The Effects of Macronutrient Composition on Oxidative Stress and Inflammation in Overweight and Obese Humans

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ABSTRACT

Two thirds of American adults are overweight and almost half of those qualify as obese. Obesity independently increases risk for cardiovascular disease (CVD), type II diabetes (T2D), and hypertension; thus, strategies to reduce risk in this population are desperately needed. Oxidative stress and inflammation are two perpetuators of these chronic diseases that are often elevated in obesity. Interventions that target reductions in oxidative stress and inflammation may help to reduce co-morbidities associated with obesity. Weight loss is shown to reduce oxidative stress and inflammation. However, the composition of and food choices within the weight loss diet may influence the response of these factors to weight loss, and has not been adequately assessed. We first tested whether there were differential effects of a conventional low-fat, high carbohydrate weight loss diet (LF) and the Atkins diet (a popular low carbohydrate, high fat diet (HF)) on oxidative stress and inflammation. We demonstrated that HF raised C-reactive protein (CRP) levels relative to LF in overweight and obese women over four weeks. This finding raises questions as to the long term safety of the HF eating plan in terms of CVD risk. We next examined the role of oxidative stress in the HF diet-induced increase in inflammation by evaluating the effects of an antioxidant supplement versus a placebo in conjunction with HF in overweight and obese men and women. Although our full hypothesis was not supported, as oxidative stress did not increase with HF, the trend for a differential effect on CRP when antioxidants were consumed is provocative. It suggests that future research on the connection between oxidative stress, the macronutrient content of the diet, and inflammation in obesity is warranted.

Regarding the effects of specific fats, epidemiological research shows that diets high in saturated fat (SFA) are associated with higher CVD risk while diets higher in omega 3 fats (n-3FA) with lower CVD risk. However, the acute effects of these fats on indices of inflammation and oxidative stress are less understood, particularly in the overweight/obese population. As the majority of the time is spent in the postprandial state, the acute responses to high fat meals are gaining attention for their contribution to endothelial dysfunction and CVD. We showed that acute meals high in SFA increased a marker of endothelial activation (ICAM-1) which could contribute to the atherogenic associations with SFA. Conversely, including n-3FA in a high fat meal acutely enhanced NF-κB activation in circulating mononuclear cells; however, there were no increases in any inflammatory proteins measured over the 6 h postprandial period. It is apparent that dietary macronutrients can influence factors associated with chronic disease in overweight and obese individuals. The evidence presented here may help to refine dietary recommendations for this population.

<center>Attributions of manuscript co-authors</center>

Dr. Janet Walberg Rankin

Dr. Rankin served as primary advisor and committee chair for this research. She is a professor in the Human Nutrition, Foods, and Exercise Department of Virginia Tech. She was involved in the development, implementation, critical analysis of results, and the preparation of all three manuscripts in this dissertation.

Dr. Yong Woo Lee

Dr. Lee served as a committee member for this research. He is an assistant professor in both the Department of Biomedical Sciences and Pathobiology and the School of Biomedical Engineering and Sciences at Virginia Tech. He assisted with the adaptation of his cell culture EMSA methodology for use with human cells and helped to analyze the results. He also advised the methods description and presentation of data for the accompanying manuscript (Chapter 5).

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<u>Chapter I. Introduction</u>

The continued increase in overweight and obesity is unsettling for numerous reasons.

Excess adipose tissue puts individuals at greater risk for chronic diseases such as type

II diabetes (T2D), hypertension, and cardiovascular disease (CVD) (1). The association

with CVD is particularly worrisome as it has been the leading cause of death in the

United States every year except 1918 since 1900. CVD was reported as the underlying

cause of 1 in every 2.8 deaths in 2004 (2). Analysis of the Framingham Heart Study

cohort indicates that obese individuals are more burdened with CVD and are projected

to have a shorter lifespan than normal weight individuals. Obesity reduces quality of life

and shortens life expectancy, for example it is estimated that a 45 year old obese

woman (without present CVD) will live 8.4 fewer years, while the same aged obese man

6.0 fewer than their lean counterparts (3).

Another important issue is the economic burden of this epidemic, which is

increasing in significance. For example, in the year 2000, $56 billion was spent in the

U.S. on healthcare expenditures associated with excess body weight (4). Costs

reportedly increase with weight as heavier individuals often require more medical care.

Accordingly, the morbidly obese category (BMI > 40 kg/m^2) accounted for over $11

billion in health care costs while the overweight category (which has 12 times the

number of people) used $17 billion (4). Employers are sure to take notice of these

expenses and loss of productivity as a recent analysis of the link between BMI and

worker's compensation showed a clear linear relationship. At the highest end, morbid

obesity was associated with 13 times as many lost work days and 7 times as much in

medical claims costs than normal weight (5). There is undoubtedly a great need to improve the health of these individuals.

Blame for the surge in obesity over the past several decades falls to a number of areas, including "the built environment," lack of daily physical activity, and inexpensive, energy dense foods (6). The combination of these factors has resulted in a heavier population with increased morbidity than in the past.   Given the facts that 1) weight loss is not easy, 2) many people are not successful in their attempts, and 3) even fewer people are able to maintain weight loss; experts recommend that an initial goal should be to simply start maintaining weight (avoiding continued weight gain) (7). Thus, developing strategies to improve the health of these individuals independent of weight loss will be necessary to reduce their risk of developing chronic diseases.

Obesity is associated with many metabolic disturbances including dyslipidemia, hyperglycemia, and hyperinsulinemia all of which increase risk for CVD, T2D, and hypertension. Excess adipose tissue also contributes to oxidative stress and inflammation; both of which perpetuate atherosclerosis and are implicated in the pathogenesis of T2D and hypertension (8-10). Therapies that target the reduction of oxidative stress and inflammation may confer a significant health benefit to overweight and obese individuals. Energy restriction is an ideal therapy as weight loss appears effective in reducing these factors, thus reducing the risk for disease (9,10). However, as mentioned, weight loss is not easy and it is estimated that only 1 in 5 overweight individuals maintain weight loss (11), thus, alternative dietary strategies to improve health are necessary.

Dietary patterns differing in macronutrient composition have been recognized for their associations with CVD risk, however, the effects of macronutrient composition on oxidative stress and inflammation has not been well studied, particularly in overweight and obese humans. A better understanding of these effects will help to determine the impact of these dietary constituents on factors related to disease risk in this large proportion of the population and will have broad reaching effects.

Purpose

The purpose of this research is to extend the existing body of knowledge regarding effects of dietary macronutrient composition on oxidative stress and inflammation in overweight and obese individuals.

Rationales and Hypotheses

Rationale for study 1: Weight loss tends to reduce oxidative stress and inflammation, the interaction between weight loss and dietary composition on oxidative stress and inflammation is not clear. As many low carbohydrate diets are high in fat and low in dietary antioxidants provided from fruits, vegetables, and whole grains, they may increase oxidative stress. Our hypothesis was that overweight and obese women consuming a low carbohydrate, high fat diet (HF) would exhibit higher markers of oxidative stress and inflammation compared to those consuming a low fat, higher carbohydrate American Heart Association (AHA) style weight loss diet (LF).

Rationale for study 2: This study was conducted to further evaluate the mechanism by which a low carbohydrate, high fat diet (HF) increased inflammation

compared to a low fat diet (LF). It was considered likely that our previous finding of an increase in serum CRP following short term HF consumption was mediated by oxidative stress induced by the dietary composition. Due to the fact that 1) there were differences in antioxidant consumption between HF and LF, 2) the high fat content of an energy restricted diet interferes with the reduction in oxidative stress in animals (12), and 3) in humans, provision of antioxidants with a high fat meal attenuates postprandial oxidative stress and inflammation. We hypothesized that provision of an antioxidant supplement concurrent to HF would attenuate the inflammatory response in comparison to placebo.

Rationale for study 3: While high fat diets in general appear atherogenic (13), specific fatty acids (FA) appear to have unique effects on CVD risk and systemic inflammation. Higher saturated fatty acid (SFA) intake is associated with increased risk of CVD and inflammation while monounsaturated (MFA) and polyunsaturated fat (PUFA) intake appear to reduce CVD risk and inflammation (14). The objective of this study was to further clarify the role of different sources of fat in a high fat meal on inflammation and oxidative stress in overweight and obese individuals. We hypothesized that the highest postprandial inflammatory response would occur in response to the meal high in SFA via activation of the transcription factor NF-κB.

Overall, this research extends our current understanding of ways that dietary patterns influence oxidative stress and inflammation. We show that dietary macronutrient content can influence oxidative stress and inflammation in overweight and obese individuals, which could impact their risk for chronic disease.

Limitations

1. Subjects were free living.

2. Subjects varied in the degree to which they were overweight or obese.

3. The results of these studies may only be generalized to overweight and obese persons with similar metabolic status.

4. No biochemical analyses of nutritional status were performed on subjects.

5. The first weight loss study was limited to four weeks, while the second to one week.

6. Timing of blood draws in the postprandial study only extended to 6 h after meal consumption.

Delimitations

1. This research applies to healthy, non-smoking, overweight and obese men and women.

Basic Assumptions

The following assumptions were made during the course of these studies:

1. Subjects complied with the dietary protocols and did not consume forbidden foods.

2. Subjects refrained from taking vitamin and mineral supplements.

3. Subjects did not change their activity levels during the studies.

4. Subjects arrived to the laboratory fasted for all studies.

Dissertation Layout

This dissertation is compiled in the following manner. Chapter 2 contains a general review of literature pertinent to the research presented. Chapters 3-5 are comprised of three stand alone manuscripts, one for each study conducted. A later version of Chapter 3 has been published in the *Journal of the American College of Nutrition*, Chapter 4 is currently under review, and Chapter 5 is in preparation for submission. As these papers are presented in their entire "to be published" form, there will be some overlap in the topics discussed. Chapter 6 contains summaries, implications, theoretical considerations of this research, and recommendations for future research.

Abbreviations / Definition of Terms

ROS – reactive oxygen species, reactive radical and non-radical forms of oxygen with cell signaling and bactericidal roles, also implicated in causing damage to biomolecules.

CRP – C-reactive protein, a protein released primarily by hepatocytes during the acute phase response by the immune system, it is considered an indicator of inflammation.

IL-6 – interleukin-6, a pro-inflammatory cytokine released by immune cells, also known as the hepatocyte stimulating factor, inducing the expression of acute phase proteins.

8-epi – 8-epi-prostaglandin-$F_{2\alpha}$, an F2-isoprostane that is a product of free radical induced peroxidation of arachidonic acid, an indicator of oxidative stress.

MCP-1 – monocyte chemoattractant protein-1, a chemokine that is a chemoattractant for monocytes to sites of inflammation.

ICAM-1 – intercellular adhesion molecule-1, an integrin found on leukocytes and vascular cells instrumental in leukocyte adhesion to the endothelium and chemotaxis.

VCAM-1 – vascular cell adhesion molecule-1, a cytokine-induced cell adhesion molecule important for the recruitment of immune cells to sites of inflammation.

SFA – saturated fatty acids, the carbon chains are fully saturated with hydrogen atoms.

MFA – monounsaturated fatty acids, the carbon chains contain one carbon-carbon double bond.

PUFA – polyunsaturated fatty acids, the carbon chains contain more than one carbon-carbon double bond.

n-3FA – omega 3 fatty acids, the last double bond is located 3 carbons from the omega end. In this text, n-3FA will refer primarily to the long chained omega 3 fats (EPA, eicosapentaenoic acid and DHA, docosahexaenoic acid).

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Chapter II. Review of Literature

A. Inflammation

The innate branch of the immune system is the first line of defense against disease or injury and involves the production and release of inflammatory cytokines (such as interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) by tissues and immune cells into the systemic circulation. IL-6 is a proinflammatory cytokine that initiates and regulates the hepatic acute phase response to mediate inflammation. IL-6 increases C - reactive protein (CRP) production and release by the liver (1); which is considered both a marker for infection and inflammation (due to a relatively short half life, 4-9 h), as well as contributor to inflammatory processes. CRP activates complement, cytokine production (2), endothelial cell adhesion molecule expression (3), and binds to low-density lipoprotein (LDL), which induces cholesterol uptake by macrophages (4). It is also perceived as a reliable predictor of heart disease (5), independent of blood lipids.

The cellular fraction of the blood known as peripheral blood mononuclear cells (PBMCs) is typically comprised of 85-90% lymphocytes and 10-15% monocytes (6). These cells are instrumental in carrying out functions of the immune system by traveling to sites of inflammation and infection and alerting other immune cells. Phagocytic cells (such as macrophages and neutrophils) remove and destroy pathogens and necrotic cells in addition to releasing inflammatory cytokines to stimulate surrounding immune cells. For protection from pathogens, immune cells play critical roles; however, chronically elevated production and release of inflammatory mediators can unnecessarily activate endothelial cells, an undesirable occurrence.

Inflammation is a key factor in atherosclerosis, a condition characterized by the deposition of cholesterol in the arterial wall with subsequent hardening of the arteries and a narrowing of the lumen which reduces blood flow. Atherosclerosis is a primary contributor to cardiovascular disease (CVD). Several components of the immune system, notably monocytes/macrophages, T lymphocytes, cytokines, and chemokines contribute to its development (7,8). Cytokines (such as TNF-α) activate vascular endothelial cells. This activation results in adhesion molecule expression, which enables the tethering of activated leukocytes to the endothelial cell surface and transmigration into the subendothelium. Inside the tissue, monocytes differentiate into macrophages and can accumulate lipids (i.e. oxLDL), leading to foam cell formation and lipid deposition within the vessel wall (9). Cytokines and chemokines released by the macrophage/foam cell recruit other immune cells, thereby perpetuating inflammation and damage to the vessel. Growth factors released stimulate smooth muscle cell proliferation and the culmination of these vascular changes is plaque formation (10). Macrophage derived metalloproteinases can subsequently degrade the support structures of the plaque leading to rupture and thrombosis, resulting in stroke or myocardial infarction (11). A critical step in the atherosclerotic process is the initial activation of the endothelium, which can occur as a result of elevations in systemic pro-inflammatory mediators.

B. Oxidative Stress

Free radicals and reactive oxygen species (ROS) are reactive molecules that are characterized by an incomplete outer shell of electrons, making them highly unstable

and damaging to molecules or compounds in close proximity. The damage inflicted by free radicals often occurs at cell membranes as double bonds present in unsaturated fatty acid chains are easy targets for electron removal (see Figure 1 for an example of this process). Allylic hydrogen molecules (those bound to carbon molecules on either side of a carbon-carbon double bond) are weakly held and abstracted relatively easily. The carbon centered radicals formed by hydrogen abstraction are electron rich, and extremely susceptible to oxidation (oxygen is very electronegative). Lipid peroxidation is a self-perpetuating process that involves continuous formation of free radicals until the chain is broken by an antioxidant. Lipid peroxidation can increase membrane permeability and inactivate important membrane transporters, required for maintaining the balance of solutes, resulting in cell death.

Exogenous sources of free radicals include pollution and radiation, while cellular metabolism is a primary endogenous source with mitochondria contributing a significant amount of ROS. The single electron reduction of oxygen producing the ROS superoxide (O_2^-) can occur along the electron transport chain (ETC) located in the inner mitochondrial membrane (complexes I and III primarily). Overproduction of O_2^- by this manner has been implicated in the pathogenesis of diabetes (for a review, see Brownlee (12)). Superoxide is also formed via the enzyme complex NADPH oxidase in phagocytic (macrophages, neutrophils) and endothelial cells.   Excess O_2^- produced by endothelial cells in the vasculature can contribute to atherosclerosis by reducing nitric oxide (NO) bioavailability. As NO is instrumental in maintaining vessel compliance, preventing platelet activation, and preventing endothelial cell activation, reducing NO reduces vasodilation, increases blood pressure, and the likelihood of vessel damage

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(13). Superoxide binds to NO forming peroxynitrite (ONOO-) which is problematic as, in addition to removing NO, ONOO- is another ROS that can damage cellular structures. Injury to the vessel wall can be an initiating event for atherosclerotic processes.

The term "oxidative stress" refers to an imbalance between oxidants and endogenous antioxidant defenses, favoring the oxidants. *In vivo* antioxidant defenses include small molecular weight compounds (i.e. vitamins C and E, glutathione (GSH)), and antioxidant enzymes such as superoxide dismutase (SOD, copper-zinc SOD (Cu-ZnSOD) in cytosol, manganese SOD (Mn-SOD) in mitochondria), glutathione peroxidase (GSH-Px), and catalase. Cu-Zn SOD is the primary enzyme that scavenges O_2^- by forming hydrogen peroxide (H_2O_2). As H_2O_2 can react with metal cations such as Fe^{2+} (via the Fenton reaction) and produce the dangerous hydroxyl radical (OH^-) minimizing H_2O_2 levels is critical. GSH-Px plays an important role in reducing H_2O_2 concentration and maintaining cellular integrity ($2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$).

Assessing the level of oxidative stress *in vivo* is challenging as the highly reactive ROS are short lived and difficult to measure directly. Therefore, "markers" of oxidative damage to biomolecules such as DNA, protein, and lipid are often used. Common markers of DNA damage include auto-antibodies to oxidized DNA and 8-hydroxydeoxyguanasine (8-OHdG). Indicators of protein oxidation include protein carbonyls, nitrotyrosine, and advanced glycation end products (AGEs). Products of lipid peroxidation include lipid peroxides (LPO), malondialdehyde (MDA), and F2-isoprostanes. F2-isoprostanes are considered reliable measures of oxidative stress *in vivo* (14) as they are biologically stable end-products of the free radical induced oxidation of arachidonic acid (AA) (for formation, see Figure 1). These products are not

only markers of oxidative stress, but they can also be bioactive. For example, 8-epi-

prostaglandin $F_{2\alpha}$, (8-epi), enhances vasoconstriction, platelet aggregation, and smooth

muscle proliferation. These effects can negatively affect vascular conditions (15) and

may contribute to atherosclerosis (16).

Figure 1. Lipid peroxidation schematic for production of F2-isoprostanes (i.e. 8-epi-prostaglandin $F_{2\alpha}$). Reprinted from The Lancet, vol. 1345, Roberts and Morrow, "The generation and action of isoprostanes" 1997 (14). With permission from Elsevier.

1) Arachidonic Acid (20:4n-6)

2) Proton abstraction by from lipid (RH) by free radical (OH·), see carbon centered free radical (R·) formed:
$$OH^. + RH \rightarrow R^. + H_2O$$

3) Oxygen reacts with carbon centered radical, lipid peroxide formed $R^. + O_2 \rightarrow ROO^.$

4) Rearrangement of electrons within the molecule for stabilization; see endocyclization

5) Another oxygen molecule reacts in the electron dense area, see a prostaglandin (PG)-G2 like compound form

6) Reduction of the ring forms PG-F2

Another method of assessing oxidative status is to measure the antioxidant

capacity of a tissue or bodily fluid (such as serum). The oxygen radical absorbance

capacity (ORAC) assay for example is based on hydrogen atom transfer reactions and has been used in a variety of food and biological samples (17). This assay provides an indication of how well a sample scavenges peroxyl radicals, evident by the extent to it prevents a fluorescent compound from degradation by ROS.

C. Obesity and Chronic Disease

The prevalence of overweight and obesity has steadily increased over the last several decades. Data from the National Health and Nutrition Examination Survey (NHANES) indicate that the age-adjusted prevalence of overweight increased from 55.9% in NHANES III (1988- 94) to 66.3% in 2003-2004 while obesity rose from 22.9% to 32.2% and extreme obesity (BMI ≥ 40.0) increased from 2.9% to 4.8% (18). Overweight and obesity are considered independent risk factors for CVD, T2D, and hypertension (19,20) due in part to metabolic disturbances associated with obesity such as hyperglycemia, hyperinsulinemia, and dyslipidemia. Chronic hyperglycemia can negatively affect pancreatic beta cells, which are responsible for the production and release of insulin. This results in an initial increase in fasting insulin levels, followed by an insulin resistant state in which insulin sensitive cells display reduced responsiveness to insulin. Hyperglycemia induced beta cell dysfunction may ultimately lead to T2D. Additionally, in obesity, dyslipidemia (specifically, a lower level of high density lipoproteins (HDL) coupled with elevated small, dense low density lipoproteins (LDL), and circulating triglycerides (TG)) can also increase risk for CVD. In conjunction with the increase in these metabolic risk factors, expanded fat depots and ectopic fat storage contribute to systemic inflammation and oxidative stress. Elevations in inflammation and oxidative

stress in obesity may contribute to endothelial dysfunction and hypertension, potentially

increasing CVD risk (21).

D. Obesity and Inflammation

Overweight and obese individuals often exhibit elevated markers of inflammation (i.e.

IL-6, TNF-α, CRP) (22,23) compared to age and sex matched lean individuals. Adipose

tissue itself contributes to the inflammatory state by secreting many inflammatory

cytokines and "adipokines" that act both locally and systemically (i.e. TNF-α, IL-6, leptin)

(24). Excess fat storage results in adipocyte enlargement and increased secretion of

pro-inflammatory mediators (25). Body fat distribution also plays a role, as visceral fat

appears to release more cytokines than subcutaneous fat (26), particularly IL-6 (27).

Elevations in TNF- α and IL-6 increase production and release of acute phase proteins

such as CRP from the liver, while reducing release of the anti-inflammatory protein

adiponectin (28). Circulating mononuclear cells are also in a pro-inflammatory state in

obese individuals. This is evident by increased levels of nuclear factor k-B (NF-kB)

activation and TNF-α and IL-6 expression in PBMCs (29). Monocyte activation

combined with monocyte chemoattractant protein (MCP-1) release by adipocytes

contributes to the tethering and transmigration of monocytes through the vascular

endothelium and into adipose tissue beds where they take up residence and mature into

macrophages (30-32). These activated phagocytic immune cells further contribute to

the release of inflammatory cytokines, ROS, and additional chemokines from adipose

tissue; attracting additional macrophages and perpetuating their infiltration (31,33,34).

Unfortunately, inflammation can also encourage metabolic abnormalities. For example, TNF-α plays a role in insulin resistance in adipocytes and skeletal muscle cells by inducing phosphorylation of serine residues of insulin receptor substrate-1 (IRS-1) (35), thus inhibiting the insulin receptor tyrosine kinase activity necessary for insulin stimulated activation. TNF-α also impairs phosphorylation of Akt substrate 160, important in the regulation of GLUT4 (glucose transporter) translocation and glucose uptake by skeletal muscle cells (36). Insulin resistance perpetuates hyperglycemia and increases oxidative stress and inflammation contributing to CVD as well as increased risk of hypertension and diabetes. Therefore, reducing the inflammatory state in obese individuals would theoretically help to reduce their risk of several chronic diseases, however, this has not been conclusively proven.

E. Obesity and Oxidative Stress

Oxidative stress is also common in obesity, stemming from a combination of insults (for a review see Vincent and Taylor (37)). Sources include hyperglycemia, hypertrophied adipocytes, increased metabolic production of reactive oxygen species (ROS), endothelial activation, and chronic inflammation. Additionally, there reduced antioxidant defenses are common in obesity. Body mass index (BMI) has been inversely related to levels of red blood cell GSH, GSH-Px, and Cu-Zn SOD, further increasing the risk for oxidative damage in this population (38-40). Adipocytes influence levels of oxidative stress via the production of H_2O_2 (41) and by producing TNF-α and IL-6, which stimulate ROS production by immune cells in circulation. Macrophages residing in adipose tissue also produce and release ROS (31). Metabolic disturbances associated with obesity

also contribute. Hyperglycemia in particular correlates strongly with markers of oxidative stress and individuals with poor glycemic control have higher levels of 8-epi than age matched controls (38,42). It is theorized that high blood glucose contributes to excess mitochondrial O_2^- production by a number of mechanisms, including increased flux through the polyol and hexosamine pathways, activation of protein kinase C (PKC), and advanced glycation end product (AGE) formation (for review, see Brownlee (12)). Free fatty acids (FFA) can increase ROS production by immune and endothelial cells by stimulating NADPH oxidase activity (43), which is associated with increased endothelial cell adhesion (44). Further, triglyceride rich lipoproteins (TRL) enhance macrophage lipid uptake and foam cell formation (45), which can increase ROS release. Postprandial TRL isolated from individuals with hypertriglyceridemia are also shown to activate NF-kB in endothelial cells, stimulating expression of adhesion molecules (46).

Many cross-sectional studies have confirmed the relationship between obesity and oxidative stress. In fact, it was shown in subjects from the Framingham Heart Study that every 5 kg/m^2 increase in BMI was linked to almost a 10% increase in a marker of lipid peroxidation, urinary 8-epi (38). Another study confirmed this association and showed that waist circumference was also related to urinary 8-epi in and to plasma TBARS, another indicator of lipid peroxidation (47). Body fat distribution has likewise been shown to play a role in oxidative stress. Obese women had higher platelet activation and 8-epi levels than non-obese, and women with android obesity had even higher 8-epi than those with gynoid obesity (48). Recently, a study reported that total body fat was positively associated with levels of vascular endothelial cell NAD(P)H oxidase expression and with nitrotyrosine, an indicator of protein oxidation (49). As

increased oxidative stress contributes to pancreatic beta cell dysfunction (50) (impairing insulin secretion and perpetuating hyperglycemia) and atherosclerosis, reducing these levels could reduce the risk of T2D and CVD in obesity.

F. Relationship between Oxidative Stress and Inflammation

Redox sensitive transcription factors link oxidative stress to the inflammatory response (51). For example, NF-κB comprises a family of transcription factors involved in inflammatory, acute phase, and immune responses (Lavrosky), with several subunit combinations possible. It is understood that NF-κB exists as an inactive heterodimer (often with subunits P65 and P50) bound to an inhibitor protein in the cytoplasm. ROS are instrumental in activating NF-κB by either activating the inhibitor kappa beta kinases (IKK), or by inhibiting the IKB phosphatases (52). IKK phosphorylate and ubiquitinate IKBα, targeting it for degradation by the 26S proteasome and allowing the NF-κB dimer to translocate to the nucleus. There it initiates the transcription of genes, many of which encode inflammatory proteins such as TNF-α, IL-1, IL-6, IL-8, and adhesion molecules (53). The electrophoretic mobility shift assay (EMSA) is one of the most powerful methods of measuring protein-DNA interactions, very useful for assessing NF-κB activation (54). Another important redox-sensitive and inflammation related transcription factor family is activator-protein-1 (AP-1). AP-1 is also a dimer, comprised of proteins from the c-fos and c-jun families of proteins, that is activated by many cytokines and ROS producing processes. It induces the gene expression of pro-inflammatory mediators and protective antioxidant genes (55).

Positive associations between oxidative stress and inflammation have been reported in obese humans (48) as lipid peroxidation was positively associated with the 3rd and 4th quartiles of CRP levels (highest lipid peroxidation and highest CRP). Further, CRP levels and android obesity were both independent predictors of urinary 8-epi, indicating links between obesity, lipid peroxidation and inflammation. As mentioned above, mononuclear cells in the obese display elevated NF-kB levels (29), providing a means by which oxidative stress can increase inflammatory protein expression.

G. Weight Loss Effects on Inflammation and Oxidative Stress

Weight loss reduces the risk of CVD, T2D, and hypertension, in part by improving metabolic factors, including glycemic control, insulin sensitivity, and blood lipids. Reductions in weight also lower indices of inflammation such as systemic cytokines (CRP, IL-6, TNF-α) and chemokines (MCP-1), in addition to the macrophage infiltration of adipose tissue (56-59). (See Table 1 for examples). It has been established that weight loss is consistently effective in reducing CRP, one of the more important indicators of inflammation, as it is highly associated with CVD (60). A recent review (61) reported that for every 1 kg of body weight lost, there was a corresponding reduction in CRP of -0.13 mg/L. Depending on the magnitude of weight loss, this could significantly reduce one's risk for CVD. However, the relationship between weight loss and other inflammatory markers is complex and studies frequently show reductions in one marker, but not another (61-63). Discrepancies within the same marker between different studies could be due to magnitude of weight loss, dietary compliance, or even medication usage.

Oxidative stress is also sensitive to weight loss, with markers such as 8-epi

$PGF_{2\alpha}$, TBARS, MDA, and 8-OHdG shown to be responsive (see Table 1 for examples).

The time course for this effect appears short, for example one study measured *ex vivo*

ROS production by both polymorphonuclear leukocytes and mononuclear cells in

subjects following a very low calorie diet (1000 kcal/d) and reported that weight loss

resulted in decreased ROS production as soon as one week (64). Similarly, a lifestyle

intervention in overweight men following a diet and exercise program reduced 8-epi in

as little as 3 weeks (65). While many studies concur, the literature is not entirely

supportive of the link between weight loss and reduction in markers of oxidative stress.

For example, Samuelsson et al (66) did not observe any association between the

decrease in BMI and changes in 8-epi levels of 376 obese subjects, over 54 weeks.

Again, differences in study methodology, medication, supplement usage, and subject

characteristics can all play a role in different reports.

Table 1. Weight loss effects on oxidative stress and inflammation

References - Chronological	Subjects	Diet / Protocol	Time/Wt. loss	Inflammatory or Ox stress meas.
Bastard, 2000 (62)	21 Obese (Ob), 8 lean, 14 Ob 45± 1.1y & 7 T2D Ob (58±2y)	VLCD 941± 27 kcal/d, 45%CHO, 20%Fat, 35%Pro	3 weeks Lost 3 kg fat BMI from 39.5 to 37.4kg/m^{2*}	IL-6 ↓* 22±9% Leptin ↓* 36±4% CRP trend ↓ (6.3-4.3 mg/L, p=0.14) No Δ TNF-α
Dandona, 2001(64)	7F/2M Ob, 45.3 ± 13.2y, BMI: 32.5-64.4	1000kcal/d, 4 wk, Shake for B/L, 600 kcal dinner	Lost 4.5± 2.8 kg	ROS by PMN↓* ROS by MNC↓* TBARS also ↓* NO Δ TNF-α
Heilbronn, 2001 (67)	83F Ob, BMI: 33.8±.4 kg/m^2 Sedentary	Very LF diet 1361kJ, 61.4% CHO, 14.2% Fat	12 wks, lost 7.9±0.4 kg	CRP ↓* 26% w/ wt. loss p<0.001
Davi, 2002 (48)	20F, android Obesity, BMI= 45±6kg/m^2	1200kcal/d	12 weeks, 11 lost wt (15.3±10.5kg), 9 ↑wt. (~2kg)	8-epi ↓* 32%, also 11-dehydro-TxB$_2$ ↓ 54% CRP↓* (23%)

Laimer, 2002 (61)	20F Ob, BMI: 41.6±5.4 kg/m^2,40.5±9.1y	Gastric banding procedure	Lost 24.1kg fat, BMI ↓ to 30.8±6.1 kg/m^2	CRP ↓ (1.33 ±1.21 to 0.4±0.61 mg/dl) But no sig. Δ IL-6 or TNF-α
Roberts, 2002 (65)	11M, 38-72y, 7 hypertensive, 3 T2D.	21 d resid. diet & ex. ad libit., ↓fat, ↑fiber, ↑grains	BMI ↓ *3.9%, lost 3.7% body wt.	↓8-epi
Tchernof, 2002 (68)	61F Ob, BMI: 35.6±5.0 kg/m^2	1200 kcal/d, AHA step II diet, medifast supp.	Fat loss: 10.4 ± 5.4 kg over 13.9±2.6 mo.	CRP ↓ 32.3%
Ziccardi, 2002 (69)	56F 25-44y, BMI: 37.2 ± 2.2, 40 cont.	Multi-disc. prog 1300 kcal/d, sim. To medit. Step I diet	1yr, lost ≥ 10% body wt (9.8±1.5kg) BMI ↓* 4.7	TNF-α, IL-6 ↓ (1.8 and 1.48 pg/ml resp.) w/ wt. loss
Esposito, 2003 (70)	120 F Ob/sed, BMI 30-49 20-46y	1300-1500 kcal/d 50-60% CHO, <30% fat, 15-20% PRO	2 y program Interv. Grp wt. loss > Con	IL-6, IL-18, CRP ALL ↓
Desideri, 2003 (71)	36M Ob, 26M lean controls 53.4±6y	VLCD 800-1200 kcal/d, 16 weeks	BMI ↓* 3.5±0.7 kg/m^2	8-epi↓ 142.2 ± 41.2 pg/ml, CD40L ↓
Kopp, 2003 (72)	34F/3M Ob, BMI>40 kg/m^2 41±7y	Gastric surgery	BMI ↓* from 49±7 to 33±6	CRP ↓ (8.6 to 2.5), IL-6↓ (5.13 to 3.95 pg/ml) No Δ TNF-α
Samuelsson, 2003 (66)	376 M/F, 27-75 BMI 33.2±3.0	600 kcal deficit w/ orlistat vs placebo	54 wks, wt loss: 5.6±5.2kg (orl) & 4.3± 5.9kg (P)	↓IL-6, ↓TNF-α, NO Δ 8-epi
Clifton, 2005 (73)	55 M/F, BMI: 27-40 kg/m^2 high blood TG	2 LF/HC wt loss strategies, w/ or w/o slimfast prod. (6000 kJ/d)	3 months, lost 6.3 ± 3.7 kg no diff b/w grps	CRP, ICAM, PAI-1 dec. with wt loss, No Δ IL-6, VCAM-1
Dvorakova, 2006 (63)	40 Ob F, 25-35y	Diet and phys activity	BMI dec > 7%	CRP decreased, No chg IL-6 or adiponectin
Heilbronn, 2006 (74)	48 M/F healthy, overwt 26-49y, BMI range 24.7-31.3 kg/m^2	4 grps - Cal restr, CR + ex, VLCD (890 kcal/d), or control	6 months, CR ↓10%, CR + ex ↓10.4%, VLCD ↓13.7%	DNA damage dec. in all groups, but no Δ protein carbonyls

BMI = body mass index, T2D = type two diabetics, VLCD = very low calorie diet, CHO = carbohydrate, LF = low fat, PRO = protein, ROS = reactive oxygen species, PMN = polymorphonuclear cells, MNC = mononuclear cells, TBARS = thiobarbituric acid reactive substances, TNF-α = tumor necrosis factor-alpha, ICAM-1 = intercellular adhesion molecule-1, VCAM-1 = vascular cell adhesion molecule-1, IL-6 = interleukin-6, MDA = malondialdehyde, TG = triglycerides, CRP = C-reactive protein, 8-epi = 8-epi=prostaglandin-F2-alpha, AHA = American Heart Association diet, PAI-1 = plasminogen activator inhibitor-1.

H. Macronutrient Intake

Dietary macronutrient content effects on oxidative stress and inflammation

The American Heart Association (AHA) recommends the consumption of a diet rich in carbohydrate sources (fruits, vegetables, whole-grain high fiber foods), and low in saturated fat but including fatty fish to reduce the risk of CVD (75). In regards to carbohydrates, Nurse's Health Study data showed that increasing dietary intake of fruits and vegetables is associated with a reduced risk of obesity over a 12 year period (76) and increased grains and dietary fiber relate to a less weight gain (77). Reductions in obesity and weight gain surely help to lower the risk for CVD, however, it has also been shown that carbohydrate quality can influence inflammation and oxidative stress. For example, diets high in unrefined carbohydrates (fruits, vegetables, whole grains) (HC) are associated with lower markers of endothelial dysfunction (78). They also provide protection against artificially induced oxidative stress (79) compared to typical "Western" style diets. This protection may be due in part to the observation that diets rich in fruits and vegetables can increase levels of the antioxidant enzyme GSH-Px (80).

On the other hand, diets high in fat are associated with increased risk for chronic diseases such as CVD, cancer, hypertension, obesity, diabetes, and gall bladder disease (81,82). The evidence that total dietary fat is associated with elevated levels of inflammatory markers is not as strong as the evidence for specific fatty acids (discussed below in Section K). Although, in men matched for TG levels, those with higher fat intake had higher levels of the adhesion molecules ICAM-1 and e-selectin than those with lower fat intake (83). Further, a study examining children showed that, independent of BMI, total fat intake and the percent energy from fat were both

predictors of CRP (84). It has also been shown that feeding animals high fat, high cholesterol diets results in increased inflammation and endothelial dysfunction (85,86). Several groups have demonstrated that high fat diets increase markers of oxidative stress in both animals (87,88) and humans (89) which can stimulate an inflammatory response. In humans, Erhardt et al determined that a diet high in fat (50%) and low in fiber increased ROS in excrement and plasma MDA compared to a diet low in fat (20%) and high in fiber, showing that even relatively brief periods (12 d) of dietary modification can alter oxidative stress (89). A potential problem with high fat diets is the accompanying increase in free fatty acid (FFA) levels compared to isocaloric high carbohydrate diets (90). Elevations in FFA have been shown to enhance oxidative stress *in vitro* (43) and to impair endothelium dependent vasodilation as measured by flow mediated dilation (FMD) *in vivo* (91) (achieved via intralipid and heparin infusions). Increases in plasma FFA that occurs with high fat intakes is also associated with reduced effectiveness of insulin and may interfere with glucose disposal (92), which could contribute to an insulin resistant state of obese individuals.

Weight Loss Diet Composition Effects on Inflammation and Oxidative Stress

To ensure that overweight individuals attain the disease-reducing benefits of weight loss, the composition of weight loss diets should be considered. Many studies have utilized an AHA style weight loss diet emphasizing a low fat, high complex carbohydrate (LF/HC) eating plan in order to study the effects of weight loss on CVD risk factors. Due to the popularity of non-traditional, non-AHA style weight loss diets, studies have tested the effects of diets differing in macronutrient composition on weight loss and traditional

CVD risk factors. Low carbohydrate, high fat weight loss diets (LC/HF), have been shown to result in greater short term weight loss (93,94), while other studies show that as time progresses (up to 1 year), there are no differences between LC/HF and LF/HC (95). Studies comparing the effects of LC/HF versus LF/HC on blood lipids are surprisingly supportive of LC/HF (93,95,96), in lieu of associations between high fat intakes and CVD. For example, some studies have shown that LC/HF can result in greater reductions in TG (93,95,96) and increases in HDL (95,96) compared to LF/HC, but they may also increase LDL to a greater extent (96).

Less is known, however, about the effects of weight loss diet macronutrient composition on measures of inflammation and oxidative stress. LF/HC (67,97) and Mediterranean style (98) energy restricted diets are reported to reduce CRP, while the effects of LC/HF are mixed. Some authors have reported no difference in effects on CRP between LC/HF and LF/HC diets (99,100), while others showed that CRP decreased more for high risk subjects consuming LC, while low risk subjects consuming LC/HF experienced increases in CRP (101). A study funded by the Atkins foundation reported that IL-6, TNF-α, and ICAM-1 all decreased to a similar extent on both LC/HF and LF/HC diets (100). However, in this study, the diets were given in succession to the same subjects, with no mention of a wash out period, making a comparison difficult due to the potentially confounding effects of weight loss on the second diet.

A larger comparison across 4 types of diets (Atkins, Ornish, Weight watchers, Zone) concluded that weight loss was the key factor, with no influence of diet type (102). However, as subjects reported low adherence to the diets by the last time point, the actual comparison between dietary compositions is weakened. This poses a

significant issue. It is difficult to determine the interaction between weight loss and macronutrient composition due to confounding factors such as poor dietary compliance, supplement usage, or allowable carbohydrate intakes for diet comparisons. For example, subjects in one LC diet study were consuming up to 32% of energy from carbohydrates (CHO) when the last samples were collected (93,101). As this is a three fold higher CHO intake than was intended for the LC group (10% energy from CHO is a common goal for LC diets (100,103)), it could certainly confound comparisons made between LC and HC diets. Interestingly, a study in rheumatoid arthritis patients (non-obese) showed that compared to a modified fast, a ketogenic diet (6-8% energy from CHO) was unsuccessful in reducing inflammation (IL-6, CRP). This is opposed to the reduction that occurred with fasting, even though the diets resulted in similar weight loss (104), indicating that the ketogenic diet somehow impeded the reduction in inflammation associated with weight loss.

It does not appear as though altering the protein content of a weight loss diet has any impact on CRP. Due et al (105) assigned subjects to an *ad libitum* lower fat diet (< 30% of energy) that was either high in protein and low in CHO (25 and 45% energy respectively) or low in protein and high in CHO (12 and 58% energy) for 6 months. Following this dietary intervention, the authors concluded that the ratio of dietary protein to CHO had no effect on CRP, which tended to decrease slightly for all subjects with no difference between groups. Similarly, a recent study (106) compared isocaloric energy restricted diets (1000 kcal), with a vegetarian base, by adding 250 kcals of either protein from chicken or beef or CHO (meat groups had 7-8% greater protein intake). Even

though the chicken group lost more weight than the CHO group, there were no differences in CRP responses after 8 weeks of the dietary intervention.

The impact of the macronutrient composition of reduced energy diets on oxidative stress deserves attention. Overweight men (38-72y) following an ad libitum diet low in fat (10% of calories) and high (70-75% of calories) in unrefined CHO (rich in whole grains and fiber) saw reductions in weight and oxidative stress (8-epi) in as little as 3 weeks (65). However, as this study also included daily exercise, it is difficult to tease out the specific effects of dietary composition. Another study instructed overweight women to consume a fruit rich (15% of energy), reduced energy diet and observed an improvement in oxidative status (107) compared to women consuming a lower fruit diet (5% of energy). Currently, there is little information regarding the effects of a LC/HF weight loss diet on oxidative stress in humans. A recent study tested the effects of a LC/HF weight loss diet on blood antioxidant capacity in healthy women (BMI 21.45 ± 2.05), and reported that LC/HF did not induce oxidative stress and in fact it increased antioxidant capacity of the blood (108). These subjects, however, appeared quite healthy and likely had sufficient endogenous defenses initially. Thus, they were less susceptible to any stressful effects of a short term (2 wk) LC/HF diet. On the other hand, evidence from an animal study disagrees, showing that the beneficial effects of a reduced energy diet in reducing oxidative stress are negated when the diet is high in fat (109). Rats characterized by a self-induced low caloric diet were placed on either a HF or a HC diet. The low intake of calories was similar between groups, and both had reduced weights compared to normal rats, however, the HC group experienced decreased mitochondrial ROS, compared to those in HF. As this could also occur in

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humans, additional study is warranted in this area to further clarify the effects of macronutrient composition of a reduced energy diet on oxidative stress in humans.

I. Acute High Fat Meal Effects on Inflammation, Role of Oxidative Stress

High fat meals can increase inflammation and reduce endothelial function in the postprandial period in comparison to isoenergetic high CHO meals in a variety of populations (110-112) (see Table 2). For example, high fat meals increased IL-6, TNF-α, ICAM-1, and VCAM-1 in diabetics (111) and decreased endothelial function (as measured by flow mediated vasodilation (FMD)) in healthy subjects compared to high carbohydrate meals (113). Additional reports include increased plasma CRP in healthy subjects after a high fat mixed meal (114). However, not all studies in this area agree. For example, an oral fat load (predominantly soybean oil) did not increase markers of inflammation in either diabetics or normal individuals (115). Another study reported decreases in IL-6 and FFA 1 h after a HF meal, while TNF decreased by 4 and 6 h in diabetics (116). The lack of agreement indicates a need to determine the mechanism by which high fat meals can affect inflammation.

It is hypothesized that inflammatory responses are linked to oxidative stress, as evident by attenuations in cytokine levels and improvements in endothelial function when antioxidants are provided with high fat meals (111,113). High fat meals are also shown to activate NF-κB, although the time frame for activation is wide, increasing after 1- 2 h and returning to baseline by 3 h in one study (114) but taking 6-9 h to peak in another (117). Elevated blood lipids from acute high fat meals can contribute to oxidative stress and vascular inflammation. For example, hypertriglyceridemia is

associated with O_2^- production by stimulated neutrophils *ex vivo*, and reduced GSH and

FMD *in vivo* (118). Likewise, FFA are implicated in endothelial dysfunction associated

with oxidative stress. For example, Tripathy et al (119) showed that FFA elevation in

healthy individuals resulted in mononuclear cell activation (increased nuclear NF-kB)

and increased ROS generation by these cells *ex vivo* which corresponded to decreased

FMD. FFA can also inhibit endothelial cell NO production by activating IKKβ (the kinase

responsible for activating NF-κB) (120). The FFA are then quickly (within 2 h of lipid

and heparin infusion) associated with increased free radical production and lipid

peroxidation (121). Of note, these effects were more pronounced in obese than lean

individuals indicating that they may be more sensitive to the effects of FFA (121). As

few studies have examined the response of healthy overweight and obese individuals

(without T2D or CHD), there is a great need for information on this population.

Table 2: High fat meals, inflammation, oxidative stress, effects of antioxidants

Reference Chronological	Subjects	Meal Challenge	Measures Made	Results
Plotnick, 1997 (110)	20M/F, 24-54y, healthy	900 kcal, HF (50 g fat), LF (0 g fat), or HF + 1 g Vit C, 800 IU vitE	FMV	FMV ↓ w/ HF p<0.001, no Δ LF, HF w/ vitamins
Blanco-Colio, 2000 (117)	16 Healthy (8M/8F), 22-33y	Red wine w/ HF meal (no, low or mod dose), 602 kcal/m², 57% fat	NF-κB, 0, 3, 6, 9 h PP	Red wine prevented incr. in NF-κB after meal
Schinkovitz, 2001 (122)	11 healthy, age: 32y, BMI: 25.4	HF (90 g fat) meal, 1200 kcal	RH, FBF, AOX capacity, plasma peroxides, 0-8 h	RH ↓ 2h PP, no Δ AOX cap., FBF, or peroxide levels
Kay, 2002 (123)	8 healthy Males	HF (~50% fat) meal (853 kcal) with or w/o 100 g dried blueberry	ORAC, TAS 0, 1, 2, 3, 4 h PP	ORAC ↑ w/ blueberry at 1 and 4 h PP

Ling, 2002 (124)	74 CHD patients 50 NO CHD	HF meal 800 kcal 50 g F with or w/o 2 g Vit C	FMV	For CHD, HF meal ↓ FMV, which was prev. by Vit C
Nappo, 2002 (111)	20 diab 44 ± 5y, 20 controls BMI:26.8	HF vs HC meal, 760 kcal +/- 800 IU E, 1gC	TNF-α, ICAM-1, VCAM-1, IL-6	HF ↑ cytokines for both, vit supp. prev. negative Δ
Bae, 2003 (112)	10 healthy, 26y, acute	HF, HC, and HF+Vit E meals (800 kcal each),	FMD, MDA, TG	TG↑* after HF w/o Vit E, FMD↓* after HF also. NO Δ MDA any group
Carroll, 2003 (125)	11 Type II diabetics	HF dinner with 800IU E, 1g C Pre-break. or Pre-Supper	CRP, IL-6, MDA, PAI-1, TRAP	Vits prev CRP↑ , but no chg with meal for IL-6, MDA, or TRAP
Plotnick, 2003 (126)	N=38, 36.4+/- 10y, healthy	900 kcal, 50 g fat before and after 4 wk juice plus supplement (JP) or JP + Vitamins	FMV, LDL, HDL, TG 3h PP	JP & JP+V blunted eff. of HF meal on FMV
Aljada, 2004 (114)	9 Healthy subj, 8 controls (water)	900 kcal mixed HF meal vs. water	NF-κB, ROS generation, ICAM-1, CRP For 3h PP	NF-κB ↑ 2x, ROS gen inc., insulin ↑ 1-3 h TG ↑ 2,3h PP, ICAM-1 no chg, CRP ↑
Tsai, 2004 (127)	N=16, 30±5y, healthy, acute	Samples 2, 4, 6h post HF meal (50g fat)	GSH-Px, CRP, ICAM-1, urinary 8-epi	8-epi ↑ 4h after HF, no ΔCRP or ICAM-1
Blackburn, 2006 (128)	38 Overwt men, w/ abdominal obes. 19-62y	60 g fat/m^2 body surface = 1800- 2200 kcal, 65% fat	IL-6, TNF-α, CRP, TG, Insulin 0, 4, 8 h PP	IL-6 ↑, TNF ↓ PP, no Δ CRP. TNF-α correl with insulin AUC
Lundman, 2007 (129)	41 overwt CHD (41-55y), 26 controls	1000 kcal, 65 g fat	0, 2, 4, 6 h PP	IL-6 ↑ in both groups 4h PP, TG inc more for CHD, No chg ICAM-1, eSelectin VCAM-1

HF = high fat, LF = low fat, HC = high carbohydrate, PP = postprandial, FMV/D = flow mediated (vaso)dilation, NF-κB = nuclear factor κB, RH = Reactive hyperaemia, FBF = forearm blood flow, ORAC = oxygen radical absorbance capacity, TAS = total antioxidant status, CHD = coronary heart disease patients, TNF-α = tumor necrosis factor-alpha, ICAM-1 = intercellular adhesion molecule-1, VCAM-1 = vascular cell adhesion molecule-1, IL-6 = interleukin-6, MDA = malondialdehyde, TG = triglycerides, CRP = C-reactive protein, PAI-1 = plasminogen activator inhibitor-1, TRAP = total radical trapping antioxidant potential, LDL = low density lipoprotein, HDL = high density lipoprotein, ROS = reactive oxygen species, GSH-Px = glutathione peroxidase, 8-epi = 8-epi=prostaglandin-F2-alpha.

J. Antioxidants, Value in Obesity and Diet Induced Oxidative Stress

Reductions in endogenous antioxidants are associated with increased risk for oxidative damage. For example, plasma ascorbic acid and carotenoid levels were shown to be inversely related to both F_2-isoprostanes and MDA in an analysis of almost 300 Californian adults (130). As mentioned above, obese individuals often have lower antioxidant defenses and thus may benefit from antioxidant supplements to reduce the risk of chronic diseases associated with oxidative stress. However, the value of antioxidant supplementation as a mechanism for reducing chronic diseases associated with oxidative stress has been questioned. Several large scale antioxidant supplementation trials have shown lack of a clear reduction in atherosclerosis or CVD risk (for review, see Riccioni et al, (131)). However, it was suggested that the lack of a clear benefit may be due to a number of reasons, including the gender, age, and body weight of the patients studied combined with the treatment duration, dosage used, and dietary habits.

The suggestion has also been raised that a positive effect of antioxidants may not occur in individuals without elevations in oxidative stress initially (132). Populations with known oxidative stress, such as diabetics, have been shown to reap a benefit from antioxidant supplementation. For example, provision of T2D subjects with vitamin E (600 mg) for as little as 2 wk reduced urinary 8-epi by 37% (133). Recently, another trial showed 500 mg vitamin E (either as alpha-tocopherol or mixed tocopherols) for 6 wk reduced plasma F2-isoprostanes, although without change in 24 h urine F2-isoprostanes (134). Importantly, in overweight and obese individuals, a recent study showed that 6 months of vitamin E supplementation (800 IU for 3 months, then 1200 IU

for next 3), reduced plasma 8-epi levels by 11% (135). An earlier study by this laboratory (same protocol) showed a decrease in plasma peroxides with this vitamin E regimen, in addition to a slight improvement in insulin resistance (136). The authors pointed out that vitamin E may help to reduce oxidative stress in obese individuals unable to lose weight.

In relation to diet induced oxidative stress, several research groups have tested whether oxidative damage induced by excess dietary fat can be alleviated by antioxidant supplementation. Sreekumar et al (137) fed rats either a high fat or a high fat + antioxidant diet for 36 weeks and observed that the high fat diet reduced the gene expression of antioxidant enzymes important for scavenging free radicals. Specifically, Cu-Zn SOD, Mn SOD and GPx mRNA expressions were 3.6 to 4.5 times lower than controls. Antioxidants were shown, by this group and in studies by others, to attenuate some of the oxidative damage caused by the chronic ingestion of high fat diets (138).

K. Effects of Dietary Fat Type on CVD Risk, Inflammation, and Oxidative Stress

Specific Types of Fatty Acids

Fatty acids are chemically comprised of a carboxyl head group and a long non-polar, hydrocarbon tail. "Saturated" fatty acids (SFA) contain hydrocarbon tails that are fully saturated with hydrogen atoms, with no double bonds present in the chains. "Unsaturated" fatty acids contain carbon to carbon double bonds, which displace hydrogen atoms and are typically in the "cis" conformation, giving them a bent appearance and the ability to enhance cell membrane fluidity. Mono-unsaturated fatty acids (MUFA) have a single double bond while poly-unsaturated fatty acids (PUFA)

have multiple double bonds. Some PUFA are essential to the diet as the body lacks

enzymes capable of placing double bonds closer than 9 carbons to the methyl (omega)

end of the fatty acid chain. Therefore, fatty acids with double bonds closer to the

omega end (omega 6 (n-6) and omega 3 (n-3)) must be consumed in the diet. Common

n-6 fatty acids include linoleic (LA, 18:2n-6) and arachidonic (AA, 20:4n-6) and the most

common vegetable source of n-3 is alpha-linolenic acid (ALA, 18:3n-3), while "long

chain" n-3s (n-3FA) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic (DHA,

22:6n-3) are found in high quantities in cold water fish. n-3FA are best obtained through

the diet, as the body does not produce them very efficiently.

A coordinated sequence of events results in the metabolism of fatty acids,

yielding a variety of bioactive molecules important for the cell-to-cell communication and

the regulation of immunological processes (139). For example, the cytoplasmic

phospholipase A2 (PLA2) releases AA from intracellular membrane phospholipids,

enabling the metabolism of AA by cyclooxygenase (COX), lipoxygenase (LOX), and

cytochrome P450 enzymes. The family of resulting biomolecules are termed

"eicosanoids" and include the COX derived prostaglandins (PG) and thromboxanes

(TX), leukotrienes (LT) and lipoxins from LOX, and epoxyeicosatrienoic acids (EETs)

from cyt-P450. COX produced eicosanoids are known for regulating cardiovascular

effects (vasoconstriction and vasodilation), blood coagulation, and play a role in

inflammatory processes while LOX products are mostly involved in the tissue responses

to inflammation, and EETs modulate vascular resistance (139).

Fatty Acid Specific Effects on CVD Risk and Blood Lipids

In addition to the *amount* of fat in the diet that is associated with health risks, the *type* of fat plays a role as well; arguably a more important one. Diets high in SFA and trans fatty acids (TFA) are associated with a greater risk of CVD, while PUFA intake appears to reduce risk (140,141). Further, diets high in long chained n-3FA are generally associated with favorable effects on blood lipid profiles (reduced triglycerides (TG) and increased high density lipoprotein (HDL) levels), while SFA appear to have negative effects on blood lipids (raising total and LDL cholesterol) (141,142) . Interestingly, Nurse's Health Study data showed that while TFA intake was positively correlated with CVD risk for all subjects, the association between PUFA intake and reduced CHD was strongest for overweight individuals (140). This population may especially benefit from the protective effects of PUFA.

Effects of Dietary Fat Type on Inflammation and Oxidative Stress

Dietary SFA and TFA have been linked in epidemiological studies to higher levels of inflammation, while n-3FA are associated with lower levels (83,143-145). The increased association between SFA and inflammation may be due in part to the fact that diets high in SFA appear to negatively impact insulin sensitivity compared to PUFA (146). This could in turn reduce the anti-inflammatory effects of insulin (147), or consequent to the increase in blood lipids as discussed above. *In vitro*, SFA are also shown to increase activation of NF-kB macrophages (148). SFA may also increase oxidative stress *in vivo* as a study in pigs showed that feeding them beef tallow resulted in higher plasma F2-isoprostanes compared to omega-3 rich fish oil (149). Additionally,

in humans, increased SFA intake has been tied to impaired FMD, a process influenced by oxidative stress (150).

Omega-3 fats on the other hand are shown to decrease inflammation in a number of "at risk" populations, reducing their risk for CVD (151). There is evidence that DHA and EPA are more effective than ALA for anti-inflammatory benefits, thus, the American Heart Association recommends 0.5-1.8 g/d EPA/DHA, or a higher dose of 1.5-3.0 g/d if ALA is the source (152). A well described mechanism of EPA and DHA anti-inflammatory activity is subsequent to cell membrane incorporation. EPA and DHA levels inversely relate to cytokine production *in vitro* (153) and CRP levels *in vivo* (154). Competition with AA for metabolism by metabolic pathways results in the production of less bioactive eicosanoids from n-3FA (155). For example, the leukotriene LTB5 derived from EPA is produced in place of the strong leukocyte chemotactic factor LTB4 (from AA) thereby reducing LTB4 stimulation of cytokines such as TNF-α and IL-1 and IL-6 by monocytes and macrophages (6). However, short exposures to n-3 FA have also yielded anti-inflammatory effects. In cell culture, a 4 h n-3FA exposure reduced NF-kB activity and TNF-α by almost 50% (156), while 3 h decreased neutrophil O_2^{-} (157,158) and TNF-α production by macrophages. This indicates that an acute n-3FA effect *in vivo* may also occur. In regards to oxidative stress, some studies have reported that fish oil supplementation increases markers of lipid peroxidation (159,160), while others report decreases (161) or mixed results (162). Four weeks of 2.4 g/d EPA supplementation reduced *ex vivo* O_2^{-} production by neutrophils (163), as did 5.4 g/d EPA + 3.2 g/d DHA (164) showing that while n-3FA may be more susceptible to oxidation, their byproducts may not result in free radical production or oxidative stress.

In addition, fish oil supplementation is shown to upregulate antioxidant enzymes in diabetics (165) which could help to protect against oxidation and thereby also attenuate inflammation.

L. Acute Effects of Fat Type on Endothelial function, Inflammation, and Oxidative Stress

Americans spend the majority of time each day (17 of 24 hours) in a "postprandial" as opposed to a postabsorptive (or fasted) state (166). The lingering presence of postprandial dietary factors is recognized as a likely contributor to CVD. In particular, it is noted that prolonged elevations in blood lipids contribute to endothelial dysfunction, which can lead to atherosclerosis and eventually CVD (167). Therefore, studying the acute effects of meals varying in fat composition on endothelial function, inflammation, and oxidative stress may be useful in determining the potential "atherogenicity" of the meal composition.

Several groups have reported that meals high in SFA decrease flow mediated dilation (FMD), a measure of endothelial function related to oxidative stress (110,168), while others report no change with meals high in either SFA or MFA (169) (see Table 3). A comparison between the unsaturated fats showed that a meal rich in olive oil (MFA) reduced FMD in comparison to canola oil (PUFA) or salmon oil (n-3FA) (170). This reduction in FMD was prevented by the inclusion of antioxidants with the meal, indicating a role for oxidative stress following consumption of the olive oil. Another group credited the omega-3 fat content of walnuts (PUFA) with attenuating the reduction in FMD associated with a Mediterranean style meal (171). This indicates that there may also be an acute protective effect of omega-3 fat on endothelial function.

Acute fatty acid specific effects on inflammation in the postprandial period are not well defined. One study reported no difference between MFA and SFA rich meals in T2D patients, in fact, both IL-6 and TNF *decreased* after these meals (116). Similarly, Cortes et al reported decreases in inflammatory markers in both healthy patients and patients with high blood cholesterol (171) after PUFA or MFA meals. Another study reported that HDL isolated after PUFA ingestion were more anti-inflammatory than HDL after SFA ingestion in terms of their ability to inhibit endothelial cell expression of ICAM and VCAM *ex vivo* (168). This group reported similar FMD findings, indicating that there may be a relationship between the reduced ability of SFA HDL to prevent adhesion molecule expression and an increase in endothelial dysfunction.

The effect of different fat sources on oxidative stress has been indirectly tested by examining the activation of the redox sensitive transcription factor NF-kB (172). A high fat meal comparison using 3 different fat sources (butter (high in SFA), olive oil (MFA), and walnuts (PUFA)) showed that SFA increased NF-kB activation by 3 h postprandial and subsequently higher ICAM-1 concentrations were noted by 9 h compared to both MFA and PUFA. PUFA also increased NF-kB, but not until 9 h, while MFA had no effect. Therefore, SFA appeared to increase inflammation acutely, possibly mediated by oxidative stress, however no additional markers of oxidative stress were used to confirm these results. The use of different whole foods in testing the relationship between dietary fats and inflammation or oxidative stress is useful; however it does not efficiently remove extraneous influences such as the contribution of other components present in different whole foods (fiber, protein, micronutrients).

Additionally, the specific effects of (long chain) n-3FA have yet to be addressed in terms of their acute effects on the relationship between oxidative stress and inflammation.

Table 3: High fat meal effects on flow mediated dilation (FMD), oxidative stress, inflammation in healthy subjects

Reference Chronological	Subjects	Meals, type of fat used	Measures	Time frame, start results	Results
Raitakari, 2000 (169)	12 healthy 7M/5F, 33±7y BMI: 24.3±3.1	SFA vs MFA: 1030 kcal, 61g fat -oil or tallow	FMD, forearm blood flow (FBF)	0, 3, 6h PP 1 wk b/w trials	FBF ↑ No chg in FMD
Vogel, 2000 (170)	10 healthy, 5M/5F; 28-56y	900 kcal, 50g fat, olive oil, salmon, canola	FMD	0, 3h PP	FMD ↓ only for olive oil meal
Bellido, 2004 (172)	8 M, healthy not obese	50-66% daily kcal, 1g/kg fat, butter, olive oil, or walnuts	PBMC NF-kB, plasma ICAM-1	0, 3, 6, 9h sep by 1 wk	Butter ↑ NF-kB 3h, waln. 9h; ICAM-1 ↑ for SFA 9 h
Manning, 2004 (116)	18 T2D, 61 ± 10y, BMI: 28.5 ± 4.4	0.6g/kg BW SFA cream vs MFA olive oil vs Cont (LF)	Plasma IL-6, TNF-α, TG, FFA, insulin	1, 4, 6h PP 1wk b/w trials	↓ IL-6 1h PP ↓ TNF-α 4 h PP
West, 2005 (173)	18 healthy T2D, rand crossover	50g fat (MFA), or subst 3-5g of n- 3s (EPA & DHA or ALA)	FMD	0, 2, 4, 8h PP 3wk crossover	FMD inc 17% but inc. 50-80% after 3-5g EPA/DHA
Blum, 2006 (174)	10 healthy M, 26.6±1.1y BMI: 24.8± 0.8	975 kcal, 50gF Mediter. meal, Western meal after1 wk	Serum CRP, PON-1	0, 2, 4, 7h PP	PON-1 ↑ w/ Med, not Wes CRP ↓ w/ Mediterran.
Cortes, 2006 (171)	12 normal 12 high chol.	1200 kcal, 80g fat - sources: walnuts or olive oil	FMD, ICAM-1, VCAM-1, e-selectin, oxLDL	FMD worse after olive oil than walnuts	↓ oxLDL, ICAM, VCAM after meals
Nicholls, 2006 (168)	14 healthy 8M/6F, 29.5 ± 2.3y, BMI: 23.6 ± 0.8	MFA olive oil PUFA walnuts	FMD, PP HDL eff. on stimulated ICAM,VCAM in HUVEC	0-6h PP FMD dec 3 h PP for SFA	SFA HDL did not prevent stimul. ICAM & VCAM expression

SFA = saturated fat, MFA = mono-unsaturated fat, PUFA = polyunsaturated fat, FMD = flow mediated dilation, FBF = forearm blood flow, PP = postprandial, PBMC = peripheral blood mononuclear cells, NF-κB = nuclear factor-κB, ICAM-1 = intercellular adhesion molecule-1, IL-6 = interleukin-6, TNF-α = tumor necrosis factor-alpha, TG = triglycerides, FFA = free fatty acids, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ALA = alpha-linolenic acid, CRP =

C-reactive protein, PON-1 = paraoxonase-1, oxLDL = oxidized low density lipoprotein, HDL = high density lipoprotein

M. Summary

Obesity is an independent risk factor for chronic diseases such as CVD and T2D. It is also associated with inflammation and oxidative stress, both of which contribute to the development and perpetuation of these diseases. Diets differing in macronutrient content impart different levels of CVD risk, potentially due to effects on oxidative stress and inflammation. In particular, high carbohydrate diets are associated with lower CVD risk and lower inflammation while high fat diets are linked to higher CVD risk and inflammation.

Due to metabolic disturbances (such as insulin resistance and dyslipidemia) and their pro-inflammatory pre-disposition, obese individuals may be more sensitive to dietary stressors. It is known that weight loss can reduce oxidative stress and inflammation, but the interaction between weight loss and macronutrient composition of a weight loss diet has not been adequately assessed for effects on these factors. Similarly, the chronic effects of dietary fat on oxidative stress and inflammation are shown to be fatty acid specific, but since most of the day is spent in the postprandial state, clarifying the acute postprandial effects of different fat sources would be valuable. Obese individuals display exaggerated inflammatory responses to elevations in blood lipids, therefore, they may be particularly susceptible to differential effects imparted by specific fatty acids. Information regarding the effects of macronutrients on oxidative stress and inflammation will help to refine dietary recommendations for this population.

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Chapter III.

"High Fat, Low Carbohydrate Diet Increases C-Reactive Protein during Weight Loss"

Title: High Fat, Low Carbohydrate Diet Increases C-Reactive Protein during Weight Loss

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Abbreviations: 8-epi = 8-epi-prostaglandin F2α, CRP = C-reactive protein, HF = high fat,

low carbohydrate diet, LF = low fat, high carbohydrate diet, IL-6 = interleukin-6, BMI =

body mass index, FFA = free fatty acids, ROS = reactive oxygen species

Abstract

Objective: Cardiovascular disease (CVD) is considered an inflammatory condition of complex etiology, although oxidative stress appears to play a role. Obesity is associated with elevated inflammation and oxidative stress, which may contribute to CVD risk. The purpose of this study was to determine the effects of macronutrient composition of a weight loss diet on oxidative stress and inflammation in overweight women. *Methods:* Thirty-two overweight (BMI > 25 kg/m^2) women were randomly assigned to follow an *ad libitum* low carbohydrate, high fat (HF) or an energy restricted low fat, high carbohydrate (LF) weight loss diet for 4 weeks. Fasted blood samples were analyzed for markers of inflammation (C-reactive protein, CRP; interleukin-6, IL-6) and metabolic factors (glucose, free fatty acids, FFA) while urine was analyzed for oxidative stress (8-epi-prostaglandin-F$_{2\alpha}$, 8-epi). *Results:* Both groups lost a significant amount of weight (p<0.01), although HF lost more (3.8 ± 1.2 kg HF vs. 2.6 ±1 .7 LF, p=0.04). CRP increased over the 4 weeks for HF but decreased for LF dieters (p<0.01). IL-6 increased for both groups over time (p<0.05) while 8-epi changed differently over time for the two groups (p=0.035), but with no consistent pattern. Glucose decreased over time (p<0.05) with no difference between groups, while FFA increased for both groups with a greater increase in HF (p<0.05). *Conclusion:* Composition of a weight loss diet differentially affected serum CRP levels, which was apparently unrelated to oxidative stress. As differences in CRP response could indicate differences in CVD risk, a low fat weight loss diet is recommended.

Keywords: C-reactive protein, obesity, energy restriction, weight loss

Introduction

      The number of overweight and obese US adults and children continues to rise with each national survey conducted. The most recent data from the NHANES survey shows that 66.3% of adults are overweight and 32.2% are obese (2). Low carbohydrate diets are currently one of the more popular weight loss strategies as an estimated 20 million people are using this type of diet worldwide (3). A low carbohydrate approach to weight loss has been touted since the 1800's but most recently was introduced by Dr. Robert Atkins in his popular diet books first published in the 1970's and revised in the 1990's (4).

      Traditionally, nutritionists have cautioned against use of low carbohydrate diets because the high fat and cholesterol intake was thought to cause an increase in blood lipids and thus heart disease risk. Recently, however, a number of laboratories have reported that weight loss and blood lipid changes are at least comparable or even superior with a low carbohydrate compared to a low fat approach (5-8). Although important, elevated blood lipids are only risk factor or heart disease risk. Many people who develop heart disease have normal blood lipids. One marker, C- reactive protein (CRP), appears to increase heart disease risk irrespective of blood lipids (9,10). Elevated CRP, an acute phase protein, can be an index of chronic, low level inflammation. Increased levels of pro-inflammatory factors have been noted in adipose tissue, macrophages in adipose tissue, and circulating mononuclear cells in obese individuals (11). It has been suggested that these inflammatory mediators may activate endothelial cells, stimulating expression of adhesion molecules and further release of cytokines associated with migration of leukocytes between smooth muscle cells and into

vessel walls. Obesity is associated with higher concentrations of serum CRP (10,12), suggesting one mechanism linking obesity to increased heart disease risk. The causes of high CRP are not entirely clear, but weight loss usually reduces concentration of this protein (13). Although specific dietary effects on CRP have not been fully elucidated, diets rich in fruits, vegetables and grains (14,15) and high in fiber (16) have been shown to be associated with lower CRP while high trans fat and glycemic load diets are associated with higher CRP in epidemiological studies. The 20 g of carbohydrate allowed per day in the early part of the described diet and 5-10% of total calories later in the plan, requires elimination of many foods high in carbohydrate and relies on foods high in protein and fat.

Studies have reported an association between obesity and higher oxidative stress markers (17). It is thought that the accumulation of cellular reactive oxygen species (ROS) is a perpetuator of inflammation via activation of redox-sensitive nuclear transcription factors such as AP-1 and NF-κB and subsequent increase in expression of inflammatory factors (18). The connection between a chronic inflammatory state and oxidative stress is shown in obese individuals displaying elevated levels of NF-kB in circulating immune cells (19).

Although research is limited, diets high in lipid content (particularly saturated fat and cholesterol) are shown in several studies to be associated with higher markers of oxidative stress in both animals (20,21) and humans (22). As the Atkins diet is high in fat and low in dietary antioxidants provided from fruits, vegetables, and whole grains, those who follow the diet may be more susceptible oxidative stress. The connection of these changes in oxidative stress to inflammation has not been studied.

Our hypothesis was that subjects following a low carbohydrate, high fat diet would demonstrate higher markers of oxidative stress and inflammation compared to similar individuals following a low fat, higher carbohydrate traditional weight loss diet (1).

Research Methods and Procedures

Subjects

Thirty two female subjects, aged 32-45, were recruited from a local community and screened for contraindications to energy restriction and weight loss as well as for medical conditions or medications known to influence inflammation. All subjects were pre-menopausal, weight stable for at least 6 months, nonsmokers, overweight >25 kg/m^2 (BMI 32.1±5.4 kg/m^2), sedentary (less than 2 hrs exercise per week), but otherwise healthy. Subjects were requested to immediately discontinue any supplement use upon acceptance into the study so that no subjects consumed any supplements within 2 wks of the start of the study. The study was approved by the Institutional Review Board of Virginia Tech. Three subjects discontinued the study of their own volition during the first week so the results for 29 women are presented.

Study Design and Procedures

Subjects were randomly assigned to follow either an *ad libitum* low carbohydrate, high fat (HF) diet or a calorie restricted low fat (LF) diet for 4 wks. HF dieters were provided with a copy of "The New Diet Revolution" by Robert Atkins (ref,2001) as well as additional handouts prepared by the experimenters to help them to follow the diet. LF diet subjects were provided with an exchange system plan based on their body weight and were instructed in how to prepare and record selections and serving sizes. Energy goals for the LF diets were determined by using estimated resting metabolic rate and a

factor for low daily physical activity to achieve weight loss of 0.5-1.0 kg/wk (RDA subcommittee, 1989). Subjects were assigned to the closest of three energy levels (1300, 1500, 1700 kcal/d) with 20-25% fat, 55-60% carbohydrate, and 15-20% protein. All subjects attended a weekly education and social support session (separate sessions for the two diet groups). Subjects were asked to complete 4 d food records during each week that were examined to assess compliance. Diet records were analyzed using nutrition analysis software (Food Processor® dietary analysis software (ESHA Research, Version 8.1; 2003).

Subjects came to the laboratory each week between 7:00 and 9:30 a.m. in a fasted state. They were weighed on a calibrated scale, waist and hip circumferences were measured, and blood samples were collected via venipunture by a certified phlebotomist. The blood samples were allowed to clot and then centrifuged at 1000 x g for 15 min. Serum was collected, divided into separate aliquots, and frozen at -80°C for later analysis. Subjects were also asked to collect all of their urine for 24 h prior to the laboratory visit. Total volume of the urine collection was measured and the urine was immediately tested for ketones (Acetest Reagent Tablets (Bayer Corporation, Elkhart, IN). Two 2 ml samples of the urine were frozen at -80° C for later analysis of 8-epi-prostaglandin-$F_{2\alpha}$ (8-epi) and creatinine.

<u>Sample Analysis</u>

Serum was analyzed for high sensitivity C-reactive protein (CRP) and interleukin-6 (IL-6) using ELISA (United Biotech, Inc.; Mountain View, CA and R&D systems; Minneapolis, MN, respectively). Serum glucose was measured by an enzymatic colorimetric assay (Stanbio, Boerne, TX) and serum non-esterified (free) fatty acids

(FFA) were analyzed via a spectrophotometric method adapted for microplate (Wako, Richmond VA). All analyses were done in duplicate.

Urine collections (24 h) were analyzed in duplicate for urinary 8-epi using a competitively linked ELISA (Oxis International, Portland, OR). Urinary creatine was measured in each urine sample (Stanbio, Boerne, TX) and the amount of 8-epi was normalized to urinary creatinine.

<u>Statistics</u>

Baseline characteristics of subjects were compared using t-tests. All other dependent measures were analyzed using mixed model repeated measures ANOVA (SPSS, version 13.0) and $p < 0.05$ was considered significant. Changes in dependent measures from baseline to week 4 were also calculated and compared using t-tests. Associations between dependent measures were analyzed using Pearson's Product moment correlation.

Results

<u>Body weight</u>

All subjects lost a significant amount of weight ($p < 0.01$), while HF lost more weight than LF over the dietary period (3.8±1.2 HF vs. 2.6±1.7 kg LF, $p = 0.04$).

<u>Diet records</u>

There were no significant differences in the baseline diet of the two groups (Table 2). Reported caloric intake between the groups was remarkably similar (1376±231 and 1356±204 kcal/d HF and LF respectively) during the weight reduction period in spite of very different macronutrient composition of the diets. The percent energy consumed from fat, carbohydrate, and protein was 24, 59, 18 for the LF group and 58, 12, 30 for

HF (p<0.01 for all). Among the antioxidant vitamins, the reported vitamin C and vitamin A intakes were significantly higher for the LF diet group (p<0.05) but there were no differences in vitamin E (Table 3).

<u>Serum measures</u>

Initial serum CRP was positively correlated with FFA (r=0.44, p=0.01), glucose (r=0.52, p=0.004), waist circumference (r=0.45, p=0.02), and IL-6 (r=0.46, p=0.01). Baseline serum glucose correlated with serum IL-6 (r=0.54, p=0.002) and FFA (r=0.38, p=0.04).

Serum glucose decreased 4% over the 4 weeks of dieting for both groups with no difference between the groups (85.4 baseline vs. 82.1 mg/dL week 4, p<0.01). Although the reduction in glucose was negatively correlated with the change in serum FFA (r= -0.55, p=0.002). IL-6 increased about 16% for groups combined (1.39 to 1.62 pg/mL, p = 0.04) with no difference in the response by diet. FFA increased for both groups (p<0.05) but the increase was greater from baseline to week 4 for HF than LF (p<0.05).

There was a significant interaction of groups over time for the CRP response to the different diets (Table 2, p<0.01). By week 4, CRP had increased for HF and decreased for LF dieters (+25% vs. -43% respectively, p=0.02). Figure 1 demonstrates the individual responses in CRP to the two diets from baseline to week 4, where it is evident that almost all subjects in LF experienced decreases while many in HF increased from baseline to week 4.

<u>Urine measures</u>

There was no consistent effect of weight loss on 8-epi excretion when groups were combined (Table 2). A significant interaction between groups over time did not appear

to be meaningful as concentrations for both groups oscillated over time with no clear pattern. Neither baseline nor change in 8-epi correlated with any other variable measured (Table 4).

Discussion

The most important finding from this study is that a low carbohydrate, high fat weight loss diet was associated with an increase while a low fat diet was associated with a reduction in serum CRP. This was in spite of the fact that, as in other studies (5,6,8), the low carbohydrate diet group lost more weight than the low fat diet group. Many studies have shown a strong association between BMI and blood CRP (23,24). The mechanism for higher serum CRP in obesity is not entirely clear, but has been suggested to be secondary to factors such as increased cytokine release from adipocytes, insulin resistance, and oxidative stress.

The effect of weight loss on inflammatory markers, particularly CRP, has recently gained attention. Most, but not all (25), studies have demonstrated a reduction of this inflammatory marker in response to diet-induced weight loss. For example, two studies reported 26-32% reductions in serum concentrations of CRP in obese women who lost a modest amount of weight over 3 to 14 months (26,27). The declines in serum CRP were correlated with the magnitude of weight change. Similarly, a study in obese men showed significant reductions in various inflammatory markers including CRP, IL-6, soluble TNF receptors (sTNF-R55, sTNF-R75), and PAI-1 antigen following 8 wks of energy intake reduction that caused a 9.4 kg weight loss (28). Kopp et al (29) studied 37 morbidly obese patients before and 14 months following gastric bypass surgery and noted that the effects of weight loss were not uniform. CRP and IL-6, but not tumor

necrosis-alpha (TNF-α) fell with weight loss and the reduction in CRP correlated with a decrease in BMI, while the change in IL-6 correlated with improvements in insulin resistance. Disconnect between the responses of CRP and IL-6 have been reported in other studies as well (30) demonstrating the complexity of the relationship between weight loss and markers of inflammation.

The potential effect of dietary composition on CRP can not be determined from most studies, due to lack of dietary control and/or comparison between various compositions. Interestingly, one study reported that in contrast to weight loss induced by fasting, a ketogenic diet (<40 g/d carbohydrate, high fat and protein) in rheumatoid arthritis patients did not reduce CRP and IL-6. As weight loss was similar between the two treatments, it must be considered that the ketogenic diet interfered with the normal reduction in inflammation associated with weight loss (31). Several clinical trials comparing the effectiveness of low carbohydrate to low fat diets measured effects on serum CRP and other inflammatory indicators in obese individuals. Two studies found no specific effect of diet composition in that reduction in serum CRP was associated with only the amount of weight lost (32). A third study found that both a low carbohydrate and a low fat diet reduced serum CRP but that the low carbohydrate diet was actually superior for reduction in this factor when subjects began with elevated serum CRP (34). On the other hand, subjects who began their study with low or intermediate serum CRP and were assigned to the low carbohydrate diet experienced increases in their average serum CRP. The reason for the differential effect based on initial serum CRP is unclear, and the authors did not provide any theoretical basis for these findings.

Our hypothesis was that the low carbohydrate, high fat diet composition would influence oxidative stress that would subsequently increase inflammation as illustrated by elevated serum CRP. Several diet manipulation studies in rodents or rabbits support a connection between high fat diets (high in saturated fats and cholesterol) and oxidative stress (20,21). There is limited evidence that a high fat diet increases oxidative stress in humans. Subjects who consumed a high fat (50%), low fiber diet for one 12 day trial and a low fat (20%), high fiber diet in another had 13 fold higher hydroxyl radical production in feces during the high fat diet phase (22). In addition, plasma malondialdehyde concentrations were 47% higher after the high fat diet. This study shows that relatively brief periods of dietary modification, specifically high fat diet, can alter oxidative stress in humans. Although they were not measured, this increase in oxidative stress could theoretically stimulate expression of inflammatory factors.

Although we observed an increase in serum CRP with the higher fat, lower carbohydrate diet, the marker of oxidative stress used was not higher in that group. This initially does not support our hypothesis, but it is possible that our urinary 8-epi measurement was not sensitive enough or not the ideal marker to detect a change in oxidative stress in these individuals. Although 8-epi is touted as an excellent indicator of oxidative stress associated with atherosclerosis (35), there is uncertainty as to which is the best marker for oxidative stress *in vivo* (36). Inconsistencies exist among different markers of oxidative stress to the same intervention, and differences in methodology between studies may also play a role. For example, the ELISA method for measuring 8-epi has recently been criticized as less specific than the GC/MS method (37). Further

research will need to be conducted to clarify whether diet composition influences other markers of oxidative stress.

Our results may differ from those of other studies due to differences in supplements, medications, and dietary compliance. For example, Yancy et al (8) provided vitamin and fish oil supplements to the Atkins group, while lipid lowering medications were allowed by Seshadri et al (34) which could have confounded results. Studies that did not provide, but did not prohibit supplements are equally suspect as differential use by subjects could influence oxidative stress and inflammation. Therefore, if oxidative stress is the link between diet composition and inflammation, studies allowing supplements would be expected to have different results. Differences in dietary compliance also make it difficult to compare studies examining the effect of diet composition of a weight loss diet on inflammation. For example, although both diet groups studied by Seshadri et al experienced a reduction in serum CRP as a result of weight loss, the drop was greater for those in the low carbohydrate group if they began with higher CRP levels. The diet reported via 24-hour dietary recall in the subjects from the same trial in another publication (7), suggests that dietary compliance to the low carbohydrate diet was poor by the 6 month blood measurement point. Although carbohydrate intake was different between groups (37% and 51% of energy for low carbohydrate and low fat, respectively) it was not close to the goal of less than 30 g per day carbohydrate for the low carbohydrate diet group requested by the investigators. Similarly, O'Brien et al (32) reported no effect of dietary composition on serum CRP, however, their data also shows that dietary compliance was compromised by the 6 month time point when blood was measured for this inflammatory marker (5). Although

the LC group was consuming within the goal of up to 60 g/d of carbohydrate per day at 3 months (~43 g/d and 15% of energy), it was up to about 98 g/d and 30% of energy at 6 months. Therefore, any differences in the treatments in both these studies may have been confounded by the 6 month point when blood CRP was measured. It would be less likely that there would be a different metabolic response to diets with such a lower degree of dietary compliance. In contrast, the reported carbohydrate intake by our HF diet group was much closer to the target value of 10% carbohydrate consumption (12%) while the LF group was on target with their fat consumption (24%). This may have enhanced our ability to detect differential responses of CRP to diet composition.

A shorter study (two 6 wk dietary periods) by Sharman et al (38) had better dietary compliance as the data from 7 d diet records showed that dietary carbohydrate intake was within the goal of < 10% of energy (56% and 8% of energy for low fat and low carbohydrate, respectively). However, the cross-over design of this study with no wash out between trials makes the interpretation difficult as subjects would theoretically be in different metabolic states at the start of each diet period. In addition, as it was not mentioned that the treatments were counterbalanced, it is assumed that all subjects followed the two diets in the same order. Although the total dietary fat was significantly different between diet groups (23 and 63% of energy, for low fat and low carbohydrate, respectively), the proportion of the various types of fats (saturated, monounsaturated, polyunsaturated) was remarkably similar (45%, 34%, 21% vs 40%, 42%, and 17% for LF and LC, respectively). There was no provided estimate of more specific types of fat (i.e. trans or omega 3 fat) consumed, but data from the Nurse's Health Study suggests that trans fat increases and omega 3 fats reduce CRP (39,40). Therefore, future

studies should attempt to control or measure dietary content of these fatty acids when examining the effect of diet composition on inflammation.

The significance of a differential effect in CRP in otherwise healthy individuals deserves consideration. The elevation experienced by the HF dieters pushed them further into the high risk category for CRP (>3 mg/L) (41). This is not desirable, as a recent analysis of the relationship between CRP levels and CVD risk showed that CRP in the highest quartile was associated with a 3 fold risk in MI and 2 fold risk in stroke and peripheral artery disease compared to the lowest quartile (42). On the other hand, the women in LF decreased from 4.8 to 2.6 mg/L, which re-categorized them from a high risk to a moderate risk group (41). It is difficult to say whether the increase in CRP or the prevention of a decrease with weight loss is of greater consequence for the women following HF. Longer term studies are necessary to determine whether the CRP reducing effect continues with LF diet induced weight loss and whether HF dieters remain elevated.

Although we have focused on the differences between fat and carbohydrate contents of the two diets, protein intakes differed as well. However, it does not appear from the literature as though altering the protein content of a weight loss diet has any impact on CRP. Due et al (43) assigned subjects to an *ad libitum* lower fat diet (< 30% of energy) that was either high in protein and low in CHO (25 and 45% energy respectively) or low in protein and high in CHO (12 and 58% energy respectively) for 6 months. Following this dietary intervention, the authors concluded that the ratio of dietary protein to CHO had no effect on CRP, which tended to decrease slightly for all subjects with no difference between groups. Similarly, a recent study (44) compared

isocaloric energy restricted diets (1000 kcal), with a vegetarian base, by adding 250 kcals of either protein from chicken or beef or CHO (meat groups had 7-8% greater protein intake). Even though the chicken group lost more weight than the CHO group, there were no differences in CRP responses after 8 weeks of the dietary intervention. The difference in protein intakes between groups in our study was similar to these studies, indicating that the differential response in CRP is likely due to the greater differences in fat and CHO content.

Our study was not without limitations. Subjects were free living, and although they received weekly instructional and diet support sessions, they ultimately chose their own foods. However, as mentioned, our subjects reported good compliance with the dietary restrictions of their assigned group. We also examined a relatively small number of subjects, and we assessed the diet effects for only 4 weeks. Thus, longer term studies are necessary to more fully understand the effects of consuming HF over time, and whether the trend for an increase continues or whether an adaptive response occurs.

The most important result of this study was that CRP increased in overweight women after consumption of a HF diet while it decreased in women consuming a LF diet. This was in spite of the fact that the HF dieters lost more weight. This study only examined one indicator of oxidative stress which may have been insufficient to detect changes in these individuals, thus additional work to test the role of oxidative stress following HF is necessary. In the mean time, a LF weight loss diet appears more prudent for reducing CVD risk.

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Table 1. Baseline subject characteristics (Printed in Rankin & Turpyn 2007 (1))

Diet	Age	Wt (kg)	BMI (kg/m^2)	W:H ratio
HF	38.8 ± 3.8	87.3 ± 15.2	32.7 ± 5.5	0.78 ± 0.06
LF	40.1 ± 3.0	79.2 ± 16.0	31.4 ± 5.4	0.81 ± 0.05

Values are averages ± SD. HF indicates high fat, low carbohydrate diet; LF indicates

low fat, high carbohydrate diet; W:H indicates waist to hip ratio.

There were no significant differences between the groups for these factors.

Table 2. Body weight, serum and urinary metabolites at baseline and each week of the intervention (1).

Measure	Group	Week 0	Week 1	Week2	Week 3	Week 4
Body Weight (kg) [1]	HF	87.3±15.2	85.0±15.0**	84.4±15.1**	83.8±14.9**	83.5±14.8**
	LF	79.2±16.0	78.3±16.0**	78.0±15.7**	77.1±15.9**	76.6±15.7**
CRP (mg/L) [2]	HF	5.7±5.5	7.5±4.4*	6.1±4.9	6.1±5.8	7.1±6.1*
	LF	4.8±4.2	4.2±3.8†	4.3±4.4	4.1±4.5	2.7±2.9†**
IL-6 (pg/mL) [3]	HF	1.60±0.78	1.55±0.77	1.55±0.75	1.25±0.44	1.78±0.73
	LF	1.20±0.76	1.23±0.74	1.11±0.67	1.18±0.75	1.47±1.03
8-Epi (pg/mg creatinine) [1]	HF	1576±511	1182±661*	1365±921	1124±538*	1495±797
	LF	1246±543	1541±1094	1227±736	1323±693	1064±624
Glucose [4] (mg/dL)	HF	85.6±5.5	79.7±8.3	78.7±5.7	81.6±7.3	81.6±7.5
	LF	84.7±10.7	83.6±6.9	79.0±6.3	81.7±6.0	83.3±4.7
FFA [5] (mEq/L)	HF	0.32±0.13	0.58±0.21	0.57±0.13	0.54±0.20	0.56±0.24
	LF	0.27±0.10	0.39±0.11	0.37±0.16	0.41±0.10	0.36±0.14

Mean ± SD

CRP = C-reactive protein, BMI = body mass index, IL-6 = interleukin 6, FFA = free fatty acids, 8-epi = 8-epi-prostaglandin F2α

* Different from baseline $p < 0.05$, ** $p < 0.01$

† Different from HF diet $p < 0.05$, ‡ $p < 0.001$

[1] Group by time interaction $p < 0.05$

[2] Group by time interaction $p < 0.01$

[3] Main effect of time $p < 0.05$, (week 4 differs from week 0)

[4] Main effect of time $p < 0.01$ (weeks 1, 2, and 3 different from baseline)

[5] Main effect of group $p < 0.01$, main effect of time $p < 0.001$ (all wks different from wk 0)

Table 3. Average dietary intake obtained from repeated 4 d diet records by group (1).

Nutrient	Grp	Wk 0	Wk1	Wk 2	Wk 3	Wk 4
kcals/day[1]	HF	2001+24	1251+337	1357+283	1442±304	1453±304
	LF	2274±1095	1283+200	1462±191	1322±297	1395±325
kcal/kg[1]	HF	24.0± 8.2	15.0± 4.3	17.0±5.7	17.0±4.8	18.2±5.9
	LF	30.0±16.0	17.0 ± 4.1	19.6±4.9	17.6±4.2	18.7±5.2
Fat kcal/kg[2]	HF	8.9 ±3.1	8.2 ±2.6	10.1 ±3.2	10.5 ±2.8	11.2±4.0*
	LF	10.9 ± 8.9	4.3 ± 1.2*‡	4.9±1.6*‡	4.3±1.8*‡	4.4±1.9*‡
CHO kcal/kg[2]	HF	12.0 ±4.6	1.4 ±0.6**	1.6±0.8**	1.9 ± 0.8**	2.0±0.9**
	LF	15.2 ±7.2	9.9 ±2.6*‡	11.1±3.6‡	10.5 ± 2.4*‡	11.2±3.4*‡
PRO kcal /kg [3]	HF	3.6 ±1.3	5.0 ±1.7*	5.15±2.1**	5.1 ± 1.7*	5.2±1.8**
	LF	4.2 ±1.2	3.3 ±0.8*‡	3.5±0.9‡	3.2 ±0.9*‡	3.4±1.1‡
Fat % of total energy [2]	HF	37 ± 4	55 ± 7**	60 ± 6**	60 ± 9**	61 ± 4**
	LF	34 ± 7	25 ± 4**‡	26 ± 6**‡	24 ± 6**‡	23 ± 8**‡
CHO % of total energy [2]	HF	49 ± 6	9 ± 3**	10 ± 4**	11 ± 5**	12 ± 5**
	LF	51 ± 7	58 ± 4*‡	57 ± 7*‡	60 ± 5**‡	60 ± 10*‡
PRO % of total energy [2]	HF	15 ± 3	34 ± 12**	30 ± 4**	28 ± 6**	29 ± 5**
	LF	15 ± 4	19 ± 3**‡	18 ± 2**‡	18 ± 3**‡	18 ± 2*‡
Vit C (mg/d) [4]	HF	60.1 ± 34.6	62.6 ± 44.5	63.4 ± 40.6	65.6 ± 40.7	60.1 ± 44.1
	LF	105.9 ± 105.7	130.2 ± 54.2*	140.0 ± 55.9*	132.0 ± 69.0*	124.4 ± 69.5*
Vit E (mg/d)	HF	31.5 ± 18.0	33.2 ± 29.8	24.8 ± 10.4	37.2 ± 20.3	36.5 ± 19.6
	LF	33.2 ± 12.1	33.0 ± 26.5	30.9 ± 20.4	29.8 ± 26.9	35.4 ± 16.8
Vit A (RE) [5]	HF	787.2 ± 392.2	788.7 ± 370.4	790.6 ± 361.2	721.4 ± 275.9	837.6 ± 414.9
	LF	762.0 ± 194.3	1447.6 ± 661.1**‡	1414.7 ± 516.4**‡	1568.0 ± 621.3**‡	1498.6 ± 826.7**†

HF = low carbohydrate, high fat diet, LF = low fat, high carbohydrate diet, Kcal = kilocalories, CHO = Carbohydrate, PRO = Protein, Vit = vitamin

Mean ± SD

* Change from baseline $p < 0.05$, ** $p < 0.01$

† Different from HF diet $p < 0.05$, ‡ $p < 0.01$

[1] Main effect of time $p < 0.01$, all weeks different from baseline (week 0).

[2] Main effect of time, group $p < 0.001$ and group x time interaction all $p < 0.001$, all time points different from week 0, groups different weeks 1-4.

[3] Main effect of group $p < 0.01$, group x time interaction $p < 0.001$, groups different weeks 1-4.

[4] Main effect of group $p < 0.001$.

[5] Main effect of time $p < 0.05$, group x time interaction $p < 0.05$, all weeks different from week 0, groups different weeks 1-4.

Table 4: Correlations between change scores in dependent measures and selected values.

Measure	Pearson Correlation P values	BMI 0	CRP 0	Gluc 0	8epi Δ	CRP Δ	Gluc Δ	IL6 Δ	FFA Δ	Wt Δ
BMI 0	Correlation	1								
	p-value									
CRP 0	Correlation	.359	1							
	p-value	.056								
Gluc 0	Correlation	.086	.523**	1						
	p-value	.658	.004							
8-epi Δ	Correlation	-.336	-.116	-.029	1					
	p-value	.080	.556	.882						
CRP Δ	Correlation	.049	-.152	-.324	.149	1				
	p-value	.802	.430	.086	.450					
Gluc Δ	Correlation	.117	-.366	-.730**	-.206	.044	1			
	p-value	.544	.051	.000	.294	.819				
IL-6 Δ	Correlation	.136	.241	.131	.071	-.214	.030	1		
	p-value	.481	.207	.497	.718	.266	.879			
NEFA Δ	Correlation	-.053	.038	.215	.108	.282	-.546**	-.162	1	
	p-value	.784	.844	.262	.585	.138	.002	.401		
Weight Δ	Correlation	-.240	-.241	-.416*	.107	-.232	.315	-.082	-.185	1
	p-value	.209	.207	.025	.587	.227	.096	.671	.337	

"0" indicates baseline value, "Δ" indicates change in measure.

CRP, C-reactive protein; BMI, body mass index; IL-6, interleukin 6; FFA, free fatty acids;

8 –epi, 8-epi-prostaglandin $F_{2\alpha}$

* indicates significance p<0.05

** indicates significance p<0.01

Figure 1. Individual change in C-reactive protein by group.
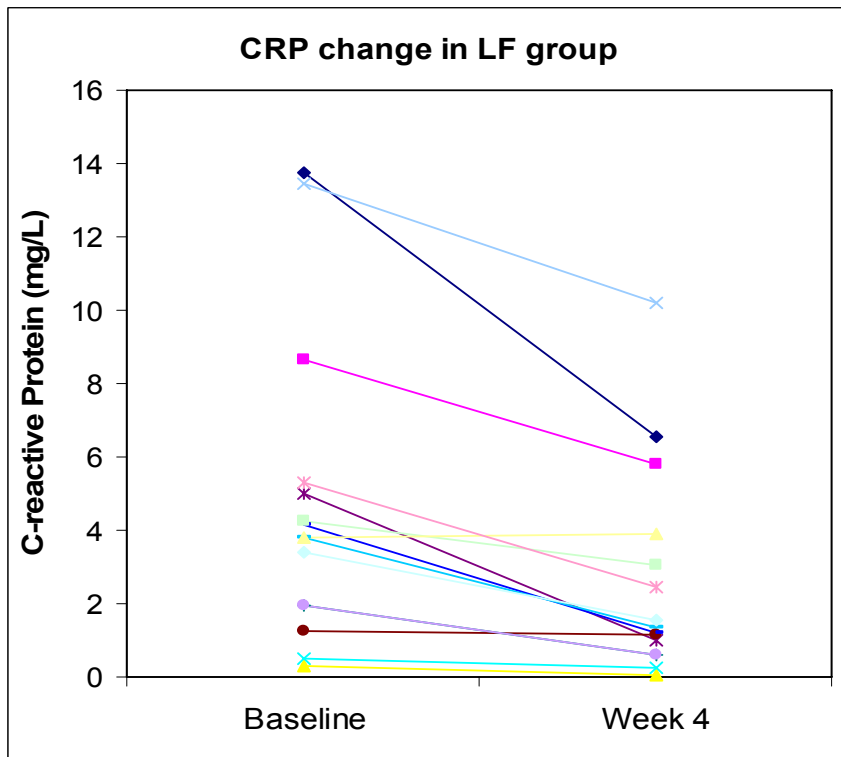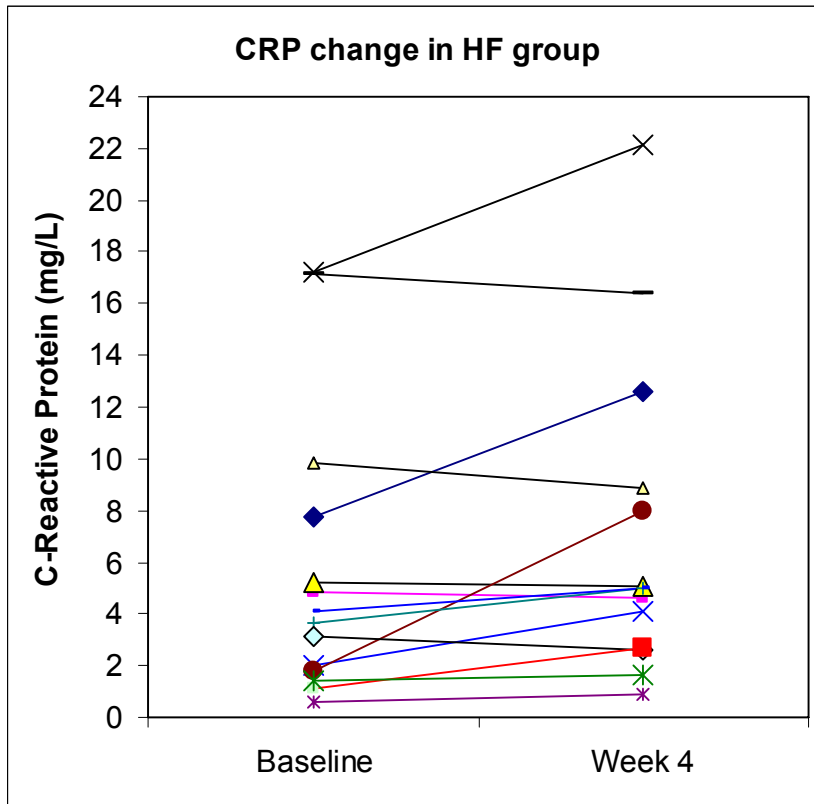
Chapter IV.

"High fat, low carbohydrate weight loss diet and inflammation: the role of oxidative stress"

Title: High fat, low carbohydrate weight loss diet and inflammation: the role of oxidative stress.

Running title:
High fat weight loss diet, inflammation

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Abbreviations used: BMI = Body mass index, HF = High fat, low carbohydrate weight loss diet, AS = Antioxidant supplement, P = Placebo, CRP = C-reactive Protein, IL-6 = Interleukin-6, MCP-1 = Monocyte chemoattractant protein-1, 8-epi = 8-epi-prostaglandin-$F_{2\alpha}$, ORAC = Oxygen radical absorbance capacity.

Abstract

Objective: This study tested the hypothesis that the inflammatory response to a high fat, low carbohydrate weight loss diet (HF) we previously observed was due to oxidative stress. *Research Methods and Procedures*: Nineteen overweight subjects (BMI > 27 kg/m^2) were randomly assigned to either an antioxidant supplement (AS) (1 g Vit C/ 800 IU Vit E) or a placebo (P) group and placed on a high fat, low carbohydrate weight loss diet (HF) for 7 d. Subjects were provided with all food throughout the dietary period. Pre and post samples were measured for markers of inflammation (C-reactive protein (CRP), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1)), oxidative stress (8-epi-prostaglandin-F$_{2\alpha}$ (8-epi) and oxygen radical absorbance capacity (ORAC)), and glucose. *Results*: HF resulted in significant reductions in weight, glucose, and MCP-1 ($p<0.01$), with no difference between groups. There was a trend for a differential effect between groups for CRP as this protein was decreased 32% in the AS group but increased 50% for P ($p=0.119$). Inverse correlations were noted between initial values and changes in several inflammatory and oxidative stress markers including CRP ($r = -0.501$), 8-epi ($r= -0.863$), and ORAC ($r = -0.546$) (all $p<0.05$). *Discussion*: Weight loss on a low carbohydrate, high fat diet caused reduction of some but not all markers of inflammation. Improvement in biomarkers was greater in those with initially high values. The trend for an effect of antioxidant supplementation on serum CRP response suggests further study of the connection between inflammation and oxidative stress during weight loss may be warranted.

Key words: antioxidants, energy restriction, macronutrient composition, inflammation, C-reactive protein

Introduction

Inflammation is an underlying component of several debilitating chronic diseases, most notably atherosclerosis and type 2 diabetes. Obesity is an independent risk factor for both of these conditions (1) and is often associated with elevated levels of inflammatory markers such as c-reactive protein (CRP) and interleukin-6 (IL-6) (2). Blood CRP and IL-6 are considered independent predictors of future coronary events (3,4). CRP may be actively involved in the atherosclerotic process (5) therefore, lowering these levels in at-risk individuals may help to prevent chronic disease development. Fortunately, weight loss is shown to decrease inflammation with markers such as CRP, IL-6, and monocyte chemoattractant protein-1 (MCP-1) often responsive (6,7).

There is uncertainty as to whether the macronutrient composition of a weight loss diet influences inflammation. Of particular interest is the contribution of dietary fat, as high fat diets in animals (8,9) and acute high fat meal challenges in both diabetic and healthy humans have been shown to increase inflammation (10). In comparisons with low fat diets, high fat reduced energy diets have been reported to confer either no additional benefit (11), a greater benefit for high risk subjects (12), or (as recently shown by our lab) a detriment to inflammatory levels as illustrated by blood CRP (13). The reason for the discrepancy among studies is not clear. It is possible that differences in degree of dietary control, weight loss, duration of energy restriction, or medication usage may confound results among studies. Additional study is necessary to explain these observations.

One stimulus for inflammation is oxidative stress (14), a condition when production of reactive oxygen species (ROS) exceeds the ability to remove them. The effect of dietary composition on oxidative stress has received modest research attention. There is limited evidence that a high fat diet is associated with higher markers of oxidative stress in rats and rabbits relative to lower fat diets (15,16). One study in humans showed that a short-term high fat, low fiber diet increased ROS content in feces (17). Notably, although hypocaloric diets often reduce oxidative stress levels (Desideri (18), Dandona (19)), a recent animal study showed that while a high carbohydrate hypocaloric diet reduced mitochondrial ROS production, no such reduction occurred with a high fat hypoenergy diet (20).

We hypothesized that our previous finding of an increase in serum CRP following a short term low carbohydrate, high fat weight loss diet in overweight women was mediated by oxidative stress induced by the diet. Research has shown that acute increases in oxidative stress and inflammation following high fat meal ingestion can be reduced by co-ingestion of antioxidants (10,21). The purpose of this study was to further study the effect of a low carbohydrate, high fat diet on inflammation and to determine whether oxidative stress mediated this effect.

Research Methods and Procedures

<u>Subject Recruitment</u>

A total of nineteen overweight (BMI> 27 kg/m^2), non-smoking, sedentary, weight stable (for at least 6 months), men and women were recruited for this study. The study was approved by the Institutional Review Board for human subjects prior to subject recruitment, and all subjects signed an informed consent prior to any study procedures.

Subjects were excluded if they had any past or present cardiovascular disease, diabetes, inflammatory condition (i.e. Crohn's disease), or high blood pressure. Any subjects who reported use of antioxidant supplements were asked to cease at least 2 weeks prior to starting the study.

Dietary Intervention

All subjects were fed the same low carbohydrate, energy restricted weight loss diet (HF) for one week (breakfast and lunch at our laboratory and given a take away dinner and snacks). Each subject received 16 kcal/kg rounded to the nearest of five calorie levels (1200, 1500, 1800, 2100, 2400 kcal/d). This energy intake was based on the average ad libitum intake of a similar diet by subjects in a previous study by our lab (Rankin & Turpyn, 2007). HF provided less than 10% of energy from carbohydrates, was devoid of grain products and fruits, and high in meats, cheese, eggs, and low carbohydrate vegetables. It contained on average (across all calorie levels) 63.6 ± 1.6 % calories as fat, 31.2 ± 1.2 % protein, 5.1 ± 0.5 % carbohydrate, 5.8 ± 1.1 mg vitamin E (as alpha-tocopherol), and 14.2 ± 2.0 mg vitamin C.

The subjects were randomly assigned and blinded to treatment groups: antioxidant (AS) or placebo (P) supplement. AS received 1 g vitamin C and 800 IU vitamin E in a combination supplement (Leiner Health Products, Carson, CA) each morning with breakfast, while the placebo group (P) received lactose pills. The consumption of all supplements was observed by the experimenters. Dietary intake was analyzed using Nutritionist Pro™ software (version 2.4). Subjects were requested to complete a follow up survey after the intervention to help assess dietary compliance.

Compliance to the diet was assessed by the combined results of the post-intervention survey, presence of urinary ketones, and loss of body weight.

<u>Measurements and Biochemical analyses</u>

Subjects arrived to the laboratory on days 0 and 8 for blood and urine collections after an overnight fast. A single void urine sample was collected into a sterile cup and immediately refrigerated until it was aliquoted and frozen at -80°C until analysis for 8-epi prostaglandin F2α (8-epi) and creatinine. The urine was tested for ketone levels with ketostix as an assessment of dietary compliance. Blood samples were collected without anticoagulant, allowed to clot, and centrifuged at 2500 rpm for 15 minutes at room temperature. Serum was stored in separate aliquots at -80°C until later analysis for CRP, IL-6, MCP-1, oxygen radical absorbance capacity (ORAC), and glucose. Body weight was measured in kilograms on the same calibrated scale each morning.

IL-6, MCP-1 (R&D, Minneapolis, MN) and CRP (United Biotech, Mountain View, CA) were analyzed by ELISA while glucose was analyzed by an enzymatic colorimetric assay (Stanbio, Boerne, TX). All analyses except for MCP-1 were done in duplicate. Serum ORAC determined as described by Ou et al (22), using a FLUOstar OPTIMA plate reader with fluorescence filters with wavelengths of 485 nm and 520 nm for excitation and emission respectively. The area under the curve (AUC) was considered an indication of antioxidant capacity as it represents the time course and degree to which the intensity of a fluorescent compound (fluoresceine, Sigma Aldrich) decays after exposure to a free radical generator (2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), Wako, Richmond, VA). Samples were analyzed in duplicate, all samples for each subject were analyzed within the same run.

Fasted urine samples were analyzed in duplicate for urinary 8-epi-prostaglandin-$F_{2\alpha}$ (8-epi) by ELISA (Oxis International, Portland, OR). Urinary creatinine was measured in each sample (Stanbio, Boerne, TX) and the amount of 8-epi was normalized to urinary creatinine.

<u>Statistics</u>

Data are presented as mean ± SEM for all measures. Baseline characteristics of subjects were compared using t-tests. Repeated measures ANOVA was employed to test differences between groups over time for dependent measures. Changes (both absolute and percent changes) from day 0 to day 8 were determined and compared by t-tests for independent samples. Associations between measures were determined using the Pearson product moment correlation. The level for significance was set at $p<0.05$. All analyses were carried out using SPSS version 15.0.

Results

Subjects who reported that they had consumed foods not included in the intervention, lacked sufficient urinary ketones on day 8, and/or did not lose weight over the intervention were excluded from analysis. Based on these criteria, one subject was excluded from analyses for non-compliance.

There were no significant differences in subject characteristics or blood and urine measurements between groups at baseline (Table 1 and Table 2). Baseline measures of adiposity (BMI and waist circumference) were correlated with IL-6 ($p<0.05$), but not other measures. Serum glucose was inversely associated with serum CRP and urinary 8-epi ($r= -0.51$, $p=0.044$ and $r= -0.62$, $p<0.01$ respectively). Measures of oxidative stress and inflammation were not correlated.

After one week on HF, all subjects lost a significant amount of weight (3.0 ± 1.4 kg for AS and 3.6 ± 1.3 kg for P, p<0.01) with no difference between groups (Table 2). The absolute change in weight was inversely related to the starting weight (r= -0.599, p<0.01). Although weight loss was similar, there was a trend for serum CRP to change differently during the dietary intervention by group (p = 0.119). Average serum CRP increased by 50% in P, and decreased by 32% in AS (Figure 1). Both serum glucose and MCP-1 decreased significantly over the weight loss period for both groups (p < 0.01) with no difference in the change by group. There were no overall effects of weight loss or the specific intervention on urinary 8-epi or serum ORAC, although there was a strong significant inverse correlation between baseline 8-epi and the change in 8-epi (Table 3 and Figure 2) and likewise between baseline ORAC and change in ORAC (Table 3).

Although there was a numerical drop in average serum IL-6 after weight loss, this was not significant. However, there was a positive correlation between change in IL-6 and percent change in weight (see Table 3 for correlations). The change in IL-6 was also negatively correlated with changes in urinary 8-epi and tended to be negatively correlated with initial IL-6 levels. The change in urinary 8-epi was negatively correlated with percent change in weight and tended to be negatively correlated with initial CRP. Initial CRP was negatively correlated with the change in CRP, but positively correlated with the change in glucose, MCP-1, and the percent change in weight.

Discussion

This study provided some insight into the changes in biomarkers related to inflammation and oxidative stress over a brief intervention period. This low carbohydrate, weight loss diet caused a significant decrease in some indicators of inflammation (e.g. MCP-1) but not others (e.g. IL-6, CRP) within 7 d. The reduction in serum MCP-1 with weight loss is consistent with observations in other longer term studies, where weight loss was induced by either surgery (23) or dietary intervention (7,24). The reported association between adiposity and MCP-1 is believed to be related to increased release of this compound by not only adipocytes, but also macrophages that have infiltrated adipose tissue in the expanded adipose mass in obese individuals (25). MCP-1 plays a key role in recruiting monocytes to sites of inflammation (i.e. adipose tissue or vascular endothelium). MCP-1 further contributes to scavenger receptor expression and the differentiation of monocytes into atherogenic foam cells (26). As monocytes/macrophages have been implicated in atherosclerotic processes (27), it is not surprising that elevated levels of MCP-1 have been associated with atherosclerotic risk factors and an increased risk of myocardial infarction or death in patients with acute coronary syndromes (28). Reducing circulating MCP-1, therefore, has potential health benefits to obese individuals who are already at elevated risk for atherosclerosis and cardiovascular disease. Of interest, the reduction that we observed in serum MCP-1 was not directly correlated to the amount of weight lost. Thus, it may be that negative energy balance rather than a reduction in body fat contributes to reduction in this factor. Christiansen et al (7) conducted a longer term (12 week) program for 23 non-diabetic morbidly obese subjects that resulted in weight loss and a 20% reduction in MCP-1. This is comparable to the 15% decrease that we observed,

and, similar to our study, no direct correlation was noted between circulating MCP-1 and any anthropometrical measures.

In contrast to MCP-1, the positive correlation between changes in weight and IL-6 suggests that although modification of this factor was too variable among subjects losing different amounts of weight to demonstrate a significant overall change, IL-6 was connected to the magnitude of weight (and likely fat) lost. Adipose tissue is considered a significant source of circulating IL-6 in obese individuals. It is possible that our study was too brief and not enough fat was lost to see a significant reduction in IL-6. Our subjects lost a significant amount of weight in a very short time, but the absolute amount of fat loss was likely only a modest proportion of the total weight lost. Krotkiewski (29) showed that one week of rapid weight loss on a VLCD resulted in weight loss comparable to our study (-2.9 +/- 0.5 kg), however the composition of the loss was determined to be predominantly (1.9 +/- 0.3 kg) fat free as opposed to fat mass. However, even when fat loss is substantial in longer-term studies, the effects on inflammatory markers are not uniform. For example, after 24 weeks of dietary restriction and an impressive amount of weight loss (about 30% of adipose tissue, 15 kg), decreases in IL-6 and TNF-α were observed, but an unexpected increase in the inflammatory chemokine IL-8 also occurred (30). Similarly, Xydakis et al (31) noted that although CRP decreased with an average weight loss of 8 kg over 4-6 weeks, TNF- α did not. It is becoming apparent that the response of various inflammatory markers to weight loss is complex and inconsistencies noted between studies have yet to be explained. Even the relationship between CRP and IL-6 is not steadfast.   Bastard et al (32) reported that IL-6 decreased significantly while CRP did not, despite a 3 kg fat

mass loss, while other studies (33,34) reported decreases in CRP but no change in IL-6 with weight loss, suggesting some disconnect between these markers. The reason for the variability in response of these inflammatory markers to weight loss is unclear.

The starting level of inflammation may play a significant role in the response of inflammatory markers to weight loss. Subjects who began our study with higher markers of inflammation, in most cases, had a greater reduction following weight loss. For example, the inverse relationships observed between initial serum CRP and change in CRP as well as the strong trend for a similar relationship between initial concentration and change in IL-6, demonstrates that those with higher initial levels of these two inflammatory factors had the greatest reductions in response to the diet. This relationship has been observed by others, as Seshadri et al (12) noted that when obese subjects consumed a low carbohydrate weight loss diet, those with higher initial CRP experienced a greater reduction in CRP, while those who started low actually showed a trend for an increase in CRP (12). No possible explanation was suggested by this group; however, the heterogeneity present in the obese population may play a role (35), as it has been noted that not all obese individuals are "metabolically abnormal" (36). It appears that "metabolically healthy obese" individuals do not have elevated inflammatory levels at baseline (37), nor do they experience reductions in inflammation with weight loss (38). We may have selected a study population that contained a subset of these obese individuals "too healthy" to see significant reductions in CRP, as in our subject pool, 44% had CRP < 1 mg/L at baseline, and none had impaired fasting glucose.

Contrary to our hypothesis, there were no overall effects of the weight loss diet on measures of oxidative stress (urinary 8-epi and serum ORAC). Although 8-epi is touted as an excellent marker of oxidative stress in vivo (39), and other studies have reported that both urinary and serum levels decrease with weight loss (18,40), we did not observe these associations. Our results are, however, in line with Samuelsson et al (41), who reported that plasma 8-epi did not decrease with weight loss, nor was there any relationship between 8-epi and BMI.

There is room for speculation that our measures of oxidative stress were not ideal or sensitive to the intervention, and did not detect changes in oxidative stress that occurred. Methodological differences can also be consequential, as a discrepancy between the GC/MS and ELISA methods for measuring 8-epi has recently been reported (42). Choosing optimal markers of oxidative stress is difficult as there is disparity between the responses of different markers to the same intervention. For example, Heilbronn et al (43) saw no change in protein carbonyl levels but they did observe reductions in oxidative DNA damage following 6 months of weight loss. Crujeiras et al also noted a disjointed response in markers, as MDA, but not oxidized LDL, decreased in response to similar weight loss (44). The ideal marker of oxidative stress has yet to be determined, therefore future dietary studies would benefit from incorporating a battery of tests to assess markers of oxidative stress on a wide selection of macromolecules (i.e. indices of DNA and protein damage, as well as lipid peroxidation) (45).

Although we did not see overall changes in oxidative stress, further examination of our data showed that, similar to the pattern that we observed with inflammatory

markers, those individuals with higher initial levels of oxidative stress improved while those with lower initial levels did not benefit from short term weight loss. This is evident by the negative correlations noted between initial levels of oxidative stress markers and change in those markers. For example, as shown in Figure 2, individuals with higher initial 8-epi experienced greater decreases after weight loss. Similar effects of various dietary interventions on oxidative stress markers have been reported by others. For example, 14 days of a high antioxidant diet rich in fruits and vegetables reduced urinary 8-epi primarily in those subjects with higher initial levels (46). This differential effect could potentially explain the variable results in the literature regarding the effect of dietary interventions on oxidative stress. If oxidative stress levels are not initially high, an effect is unlikely.

The effects of antioxidant supplementation were not as hypothesized. Serum CRP was the only inflammatory marker that showed a trend for a differing response over the weight loss period between those who consumed an antioxidant versus placebo supplement. As mentioned, it has been established that high fat meals transiently increase inflammatory markers in healthy, normal weight as well as obese individuals and diabetics. Studies have implicated oxidative stress as a mechanism by which the elevation in inflammation occurs by demonstrating activation of the redox sensitive transcription factor nuclear factor-κB (NFκB) (47). Further evidence includes the attenuation of the increase in inflammatory markers and improvement in endothelial function when antioxidant supplements (10,21) or high antioxidant foods (48,49) are consumed concurrently with a high fat meal. Although we did not include a meal challenge in our study (so we cannot exclude the possibility that postprandial oxidative

stress or inflammation would be higher on a low carbohydrate, high fat diet), our study does not provide evidence of oxidative stress in the post absorptive state in individuals consuming a high fat diet.

In conclusion, it is evident from this study that even brief weight loss can alter some markers of inflammation and that MCP-1 may be more responsive to a negative energy balance while IL-6 is more tied to loss of body fat. Additionally, it is apparent that individuals with high inflammatory states are likely to reap the greatest benefit with weight loss. Our findings do not initially support our hypothesis that oxidative stress is the mechanism for the increase in inflammation that we observed in a previous study (Rankin & Turpyn, 07 (13)). However, the trend for a differential response of serum CRP when antioxidants were consumed suggests that more research could explore a role for oxidative stress in the inflammatory response to a low carbohydrate, high fat diet in obesity. Longer term, diet-controlled studies are necessary to determine whether the trend for a differential response in CRP continues. Overall, it is noted that a short term low carbohydrate, high fat diet results in rapid weight loss and reduction in some biomarkers related to heart disease risk.

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       1026.

Table 1: Baseline subject characteristics

Group	n	M	F	Age	Weight (kg)	BMI (kg/m^2)	Waist (M) (cm)	Waist (F) (cm)
AS	10	5	5	31.6 ± 2.0	96.8 ± 8.5	33.2 ± 2.6	102.1 ± 7.7	95.4 ± 9.0
P	8	4	4	29.9 ± 3.0	105.4 ± 9.1	35.1 ± 2.7	110.0 ± 7.1	99.2 ± 9.6

AS= antioxidant supplement group, P= placebo group

All values are mean ± SEM or number of subjects. No significant differences between groups at baseline for any measures.

Table 2: All measures before and after the 7 d dietary intervention

Measure (units)	AS pre (d 0)	AS post (d 8)	P pre	P post
Body Weight (kg) *	96.8 ± 8.5	93.8 ± 8.3	105.4 ± 9.1	101.9 ± 8.7
BMI (kg/m^2) *	33.2 ± 2.6	32.3 ± 2.6	35.1 ± 2.7	33.9 ± 2.6
Waist (cm) *	98.8 ± 5.7	95.9 ± 5.8	104.6 ± 5.9	101.4 ± 5.9
CRP (mg/L) †	2.63 ± 1.4	1.80 ± 0.6	2.66 ± 0.5	3.98 ± 1.2
IL-6 (pg/mL)	0.87 ± 0.15	0.70 ± 0.12	1.30 ± 0.26	1.15 ± 0.24
MCP-1 (pg/mL) *	385 ± 40	330 ± 42	329 ± 57	271 ± 42
8-epi (pg/mg Creatinine)	2320 ± 404	2250 ± 195	1891 ± 312	1805 ± 132
ORAC (µM TE/L)	11530 ± 336	11858 ± 285	12102 ± 405	12098 ± 442
Glucose (mmol/L) *	4.4 ± 0.1	3.5 ± 0.1	4.7 ± 0.1	3.9 ± 0.2

AS= Antioxidant supplement group, P= Placebo group, BMI = body mass index, CRP =

c-reactive protein, IL-6 = interleukin-6, 8-epi = 8-epi-prostaglandin F2α, ORAC =

Oxygen radical absorbance capacity, TE = Trolox equivalents. Values are Mean ± SEM

* time effect $p<0.01$

† trend for group x time interaction $p=0.119$

Table 3: Associations between changes in selected dependent measures

Measure	Pearson Correlation P value	8-epi Δ	CRP Δ	IL-6 Δ	ORAC Δ	Wt % Δ
Wt 0	Correlation	0.078	0.429	-0.221	-0.181	0.010
	p-value	0.765	0.097	0.410	0.471	0.967
CRP 0	Correlation	-0.507	-0.501*	0.372	-0.246	0.636*
	p-value	0.054	0.048	0.155	0.358	0.008
Glucose 0	Correlation	0.549*	0.112	-0.253	-0.328	-0.631†
	p-value	0.022	0.679	0.345	0.184	0.005
8-epi 0	Correlation	-0.863†	-0.241	0.636*	0.125	0.501*
	p-value	0.000	0.387	0.011	0.621	0.041
IL-6 0	Correlation	0.055	0.504*	-0.462‡	-0.076	-0.355
	p-value	0.845	0.047	0.072	0.780	0.177
ORAC 0	Correlation	-0.030	0.042	-0.045	-0.546*	-0.208
	p-value	0.908	0.878	0.868	0.019	0.407
8-epi Δ	Correlation	1	0.311	-0.599*	0.010	-0.492*
	p-value		0.259	0.018	0.989	0.045
CRP Δ	Correlation		1	0.090	0.274	-0.205
	p-value			0.740	0.305	0.446
IL-6 Δ	Correlation			1	0.064	0.677†
	p-value				0.813	0.004

*indicates p<0.05

† indicates p<0.01

‡ indicates 0.05 < p < 0.10

Baseline measure is designated by "0" while change from baseline after 7 d HF by "Δ".

CRP = C-reactive protein, BMI = Body mass index, Waist = Waist circumference, IL-6 = interleukin-6, 8-epi = 8-epi prostaglandin F2α, MCP-1 = Monocyte chemoattractant protein-1, ORAC = Oxygen radical absorbance capacity.

Figure legends:

Figure 1: CRP concentrations before and after 7 d HF with or without antioxidant

supplementation

Figure 2: Relationship between change in urinary 8-epi after 7 days HF diet and

baseline levels

Figure 1
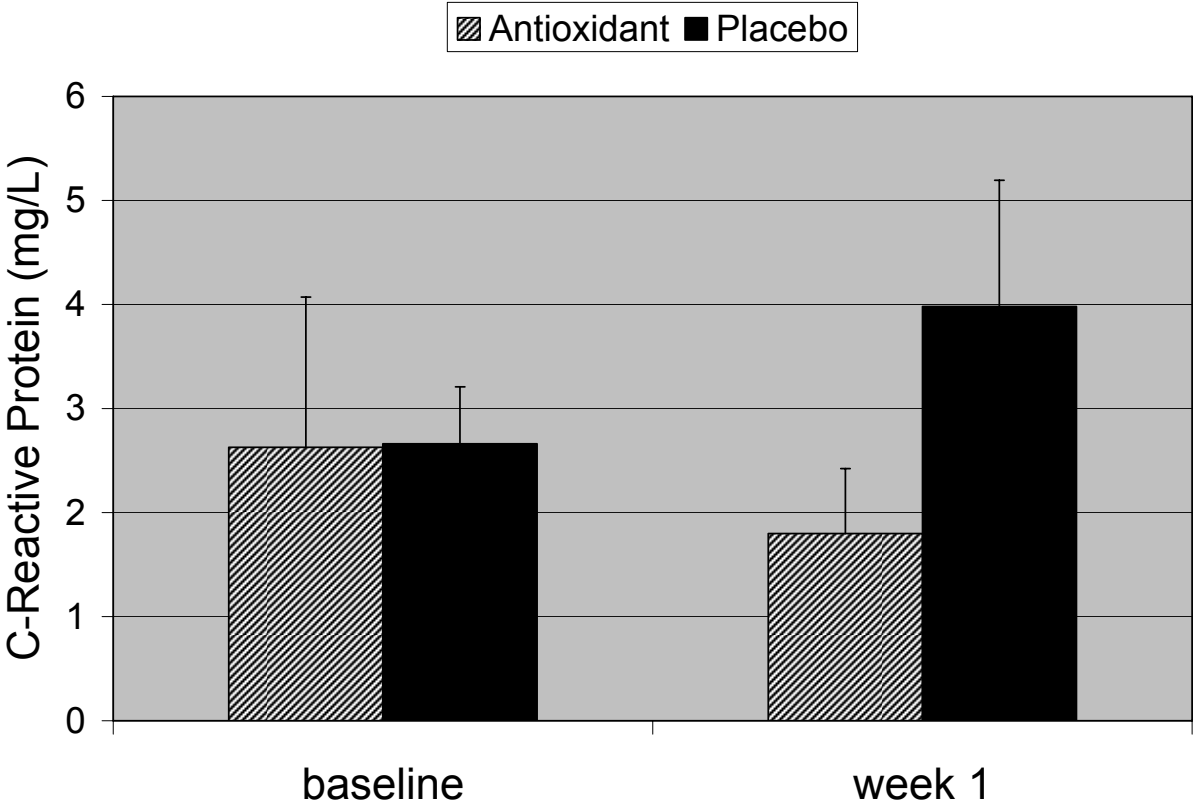
Trend for a difference between group responses (p=0.119)

Figure 2
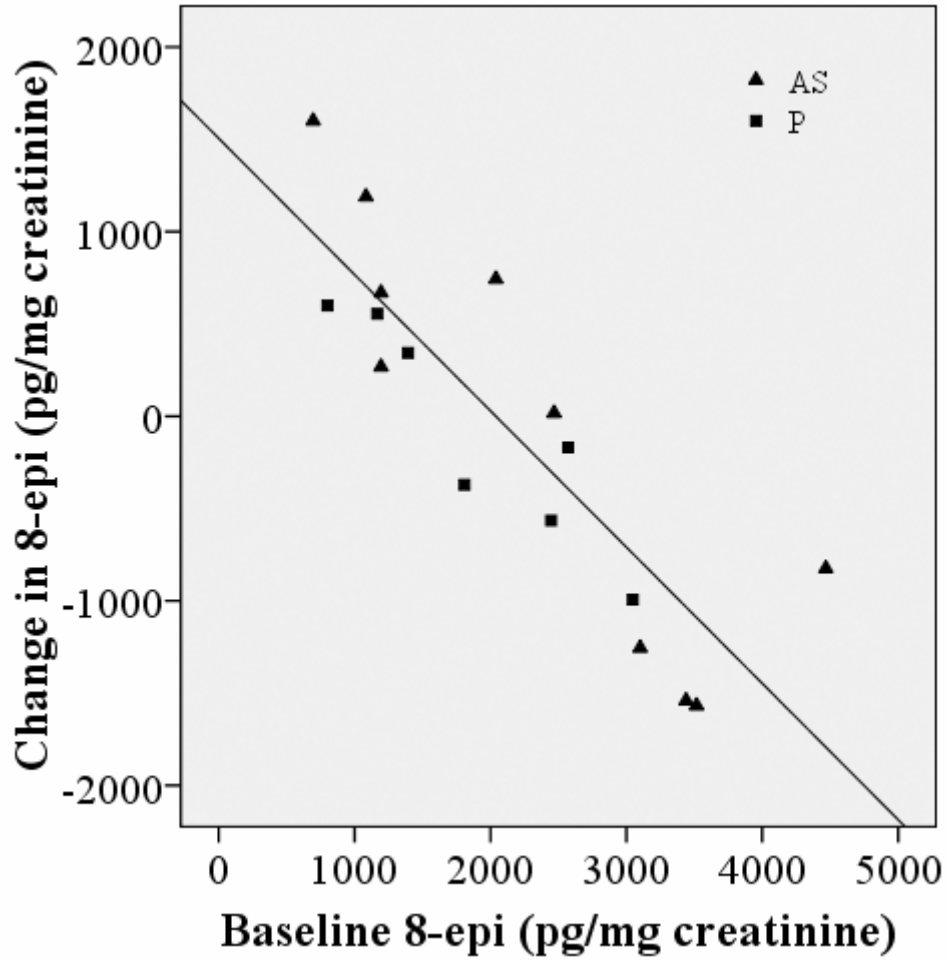
8-epi = 8-epi-prostaglandin-$F_{2\alpha}$

AS = Antioxidant Supplement group

P = Placebo group

Correlation (r = -0.863, p < 0.001)

Chapter V.

"Effects of acute ingestion of various fat sources on oxidative stress and inflammation in overweight individuals"

Effects of acute ingestion of various fat sources on oxidative stress and inflammation in overweight individuals

Running Title: Fat source and acute inflammation in obese

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Abbreviations used: BMI = body mass index, FO = fish oil, PO = palm oil, OO = olive oil, n-3FA = long chain omega 3 fatty acids, SFA = saturated fatty acids, MFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, CRP = C-reactive protein, TNF-α = tumor necrosis factor-α, ICAM-1 = soluble intercellular adhesion molecule-1, VCAM-1 = soluble vascular cell adhesion molecule-1, 8-epi = 8-epi-prostaglandin-$F_{2\alpha}$, NF-κB = nuclear factor - κB

Abstract

Objective: To further clarify the role of different fat sources in a high fat meal on inflammation and oxidative stress in overweight and obese individuals. *Methods:* Eleven overweight and obese, but otherwise healthy individuals consumed three high fat milkshakes in random order, differing in fat source; olive oil (OO, high in monounsaturated fat), palm oil (PO, high in saturated fat SFA), or olive oil with fish oil (FO, containing 4 g omega 3 fats). Blood samples were measured for markers of oxidative stress (8-epi-prostaglandin-$F_{2\alpha}$, 8-epi, nuclear factor-κB, NF-κB), inflammation (intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), tumor necrosis factor-α (TNF-α), C-reactive protein (CRP)), and metabolism (insulin, glucose, triglycerides (TG), free fatty acids (FFA)) before, 1, 2, 4, and 6 hours postprandial. *Results*: FO enhanced NF-κB activation compared to PO, but did not result in increased expression of any inflammatory factors measured over the 6 h postprandial period. Conversely, PO led to higher ICAM-1 than OO, while OO increased TG more than PO (all $p < 0.05$). *Discussion:* As FO acutely activated a transcription factor related to inflammation, further research is necessary to determine whether this activation spurs adaptation and results in the anti-inflammatory effects observed by others with chronic ingestion. OO reduced ICAM-1, which could prove cardio-protective, while PO increased ICAM-1, which could contribute to the atherogenic associations with saturated fats. Therefore, meals high in SFA should be approached with caution due to the associations between endothelial activation (ICAM-1) and CVD.

Key words: acute, postprandial, endothelial activation, high fat, obesity, NF-κB

Introduction

Cardiovascular disease (CVD) and type II diabetes are debilitating chronic diseases associated with obesity. Both diseases are perpetuated by inflammation and oxidative stress (damage by reactive oxygen species, ROS) (1). As excess adipose tissue contributes to elevations in oxidative stress and inflammation, it thereby increases health risks (2,3). Weight loss is effective in reducing these conditions (2,3), however, as only 1 in 5 overweight people successfully maintain weight loss (4), alternative dietary strategies to improve are health necessary.

Previous research has suggested that high fat diets are atherogenic. Further, specific fatty acids (FA) have been shown to have unique effects on the risk of CVD. Higher saturated fatty acid (SFA) intake is associated with increased risk of CVD while monounsaturated (MFA) and polyunsaturated fat (PUFA) intake appear to reduce risk (5). Dietary omega 3 PUFAs in particular appear to lower CVD risk, with the long chain omega 3 PUFAS (n-3FA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) imparting greater benefit than the plant derived α-linolenic acid (ALA) (6). In fact, the American Heart Association (AHA) recommends consumption of two fish meals per week for prevention of CVD, 1 g/d EPA+DHA for those with diagnosed CVD, and 2-4 g/d EPA+DHA for those with hypertriglyceridemia (7). The mechanism by which n-3FA reduce CVD risk is not entirely clear. Studies show that long term n-3FA supplementation reduces fasting and postprandial triglyceride (TG) levels (8,9), which is associated with reduced risk of CVD (10). Chronic n-3FA ingestion is also shown to upregulate antioxidant defenses, reduce systemic markers of inflammation, and improve endothelial function all of which could contribute to their cardio-protective effects (6,11).

Most research in this area has focused on measuring the effects of chronic or acute dietary fat change on blood lipids (12-15) or endothelial function by flow mediated vasodilation (FMV) (16,17). Less has been done examining the connection between consumption of specific dietary fats to inflammation and oxidative stress. There is evidence that dietary trans fat (TFA) and SFA are pro-inflammatory in comparison to MFA (18) and the addition of n-3FA to the daily diet can reduce mononuclear cell cytokine production (19). Although many studies have examined the effects of n-3FA in individuals at risk of CVD, they have not focused on obese subjects. As obese individuals are also at risk of an elevated inflammatory state, it would be valuable to confirm whether n-3FA ingestion affects inflammation in this population. In addition, as most of the day (17 of 24 h) is spent in the postprandial state (20), it is also of interest to determine whether some of the effects of n-3FA reported for chronic ingestion are observed with acute meal ingestion.

In addition to their effects on inflammation, specific types of FA can differentially affect oxidative stress due to differences in chemical susceptibility to oxidation (21). As oxidative stress can disrupt endothelial function via reduced nitric oxide availability, meals with varying FA content may also affect CVD risk through this mechanism. Bellido et al (22) reported that a redox-sensitive transcriptional factor, NF-kB, was increased following a meal high in butter or walnuts but not olive oil in healthy, lean individuals. However, the acute effect of n-3FA was not evaluated. While the acute effects of n-3FA on postprandial lipemia (9) and chronic effects on oxidative stress are mixed (23-25), their acute effects on inflammation and oxidative stress have not been studied. The objective of this study was to further clarify the role of different sources of

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fat in a high fat meal on inflammation and oxidative stress in overweight and obese individuals.

Research Methods and Procedure

<u>Subject Selection</u>

A total of eleven overweight (BMI>27kg/m^2), non-smoking, sedentary, weight stable subjects were recruited for this study. The study was approved by the Institutional Review Board for human subjects at Virginia Tech, prior to subject recruitment. All subjects signed informed consent prior to any study procedures. Subjects were excluded if they had any past or present cardiovascular disease, diabetes, a known inflammatory condition, or were taking medications known to reduce inflammation. Any subjects who reported use of antioxidant supplementation ceased at least 2 weeks prior to starting the study.

<u>Study protocol</u>

Subjects were randomly assigned to treatment in a counter-balanced fashion to consume each of the three test meals, at least one week apart. Subjects were instructed to follow the same pattern of eating prior to each test day. Each test day, subjects arrived to the lab at 7 a.m. after an overnight fast. Blood was collected in the fasted state (time 0), followed by provision of the meal, which was to be consumed within 10 minutes. Subsequent blood samples were collected 1, 2, 4, and 6 hours after meal consumption. Subjects were not allowed to consume any additional foods in the postprandial period, but water was allowed ad libitum.

<u>Meal composition</u>

All meals were high energy, high fat milkshakes, developed to be almost identical in energy and macronutrient composition, with the exception of the source and type of fat. The meals were high in MFA, SFA, or contained a significant dose of n-3FA. The fat source was blended with 1% milk, strawberry flavored syrup, low fat frozen yogurt, and non-fat dry milk powder. The fat sources were the following: refined olive oil (OO) for the high MFA meal, refined palm oil (PO) for the high SFA meal, and refined olive oil plus 4 g of omega 3 fatty acids from fish oil (FO) (Vitamin World Super Omega-3 Fish Oil) for the n-3FA meal. The milkshakes contained approximately 12.8 kilocalories per kilogram body weight with 59% fat, 30% carbohydrate, and 11% protein. The meals averaged 1267 calories with the n-3FA containing an additional 36 calories from the fish oil.

Blood sample collection

Blood was collected into vacutainers containing anticoagulant (EDTA for all except the harvest of peripheral blood mononuclear cells (PBMC) which contained heparin) at 0, 1, 2, 4, and 6 hours postprandial and immediately placed on ice until processing. Blood was centrifuged (1000 x g), to obtain plasma for analysis of inflammatory, oxidative stress, and metabolic parameters. Plasma was aliquoted into separate cryovials for each measure and immediately stored at -80°C.

Inflammatory markers

Plasma was analyzed in duplicate for soluble intercellular adhesion molecule-1 (ICAM-1), soluble vascular cell adhesion molecule-1 (VCAM-1), tumor necrosis factor- α (TNF-α) (all R&D systems, Minneapolis, MN), and C-reactive protein (CRP) (United Biotech, Mountain View, CA) via enzyme linked immunosorbent assays (ELISA).

Oxidative stress markers

Markers of oxidative stress were analyzed for 4 h due to previously reported rapid activation of NF-κB (within 1-4 h (26,27)). In order to correlate the two measures of oxidative stress, we measured 8-epi for the same interval. 8-epi-prostaglandin-$F_{2\alpha}$ (8-epi) was determined using gas chromatography mass spectrometry (GC/MS) according to the methodology of Morrow and Roberts (28). Free F_2-isoprostanes were extracted from 1 ml of plasma. Deuterated [$^2H_4$]-8-iso-PGF$_{2\alpha}$ internal standard was added, vortexed, applied to a C_{18} Sep-Pak column, and extracted. The F_2-isoPs were converted into pentafluorobenzyl esters, subjected to thin-layer chromatography, extracted from the silica gel with ethyl acetate, converted into trimethylsilyl ether derivatives and analyzed by negative ion chemical ionization GC/MS using an Agilent 5973 mass spectrometer with a computer interface.

PBMC isolation

Briefly, heparinized blood was mixed 1:1 with cold PBS and layered over lymphocyte separation media (LSM) (Mediatech) for density gradient separation of PBMCs. PBMCs were harvested, rinsed twice, and subjected to the nuclear extraction protocol for measurement of nuclear factor - κB (NF-κB). Cells were kept on ice throughout the procedure.

Electrophoretic mobility shift assay (EMSA) procedures:

Nuclear extracts from PBMCs were prepared according to the method of Andrews and Faller (29) with minor modifications. PBMCs were lysed in 400 μL buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF), followed by centrifugation to obtain the nuclear pellet which was incubated in 20-40 μL

(depending on the pellet size) of buffer C (20 mM HEPES K-OH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) on ice. After centrifugation, the supernatant was collected, aliquoted, and immediately frozen at -80°C.

The EMSA procedure was performed as described (30), with modifications (31). Binding reactions were conducted in a 20 μl volume containing 2-4 μg (4μL) of nuclear protein extracts, 10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10 % glycerol, 2 μg of poly[dI-dC] (nonspecific competitor) and 40,000 cpm of ^{32}P-labeled specific oligonucleotide probe. Double-stranded oligonucleotides with the consensus sequence of the binding site for transcription factor NF-κB(5'-AGTTGAGGGGACTTTCCCAGG-3',(Promega, Madison, WI)) were labeled with [γ-^{32}P]-ATP (Amersham Pharmacia Biotech, Piscataway, NJ) using T4 polynucleotide kinase. Resultant protein-DNA complexes were resolved on native 5 % polyacrylamide gels using 0.25 × TBE buffer (50 mM Tris-Cl, 45 mM boric acid, 0.5 mM EDTA, pH 8.4). Competition studies were performed by the addition of a molar excess of unlabeled oligonucleotide to the binding reaction. Gels were dried and exposed to x-ray film. Band intensity corresponding to specific NF-κB-DNA binding was determined using Syngene GeneTools software (Imgen, Alexandria VA). The relative intensity units were calculated in relation to the basal value for each subject for each trial.

Metabolic variables

Plasma glucose was measured by spectrophotometric method (Stanbio, Boerne, TX), as were free fatty acids (FFA) and triglycerides (TG) the two of which were analyzed

using a technique adapted for microplate (Wako, Richmond, VA). Insulin was measured by ELISA (Mercodia, Alpco Diagnostics).

<u>Statistics</u>

Data were analyzed for the effects of treatment, time, and the treatment by time interaction by a two factor RM-ANOVA using a mixed linear model and the baseline values as covariates. Treatment and time were fixed effects and subject was a random effect. Non-normally distributed data were log transformed prior to analysis (CRP, 8-epi, TG). For clarity, the measured data are presented in figures. The area under the curve (AUC) for each dependent measure was calculated using the linear trapezoidal method. Data were analyzed for difference by treatment via a 1- factor ANOVA using a mixed linear model with the baseline values as covariates. Data are presented as mean ± SEM, a p-value< 0.05 was considered significant, and all analyses were carried out using SPSS version 15.0.

Results

The milkshakes were well tolerated. Two subjects were unable to finish the entire milkshake the first day, therefore, the remaining milkshakes for those two subjects were adjusted to match the amount consumed the first day. Subjects with missing samples were included in the overall analysis. However, any subject with more than one missing data point (time) in more than one trial (OO, PO, FO) for more than one measure (8-epi, NF-κB, etc) was excluded from the overall analysis. This resulted in one subject being excluded, therefore data are presented for n = 10.

Initial subject characteristics and average inflammatory, oxidative stress, and metabolic variables are located in Table 1. Waist circumference was positively

correlated with both average fasted plasma glucose (r =0.713, p =0.014) and TG
(r=0.645, p=0.032) levels, while body mass index was positively correlated with several
markers of inflammation including fasted plasma levels of TNF-α (r =0.661, p=0.027),
CRP (r=0.857, p=0.007), and ICAM-1 (r=0.778, p=0.008). Fasted glucose was strongly
correlated with TG (r=0.930, p< 0.001) while insulin tended to be correlated with ICAM-1
(r=0.652, p=0.052). CRP was positively correlated with both ICAM-1 (r=0.838, p=0.002)
and the oxidative stress marker 8-epi (r=0.734, p=0.016).

Inflammatory Markers

Plasma TNF-α decreased and VCAM-1 had a trend for a decrease following the meals
(p<0.01 and p=0.085 respectively) with no difference between treatments. Plasma
ICAM-1 did not change significantly following the meals, but remained lower for the OO
treatment (p=0.031) than both FO and PO and the ICAM-1 AUC was greater for PO
than OO (p=0.051) (Figure 1). CRP increased slightly over time (p=0.045) with no
difference between meals.

Oxidative Stress Markers

Although there was no significant combined-meal postprandial effect on NF-κB, it was
higher throughout the 4 h postprandial period for FO than PO, resulting in a greater
AUC (p=0.046, Figure 2). 8-epi decreased during the postprandial period (p=0.019)
with no difference between treatments.

Metabolic Variables

As expected, there were significant effects of meal ingestion on plasma insulin
(p<0.001), glucose (p=0.019), FFA (p<0.001), and TG (p<0.001). Insulin and glucose
increased 1h postprandial and then returned to baseline levels. FFA had the opposite

trend, decreasing initially and then rebounding to baseline, while TG increased steadily over time. Type of fat in the meal did not influence the plasma glucose or FFA response. However, the insulin AUC tended to be lower for SFA than n-3FA (p=0.076, Figure 4) while TG were lower for SFA than MFA (p=0.019).

Discussion

The key findings in this study were that the type of fat included in a high fat meal differentially influenced levels of the redox sensitive transcription factor NF-κB, and the soluble adhesion molecule ICAM-1 in overweight and obese individuals. The observation that a meal containing FO lead to higher levels of NF-κB than a meal high in PO is interesting as FO are typically thought to be anti-inflammatory. However, FO have been shown previously to act as oxidative stressors chronically *in vivo* (24,25,32). For example, Pedersen et al noted increased MDA *in vivo* and *ex vivo* LDL oxidation susceptibility (32) following chronic FO supplementation in diabetics, while Grundt et al (25) observed greater TBA-MDA (lipid peroxidation measure) in patients with prior myocardial infarction supplemented with FO compared to corn oil. Thus, recommendations have been made that the addition of n-3FA to the diet should be accompanied by antioxidant supplementation (33). The presence of many double bonds susceptible to oxidation in the highly unsaturated n-3FA in FO provides a rationale for increased acute oxidative stress. However, levels of 8-epi, a measure of lipid peroxidation, did not increase with FO in our study, and in fact, decreased during the postprandial period for all trials, indicating a reduction in oxidative stress. It is possible that the duration of sampling was not long enough to see an increase in 8-epi.

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The dosage of n-3FA could have played a role in activating NF-κB. Higher daily

doses of fish oil (15 g) have been shown to increase oxidative stress in post

menopausal women (34), whereas lower doses were less likely to do so in overweight

hypertensive diabetic patients (23). Our dose (8 g of FO to achieve 4 g n-3FA) may

more closely resemble boluses taken twice daily in the higher dose study. The

conundrum lies in the fact that, while the activation of NF-κB is considered a pro-

inflammatory event, we did not observe a subsequent increase in inflammatory marker

expression following the FO meal. The disconnect between the upregulation of NF-κB

by FO and expression of inflammatory markers may be related to the brief time course

of the postprandial period. It may be that other unmeasured markers of inflammation

were increased (i.e. perhaps IL-6), or that some unmeasured factor suppressed the

expression of these markers.

The acute increase in NF-κB with FO is counter to the effects of chronic ingestion

of FO tending to reduce the inflammatory state *in vivo* (35,36), and *in vitro* studies show

pre-exposure to n-3FA reduces pro-inflammatory responses in vascular endothelial cells

(37). The difference in response may be due to the fact that chronic supplementation

allows for the incorporation of n-3FA into cell membranes, which is shown *in vitro* to

play a key role in the anti-inflammatory effect (38). Incorporation of n-3FA into cell

membranes replaces arachidonic acid (AA) and allows for the production of the less

bioactive n-3FA derived metabolites in place of the inflammatory mediators released

from AA by enzymatic cleavage (39). In fact, chronic supplementation with a high dose

of n-3FA (18 g/d of FO) and associated reduction in cell membrane AA content was

implicated in the reduced *ex vivo* PBMC IL-1 and TNF-α production (40), likewise,

increased cell membrane EPA content also corresponded to reduced cytokine production (19).

The acute transcriptional activation associated with the inflammatory response to n-3FA and other PUFAs is less clear, and discrepancy exists among studies. *In vitro* studies have shown that short incubation with PUFAs (including n-3FA) reduces NF-κB activation compared to the SFA palmitate (41), while longer incubation studies show that palmitate induced increases in NF-κB and IL-6 can be partially attenuated by DHA, but the SFA induced IL-6 increase is independent of the NF-κB pathway (42). Still others report that exposure to EPA increases or prolongs cell stimulated NF-κB activation, with concomitant upregulation in genes such as toll-like receptor 4 and TNF-α receptor associated factor -6 (43,44). Little information is available regarding acute inflammatory and signaling responses to different fatty acids in humans. Similar to our study, Bellido et al (22) reported that a high fat meal containing PUFA increased NF-κB. However, unlike our study; they noted the same effect 3 h after ingestion of a high SFA meal. It is possible that differences in SFA sources played a role, as they used butter, while we used palm oil. Dairy fats also contain cholesterol and significant levels of medium chain fatty acids along with palmitate, while palm oil contains no cholesterol and approximately twice the palmitate. Interestingly, *in vitro* studies have shown that while lower levels of palmitic acid induce NF-κB activation, higher levels can result in suppression (42).

Chronic FO intake enhanced activity of the antioxidant enzyme glutathione peroxidase in diabetics and reduced lipid peroxide levels, indicating that n-3FA can improve overall in oxidative status (11). Therefore, it is possible that the acute increase

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in NF-κB observed here is transient and serves to upregulate immune defenses. Following chronic supplementation, the adaptations may be beneficial by reducing the overall inflammatory state. This is similar to the effects of acute bouts of exercise which are shown by some studies to acutely increase inflammation and oxidative stress, but the chronic effect of physical activity is one of reduced systemic inflammation (45). This reduction is likely due to adaptive responses such as enhanced antioxidant defenses which over time, resulting in a lower level of inflammation which may reduce CVD risk (46). The hypothesis that repeated acute oxidative stress from n-3FA ingestion enhances antioxidant capacity in obese individuals requires confirmation.

Higher ICAM-1 levels following SFA ingestion have been reported previously in lean individuals consuming butter (22), but not until 9 hours postprandial. It has also been noted that HDL isolated 6 h after a high SFA meal displayed "impaired" anti-inflammatory properties compared to a PUFA meal. Specifically, the HDL after SFA were less able to inhibit the expression of ICAM-1 and VCAM-1 in stimulated endothelial cells. The SFA meal also resulted in a greater decrease in FMD, indicating endothelial dysfunction (47). The difference in ICAM-1 between our groups became apparent within 1 h of meal consumption, indicating that perhaps a rapid activation of the endothelium resulted in the cleavage of existing ICAM-1 from endothelial cell membranes, rather than an increase in transcription, production, and release. ICAM-1 is an adhesion molecule involved in the tethering of monocytes and other immune cells to the endothelium. It is constitutively expressed on the surface of endothelial cells and its release into the blood increases in response to inflammatory stress (48). This study extends the observation by others that dietary SFA intake significantly contributed to

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fasted ICAM-1 and VCAM-1 levels in an examination of healthy, sedentary men (49).
As ICAM-1 has been associated with future myocardial infarction risk (50), the
mechanism by which ICAM-1 increased to a greater extent with SFA in this short time
frame deserves future study.

In regards to metabolic factors, insulin and TG were the most differentially
responsive to the type of FA source in the meal. The insulin AUC tended to be higher
for FO than PO, but not different than OO. Insulin differences may have been due to
higher TG following the primarily unsaturated fat meals compared to saturated, as
lipolysis of TG could directly provide fatty acids to enhance glucose stimulated insulin
secretion. The anti-inflammatory properties (51) of insulin may have played a role in
suppressing ICAM-1 following OO in comparison to PO. The fact that plasma TG were
acutely lower for PO than OO or FO, is in line with other studies showing that high PO
meals resulted in lower TG responses than meals with unsaturated fats (52,53). While
SFA have been shown by others to result in a relatively lower lipemic response than
unsaturated fats, they have also been shown to take longer to return to fasting levels
(53,54) which could increase CVD risk (10). Mekki et al also showed that while TG
were lower following SFA, the chylomicrons were smaller and more dense (52).
However, as TG did not return to postabsorptive levels during our sample collection
period and we did not analyze chylomicrons, these suggestions remain speculative.

Regarding the FO TG response, our findings are contrary to Zampelas et al who
showed that a meal high in fish oil reduced postprandial TG (55). There may be both
dose and time effects involved, which could explain differences in our findings.
Zampelas et al (55) showed reduced TG after a high dose of fish oil (12 g n-3FA) was

incorporated into an evening meal, in measurements taken over 11 h, which is 3 times

our dose (4 g n-3FA) and almost twice our sampling time. However, similar to our

study, Jackson et al were unable to show differences in acute postprandial TG levels

following n-3FA rich meal in comparison to other fats (56). Interestingly, West et al

showed that replacing some of the MFA in a high fat meal with omega-3 fat ALA

reduced postprandial TG in comparison to the MFA meal (57), whereas there was no

effect of replacing MFA with n-3FA (DHA and EPA). Clearly, there is insufficient

evidence in this area; and our results add to the little information available regarding the

acute effects of n-3FA on postprandial TG.

It is well accepted that chronic ingestion of FO is associated with reduced fasting

and postprandial TG levels. Potential mechanisms leading to the hypotriglyceridemic

effects of n-3FA have been described in terms of changes in the gene expression of

several key proteins involved in fat metabolism (8). The net effect is a reduction in TG

storage and increase in oxidation, an adaptation that occurs over a period of chronic

supplementation. It has also been suggested that intestinal cells may also respond to

n-3FA by reducing triglyceride release, but again chronic exposure appears to be

required (58). Additionally there is evidence that adaptations to diets higher in PUFA

result in enhanced chylomicron clearance compared to diets higher in SFA (54), which

would reduce circulating TG as well.

Overall, high fat meal consumption by overweight subjects resulted in mixed

effects on inflammatory markers. All of the meals induced some inflammation as

indicated by a modest increase in CRP; on the other hand, they reduced TNF-α and

tended to reduce VCAM. Additionally, a marker of oxidative stress, 8-epi, decreased

following the meals. While some studies have shown that high fat meals lead to acute

increases in multiple markers of inflammation and oxidative stress (59,60), others agree

with our findings that the response is not consistently inflammatory (61-63). It is

possible that these markers were more influenced by acute changes in either insulin

(depressing effect) (51) or FFA (reduction in this factor's stimulatory effect) (64). It is

also possible that a longer sampling period would have been necessary to see an

increase in the inflammatory proteins, or ones that we did not measure (i.e. IL-6) did

increase. Finally, inflammatory and oxidative postprandial responses have been shown

to be higher for individuals with insulin insensitivity or diabetes (59,65).

The key new findings of this study are that inclusion of FO (4 g n-3FA) in a high

fat meal acutely increases NF-κB and that there were negative effects of PO on ICAM-1

that were independent of blood TG. Future research into the mechanisms by which FO

increased NF-κB acutely and whether this response changes over time with repeated n-

3 ingestions are necessary to better understand the chronic effects of FO

supplementation. Meals high in SFA should be approached with caution due to the

associations between ICAM-1 and CVD.

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Table 1: Subject characteristics and average fasting values for dependent measures

Measure	Mean (or number) ± SEM
Gender	(4M, 6F)
Age	31.9 ± 3.2
Weight (kg)	98.6 ± 5.7
Waist (cm)	99.5 ± 4.8
Males	108.8 ± 5.4
Females	93.3 ± 6.2
BMI (kg/m^2)	34.6 ± 2.0
CRP (mg/L)	3.5 ± 1.4
TNF-α (pg/mL)	1.30 ± 0.12
ICAM-1 (ng/mL)	210 ± 11
VCAM-1 (ng/mL)	677 ± 42
8-epi (ng/mL)	0.08 ± 0.01
Glucose (mg/dL)	108 ± 6
Insulin (mU/L)	11.0 ± 2.1
Triglycerides (mg/dL)	140 ± 42
Free Fatty Acids (meq/L)	0.33 ± 0.04

Table 2: Postprandial inflammatory, oxidative stress, and metabolic measures (mean ± SEM) following three meals with varying fat sources (n=10).

Measure	Baseline	1 h	2h	4h	6h
CRP (mg/L)					
n-3FA	4.0 ± 1.3	3.8 ± 1.2	4.0 ± 1.3	4.3 ± 1.4	4.2 ± 1.4
SFA	3.4 ± 1.3	3.3 ± 1.2	3.2 ± 1.2	3.8 ± 1.3	3.6 ± 1.3
MFA	3.2 ± 1.1	3.2 ± 1.0	3.2 ± 1.1	3.6 ± 1.2	3.6 ± 1.1
TNF-α (pg/mL)*					
n-3FA	1.18 ± 0.11	1.13 ± 0.11	1.10 ± 0.10	1.11 ± 0.10	1.08 ± 0.09
SFA	1.32 ± 0.12	1.18 ± 0.11	1.13 ± 0.09	1.14 ± 0.10	1.19 ± 0.10
MFA	1.39 ± 0.15	1.30 ± 0.13	1.07 ± 0.11	1.16 ± 0.14	1.22 ± 0.14
ICAM-1 (ng/mL)[a]					
n-3FA	205 ± 11	208 ± 10	206 ± 11	208 ± 11	210 ± 11
SFA	213 ± 11	219 ± 14	217 ± 13	216 ± 12	219 ± 14
MFA	212 ± 12	204 ± 11	199 ± 10	206 ± 12	209 ± 14
VCAM-1 (ng/mL)					
n-3FA	674 ± 42	642 ± 47	635 ± 45	652 ± 47	669 ± 45
SFA	681 ± 43	667 ± 44	663 ± 41	667 ± 36	677 ± 43
MFA	676 ± 47	660 ± 39	660 ± 47	652 ± 43	691 ± 44
8-epi (pg/mL)					
n-3FA	70 ± 7	71 ± 9	67 ± 7	83 ± 11	--
SFA	99 ± 17	82 ± 11	81 ± 9	87 ± 12	--
MFA	84 ± 10	82 ± 11	78 ± 10	83 ± 8	--
NF-κB (relative intensity)[b]					
n-3FA	1.00±0.0	1.14 ± 0.07	1.08 ± 0.10	1.19 ± 0.09	--
SFA	1.00±0.0	1.01 ± 0.08	0.98 ± 0.05	0.81 ± 0.05	--
MFA	1.00±0.0	1.02 ± 0.10	1.06 ± 0.15	1.16 ± 0.19	--
Glucose (mg/dL)*					
n-3FA	107 ± 6	121 ± 8	110 ± 8	102 ± 5	113 ± 9
SFA	112 ± 8	111 ± 12	115 ± 10	106 ± 9	106 ± 7
MFA	105 ± 4	112 ± 9	97 ± 6	102 ± 3	106 ± 4
Insulin (mU/L)*					
n-3FA	12.6 ± 3.8	54.8 ± 14.8	43.4 ± 10.9	16.4 ± 3.2	11.5 ± 1.9
SFA	8.9 ± 1.2	46.9 ± 11.9	29.0 ± 6.2	10.7 ± 1.0	9.8 ± 2.3
MFA	11.5 ± 2.2	40.2 ± 10.5	33.1 ± 8.3	18.6 ± 4.4	14.7 ± 4.3
Triglycerides (mg/dL)* [c]					
n-3FA	132 ± 40	159 ± 40	143 ± 36	170 ± 34	202 ± 52
SFA	126 ± 33	119 ± 24	109 ± 23	145 ± 26	146 ± 26
MFA	161 ± 56	201 ± 58	199 ± 51	214 ± 52	247 ± 66
Free Fatty Acids (meq/L)*					
n-3FA	0.33 ± 0.05	0.17 ± 0.04	0.10 ± 0.02	0.20 ± 0.02	0.39 ± 0.05
SFA	0.37 ± 0.06	0.10 ± 0.02	0.08 ± 0.02	0.21 ± 0.03	0.39 ± 0.05
MFA	0.29 ± 0.05	0.15 ± 0.06	0.14 ± 0.05	0.20 ± 0.02	0.32 ± 0.04

* Effect of time $p < 0.05$
Effect of treatment $p < 0.05$, [a] SFA > MFA, [b] n-3FA > SFA, [c] MFA > SFA

<u>Figure Legends</u>

Figure 1: Soluble intercellular adhesion molecule-1 (ICAM-1) area under the curve (AUC) following three meals with varying fat sources.

Figure 2a: NF-κB activation area under the curve (AUC) following three meals with varying fat sources.

Figure 2b: Representative electrophoretic mobility shift assay (EMSA) analysis of high fat meal effects on NF-κB activation in peripheral blood mononuclear cells (PBMC).

Figure 3: Insulin area under the curve (AUC) following three meals with varying fat sources.

Figure 4: Triglyceride area under the curve (AUC) following three meals with varying fat sources.

Figure 1: Soluble intercellular adhesion molecule-1 (ICAM-1) area under the curve (AUC) following three meals with varying fat sources.
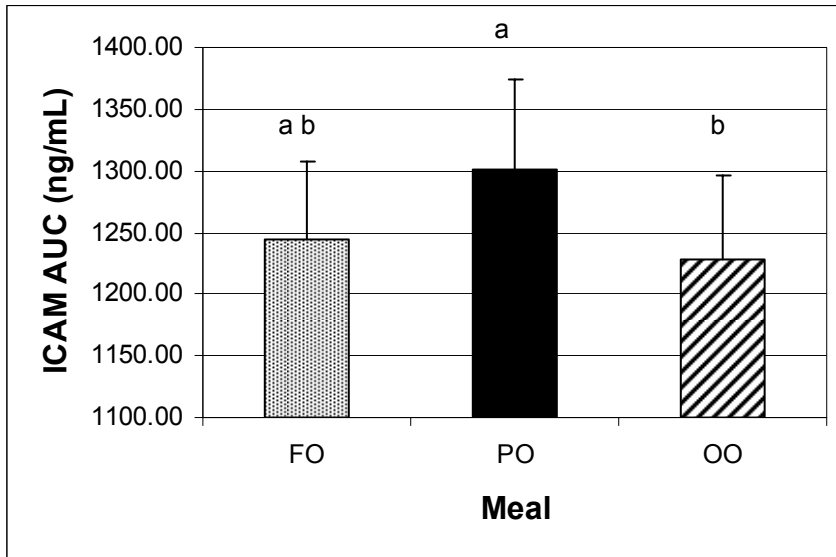
* PO higher than OO (p=0.051)

Difference in superscript denotes difference between meals

FO = fish oil, omega 3 fatty acid enriched meal

PO = palm oil, high saturated fat meal

OO= olive oil, high monounsaturated fat meal

Figure 2a: NF-κB activation area under the curve (AUC) following three meals with varying fat sources.
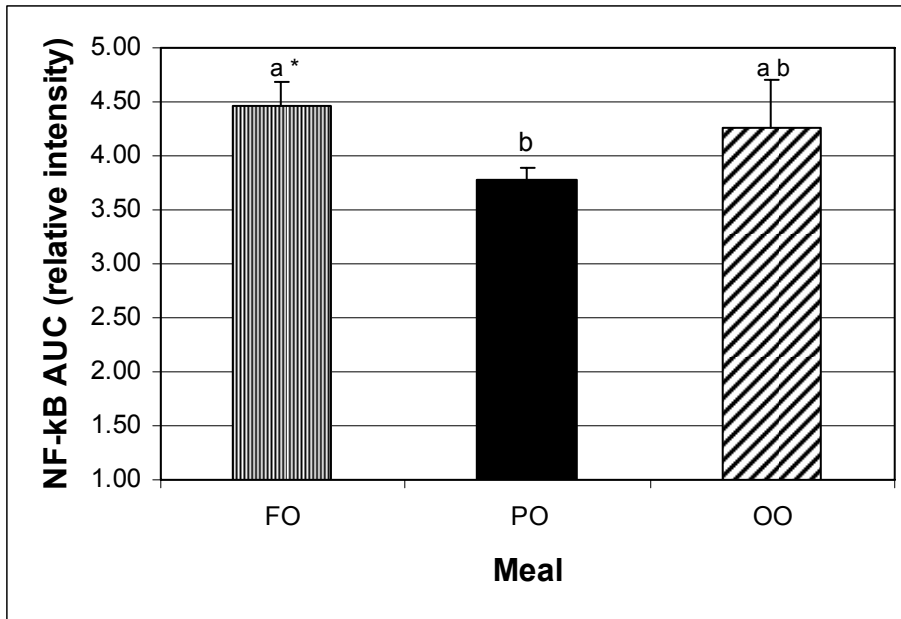
* FO AUC > PO AUC, p= 0.046

Difference in superscript denotes difference between meals

FO = fish oil, omega 3 fatty acid enriched meal, PO = palm oil, high saturated fat meal

OO = olive oil, high monounsaturated fat meal

Figure 2b: Representative electrophoretic mobility shift assay (EMSA) analysis of high

fat meal effects on NF-κB activation in peripheral blood mononuclear cells (PBMC).

NOTE: All trials are normalized to their own baseline intensity. Therefore, time points

after baseline are expressed and analyzed in terms of relative intensity (arbitrary units).
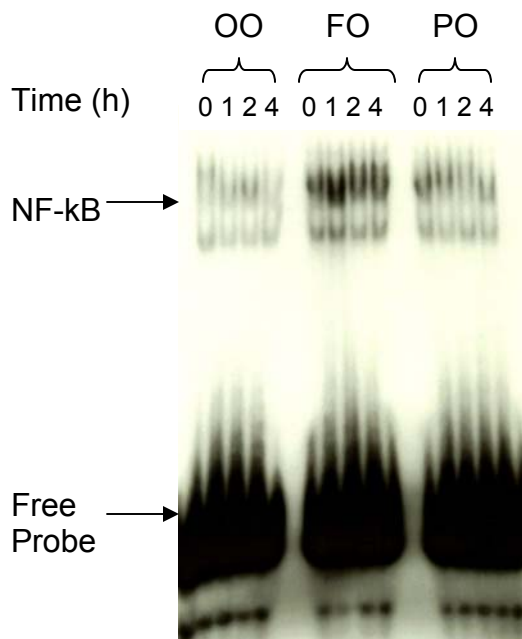
FO = fish oil, omega 3 fatty acid enriched meal, PO = palm oil, high saturated fat meal

OO = olive oil, high monounsaturated fat meal

Figure 3: Insulin area under the curve (AUC) following three meals with varying fat sources
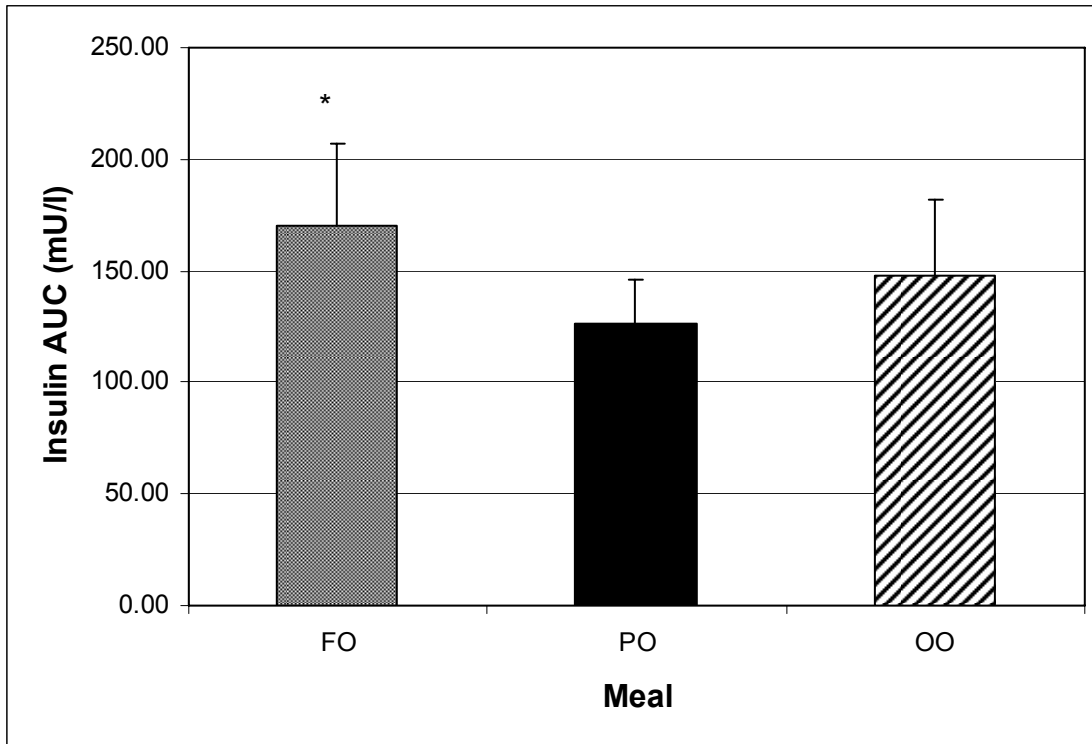
* Trend for FO AUC to be greater than PO (p=0.076)

FO = fish oil, omega 3 fatty acid enriched meal, PO = palm oil, high saturated fat meal

OO= olive oil, high monounsaturated fat meal

Figure 4: Triglyceride area under the curve (AUC) following three meals with varying fat sources.
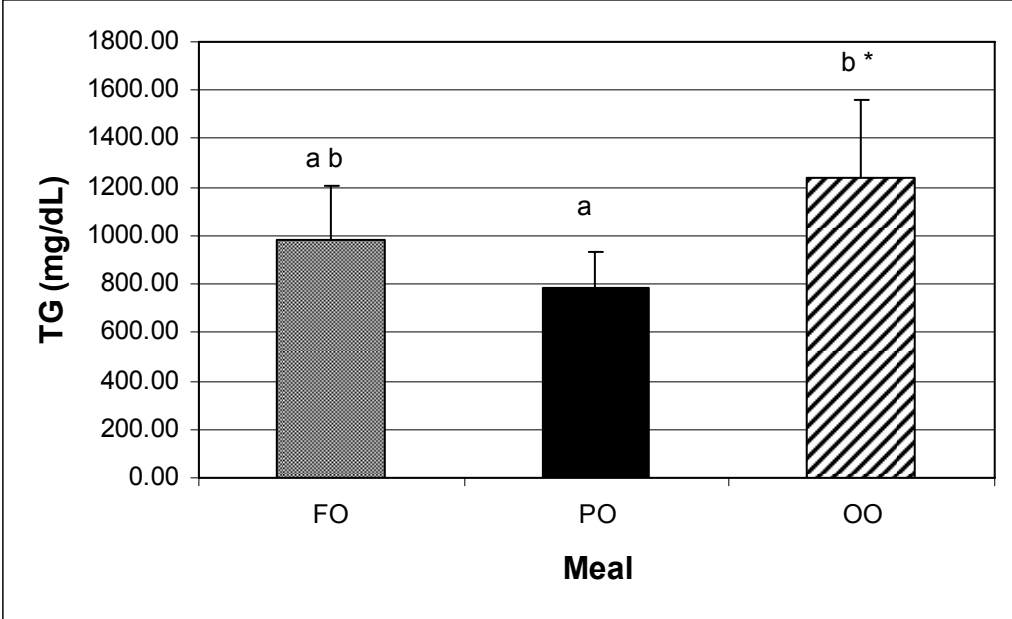
* OO AUC > PO AUC (p=0.019)

Difference in superscript denotes difference between meals

FO = fish oil, omega 3 fatty acid enriched meal, PO = palm oil, high saturated fat meal

OO= olive oil, high monounsaturated fat meal

Chapter VI. Summaries, Contributions, Theoretical Issues, Recommendations for
Future Research, and Practical Implications.

Summaries and contributions of this research are outlined in the following section,
followed by theoretical issues, suggestions for future research, and practical
implications.

Summaries and Contributions of this research

High Fat, Low Carbohydrate Diet Increases Inflammation

Nutritionists were unsettled by a recent resurgence in popularity of the Atkins diet
approach to weight loss, as although the low carbohydrate eating plan boasts rapid
weight loss, suggested menus are high in fat (particularly saturated fat) and cholesterol
(1) raising concerns regarding cardiovascular impact. Studies showing that these diets
do not negatively impact traditional risk factors such as cholesterol levels (2-4) led us to
investigate their effects on oxidative stress and inflammation, two contributing factors to
atherosclerosis. We randomly assigned 32 healthy overweight and obese women to
follow either an *ad libitum* Atkins (high fat, low carbohydrate (HF)) or a calorie restricted
high complex carbohydrate, low fat weight loss diet (LF) for 4 weeks.

The most significant contribution of this study was that we showed HF increased
inflammation in overweight and obese individuals in comparison to LF (Chapter 3). This
adds to the current literature showing that high fat, high cholesterol diets are pro-
inflammatory in animals (5,6), and expands the growing literature comparing the health

effects of HF to LF weight loss diets. The few reported studies on the effects of low

carbohydrate diets on inflammatory markers have shown mixed results on CRP (7-9).

The lack of a consensus among studies may be due to differences in dietary

compliance, supplements, or medication use affecting lipid levels or inflammation.

While this study raises questions regarding the appropriateness of low carbohydrate

diet plans, it also underscores the beneficial effects of LF on a marker of CVD risk. It is

evident that continued research in this area is needed to determine long term effects of

these diets on systemic inflammation and the role of oxidative stress.

High Fat, Low Carbohydrate Diet, the Role of Oxidative Stress

A follow up study was conducted to delineate how HF increased inflammation (Chapter

4). Compared to the large intake of fruits and vegetables by LF, HF reported lower

intakes and subsequently, lower intakes of the antioxidant vitamins C and A (largely

from beta carotene). We were not convinced that oxidative stress did not play a role as

these dietary differences could have easily removed protection from damaging ROS

caused by a high fat intake. Animal studies have also shown that a high intake of

dietary fat may negate the positive effects of an energy restricted diet on oxidative

stress (10). Moreover, inclusion of antioxidants with acute high fat meals is shown to

attenuate inflammatory responses (11,12) indicating a role for oxidative stress. As

repeated oxidative stress could theoretically elevate inflammation, it was of interest to

further study the role of oxidative stress in HF diet-induced inflammation. Therefore, we

tested the ability of antioxidants to attenuate the elevation in inflammation in overweight

and obese men and women consuming a controlled low carbohydrate high fat diet (HF)

with (AS) or without (P) antioxidant supplements (1 g vitamin C, 800 IU vitamin E) for 7 d (matching the time frame within which HF previously increased inflammation).

The negative energy balance resulted in rapid weight loss and reduction in MCP-1, with a positive relationship between weight loss and IL-6, but not MCP-1. The inclusion of supplemental antioxidants did not impart a differential effect on most markers of inflammation (IL-6, MCP-1) or oxidative stress (8-epi, ORAC), however, there was a trend for a differential effect on CRP, as P increased by 50% while AS decreased by 32% (p=0.119). It is possible that the rapid rate of weight loss confounded any effects induced by HF and that (at least in the short term) weight loss is the dominant influence on inflammatory markers. However, these results do not support the hypothesis that oxidative stress is a mechanism for the previously observed increase in inflammation. While the trend for a differential response of serum CRP when antioxidants were consumed is provocative, the role for oxidative stress in HF was not confirmed and requires additional research to validate.

High Fat Meals Differing in Fat Source; Effects on Oxidative Stress and Inflammation
HF dieters also consumed a significantly higher proportion of saturated fat (SFA) and lower polyunsaturated fat (PUFA) than LF (both p<0.05) which could have impacted inflammation. Epidemiological research shows that SFA are associated with higher while PUFA such as long chain omega-3 fats (n-3FA) with lower levels of inflammatory markers (13). Acute high fat meals in some studies are shown to be pro-inflammatory, with a role for oxidative stress (11,12), however less is known about the contribution of the type of fatty acid on these acute postprandial effects. As a majority of time (17 of

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the 24 h) is spent in the postprandial state (14), clarifying the acute effects of different fats helps to refine dietary recommendations to reduce the risk of chronic disease. We therefore examined the acute responses in inflammation and oxidative stress following three high fat meals differing in fat source; palm oil (PO, high in SFA), olive oil (OO, high in MFA) or olive oil + fish oil (FO, containing 4 g n-3FA). Eleven overweight and obese men and women consumed all 3 meals in a crossover design at least one week apart and provided blood samples over a 6 h postprandial period (Chapter 5).

The novel finding of this study was that FO significantly increased activation of the redox sensitive transcription factor NF-κB compared to PO ($p<0.05$). This could indicate oxidative stress, yet another marker of oxidative stress (plasma 8-epi) did not follow the same trend, but rather, it decreased for all meals. Despite increased NF-κB, there was no associated increase in any inflammatory markers measured for FO. The only inflammatory marker to exhibit a differential effect between meals was ICAM-1. Higher ICAM-1 levels were detected following PO than OO ($p<0.05$), indicating an acute inflammatory effect of PO that may be independent of oxidative stress. The PO effects on ICAM-1 also appeared to be independent of plasma TG as TG were actually lower following PO than OO ($p<0.05$). The acute increase in NF-κB following FO may be a transient occurrence that subsides with repeated exposures, as would occur with chronic supplementation. This study raises questions regarding the acute effects of fatty acids on oxidative stress and inflammation which may be independent of their effects on blood lipids in overweight and obese individuals.

Theoretical Issues Related to the Effects of High Fat Diets and Acute Meals

Metabolite Contributions to Oxidative Stress and Inflammation

While both glucose and FFA are shown to increase oxidative stress and inflammation via mechanisms such as increased NADPH-oxidase activity (15), it appears that FFA played a more instrumental role in the findings of our studies. For example, although glucose declined in both weight loss studies, FFA increased to a greater extent with HF than LF, with a concomitant increase in CRP (Chapter 3). It is reasonable to assume that FFA increased substantially with the controlled HF diet in the second study (Chapter 4) as well. Thus, elevations in ROS may have occurred, with antioxidants helping to some degree, which led to the provocative trend of CRP attenuation for AS. FFA elevations are shown to elevate markers of oxidative stress *in vivo* (16) which may be linked to the fact that monocytes and neutrophils appear sensitive to activation by elevations in blood lipids (17,18). When activated, these immune cells can release both inflammatory mediators and ROS which could then stimulate an inflammatory response.

Further evidence is found in the fact that several markers of inflammation and oxidative stress (TNF, VCAM-1, 8-epi) decreased after the high fat, high energy meals (Chapter 5) in spite of an increase in blood glucose. As FFA also diminished during this period, the decrease in these markers signifies that they may have been more affected by the change in FFA than the change in glucose. The decline in FFA removed a powerful stimulus for inflammatory mediator release by immune and endothelial cells. It would be worthwhile to delve further into the effects of these high fat, high energy meals on inflammatory markers to determine the reason for this discrepancy; as several

studies agree with ours in that inflammation declined, while others have reported increases which are also supported by evidence from *in vitro* and animal model studies.

Heterogeneity of Overweight and Obese Subjects

Overall, the subjects in the second study (Chapter 4) did not respond as expected to the intervention, possibly due to the fact that subjects varied more widely at baseline than anticipated. These subjects also appeared healthier than the other studies, for example, 44% of these subjects started had a fasting CRP < 1 mg/L, qualifying them as low risk compared to 10 and 18% of subjects in the first and last studies respectively. Similarly, subjects varied considerably in 8-epi, however, there are no defined "risk" categories for that marker. Using above and below the median in 8-epi, we noted an inverse relationship between baseline and change scores, indicating that overall, the higher subjects responded favorably to weight loss. However, the "lower risk" subjects may have cancelled out any trends that would have been evident in the higher risk group alone. While the subjects did not differ in fasting blood glucose levels, it is possible that they did in blood lipids. In the acute meal study, for example, we identified two individuals as hypertriglyceridemic, though they did not respond differently than other subjects to the meals. Since they served as their own controls in that study, individual differences were less likely to affect the results or interpretations of treatment effects. However, these factors could have influenced the results of studies testing the effects of an intervention on distinct groups.

The heterogeneity present amongst the obese population is becoming better characterized as "metabolically healthy obese" (MHO) and "metabolically abnormal

obese" (MAO) (19,20) are shown to differ both in metabolic and inflammatory factors.

MHO appear to be less "inflamed" (21) and thus, do not experience decreases in

inflammation with weight loss (22), the opposite of which is true for MAO. The

difference between these two groups in oxidative stress, however, has not been

reported. The fact that not all obese individuals are inflamed challenges our current

view of obesity, where we assume that all excess fat is pro-inflammatory. The reason

that some people are MHO while others are MAO is likely due to a combination of

genetics and lifestyle, and possibly the length of time that an individual has been

overweight or obese. The existence of these subgroups complicates matters from a

research stand point and it is suggested that future studies include a pre-assessment

biochemical screening of obese subjects to ensure a more homogenous population for

dietary and weight loss studies, as mixing MHO and MAO may influence outcomes in

studies with distinct groups designed to test effects on inflammation.

Methodology for Oxidative Stress Assessment

Direct assessment of ROS *in vivo* is difficult, as high reactivity results in a very transient

nature. However, in order to discuss "oxidative stress," it is necessary to consider that

endogenous antioxidants help to protect cellular components from damage. Thus,

measuring markers of free radical damage is valuable not only because the markers are

more stable (longer-lived), but they also provide information regarding the actual

damage that has occurred. However, the marker of oxidative stress used in the first two

studies (urinary 8-epi) proved inconclusive, and did not yield meaningful or consistent

results. This may be due to the fact that the method used to measure 8-epi (urinary

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ELISA) has been criticized by some experts as being less reliable than the GC/MS method (23). However, the GC/MS method utilized later in plasma samples (Chapter 5) still did not provide an indication of the previously reported oxidative stress that follows acute high fat meals. It is, therefore, recommended that more comparisons be made between the response of 8-epi to other markers of oxidative stress in human dietary interventions. Future studies would also benefit from incorporating additional measures such as DNA damage and protein carbonyls, to obtain more information regarding the impact of oxidative stress on a wider selection of biomolecules.

Recommendations for Future Research

Inflammatory Marker Response to Dietary Modification

These studies have shown that while both acute and chronic modification of dietary macronutrients influences inflammation in overweight and obese individuals, the effects on various markers were not uniform. For example, IL-6 correlated better with weight loss in one study (Chapter 4) but not another (Chapter 3), while MCP-1 appeared more responsive to a negative energy balance than weight loss per se. CRP was more affected by different macronutrient type (high carbohydrate vs. high fat diet), while ICAM-1 was more sensitive to acute exposure to different fat sources. These differences may be due to differences in how the dietary interventions affected the system as a whole or which tissues/cells were directly impacted. For example, a primary source of IL-6 in obese individuals is adipose tissue, thus reducing the amount of body fat should correlate well with reductions in this marker, as we have shown. Following this line of reasoning, one possible explanation for why MCP-1 decreased

with the energy deficit but did not correlate with weight loss is that there was an acute depression of the innate immune system. It was shown that obese women consuming a low calorie diet experienced a degree of immunosuppression; specifically, reduced monocytic oxidative burst capacity (24). Reduction in the activity of cells such as circulating monocytes and tissue macrophages could theoretically decrease MCP-1 levels; as these cells play a key role in the release of this chemokine. Similarly, ICAM-1 levels may have been more responsive to an acute high fat meal due to direct exposure of endothelial cells to postprandial lipids, resulting in cell activation and subsequent release of ICAM-1. Additional work comparing the responses of these and other inflammatory markers to different dietary interventions is warranted. Further, it would be useful to test these effects using a homogenous group of overweight or obese individuals (i.e. MAO).

Fatty Acid Effects on Inflammatory Pathways

Inclusion of fish oil (FO) in a high fat meal was associated with a higher level of NF-kB activation compared to palm oil (PO), although no corresponding increase in inflammatory proteins occurred. It is well reported that chronic FO supplementation reduces inflammation; however, the chemical nature of the n-3FA in FO increases their susceptibility to oxidation, thus an acute increase in NF-kB is not entirely surprising. However, there is debate as to whether n-3FA oxidation products cause oxidative stress, and as 8-epi did not increase, it should be considered that NF-kB may have been activated by another signaling pathway. For example, it is possible that n-3FA inhibited phosphatidylinositol-3-kinase (PI3K)-akt (PI3K), and thereby activated NF-κB,

as has been shown *in vitro*. PI3K is a negative regulator of NF-κB, thus, inhibition of PI3K is shown to increase NF-κB activation in many cell types (25-28). In spite of this, inhibition of PI3K is also associated with reduced monocyte respiratory burst, NADPH oxidase activity, adhesion to endothelial cells, and chemotaxis (29-31). This is noteworthy as it has been suggested that this pathway may be a means by which n-3FA protect endothelial cells under conditions of elevated inflammation (25) and it could help to explain the reduced atherosclerosis and CVD noted with n-3FA.

As chronic ingestion of FO is often anti-inflammatory, the increase in NF-κB may be transient, subsiding with adaptation to supplementation. FO is shown to upregulate antioxidant enzymes over time in diabetics (32), which may help to prevent continued activation of this factor. Additional study in this area is warranted to better understand the changes that occur with chronic supplementation. Future research could investigate the acute dose responses to FO to determine whether effects on NF-κB are dose dependent, followed by a supplementation period (8 wk appears typical) of each dose prior to another acute meal to provide information regarding adaptations over time.

Implications for Differential Effects of Fatty Acids on ICAM-1

Olive oil (high in MFA) reduced plasma ICAM-1 compared to palm oil (high in SFA) which is important to consider, due to the role that ICAM-1 plays in atherosclerotic processes and the fact that plasma ICAM-1 is related to future coronary events (33). This supports the association noted by Couillard et al (34) that while total dietary fat intake was associated with elevated adhesion molecules, SFA intake was the only dietary variable that contributed to fasting ICAM-1 and VCAM-1 levels. This also adds

to the *in vitro* evidence that oleic acid (35) and other unsaturated fats are able to prevent induced adhesion molecule expression in endothelial cells while SFA appear unsuccessful (36). Moreover, *in vivo,* rats fed diets rich in either olive oil or fish oil, exhibit reduced lymphocyte adhesion molecule expression and *ex vivo* adhesion of stimulated lymphocytes to untreated endothelial cells (37). While cell membrane incorporation precedes many of the aforementioned effects, examination of the acute influence of fatty acids shows that meals high in SFA result in "less anti-inflammatory" HDL compared to those isolated after PUFA. Unlike PUFA HDL, SFA HDL were unable to prevent expression of ICAM-1 or VCAM-1 on endothelial cells *in vitro* (38).

ICAM-1 is constitutively present in endothelial cell membranes, and higher release is considered a product of endothelial activation (39). ICAM-1 assists in the "firm" tethering of leukocytes to the endothelium which precedes their transmigration through the vessel wall. Inappropriate activation of immune and endothelial cells can result in vessel damage and plaque development as monocytes in the wall differentiate into macrophages, take in cholesterol laden lipoproteins, and become foam cells. Here we have shown that OO reduced plasma ICAM-1 levels (and thus endothelial activation), making OO less atherogenic than PO in this respect. However, as ICAM-1 tended to return to baseline levels 6 h after the meals, the significance of the divergent effects remains uncertain. Although, using blood glucose levels as an example, significant increases, even for brief periods, may be detrimental over time. This may be particularly true for individuals who frequently consume high SFA meals, and thus experience repeated increases in ICAM-1.

Practical Implications of Dietary Macronutrient Effects on Health

Our findings support the dietary recommendations put forth by the American Heart Association (AHA) which advocate eating diets high in unrefined carbohydrates (fruits, vegetables, whole grains), including fish or fish oil supplements, minimizing intake of SFA (< 7%), and achieving and maintaining a healthy weight (40). Although the AHA and other organizations emphasize weight reduction for decreasing risk of chronic disease, we have shown that the macronutrient composition of a weight loss diet is equally as important. Short term, a high carbohydrate, low fat weight loss diet favorably reduced the inflammatory protein CRP, while a low carbohydrate, high fat diet increased this factor (which is associated with CVD risk). In regards to dietary fat, it appears that overweight and obese individuals are sensitive to differences in the acute effects of specific fatty acids in the postprandial period. In support of the AHA recommendations, dietary substitution of MFA for SFA may be prudent as meals high in SFA were shown to have a greater endothelial activation compared to those high in MFA. As a significant dose of n-3FA from fish oil transiently activated NF-κB in circulating immune cells, more modest doses are advisable until additional information is known regarding the effects of dosage and adaptation to chronic supplementation.

Conclusions

Overweight and obese individuals are at increased risk for chronic disease and often have elevations in inflammation and oxidative stress, two perpetuators of CVD, T2D, and hypertension. We have extended our understanding of the associations between dietary patterns and chronic disease by demonstrating that macronutrients differentially

influence oxidative stress and inflammation in overweight and obese individuals. We have shown that a weight loss diet that is low in carbohydrate and high in fat can negatively impact inflammation by increasing CRP in comparison to a low fat, high carbohydrate weight loss diet. This effect on CRP could be a troubling consequence of the high fat diet as it reduces the health benefits associated with weight loss and may increase CVD risk. We further showed that a negative energy balance resulting in rapid weight loss reduces MCP-1, potentially overriding the negative effects of a high fat diet on inflammation, while the role of oxidative stress remains unresolved. We also added insight into the health consequences of high fat meals in this population by showing that the type of fat can differentially impact postprandial inflammation and oxidative stress. Inclusion of fish oil in a high fat meal activated NF-κB, without concomitant elevations in a subset of inflammatory markers. On the other hand, olive oil reduced ICAM-1 in comparison to palm oil, lending support to the notion that diets high in MFA are more cardio-protective while SFA are considered more atherogenic.

Overall, these studies provide evidence that macronutrients can contribute to chronic disease both in conjunction with and independent of weight loss via effects on inflammation and oxidative stress in overweight and obese individuals. Additional research is necessary to clarify 1) the role of oxidative stress in the low carbohydrate high fat diet induced inflammation and 2) acute dose effects of fish oil on inflammation and whether adaptation occurs with chronic supplementation. As most Americans are either overweight or obese, and obesity continues to increase worldwide, this research can have broad reaching effects and may be valuable in helping to refine future dietary recommendations to help reduce the risk of chronic disease in this growing population.

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Appendix A

Sample Labeling and Handling Procedures

<u>Labeling legend and brief handling procedures</u>

Study #1

    <u>Sample labels</u>

    Subject number (1-38)

    Week number (Baseline, 1-4)

    <u>Study 1: Steps for serum collection, 24 h urine aliquots</u>

    Collect blood into 7 mL vacutainers

    Centrifuge at 1070 x g for 15 minutes

    Aliquot serum into appropriate cryovials (pre-labeled with measure and subject)

    Measure urine volume, aliquot into 2 cryovials – creatinine and 8-epi

Study #2

    <u>Sample labels</u>

    Subject number (1-20)

    Pre or post (1 or 2)

    <u>Study 2: Steps for serum collection, fasted urine aliquots</u>

    Collect blood into 7 mL vacutainers

    Centrifuge at 1070 x g for 15 minutes

    Aliquot serum into appropriate cryovials (pre-labeled with measure and subject)

    Measure urine volume, aliquot into 2 cryovials – creatinine and 8-epi

Study #3

    <u>Sample labels</u>

    Subject number, Trial, Blood draw time point

    <u>Study 3: Detailed sample handling procedures, see below</u>

Example of detailed sample handling procedures (used for study 3)

Need: Ice bucket, dewar with liquid N2, pipets (100uL and 200uL), and tips (green)

Plasma collection
Blood will be drawn into 4 tubes
    a) 1 - 10mL plasma EDTA tube (purple top) which will be used for all other
    analyses

    b) 2 - 6mL (green top) heparin tubes which will be used for cell collection

    c) 1 - 10mL heparin tube (green top)
    Place tubes on ice as soon as they're drawn…

Procedures:
    1) Take the tubes to lab 328 (on ice).
    - **6mL heparin tubes** to spin in 328 centrifuge at 400RCF for cell collection…
    - Large (10mL) green top heparin tubes into rack in 328 **refrigerator** for Dr. Liwu
    Li.
    - **Purple top EDTA** tubes to Dr. Grange's lab centrifuge to spin at 4 deg C for 15
    mins, 1000 x g (to obtain the plasma).

    2) Plasma
    a) Remove from centrifuge and place in rack carefully (do not disturb the RBC
    layer).

    b) Remove top with kim wipe and discard in a biohazard waste container

    c) With a disposable transfer pipet, remove all plasma from the tube and place in
    a small plastic or glass tube labeled for each subject (remove all plasma from
    tube first then aliquot into cryovials).

AMOUNTS TO ADD TO EACH TUBE
    3) Do the 8epi first (1.5 mL). Drop into dewar for immediate flash freezing.
    - After all have been aliquoted, put in the appropriate bags for each subject and
    into -80 freezer.

Order of aliquoting and amount of sample to be stored:

1) 8-epi	1.5mL		6) FFA	100uL
2) TNF-alpha	1.0mL		7) TG	100uL
3) Insulin	200uL-250uL		8) sICAM	100uL
4) Glucose	100uL		9) sVCAM	100uL-150uL
5) CRP	100uL		10) MCP-1	1mL if possible...

Note: Fill in this order with the first amount listed. If extra allows, put more in….

Appendix B

Detailed Methodology

Methodology for NF-kB. Includes PBMC isolation, nuclear extraction, EMSA, and processing of films.

Methodology for PBMC Isolation

Supplies: 1x PBS, Ice bucket, 15 mL conical tubes, Lymphocyte Separation Medium (LSM) density 1.077-1.080.

Set up: Add 4 mL LSM to conical tubes – how many tubes depends on how much blood you are using. For example, if you are using 10 mL of blood, which you need to mix 1:1 with PBS, you'll have 20ish mL of blood to distribute about 7 mL blood over 3 tubes containing 4 mL LSM each.

Procedures:
1) Mix Blood in 15 mL conical tubes with PBS in a ratio = 1:1

2) Carefully layer 7 mL of Blood/PBS mixture over 4 mL LSM (don't allow to mix). There

will be 3 of these tubes for each person.

3) Spin **400** rcf for 25 mins

4) Carefully remove buffy coats with Pasteur pipet, combine each subject's cells into

one tube (labeled)

5) Fill tube up to 10 mL with PBS, spin at **200** rcf for 10 mins.

6) Decant, gently resuspend pellet in fresh PBS (about 1mL), then fill up to 10 mL and

spin 10 mins at 200 rcf.

7) Decant, resuspend in 8mL fresh PBS, spin 8 mins **200** rcf to repellet

8) Resuspend in 1mL PBS ONLY. Transfer to microcentrifuge tube.

9) remove 45-50uL and put into tube labeled with a C (for Coulter counter). Place in

fridge

10) Keep cells on ice, immediately begin nuclear extraction procedure (move to step 2)

Nuclear extract preparation (Andrews and Faller method)

Start w/ between 5x10^5 and 10^7 cells. – find that about 10 million cells is ideal

1. Collect PBMCs using Lymphocyte Separation Medium (LSM) density 1.077-1.080.

Wash 2x with cold PBS, resuspend in 1mL 1X PBS.

2. Re-pellet the cells for 10 seconds upon arrival, remove PBS (3000 rpm).

3. Resuspend cell pellets in 400 uL cold Buffer A by flicking the tube

    Buffer A (10mM HEPES-KOH pH 7.9 @ 4 deg C, 1.5mM MgCL2, 10mM KCl,

    0.5mM DTT, 0.2mM PMSF).

4. Allow cells to swell on ice 12 mins.

5. Vortex 10 seconds.

6. Centrifuge 10 seconds (3000 rpm), discard supernatant.

7. Resuspend pellet in 20-100 uL (according to starting # of cells) of cold Buffer C.

    Buffer C: 20mM HEPES K-OH pH 7.9, 25% glycerol, 420mM NaCl, 1.5mM

    MgCl2, 0.2mM EDTA, 0.5mM DTT, 0.2mM PMSF.

8. Incubate on ice for 25 min for high salt extraction

9. Remove cellular debris by centrifugation for 2 mins @ 4 deg C (3000rpm).

10. Collect supernatant fraction, store at -70 deg C. (aliquot into 4 microcentrifuge (0.6

mL) tubes. Labeled with subject #, time point, and either P, 1, 2, 3. P = protein

analysis, 1, 2, 3, will be used to run on 3 different gels so must be in separate vials)

<u>Gel Shift Assay (from lab of Dr. Yong Woo Lee)</u>

<u>Night before:</u> Wash all plates, be sure to scrub in direction that gel will be running. Rinse with DI water and allow to dry upright in test tube rack. Wash spacers and comb.

<u>Preparation:</u>
1) Make the 5% Polyacrylamide gel:

Components	Amounts	Location
Distilled water (DW)	7.5 mL	Conical tube
2X Electrophoresis Buffer	10 mL (0.5X TBE)	On counter
40% acrylamide/bis solution	2.5 mL	Fridge
10% ammonium persulfate (APS)	200 uL	-80 freezer (-20 here)
TEMED	20 ul	On counter

2) Solidify for at least 1.5h, mark wells, clamp in stand, no bubbles, wash wells, pre-run for 2-3h at 150V. (Use 0.25X TBE for running.)

3) When 1hr 15 mins left in pre-run, prepare gel loading samples.

Master Mix (per sample) multiply these out by the number of samples +1

Distilled Water	8uL x 16 = 128	Fridge – white box
5X Binding buffer	4uL x 16 = 64	Fridge – white box
Poly(dI-dC)	2uL x 16 = 32	-80 freezer (-20 here)

4) Add 14uL of master mix to each labeled sample tube

5) Add appropriate amount of NEB to each tube

6) Add appropriate amount of sample to each tube
- for probe – add 4uL DI water instead of sample
- for competitor – repeat the sample with the expected highest concentration of your TF in the same proportions as used in that sample. (Later add 1 uL unlabeled competitor).

7) **Incubate all 10 mins at RT**

8) Add 1uL of competitor (unlabeled oligonucleotide) to "competitor, inducer goup" and further **incubate all for 15 mins at RT.**

9) Add 2uL of 32P-labeled oligonucleotide probe to each sample and **incubate 25mins at RT**.

10) Load 10uL of sample to 5% polyacrylamide gel with very skinny tips – add dye first. Then **run for 3h** (180 mins) at **150V**. Use gel running buffer 0.25X TBE (pH 8.0).

11. Dry gel & use autoradiography.

<u>Directions for Processor in Vet Med Phase II to process EMSA films</u>
Developed by Abby (Turpyn) Peairs and Susie Ayers

1. Housed in room 248 Phase II. Need key for weekends

2. To the right of the processor, see 3 water valves. The only one we will use is the top one. It should start off perpendicular to the pipe (off).

3. Close the lids, top down, paper lid to the right, PLUG in red light.

4. Turn the white on/off switch to on – located on the far right side of the processor (top)

5. Turn the big water valve on the wall ON (top one, turn up so is in line with pipe).

6. Turn the small blue water valve on the processor DOWN, to close the water valve.

7. When water stops running, processor is ready. Really – after this, wait about 5-10 mins. Should warm up for a total of 15 minutes before you run any films through.

Make sure the lights are off and red light on before running your films through. Turn on red ceiling lights, also. Light switches are labeled.

8. Once warmed up, run through 4 of the pre-exposed films (on top of the bin). The film goes in length wise – will only go in one way. They should come out the same way they went in. If you see streaks, etc, call Susie.

9. Then, run 1 unexposed film through. **Make sure room is dark and red light on before opening the bin or else will expose all the films to light**.

10. Look at the film after processing. If it looks ok, then it is safe to run your film through. (If not, call Susie).

11. Run your film through.

12. After get your film, turn the machine OFF, turn water OFF (perpendicular to hose), open the valve on the processor again (turn in line with the hose), prop open to air out.

<u>Notes</u>:
1. When chemistry level is 1 gallon, need to contact Susie Ayers in radiology for assistance in changing the chemicals. She will send someone over to mix chemistry.

Assistance Numbers:
Radiology: 231-4628
Susie Ayers: 231-2607

Oxygen Radical Absorbance Capacity (ORAC) Abbreviated Materials

Abbreviated Steps for ORAC procedure.

Training received at Tufts University under Drs. Blumberg and Chen (Summer 2006), proprietary - thus abbreviated steps are printed here.

For use on a FluoSTAR Optima Plate reader

Reagents:

a) ORAC buffer (0.75M K_2HPO_4, 0.75M NaH_2PO_4)

b) 100uM Trolox Standard Stock Solution for standards

c) 200nM Fluorescein Solution

d) 2,2'-azobis (2-amidinopropane) dihydrochloride AAPH (Wako)

Experimental Protocol

1. Prepare plasma or serum

2. Prepare trolox standards (50uM down to 5uM).

3. Load samples and standards into microplate

4. Add fluorescein

5. Add AAPH using an injection pump

6. Fluorescence filter - excitation wavelength of 485 nm and 520 nm for emission

7. Determine the area under the curve for the magnitude and time for complete

fluorescein oxidation.

C-Reactive Protein

Company: United Biotech

Solid phase enzyme linked immunosorbent assay (ELISA) technique, catalog # AD-401

NOTE: You will need to purchase additional diluents for this assay (difficult to tell ahead of time how much you will need to dilute your samples).

NOTE: Pre-label your sheet with your samples to keep you on track.

Procedure:

1. Bring all samples and reagents to room temperature, mix gently.

2. Prepare 1:100 dilutions of test samples by adding 5 uL of sample to 0.5 mL sample diluent in separate tubes. (you may need to do a 1:250 or 1:500 dilution later if 1:100 does not work). Be sure to leave blank wells.

3. Dispense 10 uL of standards or diluted samples in duplicate into appropriate wells.

4. Dispense 100 uL of enzyme conjugate (anti-CRP) antibody conjugated with horseradish peroxidase) into each well, mix for 5 seconds

5. Incubate at room temperature (RT) for 30 minutes.

6. Remove all mixture and rinse wells 5 times with water, wash thoroughly and completely dry wells.

7. Dispense 100 uL of solution A (substrate) and then 100 uL of solution B (chromogen) into each well, mix 5 seconds

8. Incubate in the dark for 15 minutes.

9. Stop reaction by adding 50 uL of 2 N HCl solution to each well, read at 450 nm with microwell reader against blank well (which contains only solution A and B).

10. Generate standard curve, plot unknowns to determine sample concentration.

Monocyte chemoattractant protein-1 (MCP-1)

Company: R&D Systems

Quantitative sandwich enzyme immunoassay (ELISA) technique, catalog # DCP00

<u>Procedure</u>

1. Dilute serum samples 1:1 (250 uL diluent: 250 uL sample)

2. Add 500 uL diluent to tubes for standards.

3. Do serial dilution for standards (start at 2000 pg/mL, transfer 500 uL to each, get

1000, 500, 250, 125, 62.5, 31.25 pg/mL for standards).

4. Add 50 uL assay diluent to each well.

5. Add 200 uL standard, sample, or control to each well, cover with adhesive strip,

incubate 2 h RT.

6. Aspirate, wash 3 x with wash buffer, using a squirt bottle. After last wash, invert and

rap smartly on to paper towels to dry.

7. Add 200 uL MCP-1 conjugate (polyclonal antibody to MCP-1 conjugated with HRP)

to each well, cover, incubate 2 h RT.

8. Repeat aspiration and wash step

9. Add 200 uL substrate solution (hydrogen peroxide and chromogen), place in dark for

30 minutes at RT.

10. Add 50 uL stop solution (2 N sulfuric acid).

11. Determine optical density within 30 minutes, plate reader set to 450 nm,

wavelength correction 540 nm.

12. For standard curve, use a 4 parameter logistic curve fit.

Soluble Intercellular Adhesion Molecule-1 (sICAM-1)

Company: R&D Systems

Quantitative sandwich enzyme immunoassay (ELISA) technique, catalog # BBE 1B

<u>Procedure</u>

1. Dilute samples 15 uL serum/plasma + 285 uL diluent (20 fold)

2. Add 100 uL diluted conjugate (antibody to sICAM-1 conjugated to HRP) to each well.

3. Add 100 uL sample, standard, or control to each well

4. Incubate 1.5 h at RT.

5. Wash 6 times with wash buffer, rap on paper towels after last one.

6. Add 100 uL substrate solution (hydrogen peroxide and chromogen) to each well

7. Incubate 30 min at RT

8. Add 100 uL stop solution (2N sulfuric acid)

9. Read within 30 min. Use 450 nm with wavelength correction of 620 nm.

10. Use 4-parameter logistic curve fit for standard curve.

Soluble Vascular Cell Adhesion Molecule-1 (VCAM-1)

Company: R&D Systems

Quantitative sandwich enzyme immunoassay (ELISA) technique, catalog # DVC00

<u>Procedure</u>

1. Dilute samples 20 uL serum/plasma + 380 uL diluent (20 fold)

2. Add 100 uL conjugate (monoclonal Ab against sVCAM-1 conjugated to HRP) to each well.

3. Add 100 uL sample, standard, or control to each well

4. Incubate 1.5 h at RT.

5. Aspirate, wash 4 times with wash buffer, rap on paper towels after last one.

6. Add 100 uL substrate solution (hydrogen peroxide, chromogen) to each well

7. Incubate 20 min at RT in the DARK

8. Add 50 uL stop solution (2N sulfuric acid)

9. Read within 30 min. Use 450 nm with wavelength correction of 540 nm.

10. Use best curve fit for standard curve (optical density vs. concentration), log/log.

Tumor Necrosis Factor-alpha

Company: R&D Systems

Quantitative sandwich enzyme immunoassay (ELISA) technique, catalog # HSTA00C

<u>Procedure</u>

1. Serial dilution of standards from 32 pg/mL to 0.5 pg/mL (32, 16, 8, 4, 2, 1, 0.5).

2. Add 50 uL assay diluent to each well.

3. Add 200 uL standard, sample, or control to each well, cover with adhesive strip.

4. Incubate 3 h at RT.

5. Wash 6 times with wash buffer. For each wash: fill each well with buffer with squirt
bottle, let soak for about 30 seconds, aspirate, rap smartly on paper towels, repeat all.

6. Add 200 uL conjugate (polyclonal Ab against TNF-a, alkaline phosphatase) to each
well, cover.

7. Incubate 2 h at RT.

8. Wash 6 times as in step 5.

9. Add 50 uL substrate solution (NADPH).

10. Incubate 1 h at RT.

11. Add 50 uL amplifier solution.

12. Incubate 30 min at RT.

13. Add 50 uL stop solution (2N sulfuric acid)

14. Read within 30 min at 490 nm, correction of 650 or 690 nm.

15. Average duplicate readings and subtract the zero standard optical density.

16. Plot optical density vs. concentration of standards, draw best curve, can use log/log
to linearize and use regression equation

Urinary 8-epi-prostaglandin-F2-alpha

Company: Oxis International

Competitive enzyme-linked immunoassay (ELISA) technique, catalog # 21048

<u>Procedure</u>

1. Prepare standards – serial dilution 100 to 0.05 ng/mL (100, 50, 10, 5, 1, 0.1, 0.05).

2. Dilute urine samples 1:2, 1:4, or 1:8 depending on expected concentration.

3. Add 100 uL standard or sample to each well.

4. Add 100 uL conjugate (8-epi conjugated to HRP) to all wells EXCEPT blank.

5. Seal plate, incubate 2 h at RT.

6. Aspirate, blot on paper towel, fill with wash buffer, let sit 2-3 minutes, repeat 2 times.

7. Add 200 uL substrate (TMB - chromogen), incubate 20-40 minutes until blue hue in standard 0.

8. Add 50 uL stop solution, see change to yellow.

9. Read at 450 nm within 30 minutes.

10. Use logistic curve fit. Subtract blank.

11. Report as pg/mg creatinine.

NOTE: As this is a competitive assay, the color intensity will be inversely proportional to the amount of unconjugated-F2a in the sample or standard (curve looks backwards).

Glucose

Company: Stanbio

Colorimetric assay, Enzymatic Glucose 1075

<u>Procedure</u>

1. Pipet 1 mL of reagent (glucose oxidase, peroxidase, 4-aminoantipyrine, p-

hydroxybenzene sulfonate) into glass tubes.

2. Add 0.01 mL of standard or sample to appropriate tubes.

3. Incubate at 37 deg for 10 minutes or at RT for 20 minutes.

4. Read at 500 nm within 15 minutes.

5. Calculate by hand: Glucose (mg/dL) = Abs of unknown/ Abs of std * 100

Non-esterified Free Fatty Acids (FFA)

Company: Wako

Colorimetric enzymatic assay, Catalog 994-75409

<u>Procedure</u>

1. Add 5 uL sample or calibrator to appropriate wells.

2. Add 100 uL color reagent A (acyl-coenzyme A synthetase, ascorbate oxidase, coenzyme A, ATP, 4-aminoantipyrine).

3. Mix well, incubate 5 min at RT.

4. Measure absorbance at 550 nm (correction 660).

5. Add 200 uL color reagent B (acyl-coA oxidase, peroxidase, 3-methyl-N-(B-hydroxyethyl)-aniline).

6. Mix well, incubate 5 min at RT.

7. Measure absorbance at 550 nm (correction 660).

8. Plot absorbance vs. concentration of calibration curve.

Triglycerides (TG)

Company: Wako

Colorimetric assay, Catalog 994-75409

<u>Procedure</u>

1. Add 5 uL sample or calibrator to appropriate wells.

2. Add 80 uL color reagent A (glucose kinase, glycerol-3-phosphate oxidase, 2-hydroxy-3-sulfopropyl)-3,5-dimetoxyaniline, ascorbate oxidase, ATP).

3. Mix well, incubate 5 min at RT.

4. Measure absorbance at 600 nm (correction 700).

5. Add 40 uL color reagent B (lipoprotein lipase, peroxidase, 4-aminoantipyrine).

6. Mix well, incubate 5 min at RT.

7. Measure absorbance at 600 nm (correction 700).

8. Plot absorbance vs. concentration of calibration curve.

Insulin

Company: Alpco Diagnostics

Quantitative sandwich enzyme immunoassay (ELISA) technique, Mercodia assay.

Procedure

1. Add 25 uL sample or standard to each well in duplicate.

2. Add 100 uL enzyme conjugate (anti-insulin Ab conjugated to peroxidase) to each well.

3. Incubate on plate shaker 1 h.

4. Wash 6 times with wash buffer, be sure to completely empty plate between each wash, rap smartly on paper towels at end.

5. Add 200 uL substrate (TMB – chromogen) to each well.

6. Incubate 15 min RT.

7. Add 50 uL stop solution (0.5M H_2SO_4), gently mix.

8. Read at 450 nm within 30 min.

9. Use cubic spline regression for standard curve.

Appendix C

Institutional Review Board Informed Consent Forms.

Study 1: Informed consent

Virginia Polytechnic Institute and State University
Informed Consent for Participants of Investigative Projects

Title of Project: Effects of a Low-Carbohydrate/High-Protein diet on Acid-Base Balance, Calcium metabolism, and Oxidative stress in premenopausal women.

Investigators: Mary Dean Coleman, M.S. R.D., Abigail D.Turpyn, B.S., Sharon M. Nickols-Richardson, Ph.D., R.D., and Janet W. Rankin, Ph.D.

I. Purpose: The purpose of this project is to determine the effects of long-term adherence to several weight loss diets on bone mineral density, markers of bone changes, acid/base metabolism, oxidative stress, and indicators of inflammation in a group of premenopausal women ages 32-45 over a 12-week period of time.

II. Procedures: Prior to being included in this research study, you will complete a Screening Questionnaire that will help determine if there are reasons why you should not participate in this study. If results of this Screening Questionnaire indicate that you are an appropriate participant for this study, asked to read, review, and sign this Informed Consent Form and to obtain authorized medical clearance from your primary care physician.

Following your acceptance into the study, you will be randomized into one of two dietary treatment groups. The investigators will then contact you to inform you of your assigned dietary intervention protocol. You will be scheduled to attend an informational session held in a classroom in Wallace Hall on the Virginia Tech campus. Group 1 will be assigned to the control group in which the members will be asked to maintain their current dietary habits throughout the duration of the study. Other members selected to participate in the dietary intervention protocol will be asked to attend the same meeting. During this session, the dietary regimen that the investigators will expect you to follow will be explained in further detail. If you are assigned to Group 2, you will follow a low-carbohydrate/high-protein Atkins Diet) in which carbohydrate intake will be limited to 20 grams per day for a period of 2-weeks. After the 2-week period, you will increase your carbohydrate intake by 5 grams per week until the end the study at 12-weeks. You will be asked to consume a calcium supplement that contains no more than 250 mg of calcium per tablet. Group 3 will be asked to follow a moderately energy restricted, low fat diet (similar to a Weight Watchers approach). Books and handouts that explain the requirements of the assigned dietary protocol, and that include recipes and lists of acceptable and non-acceptable foods will be provided during this time. Once the dietary protocol has been explained, an education session will be given to explain the proper method for filling out a 4-day dietary food record. You will be asked to take the 4-day dietary food record home and to fill it out four days prior to coming in for the first scheduled testing date. You will also be given a 24-hour urine specimen container and instructions explaining how to collect your urine by the investigators.

If you are selected to be in this study, you will be scheduled for the first (baseline) testing date. You will do the listed procedures, in order, during one, 2-hour ($\pm$ 30 minute) session at 7 time points (baseline, 1-week, 2-weeks, 3-weeks, 4-weeks, 6-weeks, and 12-weeks after baseline). You will be asked to do the following during each testing session:

(1) arrive in Room 229 Wallace Hall on the Virginia Tech campus at your scheduled day/time after having fasted overnight (minimum 12 hour fast; not having consumed foods or beverages except water);
(2) provide investigators with a 24-hour urine specimen collected during the 24-hours prior to appointment (collected in containers provided by investigators);
(3) provide a 4-day dietary record which was kept during the week prior to your appointment. The investigator will review the 4-day recall with each participant to clarify any questions the investigator may have regarding the recorded information (30 minutes);
(4) provide a second-void urine sample for pregnancy testing (all participants) and to detect the presence urinary Ketones (Atkins diet group only; 5 minutes);
(5) in an interview fashion, complete the Food Frequency Questionnaire (20 minutes); and KIHD Leisure-Time Activity History (5 minutes);—baseline testing only;
(6) have 44 mL (~4 Tablespoons) of whole blood drawn from your arm by a Licensed Medical Technician (10 minutes);
(7) provided with breakfast foods and beverages if desired;
(8) have your height and weight measured by an investigator (to determine your body mass index; 5 minutes);
(9) in an interview fashion, complete the 7-Day Physical Activity Recall (5 minutes);

You will be asked to have four DXA scans done at 3 times throughout the study. The initial scans will be done during the baseline testing session, while the other two scans will be done at week 6 and at week 12 following baseline testing. To complete the scans, you will be asked to:
(10) lie on or sit next to the dual-energy X-ray absorptiometer (DXA) as directed by a Licensed Radiologic Technologist – Limited who will conduct DXA scans of your whole body, lumbar spine, hip, and forearm for bone mineral density testing (15 minutes).

If you are selected for this study, you understand that your participation will require approximately 2-hours (± 30 minutes) of your time at each of seven testing times. You also understand that you may require more or less time than estimated to complete each procedure and that you will be given ample opportunity to complete all procedures in an unhurried manner. You will be expected to discontinue the use of any vitamin or mineral supplements unless the investigator asks you to take a supplement as part of the dietary regimen. If at anytime during the study there are any changes to your present health or medical status, or you experience any unusual symptoms, you understand you need to inform the investigator immediately.

Throughout the study, you will participate in weekly group sessions that will last approximately 1 hour (± 30 minutes). During this time, the investigators will offer motivational support and additional educational materials related to the dietary intervention being followed by the low-carbohydrate/high-protein and low-fat diet group. These sessions will be offered several times during the week to ensure each participant can attend. The investigators will also review the proper techniques for filling out the 4-day food record to ensure the participants are recording their intake as accurately as possible. The investigator will be available to answer any questions the participants may have regarding the diets during the weekly group sessions and individual testing sessions.

At the end of this study, the participants selected to be in the wait-list control group, will be given the option to follow the weight loss diet for a period of 12 weeks. The investigators will assign you to either the low-carbohydrate/high-protein diet (Atkins diet) or the conventional weight loss, low-fat diet with the supervision of the investigators of this study.   If you choose to follow the weight loss diet, you will be scheduled for the first (baseline) testing date and you will complete the listed procedures (as explained above), in order, during one, 2-hour (± 30 minute) session at

7 time points (baseline, 1-week, 2-weeks, 3-weeks, 4-weeks, 6-weeks, and 12-weeks after baseline).

III. Risks: The researchers are not aware of any specific risks associated with following either of the weight loss interventions included in this study.   Weight loss and low energy intake, in general can cause fatigue, dizziness, and changed in menstrual function in some women. Should these side effects occur, they should disappear after the dieting period.   If you experience any of the above or unusual symptoms during the dietary intervention, we ask you report those symptoms to the investigators immediately.

You understand that potential risks while participating in this study exist during blood draws and DXA scans. You understand that there is minimal risk involved in blood draws. A bruise may result from blood collection procedures with no known detrimental effects to your health or well being. You understand that to avoid or minimize bruising, a certified medical laboratory technician will draw your blood. Additionally, you will be allowed to sit or recline in the most comfortable position for yourself during your blood draw. You may rest for as long as needed after your blood is drawn and will be provided with breakfast foods and beverages after your blood is collected. Two attempts to draw your blood (or two needle sticks) will be allowed. If a second attempt is unsuccessful, no further tries for collection of your blood will be performed. You understand that all personnel involved in drawing and handling blood have undergone training for Blood Borne Pathogen Exposure Control administered by the Environmental Health and Safety Services of the Occupational Health Lab Safety Division at Virginia Tech. You understand that precautions will be taken by research personnel during handling of your blood (and urine) samples. You further understand that the standard operating procedures set by Virginia Tech's governing body will be executed in the event that blood or urine exposure occurs and includes HIV and hepatitis testing of your blood.

You understand that exposure to radiation will occur during DXA scans for measurement of your bone mineral density. Radiation exposure will occur from the DXA scans because the DXA machine uses x-ray technology. Radiation exposure is measured in millirads (or mR). Your total amount of exposure is 20 mR (whole body = 1mR, lumbar spine = 7 mR, hip = 7 mR; forearm = 5 mR) during each testing time and your cumulative total exposure is 60 mR if you complete all DXA scans throughout the 12 weeks of this study. Because your combined total exposure for the entire study represents 6% of the estimated exposure to increase cancer risk in only 0.03% or the population, you understand that this dose is very small, and poses minimal risk compared to radiation doses from dental bite-wing films (334 mR) and environmental background exposure (100 to 400 mR per year) expected to occur in one 12-week period. The following table lists the radiation limits for an adult research participant according to the National Institutes of Health, Office for Protection from Research Risk (NIH-OPRR), compared to your exposure during this study.

NIH-ORPP Radiation Limits for an Adult Research Participant	My Exposure During Participation in this Research Study
Whole body (single dose) = 3,000 mR	Whole body (single dose) = 1 mR
Whole body (annual cumulative dose) = 5,000 mR	Whole body (annual cumulative dose) = 3 mR
Lumbar spine (single dose) = 5,000 mR	Lumbar spine (single dose) = 7 mR
Lumbar spine (annual cumulative dose) = 15,000 mR	Lumbar spine (annual cumulative dose) = 21 mR
Hip (single dose) = 5,000 mR	Hip (single dose) = 7 mR
Hip (annual cumulative dose) = 15,000 mR	Hip (annual cumulative dose) = 21 mR
Forearm (single dose) = 5,000 mR	Forearm (single dose) = 5 mR
Forearm (annual cumulative dose) = 15,000 mR	Forearm (annual cumulative dose) = 15 mR
	ANNUAL CUMULATIVE EXPOSURE (whole body + lumbar spine + hip + forearm) = 60 mR during 12 weeks

You have been informed of the risks and may choose to not complete any one, combination, or all of these DXA scans. If in the event that any scan is unreadable or unusable, a replacement scan will not be conducted to avoid further exposure. **You further understand that if you are pregnant or think that you may be pregnant that you should not undergo DXA scans because radiation exposure from DXA scans may cause harm to your unborn fetus.**
These DXA scans will be conducted in the BONE Laboratory, Room 229 Wallace Hall, on the Virginia Tech campus by one of the Principal Investigators who are both Licensed Radiologic Technologists – Limited in the Commonwealth of Virginia.

IV. Benefits of this Project: The researchers do not guarantee any specific benefit to you as a result of following the dietary intervention or from your participation in the study, however, you will receive the following tests and results during your participation in the study: (1) analysis of your dietary intake, (2) measurement of your body composition; (3) analysis of your bone formation and resorption status; (4) measurement of your bone mineral density of your whole body, lumbar spine, hip, and forearm (5) analysis of your blood lipid profile; (6) analysis of your acid/base metabolism; (7) analysis of your calcium metabolism; and (8) analysis of indicators of oxidative stress, At completion of this study, you will be provided with your individual results from every procedure at each time point and may contact the investigator at a later date for a summary of the overall research results. You will be referred to an appropriate health care professional, if necessary, based on your individual results. Any and all costs related to such referral will be borne by you and not by Virginia Tech. The general public will benefit from your participation in this research as new understandings of links among dietary practices and chronic disease identified from this study will extend osteoporosis prevention knowledge and applications. Information concerning the effects of the diets on oxidative stress and inflammatory indicators will provide insight into the health risks or benefits of various dietary approaches to weight loss.

V. Extent of Anonymity and Confidentiality: Due to the inability to assure anonymity, you understand that confidentiality of your results will be preserved. You understand that this means that all of your answers to questions that you are asked, measurement and laboratory values, and DXA scans results will be kept confidential. A three-digit code number will be assigned to you. All questionnaires, data collection sheets, data analysis sheets, blood and

urine collection and storage containers, and DXA scan sheets will be identified by code number only and not by your name. You understand that a master list of participants' code numbers will be kept in a locked filing cabinet separate from completed data which will also be maintained in a locked filing cabinet. You further understand that only the investigators of this study will be allowed access to any data.

VI. Compensation: You will not be compensated or paid to be in this research project. However, you will receive your individual results from each procedure that you complete at the end of this study.

VII. Freedom to Withdraw: You understand that you can withdraw from this study at any time without penalty. You are free to not answer any questions or to not participate in any procedure included in this study without penalty. You understand that there may be circumstances under which the investigator may determine that you should not continue to participate in this project.

VIII. Emergency Procedure: If a minor emergency arises during your participation in this study, you will discontinue your participation and seek care from your personal physician. If a major emergency arises during your participation in this study, emergency personnel will be called (911), and they will care for you.

IX. Approval of Research: This research project has been approved, as required, by the Institutional Review Board for Research Involving Human Subjects at Virginia Polytechnic Institute and State University and by the Department of Human Nutrition, Foods and Exercise.

X. Subject's Responsibilities: You voluntarily agree to participate in this study. You have the following responsibilities:

For each dietary protocol:
(1)   provide consent to complete procedures for this study;
(2)   provide authorized medical clearance from your primary care physician (baseline testing only);
(3)   follow the guidelines established by the investigators for the dietary protocol assigned to you in this study;
(4)   attend the scheduled weekly group support and educational meetings;
(5)   arrive at Wallace Hall on the scheduled day after having fasted overnight for a minimum of 12 hours;
(6)   provide a 24-hour urine sample;
(7)   provide a 4-day dietary intake record;
(8)   provide a second-void urine sample;
(9)   be tested for pregnancy status;
(10)   honestly, and to the best of your knowledge, answer questions from the Food Frequency Questionnaire and KHID 12-month Leisure-Time Physical Activity History (baseline testing only);
(11)   have 44 mL (~ 4 Tbsp) of venous blood drawn by a Certified Medical Technician;
(12)   consume breakfast foods and beverage if desired;
(13)   have your height and weight measured by an investigator;
(14)   honestly, and to the best of your knowledge, answer questions from the 7-day Physical Activity Recall;
(15)   have your bone mineral density of the whole body, lumbar spine, hip, and forearm measured by DXA;
(16)   follow directions of the investigator as related to this project.

VI. Subject's Permission: You have read and understand the Informed Consent and conditions of this project. You have had all of your questions answered. You hereby acknowledge the above and give your voluntary consent for participation in this project. If you participate, you may withdraw at any time without penalty. You agree to abide by the rules of this project.

_____

Participant's Signature   Date

_____

Investigator's Signature   Date

Should you have any questions about this research or its conduct, you may contact:

Sharon M. (Shelly) Nickols-Richardson, Investigator
(540) 231-5104

Janet W. Rankin, Investigator
(540) 231-6355

OR

Dr. David M. Moore, IRB Chair
(540) 231-4991

Study 2 Informed Consent

Virginia Polytechnic Institute and State University
Informed Consent for Participants of Investigative Projects

Title of Project: Value of Dietary Antioxidants for Reduction of Oxidative Stress and Inflammation in Obesity.

Investigators: Janet W. Rankin, Ph.D (PI), Abigail D. Turpyn, B.S., Mary Whitlock, B.S.

I. Purpose: The purpose of this study is to examine the effects of antioxidant supplementation on levels of oxidative stress and inflammation in overweight/obese people following an Atkins diet. Various processes are known to increase the amount of chemicals that cause oxidation of chemicals in the body (e.g. smoking, strenuous exercise in unfit people, high fat and calorie meals). Oxidative stress and inflammation can be estimated by measuring several factors in the blood. Some research suggests that oxidative stress and inflammation may be linked to increased risk of heart disease, cancer and other chronic diseases. Overweight people usually have higher oxidative stress markers compared to lower weight individuals. It is possible that changes in diet may reduce the oxidative stress levels in these people. We will be specifically testing the effect of an antioxidant supplement on measures of oxidative stress and inflammation.

II. Procedures: Prior to being included in this research study, you will complete a Screening Questionnaire that will help determine if there are reasons why you should not participate in this study. These will include evidence of various health problems but also issues such as schedule and availability. If you meet the initial qualifications for the study criteria will be invited to attend an informational session held in a classroom on the Virginia Tech campus. During this session, the dietary regimen that the investigators will expect you to follow will be explained in further detail. Handouts that explain the requirements of the assigned dietary protocol, and that include lists of acceptable and non-acceptable foods will be provided during this time. If you are still interested, we will weigh you and measure the circumference of your abdomen. Experimenters will notify those subjects who qualify for the study within two weeks. If you qualify and you volunteer to participate, you will be provided with an Atkins diet for one week. All your food will be provided by the experimenters. You will have to come to Wallace Hall for two meals a day and given lunch and snacks to eat away from the facility. This diet will be designed around the recommendations for the first week of the Atkins diet [Dr. Atkins New Diet Revolution, 2002] and will consist of foods high in protein and fat but low in carbohydrates at less than 25 grams per day (e.g. meat, eggs, cheese but not breads, fruits). It is important that you do not consume any other foods from those provided during this week unless we have given you permission ahead of time. This includes beverages except for water—please ask us first. You can expect to lose between 2 and 4 pounds over the week but this will be variable by the person. In addition to the diet, we will provide you with a dietary supplement that you should consume each day. The supplement will be either an antioxidant supplement (vitamins E and C, lipoic acid) or a placebo (dummy pill).

There will also be two occasions where we will have morning fasting blood draws (~ 2 T per draw) and urine collection; one before the feedings (baseline) and one after the 7d diet. The blood draws will be done by a certified medical technician.

To summarize, if you are selected for this study, you understand that your participation will require approximately 2-hours (± 30 minutes) of your time at each of the two testing times, in addition to your attendance for meals each day at the laboratory facility. You also understand

that you may require more or less time than estimated to complete each procedure and that you will be given ample opportunity to complete all procedures in an unhurried manner. You will be expected to discontinue the use of any vitamin or mineral supplements for at least two weeks before and during the entire study. If at anytime during the study there are any changes to your present health or medical status, or you experience any unusual symptoms, you understand you need to inform the investigator immediately.

III. Risks: There is minimal risk involved in blood draws. A bruise may result from blood collection procedures with no known detrimental effects to your health or well- being. You understand that to avoid or minimize bruising, a certified medical laboratory technician will draw your blood. Additionally, you will be allowed to sit or recline in the most comfortable position for yourself during each blood draw. You may rest for as long as needed after your blood is drawn and will be provided with breakfast foods and beverages after your blood is collected. Two attempts to draw your blood (or two needle sticks) will be allowed. If a second attempt is unsuccessful, no further tries for collection of your blood will be performed. You understand that all personnel involved in drawing and handling blood have undergone training for Blood Borne Pathogen Exposure Control administered by the Environmental Health and Safety Services of the Occupational Health Lab Safety Division at Virginia Tech. You understand that precautions will be taken by research personnel during handling of your blood samples. You further understand that the standard operating procedures set by Virginia Tech's governing body will be executed in the event that blood or urine exposure occurs and includes HIV and hepatitis testing of your blood.

IV. Benefits of this Project: It is likely that you will benefit from participation in this research in several ways including: (1) receipt of information on blood and urine markers of oxidative status and inflammation status (2) all meals for one week. At completion of this study, you will be provided with your individual results from every procedure at each time point and may contact the investigator at a later date for a summary of the overall research results. You will be referred to an appropriate health care professional, if necessary, based on your individual results. Any and all costs related to such referral will be borne by you and not by Virginia Tech. The general public will benefit from your participation in this research as new understandings of links among dietary practices and chronic disease identified from this study.

V. Extent of Anonymity and Confidentiality: Due to the inability to assure anonymity, you understand that confidentiality of your results will be preserved. You understand that this means that all of your answers to questions that you are asked and measurements and laboratory values will be kept confidential. A code number will be assigned to you. All questionnaires, data collection sheets, data analysis sheets, blood and storage containers will be identified by code number only and not by your name. You understand that a master list of participants' code numbers will be kept in a locked filing cabinet separate from completed data, which will also be maintained in a locked filing cabinet. You further understand that only the investigators of this study will be allowed access to any data.

VI. Compensation: You will be compensated for participation in this research project. Specifically, you will be paid $10 for completion of the pre testing and an additional $65 for the post testing, for a total of $75 for completion of the study.

VII. Freedom to Withdraw: You can withdraw from this study at any time. You are free to not answer any questions or to not participate in any procedure included in this study. You understand that there may be circumstances under which the investigator may determine that

you should not continue to participate in this project (e.g. evidence of health risk or non-compliance to procedures).

VIII. Emergency Procedure: If a medical problem arises during your participation in this study, you should discontinue your participation, seek care from your personal physician, and inform the investigators. If a major emergency arises during your participation in this study, emergency personnel will be called (911), and they will care for you.

IX. Approval of Research: This research project has been approved, as required, by the Institutional Review Board for Research Involving Human Subjects at Virginia Polytechnic Institute and State University and by the Department of Human Nutrition, Foods and Exercise.

X. Subject's Responsibilities: You voluntarily agree to participate in this study. You have the following responsibilities:

(1)   Follow the dietary recommendations established by the investigators
(2)   Eat only the foods provided on the controlled feeding weeks
(3)   Consume no vitamins or mineral supplements for two weeks before and during the course of the study
(4)   Inform the experimenters of any medications or medical conditions that are pre-existing or begin during the study
(5)   Arrive at the laboratory on the scheduled testing days after having fasted overnight for a minimum of 12 hours;
(6)   Consume all of the dietary treatment each day of the intervention, or record and return any unused portions.
(7)   Allow the measurements to be conducted (weight, abdominal circumference, blood draws)

XI. Subject's Permission: You have read and understand the Informed Consent and conditions of this project. You have had all of your questions answered. You hereby acknowledge the above and give your voluntary consent for participation in this project. If you participate, you may withdraw at any time without penalty. You agree to abide by the rules of this project.

_____
Participant's Signature   Date

_____
Investigator's Signature   Date

Should you have any questions about this research or its conduct, you may contact:
    Janet W. Rankin, Principal Investigator
    (540) 231-6355
OR
    Dr. David M. Moore, IRB Chair
    (540) 231-4991

Study 3 Informed Consent

<div align="center">
Virginia Polytechnic Institute and State University
Informed Consent for Participants of Investigative Projects
</div>

Title of Project: The Acute Effects of Different Types of Fat on Oxidative Stress and Inflammation in Overweight and Obese Individuals

Investigators: Janet W. Rankin, Ph.D. (PI), Abigail D. Turpyn (Ph.D. Candidate)

I. Purpose: Overweight people usually have higher inflammation and oxidative stress markers compared to lower weight individuals. Oxidative stress is the damage caused by free radicals produced (reactive oxygen species, ROS) in the body in response to over-nutrition, sun exposure, and many other environmental factors, over and above the body's antioxidant capabilities. Free radicals can damage biological structures such as lipids, proteins, and DNA in the body. As antioxidants help to protect from free radical damage, the imbalance between free ROS damage and antioxidant capabilities is termed "oxidative stress". Oxidative stress and inflammation can be estimated by measuring several factors in the blood. These factors are related to increased risk of cardiovascular disease, cancer and other chronic diseases. It is possible that changes in diet may reduce elevated oxidative stress and inflammation and thus reduce risk of cardiovascular disease. For example, some types of dietary fat have different effects on metabolism and may differentially affect inflammation.   The purpose of this study is to determine whether the type of fat present in a high fat meal influences levels of oxidative stress and inflammation in overweight/obese individuals in the 6 hours after consumption. The activation of a particular gene regulator (NF-kB) will also be measured in the blood as it is indicative of oxidative stress and can increase the inflammatory response.

II. Procedures: Prior to being included in this research study, you will be provided with an informed consent and complete a brief screening questionnaire that will help to determine if you meet our initial selection criteria (overweight, non-smoking, sedentary, otherwise healthy, lactose tolerant, weight stable, not pregnant, without bleeding disorders, and available for testing). If you meet the initial qualifications for the study criteria you will be invited to attend an informational session held in a classroom on the Virginia Tech campus in which we will explain the informed consent form and study in greater detail. If you are still interested, we will assign to you a code number and ask that you fill out a medical history questionnaire. We will then weigh you, measure your height, and measure the circumference of your abdomen and hip. Experimenters will notify those subjects who qualify for the study within one week.
        If you qualify and you volunteer to participate, you will be asked to complete a 3 day food record (we will show you how to do this) and cease any antioxidant supplementation and to avoid foods high in antioxidants for the duration of the study. After 2 weeks without antioxidant supplements, you will be asked to participate in 3 meal challenges that will consist of a milkshake at least 1 week apart. On the mornings of the test meals, you will arrive at the laboratory fasted for at least 12 hours. You may not eat or drink anything besides water the morning of the test meal. You will arrive at the laboratory and fill out a questionnaire to insure that you are not currently sick or have an infection. You will then be measured for height, weight, and waist circumference followed by taking a blood sample (about 2 tablespoons) from your arm. We will provide you with a milkshake to consume within 10 minutes. The milkshake will contain milk, powdered milk, frozen yogurt, flavoring syrup, and an inert test fat. If you have an allergy to any of these components, you will not be able to participate There will be tests of your physiological and metabolic response to this high-fat, high calorie milkshake meal. More blood samples (each approximately 2 tbsp) will be taken at 1, 2, 4 and 6h after the meal.

To summarize, if you are selected for this study, you understand that your participation will require approximately 6 hours (± 30 minutes) of your time at each testing time. You also understand that you may require more or less time than estimated to complete each procedure and that you will be given ample opportunity to complete all procedures in an unhurried manner. You will be expected to discontinue the use of any vitamin or mineral supplements for the entire duration of this study and to check with the investigators before any over the counter or prescription medications are taken. You will arrive at the laboratory fasted for each test meal and complete the entire protocol each time. If at anytime during the study there are any changes to your present health or medical status, or you experience any unusual symptoms, you understand that you need to inform the investigators immediately.

III. Risks: There is minimal risk involved in blood draws. A bruise may result from blood collection procedures with no known detrimental effects to your health or well- being. You understand that to avoid or minimize bruising, a certified medical laboratory technician or nurse will draw your blood. You will be allowed to sit or recline in the most comfortable position for yourself during each blood draw. You may rest for as long as needed after your blood is drawn and will be provided with water after your blood is collected. Two attempts to draw your blood (or two needle sticks) will be allowed. If a second attempt is unsuccessful, no further tries for collection of your blood will be performed. There is also a small risk of fainting prior to, during, or after a blood draw. If this occurs, appropriate medical personnel will be contacted (911) and if you choose to be transported to a medical care facility, any costs associated with transportation or care will be borne by you and not by Virginia Tech.

You understand that all personnel involved in drawing and handling blood have undergone training for Blood Borne Pathogen Exposure Control administered by the Environmental Health and Safety Services of the Occupational Health Lab Safety Division at Virginia Tech. You understand that precautions will be taken by research personnel during handling of your blood samples. You further understand that the standard operating procedures set by Virginia Tech's governing body will be executed in the event that blood exposure occurs (blood spilled onto open skin of researcher) and includes HIV and hepatitis testing of your blood. There are two HIV/AIDS test sites in the area that offer HIV testing. If you are a Virginia Tech student, you have access to the Schiffert Health Center services, otherwise, you must use the Montgomery County Health Department. You will have the option of an anonymous test or a confidential test. The confidential test requires that you give your name and social security number to the testing facility, if you are positive, your name will be sent to the State Health Department (state law requires this). Your name will remain confidential, but this will be on your medical record. Both sites require pre-test and post-test counseling, and you will have to return in person 2 weeks later to get your results. You will not be allowed to call in for your results.

There is also the remote possibility that you may have a food allergy that is unknown to you. The ingredients have been made known to you ahead of time to avoid allergies, but if an allergic reaction were to occur, 911 would be called immediately and you may accept or decline their care, again all expenses would be your responsibility and not borne by Virginia Tech.

IV. Benefits of this Project: You will be provided with the results of your measurements, if you desire, including markers of oxidative stress and inflammation, blood triglyceride, glucose, insulin. You will be referred to an appropriate health care professional, if necessary, based on your individual results. For example, if any of your blood values appear to be above clinical guidelines, you will be informed and encouraged to have further evaluation by your personal physician at your own expense. The general public may benefit from your participation in this research as new understandings of links among dietary practices and chronic disease could be identified from this study.

V. Extent of Anonymity and Confidentiality: Due to the inability to assure anonymity, you understand that confidentiality of your results will be preserved. You understand that this means that all of your answers to questions, measurements and laboratory values will be kept confidential. A code number will be assigned to you. All questionnaires, data collection sheets, data analysis sheets, blood and storage containers will be identified by code number only and not by your name. You understand that a master list of participants' code numbers will be kept in a locked filing cabinet separate from completed data, which will also be maintained in a locked filing cabinet. You further understand that only the investigators of this study will be allowed access to any data.

VI. Compensation: You will be compensated for participation in this research project. Specifically, you will be given a $25 gift card for a local super store for completion of all procedures involved with each meal challenge (meal consumption and blood draws) and $25 upon completion of the study for a total of $100 for completing all three meal challenges.

VII. Freedom to Withdraw: You can withdraw from this study at any time. You are free to not answer any questions or to not participate in any procedure included in this study. You understand that there may be circumstances under which the investigator may determine that you should not continue to participate in this project. This could include evidence of health risk, weight loss, or non-compliance to procedures.

VIII. Emergency Procedure: If a minor emergency arises during your participation in this study, you will discontinue your participation and seek care from your personal physician. If a major emergency arises during your participation in this study, emergency personnel will be called (911), and they will care for you. Any costs associated with medical care received or transportation to a medical facility will be at the expense of the individual, and not Virginia Tech.

IX. Approval of Research: This research project has been approved, as required, by the Institutional Review Board for Research Involving Human Subjects at Virginia Polytechnic Institute and State University and by the Department of Human Nutrition, Foods and Exercise.

X. Subject's Responsibilities: You voluntarily agree to participate in this study. You have the following responsibilities:

(1)   Follow the dietary recommendations established by the investigators
(2)   Eat only the meals provided during the meal challenge measurement periods
(3)   Maintain your weight within 2 pounds through the study.
(4)   Consume no vitamins or mineral supplements during the course of the study
(5)   Arrive at the laboratory on the scheduled days after having fasted overnight for a
          minimum of 12 hours;
(6)   Consume all of the dietary treatment provided as a meal challenge.
(7)   Allow for measurements to be made (height, weight, abdominal circumference)
(8)   Allow for blood to be drawn at all time points (0, 1, 2, 4, 6h).

VI. Subject's Permission: You have read and understand the Informed Consent and conditions of this project. You have had all of your questions answered. You hereby acknowledge the above and give your voluntary consent for participation in this project. If you participate, you may withdraw at any time without penalty. You agree to abide by the rules of this project.

_____

Participant's Signature   Date

_____

Investigator's Signature   Date

Should you have any questions about this research or its conduct, you may contact:

Abigail Turpyn, Principal Investigator.   Janet W. Rankin, Faculty Advisor
(540) 231-7708   (540) 231-6355

OR

Dr. David M. Moore, IRB Chair
(540) 231-4991

Appendix D

IRB Approval Letters

Study 1 IRB Approval Letter

Virginia
[[[Tech
VIRGINIA POLYTECHNIC INSTITUTE
AND STATE UNIVERSITY

Department of Human Nutrition, Foods and Exercise

College of Human Resources and Education
338 Wallace Hall (0430), Blacksburg, Virginia 24061
(540) 231-4672   Fax: (540) 231-3916
Homepage: http://www.chre.vt.edu/HNFE/

Memorandum

Date: May 23, 2003

To: Dr. David M. Moore, Chair IRB
THRU: Dr. William G. Herbert, IRB Reviewer, HNFE Department

From: Sharon M. Nickols-Richardson, PhD, RD *Srf*
              HNFE (0430)

Subj: " Effects of a Low-Carbohydrate High-Protein diet on Acid-Base Balance, Calcium
         metabolism, and Oxidative stress in premenopausal women"

Enclosed is an application package for the project, " Effects of a Low-Carbohydrate High-
Protein diet on Acid-Base Balance, Calcium metabolism, and Oxidative stress in premenopausal
women" being conducted by Drs. Nickols-Richardson and Janet Rankin and graduate students,
Mary Dean Coleman, M.S., R.D., and Abigail Turpyn of the HNFE Department. Thank you for
your attention to this request. Should additional information be required, please contact Dr.
Nickols-Richardson by phone (1-5104) or e-mail (snrichar@vt.edu).

Study 2 IRB Approval letter

Virginia Tech
VIRGINIA POLYTECHNIC INSTITUTE
AND STATE UNIVERSITY

Institutional Review Board

Dr. David M. Moore
IRB (Human Subjects)Chair
Assistant Vice President for Research Compliance
CVM Phase II- Duckpond Dr., Blacksburg, VA 24061-0442
Office: 540/231-4991; FAX: 540/231-6033
email: moored@vt.edu

DATE: May 31, 2004

MEMORANDUM

TO: Janet W. Rankin Human Nutrition, Foods, & Exercise 0430
           Abigail Turpyn
           Mary Whitlock

FROM: David Moore

SUBJECT: **IRB Expedited Approval:** "Value of Dietary Antioxidants for Reduction of
           Oxidative Stress and Inflammation in Obesity" IRB # 04-286

This memo is regarding the above-mentioned protocol. The proposed research is eligible for
expedited review according to the specifications authorized by 45 CFR 46.110 and 21 CFR 56.110.
As Chair of the Virginia Tech Institutional Review Board, I have granted approval to the study for a
period of 12 months, effective May 28, 2004.

cc: File
    Department Reviewer Kevin Davy 0351

Study 3 IRB Approval letter
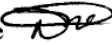
Virginia Tech

VIRGINIA POLYTECHNIC INSTITUTE
AND STATE UNIVERSITY

Institutional Review Board

Dr. David M. Moore
IRB (Human Subjects) Chair
Assistant Vice President for Research Compliance
1880 Pratt Drive, Suite 2006(0497), Blacksburg, VA 24061
Office: 540/231-4991; FAX: 540/231-0959
email: moored@vt.edu

DATE: October 14, 2005

MEMORANDUM

TO: Janet W. Rankin Human Nutrition, Foods, & Exercise 0430
            Abigail Turpyn HNFE

FROM: David Moore

SUBJECT: **IRB Expedited Approval:** "The Acute Effects of Different Types of Fat on
            Oxidative Stress and Inflammation in Overweight and Obese Individuals" IRB #
            05-609

This memo is regarding the above-mentioned protocol. The proposed research is eligible for
expedited review according to the specifications authorized by 45 CFR 46.110 and 21 CFR 56.110.
As Chair of the Virginia Tech Institutional Review Board, I have granted approval to the study for a
period of 12 months, effective October 14, 2005.

Virginia Tech has an approved Federal Wide Assurance (FWA00000572, exp. 7/20/07) on file with
OHRP, and its IRB Registration Number is IRB00000667.

cc: File
    Department Reviewer: William G. Herbert

3

Appendix E

Medical Health History Form

MEDICAL AND HEALTH HISTORY

Name: _____ Age: _____ Birth Date: _____

Address: _____ e-mail: _____

Phone Numbers: Home: _____ Work :_____

Person to Contact in Case of an Emergency: _____

Relationship: _____Phone: _____

Primary Care Physician: _____Phone: _____

Medical Insurance Carrier: _____

Are you employed by Virginia Tech? _____

Current Body Weight: _____ Height: _____

Are you a US citizen? _____ (added for study 3)

Subject #_____

MEDICAL HISTORY

Please indicate any current or previous conditions or problems you have experienced or have been told by a physician you have had:

	Yes	**No**
Heart disease or any heart problems:	_____	_____
Rheumatic Fever:	_____	_____
Respiratory disease or breathing problems (e.g. asthma):	_____	_____
Circulation problems:	_____	_____
Kidney disease or problems:	_____	_____
Urinary problems:	_____	_____
Musculoskeletal problems:	_____	_____
(i.e. Orthopedic injuries, osteoporosis)		
Fainting and Dizziness:	_____	_____
High Cholesterol:	_____	_____
Diabetes:	_____	_____
Thyroid problems:	_____	_____
Mental illness:	_____	_____
Hypoglycemia:(i.e. low blood sugar)	_____	_____
Epilepsy or seizures:	_____	_____
Blood clotting problems (e.g. hemophilia**):**	_____	_____
Anemia	_____	_____
Liver disorders (e.g. hepatitis B)	_____	_____
Rheumatoid arthritis	_____	_____
Lupus	_____	_____
Crohn's Disease	_____	_____

If you answered "yes" to any of the previous questions, please indicate the date and describe:
_____
_____
_____
_____

Please list any hospitalizations/operations/recent illnesses (type/date):
_____
_____
_____
_____

	YES	NO
Have you ever been diagnosed as having high blood pressure?	_____	_____
Are you currently being treated for high blood pressure?	_____	_____

If "yes", please explain:
_____
_____

Please list all medications (prescription and over-the-counter) you are currently taking or have taken in the past week:

_____
_____

For what reason(s) are you taking this medication?

_____
_____
_____

Health Habits

	Yes	No
Do you drink alcoholic beverages?	_____	_____
How many drinks per week? _____		
Do you smoke cigarettes?	_____	_____
Packs per day: _____		

	Yes	No
Do you engage in regular exercise?	_____	_____

If "yes", please list:

Activity	Frequency (times per week)	Duration (minutes)
_____	_____	_____
_____	_____	_____
_____	_____	_____

Do you ever faint, experience shortness of breath or chest discomfort with exertion? _____

If "yes", please explain: _____
_____

Are there any orthopedic limitations you have that may restrict your ability to perform exercise and if "yes", please explain:
_____
_____

Family History

Has anyone in your family been diagnosed or treated for any of the following?

	Yes	No	Relationship	Age
Heart attack	____	____	_____	____
Heart disease	____	____	_____	____
High blood pressure	____	____	_____	____
Stroke	____	____	_____	____
Kidney disease	____	____	_____	____
Diabetes	____	____	_____	____

Schedule Fall 2005 semester (indicate those times you have classes, work etc that you CANNOT be involved in study activities):

	Mon	Tue	Wed	Thurs	Fri	Sat	Sun
6:00-7:00am							
7:00-8:00							
8:00-9:00							
9:00-10:00							
10:00-11:00							
11:00-12:00							
12:00-1:00pm							
1:00-2:00							
2:00-3:00							
3:00-4:00							
4:00-5:00							
5:00-6:00							
6:00-7:00							
7:00-8:00							

Any explanation required for above _____

_____

Please sign to indicate that the above information is correct:

_____ _____   _____

 Print name   Signature   Date

Supplemental questions

Food Habits and Allergies

1. Are you allergic to any foods? _____ If yes, which ones?

2. Are you on any kind of special diet? _____ If so, what kind?

3. Do you take any dietary supplements?_____ If so, what kind and how often?

4. Has your weight been stable over the past year?_____ past 3 months?_____
    If you have not been weight stable, <u>how</u> has it changed? Please explain.

Comfort with procedures

5. Do you have a fear of needles or having blood withdrawn?

Appendix F

Diet Record Forms

Study 1: Food record for the low-carbohydrate diet group (developed by Mary Dean Coleman)

Date _____   Code # _____

4-Day Diet Record (3 Week Days + 1 Weekend Day)

Day of week Taken: M   T   W   Th   F   Sat   Sun   (Circle)

Meal	Time of Day	Food Eaten	Amount	# of Carbs	Cooking Method
Breakfast					
Snack					
Lunch					
Snack					
Dinner					
Snack					

Study 1: Diet record to fill in for the low-fat diet group, to help keep track of exchanges
4-Day Diet Record (3 Week Days + 1 Weekend Day)

Day of week: **M   T   W   Th   F   Sat   Sun   (Circle)**

Meal	Time of Day	Food Eaten	Cooking method	Amt	Starch	Fruit	Veg	Meat	Milk	Fat
Brkfst										
Snack										
Lunch										
Snack										
Dinner										
Snack										

Appendix G

Exit survey to assess compliance to controlled feeding portion of study 2

Exit survey

This survey is necessary so that we can accurately assess the study and analyze your data. Information obtained by this research may be published and used for future medical advice, so it is important that we know whether or not we should use your data in analyzing our results. Remember, the IRB informed consent that you signed before the study began was a contract. By participating in the entire study and performing both sample collections, you WILL receive the full payment, regardless of your answers here. It is very important that you answer these honestly. Thank you for working with us, and we hope that you have gotten something out of the study.

Subject #_____

1) How satisfied are you with the amount of weight lost?

2) Do you intend to lose more weight?
        If so, what is your plan? (Specific diet? Exercise?)

3) Did you like the Atkins type diet?

4) What was your favorite meal?

5) What was your least favorite meal?

6) Any comments or suggestions as to how to make this type of study run more smoothly?

7) On a scale of 1-5, with 5 being the utmost, how compliant were you during this study?
        Explain….

8) Did you comply (eating ONLY the foods given) every day?
        If not, how many days?

9) If you ate any other foods, or consumed any forbidden substances, please list them here, along with amounts and frequencies.
Appendix G

Appendix H

Summaries of Statistical Analyses

Table 1: Repeated Measures Analysis of Variance Summary for dependent measures in HF versus LF weight loss diet effects on inflammation and oxidative.

Measure	Group p-value	Time p-value	Group x Time p-value
Weight	0.231	0.000	0.000
Post hoc		All wks diff except wk 3 and 4	HF lost more wt than LF at wk 1
CRP	0.109	0.015	0.001
Post hoc		Wk 1 diff from other wks	HF inc. at wk 1, while LF dec.
Glucose	0.612	0.001	0.486
Post hoc		All wks diff from wk 0	
FFA	0.001	0.000	0.08
Post hoc	HF higher than LF	All wks diff from wk 0	
IL-6	0.177	0.041	0.711
Post hoc		Wk 4 diff from 2 and 3	
8-epi	0.669	0.609	0.035
Post hoc			No diff b/w grps at any wk, no sig. pattern

Group: Low carbohydrate, high fat (HF) or low fat, high carbohydrate (LF)
Time: Samples measured over 4 weeks (baseline, week 1, week 2, week 3, week 4)

CRP = C-reactive protein
FFA = Non-esterified fatty acids
IL-6 = Interleukin 6
8-epi = 8-epi-prostaglandin F2α

Table 2: Repeated Measures Analysis of Variance for dietary intake for low carbohydrate versus low fat weight loss diet study.

Measure	Group p-value	Time p-value	Group x Time p-value
Kcals	0.662	0.000	0.568
Post hoc		All wks diff. from wk 0, (all wks < wk 0)	
Kcal/kg	0.205	0.001	0.479
Post hoc		All wks diff. from wk 0 (all wks < wk 0)	
Fat	0.000	0.002	0.000
Post hoc	HF > LF	All wks diff. from wk 0	Grps diff. all wks > wk 0
Carbohydrate	0.000	0.000	0.000
Post hoc	LF > HF	All wks diff. from wk 0	Grps diff. all wks > wk 0
Protein	0.000	0.569	.000
Post hoc	HF > LF		Grps diff. all wks > wk 0
Vitamin C	0.000	0.673	0.845
Post hoc	LF > HF		Grps diff. all wks > wk 0
Vitamin A	0.000	0.017	0.012
Post hoc	LF > HF		Grps diff. all wks > wk 0
Vitamin E	0.951	0.507	0.855

Group: Low carbohydrate, high fat (HF) or low fat, high carbohydrate (LF)
Time: Samples measured over 4 weeks (baseline, week 1, week 2, week 3, week 4)

Table 3: Repeated Measures Analysis of Variance Summary for dependent measures before and after 1 wk HF with or without antioxidant supplementation.

Measure	Group p-value	Time p-value	Group x Time p-value (t-test between change)
Weight	0.507	0.000	0.344
Post hoc		Wk 1 < wk 0	
CRP	0.409	0.711	0.119
IL-6	0.113	0.183	0.939
MCP-1	0.322	0.006	0.929
Post hoc		Wk 1 < wk 0	
8-epi	0.241	0.75	0.975
Glucose	0.07	0.000	0.573
Post hoc		Wk 1 < wk 0	
ORAC	0.374	0.544	0.534

Group: Antioxidant Supplement (AS) or Placebo (P)
Time: Baseline (day 0), after 1 week on diet (day 8)

HF = low carbohydrate, high fat diet
CRP = C-reactive protein
IL-6 = Interleukin-6
MCP-1 = Monocyte chemoattractant protein-1
8-epi = 8-epi-prostaglandin F2α
ORAC = Oxygen radical absorbance capacity

Table 4: Repeated Measures Analysis of Variance for dependent measures following high energy, high fat meals differing in the source of fat.

Measure	Treatment p-value	Time p-value	Treatment x Time p-value
CRP (ln)	0.222	0.045	0.056
Post hoc		4h and 6h PP > time 2h	
TNF	0.393	0.000	0.421
Post hoc		All time points < time 0	
ICAM	0.031	0.315	0.654
Post hoc	PO > OO		
VCAM	0.574	0.085	0.948
8-epi (ln)	0.374	0.019	0.459
Post hoc		4 h > 2h	
NF-κB	0.146	0.759	0.315
FFA	0.369	0.000	0.14
Post hoc		Time 0 > all but 6 h	
TG (ln)	0.017	0.000	0.214
Post hoc	OO > PO	All time points > time 0	
Glucose	0.157	0.019	0.115
Post hoc		1 h PP > all	
Insulin	0.856	0.000	0.781
Post hoc		1 h PP > 2 h PP > all other time points	

(ln) = natural logarithm used due to non-normal data distribution

CRP = C-reactive protein, TNF = Tumor necrosis factor-α, ICAM = Intercellular adhesion molecule-1, NF-κB = nuclear factor κ-B, FFA = Free fatty acids, TG = Triglycerides.

Treatment: PO = palm oil, OO = olive oil
Time: over 6 hours (baseline (time 0), 1, 2, 4, 6 h postprandial (PP)).

Table 5: One factor Analysis of Variance for dependent measures area under the curve following high energy, high fat meals differing in the source of fat.

Measure	Treatment p-value	Post hoc
CRP (ln)	0.106	
TNF	0.773	
ICAM	0.051	PO > OO
VCAM	0.83	
8-epi (ln)	0.339	
NF-kB	0.046	FO > PO
FFA	0.331	
Tg (ln)	0.02	OO > PO
Glucose	0.347	
Insulin	0.076	FO > PO

(ln) = natural logarithm used due to non-normal data distribution

CRP = C-reactive protein, TNF = Tumor necrosis factor-α, ICAM = Intercellular adhesion molecule-1, NF-κB = nuclear factor κ-B, FFA = Free fatty acids, TG = Triglycerides.

Treatment: OO = olive oil, PO = palm oil, FO = fish oil
Time: over 6 hours (baseline (time 0), 1, 2, 4, 6 h postprandial (PP)).

Appendix I

Raw Data Tables

Table 1. Anthropometrics for LC/HF versus HC/LF weight loss diet study

Group	Subj #	Age	Weight (kg)	BMI (kg/m^2)	Waist (cm)
LC/HF	1	33	61.0	24.6	77.5
	3	42	73.6	25.5	81.3
	4	39	96.8	37.8	96.5
	5	40	86.8	30.9	87.6
	6	39	75.0	30.2	94.0
	7	40	74.1	29.9	91.4
	9	43	83.2	30.6	81.3
	10	32	84.5	31.1	82.6
	11	38	92.5	33.0	94.0
	12	37	88.6	33.6	*
	14	38	91.4	35.1	88.9
	16	34	118.6	42.3	101.6
	17	44	112.7	43.4	111.8
	24	44	82.7	30.4	91.4
Mean +/-		38.8	87.3	32.7	90.8
SEM		1.0	4.1	1.5	2.6
HC/LF	19	38	55.5	26.6	80.0
	20	40	70.0	26.5	88.9
	21	34	56.4	25.1	71.1
	22	40	69.5	26.3	80.0
	23	42	69.5	27.2	77.5
	25	41	75.0	29.8	83.8
	26	41	73.2	28.6	88.9
	27	40	72.3	30.1	87.6
	28	36	81.8	32.0	90.2
	29	43	93.6	33.4	87.6
	30	40	75.5	31.4	*
	33	37	109.5	37.9	105.4
	34	40	101.4	39.6	106.7
	35	45	101.4	43.6	100.3
	36	45	83.4	33.6	87.6
Mean +/-		40.1	79.2	31.4	88.3
SEM		0.8	4.2	1.4	2.7

* not determined or missing data point

LC/HF = low carbohydrate, high fat diet
HC/LF = high carbohydrate, low fat diet

Table 2. Weights (kg) for LC/HF versus HC/LF weight loss diet study.

Group	Subj #	Wk 0	Wk 1	Wk 2	Wk 3	Wk 4
LC/HF	001	61.0	59.00	58.32	57.73	56.82
	003	73.6	70.68	70.23	69.66	70.00
	004	96.8	94.09	93.64	92.73	92.27
	005	86.8	85.45	85.23	85.00	84.68
	006	75.0	72.45	71.36	71.02	70.23
	007	74.1	72.05	70.68	71.14	71.59
	009	83.2	81.36	81.14	80.36	79.55
	0010	84.5	82.95	82.27	81.14	81.36
	0011	92.5	89.23	88.30	87.50	87.73
	0012	88.6	86.36	84.32	84.00	83.64
	0014	91.4	89.77	89.77	89.77	88.55
	0016	118.6	115.91	115.68	114.32	114.00
	0017	112.7	109.68	108.77	107.86	107.27
	0024	82.7	81.36	81.36	80.91	81.02
Mean +/-		87.3	85.03	84.36	83.80	83.48
SEM		4.1	4.01	4.04	3.97	3.96
HC/LF	0019	55.5	54.20	54.66	54.09	52.95
	0020	70.0	69.09	69.55	67.95	66.70
	0021	*56.4*	*55.23*	*56.59*	*55.45*	*55.68*
	0022	69.5	69.09	67.95	67.16	67.05
	0023	69.5	68.64	67.95	67.73	66.70
	0025	75.0	74.55	74.09	73.18	72.73
	0026	73.2	71.93	71.36	70.00	68.30
	0027	72.3	71.36	71.25	70.23	69.66
	0028	81.8	81.36	80.45	78.75	79.09
	0029	93.6	92.27	90.91	89.55	89.09
	0030	75.5	74.09	75.23	*	73.86
	0033	109.5	107.73	107.50	104.77	103.64
	0034	101.4	100.91	100.91	100.23	100.91
	0035	101.4	100.91	99.77	98.18	98.52
	0036	83.4	82.73	81.70	81.59	83.64
Mean +/ -		79.2	78.27	77.99	77.06	76.57
SEM		4.1	4.15	4.05	4.26	4.05

* not determined or missing data point

LC/HF = low carbohydrate, high fat diet
HC/LF = high carbohydrate, low fat diet

Table 3. Serum C-reactive protein (mg/L) for LC/HF versus LF/HC weight loss diet study.

Group	Subj #	Wk 0	Wk 1	Wk 2	Wk 3	Wk 4
LC/HF	001	7.730	**	17.258	10.031	12.573
	003	4.824	7.244	6.943	3.134	4.638
	004	5.215	8.297	5.043	4.410	5.070
	005	2.024	4.266	3.786	3.627	4.136
	006	0.611	4.340	1.170	1.029	0.916
	007	1.785	4.243	2.583	1.149	7.950
	009	3.655	12.231	7.464	5.191	4.996
	0010	4.078	9.317	5.502	6.066	4.966
	0011	17.123	15.630	8.394	13.382	16.389
	0012	3.115	1.698	1.086	1.611	2.608
	0014	1.144	9.542	3.390	2.471	2.700
	0016	9.872	11.422	6.513	12.354	8.843
	0017	17.250	**	14.872	20.202	22.105
	0024	1.426	1.647	1.442	1.136	1.611
Mean +/-		5.704	7.490	6.103	6.128	7.107
SEM		1.47	1.26	1.30	1.54	1.63
HC/LF	0019	13.727	6.478	15.989	12.516	6.566
	0020	8.666	7.206	†	8.009	5.817
	0021	0.281	0.069	0.294	0.198	0.074
	0022	0.510	0.279	0.211	0.198	0.237
	0023	4.995	4.240	4.518	1.563	1.009
	0025	1.260	0.837	1.138	0.961	1.150
	0026	1.950	0.756	2.489	1.173	0.609
	0027	4.155	3.456	2.448	2.716	1.183
	0028	3.812	4.713	*	1.308	1.373
	0029	3.382	2.667	2.510	2.066	1.539
	0030	4.268	2.121	2.717	*	3.031
	0033	3.821	7.349	2.403	1.796	3.913
	0034	13.461	14.516	9.971	14.004	10.186
	0035	5.307	6.488	4.597	8.342	2.439
	0036	1.930	2.402	6.036	1.416	0.596
Mean +/-		4.768	4.238	4.255	4.048	2.648
SEM		1.07	0.98	1.22	1.16	0.74

* not determined or missing data point
** too high to be accurately determined
† too high due to recorded illness

LC/HF = low carbohydrate, high fat diet
HC/LF = high carbohydrate, low fat diet

Table 4. Serum interleukin-6 (pg/mL) for LC/HF versus HC/LF weight loss diet study.

Group	Subj #	Wk 0	Wk 1	Wk 2	Wk 3	Wk 4
LC/HF	001	0.547	0.512	2.69	1.493	0.887
	003	0.535	0.507	0.728	0.706	1.061
	004	1.506	2.87	1.968	1.416	1.59
	005	2.243	1.326	2.014	1.681	3.087
	006	1.977	1.946	1.758	1.479	2.21
	007	2.029	1.438	1.222	1.455	1.339
	009	2.722	1.36	1.222	1.193	1.207
	0010	0.855	1.3	0.701	0.676	1.395
	0011	2.748	2.157	1.61	1.6	2.53
	0012	0.802	0.641	1.022	0.905	2.815
	0014	0.847	1.035	0.499	0.491	1.075
	0016	2.195	1.814	1.396	1.714	2.07
	0017	2.015	2.815	1.765	1.825	2.467
	0024	1.412	2.045	3.125	0.913	1.165
Mean +/-		1.6024	1.55	1.55	1.25	1.78
SEM		0.21	0.20	0.20	0.12	0.20
HC/LF	0019	3.082	1.968	2.776	2.794	4.013
	0020	1.754	1.9636	†	1.586	1.903
	0021	0.327	0.289	0.242	0.35	0.25
	0022	0.715	0.654	0.841	0.455	0.755
	0023	1.395	1.797	1.277	0.895	1.033
	0025	0.29	0.515	0.437	0.589	0.518
	0026	0.727	0.514	1.254	2.45	1.381
	0027	0.632	0.758	0.678	0.393	0.539
	0028	1.086	1.691	*	1.077	0.908
	0029	1.203	0.81	0.841	1.238	1.896
	0030	0.615	0.701	0.636	*	1.353
	0033	2.217	2.566	1.694	1.732	2.245
	0034	0.829	0.88	0.894	0.676	3.01
	0035	1.343	1.01	1.103	1.116	1.632
	0036	1.737	2.356	1.743	1.146	0.553
Mean +/-		1.20	1.23	1.11	1.18	1.47
SEM		0.20	0.19	0.19	0.20	0.27

* not determined or missing data point
† too high due to recorded illness

LC/HF = low carbohydrate, high fat diet
HC/LF = high carbohydrate, low fat diet

Table 5. Urinary 8-epi-prostaglandin F2α (pg/mg creatinine) for LC/HF versus LF/HC weight loss diet study.

Group	Subj #	Wk 0	Wk 1	Wk 2	Wk 3	Wk 4
LC/HF	001	715	1652	932	1505	1648
	003	601	1175	1667	1307	986
	004	1711	484	2199	896	785
	005	*	2905	2675	2320	3691
	006	1161	499	873	1169	1101
	007	1587	1499	681	1284	1771
	009	2250	1123	1919	1047	1168
	0010	1767	960	1000	697	1620
	0011	2032	961	640	671	2427
	0012	1623	885	713	496	1031
	0014	1752	*	633	1009	1923
	0016	2277	1736	3604	800	880
	0017	1583	508	691	515	789
	0024	1428	978	876	2014	1115
Mean +/-		1576	1182	1365	1124	1495
SEM		142	183	246	144	213
HC/LF	0019	1382	1078	624	596	710
	0020	1131	1037	388	1048	778
	0021	1523	2630	1346	1639	1246
	0022	1193	1089	457	1726	660
	0023	671	641	606	361	406
	0025	2163	4754	977	2550	1705
	0026	1545	1786	1745	1337	1647
	0027	961	1019	822	841	458
	0028	946	1362	644	1890	2205
	0029	1529	1413	1756	2344	947
	0030	791	610	991	*	1606
	0033	419	746	1290	715	118
	0034	1002	1020	1622	1770	590
	0035	973	1167	2024	441	948
	0036	2459	2756	3115	1268	1933
Mean +/-		1246	1541	1227	1323	1064
SEM		140	283	190	185	161

* not determined or missing data point

LC/HF = low carbohydrate, high fat diet
HC/LF = high carbohydrate, low fat diet

Table 6. Serum non-esterified free fatty acids (FFA) (mEq/L) for LC/HF versus LF/HC weight loss diet study.

Group	Subj #	Wk 0	Wk 1	Wk 2	Wk 3	Wk 4
LC/HF	001	0.31	0.94	0.59	0.45	1.10
	003	0.45	0.50	0.58	0.46	0.36
	004	0.34	0.42	0.62	0.66	0.46
	005	0.25	0.65	0.57	0.55	0.49
	006	0.48	0.96	0.67	0.98	0.79
	007	0.22	0.29	0.47	0.26	0.47
	009	0.18	0.45	0.62	0.44	0.65
	0010	0.31	0.55	0.80	0.69	0.79
	0011	0.48	0.80	0.72	0.74	0.59
	0012	0.27	0.62	0.53	0.67	0.25
	0014	0.18	0.49	0.42	0.54	0.42
	0016	0.17	0.67	0.58	0.39	0.80
	0017	0.58	0.55	0.56	0.46	0.50
	0024	0.22	0.26	0.27	0.24	0.21
Mean +/-		0.32	0.58	0.57	0.54	0.56
SEM		0.04	0.06	0.03	0.05	0.06
HC/LF	0019	0.28	0.43	0.49	0.35	0.55
	0020	0.22	0.47	0.21	0.35	0.32
	0021	0.30	0.32	0.20	0.22	0.23
	0022	0.17	0.40	0.58	0.30	0.21
	0023	0.38	0.50	0.32	0.36	0.25
	0025	0.24	0.44	0.35	0.37	0.26
	0026	0.14	0.15	0.21	0.32	0.29
	0027	0.19	0.49	0.48	0.49	0.26
	0028	0.46	0.52	*	0.48	0.70
	0029	0.31	0.14	0.71	0.54	0.52
	0030	0.15	0.41	0.19	*	0.31
	0033	0.38	0.39	0.38	0.45	0.39
	0034	0.35	0.44	0.27	0.57	0.37
	0035	0.35	0.35	0.38	0.51	0.41
	0036	0.20	0.36	0.45	0.44	0.29
Mean +/-		0.27	0.39	0.37	0.41	0.36
SEM		0.03	0.03	0.04	0.03	0.04

* not determined or missing data point

LC/HF = low carbohydrate, high fat diet
HC/LF = high carbohydrate, low fat diet

Table 7. Serum glucose (mg/dL) for LC/HF versus LF/HC weight loss diet study.

Group	Subj #	Wk 0	Wk 1	Wk 2	Wk 3	Wk 4
LC/HF	001	87.0	59.0	69.0	64.0	61.0
	003	83.0	77.0	91.0	82.0	88.0
	004	84.0	84.0	82.0	75.0	78.0
	005	77.0	79.0	78.0	78.0	81.0
	006	93.0	75.0	74.0	95.0	86.0
	007	82.0	92.0	83.0	90.0	87.0
	009	87.3	86.0	80.0	82.0	79.0
	0010	86.8	85.0	76.0	84.0	82.0
	0011	94.1	79.0	74.0	85.0	86.0
	0012	86.6	89.0	83.0	80.0	90.0
	0014	75.1	70.0	72.0	86.0	73.0
	0016	84.7	84.0	84.0	76.0	86.0
	0017	92.4	77.0	80.0	81.0	84.0
	0024	86.0	80.0	76.0	84.0	82.0
Mean +/-		85.6	79.7	78.7	81.6	81.6
SEM		1.5	2.2	1.5	2.0	2.0
HC/LF	0019	113.0	89.0	92.0	93.0	90.0
	0020	83.0	86.0	78.0	72.0	84.0
	0021	71.4	74.0	72.0	71.0	81.0
	0022	69.3	70.0	69.0	79.0	75.0
	0023	85.3	81.0	82.0	80.0	85.0
	0025	74.0	84.0	75.0	80.0	78.0
	0026	87.0	96.0	73.0	81.0	80.0
	0027	85.0	89.0	80.0	82.0	81.0
	0028	90.0	84.0	*	84.0	81.0
	0029	98.5	75.0	86.0	81.0	89.0
	0030	82.0	81.0	80.0	*	80.0
	0033	85.6	91.0	86.0	86.0	92.0
	0034	81.0	88.0	82.0	91.0	88.0
	0035	86.7	84.0	77.0	82.0	83.0
	0036	79.3	82.0	74.0	82.0	82.0
Mean +/-		84.7	83.6	79.0	81.7	83.3
SEM		2.8	1.8	1.7	1.6	1.2

* not determined or missing data point

LC/HF = low carbohydrate, high fat diet
HC/LF = high carbohydrate, low fat diet

Table 8. Baseline subject characteristics of antioxidant supplement (AS) and placebo (P) groups.

Group	Subj #	Age	Height (m)	Weight (kg)	BMI (kg/m^2)	Waist (cm)
AS	1	26	1.82	92.4	27.9	91.5
	3	39	1.56	83.8	34.4	97.5
	4	35	1.74	96	31.9	96.0
	5	25	1.55	66.5	27.7	74.0
	8	34	1.80	92.4	28.5	95.5
	10	40	1.66	80.1	29.1	82.5
	11	24	1.71	79.9	27.5	92.5
	15	25	1.84	152.5	45.3	132.5
	19	37	1.71	86.3	29.7	98.5
	20	31	1.65	137.6	50.5	127.0
Mean +/- SEM		31.6	1.71	96.8	33.3	98.8
		2.0	0.03	8.3	2.6	5.7
P	2	27	1.78	89.2	28.3	90.5
	6	47	1.79	130.5	40.7	126.0
	7	31	1.72	142.3	48.4	117.5
	9	21	1.81	126.1	38.7	117.5
	12	30	1.77	88.9	28.5	95.5
	14#	30	1.68	81.4	29.0	93.0
	16	21	1.60	68.9	27.1	76.5
	17	27	1.70	88.3	30.6	101.0
	18	35	1.69	109.3	38.3	112.5
Mean +/- SEM		29.9	1.73	105.4	35.1	104.6
		3.0	0.02	9.1	2.7	5.9

subject removed from data analysis due to non-compliance

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Table 9. Body weight (kg) following HF for one week with antioxidant (AS) vs. placebo (P).

Group	Subject	Baseline	Week1	Change	% chg
AS	1	92.4	88.6	-3.8	-4.1
	3	83.8	82.3	-1.5	-1.8
	4	96	92.9	-3.1	-3.2
	5	66.5	65.1	-1.4	-2.1
	8	92.4	89.4	-3	-3.2
	10	80.1	76.8	-3.3	-4.1
	11	79.9	77.3	-2.6	-3.3
	15	152.5	146.5	-6	-3.9
	19	86.3	82.8	-3.5	-4.1
	20	137.6	136.3	-1.3	-1.0
Mean +/-		96.8	93.8	-3.0	-3.1%
SEM		8.5	8.3	0.4	0.4
P	2	89.2	86.2	-3	-3.4
	6	130.5	125	-5.5	-4.2
	7	142.3	136.5	-5.8	-4.1
	9	126.1	123	-3.1	-2.5
	12	88.9	85.9	-3	-3.4
	14#	81.4	81.3	--	--
	16	68.9	66.6	-2.3	-3.3
	17	88.3	84.7	-3.6	-4.1
	18	109.3	106.9	-2.4	-2.2
Mean +/-		105.4	101.9	-3.6	-3.4%
SEM		9.1	8.7	0.5	0.3

this subject removed from data analysis due to non-compliance

HF = low carbohydrate, high fat diet

Table 10. Serum C-reactive protein (mg/L) following HF for one week with antioxidant (AS) vs. placebo (P).

Group	Subject	Baseline	Week1	Change	% chg
AS	1	0.7	0.3	-0.5	-62.5
	3	12.4	5.0	-7.4	-59.9
	4	3.2	3.9	0.7	20.8
	5	1.6	†	--	--
	8	0.2	0.4	0.2	70.0
	10	0.9	1.3	0.3	34.3
	11	0.3	0.2	-0.1	-47.9
	15	1.0	2.0	1.0	107.2
	19	2.3	1.5	-0.8	-34.0
	20	**	**	**	**
Mean +/-		2.6	1.8	-0.8	3.5
SEM		1.4	0.6	1.0	22.7
P	2	2.7	4.4	1.7	63.6
	6	3.4	6.4	3.0	87.7
	7	3.2	4.0	0.8	24.3
	9	2.1	3.0	1.0	46.7
	12	0.6	0.5	-0.1	-10.2
	14#	0.5	0.5	0.0	0.0
	16	4.2	2.2	-2.0	-47.1
	17	0.4	0.4	-0.1	-11.2
	18	4.7	10.9	6.2	133.7
Mean +/-		2.7	4.0	1.3	35.9
SEM		0.5	1.2	0.9	20.9

** too high to be accurately determined
† too high due to recorded illness
# not included due to non-compliance with diet

HF = low carbohydrate, high fat diet

Table 11. Serum interleukin-6 (pg/mL) following HF for one week with antioxidant (AS) vs. placebo (P).

Group	Subject	Baseline	Week 1	Change	% Change
AS	1	0.47	0.30	-0.17	-35.8
	3	0.33	0.78	0.45	136.8
	4	1.15	1.23	0.08	7.1
	5	0.6	3.7†	--	--
	8	0.93	1.11	0.18	19.3
	10	0.41	0.52	0.11	27.1
	11	1.07	0.71	-0.35	-33.2
	15	1.08	0.54	-0.54	-49.9
	19	1.55	0.42	-1.13	-72.6
	20	**	**	**	**
Mean +/-		0.87	0.70	-0.17	-0.2
SEM		0.15	0.12	0.18	23.15
P	2	1.33	0.77	-0.56	-42.3
	6	2.62	2.46	-0.17	-6.3
	7	2.04	1.26	-0.78	-38.3
	9	0.60	0.78	0.18	30.4
	12	0.49	0.60	0.11	22.9
	14#	0.19	0.25	--	--
	16	1.04	1.13	0.10	9.2
	17	0.90	0.38	-0.51	-57.1
	18	1.35	1.82	0.47	34.8
Mean +/-		1.30	1.15	-0.15	-5.8
SEM		0.26	0.24	0.15	12.7

** too high to be accurately determined
† too high due to recorded illness
# not included due to non-compliance with diet

HF = low carbohydrate, high fat diet

Table 12. Serum monocyte chemoattractant protein-1 (pg/mL) following HF for one week with antioxidant (AS) vs. placebo (P).

Group	Subject	Baseline	Week 1	Change	% Change
AS	1	472	379	-94	-19.8
	3	287	325	38	13.3
	4	337	320	-17	-5.0
	5	133	57	-76	-56.9
	8	429	340	-89	-20.8
	10	494	356	-138	-27.9
	11	325	197	-128	-39.4
	15	534	563	29	5.5
	19	517	413	-104	-20.2
	20	318	348	30	9.4
Mean +/-		385	330	-55	-16.2%
SEM		40	40	22	7.1
P	2	334	257	-77	-23.0
	6	475	342	-133	-27.9
	7	176	176	0	0.0
	9	415	340	-75	-18.1
	12	211	162	-49	-23.4
	14#	235	181	--	--
	16	237	312	75	31.7
	17	454	307	-147	-32.4
	18	*	*	*	*
Mean +/-		329	271	-58	-13.3%
SEM		40	28	29	8.4

* not included due to sample issue
# not included due to non-compliance with diet

HF = low carbohydrate, high fat diet

Table 13. Urinary 8-epi prostaglandin F2α (pg/mg creatinine) following HF for one week with antioxidant (AS) vs. placebo (P).

Group	Subject	Baseline	Week 1	Change	% Change
AS	1	1084	2274	1190	110
	3	3439	1898	-1542	-45
	4	4468	3643	-825	-18
	5	3101	1847	-1254	-40
	8	3516	1948	-1568	-45
	10	1194	1863	669	56
	11	695	2295	1600	230
	15	2040	2783	743	36
	19	1194	1462	267	22
	20	2468	2485	17	1
Mean +/-		2320	2250	-70	31
SEM		404	195	366	27
P	2	800	1399	599	75
	6	2447	1882	-565	-23
	7	1394	1737	343	25
	9	3048	2055	-993	-33
	12	1808	1437	-371	-21
	14#	1906	3134	--	--
	16	2571	2401	-170	-7
	17	1168	1722	555	47
	18	2318*	1904*	*	*
Mean +/-		1891	1805	-86	9
SEM		312	132	229	15

* not included due to sample issue
# not included due to non-compliance with diet

HF = low carbohydrate, high fat diet

Table 14. Serum Oxygen Radical Absorbance Capacity (ORAC) (Trolox Equivalents/L) following HF for one week with antioxidant (AS) vs. placebo (P).

Group	Subject	Baseline	Week 1	Change	% Change
AS	1	9835	10507	671	6.8
	3	12322	10991	-1332	-10.8
	4	11055	11642	586	5.3
	5	10612	12198	1586	14.9
	8	11086	11908	821	7.4
	10	11799	12689	890	7.5
	11	11990	12048	58	0.5
	15	13168	13374	205	1.6
	19	12808	12459	-349	-2.7
	20	10625	10764	139	1.3
Mean +/-		11530	11858	328	3.2
SEM		336	285	251	2.2
P	2	10640	12023	1382	13.0
	6	12802	12378	-424	-3.3
	7	11005	9820	-1186	-10.8
	9	12521	13930	1409	11.2
	12	13752	11092	-2660	-19.3
	14#	--	--	--	--
	16	10838	11868	1030	9.5
	17	13044	13117	73	0.6
	18	12209	12552	344	2.8
Mean +/-		12102	12098	-4	0.5
SEM		406	442	495	4.0

not measured due to non-compliance with diet

HF = low carbohydrate, high fat diet

Table 15. Serum glucose following HF for one week with antioxidant (AS) vs. placebo (P).

Group	Subject	Baseline	Week 1	Change	% Change
AS	1	92.6	67.3	-25.3	-27.3
	3	76.4	75.3	-1.1	-1.5
	4	71.6	67.9	-3.7	-5.2
	5	65.9	49.4	-16.5	-25.0
	8	81.3	60.5	-20.7	-25.5
	10	86.9	60.2	-26.7	-30.7
	11	82.5	61.9	-20.6	-25.0
	15	84.4	66.5	-17.9	-21.2
	19	81.5	55.4	-26.1	-32.1
	20	73.3	66.5	-6.8	-9.3
Mean +/-		79.6	63.1	-16.5	-20.3
SEM		2.5	2.3	3.0	3.5
P	2	78.1	69.9	-8.2	-10.5
	6	79.3	70.5	-8.8	-11.1
	7	89.1	91.2	2.1	2.4
	9	83.4	66.5	-16.9	-20.3
	12	88.6	66.8	-21.9	-24.7
	14#	86	75.8	--	--
	16	80.3	57.4	-22.9	-28.5
	17	90.9	69.6	-21.3	-23.4
	18	82.2	67.6	-14.6	-17.8
Mean +/-		84.0	69.9	-14.1	-16.7
SEM		1.7	3.4	3.1	3.5

not included due to non-compliance with diet

HF = low carbohydrate, high fat diet

Table 16. Subject characteristics for acute high fat meal challenges.

Subject	Age	Height (m)	Weight (kg)	BMI (kg/m^2)	Waist (cm)
1	27	1.83	96.2	28.76	96.0
2	22	1.69	124.8	43.70	104.13
3	21	1.63	70.4	26.64	78.0
4	27	1.56	75.8	31.06	81.0
5	40	1.70	102.4	35.64	106.0
6	41	1.53	106.9	45.67	115.0
7	40	1.80	109.5	33.98	122.0
8 #	20	1.87	131.9	37.84	108.0
9	47	1.93	124.9	33.52	111.0
10	21	1.58	93.8	37.57	100.0
11	45	1.72	85.2	28.80	82.0
Mean +/-	31.9	1.71	98.64	34.6	99.5
SEM	3.2	0.13	5.7	2.0	4.8

Subject not included in final analysis

Table 17. Plasma C-reactive protein following fish oil enriched high fat meal (mg/L).

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	0.4	0.4	0.4	0.4	0.4
2	12.2	11.6	12.6	13.1	12.8
3	6.1	4.6	6.3	6.1	4.9
4	1.5	1.6	1.6	1.2	1.8
5	1.6	1.6	1.5	1.2	1.4
6	17.8**	22.5**	22.0**	19.5**	29.8**
7	5.7	5.8	5.1	7.4	6.6
8 #	1.3	1.0	1.2	1.6	0.9
9	1.8	1.7	1.3	1.4	1.5
10	5.4	6.2	5.8	6.4	6.8
11	1.4	1.3	1.2	1.3	1.3
Mean +/-	4.0	3.8	4.0	4.3	4.2
SEM	1.3	1.2	1.3	1.4	1.4

** too high due to acute illness
# subject not included in final analysis
PP = Postprandial

Table 18. Plasma C-reactive protein following high fat palm oil meal (mg/L).

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	0.3	0.3	0.3	0.4	0.3
2	10.4	9.5	9.0	10.2	10.3
3	34.8**	29.9**	33.9**	44.7**	39.2**
4	1.0	0.9	0.9	0.9	1.0
5	1.4	1.3	1.2	1.7	1.8
6	58.4**	64.9**	69.1**	56.8**	50.2**
7	6.7	7.7	7.2	6.9	7.6
8 #	2.0	*	2.2	1.7	1.4
9	1.8	1.8	2.1	3.4	2.0
10	4.8	4.3	4.5	5.7	4.8
11	0.9	0.8	0.8	0.9	0.6
Mean +/-	3.4	3.3	3.2	3.8	3.6
SEM	1.3	1.2	1.2	1.3	1.3

* no sample
** too high due to acute illness
# subject not included in final analysis

PP = Postprandial

Table 19. Plasma C-reactive protein (mg/L) following high fat olive oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	0.2	0.2	0.2	0.2	0.2
2	9.8	8.9	10.4	10.1	10.1
3	3.2	3.1	3.1	3.7	4.0
4	1.7	1.7	1.0	1.3	1.6
5	49.6**	38.0**	36.1**	40.6**	44.9**
6	6.7	6.0	6.3	8.8	7.3
7	4.2	5.7	4.8	4.9	4.8
8 #	3.8	3.5	3.7	3.2	3.3
9	0.9	1.0	0.8	0.6	0.9
10	1.6	2.1	2.0	2.3	2.2
11	0.4	0.5	0.5	0.7	0.9
Mean +/-	3.2	3.2	3.2	3.6	3.6
SEM	1.1	1.0	1.1	1.2	1.1

** too high due to acute illness
# subject not included in final analysis

PP = Postprandial

Table 20. Plasma tumor necrosis factor-α (pg/mL) following fish oil enriched high fat meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	1.00	0.71	0.93	1.07	0.94
2	1.39	1.01	1.00	1.24	1.25
3	0.91	0.97	0.88	1.22	0.90
4	0.72	0.74	0.74	0.51	0.56
5	1.83	1.79	1.52	1.37	1.40
6	1.09	1.47	1.55	1.12	1.19
7	1.57	1.40	1.48	1.68	1.48
8 #	0.98	0.92	1.20	1.08	0.70
9	0.83	0.79	0.86	0.80	0.83
10	1.20	1.21	0.95	1.16	1.19
11	1.30	1.20	1.10	0.96	1.08
Mean +/-	1.18	1.13	1.10	1.11	1.08
SEM	0.11	0.11	0.10	0.10	0.09

subject not included in final analysis

PP = Postprandial

Table 21. Plasma tumor necrosis factor-α (pg/mL) following high fat palm oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	0.93	1.11	1.26	0.98	1.18
2	1.57	1.69	1.30	1.43	1.50
3	1.12	0.97	1.10	1.10	1.02
4	0.79	0.56	0.64	0.60	0.54
5	1.60	1.26	1.33	1.61	1.61
6	1.66	1.47	1.35	1.37	1.28
7	1.46	1.46	1.04	1.36	1.35
8 #	1.35	*	1.27	1.33	1.27
9	0.85	0.81	0.60	0.67	0.78
10	1.89	1.38	1.34	1.20	1.17
11	1.34	1.15	1.36	1.08	1.44
Mean +/-	1.32	1.19	1.13	1.14	1.19
SEM	0.12	0.11	0.09	0.10	0.11

* no sample
# subject not included in final analysis

PP = Postprandial

Table 22. Plasma tumor necrosis factor-α (pg/mL) following high fat olive oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	1.03	0.90	0.93	0.91	0.91
2	1.97	1.82	1.73	1.94	2.05
3	1.01	1.16	0.70	0.80	0.80
4	0.74	0.68	0.55	0.57	*
5	1.53	1.62	1.03	1.02	1.02
6	2.11	1.63	0.84	1.27	1.36
7	1.65	1.56	1.35	1.67	1.26
8 #	1.18	1.16	1.09	1.09	1.11
9	0.87	0.99	0.95	0.86	0.86
10	1.80	1.69	1.45	1.57	1.73
11	1.23	0.95	1.12	0.98	0.97
Mean +/-	1.39	1.30	1.07	1.16	1.22
SEM	0.15	0.13	0.11	0.14	0.14

* no sample
# subject not included in final analysis

PP = Postprandial

Table 23. Plasma soluble intercellular adhesion molecule-1 (ng/mL) following fish oil enriched high fat meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	173	156	150	152	165
2	278	266	277	260	242
3	192	194	192	188	189
4	203	221	196	196	208
5	198	209	215	217	207
6	243	236	231	240	241
7	226	225	233	262	271
8 #	240	240	226	237	232
9	178	195	191	197	210
10	184	184	195	188	198
11	173	196	176	179	168
Mean +/-	205	208	206	208	210
SEM	11	10	11	11	11

subject not included in final analysis

PP = Postprandial

Table 24. Plasma soluble intercellular adhesion molecule-1 (ng/mL) following high fat palm oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	157	164	157	156	165
2	265	266	266	274	274
3	212	202	201	198	203
4	206	189	211	234	232
5	208	230	193	200	192
6	272	315	304	269	288
7	215	222	225	231	267
8#	237	*	239	236	244
9	191	194	183	182	183
10	209	198	201	192	184
11	193	209	230	222	204
Mean +/-	213	219	217	216	219
SEM	11	14	13	12	14

* no sample
# subject not included in final analysis

PP = postprandial

Table 25. Plasma soluble intercellular adhesion molecule-1 (ng/mL) following high fat olive oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	161	153	145	151	134
2	265	254	254	254	253
3	195	181	184	179	170
4	250	215	198	202	*
5	191	176	177	180	195
6	259	250	238	255	267
7	249	251	219	261	242
8#	239	247	222	231	224
9	189	200	203	184	215
10	185	187	200	209	219
11	179	178	174	181	184
Mean +/-	212	204	199	206	209
SEM	12	11	10	12	14

* no sample
# subject not included in final analysis

PP = postprandial

Table 26. Plasma soluble vascular cell adhesion molecule-1 (ng/mL) following fish oil enriched high fat meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	741	650	669	708	768
2	666	638	623	626	707
3	604	617	595	644	545
4	491	419	417	407	423
5	555	497	500	547	609
6	674	625	635	601	603
7	771	761	768	795	824
8#	868	831	882	924	933
9	579	551	540	556	588
10	698	702	682	692	720
11	963	962	923	947	900
Mean +/-	674	642	635	652	669
SEM	42	47	45	47	45

subject not included in final analysis

PP = postprandial

Table 27. Plasma soluble vascular cell adhesion molecule-1 (ng/mL) following high fat palm oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	660	698	640	687	730
2	713	643	633	620	639
3	657	627	610	632	645
4	429	391	420	465	427
5	583	562	596	590	569
6	697	697	707	691	682
7	817	773	812	801	811
8#	907	*	887	838	948
9	558	617	591	608	595
10	808	739	749	711	754
11	885	920	873	867	919
Mean +/-	681	667	663	667	677
SEM	43	44	41	36	43

** no sample
# subject not included in final analysis

PP = postprandial

Table 28. Plasma soluble vascular cell adhesion molecule-1 (ng/mL) following high fat olive oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	773	674	703	646	633
2	623	679	713	661	638
3	609	581	559	541	552
4	421	437	426	429	*
5	588	548	484	539	539
6	721	702	655	702	725
7	721	739	723	813	687
8#	768	764	780	752	816
9	548	599	589	561	645
10	830	762	854	769	928
11	924	875	896	859	866
Mean +/-	676	660	660	652	691
SEM	47	39	47	43	44

* no sample
# subject not included in final analysis

PP = postprandial

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Table 29. Plasma 8-epi-prostaglandin-F2α (ng/mL) following fish oil enriched high fat meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP
1	0.051	0.047	0.052	0.053
2	0.078	0.099	0.067	0.108
3	0.090	0.073	0.066	0.064
4	0.058	0.055	0.054	0.055
5	0.067	0.061	0.071	0.073
6	0.094	0.085	0.078	0.135
7	0.060	0.058	0.056	0.067
8 #	*	*	*	*
9	0.039	0.043	0.047	0.052
10	0.091	0.120	0.114	0.136
11	**	**	0.0611†	0.0603†
Mean +/-	0.070	0.071	0.067	0.083
SEM	0.020	0.026	0.020	0.034

* no sample
** sample unable to be analyzed
† samples not included in analysis due to others missing in time series
# subject not included in final analysis

PP = Postprandial

Table 30. Plasma 8-epi-prostaglandin-F2α (ng/mL) following high fat palm oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP
1	0.039	0.057	0.054	0.043
2	0.147	0.119	0.109	0.128
3	0.136	0.102	0.099	0.103
4	0.063	0.051	0.063	0.060
5	0.069	0.065	0.057	0.066
6	0.153	0.101	0.121	0.133
7	0.056	0.063	**	0.066
8#	*	*	*	*
9	0.054	0.053	0.057	0.052
10	0.190	0.152	0.111	0.148
11	0.083	0.060	0.057	0.072
Mean +/-	0.099	0.082	0.081	0.087
SEM	0.053	0.034	0.028	0.038

* no sample
** sample unable to be analyzed
# subject not included in final analysis

PP = Postprandial

Table 31. Plasma 8-epi-prostaglandin-F2α (ng/mL) following high fat olive oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP
1	0.050	0.039	0.046	0.064
2	0.127	0.117	0.158	0.126
3	0.098	0.109	0.055	0.071
4	0.062	0.065	0.052	0.056
5	0.067	0.065	0.071	0.075
6	0.118	0.088	0.089	0.105
7	0.075	0.092	0.082	0.083
8#	*	*	*	*
9	0.045	0.047	0.062	0.063
10	0.128	0.144	0.099	0.118
11	0.067	0.054	0.061	0.066
Mean +/-	0.084	0.082	0.078	0.083
SEM	0.032	0.034	0.033	0.025

* no sample
# subject not included in final analysis

PP = Postprandial

Table 32. Peripheral blood mononuclear cell (PBMC) NF-kB expression (relative intensity units) following fish oil enriched high fat meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP
1	1.00	1.22	0.93	1.07
2	1.00	1.09	1.37	1.54
3	1.00	1.44	1.29	1.34
4	1.00	0.99	1.37	1.03
5	1.00	1.41	0.61	1.15
6	1.00	0.99	0.95	0.93
7	1.00	0.86	0.73	0.71
8#	1.00	1.23	1.65	**
9	1.00	0.97	1.42	1.57
10	1.00	1.03	**	1.12
11	1.00	1.42	1.08	1.48
Mean +/-	1.00	1.14	1.08	1.19
SEM	0.00	0.07	0.10	0.09

** sample unable to be analyzed
# subject not included in final analysis

PP = Postprandial

Table 33. Peripheral blood mononuclear cell (PBMC) NF-kB expression (relative intensity units) following high fat palm oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP
1	1.00	0.87	1.07	0.78
2	1.00	0.66	**	0.97
3	1.00	1.46	0.81	0.86
4	1.00	1.10	0.86	**
5	1.00	1.15	1.22	0.82
6	1.00	0.88	0.89	0.70
7	1.00	0.89	0.80	0.68
8#	1.00	1.41	**	1.15
9	1.00	1.17	1.14	1.03
10	1.00	1.28	1.08	0.56
11	1.00	0.67	0.90	0.94
Mean +/-	1.00	1.01	0.98	0.81
SEM	0.00	0.08	0.05	0.05

** sample unable to be analyzed
# subject not included in final analysis

PP = Postprandial

Table 34. Peripheral blood mononuclear cell (PBMC) NF-kB expression (relative intensity units) following high fat olive oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP
1	1.00	0.92	1.52	1.00
2	1.00	1.00	1.84	1.75
3	1.00	1.51	1.34	2.39
4	1.00	0.45	0.29	0.36
5	1.00	1.28	1.32	1.29
6	1.00	1.34	0.58	1.26
7	1.00	0.95	0.73	0.54
8#	1.00	1.18	1.28	0.99
9	1.00	0.67	0.93	0.59
10	1.00	1.08	0.96	1.30
11	1.00	1.02	1.04	1.15
Mean +/-	1.00	1.02	1.06	1.16
SEM	0.00	0.10	0.15	0.19

# subject not included in final analysis
PP = Postprandial

Table 35. Plasma triglycerides (mg/dL) following fish oil enriched high fat meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	106	106	113	193	394
2	115	81	85	195	200
3	54	46	40	61	55
4	67	113	62	50	55
5	87	169	165	173	142
6	108	157	156	156	156
7	472	465	429	433	577
8#	73	120	126	221	207
9	198	277	192	182	215
10	44	75	55	99	69
11	66	96	131	160	155
Mean +/-	132	159	143	170	202
SEM	40	40	36	34	52

subject not included in final analysis

PP = postprandial

Table 36. Plasma triglycerides (mg/dL) following high fat palm oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	77	104	103	130	163
2	85	95	66	104	138
3	49	42	49	51	55
4	47	61	49	81	62
5	80	133	134	157	148
6	117	137	141	187	133
7	350	302	289	341	345
8#	74	*	98	208	186
9	291	166	106	193	192
10	67	55	53	79	74
11	97	95	104	124	148
Mean +/-	126	119	109	145	146
SEM	33	24	23	26	26

* no sample
# subject not included in final analysis

PP = Postprandial

Table 37. Plasma triglycerides (mg/dL) following high fat olive oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	69	83	111	126	170
2	130	191	148	171	190
3	104	130	150	119	104
4	55	67	63	57	*
5	80	131	142	141	113
6	127	178	180	240	195
7	647	694	633	617	695
8#	221	221	204	118	48
9	217	286	272	333	422
10	56	104	110	87	66
11	129	150	180	246	270
Mean +/-	161	201	199	214	247
SEM	56	58	51	52	66

* no sample
# subject not included in final analysis

PP = postprandial

Table 38. Plasma free fatty acids (mEq/L) following fish oil enriched high fat meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	0.36	0.14	0.11	0.18	0.46
2	0.22	0.15	0.08	0.20	0.56
3	0.62	0.09	0.08	0.13	0.34
4	0.25	0.24	0.07	0.18	0.40
5	0.10	0.11	0.10	0.16	0.13
6	0.35	0.04	0.05	0.12	0.20
7	0.53	0.49	0.27	0.28	0.73
8#	0.12	0.05	0.04	0.18	0.45
9	0.21	0.22	0.11	0.15	0.32
10	0.34	0.16	0.06	0.36	0.38
11	0.29	0.04	0.09	0.20	0.43
Mean +/-	0.33	0.17	0.10	0.20	0.39
SEM	0.05	0.04	0.02	0.02	0.05

subject not included in final analysis

PP = postprandial

Table 39. Plasma free fatty acids (meq/L) following high fat palm oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	0.26	0.09	0.08	0.19	0.14
2	0.59	0.26	0.25	0.36	0.59
3	0.36	0.10	0.10	0.15	0.38
4	0.10	0.03	0.02	0.13	0.36
5	0.17	0.10	0.06	0.13	0.28
6	0.43	0.07	0.07	0.27	0.35
7	0.42	0.16	0.08	0.29	0.40
8#	0.09	*	0.01	0.22	0.33
9	0.75	0.13	0.10	0.17	0.65
10	0.23	0.05	0.03	0.31	0.45
11	0.35	0.02	0.00	0.14	0.35
Mean +/-	0.37	0.10	0.08	0.21	0.39
SEM	0.06	0.02	0.02	0.03	0.05

* no sample
# subject not included in final analysis

PP = postprandial

Table 40. Plasma free fatty acids (meq/L) following high fat olive oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	0.45	0.14	0.09	0.29	0.43
2	0.46	0.29	0.41	0.18	0.40
3	0.30	0.01	0.03	0.07	0.21
4	0.15	0.04	0.03	0.15	*
5	0.10	0.10	0.14	0.16	0.11
6	0.29	0.09	0.08	0.18	0.22
7	0.53	0.61	0.44	0.32	0.48
8#	0.19	0.20	0.19	0.29	0.05
9	0.18	0.13	0.07	0.23	0.36
10	0.39	0.12	0.08	0.19	0.26
11	0.08	0.03	0.05	0.21	0.42
Mean +/-	0.29	0.15	0.14	0.20	0.30
SEM	0.05	0.06	0.05	0.02	0.04

* no sample
# subject not included in final analysis

PP = postprandial

Table 41. Plasma insulin (mU/L) following fish oil enriched high fat meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	4.4	21.7	60.1	9.2	12.5
2	41.5	138.1	81.3	42.4	26.4
3	3.8	19.3	4.7	17.4	10.7
4	15.3	136.6	97.4	16.2	12.5
5	9.1	32.3	21.1	20.6	9.0
6	5.2	15.5	18.8	9.3	13.6
7	11.3	61.7	85.3	17.6	12.1
8#	17.6	56.3	29.9	18.3	18.9
9	23.9	29.9	25.1	12.4	5.7
10	6.7	68.3	31.3	12.0	6.0
11	4.5	24.2	9.4	7.4	6.2
Mean +/-	12.6	54.8	43.4	16.4	11.5
SEM	3.8	14.8	10.9	3.2	1.9

subject not included in final analysis

PP = postprandial

Table 42. Plasma insulin (mU/L) following high fat palm oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	3.4	7.5	21.8	8.7	6.8
2	11.8	35.4	29.8	18.4	29.4
3	7.3	22.3	22.3	8.1	5.2
4	14.3	134.6	32.3	10.2	13.4
5	4.3	21.6	19.5	7.5	6.1
6	7.2	35.7	16.1	10.4	7.9
7	10.6	87.2	82.4	12.3	10.0
8#	11.2	*	16.9	12.6	13.8
9	11.0	28.2	27.5	11.4	6.6
10	12.3	54.9	19.8	12.3	9.0
11	6.4	41.1	18.2	8.1	3.9
Mean +/-	8.9	46.9	29.0	10.7	9.8
SEM	1.2	11.9	6.2	1.0	2.3

* no sample
# subject not included in final analysis

PP = postprandial

Table 43. Plasma insulin (mU/L) following high fat olive oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	6.7	29.8	10.6	13.1	8.8
2	25.9	96.9	90.5	43.3	29.1
3	7.0	8.7	13.5	4.8	6.9
4	17.7	101.0	42.9	20.8	*
5	10.9	21.5	18.6	13.1	9.2
6	6.7	12.8	24.9	17.7	13.8
7	18.6	48.5	68.1	43.0	43.0
8#	20.5	36.0	24.4	14.5	10.2
9	6.5	15.9	17.6	13.7	5.0
10	8.6	34.2	26.0	7.8	9.9
11	6.1	33.2	18.8	8.7	6.7
Mean +/-	11.5	40.2	33.1	18.6	14.7
SEM	2.2	10.5	8.3	4.4	4.3

* no sample
# subject not included in final analysis

PP = postprandial

Table 44. Plasma glucose (mg/dL) following fish oil enriched high fat meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	96	123	131	102	133
2	108	144	97	104	97
3	93	106	91	85	89
4	88	115	108	84	89
5	114	104	118	130	111
6	100	100	106	97	104
7	146	186	172	120	179
8#	100	110	93	109	118
9	125	127	102	105	127
10	94	103	96	89	85
11	105	104	76	105	110
Mean +/-	107	121	110	102	113
SEM	6	8	8	5	9

subject not included in final analysis

PP = postprandial

Table 45. Plasma glucose (mg/dL) following high fat palm oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	101	106	113	107	102
2	98	77	107	94	98
3	93	92	113	87	95
4	85	109	86	76	89
5	118	91	110	110	107
6	104	77	111	97	101
7	177	210	201	179	165
8#	94	*	76	104	101
9	130	118	124	109	104
10	106	122	102	101	93
11	110	106	89	97	104
Mean +/-	112	111	115	106	106
SEM	8	12	10	9	7

* no sample
# subject not included in final analysis

PP = postprandial

Table 46. Plasma glucose (mg/dL) following high fat olive oil meal.

Subject	Fasting	1 hour PP	2 hour PP	4 hour PP	6 hour PP
1	101	116	91	98	103
2	106	143	126	109	104
3	90	87	73	87	91
4	86	91	76	90	*
5	110	81	104	109	103
6	105	105	88	100	102
7	137	171	118	106	111
8#	108	104	104	100	96
9	114	113	93	112	124
10	100	108	116	96	96
11	104	106	89	111	120
Mean +/-	105	112	97	102	106
SEM	4	9	6	3	4

* no sample
# subject not included in final analysis

PP = postprandial

Table 47. Plasma C-reactive protein area under the curve (AUC) following acute high fat meals differing in fat source.

Subject	FO	PO	OO
1	2.2	1.9	1.1
2	75.5	58.9	59.7
3	34.1	**	20.8
4	8.9	5.5	8.2
5	8.2	9.0	**
6	**	**	43.9
7	37.7	43.3	29.5
8#	7.4	11.0	20.6
9	8.9	14.6	4.9
10	37.3	29.7	12.6
11	7.7	4.6	3.9
Mean +/-	24.5	20.9	20.5
SEM	8.0	7.4	6.7

** too high due to acute illness
# subject not included in final analysis

FO = fish oil enriched meal
PO = palm oil meal
OO = olive oil meal

Table 48. Plasma tumor necrosis factor- α area under the curve (AUC) following acute high fat meals differing in fat source.

Subject	FO	PO	OO
1	5.69	6.60	5.55
2	6.91	8.78	11.33
3	6.08	6.41	5.11
4	3.78	3.65	3.57
5	9.12	8.87	6.99
6	7.77	8.34	7.83
7	9.24	7.81	9.02
8#	6.07	7.81	6.67
9	4.93	4.25	5.42
10	6.75	7.91	9.63
11	6.50	7.46	6.17
Mean +/-	6.68	7.01	7.06
SEM	0.54	0.57	0.75

subject not included in final analysis

FO = fish oil enriched meal
PO = palm oil meal
OO = olive oil meal

Table 49. Plasma soluble intercellular adhesion molecule-1 area under the curve (AUC) following acute high fat meals differing in fat source.

Subject	FO	PO	OO
1	937	954	887
2	1582	1620	1528
3	1143	1209	1082
4	1217	1309	1242
5	1270	1215	1091
6	1425	1732	1513
7	1483	1395	1466
8#	1403	1431	1384
9	1175	1111	1182
10	1142	1171	1216
11	1073	1297	1073
Mean +/-	1245	1301	1228
SEM	63	73	67

subject not included in final analysis

FO = fish oil enriched meal
PO = palm oil meal
OO = olive oil meal

Table 50. Plasma soluble vascular cell adhesion molecule-1 area under the curve (AUC) following acute high fat meals differing in fat source.

Subject	FO	PO	OO
1	4209	4091	4041
2	3865	3826	4020
3	3645	3779	3359
4	2527	2592	2573
5	3228	3498	3185
6	3721	4172	4174
7	4713	4813	4497
8#	5368	5304	4637
9	3351	3593	3522
10	4178	4443	4925
11	5622	5324	5264
Mean +/-	3906	4013	3956
SEM	269	239	259

subject not included in final analysis

FO = fish oil enriched meal
PO = palm oil meal
OO = olive oil meal

Table 51. Plasma 8-epi-prostaglandin-F2α area under the curve (AUC) following acute high fat meals differing in fat source.

Subject	FO	PO	OO
1	204	200	197
2	347	484	544
3	282	422	312
4	220	237	231
5	273	251	280
6	383	492	385
7	238	254	335
8#	*	*	*
9	186	216	226
10	473	562	475
11	*	260	246
Mean +/-	290	340	320
SEM	10	20	10

* not determined
# subject not included in final analysis

FO = fish oil enriched meal
PO = palm oil meal
OO = olive oil meal

Table 52. Peripheral blood mononuclear cell (PBMC) nuclear factor-kB (NF-kB) area under the curve (AUC) following acute high fat meals differing in fat source.

Subject	FO	PO	OO
1	4.18	3.74	4.69
2	5.19	3.36	6.01
3	5.21	4.03	6.42
4	4.58	3.76	1.75
5	3.97	4.29	5.04
6	3.85	3.41	3.97
7	3.18	3.28	3.08
8#	5.86	4.99	4.59
9	5.18	4.40	3.16
10	4.26	3.96	4.32
11	5.02	3.46	4.23
Mean +/-	4.46	3.77	4.27
SEM	0.22	0.13	0.44

subject not included in final analysis

FO = fish oil enriched meal
PO = palm oil meal
OO = olive oil meal

Table 53. Plasma free fatty acids area under the curve (AUC) following acute high fat meals differing in fat source.

Subject	FO	PO	OO
1	1.29	0.86	1.51
2	1.33	2.23	1.89
3	1.12	1.11	0.55
4	1.24	0.70	0.61
5	0.76	0.82	0.79
6	0.74	1.28	0.92
7	2.45	1.48	2.65
8#	0.98	0.87	1.21
9	1.10	1.63	1.14
10	1.53	1.27	1.07
11	1.14	0.82	0.99
Mean +/-	1.27	1.22	1.21
SEM	0.15	0.15	0.20

subject not included in final analysis

FO = fish oil enriched meal
PO = palm oil meal
OO = olive oil meal

Table 54. Plasma triglycerides area under the curve (AUC) following acute high fat meals differing in fat source.

Subject	FO	PO	OO
1	1109	722	706
2	856	583	1011
3	310	297	747
4	394	383	361
5	948	837	779
6	913	914	1187
7	2787	1937	3896
8#	994	873	920
9	1243	1048	1891
10	446	400	536
11	802	694	1247
Mean +/-	981	781	1236
SEM	223	150	326

subject not included in final analysis

FO = fish oil enriched meal
PO = palm oil meal
OO = olive oil meal

Table 55. Plasma glucose area under the curve (AUC) following acute high fat meals differing in fat source.

Subject	FO	PO	OO
1	706	642	603
2	647	571	707
3	549	577	504
4	579	522	518
5	709	641	613
6	607	590	593
7	936	1124	740
8#	634	554	610
9	678	691	658
10	558	623	620
11	590	593	633
Mean +/-	656	657	619
SEM	36	54	23

subject not included in final analysis

FO = fish oil enriched meal
PO = palm oil meal
OO = olive oil meal

Table 56. Plasma insulin area under the curve (AUC) following acute high fat meals differing in fat source.

Subject	FO	PO	OO
1	145	66	84
2	392	152	361
3	74	81	49
4	335	224	237
5	119	74	90
6	78	92	103
7	242	251	289
8#	166	84	122
9	110	104	78
10	148	124	103
11	61	92	89
Mean +/-	171	126	148
SEM	36	20	34

subject not included in final analysis

FO = fish oil enriched meal
PO = palm oil meal
OO = olive oil meal

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EDUCATION

Virginia Polytechnic Institute and State University, Blacksburg, VA **Ph.D. in Human Nutrition, Foods, and Exercise** Nutrition in Sports and Chronic Disease option in Department of Human Nutrition, Foods, and Exercise. Title of dissertation: "The effects of dietary macronutrients on oxidative stress and inflammation in overweight and obese individuals."	**Fall 2007**
Virginia Polytechnic Institute and State University, Blacksburg, VA **Started in Master's Program, "fast tracked" to doctoral program** Human Nutrition, Foods, and Exercise Dept. Degree option: Nutrition in Sports and Chronic Disease	**2002-2003**
Mary Washington College, Fredericksburg, VA **B.S. in Biology** Areas of Concentration: Exercise, nutrition. QCA 4.0.	**Spring, 2000**

AWARDS/ CERTIFICATES

• Raville Award, Outstanding Graduate Student Award from the HNFE Department.	**Summer 2007**
• MILES certificate – Macromolecular Interfaces with Life Science, program designed to increase the understanding of oxidation and the effects across disciplines, from polymer chemistry to human and veterinary sciences (see Traineeship below).	**Requirements met**
• Teaching certificate – from the Graduate School of Virginia Tech. The goal of the program is to increase the understanding of pedagogical practices, how to implement effective teaching strategies, and the professoriate itself.	**Requirements met**
• MILES-IGERT Traineeship – funded by the National Science Foundation. The MILES traineeship provided a 2 year stipend and the opportunity to receive training in molecular and biochemical techniques through additional course and lab work.	**2004-2006**
• GSA Travel Grant - Graduate Student Assembly at Virginia Tech	**Summer, 2005**
• Hepler Summer Research Scholarship, HNFE Department, Virginia Polytechnic Institute and State University.	**Summer, 2004**

TEACHING EXPERIENCE

Virginia Polytechnic and State University, Blacksburg, VA **Instructor –** Metabolic Nutrition (HNFE 3026). Large upper level course, required for HNFE majors (165 students), involved development and implementation of all class lectures, assignments, and assessments.	**Spring, 2007**
Teaching Assistant – Metabolic Nutrition (HNFE 3025). Led a weekly recitation section. Developed lesson plans, quizzes, and evaluated student performance on all assignments, quizzes, and tests.	**Fall, 2006**
Instructor – Racquetball (HNFE). Developed overall course structure, evaluated student performance.	**Fall 2003-Spring 2004**

Instructor – Weight Training (HNFE).
Developed overall course structure, evaluated student performance.

Summer 2003

Teaching Assistant – Introduction to Nutrition (HNFE 1004).
Collaborated on curriculum, developed all exams and quizzes, led
problem solving activities, met with students upon request, graded all
written work, guest lecturer.

Spring, 2003

Teaching Assistant – Exercise Physiology (HNFE 3804).
Provided assistance to students, graded all written work, guest
lecturer.

Fall, 2002

Teaching Assistant – Kinesiology (HNFE 3824)
Provided assistance to students, graded all written work.

Fall, 2002

PUBLICATIONS, PAPERS, AND PRESENTATIONS

PUBLICATIONS

- In review: Peairs, Abigail D. and Janet W. Rankin. "High fat low carbohydrate weight loss diet and inflammation: the role of oxidative stress."
- Rankin, Janet W. and Abigail D. Turpyn. ""Low carbohydrate, high fat diet increases C-reactive protein during weight loss" *Journal of the American College of Nutrition*. Vol. 26, no. 2, pp. 163-169, 2007.
- Turpyn, Abigail D., Rankin, Janet W., and Brenda M. Davy. "Diabetes: something to stress about." *Scan Pulse. A publication for sports, cardiovascular, and wellness nutritionists*, ADA Vol. 25, no. 3, Summer 2006. (extension article, non-peer rev.).

ABSTRACTS and PRESENTATIONS

- Abstract, Poster, and Oral presentations at the MILES symposium, "The Effects of High Fat Diets on Inflammation and Oxidative Stress in Obese Individuals and the Role of Antioxidant Supplementation." August 2005, Blacksburg, VA.
- Poster presentation for Macromolecular Interfaces Institute conference, August 2005, Blacksburg, VA.
- Abstract and Poster presentation National American College of Sports Medicine conference, "Role of Antioxidant Supplements on Inflammation Induced by a Low Carbohydrate, High Fat Diet." June 2005, Nashville, TN.
- Abstract and Poster presentation Southeast American College of Sports Medicine conference, "The Effects of a High Fat or Low Fat Weight Loss Diet on Markers of Inflammation and Oxidative Stress in Overweight Women." January 2005, Charlotte, NC.
- Abstract and Poster presentation in the Graduate Student Assembly Research Symposium, "The Effects of a High or Low Fat Weight Loss Diet on Markers of Inflammation and Oxidative Stress in Overweight Women" Spring 2004, Virginia Tech.

RESEARCH EXPERIENCE

Virginia Polytechnic Institute and State University, Blacksburg, VA

Research Study: Investigating the effect of different types of fat in a high fat meal challenge on oxidative stress and inflammation in overweight and obese individuals. Subject recruitment, all data collection and coordination. Biochemical and molecular (EMSA) analyses

Fall, 2005

Research Study Assistant: Raisin supplementation effects on oxidative stress and inflammation in overweight and obese individuals. Assisted

Spring/ Summer 2005

in subject recruitment, meal preparation, and sample collection for study run by Janet Rankin and Mary Andreae.

Research Study: Investigating the mechanism by which high fat diets increase levels of systemic inflammation in obese individuals and the role of dietary antioxidants in this process. Subject recruitment, data collection coordination, diet development and preparation. Biochemical analyses of blood and urine, ELISAs and colorimetric assays.	**Summer, 2004**
Research Study: Investigating the effects of Atkins vs. Low-Fat diet on markers of inflammation and oxidative stress in overweight women. Study involved recruitment of subjects, diet education, blood draw and measurements, biochemical analysis of blood and urine samples. Laboratory techniques: enzyme linked immuno-sorbent assays (ELISA) and colorimetric assays.	**Fall, 2003**

Jean Mayer Clinic, USDA Antioxidants Research Laboratory, Boston, MA	**July/Aug 2006**

Internship: Gained experience in conducting several antioxidant capacity assays (Oxygen Radical Absorbance Capacity (ORAC), Ferric Reducing Antioxidant Power (FRAP) and Folin Ciocalteau). The primary objective was to learn the ORAC and then set up the procedure at Virginia Tech.

RELATED EXPERIENCE

SEACSM Executive Board **Student Representative** to the Southeast Region of the American College of Sports Medicine. Duties include organization of the student symposium, invitation and arrangement of speaker for the annual conferences.	**2005-2007**
Student Affairs Committee **Southeast ACSM representative** on the student affairs committee for the American College of Sports Medicine. Duties include the development of the student symposium for the annual meeting for the National organization - June 2007.	**2005-2007**

LANGUAGES

- English – native language
- Spanish – conversational

MEMBERSHIPS

- SEACSM, American College of Sports Medicine, Southeast Regional Chapter (2003-present)
- ACSM, American College of Sports Medicine, National membership (2005-present)
- AFAA, Aerobics and Fitness Association of America (Personal Trainer)
- USAT, U.S. National Governing Body for Triathlon

HOBBIES

- Outdoor activities - biking, swimming, hiking, soccer
- Gardening (there is just something rewarding about fresh vegetables!)