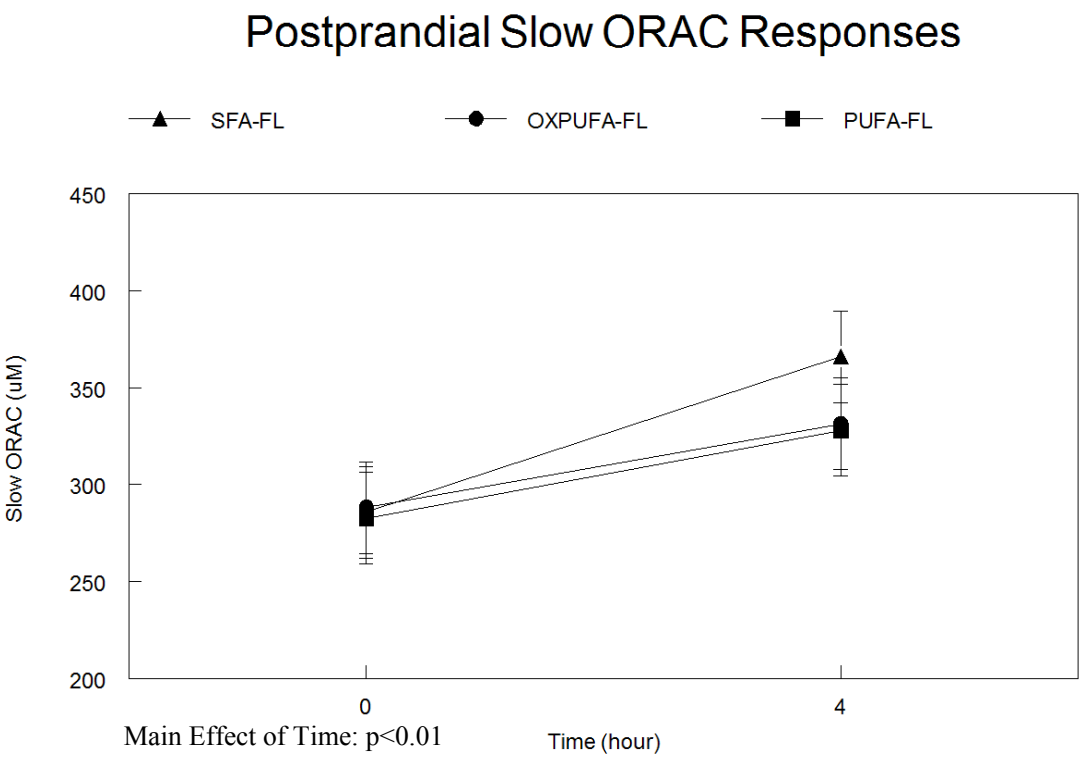




*p<0.05;
Main Effect of Time: p<0.0001

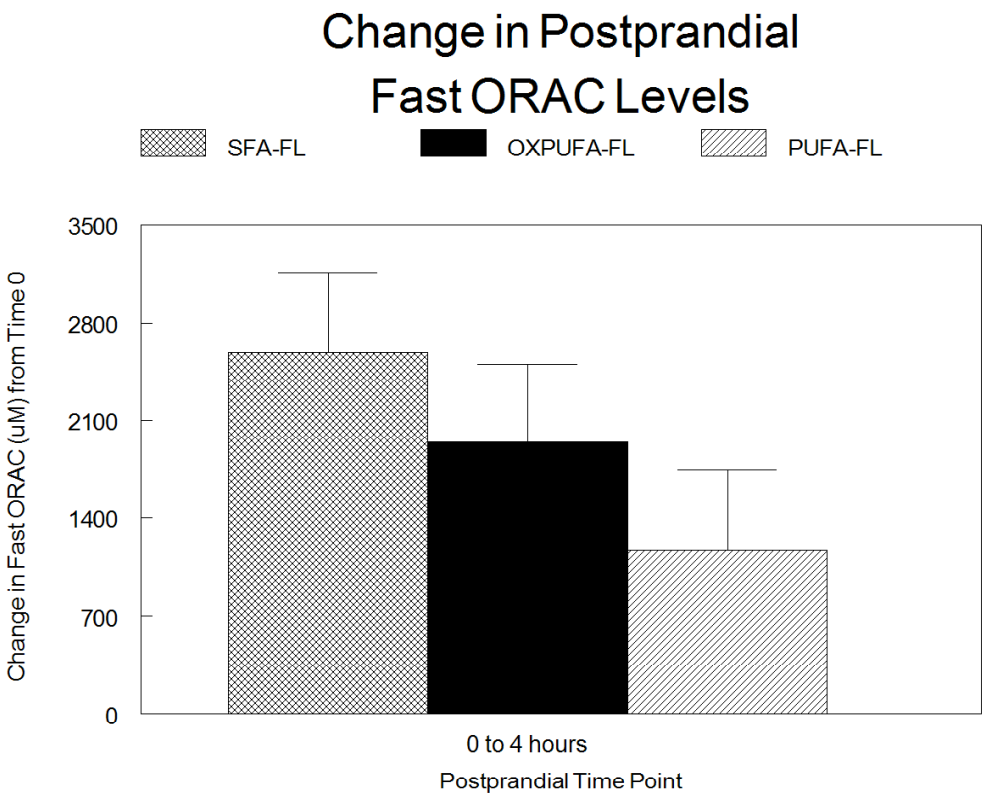
Figure 4.9: Slow ORAC responses to test fat loads

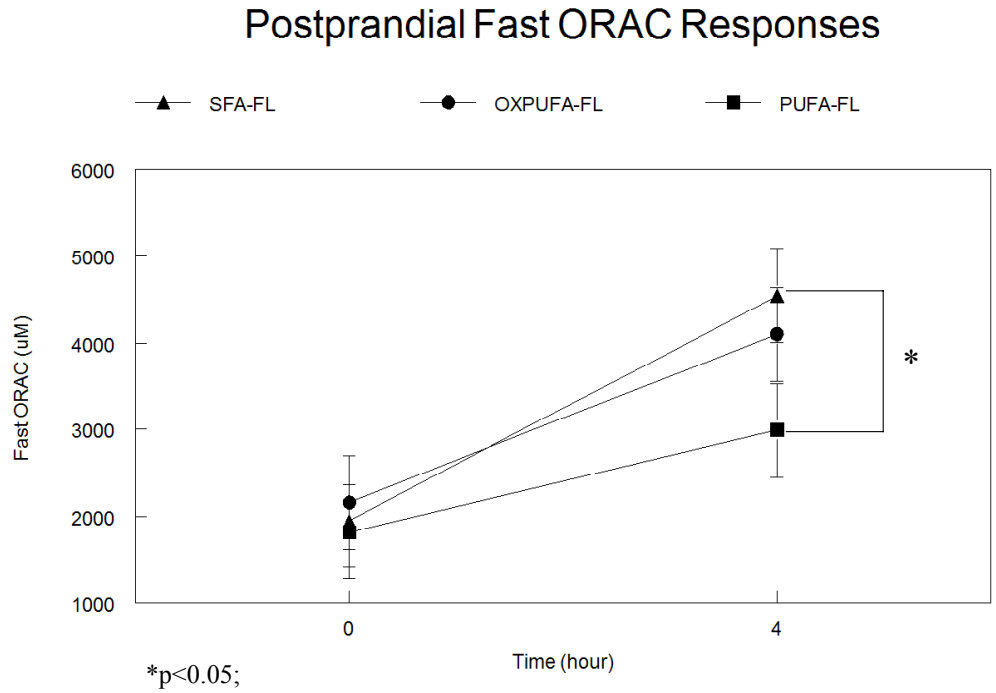




Main Effect of Time: $p<0.01$

Figure 4.10: Fast ORAC responses to test fat loads





*p<0.05;
Main Effect of Time: p<0.0001

Figure 4.11: C-reactive protein responses to test fat loads

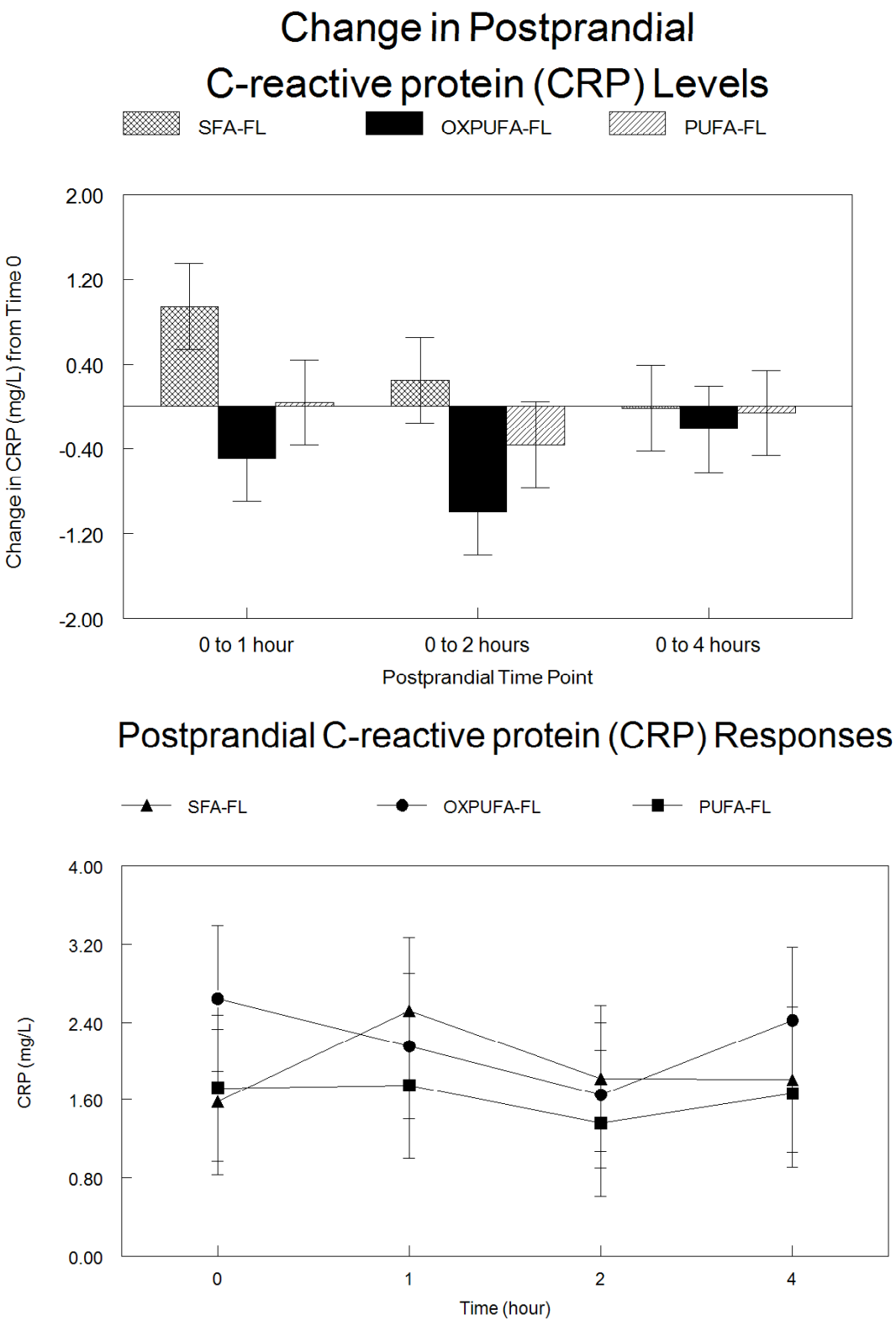
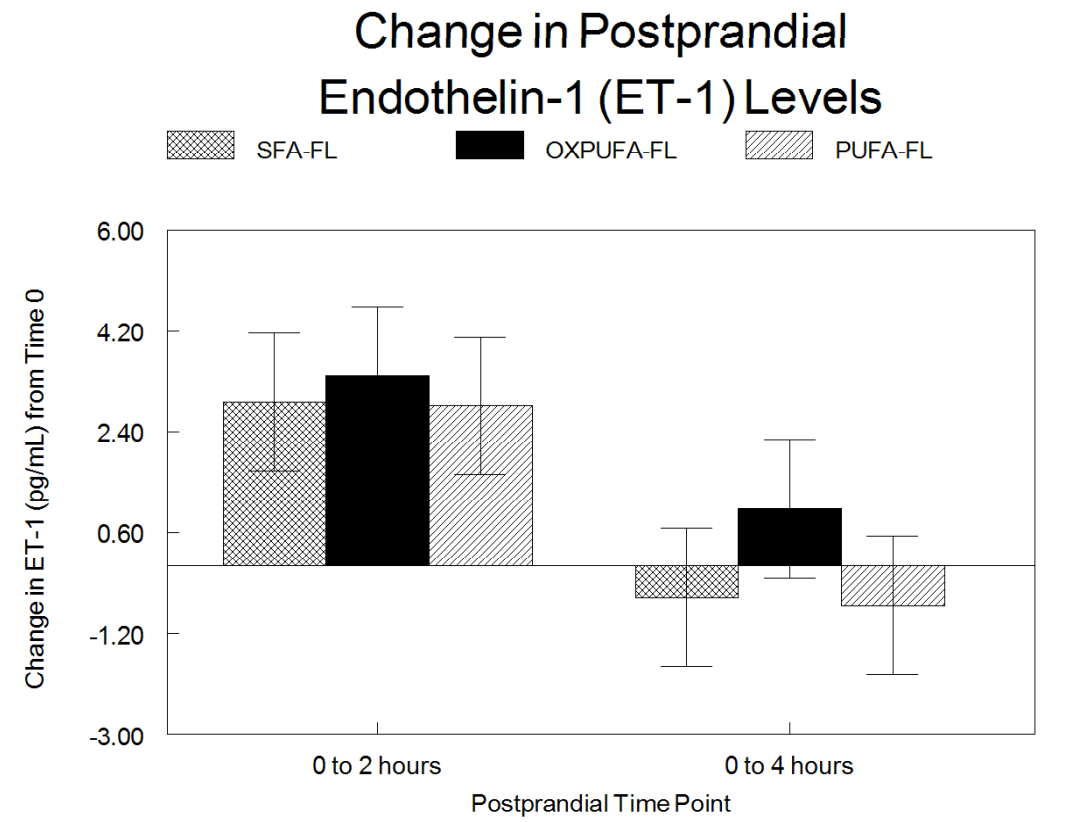
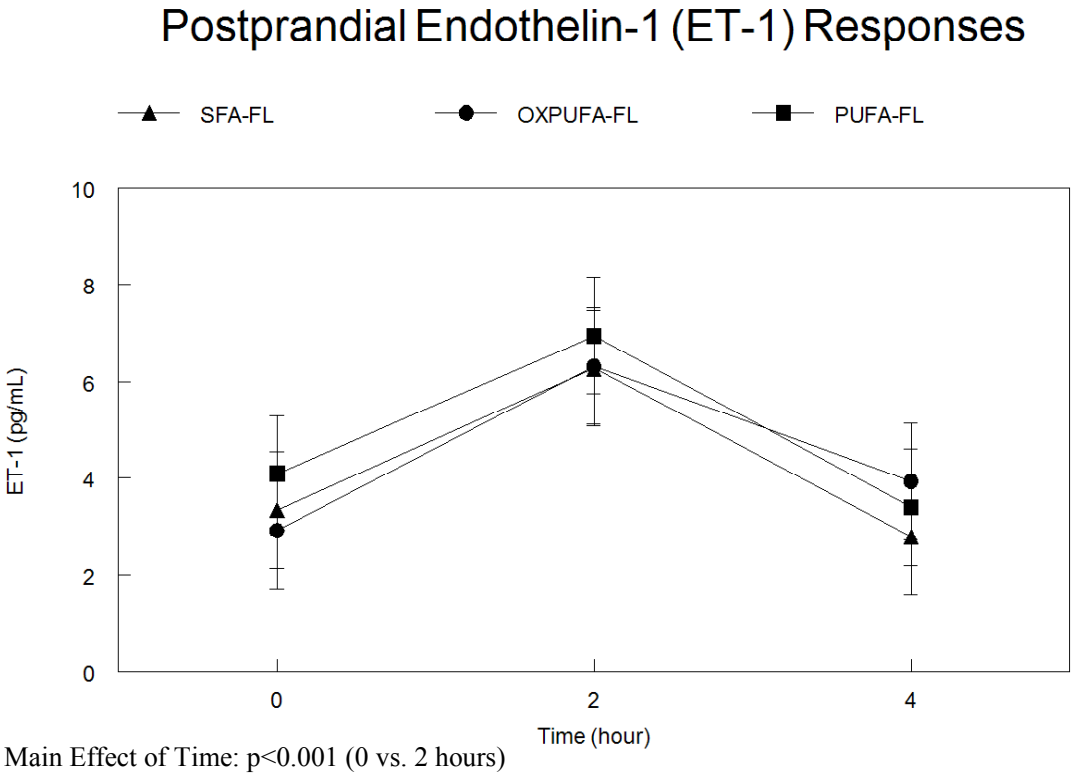


Figure 4.12: Endothelin-1 responses to test fat loads





Main Effect of Time: $p<0.001$ (0 vs. 2 hours)

Chapter 5
Summary, Limitations and Future Directions

Elevated levels of serum triglycerides alone are considered an independent risk factor for CVD. The clustering of lipid abnormalities deemed atherogenic dyslipidemia also includes low HDL-C levels, and small dense low-density lipoprotein cholesterol particles. Individuals with the atherogenic lipid phenotype also typically exhibit features of insulin resistance/hyperinsulinemia, increases in inflammation, endothelial dysfunction, the postprandial accumulation of remnant lipoproteins and a prothrombotic state. Two studies were conducted in normo- and hypertriglyceridemic individuals to determine the quantitative and qualitative effects of dietary fatty acids in the fasting and fed states, respectively. Of note, this research tests the extremes of the dietary fat recommendations (20-35% of calories) and provides guidance for the implementation of a moderate-fat diet, rich in unsaturated fatty acids, based on the acute postprandial affects of different types of fatty acids.

In the first study, a randomized controlled feeding trial was used to evaluate the effects of the chronic consumption of a low-fat versus a moderate-fat diet. The results of the studies conducted demonstrate that chronic intake of dietary patterns at either end of the recommended range of total dietary fat intake (20-35% total kcal) improves levels of serum lipids and lipoproteins. Of note in the present study is that despite a reduction in levels of serum triglycerides following a low-fat diet, individuals with elevated triglycerides at baseline experienced increased levels of the atherogenic apolipoprotein LpB:C. These results indicate that either a low-fat or moderate-fat diet (both low in saturated fats), may be recommended for individuals with normal fasting TG at baseline. On the contrary, this study suggests that a moderate-fat diet, low in saturated fatty acids

should be recommended for individuals with elevated fasting TG levels to prevent any potential increase in levels of atherogenic apolipoproteins.

The recommendation to consume a more moderate fat diet must also include a focus on the quality of the fat being consumed. The results of this postprandial study indicate that the consumption of different kinds of fatty acids can have very different affects on postprandial risk factors for CVD. In particular, the simple heating of a PUFA increased levels of individual trans fatty acid isomers and lipid hydroperoxides, resulting in diverse physiological responses, compared to the same un-heated PUFA. Consideration thus needs to be placed not only on the choice, but also on the preparation of dietary fats during the cooking process.

The studies conducted are not without limitations. One main limitation is the small sample size evaluated in both studies. Although the crossover design maximizes the power of the study by enabling each subject to serve as his/her own control, it is possible that with a greater number of total subjects, including more subjects within each group, additional significant differences may have been detected. A greater sample size also may have provided a more diverse sample, and thus elicited responses that would be more representative of the general population. A second limitation of the first study is the lack of baseline measurements for some of the biomarkers (i.e. apolipoproteins and CRP). A second limitation of the postprandial study is that the fatty acid analysis of the test fat loads was completed on multiple aliquots of only one milkshake sample. For the heated polyunsaturated-rich fat load, in particular, it is possible that the heating process produced different concentrations of the trans fatty acid isomers and lipid hydroperoxides across different test days. Multiple trials of heating the test fat and preparing multiple

OXPUFA-FL milkshake samples for analysis would provide a measure of variability in milkshake trans fatty acid isomer content.

In summary, whereas the implementation of a very healthy low-fat diet may have adverse affects on the apolipoprotein profile of individuals with elevated baseline triglyceride levels, the inclusion of higher amounts of unsaturated fatty acids in a moderate-fat diet, has a significant hypotriglyceridemic effect in individuals, regardless of baseline triglyceride status, along with no adverse changes in apolipoproteins. In making the recommendation to consume a moderate-fat diet it is important to recognize the effects of individual fatty acids on postprandial risk factors for cardiovascular disease. In particular, the heating of highly unsaturated fatty acids leads to the production of trans fatty acids and lipid oxidation end products. The diverse physiological responses observed thus indicate that a diet rich in mono- and polyunsaturated fatty acids is optimal, when compared to a diet rich in saturated and/or *trans* fatty acids.

Future research is needed to better identify the effects of altering the total amount of fat in the diet, while keeping saturated and *trans* fat within the recommended ranges on established and emerging risk factors for CVD. In addition, focus should be placed on the preparation of dietary fats with recommendations to minimally heat oils rich in polyunsaturated fatty acids. The identification and subsequent acute physiological effects of specific individual *trans* fatty acid isomers as a result of heating may provide evidence for the adverse affects observed following the chronic consumption of *trans* fatty acids in the diet.

Amy E. Griel, PhD

EDUCATION

-PhD – Graduate Program in Nutritional Sciences, The Pennsylvania State University, August 2002-August 2006
-M.Ed. – Exercise Physiology, The University of Virginia, 2002
-B.S. – Kinesiology, The Pennsylvania State University, 2000

AWARDS AND HONORS

-2006 Colonel Mary Lipscomb Hamrick Memorial Scholarship, The American Dietetic Association Foundation, 2006-2007 academic year
-Scholarship, The Pennsylvania State University Dietetic Internship Program 2006-2007
-Nutrition and Dietetics Alumni Society Travel Grant; 2005, 2006
-National Research Institute: Role of Fatty Acids in Reducing Inflammation Scholarship for 2005-2006 academic year

PUBLICATIONS

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