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NUTRITION INTERVENTIONS FOR IMPROVING CARDIOVASCULAR DISEASE RISK FACTORS

A Dissertation in

Nutrition

by

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ABSTRACT

This dissertation investigated the effects of high antioxidant spices and omega-3 fatty acids on cardiovascular risk factors. The unifying theme of these studies is adding beneficial nutrients back to a deficient diet in order to improve intermediate cardiovascular risk factors. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reduce cardiovascular mortality at a dose of about 1 g/day, and there is some evidence for reduced cardiovascular risk factors at doses that exceed current dietary recommendations (> 2 g/d). Dose response studies are rare, and little is known about underlying hemodynamic mechanisms and other mechanistic effects of omega-3 fatty acids on humans in vivo. There is much interest in the potential of dietary antioxidants to attenuate in vivo oxidative stress, but very little characterization of the time course of plasma effects is available. Culinary spices have demonstrated potent in vitro antioxidant properties, the ability blunt inflammatory responses in vitro, but it is unknown whether this effect would exist in a human model of induced inflammation. Acute psychological stress has been shown to induce inflammation in vivo.

In the first study, we compared the effects of a nutritional dose of EPA+DHA (0.85 g/d) to a pharmaceutical dose (3.4 g/d) on serum triglycerides, inflammatory markers, and endothelial function in healthy subjects with moderately elevated triglycerides. This was a placebo-controlled, double-blind, randomized, 3 period crossover trial (8 wk treatment, 6 wk washout) comparing 0.85 g/d to 3.4 g/d EPA+DHA in 26 people (23 M, 3 post-menopausal F) with moderate hypertriglyceridemia (150-500 mg/dL). The higher dose of EPA+DHA reduced triglycerides by 27% vs. placebo (173 ± 17.5 vs. 237 ± 17.5 mg/dL, p = 0.002), whereas there was no effect of the lower dose on lipids. There were no effects on cholesterol, endothelial function (as assessed by flow-mediated dilation, peripheral arterial tonometry/EndoPAT, or Doppler measures of hyperemia), inflammatory markers (IL-1β, IL-6, TNF-α, hs-CRP), or expression of inflammatory cytokine genes in isolated lymphocytes. The higher dose of EPA+DHA significantly lowered triglycerides, but neither dose improved endothelial function or inflammatory status over 8 weeks in healthy adults with moderate hypertriglyceridemia.

In the second study, we compared the effects of 0.85 g/d EPA + DHA (low dose) and 3.4 g/d EPA + DHA on heart rate, blood pressure, and impedance cardiography measures at rest and during standardized stressors (speech task and foot cold pressor) to determine whether effects were dose dependent and/or related to changes in erythrocyte fatty acid composition. The testing sessions employed standardized repeated measures during rest, stressor, and recovery tasks. Findings were overall treatment effects with the values for each of the 6 tasks as repeated measures that did not significantly differ by task. Heart rate was reduced by 2.4 bpm (low dose) and 4.0 bpm (high dose) (p < 0.003). The high dose significantly reduced mean arterial blood pressure (2 mmHg; SBP/DBP reduction of 2.1/1.7) and stroke volume (5.4 mL) and increased pre-ejection period (6.5 msec) (p < 0.05). A sub-analysis of the resting task alone demonstrated that the high dose also significantly reduced heart rate and blood pressure when only these resting values were analyzed. Reduction in mean arterial pressure was significantly predicted by
an increase in erythrocyte omega-3 fatty acids in linear regression modeling ($R^2 = 12\%, p = 0.02$). Hemodynamic effects of marine omega-3 fatty acids are dose dependent, and changes in mean arterial pressure are related to changes in membrane fatty acid composition. Therefore, increasing omega-3 intake can help reduce blood pressure and heart rate at rest and during stress.

The objective of the third study was to examine whether adding 14 g of a high antioxidant spice blend to a 1200 kcal meal exerted significant postprandial effects on markers of plasma antioxidant status and metabolism. Healthy overweight men ($n = 6$) consumed a control and spiced meal in a randomized crossover design with one week between testing sessions. Blood was sampled prior to meal and at 30 minute intervals for 3.5 h (total of 8 samples). Mixed linear models demonstrated a significant treatment by time interaction ($p < 0.05$) for insulin and triglycerides, corresponding with 21% and 31% reductions in postprandial levels with the spiced meal, respectively. Adding spices to the meal significantly increased the ferric reducing antioxidant power (FRAP), such that postprandial increases following the spiced meal were 2-fold greater than the control meal (effect of treatment $p = 0.009$). The hydrophilic oxygen radical absorbance capacity (ORAC) of plasma was also significantly increased by spices (effect of treatment $p = 0.02$). There were no changes in glucose, total thiol, lipophilic ORAC, or total ORAC. The incorporation of spices into the diet may help normalize postprandial insulin and triglycerides and enhance antioxidant defenses.

The objective of the fourth study was to examine whether adding 14.5 g of a high antioxidant spice blend to a 1200 kcal meal exerted significant effects on inflammatory markers, postprandial metabolism, and other intermediate cardiovascular risk markers in a setting of acute psychological stress. Healthy overweight men ($n = 14$) and women ($n = 6$) completed 4 testing sessions for a 2 X 2 factorial design examining the effects of spice, acute stress, and their interaction. Mixed linear models demonstrated a significant effect of the stressor for increasing plasma glucose and insulin. Spices attenuated postprandial lipemia for the rest condition and demonstrated a potential reduction in salivary cortisol that was not significant using pre-specified statistics tests. The stressor decreased levels of plasma TNF-α and showed a trend to inducing IL-6 expression in isolated peripheral blood mononuclear cells. Cortisol responses may have decreased plasma concentrations of TNF-alpha following acute psychological stress. Neither the spices nor acute stress had any effects on endothelial function (as assessed by EndoPAT) or platelet function (as assessed by closure times with a platelet function analyzer). This study confirmed our earlier finding of decreased postprandial triglycerides following spice consumption and provides evidence that spice may also decrease cortisol responses to stress. Acute psychological stress is detrimental to glucose homeostasis, as evidenced by increased levels of glucose and insulin following a standardized meal.
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Chapter 1. Introduction

1.1 Background and Significance

Despite extensive resource investment in cardiovascular research and treatment, heart disease remains the leading cause of death in United States (1) and costs are expected to exceed one trillion dollars by 2030 (2). Clearly a role for nutrition remains amongst the sophisticated surgical and pharmaceutical interventions that have been developed.

Nutritional strategies are capable of reducing many cardiovascular risk factors. Great strides have been made in designing nutritional strategies as part of therapeutic lifestyle changes for reducing traditional cardiovascular disease (CVD) risk factors such as blood pressure and low density lipoprotein cholesterol (LDL-C) (3-5). However, focusing on cholesterol and blood pressure alone fails to prevent about half of the 1.3 million individuals who develop myocardial infarction each year without significant risk from these factors alone (6), and it is clear that other intermediate risk factors, such as inflammation, are involved. This chapter briefly explores some aspects of research on nutritional interventions for cardiovascular disease and introduces the premise of this dissertation.

Several factors including safety, cost, and epidemiological evidence provide support for nutritional strategies. The vast majority of nutritional strategies can be universally applied with minimal risk (other than allergies or intolerance). In contrast, a varying percentage of patients receiving pharmaceutical therapies will experience significant side effects that necessitate discontinuation. Although financial barriers exist in applying nutritional strategies (e.g. the affordability of oily fish in low income populations) these barriers are also present in medical therapy, and medical expenses place additional financial strain on the healthcare system. Finally, strong evidence from epidemiological and animal studies supports the role of nutrition in preventing heart disease by modifying intermediate risk factors.

Because of our extensive knowledge of diet and lifestyle in cardiovascular disease, therapeutic lifestyle changes (TLC) are recommended and applied in the medical setting (4, 7). TLC recommendations are limited by a focus on LDL-C and blood pressure reduction and often are misunderstood by both patients and physicians. Current TLC recommendations call for restricting fat and cholesterol intake and increasing physical activity (7). The simplified message of “lose weight and exercise” has demonstrated several weaknesses. The instructions are often viewed as restrictive—or are misinterpreted as in the case of consumer implementation of low fat and low carbohydrate diets. Indeed, some of the relative success of medication is likely due to better rates of compliance when compared to lifestyle modification. Effects of medication are immediate and quantifiable, while dietary strategies may provide small incremental benefit over a much longer period of time. Current TLC guidelines may miss the opportunity to implement positive dietary changes, but a strong evidence base is needed before more specific recommendations can be made to patients.
Two dietary recommendations that have a strong research base guiding their use in the clinical setting are: 1) increased consumption of vegetables and fruits and 2) increased consumption of oily fish. These two recommendations are notable in that they both involve adding something to the diet that research has shown is beneficial, both dietary components are remarkably absent the average American diet, and neither recommendation is made to address an essential nutrient deficiency. The recommendation to increase vegetable and fruit consumption relates, in part, to the understanding that plant phytochemicals, such as phenolic antioxidants, ameliorate oxidative stress. Similarly, the omega-3 fatty acids in oily fish affect inflammatory pathways. The evidence base for recommending increased phenolic and omega-3 fatty acid intake is reviewed in Chapter 2.

Although there is relatively strong evidence that increasing intake of plant-derived phenolic antioxidants and omega-3 fatty acids is beneficial for preventing and treating cardiovascular disease there remain limitations for refining recommendations. The amounts and dietary sources that should be recommended, target populations that would receive most benefit, mechanism(s) of action, and strength of effect are limitations where research is most needed. Research addressing these limitations allows for more precise, evidenced-based recommendations.

1.2 Premise of this dissertation

Therefore, the purpose of this dissertation was to investigate these two dietary interventions, phenolic antioxidants and omega-3 fatty acids, using delivery forms that can be added to the diet without changing the background diet. This research approach has the advantage of producing results that can be translated to simple population messages. Like pharmacological therapies, better compliance would be expected when people are asked to make small additions to their diet than when nutritional strategies are viewed as deprivation. The phenolic antioxidant intervention was studied using a high antioxidant spice blend that was added to foods. The omega-3 intervention was studied using a prescription fish oil concentrate. Although the research designs were very different (as will be further discussed), the premise of adding beneficial nutrients back to a deficient Westernized diet in order to improve intermediate cardiovascular risk factors is a unifying theme of these studies.

1.3 Study designs for nutrition and cardiovascular research

Controlled clinical trials, epidemiological analyses, and mechanistic studies are complementary approaches to nutrition research. Controlled clinical trials with hard endpoints (such as mortality) have long been the established gold standard for demonstrating a cause and effect relationship for an intervention. Although epidemiological data is important for providing evidence of beneficial dietary patterns, results may be confounded when dietary patterns co-exist with other lifestyle factors. Mechanistic results from cell and animal models provide a highly controlled study system and reduced measurement noise, but the results may not apply in humans. The drawback to hard endpoint clinical studies is that they are very expensive and often underpowered or oversimplified for nutrition research. These trials work best for drugs
that have strong effects and are also important for characterizing side effects that nutrition interventions often do not have.

Randomized controlled trials with intermediate risk factors as outcomes provide vital information for recommendations by bridging the gap between the other types of research designs. They can demonstrate that epidemiological evidence was not confounded by other factors, provide potential mechanisms for hard endpoint studies, and demonstrate that mechanistic evidence observed in vitro or in animals can result in human benefit. A critical factor for this study design is selecting the appropriate intermediate risk factor and method of measurement.

1.4 Cardiovascular risk factors that can be studied in clinical trials

1.4.1 Lipids and lipoproteins
LDL-C concentrations are well-established as an intermediate risk factor, but increasing attention is being given to other aspects of the lipid profile. The role of triglycerides will be discussed in detail in Chapter 2. Characteristics of the lipid profile such as particle size and distribution also are becoming increasingly studied. Additionally, non-fasting triglycerides and postprandial lipemia are emerging risk factors. Triglycerides—both fasting and postprandial—are an important outcome in the studies comprising this dissertation.

1.4.2 Inflammatory markers
Inflammation is a key component in the development and progression of atherosclerosis, and the circulating pro-inflammatory biomarkers C-reactive protein (CRP), TNFα, and interleukin 6 (IL-6) are independent risk factors for cardiovascular disease (6, 8). A recent intervention trial provided evidence that statin therapy may prevent CVD mortality via anti-inflammatory effects (9). Inflammatory markers are reported for the omega-3 intervention discussed in Chapter 3 and spice intervention discussed in Chapter 6. Additionally, the study design for the spice intervention employed a model of induced inflammation. This concept is discussed in Chapter 2.

1.4.3 Endothelial function
Endothelial dysfunction is a precursor to atherosclerosis, and many other intermediate risk factors may damage vessel linings. Many techniques exist to quantify endothelial function, including techniques that infuse vasoactive compounds intra-aterially or measure nitric oxide metabolites. The most common method employs arterial occlusion as a flow-stimulus, and this approach was used in these dissertation studies. The brachial artery is occluded for 5 min using a blood pressure cuff. When the cuff is deflated, the magnitude of artery dilation indicates endothelial function. The resulting flow-mediated dilation (FMD) can be interpreted as a percent change in artery diameter from images recorded using sonography or as percent increase in reactive blood flow using fingertip plethysmography. Both methods were employed for the study of omega-3 fatty acids (Chapter 3), while only the fingertip plethysmography was employed for the study of spices (Chapter 6).
1.4.4 Hemodynamics
Resting blood pressure and heart rate are established risk factors, and more dynamic measures such as reactivity to acute stress, are also risk factors. Additionally, impedance measures inform the underlying physiology driving blood pressure elevations by providing total peripheral resistance, stroke volume, and cardiac contractility indices. These measurements were employed in the study of omega-3 fatty acids presented in Chapter 4, and blood pressure and heart rate responses were measured in the study of spices discussed in Chapter 6.

1.4.5 Additional intermediate risk measures
This dissertation also examined fasting indices of insulin sensitivity and glucose metabolism in the postprandial state. Markers of plasma oxidation and platelet function were employed in the study of spices (Chapter 6). Other markers of autonomic tone including heart rate variability and salivary cortisol were employed in these studies (cortisol is reported in chapter 6).

1.5 Conclusion
The studies presented in this dissertation contribute to the knowledge of how nutrition interventions of omega-3 fatty acids and highly antioxidant spices can modify established and emerging cardiovascular risk factors. The following chapter reviews the literature as a background to the studies of omega-3 fatty acids and highly antioxidant spices for their effects on intermediate cardiovascular risk factors. In Chapter 3, the results of the omega-3 fatty acids study for effects on lipids, inflammation, and endothelial function are presented. Chapter 4 presents the results of omega-3 fatty acids on hemodynamic responses to acute psychological stress. Chapter 5 is a study of the acute affects of a high antioxidant spice blend on postprandial plasma antioxidant status and metabolism. Chapter 6 presents results of a larger study of spices on multiple cardiovascular risk factors in the presence of acute stress or rest. In Chapter 7, the research summary, limitations, and future directions are reviewed.
Chapter 2. Review of the Literature

This chapter reviews critical evidence informing the design of the research projects in Chapters 3-6. Section 2.1 briefly reviews trials of omega-3 fatty acids with hard endpoints. Sections 2.2 to 2.8 present a background on omega-3 fatty acids and cardiovascular risk factors. Section 2.9 begins the background for the studies of spices. In addition to examining the health effects of spices, plasma bioactivity changes are discussed in Section 2.10 and models of human inflammatory challenge are reviewed in Section 2.11.

2.1 Omega-3 fatty acids in clinical trials with hard end points

Supplemental intake of the marine-derived omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is associated with reductions in cardiovascular events and mortality as shown by two large interventional studies: the GISSI-Prevenzione Study (GISSI-P) and the Japan EPA Lipid Intervention Study (JELIS). GISSI-P studied 11,323 people who had survived an MI and showed a significant 45% decrease in sudden death and 20% decrease in mortality only 4 months into the study (10). JELIS demonstrated that 1.8 grams EPA ethyl ester in combination with statin therapy reduced major coronary events by 19% versus statin alone (11). Treatment with omega-3 concentrates seems to be effective in reducing cardiovascular risk even when provided at doses below that which are indicated for lowering triglycerides. Research is ongoing to elucidate the mechanisms by which this is achieved. The recently published Alpha Omega Trial did not find a reduction in hard end points following omega-3 therapy (12). This could be due to many factors, including the increased use of pharmacological therapy (e.g. statin drugs) and surgical interventions (i.e. stenting) or differences in study population (e.g. the trial participants were older).

2.2 Omega-3 fatty acid pharmacological overview

EPA and DHA are long chain polyunsaturated omega-3 fatty acids found predominately in oily fish. Processed algae oil also is a source of supplemental DHA. EPA and DHA can be obtained over the counter as fish oil and omega-3 concentrate supplements containing varying amounts of EPA and DHA. Dietary and supplemental EPA and DHA are provided as triglycerides. Prescription omega-3 ethyl ester concentrates (P-OM3, Figure 2.1) are also available under the trade name Lovaza™1. Alpha linolenic acid (ALA) from various sources including flaxseed oil, canola oil, and walnuts is not bioequivalent to EPA and DHA, and very little is converted to EPA in vivo (13-16).

P-OM3 is more highly concentrated than most over-the-counter fish oil supplements; about 85% of its content is ethyl esters of EPA and DHA. Therefore, 4 daily capsules are needed to provide 3.4 g of EPA + DHA (versus ~11 capsules for standard fish oil containing 18% EPA and 12% DHA).

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1Lovaza™ was initially introduced in the American market under the trade name Omacor® but was renamed in 2007 due to confusion with the drug Amicar® (used for acute bleeding syndromes). P-OM3 have a longer history of being available in Europe under the trade name Lovaza®.
The FDA has advised that the unsupervised dietary intake of EPA + DHA should not be greater than 3 g per day, and that no more than 2 g/day should be provided as supplements (17).

EPA is a 20 carbon fatty acid containing 5 cis double bonds that are methylene interrupted starting from the 3rd carbon from the methyl (omega) end. DHA is 22 carbons long and has 6 double bonds—and like EPA—has methylene interrupted cis double bonds from the 3rd carbon of the omega end.

The process used to concentrate fish oil to the potency of P-OM3 requires that the triglyceride fatty acids be converted to ethyl esters. Therefore, P-OM3 contains EPA and DHA ethyl esters (Figure 2.1). Marine fatty acids provided as triglyceride and ethyl ester are believed to be equally bioavailable and efficacious in reducing triglycerides, but bioequivalence for other outcomes has been debated.

![Figure 2.1: Eicosapentaenoic acid (EPA, top) and docosahexaenoic acid (DHA, bottom) ethyl ester chemical structures. Source: Skulas-Ray AC et al. Omega-3 fatty acid concentrates in the treatment of moderate hypertriglyceridemia. Expert Opin Pharmacother 2008;9(7):1237-48.](image_url)

2.3 Omega-3 fatty acids for reducing triglyceride values

2.3.1 Triglycerides as a cardiovascular risk factor

Prevention of cardiovascular disease (CVD) has focused primarily on lowering of LDL-C. However, there is greater appreciation in recent years for the role of triglycerides as a CVD risk factor, and the treatment of severely elevated triglycerides (>500 mg/dL) has been extensively studied (18, 19). Triglycerides in this range are usually associated with genetic factors or uncontrolled diabetes. Very high triglycerides may cause acute pancreatitis, which is the basis for prompt clinical intervention.

Triglycerides greater than 150 mg/dL and less than 500 mg/dL encompass the range that exceeds normal but does not fall into Adult Treatment Panel III classifications for “very high” (7). Triglycerides in this range will be hereafter referred to as moderately elevated triglycerides. Moderately elevated triglycerides are fairly common in the adult population and usually occur in conjunction with other risk factors for CVD (high LDL-C, low HDL-C, obesity, hypertension, etc.) About 30% of the U.S. population over the age of 20 has triglycerides > 150 mg/dL (20). Isolated moderate hypertriglyceridemia is relatively uncommon, although some individuals have high
triglycerides and low LDL-C, the result of a derangement in lipid metabolism. Elevated triglycerides are more common in insulin resistance and metabolic syndrome and are characteristic of diabetic dyslipidemia (which also is characterized by small dense LDL and low HDL).

The role of triglycerides as an independent CVD risk factor has been historically debated. Current treatment guidelines focus on reducing LDL-C levels (21) and do not specifically address moderately elevated triglycerides. Some have suggested that triglyceride levels are not an independent predictor for heart disease because they are confounded by insulin/glucose, central adiposity, and low HDL-C—and it is true that risk resulting from elevated triglycerides is reduced when models are adjusted for these factors. However, many recent studies, editorials, reviews, and scientific statements agree that moderately elevated triglycerides are linked to increased cardiovascular risk (7, 22-28). The Copenhagen Male Study found that men in the highest tertile of fasting triglycerides (>142 mg/dL, average 218 mg/dL) experienced more than twice the risk for ischemic heart disease vs. those in the lowest tertile (<97 mg/dL, average 78 mg/dL) (23). Even after adjustment for HDL concentrations, each 89 mg/dL increase in triglycerides increases the risk of CVD 37% in women and 14% in men (29). Two large, long-term prospective cohort studies have shown that non-fasting triglycerides may be a more potent predictor of cardiovascular risk than fasting triglyceride levels (30-32). Non-fasting triglycerides may be a better indicator of atherogenic remnant lipoprotein concentrations, and represent a newer, more dynamic model of lipids. Most clinicians order a fasting lipid panel, and there are not yet guidelines for non-fasting triglyceride levels. It seems clear that elevated triglycerides signal cardiovascular risk, but research continues define optimal measuring and interpretation of triglyceride levels.

Despite this evidence that triglyceride lowering may be important in managing CVD risk, LDL-C remains the focus of lipid treatment guidelines, and patients with moderately elevated triglycerides may not be identified as candidates for lipid-lowering therapy. After LDL-C goals are met, lipid treatment targets non-HDL-C levels (the total of VLDL-C, IDL-C, and LDL-C) (7). The American Heart Association recommends that 2 to 4 g/d of EPA+DHA can be used under a physician’s care to lower elevated triglycerides (33).

2.3.2 Omega-3 efficacy in reducing triglycerides
The efficacy of omega-3 fatty acids in lowering triglycerides was first established with studies of fish oil capsules and dietary oily fish intake as reviewed in the Evidence Report commissioned by the National Institutes of Health (34). Briefly, most studies showed a decrease in triglycerides following high intakes of EPA + DHA. Duration does not seem to be a contributing factor to the efficacy of triglyceride lowering so long as the treatment period is greater than 2-4 weeks. Generally, the percentage of triglyceride lowering depends both on dose and baseline triglycerides values. As baseline values and dose increase, the magnitude of lowering increases.
Many published, placebo-controlled studies have reported the efficacy of omega-3 fatty acid concentrates (either triglycerides or ethyl esters) on triglyceride lowering in people with moderately elevated triglycerides (Table 2-1). In many of these studies, triglyceride lowering was not a primary endpoint. Population characteristics were highly variable across this group of randomized clinical trials. Studies of patients with coronary artery disease typically include patients with diabetes and a variety of concurrent drug therapies. These studies administered doses in the range of 0.85 g/d EPA + DHA to 5.1 g/d EPA + DHA.

For subjects at the higher end of moderate hypertriglyceridemia (mean > 250 mg/dl), the average triglyceride lowering with 4 g P-OM3 was about 30% for most studies (35-42). In a study of subjects with lower triglyceride concentrations at baseline, the reduction was 21% (43). A similar level of reduction when 3 g P-OM3 is given to subjects with higher baseline triglycerides (44). Likewise, 6 g P-OM3 decreased triglycerides by about 30% in subjects with lower baseline levels (45). Thus, pretreatment triglyceride status and dose of P-OM3 have independent and additive effects on triglyceride response. When less than 1 g of EPA + DHA is used, effects on triglycerides are minimal (10).

Effects on cholesterol concentration are of interest in treating patients with combined dyslipidemia. Most studies reported an increase in HDL-C in subjects with moderately elevated triglycerides (36, 38-41, 43-46). Many reported increases in LDL-C (37, 38, 41, 42, 46-50), which is probably due to increased particle size rather than particle number. The LDL-C increase is likely due to the particles becoming larger and more buoyant (37), an effect that may be dependent on DHA, specifically (51).

As with studies of fish oil capsules and dietary fish intake, studies of P-OM3 also indicate that the percent triglyceride lowering achieved is dependent on baseline triglyceride values in the subject population. Figure 2.2 presents the percent triglyceride lowering achieved as function of mean triglyceride values for the seven studies in Table 2-1 that administered 4 g P-OM3 (the most often studied dose and indicated prescription dose). Thus on average, patients with lower baseline triglyceride levels will be expected to experience a smaller percent reduction than people with higher triglycerides when 4 g P-OM3 is administered.

2.3.3 Mechanism of triglyceride lowering by omega-3 fatty acids
The triglyceride-lowering effect of omega-3 fatty acids is believed to be due to inhibition of the triglyceride synthetic enzyme acyl CoA:1,2-diacylglycerol acyltransferase, decreased lipogenesis, and increases in peroxisomal or mitochondrial β-oxidation (52). It also has been shown that omega-3 fatty acid intake decreases apo C-III content in plasma lipoproteins and increases lipoprotein lipase gene expression in adipose tissue (53). The mechanisms of triglyceride lowering by omega-3 fatty acids has been reviewed recently (54).
Table 2-1: Randomized controlled trials reporting efficacy of triglyceride-lowering by omega-3 concentrates in subjects with moderate elevations prior to treatment (between 150 mg/dL and 500 mg/dL). Effects on LDL-C and HDL-C varied by study and were either neutral or increased.

<table>
<thead>
<tr>
<th>Study/Year</th>
<th>Design</th>
<th>Subject Characteristics</th>
<th>Mean Baseline Triglycerides</th>
<th>N (Total)</th>
<th>Fatty Acid Preparation</th>
<th>EPA + DHA dose</th>
<th>Treatment Period Duration</th>
<th>Effect on Triglycerides</th>
<th>Effect on Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Durrington et al. 2001 (35)</td>
<td>parallel</td>
<td>CHD taking 10-40 mg simvastatin</td>
<td>409 mg/dL</td>
<td>59</td>
<td>4 g ethyl esters</td>
<td>3.4 g</td>
<td>24 weeks</td>
<td>↓ trigs 20-30%</td>
<td>↔ LDL-C, HDL-C</td>
</tr>
<tr>
<td>Davidson et al. 2007 (36)</td>
<td>parallel</td>
<td>moderately elevated trigs while taking 40 mg simvastatin</td>
<td>282 mg/dL</td>
<td>254</td>
<td>4 g ethyl esters</td>
<td>3.4 g</td>
<td>8 weeks</td>
<td>↓ trigs 29.5% (vs. 6.3% on placebo)</td>
<td>↑ HDL-C</td>
</tr>
<tr>
<td>Calabresi et al. 2000 (37)</td>
<td>crossover</td>
<td>familial combined hyperlipidemia</td>
<td>251 mg/dL</td>
<td>14</td>
<td>4 g ethyl esters</td>
<td>3.4 g</td>
<td>8 weeks</td>
<td>↓ trigs 27%</td>
<td>↑ LDL-C (more bouyant)</td>
</tr>
<tr>
<td>Mackness et al. 1994 (39)</td>
<td>parallel</td>
<td>trigs &gt; 178 mg/dL</td>
<td>355 mg/dL</td>
<td>79</td>
<td>4 g ethyl esters</td>
<td>3.4 g</td>
<td>14 weeks</td>
<td>↓ trigs 28 %</td>
<td>↑ HDL-C (subject with type IV only)</td>
</tr>
<tr>
<td>Grundt et al. 1995 (40)</td>
<td>parallel</td>
<td>trigs &gt; 178 mg/dL</td>
<td>not available</td>
<td>57</td>
<td>4 g ethyl esters</td>
<td>3.4 g</td>
<td>12 weeks</td>
<td>↓ trigs 28 %</td>
<td>↑ HDL-C</td>
</tr>
<tr>
<td>Eritsland et al. 1996 (41)</td>
<td>parallel</td>
<td>patients undergoing coronary artery bypass grafting</td>
<td>175 mg/dL</td>
<td>610</td>
<td>4 g ethyl esters</td>
<td>3.4 g</td>
<td>9 months</td>
<td>↓ trigs 19%</td>
<td>↑ HDL-C, LDL-C</td>
</tr>
<tr>
<td>Johansen et al. 1999 (45)</td>
<td>crossover</td>
<td>coronary artery disease</td>
<td>150 mg/dL</td>
<td>31</td>
<td>6 g ethyl esters</td>
<td>5.1 g</td>
<td>4 weeks</td>
<td>↓ trigs 30%</td>
<td>↑ HDL-C</td>
</tr>
<tr>
<td>Calabresi et al. 2004 (43)</td>
<td>crossover</td>
<td>familial combined hyperlipidemia</td>
<td>378 mg/dL</td>
<td>14</td>
<td>4 g ethyl esters</td>
<td>3.4 g</td>
<td>8 weeks</td>
<td>↓ trigs 44%</td>
<td>↑ HDL-C, LDL-C</td>
</tr>
<tr>
<td>Lungershausen et al. 1994 (43)</td>
<td>crossover</td>
<td>hypertension treated with diuretics and/or beta-blockers</td>
<td>150 mg/dL</td>
<td>43</td>
<td>4 g ethyl esters</td>
<td>3.4 g</td>
<td>6 weeks</td>
<td>↓ trigs 21%</td>
<td>↑ HDL-C</td>
</tr>
<tr>
<td>Bairati et al. 1992 (46)</td>
<td>parallel</td>
<td>coronary artery disease</td>
<td>204 mg/dL</td>
<td>125</td>
<td>15 g MaxEPA</td>
<td>4.5 g</td>
<td>6 months</td>
<td>↓ trigs 39%</td>
<td>↑ HDL-C, LDL-C</td>
</tr>
<tr>
<td>Study/Year</td>
<td>Design</td>
<td>Subject Characteristics</td>
<td>Mean Baseline Triglycerides</td>
<td>N (Total)</td>
<td>Fatty Acid Preparation</td>
<td>EPA + DHA dose</td>
<td>Treatment Period Duration</td>
<td>Effect on Triglycerides</td>
<td>Effect on Cholesterol</td>
</tr>
<tr>
<td>-------------------------</td>
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<td>-----------------------------------------------------------------------------------------</td>
<td>-----------------------------</td>
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<td>-----------------------</td>
</tr>
<tr>
<td>Leigh-Firbank et al. 2002 (42)</td>
<td>crossover</td>
<td>trigs &gt; 133 mg/dL and &lt; 356 mg/dL</td>
<td>223 mg/dL</td>
<td>55</td>
<td>6 g fish oil</td>
<td>3 g</td>
<td>6 weeks</td>
<td>↓ trigs 33%</td>
<td>↑ LDL-C</td>
</tr>
<tr>
<td>Sirtori et al. 1998 (44)</td>
<td>parallel</td>
<td>trigs &gt; 200 mg/dL + cad risk factors (hypertension, diabetes)</td>
<td>~300 mg/dL</td>
<td>935</td>
<td>3 g ethyl esters</td>
<td>1.7 g</td>
<td>6 months</td>
<td>↓ trigs 21.5%</td>
<td>↑ HDL-C</td>
</tr>
<tr>
<td>GISSI-P Investigators 1999 (10)</td>
<td>parallel</td>
<td>recent miocardial infarction</td>
<td>~162 mg/dL</td>
<td>11324</td>
<td>1 g ethyl esters</td>
<td>0.85 g</td>
<td>6 months</td>
<td>↓ trigs slightly (~1-6%)</td>
<td>⇔ LDL-C, HDL-C</td>
</tr>
<tr>
<td>Kelley et al. 2007 (47)</td>
<td>parallel</td>
<td>healthy middle aged men, trigs 150-400 mg/dL</td>
<td>227 mg/dL</td>
<td>34</td>
<td>7.5 g algae oil</td>
<td>~3 g (DHA only)</td>
<td>45 days</td>
<td>↓ trigs 24-25%</td>
<td>↑ LDL-C</td>
</tr>
<tr>
<td>Maki et al. 2005 (48)</td>
<td>parallel</td>
<td>HDL levels below sex-specific population median</td>
<td>179 mg/dL</td>
<td>57</td>
<td>4 g algae oil</td>
<td>1.5 g DHA only</td>
<td>6 weeks</td>
<td>↓ trigs 21%</td>
<td>↑ LDL-C</td>
</tr>
<tr>
<td>Minihane et al. 2000 (49)</td>
<td>crossover</td>
<td>atherogenic lipoprotein phenotype</td>
<td>221 mg/dL</td>
<td>55</td>
<td>6 g fish oil concentrate</td>
<td>3.0 g</td>
<td>6 weeks</td>
<td>↓ trigs 35%</td>
<td>↑ LDL-C</td>
</tr>
<tr>
<td>Mori et al. 2000 (55)</td>
<td>parallel</td>
<td>overweight men</td>
<td>190 mg/dL</td>
<td>56</td>
<td>4 g ethyl esters of EPA OR DHA</td>
<td>3.8 g EPA OR 3.6 g DHA</td>
<td>6 weeks</td>
<td>↓ trigs 20% (DHA) or 18% (EPA)</td>
<td>⇔ LDL-C, HDL-C</td>
</tr>
<tr>
<td>Meyer 2007 (56)</td>
<td>parallel</td>
<td>hyperlipidemic on stable statin therapy</td>
<td>196 mg/dL</td>
<td>40</td>
<td>8 g tuna oil</td>
<td>2.2 g</td>
<td>6 months</td>
<td>↓ trigs 27%</td>
<td>⇔ LDL-C, HDL-C</td>
</tr>
<tr>
<td>Howe et al. 1999 (50)</td>
<td>parallel</td>
<td>middle-aged men</td>
<td>174 mg/dL</td>
<td>32</td>
<td>8 g tuna oil or 8 g MaxEPA</td>
<td>~2.5 g</td>
<td>16 weeks</td>
<td>↓ trigs 26%</td>
<td>↑ LDL-C</td>
</tr>
</tbody>
</table>
2.4 Omega-3 fatty acids and endothelial function

There is growing evidence from animal and human studies that fish oil improves vascular endothelial function (57). Dysfunction of the vascular endothelium is important in the progression of CVD, and it has been shown that endothelial function is impaired in individuals with elevations in triglycerides (58) and inflammation. Many studies have shown that EPA and DHA modulate triglycerides, inflammatory pathways, and endothelial function (59).

The most consistent work on this topic has been conducted with a method that involves infusing vasoactive drugs into the peripheral circulation and measuring change in artery diameter or blood flow. These studies have consistently shown improvements in endothelial function with omega-3 fatty acids in the range of 1.16 to 6 g/d of EPA and/or DHA of duration 28 days to 8 months (60-65). Flow mediated dilation (FMD) is widely used and is regarded as an appropriate measure of endothelial function when standard quality control measures are employed (66). This technique uses ultrasound to measure the percent change in artery dilation in response to increases in blood flow created by arterial occlusion (67). Low FMD scores are a significant predictor of coronary risk in cardiac patients (68), and FMD is being tested as a predictor of coronary risk in several longitudinal studies, such as the Framingham Heart Study (69).

Although there are a handful of published studies on the chronic effects of omega-3 fatty acids on FMD, the findings are less consistent as shown in Table 2-2 (58, 70-73). A single dose of fish or plant derived omega-3 fatty acids significantly improved vascular function in diabetics with moderate elevations in triglycerides (58). Another study of longer-term omega-3 supplementation found significant improvements in FMD in children with familial hypercholesterolemia or combined dyslipidemia given 1.2 g/d DHA daily for 6 weeks (73). In contrast, one often-cited study (70) reported FMD values that are conspicuously low, with high
variability in baseline artery diameter, and others show no effects (71, 72). A recent study found improvements in postprandial FMD, but not fasting FMD, with doses of 2 g/d EPA + DHA (74).

There are no placebo controlled studies with FMD as an outcome that have been performed in adults with moderate hypertriglyceridemia, which is striking since the triglyceride-lowering effects of these fatty acids is so well substantiated. Okumura et al. did examine the effects of 1.8 g EPA daily for 3 months in 8 moderately hypertriglyceridemic men (150-500 mg/dL, average 274 mg/dL) and found that forearm blood flow response to acetylcholine improved to the level seen in normal controls (75). In this study, fasting triglycerides decreased 31% (75). Thus, there is compelling evidence that both endothelial function and hypertriglyceridemia can be improved with omega-3 fatty acids in subjects with moderate hypertriglyceridemia, although it is not clear if the reduction in triglycerides causes the improvement in endothelial function. However, this study was not placebo-controlled, and no previous studies have tested this hypothesis using flow mediated dilation prior to our study presented in Chapter 3.

2.5 Omega-3 fatty acids and the resolution of inflammation

In addition to the extensively-studied effects of omega-3 on eicosanoid pathways and cytokine production in cell and animal models (59, 76-81), omega-3 fatty acids are also precursors of novel lipid mediators with potent actions in tissue homeostasis. They were identified first in resolving inflammatory exudates and in tissues enriched with DHA (82). The name “resolvin” is an abbreviation of the phrase “resolution phase interaction product,” and was coined as such because of the potent anti-inflammatory and immunoregulatory actions. Cell-cell interactions within vessel walls—adherent platelets and neutrophils—converge on the endothelium and promote transcellular lipid mediator biosynthesis (83). Aspirin enhances the production of these compounds by altering the activity of the COX-2 enzyme to make it act as a lipoxygenase.

The beneficial effects of omega-3 polyunsaturated fatty acids have been confirmed using transgenic mice expressing genes for the conversion of omega-6 to omega-3 polyunsaturated fatty acids (84-87). Moreover, these animal studies confirm that significant levels of resolvins and protectins are generated in vivo when tissues are enriched with EPA and DHA. The transgenic mouse tissues contain omega-3 fatty acids in an approximate 1:1 ratio with omega-6 fatty acids. When colitis is induced in these mice, they produce measurable quantities of resolvin E1 and protectin D1 (0.15 ng/mL and 0.22 ng/mL, respectively) and are able to recover weight and colon tissue health (84). In contrast, their wild-type litter mates experience wasting and profound histologic changes in the colon—and produce no resolvin E1 or protectin D1. Furthermore, cardiac myocytes transfected with the gene are resistant to extracellular calcium-induced arrhythmia (87). It is clear from experiments with these mice that increasing tissue omega-3 content is beneficial via many mechanisms, including increased production of resolvins and protectins. Thus, there is mechanistic evidence to suggest that increasing tissue levels of omega-3 fatty acids may improve cardiovascular risk by one or more mechanisms, including increased production of anti-inflammatory resolvins and protectins.
<table>
<thead>
<tr>
<th>Study/Year</th>
<th>Design</th>
<th>Subject Characteristics</th>
<th>N     (Total)</th>
<th>Fatty Acid Preparation/Placebo</th>
<th>EPA + DHA daily dose</th>
<th>Duration</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kothny et al. 1998 (88)</td>
<td>parallel</td>
<td>Coronary Artery Disease</td>
<td>18</td>
<td>18 g fish oil concentrate or 18 g olive oil</td>
<td>10.3 g</td>
<td>acute, 24 hrs</td>
<td>No significant effects</td>
</tr>
<tr>
<td>Goodfellow et al. 2000 (70)</td>
<td>parallel</td>
<td>Adults with Hypercholesterolemia</td>
<td>28</td>
<td>4 g Pronova + 41 units Vitamin E or 2 g corn oil</td>
<td>3.4 g</td>
<td>4 months</td>
<td>Improved FMD and no change in response to glyceryl trinitrite</td>
</tr>
<tr>
<td>Vogel et al. 2000 (89)</td>
<td>crossover</td>
<td>Healthy Adults</td>
<td>10</td>
<td>Meals with 50 g fat made with canned salmon or extra virgin olive oil</td>
<td>6 g</td>
<td>acute (3 hrs postprandial), one week between meals</td>
<td>FMD decreased 31% with olive oil meal, but did not change with salmon meal</td>
</tr>
<tr>
<td>Yosefy et al. 2003 (90)</td>
<td>not placebo controlled</td>
<td>Healthy offspring of CHD patients and normal controls</td>
<td>36</td>
<td>9 g fish oil</td>
<td>2.7 g</td>
<td>2 weeks</td>
<td>no effect on FMD</td>
</tr>
<tr>
<td>Woodman et al. 2003 (71)</td>
<td>parallel</td>
<td>Hypertensive Type II Diabetics</td>
<td>51</td>
<td>4 g purified EPA or DHA</td>
<td>~4 g DHA or EPA</td>
<td>6 week</td>
<td>No change in FMD</td>
</tr>
<tr>
<td>West et al. 2005 (58)</td>
<td>crossover</td>
<td>Adults with Type II Diabetics</td>
<td>18</td>
<td>Shakes containing 50 g fat from sardine oil or high MUFA</td>
<td>4.8 g</td>
<td>acute (4 hrs postprandial), one week between meals</td>
<td>FMD increased 50-80% with fish oil, MUFA control shake did not affect FMD</td>
</tr>
<tr>
<td>Engler et al. 2004 (73)</td>
<td>crossover</td>
<td>Children with familial hypercholesterolemia or combined dyslipidemia</td>
<td>20</td>
<td>6 g DHASCO microalgea oil or corn/soy oil placebo, NCEP-II diet followed by both groups</td>
<td>1.2 g DHA only</td>
<td>6 weeks with 6 week washout</td>
<td>FMD increased with DHA vs. baseline and NCEP-II diet + placebo</td>
</tr>
<tr>
<td>Dyerberg et al. 2004 (72)</td>
<td>parallel</td>
<td>Healthy Males</td>
<td>79</td>
<td>33 g fat load containing either 20 g trans fat, 12 g fish oil, or all control fat in bakery products</td>
<td>4 g</td>
<td>8 weeks</td>
<td>No change in FMD</td>
</tr>
<tr>
<td>Shah et al. 2007 (91)</td>
<td>parallel</td>
<td>non-smoking medical residents</td>
<td>27</td>
<td>1 g Carlson fish oil</td>
<td>0.5 g</td>
<td>2 weeks</td>
<td>improved FMD, improved EIDV, and decreased resting heart rate</td>
</tr>
<tr>
<td>Study/Year</td>
<td>Design</td>
<td>Subject Characteristics</td>
<td>N (Total)</td>
<td>Fatty Acid Preparation/Placebo</td>
<td>EPA + DHA daily dose</td>
<td>Duration</td>
<td>Results</td>
</tr>
<tr>
<td>---------------------</td>
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<td>------------------------------------------</td>
<td>-----------</td>
<td>-------------------------------</td>
<td>----------------------</td>
<td>----------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Hill et al. 2007 (92)</td>
<td>parallel</td>
<td>overweight with CVD risk factors</td>
<td>65</td>
<td>6 g tuna oil</td>
<td>1.9 g</td>
<td>12 weeks</td>
<td>Improved in FO vs. SO groups, but exercise groups not included in analysis and FO groups appeared to have significantly lower FMD pre-intervention</td>
</tr>
<tr>
<td>Wright et al. 2008 (93)</td>
<td>parallel</td>
<td>people with systemic lupus erythmatosis</td>
<td>60</td>
<td>4 g P-OM3</td>
<td>3.4 g</td>
<td>24 weeks</td>
<td>Improved FMD</td>
</tr>
<tr>
<td>Schiano et al. 2008 (94)</td>
<td>parallel</td>
<td>people with peripheral arterial disease</td>
<td>32</td>
<td>2 g P-OM3</td>
<td>1.7 g</td>
<td>12 weeks</td>
<td>Improved FMD from 6.7% to 10%</td>
</tr>
</tbody>
</table>
2.6 Inflammation and endothelial function

Oral administration of aspirin has been shown to improve endothelial function. As discussed above, aspirin increases production of resolvins and protectins by modifying COX enzyme activity. By altering eicosanoid production (e.g. blocking thromboxane production), aspirin is a well-recognized anti-inflammatory agent and is used in low dose therapy to prevent cardiovascular mortality. In a study of 18 hypertensive patients and 10 normotensive control subjects, 8 weeks of aspirin therapy (162 mg/day) improved FMD (95). The hypertensive subjects had impaired FMD at baseline (6.4 +/- 2.0% vs. 11.3 +/- 2.3% in controls), but following aspirin therapy, FMD in hypertensive subjects was 10.4% +/- 3.5% (p < 0.0004). A potential role for nitric oxide was suggested by an increase in cGMP. Baseline hs-CRP (mg/dL) was elevated in the hypertensive subjects (0.53 +/- 12 vs. 0.11 +/- 0.19). The hs-CRP levels of the hypertensive subjects were 0.34 +/- 0.11 mg/dL following aspirin treatment. Therefore, improvements in endothelial function following aspirin therapy may be due to decreased inflammation, perhaps via increased production of resolvins and protectins, although this has not been measured.

Inflammatory cytokines such as TNF-α impair endothelial function. Monoclonal antibodies to TNF-α can be administered to patients to block the signaling of this inflammatory cytokine to treat the inflammation of chronic inflammatory diseases, such as rheumatoid arthritis and Crohn’s disease. Patients with rheumatoid arthritis (n = 10) were treated with an intra-arterial infusion of infliximab (96). Following treatment, endothelial responses to acetylcholine improved to that of healthy controls (n = 10). Endothelial function is also impaired in Crohn’s disease and can be improved following administration with infliximab (97). The converse is also true: when healthy subjects receive intra-arterial infusions of TNF-α, they experience impaired responses to acetylcholine-induced vasodilation versus placebo infusion (98). Therefore, production of inflammatory cytokines is highly linked to endothelial function, and interventions that decrease systemic inflammation could also improve endothelial health.

2.7 Blood pressure reactivity to stress as a risk factor

Several prospective studies have shown that individuals with exaggerated cardiovascular responses to acute stressors in the laboratory are at higher risk of hypertension, and exhibit more aggressive progression of atherosclerotic plaques when compared to individuals with smaller blood pressure and heart rate responses to stress (99-102). For example, West et al. assessed blood pressure and heart rate responses to stress in 103 young men, and then measured resting blood pressure 10 years later (100). In this study, men who exhibited larger cardiovascular responses to acute stress at study entry had higher blood pressure upon follow-up. Moreover, when high reactivity was combined with a positive family history of hypertension, the risk of developing high blood pressure was increased 7-fold. In a large, population-based study in Finland (102), men who showed a larger SBP response (> 20 mmHg) to a behavioral challenge had 72% greater risk of experiencing a stroke and 87% greater risk of experiencing ischemic stroke relative to less reactive individuals, and these effects persisted
after controlling for a long list of traditional stroke risk factors. Exposure to acute stress has also been shown to impair the function of the vascular endothelium for up to 90 min, and this may explain why high reactors show more aggressive growth of atherosclerotic plaques in longitudinal studies (101).

Individuals with dyslipidemia have been shown to exhibit exaggerated cardiovascular reactivity stress, and several different types of dietary changes can attenuate cardiovascular responses to laboratory stressors. For example, a high potassium diet (103), consumption of omega-3 fatty acids (104), and use of soy protein supplements (105) reduce blood pressure at rest and during stress. In addition, studies have reported significant reductions in catecholamine (106-108) and cardiovascular (109-111) responses to psychological stress after treatment with polyunsaturated fatty acids.

2.8 Omega-3 fatty acid effects on blood pressure and heart rate
The need for nutrition interventions in the management of blood pressure is increasingly acknowledged. Relatively small reductions around 5 mmHg may prevent one-third of strokes and one-fifth of coronary events in Western societies (112).

Meta-analyses of intervention studies suggest that fish oil exerts an antihypertensive effect (113-115). In 31 placebo-controlled trials on 1356 subjects, a dose-response effect of fish oil on blood pressure was found. For every 1 g omega-3 fatty acids, there was a decrease of 0.66/0.35 mmHg (113). In a separate analysis of 17 clinical trials, 11 trials enrolled 728 normotensive individuals, omega-3 supplementation led to significant reductions of diastolic blood pressure (DBP) and systolic blood pressure (SBP) in one and two trials, respectively (114). In the 6 trials that enrolled untreated hypertensives, significant reductions in of SBP and DBP were present in two and four trials, respectively. In the normotensive subjects, pooled estimates of SBP and DBP change (mmHg) with 95% confidence intervals were -1.0 (-2.0 to 0.0) and -0.5 (-1.2 to +0.2)(114). In untreated hypertensives, reductions were -5.5 (-8.1 to -2.9) and -3.5 (-5.0 to -2.1). These studies generally used high doses (>3 g/d). Geleijnse et al., in a meta analysis of 36 trials, showed that blood pressure was reduced by -2.1/-1/6 mmHg following omega-3 fatty acids. Furthermore, the blood pressure-lowering effects were greater in older (>45 years; -3.5/-2.4) and hypertensive (> or = 140/90 mmHg; -4.0/-2.5) individuals. Omega-3 fatty acids appear to be effective even in prehypertensive populations as Grundt et al. observed a 8/4 mmHg reduction in hyperlipidemic subjects with prehypertension (mean 129/84.2 mmHg at baseline) who were assigned to P-OM3 (40).

Blood pressure decreases may be dependent on changes in phospholipid omega-3 concentrations. A study by Vandongen et al. compared the effects of 12 weeks of fish meals or fish oil supplements providing a mean intake of 3.65 g/day omega-3 fatty acids in 120 men with mildly elevated blood pressure. There was a significant inverse correlation between the fall in blood pressure and heart rate and increases in omega-3 (and decreases in omega-6) fatty acids in platelet phospholipids (116). Bonaa et al. also found that blood pressure reduction (-4.6/-3
mmHg) from 6 g/day P-OM3 for 10 weeks in mild hypertensives was inversely related to baseline plasma phospholipid omega-3 fatty acids (117). Toft et al. also found that 4 g P-OM3 (n = 78, 16 weeks) was more effective in reducing blood pressure in hypertensive patients with low baseline concentrations of plasma phospholipid omega-3 (118). The effects of omega-3 fatty acids on blood pressure are additive to weight loss (119), potentiated by sodium restriction (120), and able to amplify the effects of propanolol (121) and diuretics (43).

In conclusion, there is evidence that fish oil may cause subtle decreases in resting blood pressure when administered at higher doses in people with untreated hypertension but does not affect blood pressure when consumed in moderate doses by normotensive people.

The mechanism by which omega-3s lower blood pressure is not fully understood, but it is likely to be a combination of effects on the vasculature, autonomic signaling, prostanoid production, and effects on membrane ion channels and receptor signaling. Blood pressure is influenced by arterial compliance, which in turn, is influenced by endothelial function (122). EPA and DHA improved arterial compliance in patients with dyslipidemia (123). Mozaffarian et al. found in a meta-analysis of 30 studies that omega-3 fatty acids reduced heart rate by 1.6 beats per minute (124), and omega-3 fatty acids also improve heart variability (125, 126), suggesting effects on autonomic tone. The anti-arrhythmic effects of omega-3 fatty acids are thought to be caused by their ability to inhibit the fast, voltage-dependent sodium current and the L-type calcium currents, although evidence of potassium channel modulation has also been suggested (127, 128).

2.9 Effects of culinary spices on cardiovascular risk factors

There is considerable epidemiological evidence that a diet rich in fruits and vegetables may be protective against coronary heart disease (129, 130). It is often posited that plant based antioxidants are responsible for this epidemiological observation because the oxidative stress induced by free radicals is involved in the development of so many chronic diseases. Indeed, the intake of polyphenols has been inversely correlated to the incidence of several chronic diseases including cardiovascular disease (131, 132). Although spice consumption is not usually quantified in epidemiologic studies, this suggests that phenolics such as those contained in spices may be have beneficial effects on cardiovascular health by improving indices of oxidative and inflammatory stress in vivo (133, 134).

There is also increasing experimental evidence that oxidative stress, particularly oxidation of low density lipoproteins plays a significant role in the pathogenic pathway of atherosclerosis (135, 136). Antioxidant compounds have been shown to effectively scavenge reactive oxygen species and inhibit lipid peroxidation in vitro (137). Most research to date has been conducted on fruit and vegetable interventions, with wine, tea, and cocoa also being studied for their antioxidant contributions to the diet. However, results from intervention trials on the protective effect of supplementation with micronutrient antioxidants such as beta carotene and vitamin E have not shown benefit of isolated antioxidant nutrients (138, 139) reviewed in (140). Controlled feeding
of diets enriched with fruits and vegetables increases plasma antioxidant capacity that is not explained by micronutrient antioxidant concentrations (141).

Therefore, the beneficial effects of fruits and vegetables (and perhaps spices) may not rely on the well-characterized antioxidants that they are known to contain (vitamin E and C and beta carotene) but on lesser characterized and unknown phytonutrients, or a concerted action of multiple compounds. In support of this idea, the antioxidant capacities (measured as ORAC) of some flavonoids are several times higher on a molar basis than ORAC activity for purified Vitamin E and C (142).

In the literature on fruits and vegetables, there is encouraging evidence that antioxidant action is potentiated when fruits are consumed in combination with one another (143, 144). Liu et al showed the combination of orange, apple, grape, and blueberry displayed a synergistic effect in antioxidant activity (144) (Figure 2.3). At any given amount of consumption, the mixture of fruit had a greater effect on antioxidant status than any single fruit.

![Figure 2.3](image.png)

Figure 2.3: Combining plant compounds provides synergistic effects on antioxidant capacity. Figure from Liu RH. Potential synergy of phytochemicals in cancer prevention: mechanism of action. J Nutr 2004;134(12 Suppl):3479S-3485S.

### 2.9.1 Links between antioxidants and inflammation

Accumulating evidence has linked the pathogenesis of a variety of human diseases to oxidative stress. Oxidative injury involves the modification of cellular macromolecules via by-products of oxygen metabolism (145). Immune cell activity, such as the bactericidal action of neutrophils, produces reactive oxygen species (146), linking inflammation to oxidative stress. Reactive oxygen species can be released by many cell types in response to a variety of stimuli, such as TNF-α and LPS, and can serve as intracellular signals for the activation and regulation of redox-sensitive transcription factors (147). Administration of endotoxin has been shown to allow the intracellular accumulation of reactive oxygen species and subsequently upregulate the expression and biosynthesis of TNF-α via NF-kB dependent pathways (148-150). Humans in
endotoxic shock have free radical overproduction and depleted levels of endogenous antioxidants (151). Importantly, in a mouse model of endotoxemia, preloading mice with an antioxidant-supplemented diet prior to the inflammatory challenge significantly decreased production of inflammatory cytokines and reactive oxygen species and reduced NF-kB activation (152). This provides important evidence that dietary antioxidants can be effective in suppressing an inflammatory response in vivo. Much research has demonstrated that dietary polyphenols have demonstrated anti-inflammatory activity by affecting multiple targets in inflammatory pathways (133, 134, 153).

2.9.2 Evidence that Culinary Spices Could Attenuate Inflammatory Responses
Spices are a unique delivery vehicle for adding antioxidant and anti-inflammatory compounds to the diet because they contribute few calories and make other healthy foods more attractive and flavorful. Much of the published work on spices has used in vitro assays of antioxidant capacity in order to identify spices with the most potent effects on oxidative stress. Given that inflammation and oxidative stress often co-occur and may potentiate each other, and given the detrimental effects of inflammation on vascular endothelial function (11, 154), highly antioxidant spices are a good candidate for improving multiple cardiovascular risk factors.

Nationally representative dietary surveys such as the National Health and Nutrition Examination Survey (NHANES) do not assess intake of culinary spices, so there is little epidemiological data on spice consumption and incidence of chronic disease. However, spices have a rich history of medicinal use and have been studied in many in vitro and animal models of inflammation.

Spices derive their color and flavor from phytochemicals including flavonoids and polyphenols. These compounds are often produced as plant defense compounds. Many of these compounds have wide-ranging biological activities and have served as the basis of drug development. In the USDA’s 2007 report on oxygen radical absorbance capacity (ORAC), commonly consumed culinary spices had the highest ORAC values on a per gram basis (155).

In addition to antioxidant effects, spices have demonstrated effects on glucose and lipid metabolism gene expression in inflammatory pathways. Below, sample research on these spices is presented.

2.9.3 Ginger
Ginger has been for centuries an important ingredient in herbal medicine (156, 157). Preventative and ameliorative effects of ginger have been described in the treatment of rheumatism, nervous diseases, gingivitis, toothache, asthma, stroke, constipation, and diabetes (158). Ginger has been shown to exhibit antithrombotic activity, because its extract inhibits platelet aggregation and thromboxane-B2 production in vitro (157). In addition, ginger has been shown to inhibit the formation of pro-inflammatory eicosanoids 5-hydroxyeicosatetraenoic acid and prostaglandin E2 (158). Ginger consumption does not promote platelet dysfunction that would limit its use (159).
2.9.4 Cinnamon
Many spices demonstrate some potential to affect insulin and glucose (160), but cinnamon has demonstrated the most evidence in human clinical work. Cinnamon reduced fasting glucose in people with type II diabetes (161) and post-prandial glucose response at 15, 30, and 45 min in 14 healthy subjects (162).

2.9.5 Turmeric
Turmeric has been studied to a great extent in in vitro and animal studies for its anti-inflammatory effects (163-168). Its bioactive constituent, curcumin, inhibits activation of NF-kB and is a potent antioxidant (163).

2.9.6 Black Pepper
Piperine has demonstrated antioxidant activity in vitro by inhibiting lipid peroxidation (169). The formation of 5-HETE (the product of 5-lipoxygenase) is significantly inhibited in a concentration-dependent manner by aqueous extracts of black pepper and its constituent piperine. Piperine offers protection against the oxidation of human LDL-C as evaluated by copper ion-induced lipid peroxidation (170). It has been shown to augment sulfidation and glucuronidation of curcumin (and potentially other bioactives) and thus increases plasma levels (164).

2.9.7 Garlic
The garlic compound, S-allyl cysteine (SAC) has been shown to reduce oxidant load in cells involved in the atherogenic process and to block NF-kB activation (171, 172). This suppression of NF-kB may make garlic useful for preventing atherosclerosis. Garlic may promote anti-inflammatory environment by cytokine modulation in human blood that leads to an overall inhibition of NF-kB (173, 174).

2.10 Plasma bioactivity changes following phytonutrient interventions
Very little time course work has been published on plasma bioactivity following consumption of highly antioxidant or anti-inflammatory foods. What little work has been published to date has been on changes in plasma ORAC following consumption of fruits including blueberries, dried plums, cherries, grapes, kiwifruit, and strawberries (175, 176). In these small studies, hydrophilic ORAC peaked at 1 h postprandially, and lipophilic ORAC appeared highest at 2-3 h postprandially (for blueberry and cherry interventions). However, plasma ORAC was measured at baseline and only three postprandial time points: 1 h, 2 h (or 3 h), and 4 h – 5 h, which provides limited time course information and may not have accurately captured the true peaks in hydrophilic and lipophilic ORAC. Another limitation is that some of the studies did not include a control group, and this is critical in studies of plasma ORAC (177). Blueberries and cherries have a much lower lipophilic ORAC than culinary spices as discussed above. In fact, blueberries have an H-ORAC of 6520 and L-ORAC of only 36 (155).
2.11 Models of Human Inflammatory Challenge

In the same way that postprandial measurements and glucose tolerance tests measure the human response to metabolic stress, inflammatory challenge could be useful in evaluating the ability of an intervention to attenuate inflammatory responses.

2.11.1 Human model of endotoxemia

Injection of human subjects with purified endotoxin (LPS) has shown the ability of some drugs to attenuate the inflammatory response (178). The human endotoxemia model has provided valuable information about the time course of cytokine production in an acute inflammatory state (Figure 2.5). Although the endotoxin is rapidly cleared from the blood stream, IL-6 and TNFα levels are produced in high concentrations between 1 and 3 h post-infusion, followed by the appearance of IL-8 and IL-10. A disadvantage of using this model is that it requires extensive resources to monitor research volunteers.
2.11.2 Psychological Stress-Induced Production of Cytokines

Acute psychological stress also triggers *in vivo* cytokine production as reviewed by Steptoe *et al.* (179). There is evidence from clinical studies that exposure to standardized stressors in the laboratory is associated with measurable increases in inflammatory cytokines, in particular, IL-6 (179). The literature on the IL-6 production time course has been summarized in Table 2-3.

Table 2-3: IL-6 responses to psychological stressors in human studies. Values reported in this table are significantly different from baseline values. (ND = not different, NR = not reported, TSST = Trier Social Stress Test)

<table>
<thead>
<tr>
<th>Study</th>
<th>Purpose</th>
<th>Subject Characteristics</th>
<th>Stressor</th>
<th>Baseline IL-6 (pg/mL)</th>
<th>30 min or less</th>
<th>45 min</th>
<th>60-75 min</th>
<th>90 to 105 min</th>
<th>120 min or more</th>
</tr>
</thead>
<tbody>
<tr>
<td>von Kanel <em>et al</em> 2006(180)</td>
<td>Assess Habitation to Stress</td>
<td>21 Healthy Men, mean 46 years old</td>
<td>TSST</td>
<td>0.25</td>
<td>ND</td>
<td>0.27</td>
<td>NR</td>
<td>0.37 pg/mL</td>
<td>NR</td>
</tr>
<tr>
<td>von Kanel <em>et al</em> 2008(181)</td>
<td>Aspirin/Propranolol Intervention</td>
<td>64 subjects &gt;35 years old</td>
<td>TSST</td>
<td>0.72-0.79</td>
<td>ND</td>
<td>not diff</td>
<td>NR</td>
<td>0.87 vs. 1.23</td>
<td>NR</td>
</tr>
<tr>
<td>von Kanel <em>et al</em> 2005(182)</td>
<td>fibrin and stress</td>
<td>28 healthy &gt; 35 years old</td>
<td>TSST</td>
<td>0.36</td>
<td>ND</td>
<td>0.4</td>
<td>NR</td>
<td>0.47 NR</td>
<td>NR</td>
</tr>
<tr>
<td>Pace <em>et al</em> 2006(183)</td>
<td>stress response effects of major depression</td>
<td>men w/o or w/ major depression</td>
<td>TSST</td>
<td>1.4 vs. 2.4</td>
<td>ND</td>
<td>NR</td>
<td>2.3 vs. 3, 2.3 vs. 4</td>
<td>2.6 vs. 4.9</td>
<td>NR</td>
</tr>
<tr>
<td>Kop <em>et al</em> 2008(184)</td>
<td>mental stress in healthy vs. CAD</td>
<td>28 healthy vs. 36 CAD patients</td>
<td>anger recall/ arithmetic</td>
<td>0.93 vs. 2.58</td>
<td>ND</td>
<td>NR</td>
<td>NR</td>
<td>NR NR NR</td>
<td>NR</td>
</tr>
<tr>
<td>Steptoe <em>et al</em> 2002(185)</td>
<td>assess socioeconomic / gender effects</td>
<td>230 civil servants 35-55 years old</td>
<td>color-word/mirror tracing</td>
<td>1.20 men vs. 1.32 women</td>
<td>NR</td>
<td>2.7% vs. 12.8%</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Steptoe <em>et al</em> 2001(186)</td>
<td>Pilot study</td>
<td>13 healthy, 25-51 years old</td>
<td>color-word/mirror tracing</td>
<td>1.67</td>
<td>NR</td>
<td>1.73</td>
<td>NR</td>
<td>NR 2.61</td>
<td>NR</td>
</tr>
<tr>
<td>Brydon <em>et al</em> 2004(187)</td>
<td>SES status/time course</td>
<td>38 healthy men 30-59 years old</td>
<td>color-word/mirror tracing</td>
<td>1.54 vs. 1.24</td>
<td>ND</td>
<td>NR</td>
<td>1.85</td>
<td>NR 2.65 vs. 2</td>
<td>NR</td>
</tr>
<tr>
<td>Brydon <em>et al</em> 2008(188)</td>
<td>effects of adiposity in women</td>
<td>67 women 18-25</td>
<td>Stroop and speech</td>
<td>0.71</td>
<td>0.79</td>
<td>1</td>
<td>NR</td>
<td>NR NR NR</td>
<td>NR</td>
</tr>
</tbody>
</table>

There is a clear association with chronic psychological stress and enhanced activation of the innate immune system, including increased plasma pro-inflammatory cytokines (189-191). In fact, people with major depression produce greater concentrations of IL-6 when exposed to lab stressors (183).

This acute psychological stress model poses minimal risk to the human volunteer, presents rapid recovery and provides a system in which hemodynamics can be assessed for biobehavioral outcomes. In this model, aspirin was able to significantly attenuate the inflammatory response
to acute stress (181), demonstrating that anti-inflammatory effects be demonstrated in this model. Moreover, there is evidence that suppressing an inflammatory response to acute psychological stress has positive effects on health because a large increase in TNF-α during acute stress was significantly associated with arterial stiffness measured 3 years later (192).

2.11.3 Stress Induced changes in immune cell gene expression

The stress induced production of cytokines can be measured both as changes in plasma protein concentrations and, more mechanistically, as changes in lymphocyte gene expression. Surprisingly little work has examined the effects of acute psychological stress on gene expression in inflammatory cells. Most experiments have isolated a cell population (e.g. peripheral blood mononuclear cells, PBMCs) or used whole blood, and added a stimulant such as LPS ex vivo (179, 193-196). When Brydon et al. (197) examined the effects of two 5-minute psychological stress tasks on gene expression in isolated PBMCs from 32 volunteers, they found that IL-1B expression was increased at 30, 75, and 120 min relative to IL-1B gene expression in the PBMCs of 10 unstressed control subjects. There was a correlation between heart rate response to the tasks and IL-1B gene expression increases at 30 min \( (r = 0.32, \ p = 0.05) \), 75 min \( (r = 0.44, \ p = 0.005) \), and 120 min \( (r = 0.37, \ p = 0.021) \). These correlations were independent of age, body mass index, and body fat. This suggests that the more effective a psychological stressor is at stimulating a sympathetic nervous system response, the more inflammation is induced in a human subject. Indeed, it has been shown that SNS activation by stress induces PBMC NF-kB activation, but this may be due to natural killer cell migration (198). Bierhaus et al. have shown that NF-kB is induced during stress exposure, in parallel with elevated levels of catecholamines and cortisol, and that THP-1 cells stimulated with noradrenaline have induction of NF-kB and increased NF-kB dependent gene expression (199), providing a mechanism by which psychological stress induces increases in plasma pro-inflammatory cytokine concentrations.
Chapter 3. Dose response effects of omega-3 fatty acids on triglycerides, inflammation, and endothelial function in healthy people with moderate hypertriglyceridemia

3.1 Abstract

**Background:** Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reduce cardiovascular mortality at a dose of about 1 g/day, and there is some evidence for reduced inflammation and improved endothelial function. However, since these data have come largely from studies using much higher doses, the cardioprotective mechanism is not clear.

**Objective:** The objective of this study was to compare the effects of a nutritional dose of EPA+DHA (0.85 g/d) to a pharmaceutical dose (3.4 g/d) on serum triglycerides, inflammatory markers, and endothelial function in healthy subjects with moderately elevated triglycerides.

**Design:** This was a placebo-controlled, double-blind, randomized, 3 period crossover trial (8 wk treatment, 6 wk washout) comparing 0.85 g/d to 3.4 g/d EPA+DHA in 26 people (23 M, 3 post-menopausal F) with moderate hypertriglyceridemia (150-500 mg/dL).

**Results:** The higher dose of EPA+DHA reduced triglycerides by 27% vs. placebo (173 ± 17.5 vs. 237 ± 17.5 mg/dL, p = 0.002), whereas there was no effect of the lower dose on lipids. There were no effects on cholesterol, endothelial function (as assessed by flow-mediated dilation, peripheral arterial tonometry/EndoPAT, or Doppler measures of hyperemia), inflammatory markers (IL-1β, IL-6, TNF-α, hs-CRP), or expression of inflammatory cytokine genes in isolated lymphocytes.

**Conclusions:** The higher dose of EPA+DHA significantly lowered triglycerides, but neither dose improved endothelial function or inflammatory status over 8 weeks in healthy adults with moderate hypertriglyceridemia.
3.2 Introduction
Elevated triglycerides are a risk marker for cardiovascular disease (CVD), with an estimated 30% of the adult U.S. population being affected (7, 20, 22-29). The omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reduce triglyceride levels when administered at higher doses (>3 g/d combined) (18, 54, 200). However, lower doses (about 1 g/d EPA + DHA) are recommended for cardiovascular risk reduction based on evidence from large secondary prevention trials showing that this intake can reduce cardiovascular mortality by 20-30% without significant reductions in triglycerides (10, 201). Therefore, the cardioprotective action of omega-3 fatty acids is thought to occur via multiple benefits such as anti-arrhythmic (127, 128) and anti-inflammatory effects (59, 202) secondary to changes in cell membrane properties that impact cell signaling and gene expression (59, 77, 202, 203). These benefits may also manifest in improved vascular endothelial function when omega-3 fatty acids are given acutely (58, 74, 89) or chronically (70-74, 92-94).

Despite the significant reductions in triglycerides achieved with higher, pharmacological doses of omega-3 fatty acids, establishing recommended intakes to reduce CVD risk in people with moderate hypertriglyceridemia has been complicated. Moderate hypertriglyceridemia often co-exists with high LDL-C (combined hyperlipidemia), and pharmacological doses of omega-3 fatty acids can increase LDL-C when large reductions in triglycerides are achieved (200). Due to the lack of morbidity and mortality data with high doses of omega-3 fatty acids, the current guidelines only recommend the higher dose for those who have substantial elevations in triglycerides (e.g. > 500 mg/dl), not for minimizing general CVD risk (25).

A greater understanding of the effects of different doses of EPA+DHA on intermediate CVD risk markers such as inflammation and vascular endothelial function would help to establish evidence-based recommendations, particularly for adults with moderate hypertriglyceridemia.

The objective of this study was to compare the effects of a lower dose (i.e., 0.85 g/d EPA + DHA, recommended for secondary prevention of CHD) and a higher dose (3.4 g/d EPA + DHA, indicated to lower very high triglycerides) on endothelial function, lipids, and inflammatory markers in healthy people with moderately elevated triglycerides. We hypothesized that the higher dose would significantly decrease fasting triglycerides, and that improvements in endothelial function would be dose-dependent, and accompanied by reductions in inflammation.

3.3 Subjects and methods
3.3.1 Study population
Healthy people with moderate hypertriglyceridemia (fasting triglycerides 150-500 mg/dL) were recruited for this study. Estrogen fluctuations alter endothelial function, so all women were post-menopausal (no menses > 12 months). Other inclusion criteria were: age 21-65 years, BMI 20-39 kg/m², and generally good health. Exclusion criteria were tobacco use; acute or chronic inflammatory conditions; hypertension (blood pressure equal to or greater than 150
over 95 mm Hg); liver or kidney dysfunction (self reported or abnormal screening blood work); unwillingness to discontinue nutritional supplements (except for calcium, which was allowed at a stable dose); intake of fish, flaxseed, or walnuts equal to or greater than 2 or more servings per week; use of oral contraceptives or hormone replacement therapy; use of lipid-lowering, anti-inflammatory, anti-depressant, or blood pressure medication; and abnormal screening EKG or history of heart disease. Potential participants were advised that they would be expected to maintain low consumption of omega-3 fatty acids during the study, refrain from use of all supplements, and maintain their lifestyle. A complete blood count and standard chemistry panel were obtained at screening to rule out the presence of serious illness (e.g. autoimmune disease, cancer, and immunodeficiency). Seated blood pressure was measured by nurses in a controlled environment using a calibrated mercury sphygmomanometer and appropriately sized cuffs, after a 5 minute quiet rest according to JNC 7 guidelines (204). Three readings were taken, and the average of the last 2 was used to determine eligibility for study participation and baseline characteristics. The blood pressure criterion (< 150 mmHg SBP and < 95 mmHg DBP) was established to avoid excluding people with unmedicated Stage 1 hypertension.

3.3.2 Recruitment and ethical aspects

Subjects were recruited through advertisements in the local newspaper, fliers in the community, and campus email lists. Two hundred and eighty potential subjects called to indicate interest in participating in the study. They were given information about the study and if interested, were asked a series of medical and lifestyle questions. Of the 280 respondents, 89 met the study criteria and were scheduled for a clinic screening at the Penn State General Clinical Research Center (GCRC). After providing written informed consent, a screening blood sample was drawn and a 12-lead EKG was obtained. Body weight and height were recorded to calculate BMI. From the 89 who were screened, 28 were eligible to participate in the study (Figure 3.1). Subjects with glucose > 100 mg/dL, blood pressure > or = 140/90 mmHg, or high cholesterol, were required to obtain a release form from their physicians due to the length of the trial and medication restrictions. A randomization scheme was developed in advance, and subjects were assigned to a treatment sequence at enrollment. The study protocol was approved by the Institutional Review Board of the Pennsylvania State University. The trial was registered on ClinicalTrials.gov (NCT00504309).
Figure 3.1: Schematic of subject flow and reasons for exclusion. Source: Skulas-Ray AC et al. Dose-response effects of omega-3 fatty acids on triglycerides, inflammation, and endothelial function in healthy persons with moderate hypertriglyceridemia. Am J Clin Nutr 2010.

3.3.3 Design and interventions
This was a randomized, double-blind, 3-period crossover, placebo-controlled study with 8-week treatment periods, and 6 week washout periods. Treatment was provided as 4 identical capsules per day during all periods. All capsules were provided by GlaxoSmithKline (Lovaza™ and identical corn oil placebo). Each 1 g prescription omega-3 fatty acid ethyl ester capsule contains approximately 465 mg EPA and 375 DHA (ratio of 1.2:1); most fish oil supplements contain EPA and DHA in a ratio of 1.5:1. We performed an independent analysis of one sample of active and placebo (methods previously described (205)), and the corn oil placebo contained 56% linoleic acid (C18:2n6), 28% oleic acid (C18:1n9), 12% palmitic acid (C16:0), and small amounts of other fatty acids. The fatty acid profile of these treatments as determined by our independent analysis is summarized in Table 3-1. During the three treatment periods, subjects
received in random order: 0 g/d EPA + DHA (corn oil placebo), 0.85 g/d EPA + DHA, and 3.4 g/d EPA + DHA. The lower dose was provided as 1 g prescription omega-3 fatty acid ethyl esters and 3 placebo capsules. Treatments were matched to a coded numeric identifier so that the researchers and participants were blinded to treatment assignment. Subjects were instructed to maintain their weight and activity level during the course of the study, and they were counseled to exclude fatty fish meals (including salmon, tuna, mackerel, and herring), fish oil supplements, flax products, walnuts, and omega-3-enriched eggs during the study. Subjects were contacted two weeks into each phase to determine compliance and discuss any difficulties with taking the capsules. At the midpoint of each treatment period (4 weeks), subjects reported to the GCRC to have their bottles weighed and to receive new supplies. Sample size was based on a power calculation with FMD as the primary outcome. Twenty-two subjects were estimated to provide 90% power to detect a 30% change in FMD values (73) with α of 0.05, based on variability of fasting FMD values in our previous work (67).

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>0 g/d</th>
<th>0.85 g/d</th>
<th>3.4 g/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0 Palmitic</td>
<td>464</td>
<td>348</td>
<td>1</td>
</tr>
<tr>
<td>C18:0 Stearic</td>
<td>73</td>
<td>55</td>
<td>2</td>
</tr>
<tr>
<td>C18:1n9 Oleic</td>
<td>1121</td>
<td>843</td>
<td>10</td>
</tr>
<tr>
<td>C18:2n6 Linoleic</td>
<td>2240</td>
<td>1681</td>
<td>2</td>
</tr>
<tr>
<td>C20:2n6 Eicosadienoic</td>
<td>1</td>
<td>18</td>
<td>70</td>
</tr>
<tr>
<td>C20:4n6 Arachidonic</td>
<td>0</td>
<td>15</td>
<td>62</td>
</tr>
<tr>
<td>C20:5n3 Eicosapentaenoic n-3</td>
<td>0</td>
<td>486</td>
<td>1944</td>
</tr>
<tr>
<td>C22:5n3 Docosapentaenoic n-3</td>
<td>0</td>
<td>35</td>
<td>141</td>
</tr>
<tr>
<td>C22:5n6 Docosapentaenoic n-6</td>
<td>0</td>
<td>12</td>
<td>48</td>
</tr>
<tr>
<td>C22:6n3 Docosahexaenoic</td>
<td>0</td>
<td>421</td>
<td>1686</td>
</tr>
</tbody>
</table>

Values were calculated from independent analysis of fatty acid composition a sample of active and placebo capsules (only fatty acids detected at least 1% of total fatty acids for either active treatment or placebo capsules are shown).

### 3.3.4 Blood sample collection and assays

At the beginning of the study and at the end of each treatment period blood samples were collected in the fasting state (12 h with nothing but water, 48 h without alcohol, and 2 h without vigorous exercise). A general health profile was obtained on fresh serum samples to monitor liver enzyme concentrations (Chem 24 panel, Quest Diagnostics, Pittsburgh, PA). The fasting lipid profile was measured on two separate days at the end of each period. Except for endpoints that required unfrozen samples, samples were aliquotted and stored at -80 C for batch analysis.

#### 3.3.4.1 Lipids and lipoproteins

Whole blood was drawn into serum separator tubes, allowed to clot, and centrifuged. Total cholesterol and triglycerides were determined by enzymatic procedures (Quest Diagnostics, Pittsburgh, PA; CV < 2% for both). HDL-C was estimated according to the modified heparin-manganese procedure (CV < 2%). LDL-C was calculated using the Friedewald equation: $\text{LDL-C} = \text{Total Cholesterol} - \text{HDL-C} - \frac{\text{Triglycerides}}{5}$. 


TC – (HDL-C + TG/5) except in cases where triglyceride values exceeded recommended ranges. In these cases a direct LDL-C test was ordered that directly measures LDL-C concentrations using a chromogenic reaction after removal of all non-LDL-C (N-geneous LDL-ST-C, Quest Diagnostics, Pittsburgh, PA). The between run CV of this assay is less than 3%.

3.3.4.2 Inflammatory Markers
Plasma concentrations of IL-1β, IL-6, TNF-α were measured via high sensitivity ELISA kits from R&D Systems (Minneapolis, MN) in duplicate (assay CV < 11% for all). Serum hs-CRP was measured by latex-enhanced immunonephelometry (Quest Diagnostics, Pittsburgh, PA; assay CV < 8%).

3.3.4.3 Mononuclear cell gene expression
Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient separation from EDTA-anticoagulated blood. Following isolation and washing of the buffy coat with saline, cells were counted on a hemocytometer. At least 8 x 10^6 cells were saved for RNA isolation. Cells for RNA isolation were suspended in RNALater solution before being stored at -80 °C. Following RNA isolation (RNEasy mini kit, Qiagen; Valencia, CA), high capacity cDNA Archive kit (Applied Biosystems; Foster City, CA) was used for reverse transcription. cDNA (500 ng) was amplified by SYBR Green PCR Master Mix (Applied Biosystems; Foster City, CA) and detected by ABI 7300 Sequence Detection System (Applied Biosystems; Foster City, CA). The expression of IL-1β, IL-6, TNF-α was measured using real time PCR with the following primers: IL-1β 1173F CAGGCACCACATTGGTTCTAA, IL-1β 1224R CAGAATGTGGGGAGCGAATGAC, TNF-α 803F ATCAATCAGCCGACTATCTC, TNF-α 887R TGGATGTCGTCTCTCACA, IL-6 197F GCCACCTCACCTTCAGAACG, IL-6 250R CGTCCAGGATGTACCGAATT. The expression of these genes was normalized to expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAPDH forward and reverse primers were TGGGTGTGAACCATGAGAAG and GCTAAGCAGTTGGTGTCG, respectively.

3.3.4.4 Insulin and glucose
Insulin was measured by radioimmunoassay using 125I-labeled human insulin and a human insulin antiserum (Linco Research, St. Charles, MO; cross-reactivity with proinsulin < 0.2%) (206). Glucose was determined by an immobilized enzyme biosensor using the YSI 2300 STAT Plus Glucose & Lactate Analyzer (Yellow Springs Instruments, Yellow Springs, OH) (207). The quantitative insulin-sensitivity check index (QUICKI) was calculated as 1/(log[fasting glucose] + log[fasting insulin]) (208). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as glucose x insulin / 405 (209).

3.3.4.5 Liver enzymes
Liver enzymes were measured as part a general health profile battery of blood tests (Chem 24 panel, Quest Diagnostics, Pittsburgh, PA).

3.3.4.6 Erythrocyte fatty acids
Blood samples were drawn into EDTA tubes at each visit. RBCs were separated from plasma by centrifugation, and a 0.5 mL aliquot was collected from the RBC pack. RBC were frozen at –
80 °C until analyzed. FA analysis was performed as previously described (205). Briefly, lipids were extracted, methylated to form fatty acid methyl esters (FAMEs), and analyzed by gas chromatography on a GC2010 (Shimadzu Corporation, Columbia, MD) equipped with a 100-m SP-2560 column (Supelco, Bellefonte, PA). FAME composition is reported as weight percent of total identified FAMES. The omega-3 index is the sum of EPA and DHA.

3.3.4.7 Flow mediated dilation, Doppler assessment of reactive hyperemia, and peripheral arterial tonometry

After a 12 h fast, endothelial function was assessed by FMD, using high frequency ultrasound as described previously (58, 67) in a quiet, dimly lit room at 71-75 °F. We have previously reported test retest reliability for this measurement (67). The brachial artery above the elbow of the right arm was scanned in a longitudinal section after a 15 min rest, and continuous cross-sectional images were recorded at rest (1 min), during cuff inflation (5 min), and during increased blood flow after cuff release (2 min). An automated rapid cuff inflator set to 250 mmHg (Hokanson, Bellevue, WA) was placed on the forearm distal to the ultrasound probe to induce ischemia. Changes in arterial diameter were measured by external B mode ultrasound imaging (Acuson Aspen 128XP equipped with a 10 mHz linear array transducer; Acuson, Mountain View California), by a single well-trained sonographer (P. Wagner). Images were gated using R-wave detection so that scans were assessed at end diastole. Automated edge detection software (Brachial Analyzer; MIA, Iowa City, Iowa, USA) was used to quantify artery diameter continuously, throughout the test. Peak artery diameter was determined as the largest diameter recorded in the 2 min post-deflation segment. Resting diameters were the average of all images collected over a 1 min period. FMD was calculated by 2 independent scorers as percent change in artery diameter at peak dilation vs. baseline and is reported as a percent. If FMD values differed by >2%, a third technician reviewed the scan. FMD is reported as the average of the two readings.

Average flow velocity (meters/second) across the cardiac cycle, maximum flow velocity, and velocity time integral across the cardiac cycle (meters) were measured using duplex pulsed Doppler at two time points: resting baseline and immediately after cuff release. Flow (mL/min) was calculated as described previously (67).

During the FMD test, the EndoPAT2000 (Itamar Medical, Ltd.) was used to measure relative changes in pulse wave amplitude (PWA) pre vs post occlusion (210). The EndoPAT technique has been validated (210, 211) and used in the Framingham Heart Study, which found significant, inverse associations between Endo-PAT scores and multiple CV risk factors (69). Two flexible probes were placed on the index fingers of the right (ischemic) and left (control) hands. Measurements were made during baseline (5 min), occlusion (5 min), and reactive hyperemia (5 min). The Reactive Hyperemia Index (RHI) was calculated as the ratio of the average PWA during hyperemia (60 sec to 120 sec of the post-occlusion period) compared with the average PWA during baseline in the occluded hand over the same values in the control hand multiplied by a baseline correction factor. The Framingham RHI (F-RHI) is an alternative calculation derived from the same raw data and differs in that it uses the period 90 sec to 120 sec of post-occlusion
hyperemia, does not incorporate a baseline correction factor, and has a natural log transformation applied to resulting ratio. F-RHI has been shown to correlate with other cardiovascular risk markers (69, 212). The EndoPAT device also generates the augmentation index (AI), a measure of vascular stiffness (pulse wave reflection) that is calculated from the shape of the pulse wave recorded by the probes during baseline. AI can be adjusted to a heart rate of 75 beats per minute to correct for the independent effect of heart rate on this measure (213). Both unadjusted and adjusted AI are reported here.

### 3.3.4.8 Statistical analyses
Statistical analyses were performed using SAS (Statistical Analysis System, Version 9.2, Cary, NC). The natural logarithmic transformation was used for positively skewed outcome variables. Means are reported as least squares means +/- SEM. The mixed models procedure (PROC MIXED) in SAS was used to test the effects of treatment, period, and treatment by period interactions on each outcome. Subject was treated as a random effect and the remaining factors were fixed effects. When period and treatment by period interactions were non-significant, they were removed from the model. For all outcomes, there was no treatment by period effect. When period effects were significant, they were retained in the final model of treatment effects. Tukey-Kramer adjusted p-values were used for post hoc comparisons between the three groups. Values that were measured in duplicate (lipids [separate days], plasma cytokines [assay duplicate], and FMD [2 independent scorers]) were averaged prior to analysis.

Regression modeling was performed in Minitab (version 16.1, State College, PA). Predictor by dose interactions were tested in SAS to assess whether the slope of the regression line for the predictor were equal across both treatments. Regression lines for the two treatment groups were also visually examined for equal slope. When that requirement was met, regressions were reported as pooled values, collapsing across the treatments. Graphical representations were generated in Minitab as scatterplots for outcome vs. predictor with regression line. Residual vs. fit plots were examined to ensure homoscedasticity.

### 3.4 Results
Twenty-eight people began the study and 26 completed it (23 men and 3 post-menopausal women). One male subject withdrew due to gastrointestinal symptoms that began during the placebo period and intensified during the 3.4 g/d period. One male subject died during the washout phase following the placebo dose, and this event was judged to be unrelated to study procedures. The final study population was, on average, middle-aged, overweight, and normotensive (Table 3-2). The sample was predominantly white, non-Hispanic and contained one subject of Indian descent. Compliance was excellent (>95% for all subjects during all periods) as determined by capsule logs and bottle weights. Erythrocyte EPA and DHA also increased in a dose-dependent manner in all subjects (discussed further below). Body weight did not change during the study (data not shown).
Table 3-2: Baseline characteristics of subjects who completed the study (n = 26)  

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (n = 23 M, 3F)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>44.3 ± 9.8</td>
<td>22-65</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>92.8 ± 18.2</td>
<td>59.7-137.7</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>29.0 ± 3.6</td>
<td>23.7-36.5</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>122.9 ± 9.5</td>
<td>98-139</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>81.9 ± 7.8</td>
<td>65-96</td>
</tr>
<tr>
<td><strong>Lipids and Lipoproteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>222.8 ± 56.3</td>
<td>140.5-339</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>206.2 ± 42.3</td>
<td>133-269</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>121.2 ± 38.1</td>
<td>57-188.5</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>40.4 ± 8.1</td>
<td>26-57</td>
</tr>
<tr>
<td><strong>Glucose Metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>103.5 ± 15.7</td>
<td>85-163</td>
</tr>
<tr>
<td>Insulin (µIU/mL)</td>
<td>18.0 ± 9.0</td>
<td>5-40</td>
</tr>
<tr>
<td><strong>Markers of Inflammation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hs-CRP (mg/L)</td>
<td>1.26 ± 0.83</td>
<td>&lt; 0.2 – 2.8</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>0.14 ± 0.09</td>
<td>0-0.34</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>0.77 ± 0.53</td>
<td>0.15-2.48</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>1.22 ± 0.30</td>
<td>0.55-1.71</td>
</tr>
<tr>
<td><strong>Liver Enzymes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST</td>
<td>21.5 ± 5.9</td>
<td>12-37</td>
</tr>
<tr>
<td>ALT</td>
<td>28.8 ± 12.2</td>
<td>12-56</td>
</tr>
<tr>
<td><strong>Erythrocyte Fatty Acid Content</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eicosapentaenoic acid (EPA)</td>
<td>0.49 ± 0.21</td>
<td>0.21-0.95</td>
</tr>
<tr>
<td>Docosapentaenoic acid (DPA)</td>
<td>2.68 ± 0.36</td>
<td>2.00-3.54</td>
</tr>
<tr>
<td>Docosahexaenoic acid (DHA)</td>
<td>3.97 ± 0.96</td>
<td>2.32-6.65</td>
</tr>
<tr>
<td>Omega-3 Index</td>
<td>4.46 ± 1.13</td>
<td>2.63-7.45</td>
</tr>
</tbody>
</table>

1Values are expressed as mean ± standard deviation. Values were obtained using the UNIVARIATE procedure (SAS, Version 9.2, Cary, NC). Lipids and lipoproteins are the average of two samples taken on 2 separate days. BMI = body mass index, SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure, TG = triglyceride, TC = total cholesterol, LDL-C = low density lipoprotein cholesterol, HDL-C = high density lipoprotein cholesterol, hs-CRP = high sensitivity C reactive protein, IL= interleukin, TNF = tumor necrosis factor, AST = aspartate transferase, ALT = alanine transferase.

3.4.1 Effects of lower and higher EPA + DHA doses on blood-derived measures
Compared to placebo and the low dose, triglycerides were significantly lower following the 3.4 g/d dose of EPA+DHA (Table 3-3). Relative to placebo, the reduction on the 3.4 g/d dose was 27% (p = 0.002). The 0.85 g/d dose did not alter triglyceride values. Total cholesterol, LDL-C, and HDL-L values did not differ by treatment.
<table>
<thead>
<tr>
<th>Lipids and Lipoproteins</th>
<th>0 g/d</th>
<th>0.85 g/d</th>
<th>3.4 g/d</th>
<th>p-value for treatment effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG (mg/dL)</td>
<td>237.3 ± 17.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>215.3 ± 17.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>173.7 ± 17.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.002</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>209.0 ± 7.9</td>
<td>212.1 ± 7.9</td>
<td>207.9 ± 7.9</td>
<td>0.60</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>123.3 ± 7.6</td>
<td>127.6 ± 7.6</td>
<td>130.3 ± 7.6</td>
<td>0.21</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>42.6 ± 1.9</td>
<td>42.7 ± 1.9</td>
<td>43.2 ± 1.9</td>
<td>0.76</td>
</tr>
<tr>
<td>nonHDL-C</td>
<td>166.4 ± 7.1</td>
<td>169.4 ± 7.1</td>
<td>164.7 ± 7.1</td>
<td>0.54</td>
</tr>
<tr>
<td>nonHDL-C:HDL-C</td>
<td>4.03 ± 0.2</td>
<td>4.04 ± 0.2</td>
<td>3.95 ± 0.2</td>
<td>0.74</td>
</tr>
<tr>
<td>LDL-C:HDL-C</td>
<td>2.94 ± 0.2</td>
<td>3.00 ± 0.2</td>
<td>3.11 ± 0.2</td>
<td>0.15</td>
</tr>
<tr>
<td>TC:HDL-C</td>
<td>5.03 ± 0.2</td>
<td>5.04 ± 0.2</td>
<td>4.95 ± 0.2</td>
<td>0.74</td>
</tr>
<tr>
<td>Glucose Metabolism</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>96.1 ± 2.0</td>
<td>98.0 ± 1.9</td>
<td>99.2 ± 1.9</td>
<td>0.14</td>
</tr>
<tr>
<td>Insulin (μIU/mL)</td>
<td>14.6 ± 1.4</td>
<td>15.5 ± 1.4</td>
<td>15.0 ± 1.4</td>
<td>0.31</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.55 ± 0.4</td>
<td>3.75 ± 0.4</td>
<td>3.64 ± 0.4</td>
<td>0.46</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.14 ± 0.002</td>
<td>0.14 ± 0.002</td>
<td>0.14 ± 0.002</td>
<td>0.36</td>
</tr>
<tr>
<td>Markers of Inflammation (plasma protein concentrations)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hs-CRP (mg/L)</td>
<td>1.45 ± 0.2</td>
<td>1.32 ± 0.2</td>
<td>1.29 ± 0.2</td>
<td>0.72</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>0.15 ± 0.02</td>
<td>0.15 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>0.89</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>0.87 ± 0.15</td>
<td>0.85 ± 0.15</td>
<td>0.87 ± 0.15</td>
<td>0.89</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>1.16 ± 0.07</td>
<td>1.07 ± 0.07</td>
<td>1.10 ± 0.07</td>
<td>0.20</td>
</tr>
<tr>
<td>Markers of Inflammation (normalized gene expression in isolated PBMCs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β expression</td>
<td>1.10 ± 0.09</td>
<td>1.06 ± 0.09</td>
<td>1.08 ± 0.09</td>
<td>0.92</td>
</tr>
<tr>
<td>IL-6 expression</td>
<td>0.73 ± 0.16</td>
<td>0.73 ± 0.16</td>
<td>0.69 ± 0.16</td>
<td>0.70</td>
</tr>
<tr>
<td>TNF-α expression</td>
<td>1.00 ± 0.07</td>
<td>0.94 ± 0.07</td>
<td>0.97 ± 0.07</td>
<td>0.82</td>
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<tr>
<td>Liver Enzymes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST</td>
<td>20.3 ± 1.1</td>
<td>21.1 ± 1.1</td>
<td>21.9 ± 1.1</td>
<td>0.18</td>
</tr>
<tr>
<td>ALT</td>
<td>27.4 ± 3.1</td>
<td>30.4 ± 3.1</td>
<td>32.4 ± 3.1</td>
<td>0.11</td>
</tr>
<tr>
<td>Erythrocyte Omega-3 Fatty Acid Content</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPA</td>
<td>0.57 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.15 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.30 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DPA</td>
<td>2.74 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.04 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.40 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DHA</td>
<td>4.39 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.34 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.49 ± 0.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Omega-3 Index</td>
<td>4.96 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.49 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.79 ± 0.21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values are expressed as mean ± SEM. P-values are for the main effect of treatment based on the MIXED procedure (SAS, Version 9.2, Cary, NC). Tukey adjusted p-values < 0.05 for post hoc tests for differences of means are significant for values that do not share the same superscript letter. LDL-C = low density lipoprotein cholesterol, HDL-C = high density lipoprotein cholesterol, TG = triglycerides, TC = total cholesterol, HOMA-IR = homeostatic model of insulin resistance, QUICKI = quantitative insulin-sensitivity check index, hs-CRP = high sensitivity C reactive protein, IL = interleukin, TNF = tumor necrosis factor, AST = aspartate transferase, ALT = alanine transferase, EPA = eicosapentaenoic acid, DPA = docosapentaenoic acid (n-3), DHA = docosahexaenoic acid.
Neither dose of omega-3 fatty acids had effects on glucose, insulin, inflammatory markers, or inflammatory gene expression in isolated PBMC relative to placebo (Table 3). Endpoint values for calculated measures of glucose metabolism did not differ by treatment. Liver enzymes (ALT and AST) and body weight (or BMI) were also unchanged.

3.4.2 Erythrocyte omega-3 fatty acids
The omega-3 fatty acids EPA, docosapentaenoic acid (DPA), and DHA and increased in a dose dependent manner (Table 3-3, Figure 3.2). The increase in EPA and DHA levels resulted in a significant increase in omega-3 index of 32% for the 0.85 g/d dose and 79% for the 3.4 g/d dose. This effect was achieved via modest displacement of monounsaturated and omega-6 polyunsaturated fatty acids (see Supplementary Tables at end of chapter for full fatty acid analysis).

![Figure 3.2: Changes in erythrocyte long chain omega-3 fatty acids and omega-3 index.](image)

Values are expressed as mean ± SEM. P-values are for the main effect of treatment based on the MIXED procedure (SAS, Version 9.2, Cary, NC). Tukey adjusted p-values < 0.05 for post hoc pairwise comparisons are noted by different letters. Source: Skulas-Ray AC et al. Dose-response effects of omega-3 fatty acids on triglycerides, inflammation, and endothelial function in healthy persons with moderate hypertriglyceridemia. Am J Clin Nutr 2010.

3.4.3 Endothelial function and reactive hyperemia indices
Endothelial function, as measured by FMD, RHI or F-RHI, was not affected by either dose of omega-3 fatty acids compared to placebo (Table 3-4). There were no effects on arterial stiffness (AI or AI adjusted for heart rate) or on hyperemic flow measured by Doppler ultrasound. Like FMD, the EndoPAT reactive hyperemia scores measure nitric-oxide dependent dilation (214) but may be more indicative of microvascular changes (212) than FMD, which measures changes in brachial artery diameter. No measures of vascular endothelial function or measures of artery stiffness were affected by treatment.
Table 3-4: Effects of treatment on measures of endothelial function (n = 26)\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>0 g/d</th>
<th>0.85 g/d</th>
<th>3.4 g/d</th>
<th>p-value for treatment effect</th>
<th>p-value for period effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reactive hyperemia outcomes from flow mediated dilation (FMD)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FMD (% change in artery diameter)</td>
<td>5.00 ± 0.5</td>
<td>4.03 ± 0.5</td>
<td>4.14 ± 0.5</td>
<td>0.11</td>
<td>0.73</td>
</tr>
<tr>
<td>Δ artery diameter (mm, peak-base)</td>
<td>0.24 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>0.07</td>
<td>0.80</td>
</tr>
<tr>
<td>Peak flow:resting flow(^3)</td>
<td>6.28 ± 0.44</td>
<td>6.59 ± 0.45</td>
<td>6.84 ± 0.44</td>
<td>0.55</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Reactive hyperemia indices from EndoPAT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reactive Hyperemia Index (RHI)(^3)</td>
<td>1.84 ± 0.10</td>
<td>1.82 ± 0.10</td>
<td>1.86 ± 0.10</td>
<td>0.86</td>
<td>0.02</td>
</tr>
<tr>
<td>Framingham RHI</td>
<td>0.28 ± 0.06</td>
<td>0.27 ± 0.07</td>
<td>0.33 ± 0.07</td>
<td>0.66</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Pulse wave properties and heart rate (HR) from EndoPAT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Augmentation Index (AI)</td>
<td>-9.33 ± 1.6</td>
<td>-9.52 ± 1.6</td>
<td>-9.25 ± 1.6</td>
<td>0.97</td>
<td>0.56</td>
</tr>
<tr>
<td>Al standardized for HR of 75 bpm</td>
<td>-16.9 ± 1.6</td>
<td>-17.5 ± 1.5</td>
<td>-18.1 ± 1.5</td>
<td>0.53</td>
<td>0.91</td>
</tr>
<tr>
<td>Heart rate (bpm)(^3)</td>
<td>62.9 ± 1.6</td>
<td>62.7 ± 1.6</td>
<td>61.0 ± 1.6</td>
<td>0.09</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Resting artery diameter and blood flow values (Doppler Ultrasound)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Artery diameter (mm)(^4)</td>
<td>4.83 ± 0.13</td>
<td>4.92 ± 0.13</td>
<td>4.87 ± 0.13</td>
<td>0.09</td>
<td>0.005</td>
</tr>
<tr>
<td>Velocity time integral (m)(^4)</td>
<td>0.17 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.88</td>
<td>0.003</td>
</tr>
<tr>
<td>Maximum velocity (m/s)</td>
<td>0.98 ± 0.06</td>
<td>0.95 ± 0.06</td>
<td>1.00 ± 0.06</td>
<td>0.49</td>
<td>0.10</td>
</tr>
<tr>
<td>Average flow velocity (m/s)(^4)</td>
<td>0.19 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>0.20 ± 0.02</td>
<td>0.84</td>
<td>0.01</td>
</tr>
<tr>
<td>Flow (mL/min)(^4)</td>
<td>201 ± 17.5</td>
<td>207 ± 17.7</td>
<td>206 ± 17.5</td>
<td>0.94</td>
<td>0.0007</td>
</tr>
<tr>
<td><strong>Post occlusion artery diameter and blood flow values (Doppler Ultrasound)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Artery diameter (mm)(^4)</td>
<td>5.07 ± 0.13</td>
<td>5.10 ± 0.13</td>
<td>5.06 ± 0.13</td>
<td>0.55</td>
<td>0.01</td>
</tr>
<tr>
<td>Velocity time integral (m)</td>
<td>0.97 ± 0.05</td>
<td>0.99 ± 0.05</td>
<td>1.02 ± 0.05</td>
<td>0.45</td>
<td>0.43</td>
</tr>
<tr>
<td>Maximum velocity (m/s)</td>
<td>1.76 ± 0.07</td>
<td>1.76 ± 0.07</td>
<td>1.78 ± 0.07</td>
<td>0.83</td>
<td>0.22</td>
</tr>
<tr>
<td>Average flow velocity (m/s)</td>
<td>1.02 ± 0.04</td>
<td>1.01 ± 0.04</td>
<td>1.02 ± 0.04</td>
<td>0.83</td>
<td>0.57</td>
</tr>
<tr>
<td>Flow (mL/min)</td>
<td>1193 ± 77</td>
<td>1219 ± 77</td>
<td>1238 ± 77</td>
<td>0.68</td>
<td>0.47</td>
</tr>
</tbody>
</table>

\(^1\) Values are expressed as mean ± SEM. P-values are for the main effect of treatment and period based on the MIXED procedure with both fixed effects in the model when period effects were significant (SAS, Version 9.2, Cary, NC). When period was non-significant, it was removed from the model to determine treatment effects. All Tukey adjusted p-values for pairwise comparisons for treatment effects were not significant (p > 0.05). Significance for Tukey adjusted p-values for period effects are noted by the following superscripts: \(^2\)First visit significantly less than third visit (Tukey p = 0.04), \(^3\)First visit values were significantly greater than visits 2 and 3 (Tukey p < 0.005), \(^4\)First visit values significantly less than visits 2 and 3 (Tukey p < 0.05).
3.4.4 Change in vascular function over time
Although there were no significant treatment effects on vascular function or hyperemic response, several of these parameters exhibited small but significant period effects (Table 3-4). Resting and peak brachial artery diameters increased after the first visit by about 2%, and this was accompanied by a reduction in some hyperemic indices. RHI decreased 13% after the first visit. However, there were no period effects on FMD or the absolute change in artery diameter (mm) from pre to post deflation.

3.4.5 Regression modeling

3.4.5.1 Triglyceride values as a predictor of response to 3.4 g/d EPA + DHA
Prior work has demonstrated a relationship between triglyceride values and the percent reduction in triglycerides in response to 3.4 g/d EPA + DHA (reviewed in (200)). Therefore, regression modeling was used to assess whether this relationship could be modeled on an individual basis within our sample. Log-transformed triglyceride values at the end of the placebo period were linearly related to the percent reduction in triglycerides achieved by participants following 3.4 g/d EPA + DHA ($R^2 = 41\%$, $p = 0.001$, Figure 3.3). The Pearson’s $r$ for this correlation is 0.64.

3.4.5.2 Change in triglycerides as a predictor of change in LDL-C and FMD across treatments
Change in triglyceride values relative to placebo treatment was assessed as a predictor of changes in LDL-C and FMD relative to placebo. The slope of the line did not differ by treatment. Combining across the two omega-3 treatments, the change in triglycerides was inversely correlated with change in LDL-C ($p = 0.003$); it predicted 12% of the change in LDL-C relative to placebo. The model was also tested after excluding one of the cholesterol measures that was used to calculate the average LDL-C for the placebo period for one subject (suspected technical error), and the regression R-squared increased to 19%, $p = 0.001$ (Figure 3.3). The data points for this participant had the largest residuals in the original model. The model predicts for this sample that each additional 13 mg/dL reduction in triglycerides was associated with an increase in 1 mg/dL of LDL-C.

Pooling across both doses, reduction in triglyceride values following fish oil treatment was significantly predictive of FMD improvement. Change in triglyceride value predicted about 10% of change in FMD value ($p = 0.04$). Changes in triglycerides did not predict changes in the EndoPAT RHI, F-RHI, and AI (not shown).
Figure 3.3: Scatterplots for selected regression analyses. (Minitab Version 16.1, State College, PA).

A. Percent reduction in triglycerides in response to 3.4 g/d EPA + DHA vs. triglycerides values following placebo treatment. Points (n = 26) are plotted as percent reduction in triglycerides achieved for 3.4 g/d EPA + DHA (relative to placebo) vs. log-transformed values of triglycerides following the placebo period. Triglyceride values at the end of the placebo treatment period significantly predicted the percent reduction achieved following 3.4 g/d (R² = 41%, \%Reduction in TG = -217.7 + 43.91logg(TG) p = 0.001).

B. Correlation between triglyceride reduction and increases in LDL-C. Points (n = 26/treatment group) are plotted as change relative to placebo for triglycerides and LDL-C, and the regression was modeled as change in LDL-C predicted by change in TG across both the 0.85 g/d and 3.4 g/d groups. Change in triglycerides significantly predicted change in LDL-C (R² = 19%, \DeltaLDL-C = 4.47 - 0.0784 \DeltaTG, p = 0.001). The slope of the regression did not differ significantly by treatment. One outlier data point was removed from the calculation of average placebo LDL-C for one subject in the final model (suspected technical error). TG = Triglycerides, LDL-C = Low density lipoprotein cholesterol. Source: Skulas-Ray AC et al. Dose-response effects of omega-3 fatty acids on triglycerides, inflammation, and endothelial function in healthy persons with moderate hypertriglyceridemia. Am J Clin Nutr 2010.

3.4.5.3 Other regression models examined

Changes in LDL-C, HDL-C, hs-CRP, and omega-3 index were not significant, independent predictors of the change in FMD (data not shown), and changes in the omega-3 index were not predictive of changes in inflammatory status in this sample (not shown). However, there was
very little range in within subject inflammatory changes in this study, which limits regression modeling.

3.5 Discussion
The results of this study corroborate the effectiveness of pharmacological doses of omega-3 fatty acids in reducing triglyceride concentrations in people with moderate hypertriglyceridemia. Relative to the placebo treatment, fasting triglycerides were reduced by 27% following the 3.4 g/d dose, consistent with previous reports (36, 37, 39, 40, 43, 200). The percent reduction achieved was related to subjects’ triglycerides following the placebo dose so that on average, the subjects with the highest triglycerides achieved the greatest percent reduction following the higher dose. The lower dose (0.85 g/d) did not alter lipid values, and fasting measures of glucose metabolism were not altered by either dose relative to placebo.

As expected, the omega-3 fatty acid composition of erythrocytes increased in a dose-responsive manner, confirming compliance and uptake of omega-3 fatty acids into cellular membranes. Following 8 wk supplementation, the 3.4 g/d dose achieved an average omega-3 index associated with reduced cardiovascular risk (> 8%)(205). Since we used 8 wk supplementation, these values do not reflect maximum omega-3 uptake into RBC fatty acids (215). The time between endpoint measures was at least 14 wk (8 wk treatment + 6 wk washout) to minimize potential carryover between doses.

Despite the dose-response uptake of omega-3 fatty acids into membranes, we did not observe improvements in any measure of inflammation including circulating levels of hs-CRP, inflammatory cytokines, or gene expression of inflammatory cytokines in isolated peripheral blood mononuclear cells. Although there is extensive evidence of anti-inflammatory effects of omega-3 fatty acids in epidemiological (216, 217), animal and in vitro (84, 202) and ex vivo (76, 218) studies, many clinical studies (including this one) have found no effect of supplemental omega-3 fatty acids on circulating markers of inflammation such as hs-CRP (93, 94, 219, 220). Longer treatment duration or alternate routes of administration may be necessary (221). However, most evidence suggests that omega-3 fatty acids suppress provoked inflammatory responses, and the use of a standardized inflammatory challenges for in vivo human research is a promising strategy for future studies (178, 221).

The FMD values that we obtained with lower arm occlusion (~4-5%) indicate relatively impaired endothelial function that is similar to values seen in people with type II diabetes (74, 222); however, endothelial function was not affected by either dose of omega-3 fatty acids. Our findings agree with those of Stirban et al. (74) that found no change in fasting FMD following 1.7 g/d EPA + DHA for 6 wk. However, in that study treatment with omega-3 fatty acids attenuated the postprandial decrease in endothelial function following a high fat meal (74), suggesting that the effects of omega-3 supplementation on FMD may be more evident during an oxidative, inflammatory, and/or lipemic challenge.
Earlier work reported improved fasting FMD when subjects with hypercholesterolemia were treated with 3.4 g/d EPA + DHA for 4 months (70). However, in this paper, the vasodilatory response was reported as mm change whereas the FMD% calculated from these data are unexpectedly low relative to typical values (1-3%) (69, 223). In adults with lupus (93) and peripheral arterial disease (94) FMD improved after 12-24 weeks of 2-3.4 g/d EPA + DHA despite no effect on traditional markers on inflammation (93, 94). Other studies have shown no vascular effects of 4 g/d EPA + DHA given for 6-8 weeks (71, 72).

Omega-3 fatty acids had no effect on EndoPAT scores or hyperemic responses. These findings agree with a recent report that RHI and F-RHI were not changed following 1.2 g/d EPA+DHA for 12 wk in obese adolescents (224). Since we found no effect on any vascular endpoint with omega-3 fatty acid treatment, we conclude that neither dose affected fasting endothelium dependent vasodilation, whether microvascular or macrovascular, after 8 wk.

In our study, reductions in fasting triglycerides following fish oil treatment (relative to placebo) were associated with both increases in LDL-C ($R^2 = 19\%$, $p=0.001$) and to a lesser extent improvements in FMD ($R^2 = 10\%$, $p=0.04$). The association between reductions in triglycerides and increases in LDL-C corresponds to Pearson’s correlation $r=0.44$ and is consistent with other reports (34, 200). Changes in LDL-C across the two treatments were not predictive of changes in FMD, suggesting that any increases in LDL-C within subjects did not nullify potential vascular benefits resulting from triglyceride reductions. Many exploratory regression models for predicting change in endothelial function were tested, so the finding that triglyceride reductions predict improvements in FMD is only hypothesis generating.

We were surprised to find significant period effects for hyperemic outcomes, heart rate, and artery diameter, indicating that response to the hyperemic stimulus changed in a systematic way with repeated exposure. This underscores the importance of placebo control in vascular studies and suggests that participants should be exposed to the testing conditions prior to the collection of endpoint measurements.

3.5.1 Study strengths

Our study population is unique in that our participants were recruited specifically based on the criterion of moderately elevated triglycerides. They were otherwise healthy, non-smoking, and were not taking drugs for hypertension, elevated cholesterol, or inflammation, and they were also not taking supplements. Although this sample is not typical of the population of people with moderate hypertriglyceridemia, these stringent inclusion/exclusion criteria may reduce potential variability in treatment effects. FMD values, which are highly dependent on analytical precision, were obtained following established guidelines (66). All scans were collected by one sonographer with expertise in vascular ultrasound. Our crossover design allowed us to compare the effects of two clinically important doses within the same participants.
3.5.2 Study limitations
The predominantly white male sample, treatment duration, absence of pre-treatment endothelial function testing, and sample size (although typical of endothelial function studies) are potential limitations. While triglyceride effects are known to occur very quickly (< 2 wk), it may be that > 8 wk is required to observe improvements in inflammation and endothelial function as measured by FMD and EndoPAT (93, 94). Our design required 9 months of participation from subjects (including washout periods), and in spite of this, our retention rate was high (only 1 voluntary drop out).

3.5.3 Conclusions
We found that 3.4 g/d EPA + DHA reduced fasting triglycerides by 27% in people with moderately elevated triglycerides, and this response was proportional to degree of elevation in regression modeling. In contrast, there were no effects of 0.85 g/d or 3.4 g/d EPA + DHA on endothelial function, insulin/glucose, or inflammation relative to placebo. In regression models, reductions in triglycerides predicted increases in LDL-C and FMD. Future studies should continue to examine whether omega-3 fatty acids exhibit dose-response effects on adaptive responses to postprandial and inflammatory challenges.

3.6 Supplementary Tables
Table 3-5: Baseline erythrocyte fatty acid profile1 (n=26)

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Common Name</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated Fatty Acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>Myristic</td>
<td>0.35 ± 0.10</td>
<td>0.23 – 0.67</td>
</tr>
<tr>
<td>C16:0</td>
<td>Palmitic</td>
<td>21.9 ± 1.0</td>
<td>19.9 – 23.8</td>
</tr>
<tr>
<td>C18:0</td>
<td>Stearic</td>
<td>18.3 ± 0.9</td>
<td>16.2 – 19.7</td>
</tr>
<tr>
<td>C24:0</td>
<td>Lignoceric</td>
<td>0.41 ± 0.17</td>
<td>0.20 – 0.87</td>
</tr>
<tr>
<td>Monounsaturated Fatty Acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1n7</td>
<td>Palmitoleic</td>
<td>0.26 ± 0.10</td>
<td>0.12 – 0.49</td>
</tr>
<tr>
<td>C18:1n9</td>
<td>Oleic</td>
<td>14.1 ± 1.0</td>
<td>12.2 – 15.9</td>
</tr>
<tr>
<td>C20:1n9</td>
<td>Eicosaenoic</td>
<td>0.22 ± 0.05</td>
<td>0.16 – 0.39</td>
</tr>
<tr>
<td>C24:1n9</td>
<td>Nervonic</td>
<td>0.38 ± 0.18</td>
<td>0.17 – 0.84</td>
</tr>
<tr>
<td>Trans Fatty Acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1n7t</td>
<td>Trans -7-hexa-deanoic</td>
<td>0.16 ± 0.04</td>
<td>0.10 – 0.23</td>
</tr>
<tr>
<td>C18:1t</td>
<td>Elaidic</td>
<td>1.4 ± 0.5</td>
<td>0.78 – 3.2</td>
</tr>
<tr>
<td>C18:2n6tt</td>
<td>Trans-linoleic</td>
<td>0.06 ± 0.02</td>
<td>0.03 – 0.11</td>
</tr>
<tr>
<td>C18:2n6ct</td>
<td>Trans-linoleic</td>
<td>0.08 ± 0.04</td>
<td>0.04 – 0.24</td>
</tr>
<tr>
<td>C18:2n6tc</td>
<td>Trans-linoleic</td>
<td>0.11 ± 0.02</td>
<td>0.06 – 0.16</td>
</tr>
<tr>
<td>n-6 Polyunsaturated Fatty Acids</td>
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<td></td>
</tr>
<tr>
<td>C18:2n6</td>
<td>Linoleic</td>
<td>12.4 ± 1.6</td>
<td>9.0 – 16.0</td>
</tr>
<tr>
<td>C18:3n6</td>
<td>Gamma-Linolenic</td>
<td>0.15 ± 0.05</td>
<td>0.07 – 0.26</td>
</tr>
<tr>
<td>C20:2n6</td>
<td>Eicosadienoic</td>
<td>0.31 ± 0.06</td>
<td>0.17 – 0.41</td>
</tr>
<tr>
<td>C20:3n6</td>
<td>Dihomo-y-linolenic</td>
<td>1.87 ± 0.41</td>
<td>1.1 – 2.6</td>
</tr>
<tr>
<td>C20:4n6</td>
<td>Arachidonic</td>
<td>15.9 ± 1.23</td>
<td>13.7 – 19.0</td>
</tr>
<tr>
<td>C22:4n6</td>
<td>Adrenic</td>
<td>3.61 ± 0.68</td>
<td>2.2 – 5.0</td>
</tr>
<tr>
<td>C22:5n6</td>
<td>Docosapentaenoic</td>
<td>0.66 ± 0.15</td>
<td>0.44 – 1.0</td>
</tr>
<tr>
<td>n-3 Polyunsaturated Fatty Acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty Acid</td>
<td>Common Name</td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------------</td>
<td>-----------</td>
<td>--------</td>
</tr>
<tr>
<td>C18:3n3</td>
<td>α-linolenic</td>
<td>0.17 ± 0.04</td>
<td>0.10 – 0.26</td>
</tr>
<tr>
<td>C20:5n3</td>
<td>Eicosapentaenoic (EPA)</td>
<td>0.49 ± 0.21</td>
<td>0.21 - 0.95</td>
</tr>
<tr>
<td>C22:5n3</td>
<td>Docosapentaenoic</td>
<td>2.68 ± 0.36</td>
<td>2.00 – 3.54</td>
</tr>
<tr>
<td>C22:6n3</td>
<td>Docosahexaenoic (DHA)</td>
<td>3.97 ± 0.96</td>
<td>2.32 – 6.65</td>
</tr>
<tr>
<td>EPA + DHA</td>
<td>Omega 3 Index</td>
<td>4.46 ± 1.13</td>
<td>2.63 – 7.45</td>
</tr>
</tbody>
</table>

1Values are expressed as mean ± SD. Values are derived from the UNIVARIATE procedure (SAS, Version 9.2, Cary, NC).

Table 3-6: Effects of omega-3 fatty acid supplementation on erythrocyte fatty acid profile (n=26)1

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Common Name</th>
<th>0 g/d</th>
<th>0.85 g/d</th>
<th>3.4 g/d</th>
<th>p-value for treatment effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated Fatty Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>Myristic</td>
<td>0.36 ± 0.02</td>
<td>0.39 ± 0.02</td>
<td>0.36 ± 0.02</td>
<td>0.18</td>
</tr>
<tr>
<td>C16:0</td>
<td>Palmitic</td>
<td>22.1 ± 0.2</td>
<td>22.4 ± 0.2</td>
<td>22.5 ± 0.2</td>
<td>0.23</td>
</tr>
<tr>
<td>C18:0</td>
<td>Stearic</td>
<td>18.3 ± 0.2</td>
<td>18.2 ± 0.2</td>
<td>18.5 ± 0.2</td>
<td>0.29</td>
</tr>
<tr>
<td>C24:0</td>
<td>Lignoceric</td>
<td>0.39 ± 0.03</td>
<td>0.37 ± 0.03</td>
<td>0.40 ± 0.03</td>
<td>0.60</td>
</tr>
<tr>
<td><strong>Monounsaturated Fatty Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1n7</td>
<td>Palmitoleic</td>
<td>0.28 ± 0.02</td>
<td>0.27 ± 0.02</td>
<td>0.23 ± 0.02</td>
<td>0.08</td>
</tr>
<tr>
<td>C18:1n9</td>
<td>Oleic</td>
<td>14.4 ± 0.2^a</td>
<td>14.0 ± 0.2</td>
<td>13.7 ± 0.2^b</td>
<td>0.003</td>
</tr>
<tr>
<td>C20:1n9</td>
<td>Eicosaenoic</td>
<td>0.20 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>C24:1n9</td>
<td>Nervonic</td>
<td>0.36 ± 0.03</td>
<td>0.32 ± 0.03</td>
<td>0.36 ± 0.03</td>
<td>0.39</td>
</tr>
<tr>
<td><strong>Trans Fatty Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1n7t</td>
<td>Trans -7-hexa-decanoic</td>
<td>0.14 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>0.53</td>
</tr>
<tr>
<td>C18:1t</td>
<td>Elaidic</td>
<td>1.22 ± 0.07</td>
<td>1.21 ± 0.07</td>
<td>1.24 ± 0.07</td>
<td>0.92</td>
</tr>
<tr>
<td>C18:2n6tt</td>
<td>Trans-linoleic</td>
<td>0.05 ± 0.004</td>
<td>0.06 ± 0.004</td>
<td>0.05 ± 0.004</td>
<td>0.34</td>
</tr>
<tr>
<td>C18:2n6ct</td>
<td>Trans-linoleic</td>
<td>0.07 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.25</td>
</tr>
<tr>
<td>C18:2n6tc</td>
<td>Trans-linoleic</td>
<td>0.11 ± 0.004</td>
<td>0.12 ± 0.004</td>
<td>0.11 ± 0.004</td>
<td>0.32</td>
</tr>
<tr>
<td><strong>n-6 Polyunsaturated Fatty Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:2n6</td>
<td>Linoleic</td>
<td>12.9 ± 0.3^a</td>
<td>12.9 ± 0.3^a</td>
<td>11.3 ± 0.3^b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C18:3n6</td>
<td>Gamma-Linolenic</td>
<td>0.16 ± 0.01^a</td>
<td>0.15 ± 0.01</td>
<td>0.12 ± 0.01^b</td>
<td>0.01</td>
</tr>
<tr>
<td>C20:2n6</td>
<td>Eicosadienoic</td>
<td>0.31 ± 0.01</td>
<td>0.31 ± 0.01</td>
<td>0.28 ± 0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>C20:3n6</td>
<td>Dihomo-y-linolenic</td>
<td>1.9 ± 0.07^a</td>
<td>1.8 ± 0.07^a</td>
<td>1.6 ± 0.07^b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C20:4n6</td>
<td>Arachidonic</td>
<td>15.2 ± 0.3^a</td>
<td>14.2 ± 0.3^b</td>
<td>13.6 ± 0.3^b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C22:4n6</td>
<td>Aradenic</td>
<td>3.14 ± 0.1^a</td>
<td>2.75 ± 0.1^b</td>
<td>2.61 ± 0.1^b</td>
<td>0.0003</td>
</tr>
<tr>
<td>C22:5n6</td>
<td>Docosapentaenoic</td>
<td>0.57 ± 0.02^a</td>
<td>0.46 ± 0.02^b</td>
<td>0.40 ± 0.02^c</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>n-3 Polyunsaturated Fatty Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:3n3</td>
<td>α-linolenic</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.16</td>
</tr>
<tr>
<td>C20:5n3</td>
<td>Eicosapentaenoic</td>
<td>0.57 ± 0.09^a</td>
<td>1.15 ± 0.09^b</td>
<td>2.30 ± 0.09^b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C22:5n3</td>
<td>Docosapentaenoic</td>
<td>2.74 ± 0.08^a</td>
<td>3.04 ± 0.08^b</td>
<td>3.40 ± 0.08^c</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C22:6n3</td>
<td>Docosahexaenoic</td>
<td>4.39 ± 0.2^a</td>
<td>5.34 ± 0.2^b</td>
<td>6.49 ± 0.2^c</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>OMX:3</td>
<td>Omega 3 Index</td>
<td>4.96 ± 0.2^a</td>
<td>6.49 ± 0.2^b</td>
<td>8.79 ± 0.2^c</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

1Values are expressed as mean ± SEM. P-values are for the main effect of treatment based on the MIXED procedure (SAS, Version 9.2, Cary, NC). Values sharing different superscript letters were significantly different in post hoc pairwise comparisons (Tukey adjusted p-values < 0.05).
Chapter 4. Marine-derived omega-3 fatty acids elicit dose dependent reductions in heart rate, blood pressure, stroke volume, and contractility during acute psychological stress

4.1 Abstract

Background: Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reduced heart rate and blood pressure in some studies, but effective doses (> 2 g/d) exceed current dietary recommendations. Dose response studies are rare, and little is known about underlying hemodynamic mechanisms.

Objective: The objective of this study was to compare the effects of 0.85 g/d EPA + DHA (low dose) and 3.4 g/d EPA + DHA (high dose, typically used to treat hypertriglyceridemia) on heart rate, blood pressure, and impedance cardiography measures at rest and during standardized stressors (speech task and foot cold pressor) to determine whether effects were dose dependent and/or related to changes in erythrocyte fatty acid composition.

Design: This was a placebo-controlled, double-blind, randomized, 3 period crossover trial (8 wk treatment, 6 wk washout) comparing the low and high dose in 26 adults (23 males, 3 post-menopausal females). The testing sessions employed standardized repeated measures during rest, stressor, and recovery tasks.

Results: Findings were overall treatment effects with the values for each of the 6 tasks as repeated measures that did not significantly differ by task. Heart rate was reduced by 2.4 bpm (low dose) and 4.0 bpm (high dose) (p < 0.003). The high dose significantly reduced mean arterial blood pressure (2 mmHg; SBP/DBP reduction of 2.1/1.7) and stroke volume (5.4 mL) and increased pre-ejection period (6.5 msec) (p < 0.05). A sub-analysis of the resting task alone demonstrated that the high dose also significantly reduced heart rate and blood pressure when only these resting values were analyzed. Reduction in mean arterial pressure was significantly predicted by an increase in erythrocyte omega-3 fatty acids in linear regression modeling ($R^2 = 12\%$, p = 0.02).

Conclusions: Hemodynamic effects of marine omega-3 fatty acids are dose dependent, and changes in mean arterial pressure are related to changes in membrane fatty acid composition. Increasing omega-3 intake can help reduce blood pressure and heart rate at rest and during stress.
4.2 Introduction
Elevated blood pressure and heart rate are common risk factors for heart disease and stroke (204, 225, 226). The omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) appear to reduce risk of cardiovascular death through anti-arrhythmic effects (10, 201). In contrast, their effects on heart rate and blood pressure are less understood since these have rarely been studied as primary outcomes in clinical trials.

Meta-analyses provide evidence that omega-3 fatty acids can reduce heart rate (124) and lower blood pressure (113-115). Reductions in blood pressure are more evident in people with hypertension (40, 117, 118), and may be dependent on dose (113) and changes in plasma phospholipid omega-3 content (116). Testing protocols have ranged from one to three resting measures to 24 h ambulatory monitoring with measurements recorded every 30 min (124). Cardiovascular effects may be more evident under conditions of stress due to beneficial effects of omega-3 fatty acids on autonomic tone, as evidenced by reductions in plasma norepinephrine during stress (106-108, 227) and improved heart rate variability (125, 126).

Despite evidence for the effects of omega-3 fatty acids on blood pressure and heart rate, the notable absence of dose response studies complicates interpretation of existing data. Recommendations for omega-3 intake for the prevention of cardiovascular disease typically do not exceed 1 g/d EPA + DHA (33); yet heart rate and blood pressure reductions have been reported more consistently at higher doses, i.e. 3.4 g/d EPA + DHA (115, 124). This higher dose is a therapeutic (not nutritional) dose indicated for treatment of severe hypertriglyceridemia (fasting triglycerides > 500 mg/dl).

This study compared the effects of 0.85 g/d EPA + DHA (low dose) and 3.4 g/d EPA + DHA (high dose) on hemodynamic measures during a repeated measures laboratory design that included rest, standardized mental stress (speech task), and physical challenge (food cold pressor).

We hypothesized that the high dose would be required to achieve reductions in blood pressure and heart rate. Our secondary aim was to determine whether changes in blood pressure and heart rate were predicted by increases in erythrocyte membrane omega-3 fatty acid content. Exploratory analyses were conducted to characterize other effects on systemic hemodynamics (total peripheral resistance, stroke volume, and contractility indexes) in the context of this standardized laboratory testing. A previous report described the effects of these treatments on lipids, endothelial function and inflammatory markers (228).

4.3 Methods
4.3.1 Subjects and design
Subject characteristics and effects on lipids, inflammation, glucose metabolism, and endothelial function have been described in detail previously (228). Subjects included 23 men and 3 post-menopausal women aged 22-65 (mean 44.3) who met ATP III criteria for elevated triglycerides (7) (triglycerides 150-500 mg/dL) at study entry, but were otherwise healthy. Exclusion criteria
included: tobacco use; history of cardiovascular disease; chronic inflammation; medications for hypertension, dyslipidemia, or inflammation; consumption of 2 or more omega-3 rich foods per week; use of any nutritional supplement (except calcium for postmenopausal women). The study was a randomized, double-blinded, placebo-controlled, 3-period crossover study of 8 weeks duration per treatment with a 6 week washout period between treatments. During each of the three treatment periods, participants took 4 capsules per day providing 0 g/d (placebo), 0.85 g/d (low dose), or 3.4 g/d of EPA + DHA (high dose). The placebo oil was corn oil, and omega-3 fatty acids were provided as prescription omega-3 acid ethyl ester capsules containing 465 mg EPA + 375 mg DHA (Lovaza™, GlaxoSmithKline, Philadelphia, PA). Participants were asked to maintain their low intake (< 2 servings/week) of oily fish and foods high in ALA during the study.

4.3.2 Determination of erythrocyte fatty acids
Fasting blood samples were drawn into EDTA tubes at each visit. RBCs were separated from plasma by centrifugation, and a 0.5 mL aliquot was collected from the RBC layer. RBCs were frozen at −80 °C until analyzed. Briefly, RBCs were directly methylated to form fatty acid methyl esters which were then analyzed by gas chromatography on a GC2010 (Shimadzu Corporation, Columbia, MD) equipped with a 100-m SP-2560 column (Supelco, Bellefonte, PA). The omega-3 index is the sum of EPA and DHA expressed as weight percent of total identified fatty acids (205).

4.3.3 Cardiovascular testing
Cardiovascular responses to stress were assessed during the last week of each 8 week treatment period. The 2 h testing sessions began between 12 noon and 6 pm to avoid changes in stress hormones that occur in the morning. The scheduling of testing was held constant within-subject (within 1 h). Four hours prior to cardiovascular testing, subjects consumed a light (low fat) meal and took half of their daily capsule dose (2 capsules) with this meal. For the low dose treatment, this dose included the active capsule. Subjects were provided verbal and written instructions on how to prepare for the visit. The meal was not provided. They were instructed to avoid pain relievers and alcohol (24 h), caffeine and decongestants (12 h), and exercise on the day of the visit. Compliance with pre-visit instructions was verified prior to data collection. Subjects were rescheduled if they reported symptoms of acute viral or bacterial infection.

Four band electrodes and 3 EKG electrodes were placed according to guidelines for impedance cardiography (66) as described previously (229). Blood pressure was assessed with the Dinamap Pro 100 oscillometric monitor (GE Medical Systems) in the non-dominant arm. Thoracic impedance was measured using a Hutcheson impedance cardiograph (HIC-2000, Instrumentation for Medicine, Greenwich, CT) and analyzed using the Cardiac Output Program (COP v5.08, Bio-impedance Technology, Inc., Chapel Hill, NC) (230). Total peripheral resistance (TPR) was calculated from cardiac output and mean arterial pressure as: Cardiac output (L/min) = stroke volume x heart rate; TPR (dyne * sec * cm⁻²) = mean arterial pressure x cardiac output / 80) (231). Pre-ejection period (duration of isovolumetric contraction) and cardiac contractility
indexes (Heather Index and systolic time ratio) were determined according to standard guidelines (232).

After a baseline rest period (20 min of listening to relaxing music), participants were asked to quietly prepare (2 min) and then deliver (3 min) a speech regarding a hypothetical situation (e.g., being wrongly accused of shoplifting by a security guard, with speech topics changed on subsequent visits). After a 10 min recovery period, participants underwent a cold pressor test in which they immersed one foot up to the ankle in 4° C ice water for 2.5 min. Cardiovascular variables were continuously monitored during a final 10 min recovery period. The last three measurements of the resting and recovery periods were averaged. For the speech preparation, speech delivery, and cold pressor tests, measurements were taken every minute and averaged by task. These six task averages were treated as repeated measures for each testing session.

4.3.4 Statistical analysis
Statistical analyses were performed using SAS (Statistical Analysis System, Version 9.2, Cary, NC). Chi-square analysis was performed using the FREQ procedure. The mixed models procedure in SAS was used to test the effects of treatment, period, task (e.g. baseline, speech, etc), treatment by period, and treatment by task interactions. The natural logarithmic transformation was applied to positively skewed outcome variables. Means are reported as least squares means +/- SEM. Subject was treated as a random effect and the remaining factors were fixed effects. Treatment by period effects were not statistically significant for any outcome and were removed from final models. When period effects were significant, they were retained in the model. Tukey-Kramer adjusted p-values (< 0.05) were used to determine significance for between-treatment differences. A pre-specified sub-analysis was used to additionally evaluate the effect of treatment on the resting task alone.

Regression modeling and scatterplots were performed in Minitab (version 16.1.0, State College, PA). Outcomes with repeated measures were analyzed as an arithmetic mean (average across all tasks). Change scores were calculated as the end of treatment value minus end of placebo value. Residuals and residual vs. fit plots were examined to ensure homoscedascity. Regression slopes were judged to be equal (by visual inspection and the absence of a significant treatment x predictor interaction). Therefore, regressions are reported on pooled values (collapsed across the treatments).

4.4 Results

4.4.1 Erythrocyte fatty acid composition
As reported previously (228), erythrocyte EPA + DHA content (the omega-3 index) changed in a dose dependent manner. At the end of the placebo treatment period, no subjects met the criterion for an optimal omega 3 index score (> 8% of total fatty acids (233)). On the high dose, 77% achieved an omega-3 index > 8% (compared to 1 subject on the low dose, \( \chi^2 = 43.3, p = 0.0001 \)).
4.4.2 Effects of stressor tasks on heart rate and blood pressure

All stressor tasks (preparation, speech, and cold pressor) significantly increased blood pressure and heart rate vs. the resting baseline ($p < 0.0001$, Figure 4.1). The speech task increased MAP by 16 mmHg ($\Delta$SBP/DBP = 23/13 mmHg) and heart rate by 12 bpm. The cold pressor task achieved similar increases in MAP but lower increases in heart rate. Heart rate returned to baseline during recovery periods, while blood pressure remained slightly elevated relative to baseline.

![Figure 4.1: Standardized laboratory stressors significantly increased heart rate and blood pressure. Main effects of task are graphed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC). There was a significant main effect of task for both heart rate and blood pressure ($p < 0.0001$). Tukey adjusted p-values were used to compare task means. Tasks that are annotated with different letters were significantly different from each other ($p < 0.05$).](https://example.com/image)

4.4.3 Effects of low and high doses of omega-3 fatty acids on heart rate

Heart rate was significantly different by treatment (main effect $p < 0.0001$), but the statistical test for a treatment by task interaction was not significant. Therefore, the effect of treatment is considered an overall effect, with the 6 tasks as repeated measures, and the treatment effect size does not differ significantly by task.

Treatment with omega-3 fatty acids significantly and dose-dependently reduced heart rate (main effect of treatment, $p < 0.0001$, Figure 4.2). Relative to the placebo testing session, heart rate was reduced by 2.4 bpm following the low dose ($p = 0.003$) and 4.0 bpm following the high dose ($p < 0.0001$), and the two doses significantly differed from each other ($p = 0.02$).
Figure 4.2: Omega-3 fatty acids significantly reduced heart rate.

A. Across the testing session, there was a significant dose-response effect of treatment. Assessing heart rate as a repeated measure and pooling across tasks yielded a significant main effect of treatment (p < 0.0001). The three treatment groups differed significantly from each other in post hoc comparisons (Tukey p < 0.007).

*p < 0.05 for different relative to placebo.

**p < 0.05 for different relative to placebo and lower dose.

B. Treatment by task values are graphed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC) with treatment, task, and their interaction as fixed effects. There was no significance of the treatment by task interaction term; therefore, the effect of treatment represented in (A) was not significantly different by task.

*p = 0.004 for comparison of placebo to high dose in a sub-analysis of treatment effects on resting baseline.

As a pre-specified sub-analysis, resting heart rate was also evaluated separately in a model that did not include the other tasks. Resting heart rate following the high dose was 4 bpm lower than resting heart rate after placebo (p = 0.004, Figure 4.2).

4.4.4 Effects of omega-3 fatty acids on blood pressure

Mean arterial blood pressure was also significantly different by treatment (main effect p = 0.02), without a significant treatment by task interaction. As for heart rate, the effect of treatment is interpreted across the tasks as repeated measures. Treatment with the high dose reduced mean arterial blood pressure by 2 mmHg (ΔSBP/DBP of 2.1/1.7 mmHg) relative to the placebo and low dose (Tukey p < 0.02, Table 4-1). The low dose did not reduce blood pressure.
Table 4-1: Effects of omega-3 fatty acid supplementation on blood pressure at rest and during stress (n=26).\(^1\)

<table>
<thead>
<tr>
<th>Task</th>
<th>DBP (mmHg)</th>
<th>SBP (mmHg)</th>
<th>P-value for main effect of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 g/d Placebo</td>
<td>0.85 g/d Low Dose</td>
<td>3.4 g/d High Dose</td>
</tr>
<tr>
<td>Resting(^2)</td>
<td>71.9 ± 1.8</td>
<td>72.2 ± 1.8</td>
<td>70.2 ± 1.8</td>
</tr>
<tr>
<td>Preparation</td>
<td>78.4 ± 1.8</td>
<td>79.0 ± 1.8</td>
<td>77.3 ± 1.8</td>
</tr>
<tr>
<td>Speech</td>
<td>83.7 ± 1.8</td>
<td>85.2 ± 1.8</td>
<td>83.9 ± 1.8</td>
</tr>
<tr>
<td>Recovery</td>
<td>74.1 ± 1.8</td>
<td>72.9 ± 1.8</td>
<td>72.3 ± 1.8</td>
</tr>
<tr>
<td>Cold Pressor</td>
<td>84.3 ± 1.8</td>
<td>83.0 ± 1.8</td>
<td>81.7 ± 1.8</td>
</tr>
<tr>
<td>Cold Recovery</td>
<td>74.4 ± 1.8</td>
<td>73.3 ± 1.8</td>
<td>71.6 ± 1.9</td>
</tr>
<tr>
<td>Overall</td>
<td>77.8 ± 1.6(^a)</td>
<td>77.7 ± 1.6(^b)</td>
<td>76.1 ± 1.6(^b)</td>
</tr>
<tr>
<td>Resting(^3)</td>
<td>118.3 ± 2.7</td>
<td>116.7 ± 2.7</td>
<td>114.6 ± 2.7</td>
</tr>
<tr>
<td>Preparation</td>
<td>132.1 ± 2.7</td>
<td>131.7 ± 2.7</td>
<td>129.8 ± 2.8</td>
</tr>
<tr>
<td>Speech</td>
<td>138.0 ± 2.8</td>
<td>141.7 ± 2.7</td>
<td>139.4 ± 2.8</td>
</tr>
<tr>
<td>Recovery</td>
<td>123.5 ± 2.7</td>
<td>123.1 ± 2.8</td>
<td>119.1 ± 2.7</td>
</tr>
<tr>
<td>Cold Pressor</td>
<td>136.6 ± 2.8</td>
<td>139.9 ± 2.8</td>
<td>134.8 ± 2.8</td>
</tr>
<tr>
<td>Cold Recovery</td>
<td>121.8 ± 2.8</td>
<td>120.6 ± 2.8</td>
<td>120.3 ± 2.8</td>
</tr>
<tr>
<td>Overall</td>
<td>128.4 ± 2.2</td>
<td>129.0 ± 2.2(^a)</td>
<td>126.3 ± 2.2(^b)</td>
</tr>
</tbody>
</table>

\(^1\)Values are expressed as mean ± SEM. Values and statistical results were derived from the MIXED procedure (SAS, Version 9.2, Cary, NC). Values with different superscripts are significantly from each other based on Tukey adjusted p-values (< 0.05) for post hoc pairwise comparisons.

\(^2\)There was a significant main effect of task for DBP and SBP (p < 0.0001).

\(^3\)Resting DBP and SBP values were tested separately from the other tasks for treatment effects with corresponding p-values of 0.05 and 0.08, respectively. Values with different superscripts are significantly from each other based on Tukey adjusted p-values (< 0.05) for post hoc pairwise comparisons.
In the sub-analysis of resting mean arterial blood pressure, the high dose was 2.3-2.5 mmHg lower than placebo and low dose (Tukey p < 0.05, Figure 4.3).

**Figure 4.3: Omega-3 fatty acids and changes in MAP.**

A. Across the testing session, there was a significant dose-response effect of treatment. Assessing MAP as a repeated measure and pooling across tasks yielded a significant main effect of treatment (p < 0.0001). In post-hoc analyses, the high dose was different from placebo and the low dose (Tukey p < 0.05).

* p < 0.05 for different relative to both placebo and low dose.

B. Treatment by task values are graphed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC) with treatment, task, and their interaction as fixed effects. There was no significance of the treatment by task interaction term; therefore, the effect of treatment represented in (A) was not significantly different by task.

* p = 0.02 for comparison of placebo to high dose in a sub-analysis of treatment effects on resting baseline.

### 4.4.5 Impedance cardiography measures

Due to equipment failure⁴, results from impedance cardiography could be interpreted only for the first 13 consecutive participants. There was a significant effect of the tasks on all outcome measures except stroke volume (Table 4-2). Treatment effects were significant for all measures, and the statistical test for treatment by task interaction was not significant for any measure, indicating that treatment effects were consistent across tasks. The high dose lowered myocardial contractility (as indicated by significant decreases in stroke volume and with increases in the pre-ejection period and systolic time ratio, Tukey p < 0.003). Both the low dose and high dose decreased cardiac output and the Heather Index (peak ejection velocity) relative to placebo. There was a shift to increased total peripheral resistance following omega-3 supplementation, but no *post hoc* pair-wise comparison reached significance.

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⁴ An electrical failure in the impedance cardiograph resulted in truncated impedance wave forms that could not be used in analysis. This device failure was not apparent during data collection and was discovered during analysis at the end of the trial.
Table 4-2: Effects of omega-3 fatty acid supplementation on hemodynamic measures during testing sessions (n=13).

<table>
<thead>
<tr>
<th></th>
<th>Task</th>
<th>0 g/d (Placebo)</th>
<th>0.85 g/d (Low Dose)</th>
<th>3.4 g/d (High Dose)</th>
<th>P value for treatment effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stroke Volume (mL)</strong></td>
<td>Resting</td>
<td>86.9 ± 6.6</td>
<td>84.0 ± 6.6</td>
<td>78.1 ± 6.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Preparation</td>
<td>83.8 ± 6.6</td>
<td>85.2 ± 6.6</td>
<td>78.5 ± 6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Speech</td>
<td>86.3 ± 6.6</td>
<td>89.7 ± 6.6</td>
<td>81.7 ± 6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>83.3 ± 6.6</td>
<td>84.2 ± 6.7</td>
<td>79.9 ± 6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cold Pressor</td>
<td>83.4 ± 6.6</td>
<td>85.2 ± 6.6</td>
<td>77.6 ± 6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cold Recovery</td>
<td>82.1 ± 6.6</td>
<td>81.8 ± 6.6</td>
<td>77.6 ± 6.7</td>
<td></td>
</tr>
<tr>
<td><strong>Overall</strong></td>
<td></td>
<td>84.3 ± 6.2a</td>
<td>85.0 ± 6.2a</td>
<td>78.9 ± 6.2a</td>
<td>0.0006</td>
</tr>
<tr>
<td><strong>Cardiac Output (L/min)</strong></td>
<td>Resting</td>
<td>5.47 ± 0.33</td>
<td>5.05 ± 0.33</td>
<td>4.79 ± 0.33</td>
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</tr>
<tr>
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<td>Preparation</td>
<td>6.18 ± 0.33</td>
<td>5.99 ± 0.33</td>
<td>5.64 ± 0.33</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>6.40 ± 0.33</td>
<td>6.07 ± 0.33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
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<td>5.22 ± 0.33</td>
<td>5.10 ± 0.33</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>5.09 ± 0.33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cold Recovery</td>
<td>5.30 ± 0.33</td>
<td>4.96 ± 0.33</td>
<td>4.91 ± 0.33</td>
<td></td>
</tr>
<tr>
<td><strong>Overall</strong></td>
<td></td>
<td>5.76 ± 0.29a</td>
<td>5.50 ± 0.29b</td>
<td>5.26 ± 0.29b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>Resting</td>
<td>1393.6 ± 95.6</td>
<td>1473.6 ± 95.7</td>
<td>1476.5 ± 95.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Preparation</td>
<td>1383.2 ± 95.6</td>
<td>1427.3 ± 95.7</td>
<td>1429.2 ± 97.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Speech</td>
<td>1370.3 ± 95.6</td>
<td>1393.3 ± 95.7</td>
<td>1466.4 ± 97.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>1420.5 ± 95.6</td>
<td>1472.0 ± 96.6</td>
<td>1450.1 ± 96.6</td>
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<tr>
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<td>1484.2 ± 95.6</td>
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<td>Cold Recovery</td>
<td>1463.8 ± 95.6</td>
<td>1547.5 ± 95.7</td>
<td>1467.2 ± 96.6</td>
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<tr>
<td><strong>Overall</strong></td>
<td></td>
<td>1419.2 ± 86.9</td>
<td>1477.3 ± 87.1</td>
<td>1476.0 ± 87.2</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Heather Index (ohm/s²)</strong></td>
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<td>7.46 ± 0.56</td>
<td>7.57 ± 0.56</td>
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<td>8.86 ± 0.56</td>
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<td>8.00 ± 0.57</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Task</td>
<td>0 g/d (Placebo)</td>
<td>0.85 g/d (Low Dose)</td>
<td>3.4 g/d (High Dose)</td>
<td>P value for treatment effect</td>
</tr>
<tr>
<td>---------------</td>
<td>--------</td>
<td>-----------------</td>
<td>--------------------</td>
<td>---------------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td></td>
<td>Speech</td>
<td>8.97 ± 0.56</td>
<td>8.61 ± 0.56</td>
<td>8.29 ± 0.57</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>8.14 ± 0.56</td>
<td>7.66 ± 0.56</td>
<td>7.49 ± 0.56</td>
<td></td>
</tr>
<tr>
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<td>Cold Pressor</td>
<td>7.80 ± 0.56</td>
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<td>7.44 ± 0.56</td>
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<tr>
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<td>7.93 ± 0.56</td>
<td>7.29 ± 0.56</td>
<td>7.47 ± 0.56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overall</td>
<td>8.37 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.83 ± 0.50&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.72 ± 0.50&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
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<td>115.5 ± 5.6</td>
<td>122.4 ± 5.5</td>
<td>129.6 ± 5.5</td>
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</tr>
<tr>
<td></td>
<td>Preparation</td>
<td>109.9 ± 5.6</td>
<td>114.8 ± 5.5</td>
<td>114.3 ± 5.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Speech</td>
<td>114.0 ± 5.6</td>
<td>113.5 ± 5.5</td>
<td>117.2 ± 5.6</td>
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<td>Recovery</td>
<td>119.5 ± 5.6</td>
<td>121.7 ± 5.6</td>
<td>123.7 ± 5.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cold Pressor</td>
<td>116.3 ± 5.6</td>
<td>119.0 ± 5.5</td>
<td>123.0 ± 5.6</td>
<td></td>
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<tr>
<td></td>
<td>Cold Recovery</td>
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<td>123.6 ± 5.5</td>
<td>126.3 ± 5.6</td>
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<tr>
<td></td>
<td>Overall</td>
<td>116.0 ± 5.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>119.1 ± 5.0</td>
<td>122.5 ± 5.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0006</td>
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<table>
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<tr>
<th></th>
<th>Systolic Time Ratio (PEP/LVET)&lt;sup&gt;2&lt;/sup&gt;</th>
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<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Resting</td>
<td>0.40 ± 0.02</td>
<td>0.41 ± 0.02</td>
<td>0.45 ± 0.02</td>
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<td></td>
<td>Preparation</td>
<td>0.40 ± 0.02</td>
<td>0.39 ± 0.02</td>
<td>0.41 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Speech</td>
<td>0.41 ± 0.02</td>
<td>0.39 ± 0.02</td>
<td>0.42 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>0.42 ± 0.02</td>
<td>0.41 ± 0.02</td>
<td>0.43 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cold Pressor</td>
<td>0.40 ± 0.02</td>
<td>0.39 ± 0.02</td>
<td>0.42 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cold Recovery</td>
<td>0.42 ± 0.02</td>
<td>0.41 ± 0.02</td>
<td>0.44 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overall</td>
<td>0.41 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.40 ± 0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.43 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.003</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values are expressed as mean ± SEM. Values and statistical results were derived from the MIXED procedure (SAS, Version 9.2, Cary, NC). Tukey adjusted p-values for post hoc pairwise comparisons are significant (< 0.05) for values that have different superscript letters. Due to equipment failure, data from only the 13 subjects was analyzed. Abbreviations: PEP = pre-ejection period, LVET = left ventricular ejection time.

<sup>2</sup>There was a significant main effect of task (p < 0.05) for cardiac output, total peripheral resistance, Heather Index, pre-ejection period, and systolic time ratio. Task was not a significant predictor of stroke volume.
4.4.6  Erythrocyte fatty acid changes as predictors of cardiovascular changes

Individual changes in the omega-3 index relative to placebo predicted average changes in mean arterial blood pressure during reactivity sessions relative to placebo treatment (Figure 4.4, p = 0.02). The change in omega-3 index accounted for about 12% of the variability in the change in mean arterial blood pressure ($r = 0.35$). For every 1 percentage point increase in omega-3 index, mean arterial blood pressure was reduced by 1 mmHg. The regression equation is $\Delta$mean arterial blood pressure $= 2.03 - 1.07 \Delta$omega-3 index. The slope of the regression line did not differ by treatment.

![Regression analysis of change in omega-3 index as a predictor of change in blood pressure.](image)

**Figure 4.4:** Regression analysis of change in omega-3 index as a predictor of change in blood pressure. Change in the omega-3 index (EPA + DHA of erythrocytes) relative to placebo treatment was used as a predictor of changes in mean arterial pressure during reactivity sessions relative to placebo treatment (Minitab v.16, State College, PA). The slope of the line did not differ by treatment.

4.5  Discussion

Treatment with the high dose of omega-3 fatty acids significantly reduced heart rate and blood pressure in the repeated measures model and in the sub-analysis that included only resting measures. The magnitude of reductions was similar in both models. The low dose reduced heart rate relative to placebo in the repeated measures model but had no effect on blood pressure. Systemic hemodynamics also were altered by treatment with omega-3 fatty acids. The finding that the blood pressure reduction achieved by the high dose was associated with
reductions in both heart rate and stroke volume—and not with reductions in total peripheral resistance—is unexpected relative to reports from exercise physiology studies (234, 235) and should be further investigated. The effects of omega-3 fatty acids may be very different during increased physiologic demands (235, 236). The significant increase in pre-ejection period may indicate that omega-3 fatty acids altered autonomic tone in agreement with earlier studies (106-108, 227).

The magnitude of blood pressure reduction with the high dose agrees with meta-analyses of responses to fish oil intervention (113-115). In 31 placebo-controlled trials on 1356 subjects, it was observed that for every 1 g omega-3 fatty acids, there was a decrease of 0.66/0.35 (SBP/DBP) mmHg (113). For the high dose of 3.4 g/d, this meta-analysis predicts a reduction of 2.2/1.2 mmHg and MAP of 1.5 mmHg, which is very similar to the ΔSBP/DBP of 2.1/1.7 and MAP 1.8 mmHg reduction we observed in the repeated measures model.

The reduction in heart rate that we observed was greater than earlier meta-analysis results would have predicted for our population and design but is consistent with studies that used ambulatory monitoring (124, 237). This may be due in part to combining acute and chronic effects of omega-3 fatty acids or an effect of increased statistical power due to increased measurement number (i.e., decreased variability).

Regression analysis revealed that blood pressure reductions were associated with changes in the omega-3 fatty acid content of erythrocytes. Since this is a relatively stable marker of intake that reflects changes in tissue levels, it could be speculated that blood pressure effects are dependent on changes in tissue composition whereas heart rate effects may be influenced by plasma omega-3 fatty acids and their metabolites (128, 202). A relationship between plasma DHA and heart rate reductions has been reported (238).

The reductions in blood pressure and heart rate we observed have public health implications for reducing risk of coronary events and stroke. It has been estimated that even small blood pressure reductions (around 5 mmHg) may prevent one-third of strokes and one-fifth of coronary events in Western societies (112). Although the 2 mmHg reduction for the high dose is modest, regression modeling suggests that deficient individuals (with an omega-3 index less than 4%) given a high dose could achieve average blood pressure reductions of 5 mmHg if their omega-3 index increases 6-7%. Further, all subjects in this study had blood pressure < 150/95 mmHg, and larger reductions might be achieved in people with Stage II hypertension (> 160/100 mmHg) (122) or with longer term treatment.

Omega-3 fatty acids are appealing as an adjunctive therapy because they can be safely added to multidrug regimens and therapeutic lifestyle changes. The effects of omega-3 fatty acids on blood pressure are additive to weight loss (119), potentiated by sodium restriction (120), and amplify the effects of beta-blockers (121) and diuretics (43). Other than potential issues of cost and tolerability, there are few arguments against recommending omega-3 supplementation (even at higher doses) to patients as part of a treatment plan.
Despite the evidence for health benefits of omega-3 fatty acids, the average American diet lacks rich sources of omega-3 fatty acids, with estimated daily intakes < 200 mg and omega-3 index (red blood cell EPA + DHA) values of 4-5% (216). The increase in the omega-3 index that we observed with the high dose could potentially be achieved with lower doses consumed chronically. Ongoing studies are examining this.

There are some limitations associated with our study. Our sample size was modest (n = 26) and consisted mainly of white males (n = 3 post-menopausal females). Although we were able to analyze blood pressure and heart rate data for all 26 participants, other hemodynamic data was only interpretable for half of these participants due to failure of the impedance cardiograph device. The duration of supplementation was only 8 wk per treatment period which does not allow for a new membrane fatty acid steady state to be established (239). However, by incorporating a 6 wk washout between periods, we achieved an adequate washout needed to compare these two doses (228), and had good subject retention despite the length of the trial (9 months total). Another potential limitation is that cardiovascular testing was only performed at the end of each period and not at study entry; however, mixed modeling adjusted for habituation effects when they occurred.

In summary, we demonstrated that omega-3 fatty acids have dose dependent effects on blood pressure, heart rate, and systemic hemodynamics. Reductions in blood pressure, stoke volume, and cardiac contractility were observed only with the higher dose, and the magnitude of blood pressure reduction was predicted by increases in omega-3 fatty acids in erythrocytes. In contrast, heart rate was reduced by both doses in a dose-response manner and was not predicted by changes in erythrocyte fatty acids. These findings support existing evidence that populations at risk for coronary artery disease with low omega-3 intake would achieve modest, dose-dependent benefits on blood pressure and heart rate by adding oily fish and/or fish oil capsules to their diets.
Chapter 5. A high antioxidant spice blend improves postprandial insulin, triglycerides, and plasma measures of antioxidant activity

5.1 Abstract

**Background:** There is much interest in the potential of dietary antioxidants to attenuate *in vivo* oxidative stress, but very little characterization of the time course of plasma effects is available. Culinary spices have demonstrated potent *in vitro* antioxidant properties.

**Objective:** The objective of this study was to examine whether adding 14 g of a high antioxidant spice blend to a 1200 kcal meal exerted significant postprandial effects on markers of plasma antioxidant status and metabolism.

**Design:** Healthy overweight men (n = 6) consumed a control and spiced meal in a randomized crossover design with one week between testing sessions. Blood was sampled prior to meal and at 30 minute intervals for 3.5 h (total of 8 samples).

**Results:** Mixed linear models demonstrated a significant treatment by time interaction (p < 0.05) for insulin and triglycerides, corresponding with 21% and 31% reductions in postprandial levels with the spiced meal, respectively. Adding spices to the meal significantly increased the ferric reducing antioxidant power (FRAP), such that postprandial increases following the spiced meal were 2-fold greater than the control meal (effect of treatment p = 0.009). The hydrophilic oxygen radical absorbance capacity (ORAC) of plasma was also significantly increased by spices (effect of treatment p = 0.02). There were no changes in glucose, total thiol, lipophilic ORAC, or total ORAC.

**Conclusions:** The incorporation of spices into the diet may help normalize postprandial insulin and triglycerides and enhance antioxidant defenses.
5.2 Introduction

Oxidative stress is an underlying cause of many chronic diseases (240). In addition to direct oxidative modifications of proteins, lipids, and DNA, amplified oxidative stress promotes inflammation (134, 241) and metabolic derangements (133). Thus, the USDA has generated extensive databases of the antioxidant activity of foods—more specifically the oxygen radical absorbance capacity (ORAC) (155, 242). Since antioxidant values derived from in vitro assays do not consider absorption, metabolism, disposition, and excretion, more work is warranted to directly examine the in vivo bioactivity of antioxidant-rich foods.

Based on the USDA ORAC database, spices have among the highest ORAC values on a per gram basis (155) and are potent sources of phenolic antioxidants (243). Most high antioxidant spices also have demonstrated potential benefits on glucose, lipid, and inflammatory homeostasis (174, 244, 245), which adds to their appeal as a source of dietary antioxidants. If the increased intake of spice antioxidants translates to in vivo effects, this would have great implications for nutrition. In fact, recent research has demonstrated that spices can attenuate the production of lipid peroxidation products in vitro and in vivo when they are added to ground beef (246), supporting the idea that in vitro effects translate to demonstrable benefits in humans.

Studies examining the acute in vivo plasma antioxidant effects of foods have been performed with tea (247), fruit/fruit juices (176, 248, 249), and nuts (250). Results are limited by lack of time course information and absence of control conditions in some cases. However, these studies have demonstrated that much of the antioxidant potential of plant foods is derived from phenolics rather than micronutrient vitamins and that combining these compounds results in synergistic increases in plasma antioxidant status (143, 144). Spices typically are consumed as blends, and some spice compounds increase the plasma concentrations of others (164), making spice blends well-suited for plasma antioxidant studies.

Therefore, the purpose of this study was to examine the postprandial plasma changes caused by consuming a single large dose (14 g) of a high antioxidant spice blend incorporated into a standardized test meal relative to that same meal without spices. Our aims were to characterize the time course of plasma changes on multiple measures of in vivo plasma antioxidant measures [Oxygen Radical Absorbance Capacity (ORAC), ferric reducing antioxidant power (FRAP), and total thiol] and metabolism (glucose and lipid homeostasis); and to evaluate palatability and potential gastrointestinal effects of the experimental meal. Similar to previous work in this area, we chose to study a small, homogeneous sample and use a crossover design to minimize between subject variability in characterizing these effects (141, 176, 246). We employed intensive sampling (every 30 min) to provide a detailed time course of postprandial plasma changes.
5.3 Subjects and methods

5.3.1 Study population
Men (n = 6) aged 30-65 who were free of any serious illness, did not use tobacco products, and were not taking any medications or nutritional supplements were recruited for the study. Other inclusion criteria were body mass index (BMI; in kg/m²) of 25-40, resting blood pressure <160/100 mmHg, and general good health (by self reported medical history and screening blood work). Healthy overweight men were selected as the study cohort in an effort to minimize between subject variability, while still allowing a chance to observe metabolic benefits should they exist. A complete blood count and standard chemistry panel were obtained at screening to rule out the presence of illness (autoimmune disease, cancer, and immunodeficiency). Blood pressure was measured according to JNC 7 guidelines (204).

5.3.2 Recruitment and ethical aspects
Subjects were recruited through fliers in the community and campus E-mail lists. Potential subjects called to indicate interest in participating in the study. They were given information about the study (including a description of the large dose of spices) and, if interested, were asked a series of medical and lifestyle questions. Qualified respondents were scheduled for clinic screening at the Penn State General Clinical Research Center. After written informed consent was provided, a blood sample was drawn for a complete blood count and general health profile (lipid panel, glucose, liver and kidney function). Body weight and height were measured to calculate BMI. A balanced randomization scheme was developed in advance, and subjects were assigned to a treatment sequence at enrollment. The study protocol was approved by the Institutional Review Board of the Pennsylvania State University.

5.3.3 Design and Intervention
This was a randomized, controlled, 2-period crossover study with one week separation between testing sessions. The two test conditions were: 1) a control meal (Table 5-1) and 2) the control meal with added spice (Table 5-2). The 1200 kcal control meal consisted of a coconut chicken and white rice dish, cheese bread, and dessert biscuit (Table 5-1). Spices were obtained from the McCormick Science Institute Characterized Samples Program (McCormick Science Institute, Hunt Valley, MD) and weighed with a balance accurate to one-hundredth of a gram (Mettler Toledo, Columbus, OH). For the spiced test meal, the 14 g of spices were added to these meal items to create a chicken curry, Italian herb bread, and cinnamon biscuit (Table 5-2). The meal components were weighed in advance and prepared fresh on each day of testing. The spice blend (Table 5-2) was adapted from prior research in this area (246) with the goal of meeting high ORAC contribution and phenolic content while still being palatable.
Participants were instructed to avoid high antioxidant foods (including all spices) for 48 h prior to testing and kept a dietary record for these 48 h. Four hours prior to testing, the men consumed a provided low-antioxidant meal and water *ad libitum* at home. The provided low-antioxidant breakfast was a commercially available vanilla-flavored toaster pastry providing 400 kcal and 12 g fat, primarily as enriched flour, corn syrup, and vegetable oil. Subjects were instructed not to consume any other foods or beverages (besides water) for 12 h prior to testing. The test meal was not provided after a 12 h fast for practical reasons: the amount of food and strong flavors are not typical of breakfast items, and this study was designed to inform future research in which the intervention would be provided at midday.

Subjects reported to the clinic around noon to have their dietary records reviewed. Following verification of compliance and absence of acute illness (bacterial or viral infection), an intravenous (IV) catheter was inserted and a baseline blood sample obtained.
Subjects were allowed 30 min to consume the meal and afterwards completed a questionnaire about the palatability of the meal. The four-item questionnaire asked subjects to rate fullness, satisfaction, enjoyment, and flavors on a scale of 1-10. Subjects also were asked open-ended questions about what they liked and did not like about the meal so that it could be improved for future studies.

The first postprandial blood sample was collected at the end of the 30 minute eating period. Subsequently, blood was collected every 30 min until a total of 8 samples were collected. For each sample, blood was centrifuged and portions of serum and plasma were saved for analysis.

5.3.4 Assay Methods

5.3.4.1 Serum measures
Whole blood was drawn into serum separator tubes, allowed to clot for 30 min, and centrifuged according to guidelines. Total cholesterol and triglycerides were measured by enzymatic procedures (Quest Diagnostics, Pittsburgh, PA; CV < 2% for both). HDL-C was estimated according to the modified heparin-manganese procedure (CV < 2%). LDL-C was not interpreted since calculated values are not accurate during postprandial conditions. Insulin was measured by radioimmunoassay (Quest Diagnostics). Glucose was determined by an immobilized enzyme biosensor using the YSI 2300 STAT Plus Glucose & Lactate Analyzer (Yellow Springs Instruments, Yellow Springs, OH). Serum high-sensitivity C-reactive protein was measured by latex-enhanced immunonephelometry (Quest Diagnostics; assay CV < 8%).

5.3.4.2 Plasma Hydrophilic and lipophilic Oxygen Radical Absorbance Capacity (ORAC)
Plasma aliquots were analyzed for hydrophilic, lipophilic, and total ORAC according to previously published methods (Brunswick Labs, Norton, MA) (175, 251-253).

5.3.4.3 Reducing Ability of Plasma (FRAP) assay
The FRAP assay determines the capability of antioxidants as reductants in a redox-linked colorimetric reaction of the reduction of Fe$^{3+}$-2,4,6-tripyrilid-S-triazine to a blue-colored Fe$^{2+}$ complex at low pH which is measured spectrophotometrically at 593 nm(254). Plasma was incubated at room temperature with the FRAP reagent and the absorbance recorded after 1 h. FRAP values of unknowns are calculated based on standard curves established using Trolox at 31.25 to 500 µmol/L. The reducing power is expressed as µmol TE/µmol GAE.

5.3.4.4 Total thiol in plasma
Total thiol in plasma was determined according to the method of Hu (255). Briefly, an aliquot of EDTA plasma (0.20 mL) was mixed with 0.6 mL of the Tris-EDTA buffer followed by addition of 40 µL of 10 mmol/L 2,2-dithiobisnitrobenzoic acid (DTNB) and 3.16 mL of absolute methanol. After incubation at room temperature for 15 min and centrifugation at 3000 g for 10 min at room temperature, the absorbance of the supernatant was measured at 412 nm (A) and subtracted from a DTNB blank (B) and a blank containing the sample without DTNB (C). Total SH groups as mmol/L in plasma were calculated using an absorptivity of 13,600 cm -1 M -1 as follows: (A - B - C) × (4.0/0.2)/13.6 = (A - B - C) × 1.47 mmol/L.
5.3.5 Statistical Analyses
Statistical analyses were performed using SAS (version 9.2; SAS Institute, Cary, NC). The mixed models procedure (PROC MIXED) was used to test the effects of treatment, time point, and their interaction on changes in outcomes following the meal. Model selection was based on optimizing fit statistics (evaluated as lowest BIC). Means are reported as least-squares means ± SEM. For metabolic endpoints, outcomes were modeled as doubly repeated measures with unstructured by compound symmetry for time point and visits, respectively. For antioxidant endpoints, we imposed a compound symmetry structure by designating subject as random effect. Baseline values were included as covariates. Change scores were calculated by subtracting visit baseline values from each time point. Area under the curve values were calculated using the trapezoidal rule using the premeal baseline values as the line of reference (256). Sample size was selected based on earlier studies (176).

5.4 Results
The screening and visit baseline characteristics of subjects are presented in Table 5-3 and Table 5-4. All subjects completed both visits and were able to consume the meal in less than 30 min. The meals were well-tolerated (no incidence of gastrointestinal effects), and subjects rated the control meal and spiced meal similarly in response to the meal questionnaire about fullness, satiety, enjoyment, and flavors (Table 5-5); all comparisons were not significantly different.

Table 5-3: Screening characteristics of study participants (n = 6)\(^1\).

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<td>BMI (kg/m(^2))</td>
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</tr>
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<td>SBP (mm Hg)</td>
<td>115 (3.4)</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>78.5 (3.5)</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>5.03 (0.38)</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.20 (0.06)</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>3.08 (0.31)</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.63 (0.24)</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>1.06 (0.03)</td>
</tr>
</tbody>
</table>

\(^1\)Summary statistics for screening values were derived from the Univariate procedure (SAS, V9.2). BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, TC = total cholesterol, HDL = high density lipoprotein, LDL = low density lipoprotein, TG = triglyceride.
Table 5-4: Visit baseline characteristics of study participants (n = 6)\(^1\).

<table>
<thead>
<tr>
<th></th>
<th>Control Mean (SEM)</th>
<th>Spice Mean (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mmol/L)</td>
<td>4.97 (0.36)</td>
<td>5.10 (0.36)</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.17 (0.07)</td>
<td>1.13 (0.07)</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>3.05 (0.28)</td>
<td>3.05 (0.28)</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.61 (0.36)</td>
<td>2.04 (0.36)</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.11 (0.32)</td>
<td>4.83 (0.32)</td>
</tr>
<tr>
<td>Insulin (IU/L)</td>
<td>9.0 (3.3)</td>
<td>6.5 (3.3)</td>
</tr>
<tr>
<td>Hs-CRP (mg/L)</td>
<td>1.6 (0.5)</td>
<td>1.3 (0.5)</td>
</tr>
<tr>
<td>Total ORAC (TE)</td>
<td>9927 (915)</td>
<td>10858 (915)</td>
</tr>
<tr>
<td>Lipophilic ORAC (TE)</td>
<td>321 (138)</td>
<td>620 (138)</td>
</tr>
<tr>
<td>Hydrophilic ORAC (TE)</td>
<td>4011 (403)</td>
<td>4341 (403)</td>
</tr>
<tr>
<td>FRAP</td>
<td>482 (28)</td>
<td>480 (28)</td>
</tr>
<tr>
<td>Total thiol</td>
<td>330 (17)</td>
<td>317 (17)</td>
</tr>
</tbody>
</table>

\(^1\)All comparisons between baseline values were not significant between the two treatments (\(p > 0.10\)) using the Mixed Models procedure (SAS, v9.2). TC = total cholesterol, HDL = high density lipoprotein, LDL = low density lipoprotein, TG = triglyceride, CRP = C reactive protein, ORAC = oxygen radical absorbance capacity, FRAP = ferric reducing antioxidant power.

Table 5-5: Meal questionnaire quantitative responses (n = 6)\(^1\).

<table>
<thead>
<tr>
<th></th>
<th>Full</th>
<th>Satisfied</th>
<th>Enjoy</th>
<th>Flavors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.2 ± 0.5</td>
<td>7.3 ± 0.9</td>
<td>6.5 ± 0.7</td>
<td>5.7 ± 0.9</td>
</tr>
<tr>
<td>Spice</td>
<td>7.7 ± 0.5</td>
<td>7.8 ± 0.9</td>
<td>6.7 ± 0.7</td>
<td>6.5 ± 0.9</td>
</tr>
</tbody>
</table>

\(^1\)Following the meal, subjects were asked to rate the meal on a scale of 1 – 10 (most) in response to the following questions: How full are you? How satisfied are you? How much did you enjoy the meal? How much did you like the flavors of the meal? Values are mean ± SEM based on the Mixed Models procedures (SAS, v9.2). All comparisons between the treatments were non-significant.

5.4.1 Effects on postprandial metabolism and C-reactive protein

The addition of spices significantly attenuated postprandial insulin and triglyceride responses to the meal (Figure 5.1); however, there were no effects on glucose (Figure 5.2). The significant treatment by time point interaction resulted in a 21\% reduction in postprandial insulin area under the curve and 31\% reduction in triglyceride area under the curve for the spiced meal relative to control. Cholesterol (total and HDL-C) were not affected by treatment (Figure 5.2).

There was a significant treatment by time point interaction for hs-CRP that appeared to be due to an increase immediately after the spice meal and then decrease at later time points (Figure 5.2). The overall postprandial means for hs-CRP were the same for the two meals (1.36 and 1.36 ± 0.03).
Figure 5.1: Effects of spices on postprandial insulin and triglycerides. Statistical analyses were performed using SAS (version 9.2; SAS Institute, Cary, NC). Change scores were calculated as change from pre-meal baseline values. The mixed models procedure (PROC MIXED) was used to test the effects of treatment, time point, and their interaction. Baseline values were included as covariates. The covariance structure was specified as repeated unstructured time points and with compound symmetry by visit. Means are reported as least-squares means ± SEM. Change scores were calculated by subtracting visit baseline values from each time point. TG = Triglycerides.

Figure 5.2: Effects of spices on other metabolic endpoints and hs-CRP. Statistical analyses were performed using SAS (version 9.2; SAS Institute, Cary, NC). Change scores were calculated as change from visit baseline. The mixed models procedure (PROC MIXED) was used to test the effects of treatment, time point, and their interaction. Baseline values were included as covariates. The covariance structure was specified as repeated unstructured time points and with compound symmetry by visit. Means are reported as least-squares means ± SEM. In graphs with no statistical annotations, there were no significant effects of treatment by time point or treatment as a main effect. CRP = C reactive protein, HDL = high density lipoprotein.
5.4.2 Effects on plasma antioxidant potential

Hydrophilic ORAC levels were 13% higher across time points following the spice meal \((p = 0.02)\), while lipophilic and total ORAC were not changed overall (Figure 5.3). Total ORAC appeared to increase immediately following the meal and then returned to baseline. Our \textit{a priori} statistical plan was not designed to test this individual time point, but an unadjusted, exploratory \(t\)-test demonstrated that 22% difference was significant \((p = 0.02)\). However, this effect was also observed for total cholesterol concentrations for this first postprandial sample (unadjusted \(p = 0.006\)). Postprandial increases in FRAP were 2-fold higher following the spiced meal \((p = 0.009\) for main effect of treatment, Figure 5.3). Total thiol was unchanged by treatment.

\[ \Delta \text{FRAP} \]

\[ \Delta \text{Total Thiol} \]

\[ \Delta \text{Hydrophilic ORAC} \]

\[ \Delta \text{Lipophilic ORAC} \]

\( p = 0.009 \) for effect of treatment

\( p = 0.02 \) for effect of treatment

Figure 5.3: Plasma antioxidant effects of spices. Statistical analyses were performed using SAS (version 9.2; SAS Institute, Cary, NC). Change scores were calculated as change from visit baseline. The mixed models procedure (PROC MIXED) was used to test the effects of treatment, time point, and their interaction. Baseline values were included as covariates. The covariance structure was specified as compound symmetry with subject as a random source of variance. Means are reported as least-squares means \(\pm\) SEM. In graphs with no statistical annotations, there were no significant effects of treatment by time point or treatment as a main effect. FRAP = Ferric reducing antioxidant power, ORAC = oxygen radical absorbance capacity.
5.5 Discussion
This study was designed primarily to observe time course changes in plasma antioxidant measures, and we found significant increases in FRAP and hydrophilic ORAC following spice consumption. We also observed unexpected reductions in postprandial triglycerides and insulin (p < 0.05 for treatment by time interaction) without concurrent effects on glucose concentrations.

These significant effects were likely a result of the high concentration of phenolic antioxidants in spices. It has been estimated that average daily intake of polyphenols is about 1 g for most people (257, 258). The spice dose that we examined provided an acute dose of polyphenols (554 gallic acid equivalents) that is similar to 5 oz red wine (259), 8 oz blueberry or acai juice (259), or 40 g dark chocolate (typical commercial candy bar size) (260), and the results may be hypothesis-generating for research on these products, as well.

We are not aware of prior reports on spices and postprandial triglycerides, but our findings are consistent for effects observed with tea (261, 262). Potential mechanisms include delayed gastric emptying and direct inhibition of pancreatic lipases (263). Future studies will explore this finding.

We observed an attenuation of insulin responses consistent with earlier studies of cinnamon (162), but we did not see a reduction in glucose (264). Polyphenols may improve insulin sensitivity (265-267). Our subjects had large insulin and triglyceride responses to the meal, but their glucose responses were relatively low. Peak postprandial glucose values were less than 122 mg/dL. Average fasting values were 94 mg/dL, so this is a relatively small glycemic response to the meal. In subjects who have developed insulin resistance or type II diabetes, the effects may be very different (268).

We employed multiple assays to determine changes in antioxidant activity in plasma and detected significant increases in FRAP and hydrophilic ORAC (effect of treatment p < 0.05). The ORAC assay assesses the hydrogen atom donating capability of antioxidants, the FRAP assay evaluates electron transferring capability of antioxidants (269), and the total thiol assay determines antioxidants with sulhydryl groups. An increase in hydrophilic ORAC was observed reflecting polyphenolic activity in this plasma fraction, but plasma lipophilic ORAC and total ORAC values were not improved by spices. FRAP increased after the meal, but total thiol was unaffected. The increased plasma antioxidant capacity (FRAP and hydrophilic ORAC) following consumption of spices is consistent for what has been reported in similarly powered studies of fruits (175, 176, 248, 270) and tea (247, 271), but we did not observe an increase in lipophilic ORAC that has been detected 2 h following blueberry or pecan consumption (175, 250).

5.5.1 Strengths and limitations
A strength of this study is the use of the control meal to directly compare postprandial responses with and without spices added to the meal and the use of intensive time course sampling during the postprandial period. We incorporated the spice blend into food items and
did not use capsules, which enables direct real world application of the results and a more translatable dietary message. The multiple time course measures that we used to characterize the postprandial state fill a gap in the literature since there has been very little human data reported in this area.

However, an important limitation is our small sample size (n = 6), and that is consistent with prior studies (176, 246, 248, 249). Follow up work is needed to characterize these effects in larger sample sizes and more diverse populations including people with cardiovascular risk factors. We did not incorporate cell-based protection assays (e.g. CAPe) or markers of lipid oxidation (e.g TBARS, MDA, or ox-LDL), which would have added further insight to the findings. Also, it was not possible to blind the intervention during testing, although we are not aware of psychological effects for these parameters.

There were significant improvements in postprandial triglycerides, insulin, hydrophilic ORAC, and FRAP following a meal containing 14 g of high antioxidant spices. This very high dose of spices was used for proof of concept. Future studies should assess whether lower doses also result in changes in these endpoints. Additional work may clarify whether certain spices within the blend are more potent than others with respect to these outcomes.

Studies also should examine whether postprandial antioxidant effects result in changes in intracellular gene expression. This would be especially relevant in response to oxidative and inflammatory challenge since activation of the inflammatory transcription factor NF-kB is redox sensitive (145, 241, 272, 273).

5.5.2 Conclusions
Dietary surveys typically do not assess intake of culinary spices, and thus there is little epidemiological data on spice consumption and incidence of chronic disease. However, we have demonstrated that spices significantly decrease the magnitude of postprandial increases in circulating triglycerides and insulin, with favorable effects on plasma antioxidant status (hydrophilic ORAC and FRAP). Therefore, the incorporation of spices into the daily diet may help normalize postprandial disturbances in glucose and lipid homeostasis while enhancing antioxidant defense.
6.1 Abstract

**Background:** Culinary spices have demonstrated the ability to blunt inflammatory responses in vitro, but it is unknown whether this effect would exist in a human model of induced inflammation. Acute psychological stress has been shown to induce inflammation in vivo.

**Objective:** The objective of this study was to examine whether adding 14.5 g of a high antioxidant spice blend to a 1200 kcal meal exerted significant effects on inflammatory markers, postprandial metabolism, and other intermediate cardiovascular risk markers in a setting of acute psychological stress.

**Design:** Healthy overweight men (n = 14) and women (n = 6) completed 4 testing sessions for a 2 X 2 factorial design examining the effects of spice, acute stress, and their interaction. Testing sessions were separated by at least one week. In addition to measurements performed on blood sampled at 5 time points per session, salivary cortisol was assessed at 3 time points, platelet function was assessed at 3 time points, and endothelial function was assessed twice; gene expression was assessed twice, and measurements of subjective stress, blood pressure, and heart rate were recorded during the stressor or rest period.

**Results:** Mixed linear models demonstrated a significant effect of the stressor for increasing plasma glucose and insulin. Spices attenuated postprandial lipemia for the rest condition and demonstrated a potential reduction in salivary cortisol that was not significant using prespecified statistics tests. The stressor decreased levels of plasma TNF-alpha, but showed a trend to inducing IL-6 expression in isolated peripheral blood mononuclear cells. Cortisol responses may have unexpectedly decreased plasma concentrations of TNF-alpha following acute psychological stress. Neither the spices nor acute stress had any effects on endothelial function (as assessed by EndoPAT) or platelet function (as assessed by closure times with a platelet function analyzer).

**Conclusions:** This study confirms our earlier finding of decreased postprandial triglycerides following spice consumption and provides evidence that spice may also decrease cortisol responses to stress. Acute psychological stress is detrimental to glucose homeostasis, as evidenced by increased levels of glucose and insulin following a standardized meal.
6.2 Introduction
Oxidative stress and inflammation are key players in most chronic disease (132, 133). In addition to direct oxidative modification, amplified oxidative stress promotes inflammation (134, 241) and metabolic derangements (133). Thus, interest in dietary antioxidants has increased, and extensive, publicly-available databases of in vitro antioxidant properties—more specifically oxygen radical absorbance capacity (ORAC)—have been generated (155, 242). Spices are potent sources of dietary antioxidants, and have among the highest in vitro ORAC values on a per gram basis (155). Most high antioxidant spices have also demonstrated potential benefits on glucose, lipid, and inflammatory homeostasis (174, 244, 245), which adds to their appeal as a source of dietary antioxidants. Adding spices to foods can increase antioxidant intake without appreciably increasing caloric intake. However, little work has directly examined how antioxidant supplementation may affect responses to acute provoked inflammation.

The Trier Social Stress Test (TSST) is a rigorous model of acute psychological stress that has been shown to provoke inflammatory responses (179-181). Several characteristics lead to the success of the TSST as a model of provoked inflammation in response to acute psychological stress: its combination of speech and math tasks, its duration (~30 min) that exceeds other models, and inclusion a social evaluative component (panelists who are trained to provide negative feedback during the task) in addition to subjects’ being told that their performance and is being taped and will be further evaluated. Much work has demonstrated that psychological stress is a key driver in inflammatory diseases—including heart disease—and dietary interventions capable of attenuating the negative physiological consequences of psychological state could be of great benefit to public health.

Prior to the conduct of this study, we demonstrated that a large acute dose of high antioxidant spices providing a polyphenolic dose (554 gallic acid equivalents) similar to a 5 oz glass of red wine, 40 g dark chocolate, or 8 oz glass of acai juice was well-tolerated and significantly improved postprandial triglycerides, insulin, and some measures of plasma antioxidant status (FRAP and H-ORAC).

This study was designed to assess whether the high antioxidant spice blend was able to attenuate inflammatory responses provoked by acute psychological stress, as measured by plasma levels of the inflammatory cytokines interleukin(IL)-6 and tumor necrosis factor alpha (TNFa). We also measured levels of gene expression in isolated peripheral blood mononuclear cells (PBMC) to characterize any effects on inflammation. Other aims of this study were to validate our earlier finding that spices attenuate postprandial triglycerides and insulin and to assess effects of the high antioxidant spice blend on salivary cortisol, glucose, and hs-CRP responses to stress.
6.3 Subjects and methods

6.3.1 Study population
Men and women aged 30-65 y who were free from any serious illness were recruited for the study. Other inclusion criteria included a body mass index (BMI; in kg/m^2) of 25-40, resting blood pressure < 160/100 mm Hg, fasting glucose <126 mg/dL, and willingness to discontinue all dietary supplements during the study. Exclusion criteria included acute or chronic inflammatory conditions, liver or kidney dysfunction, a history of heart disease, the use of tobacco products, training for athletic competition (>2 h aerobic activity a week), and the use of medications relating to birth control, hormone replacement therapy, lipid lowering, blood pressure, and psychosis or depression, with the exception of SSRIs. A complete blood count and standard chemistry panel were obtained at screening to rule out the presence of illness (autoimmune disease, cancer, and immunodeficiency). Blood pressure was measured according to JNC 7 guidelines (204). Briefly, after a 5-min seated rest, three readings were taken by nurses in a controlled environment using a calibrated mercury sphygmomanometer. The average of the last 2 readings was used to determine eligibility. If blood pressure was > or = 140/90, approval for study participation was requested from the subjects physician.

6.3.2 Recruitment and ethical aspects
Subjects were recruited through fliers in the community and campus E-mail lists. Potential subjects called to indicate interest in participating in the study. They were given information about the study (including the nature of the large dose of spices) and, if interested, were asked a series of medical and lifestyle questions. Qualified respondents were scheduled for clinic screening at the Penn State General Clinical Research Center. After written informed consent was provided, a blood sample was drawn for a complete blood count and general health profile (lipid panel, glucose, liver and kidney function). Body weight and height were measured to calculate BMI. A balanced randomization scheme was developed in advance, and subjects were assigned to a treatment sequence at enrollment. The study protocol was approved by the Institutional Review Board of the Pennsylvania State University.

Interested individuals (n=118) were phone screened for participation after answering advertisements from community boards, 33 met the criteria for a clinical screening procedure. Of the 31 individuals who were scheduled for a clinic screening appointment, 2 cancelled prior to clinic appointments. Of the completed clinic screenings (n = 29), 24 were approved for study protocol. Twenty two participants started the study and two participants were withdrawn during the study due to exceeding testing limits for blood pressure (n = 1) and emotional response (n =1). Thus, data are reported for 20 healthy participants (n = 14 men, n = 6 women).

6.3.3 Design and intervention
This was a randomized, controlled, 4-period crossover study with one week separation between testing sessions. The 2 factors studied were the effects of adding spice to a meal (spice) vs. the same meal without spice (control) and the effect of undergoing a psychological stressor (stress) vs. undergoing a rest condition (rest). In this 2 x 2 design, participants were exposed to the four
conditions presented in counterbalanced order including each of the following: 1) control and rest, 2) control and stress, 3) spice and rest, 4) spice and stress. Outcome measurements were timed relative to the meal and stressor. Each aspect of the testing visit is discussed in further detail below.

6.3.3.1 Visit Preparation
The four testing sessions were separated by a minimum of one week for men and postmenopausal women. Visits for premenopausal women (n = 2) were scheduled during the first seven days of the menstrual cycle to avoid the hormone effects on vascular measures and therefore, separated by one month. Participants were instructed to avoid high antioxidant foods (including all spices) for 48 h prior to testing. For 48 h prior to the “spice” testing sessions, participants consumed 3 meal items per day that total provide the dose of spices given on the spice testing day. For two days prior to the “control” testing sessions, participants consumed the same 3 snacks per day without spices along with methylcellulose placebo capsules that they were told contained an antioxidant concentrate equivalent to the amount of spices in the food. This deception was used in attempt to avoid a placebo effect in response to a “high antioxidant” intervention. Participants were told that the study was comparing the effects of antioxidants delivered in foods vs. antioxidants delivered as a concentrate in capsules.

Prior to arriving at the clinic, participants consumed a provided breakfast (finishing by 8 am). The provided low-antioxidant breakfast was a commercially available white bagel and light spread providing 400 kcal and 12 g fat, primarily as enriched flour and vegetable oil. Subjects were instructed not to consume any other foods or beverages (besides water) for 12 h prior to testing. Subjects reported to the clinic at 10 am.

6.3.3.2 Baseline Measurements
Following verification of compliance and absence of acute illness (bacterial or viral infection), subjects rested supine for 25 min, during which time baseline endothelial function testing was performed. Following this period, a baseline salivary sample for cortisol measures was obtained. Then an intravenous catheter (IV) was inserted and a baseline blood sample obtained.

6.3.3.3 Test Meal
The 14.5 g spice blend consisted of cloves, cinnamon, oregano, rosemary, ginger, black pepper, paprika, garlic powder, and turmeric. The spices were obtained from the McCormick Science Institute Characterized Samples Program (McCormick Science Institute, Hunt Valley, MD) and weighed with a balance accurate to one-hundredth of a gram (Mettler Toledo, Columbus, OH). The 1200 kcal test meal consisted of a chicken and white rice curry, corn muffin, and cinnamon pastry dessert. The spice blend incorporated into the meal was previously tested (Chapter Chapter 5). Cinnamon (0.5 g) was added to the original blend based on feedback from participants. The test meal was provided at noon, and subjects were allowed 30 min to consume the meal.
6.3.4 Stressor Task

After the subjects finished the meal, they were moved to a phlebotomy chair. Blood pressure and heart rate were assessed with a Dinamap Pro 100 oscillometric monitor (GE Medical Systems). The stress task administered was the Trier Social Stress Test (180). Briefly, participants were given instructions by DVD and then were asked to prepare and deliver a speech to 2 trained panel members who were presented to the participant as psychologists trained to evaluate the participant’s performance and ability to give a speech.

The speech task included 10 min of preparation, and 5 min of delivery, with the topic of the speech pertaining to suitability for a job (two different job tasks were provided in random order). Following this mock interview task, subjects were administered an 8 minute math task that required them to serially subtract 7 (first math task) and then 13 (second math task) from large prime number as quickly and accurately as possible. When an incorrect number was given, a manager interrupted them and asked them to start over with a new number.

Cardiovascular variables were continuously monitored during a final 10 min recovery period. Following this period, subjects were asked to rate the stressfulness of the experience by making a mark on a visual analog scale that ranged from “not at all stressful” to the “most stressful thing I have ever experienced.” The last three measurements of the resting and recovery periods were averaged. For the speech preparation, speech delivery, and math tasks, measurements were taken every minute and averaged by task. These six task averages were treated as repeated measures for each testing session.

6.3.5 Salivary Cortisol

Salivettes were used to collect saliva samples at resting baseline prior to the meal, following a rest period after the meal, 10 min after completing the stressor or rest period, and 45 min after the stressor or rest period. Analysis of cortisol concentrations in saliva was performed using commercially available enzyme-linked immunosorbent assays (Salimetrics, State College, PA).

6.3.6 Blood sample collection and assays

Blood samples were collected through the intravenous (IV) catheter that was established at the beginning of the visit. To prevent sample hemolysis incurred by vacutainers acting on the IV line, syringe transfer procedures were employed. Except for endpoints that required unfrozen samples, samples were aliquotted and stored at -80 °C for batch analysis. Blood was sampled at pre-specified time intervals relative to the meal and stressor/rest period. The baseline sample was collected following the endothelial function testing at the beginning of the visit. The second sample was collected 10 min after completing the stressor and 105 min after the first bite of food. The third sample was collected 45 min after the stressor and 140 min after the first bite of food. The fourth sample was collected 90 min after the stressor and 3 h after the meal. The final sample was 2 h post stressor and 3.5 h after the meal.
6.3.6.1 Lipids and lipoproteins
Whole blood was transferred into serum separator tubes, allowed to clot, and centrifuged. Total cholesterol and triglycerides were determined by enzymatic procedures (Quest Diagnostics, Pittsburgh, PA; CV < 2% for both). HDL-C was estimated according to the modified heparin-manganese procedure (CV < 2%). LDL-C was not interpreted since calculated values are not accurate during postprandial conditions.

6.3.6.2 Insulin and glucose
Insulin was measured by radioimmunoassay using 125I-labeled human insulin and a human insulin antiserum (Quest Diagnostics). Glucose was determined by an immobilized enzyme biosensor using the YSI 2300 STAT Plus Glucose & Lactate Analyzer (Yellow Springs Instruments, Yellow Springs, OH).

6.3.6.3 Inflammatory Markers
Serum high-sensitivity C-reactive protein was measured by latex-enhanced immunonephelometry (Quest Diagnostics; assay CV < 8%). Plasma concentrations of IL-1β, IL-6, TNF-α were measured via high sensitivity ELISA kits from R&D Systems (Minneapolis, MN) in duplicate (assay CV < 11% for all). Serum hs-CRP was measured by latex-enhanced immunonephelometry (Quest Diagnostics, Pittsburgh, PA; assay CV < 8%).

6.3.6.4 Mononuclear cell gene expression
Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient separation from EDTA-anticoagulated blood. Following isolation and washing of the buffy coat with saline, cells were saved for RNA isolation. Cells for RNA isolation were suspended in RNALater solution before being stored at -80 °C. Following RNA isolation (RNEasy mini kit, Qiagen; Valencia, CA), a high capacity cDNA Archive kit (Applied Biosystems; Foster City, CA) was used for reverse transcription. The cDNA (500 ng) was amplified by SYBR Green PCR Master Mix (Applied Biosystems; Foster City, CA) and detected by an ABI 7300 Sequence Detection System (Applied Biosystems; Foster City, CA). The expression of IL-1β, IL-6, TNF-α was measured using real time PCR with the following primers: IL-1β 1173F CACGGCCACATTGTTTCTAA, IL-1β 1224R CAGAATGTGGGAGCGAATGAC, TNF-α 803F ATCAATCGGCGACTATCTC, TNF-α 887R TGGATGTGCTGCCCTCCTCACA, IL-6 197F GCCACTCACCTTCCAGAACG, IL-6 250R CCGTCGAGGATGTACCAGAATT. The expression of these genes was normalized to expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAPDH forward and reverse primers were TGGGTGTAACCATGAGAAG and GCTAAGCAGTTGGTGTCG, respectively.

6.3.7 Endothelial function testing
Endothelial function was assessed as the beginning and end of the visit in a quiet, dimly lit room at 71-75 °F. An automated rapid cuff inflator set to 250 mmHg (Hokanson, Bellevue, WA) was placed on the forearm to induce ischemia. The EndoPAT2000 (Itamar Medical, Ltd.) was used to measure relative changes in pulse wave amplitude (PWA) pre vs post occlusion (210). Two flexible probes were placed on the index fingers of the right (ischemic) and left (control) hands. Measurements were made during baseline (5 min), occlusion (5 min), and reactive hyperemia (5
min). The Reactive Hyperemia Index (RHI) was calculated as the ratio of the average PWA during hyperemia (60 sec to 120 sec of the post-occlusion period) compared with the average PWA during baseline in the occluded hand over the same values in the control hand multiplied by a baseline correction factor. The Framingham RHI (F-RHI) is an alternative calculation derived from the same raw data and differs in that it uses the period 90 sec to 120 sec of post-occlusion hyperemia, does not incorporate a baseline correction factor, and applies a natural log transformation to resulting ratio. F-RHI has been shown to correlate with other cardiovascular risk markers (69, 212). The EndoPAT device also generates the augmentation index (AI), a measure of vascular stiffness (pulse wave reflection) that is calculated from the shape of the pulse wave recorded by the probes during baseline. AI can be adjusted to a heart rate of 75 beats per minute to correct for the independent effect of heart rate the pulse wave form (213).

6.3.7.1 Platelet function
Platelet activation was measured using a PFA-100 platelet function analyzer (Dade Behring) (274-276). Briefly, citrated whole blood (800 uL) was pipetted into cartridges that were inserted into the machine. The machine measures the amount of time in seconds that it takes for a clot to form when the whole blood is aspirated through a narrow aperture containing a collagen surface coated with adenosine diphosphate or epinephrine to stimulate thrombogenesis. Clotting time indicates the ability of platelets to form clots.

6.3.8 Statistical analyses
Statistical analyses were performed using SAS (version 9.2; SAS Institute, Cary, NC). The mixed models procedure (PROC MIXED) was used to test the effects of treatment, stressor, time point, and their interaction on changes in outcomes following the meal. Model selection was based on optimizing fit statistics (evaluated as lowest BIC). Means are reported as least-squares means ± SEM. For metabolic and cortisol endpoints, outcomes were modeled as doubly repeated measures with unstructured by compound symmetry for time point and visits, respectively. For other endpoints, we imposed a compound symmetry structure by designating subject as a random effect. Change scores were calculated by subtracting visit baseline values from each time point. Baseline values were included as covariates when change scores or post time points were analyzed. Area under the curve values were calculated using the trapezoidal rule with baseline values as the line of reference (256).

Sample size was selected using a power calculation based on a study in which aspirin reduced IL-6 reactivity to the TSST. Because IL-6 is not normally distributed, we utilized means and SDs for log transformed concentrations of IL-6 provided by Dr. von Känel. Their study was a between-subjects design, so we estimated the SD of the difference for a crossover design. To be conservative, we based estimates on the assumption that the effect of spices would be 50% smaller. We calculated sample size required to meet the following assumptions: peak effect of stress on IL-6 at 90-100 min post stress, mean difference between treatment and control = 0.28 pg/mL, SD of the difference estimated to range from 0.40 – 0.42, power = 0.80, p = 0.05, and a two tailed test. With these assumptions, we would require 19-20 participants to detect a significant difference between active treatment and placebo.
6.4 Results

6.4.1 Baseline characteristics by treatment
Baseline characteristics were analyzed to determine whether pre-treatment with spices resulted in measurable differences at baseline (Table 6-1). All comparisons were not statistically significant except for ADP clotting time, which was significantly decreased by 5 sec following pre-treatment with spices.

Table 6-1: Baseline characteristics following pre-treatment with control vs. spiced foods

<table>
<thead>
<tr>
<th></th>
<th>Control Mean (SEM)</th>
<th>Spice Mean (SEM)</th>
<th>p-value for treatment difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG (mg/dL)</td>
<td>155 ± 14</td>
<td>162 ± 14</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>90.2 ± 3.2</td>
<td>91.6 ± 3.2</td>
<td>NS</td>
</tr>
<tr>
<td>Insulin (IU/L)</td>
<td>8.92 ± 1.40</td>
<td>8.71 ± 1.41</td>
<td>NS</td>
</tr>
<tr>
<td>Hs-CRP (mg/L)</td>
<td>1.40 ± 0.24</td>
<td>1.36 ± 0.24</td>
<td>NS</td>
</tr>
<tr>
<td>Salivary Cortisol (µg/dL)</td>
<td>0.20 ± 0.04</td>
<td>0.19 ± 0.04</td>
<td>NS</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.18 ± 0.21</td>
<td>1.24 ± 0.21</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>1.47 ± 0.26</td>
<td>1.34 ± 0.26</td>
<td>NS</td>
</tr>
<tr>
<td>IL-1β gene expression</td>
<td>0.39 ± 0.04</td>
<td>0.37 ± 0.04</td>
<td>NS</td>
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<tr>
<td>IL-6 gene expression</td>
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<td>TNF-α gene expression</td>
<td>1.62 ± 0.22</td>
<td>1.38 ± 0.22</td>
<td>NS</td>
</tr>
<tr>
<td>Augmentation Index (AI)</td>
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<td>-4.74 ± 3.2</td>
<td>NS</td>
</tr>
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<td>AI Standardized to 75 bpm</td>
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<td>-11.0 ± 3.4</td>
<td>NS</td>
</tr>
<tr>
<td>RHI</td>
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<td>1.75 ± 0.10</td>
<td>NS</td>
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<td>Framingham RHI</td>
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<td>0.26 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td>ADP clotting time</td>
<td>91.1 ± 3.0</td>
<td>86.1 ± 3.0</td>
<td>0.04</td>
</tr>
<tr>
<td>EPI clotting time</td>
<td>112 ± 4.0</td>
<td>111 ± 4.0</td>
<td>NS</td>
</tr>
</tbody>
</table>

1Results based on Mixed Models procedure (SAS, v9.2). TG = triglycerides, CRP = C reactive protein, IL = interleukin, TNF = tumor necrosis factor, RHI = reactive hyperemia index, ADP = adenosine diphosphate, EPI = epinephrine.

6.4.2 Cortisol responses
The social stressor induced significant increases salivary cortisol following the stressor relative to the rest condition (p = 0.0005 for stress by time point interaction). In a model that examined cortisol levels at post-meal time points with adjustment for fasting values, there was a trend (p = 0.098) for a treatment by stressor interaction. Area under the curve analyses revealed a significant treatment by stressor interaction (p = 0.045), however; post hoc analyses were only significant for the comparison of the stress condition to rest. In an exploratory analysis of cortisol AUC from the stress visit only, the comparison of spice to control was significant (p = 0.02) in a model that adjusted for period effects.
Figure 6.1: Effect of stressor on salivary cortisol levels. Values are expressed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC).

Figure 6.2: Models for a potential treatment by trier interaction. Left) Postprandial salivary cortisol levels were modeled with adjustment for fasting values. Right) Log transformed area under the curve values revealed a significant treatment by trier effect, but only comparison of stress to rest conditions were significantly different. Values are expressed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC).
* p < 0.05 for adjusted comparison relative baseline.

Figure 6.3: Exploratory analysis of cortisol area under the curve for stress visits only. The spice area under the curve (AUC) is significantly lower in a model that adjusts for period effects (p = 0.02). Values are expressed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC).
6.4.3 Triglyceride responses

Postprandial changes in triglycerides exhibited a significant treatment by stressor interaction (Figure 6.4). Post hoc comparisons revealed a significant difference between the spice and rest condition versus the control and rest condition (Tukey p = 0.01). Overall, postprandial triglyceride concentrations were reduced by 25 mg/dL for the resting spice visit compared to the control rest visit. Other comparisons were not significantly different after adjustment for multiple comparisons.

![Graph showing postprandial triglyceride values](image)

Figure 6.4: Postprandial triglyceride values exhibited a significant treatment by stressor interaction. Left) Postprandial changes in triglycerides were modeled with adjustment for fasting values. Right) To examine the significant treatment by stressor interaction, post hoc comparisons of least-squares means were made. Different letters denote significantly different conditions (Tukey p = 0.01). Values are expressed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC).

6.4.4 Insulin Responses

Insulin values were significantly increased by stress, but not affected by treatment with spices (Figure 6.5). The effect of the stressor was evident across the postprandial time points and resulted in a significant 27% increase in insulin relative to the rest condition (p = 0.006).

6.4.5 Glucose Responses

Glucose values were significantly increased by stress in a time dependent manner (significant stressor by time point interaction p = 0.03, Figure 6.6). The effect of the stressor for increasing glucose concentrations was evident was evident during the first two postprandial samples where glucose values peaked (adjusted comparisons p < 0.05). Postprandial increases in glucose were twice as high under stress conditions.
Figure 6.5: Insulin values exhibited a significant effect of the stressor. Left) Postprandial changes in insulin were modeled with adjustment for fasting values. Right) The stressor resulted in significantly higher insulin values across visits and time points ($p = 0.006$). Values are expressed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC).

Figure 6.6: Glucose values exhibited a significant effect of the stressor. Left) Postprandial changes in glucose were modeled with adjustment for fasting values. Right) The stressor resulted in significantly higher glucose values at the first two time points, with a significant stressor by time point effect of $p = 0.03$.

*Adjusted $p < 0.05$ for comparison across conditions. Values are expressed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC).

### 6.4.6 Plasma cytokine levels

Plasma IL-6 values exhibited a trend ($p = 0.07$) for a stressor by time point effect (Figure 6.7). In an exploratory analysis of change at time point 2 (the apparent peak) increases in IL-6 concentrations following the stressor were twice that of the rest condition (1.04 vs. 0.53 ± 0.26), but this did not reach statistical significance ($p = 0.12$). Plasma TNF values showed a significant stressor by time point effect ($p = 0.02$, Figure 6.8). At the final time point, TNF concentrations were significantly lower under the stressor condition (adjusted $p < 0.05$ for stress vs. rest).
Figure 6.7: Interleukin-6 values exhibited a significant time point effect only. Values are expressed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC).

Figure 6.8: Tumor necrosis factor (TNF) concentrations exhibited a significant stressor by time effect. Left) Changes in TNF were modeled with adjustment for fasting values. Right) The stressor resulted in significantly lower TNF values at the last time point. Values are expressed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC).

6.4.7 Gene expression of inflammatory cytokines in isolated mononuclear cells
There was no effect of stressor or spices on expression of IL-1B or TNF-a in cells isolated 10 min following the stressor (Figure 6.9). However, IL-6 did exhibit a treatment by stressor interaction (p = 0.02, Figure 6.9). In post hoc comparisons, the control and stress condition was higher than the control and rest condition prior to applying penalties for multiple comparisons (unadjusted p = 0.02). No other conditions were significantly different. For TNF-a expression, there was a main effect of time point (p = 0.03) resulting from lower expression levels of expression relative to pre-treatment baselines.
Figure 6.9: Gene expression of inflammatory cytokines in isolated peripheral blood mononuclear cells. In each panel, the left figure models levels at pre-treatment baseline and 10 min after the stressor or rest period. On the right, the 10 min post stress values are modeled with adjustment for pre-treatment values. Values are expressed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC).
6.4.8 **Endothelial function**

No measure of endothelial function (RHI, F-RHI, Al, and standardized Al) was significantly affected by acute stress or spices (Figure 6.10). For a model of post treatment values with adjustment for pre-treatment values, the treatment by stressor effect is \( p = 0.18 \) for RHI (below) and \( p = 0.14 \) for F-RHI.

![Graph showing endothelial function outcomes](image)

**Figure 6.10**: Endothelial function outcomes. In each panel, line graphs model levels at pre-treatment baseline and end of visit values. The bar graph at top right models change from pre-treatment values with adjustment for pre-treatment values. No effects were statistically significant. Values are expressed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC).

6.4.9 **Platelet function**

The stressor and spices did not affect platelet function as assessed by closure times, but there was a significant main effect of time for the adenosine diphosphate-coated cartridges (Figure 6.11). Closure times were shortest from the sample taken 105 min after the first bite of food and 10 min after the stressor/rest session.
6.4.10 Perceived stress, blood pressure, and heart rate during rest or stress

The stressor task significantly increased subjective stress levels as measured by a visual analog scale (p < 0.0001, Figure 6.12) and this did not differ between spice and control. There were significant treatment by stressor by event effects for measures of blood pressure and heart rate (p < 0.0001, Figure 6.13). No post hoc comparisons were significant for blood pressure, but the spice treatment significantly elevated heart rate during the mental stress tasks relative to control.

![Figure 6.11: Platelet function. Line graphs model closure times at pre-treatment baseline, 10 min after the stressor, and end of visit. No effects were statistically significant, except for a main effect of time for the ADP cartridge. Values are expressed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC).](image)

![Figure 6.12: Perceived stress. Values are expressed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC).](image)

![Figure 6.13: Blood pressure and heart rate during stress and rest. There was a significant treatment by stressor by event effect for blood pressure and heart rate (p < 0.0001). Values are expressed as mean ± SEM based on the MIXED procedure (SAS, Version 9.2, Cary, NC).](image)
6.5 Discussion

The postprandial design was important for measuring the effects of spices on study outcomes. Pre-treatment with spices did not result in changes in any baseline value with the exception of slightly decreased closure time for the ADP-coated cartridge. This result may be a chance finding due to multiple comparisons. It is important to note that the pre-treatment period was only 48 hours as was intended as preparation for the testing visit.

The spice blend significantly decreased postprandial triglycerides on resting visits as we have previously observed (Chapter 5) but did not affect postprandial insulin and glucose levels. The reduction in postprandial lipemia by spices may have important health implications as elevated nonfasting triglycerides is becoming increasingly recognized as a cardiovascular risk factor (30, 31, 277, 278), although clinical norms have not been established. We are not aware of prior reports on spices and postprandial triglycerides, but our findings are consistent for effects observed with tea (261, 262). Potential mechanisms include delayed gastric emptying and direct inhibition of pancreatic lipases (263). Future studies will explore this finding.

Plasma concentrations of IL-6 appeared to increase only transiently at 10 min post stressor, but there was no delayed phase increase in contrast to prior reports (179-181, 185, 186, 188). Gene expression for IL-6 in isolated peripheral blood mononuclear cells from 10 min after the stressor was higher for control stress relative to control rest when no adjustment for multiple comparisons was applied, indicating that IL-6 may have been stress-responsive, but only transiently. Unexpectedly, the stressor decreased plasma TNF concentrations at the last blood draw, 2 h after the stressor. This may be due to the significant increases in cortisol resulting from the stressor. Exercise has also been shown to reduce TNF concentrations by this mechanism (279). Because we did not induce an inflammatory response, we were not able to address our primary aim of assessing whether IL-6 responses to the stressor were attenuated by spices.

A potential explanation for our lack of inflammatory induction is that perhaps we were only able to induce stress acutely relative to earlier trials (179-181, 185, 186, 188). This may suggest that the stressor was experienced more intensely and acutely relative to other the reports that may have had a greater influence of post stressor rumination on study findings. The present study involved a great deal of subject interaction due to complex visit preparation instructions. The study coordinator and nursing staff were not in the room during the stressor and rest periods and did not discuss any aspect of the stressor with the participant at any point during the visit. However, it is possible that the participants experienced more transient stress relative to previous reports due to unanticipated comfort resulting from familiarity with the study personnel and testing environment. Salivary cortisol measures, subjective responses, and hemodynamic outcomes confirm that the stressor experience was at least acutely effective. It is also noteworthy that IL-6 concentrations differed by 0.5 pg/mL at 10 minutes after the stressor. Although this comparison was not statistically significant, it exceeds the projected difference for the final time point (0.3 pg/mL) (181).
Another explanation for the lack of inflammatory response in this study is the relationship between cortisol responses and cytokine production. In a sample of ~200 participants of the Whitehall II cohort, people who did not produce cortisol in response to laboratory stressors experienced more subjective stress and were more likely to have significant inflammatory responses (280). Since our subjects were healthy with no identified negative mental health traits, the cortisol they produced in response to the stressor may have negated any delayed-phase inflammatory response. People under chronic stress or experiencing depression may be more vulnerable to inflammatory responses to stress, which also may have implications for studying the role of stress in chronic inflammatory diseases (281).

Acute psychological stress increased postprandial levels of insulin and glucose. Increased cortisol levels may explain the increase plasma glucose levels (282), and increased distress in people with type II diabetes also linked with elevated glycosylated hemoglobin (283). The increases in both glucose and insulin suggest important negative effects of psychological stress on glucose metabolism.

The spice blend showed trends to reducing cortisol responses to stress, and we were unaware of similar reports. A study of vitamin C showed improved recovery of cortisol levels, but no reduction in responses using the same psychological stressor (284). The effect of spices for increasing heart rate during psychological stress is unexpected and difficult to interpret. There was no increase in blood pressure with spices and stress.

We found no changes in endothelial function, and this may be unexpected given the abundance of literature on high polyphenolic interventions, lipemia, and endothelial function (89, 285-287). However, many earlier reports have reported endothelial function with FMD as the method of measurement, and this study used the newer endoPAT methodology. Studies using the endoPAT device to study high antioxidant interventions have significant limitations. One study of cocoa analyzed a sample size of only 3 participants (288). Other studies of cocoa did not report RHI from the endoPAT and did not incorporate a placebo control (289, 290). A study of a high flavonoid drink powder reported a significant 10% improvement for percent change using t-tests, which may not be an optimal statistical test for this outcome (291). We observed similar differences (i.e. 10% between treatment) that were not statistically significant using mixed models comparisons of treatment values. In summary, no prior reports have demonstrated convincingly that the endoPAT RHI can be improved by acute high antioxidant interventions.

This is our second report that demonstrates the ability of spices to reduce postprandial triglycerides. Future work will examine the mechanism by which this effect is achieved. The attenuation of cortisol responses is intriguing, and merits further investigation. Overall, this work confirms that spices have a hypolipidemic effect when incorporated into food items that may be realistically consumed. It is unknown whether this effect is dose dependent or due to individual spices within the blend that we examined.
Chapter 7. Summary, Limitations, and Future Directions

The studies in this dissertation were designed to explore the effects of nutrition interventions on modifying cardiovascular risk factors. Specifically, these studies investigated (1) the effects of omega-3 fatty acids on endothelial function, lipids, inflammation, glucose metabolism, and erythrocyte fatty acids, 2) the effects of omega-3 fatty acids on hemodynamics, 3) the effects of highly antioxidant spices on plasma antioxidant status and metabolism, and 4) the effects of highly antioxidant spices for attenuating the deleterious effects of psychological stress. The purpose of this chapter is to summarize the findings and explore additional avenues for research based on what has been reported in these chapters.

7.1 Omega-3 fatty acid studies

In chapters 2 and 3, the results of our clinical dose-response study of 0.85 g/d vs. 3.4 g/d EPA + DHA were presented. We found significant reductions in triglycerides and blood pressure with the high dose, and both doses reduced heart rate in a dose-response manner. Other outcomes were not changed, and these included a large range of cardiovascular risk factors that omega-3 fatty acids are thought to improve (34, 59).

For the existing samples, lipoprotein particle size would be a useful outcome. Much interest has recently evolved concerning the LDL-C raising effect of fish oils and whether this effect is due to DHA in particular (48, 292). Particle size analysis could demonstrate whether increases in particle size were demonstrable with either dose and whether changes in particle size were related to reductions in triglycerides and/or increases in lipoprotein concentrations (55). Analysis of plasma norepinephrine or catecholamine levels could provide insight to hemodynamic effects (106, 108, 227, 293-296), but a limitation is that blood samples were collected at a separate visit than hemodynamic data.

Analyses of apolipoprotein distributions and lipoprotein-associated phospholipase A2 activity and mass are forthcoming and may provide greater characterization of the effects reported in Chapter 3. Apolipoproteins are becoming increasingly recognized as adding enhanced assessment of cardiovascular risk relative to the traditional lipid profile (28, 297, 298). Similarly lipoprotein-associated phospholipase A2 is becoming increasingly recognized as a more specific marker of inflammation related to unstable plaques (299-306).

Future research should explore the effects of omega-3 fatty acids in a human model of induced inflammation, specifically by inducing systemic inflammation via injection of endotoxin (178, 307). Research in animal models has demonstrated that the anti-inflammatory effects of omega-3 fatty acids are more apparent in models of induced inflammation, and omega-3 fatty acids may enhance recovery (82-84, 308). This model system should be used to study whether omega-3 fatty acids may attenuate inflammatory responses and/or enhance the active resolution phase as measured by faster or more complete recovery from invoked inflammation (82-84).
Future research should also examine the independent contributions of EPA on the cardiovascular risk markers examined in these research studies and suggested for future studies. As well, the independent health benefits of plant-derived omega-3 fatty acids should be assessed, especially in the context of EPA and DHA sufficient diet, since health benefits of plant omega-3 are often ascribed to their conversion to the longer chain fatty acids.

7.2 Spice Studies

The effect of spices for improving postprandial triglyceride concentrations was demonstrated in both studies (Chapters 5 and 6). This may indicate the potential of spices to increase thermogenesis, enhance lipid clearance, alter gastrointestinal transit rate, or impair lipid absorption. The potential impairment of lipid absorption by spice polyphenols is appealing as a mechanism because inhibition of pancreatic lipase has been used successfully for weight loss by pharmacological agents (Orlistat) (309) and has been demonstrated in other foods having a high content of phenolic compounds (134, 310-317). In fact, tea and its phenolic isolates such as epicatechin polymers and epigallocatechin gallate have also been shown to effectively inhibit fat absorption by impairing pancreatic lipase (261-263, 313, 314, 318).

The reduction in triglycerides prompts the inquiry of whether improved fasting lipid values could be observed in the context of longer term supplementation. One 6 month study of curcumin’s effects on lipids in 36 people demonstrated no reduction in triglycerides, but this could be due to study design factors, such as not administering the intervention incorporated into foods (319).

Another potential area of future investigation is the role of spices in weight reduction interventions. If spices do indeed reduce postprandial triglyceride concentrations by any combination of the above-described mechanisms, this affect may enhance other weight loss strategies. Impaired fat absorption may also suppress appetite via affects on gastrointestinal lipid sensing (320). Spices also affect the sensory properties of food and could increase meal satisfaction. We collected mood responses to the meals using the Essense questionnaire.

Our ongoing work is examining the effects of spices in in vitro models that assess the ability of spices to inhibit digestive lipases. This work may lead to greater understanding of which spices have the most potent hypolipemic effects. Preliminary experiments performed by Yeyi Gu and Joshua Lambert have shown that the spice blend potently inhibits pancreatic lipase in vitro. In this experimental model, the spice blend was extracted with acetone: water: acetic acid (80:20:0.1), dried, and reconstituted with DMSO to make a 100 mg/mL stock solution. This stock solution was then diluted with water to test concentrations ranging from 0 to 200 µg/mL that was then incubated with porcine pancreatic lipase (100 µg/mL). The inhibition potency was determined by adding 4-NPB (0.2 mM) and a Tris-HCl buffer (0.1 M) to maintain pH of 8. Active lipase cleaved 4-NPB to release 4-nitrophenol, producing a yellow color that was read by a plate reader set for absorbance of 400 nm after a 10 min incubation period. The results were then interpreted using GraphPad software to determine the concentration that inhibits half of the pancreatic lipase activity (IC50). For the spice blend, the IC50 was determined to be 33 µg/mL. In
This model, Orlistat has demonstrated an IC$_{50}$ of 0.6 µg/mL, and ECGC isolated from green tea had an IC$_{50}$ of 7.5 µM (personal communication with Josh Lambert and Yeyi Gu).

These experiments will be repeated for the individual spices within the blend to determine which spices demonstrate the most potent lipase inhibition. These results can be used to design new postprandial experiments in which a lipid challenge is administered to human subjects and the effect on postprandial lipidemia is measured. These experiments should also measure markers of lipid oxidation than may demonstrate superior health benefits as compared to pharmaceutical fat absorption inhibitors. It would be interesting to test whether the decreased lipidemia is associated with measurable reductions in metabolic stress. In our work hs-CRP was not a valid marker of acute metabolic stress, but markers of lipid oxidation (8-isoprostane, malondialdehyde, and oxidized-LDL) could be examined as a window to postprandial metabolic stress.

Additionally, future postprandial studies could collect fecal samples as a means of directly demonstrating increased elimination of lipid in the feces and/or measure metabolic rate. This would help to confirm whether the decreased postprandial lipidemia was due to increased oxidation of lipid (increased clearance/metabolic expenditure/thermogenesis) or decreased absorption. Fecal and urine studies could also examine elimination of spice bioactives.

The notable effect of spices for inhibiting lipase has also been demonstrated in exploratory experiments with pancreatic secretory phospholipase A2 (IC50 = 16 µg/ml) and this creates another area of potential research. The ability of the spice blend to inhibit lipases combined with demonstrated effects for inhibiting NF-kB (245) and poor absorption (164, 258, 321) creates a novel agent for treating inflammatory bowel disease. Inflammatory bowel disease, the intestinal mucosa exhibits a chronic inappropriate inflammatory response. Current medications include aspirin derivatives (sulfasalazine/mesalamine) that coat the gastrointestinal tract and inhibit activation of inflammatory pathways (167). Spices may serve as protective agents by similar mechanisms (165, 174, 245, 322) to these gold standard medications, and blends and/or extracts could be developed that could be administered orally to reduce the oxidative stress and chronic inflammation of inflammatory bowel disease. Based on animal work and evidence pertaining to phenolics (134), spice blends could also reduce the incidence of colon cancer in inflammatory bowel disease patients (167, 323).

As is the case with omega-3 fatty acids, a model of human induced inflammation may be needed to demonstrate some of the health benefits of spices, and we did not observe measurable increases in inflammatory cytokine expression and plasma levels following the established model of psychological-stress induced inflammation in contrast to other reports (179, 186, 187, 197). Although other options may exist, the human model of endotoxemia is currently the most reproducible and established model of human inflammatory challenge (178).

We approached the concept of postprandial changes in blood bioactives by incorporating a serum on cells research models. The appendix describes the results of serum on cells
experiments performed in cultured macrophages treated with postprandial serum and stimulated with lipopolysaccharide as an inflammatory stimulus. Future analyses will also assess this model with oxidized low density lipoprotein as the inflammatory stimulus. A way to optimize this research model may be to extract serum (or plasma) prior to its administration to cultured cells. Although this method will require optimization and validation, one potential approach is to perform a simple solvent extraction. A phenolic extraction of the plasma performed with 80:20:0.1 (acetone: water: acetic acid) could be used to assess plasma phenol concentrations in terms of gallic acid equivalents. The phenolic extract could also be reconstituted in dimethyl sulfoxide and administered to cells in culture. This may address any variability introduced by plasma proteins. Alternatively, a total lipid extraction may be relevant in the context of the lipophilicity of spice antioxidants (as evidenced by high in vitro lipophilic ORAC values), but this method could potentially introduce some variability in the cell culture model, especially in the context of significant treatment effects on postprandial lipid concentrations. A phenolic extract methodology is recommended for follow up studies based on currently available results.

For the results reported in Chapter 5 (preliminary spice study), additional assays may be performed to assess lipid oxidation (170). These potential assays include 8-iso-Prostaglandin F2α, a non-specific marker of non-enzymatic lipid oxidation (324); malondialdehyde (thiobarbituric acid reactive substances), which is a marker of lipid peroxidation shown to be sensitive to spice treatment (246), and oxidized LDL-C (135, 136). Glutathione oxidation state (the ratio of oxidized to reduced glutathione) would provide insight to the effect of the spices on antioxidant enzyme systems. Additionally, lipoprotein associated phospholipase A2 could be examined in the postprandial state since inhibition of other lipase enzymes has been demonstrated in vitro. Current results primarily assessed the effects of the antioxidant intervention in the aqueous compartment because measurements focused on plasma changes.

For the results reported in Chapter 6, several additional analyses could be performed with the existing data and samples. It would be informative to assess whether changes in cortisol at 10 min relative to pre-stress are also related to changes in IL-6 protein concentrations and gene expression levels. Additionally, increases in cortisol may predict reductions in plasma TNFα concentrations at the last time point (2 h post stressor) (279, 280). Finally, changes in cortisol relative to reported perceived stress to examine whether perceived stress is correlated with cortisol induction would be a further analysis using these study data.

During the stressor and rest periods, blood pressure was also assessed continuously using the NexFin device, which also records hemodynamic measurements and a continuous EKG tracing. It may be possible to determine whether the increased heart rate during stressor tasks during the spice condition was related to a challenge response (increased heart rate/stroke volume) as opposed to a threat appraisal (increased total peripheral resistance) (325). Heart rate variability data could be analyzed, as well.
7.3 Conclusions

In conclusion, although these studies were intended to elaborate the potential anti-inflammatory effects of dietary omega-3 fatty acids and spices, both the omega-3 intervention and spice intervention demonstrated significant reductions in triglycerides. This effect merits future mechanistic investigation to explore the implications for human health.
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Appendix: The effect of postprandial serum samples in a cell-based assay of induced inflammation

Introduction

Studying the bioactivity time course of foods is complicated by the complexity in its composition. Rather than study bioavailability by measuring the concentration of individual compounds, an efficient bioactivity assay would be useful in screening the potential health effects of foods. This “serum on cells” model was applied as a means of assessing the potential anti-inflammatory effects of spice compounds present in postprandial serum.

Methods

Serums samples were obtained from the study protocol described in Chapter 5 from 6 healthy men following the consumption of a meal with and without spices. This serum was applied at 10% concentration to cultured THP-1 macrophages that were subsequently stimulated with LPS (10 ng/mL). Gene expression of COX-2, TNFα, and IL-6 was measured.

Results

There was no effect of the spice serum for reducing gene expression levels in this model, although TNFα showed a trend for a treatment by time point effect (p = 0.12) with an unadjusted comparison at the final time point of p = 0.01.

Discussion

Future analyses will evaluate whether oxidized LDL serves as a better stimulus for this model and whether trends in TNFα relate to trends observed in total oxygen radical absorbance capacity.
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