The Pennsylvania State University
The Graduate School
College of Health and Human Development

PLANT-DERIVED BIOACTIVES AND LONG-CHAIN OMEGA-3 FATTY ACIDS AS NUTRITIONAL INTERVENTIONS FOR CARDIOVASCULAR DISEASE RISK FACTORS

A Dissertation in
Nutritional Sciences and Clinical and Translational Sciences

by

Chesney Kirstin Richter

© 2016 Chesney Kirstin Richter

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

August 2016
This dissertation of Chesney K. Richter was reviewed and approved* by the following:

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

Gordon Jensen  
Senior Associate Dean for Research at the University of Vermont College of Medicine  
Professor of Medicine and Professor of Nutrition and Food Sciences

Sheila West  
Professor of Biobehavioral Health

Eric Loken  
Research Associate Professor of Human Development and Family Studies

Michael Green  
Interim Department Head and Professor of Nutritional Sciences

*Signatures are on file in the Graduate School
ABSTRACT
Nutritional interventions can be used to modify multiple risk factors for cardiovascular disease (CVD), which remains the leading cause of death in the United States and worldwide. This dissertation research investigated the effects of plant-derived bioactives and long-chain omega-3 fatty acids on CVD risk factors. The underlying premise of these studies was to improve intermediate CVD risk factors by adding beneficial dietary components to a deficient background diet. Excessive, non-resolving inflammation and oxidative stress are closely linked physiological processes that are key elements in the pathogenesis of many chronic diseases, particularly atherosclerotic CVD. Arterial stiffness, central blood pressure, and postprandial dysmetabolism are also emerging CVD risk factors that recent evidence suggests may be modifiable with dietary interventions. There is substantial epidemiological evidence that fruit and vegetable intake is associated with prevention of CVD, but the underlying mechanisms for these health benefits are not fully understood. Fruits and vegetables provide fiber, numerous vitamins and minerals, phytosterols, and other phytochemicals. It is likely that each component of this whole food matrix contributes protective effects; thus, a whole-food approach focused on the consumption of fruits and vegetables rather than isolated dietary compounds may be more effective for reducing CVD risk. Previous studies indicate that longer-term strawberry supplementation can improve CVD risk factors, but little is known about the postprandial effect of a single dose of strawberries incorporated into a meal. There is a large body of pre-clinical evidence demonstrating the anti-inflammatory potential of omega-3 fatty acids. However, results from clinical omega-3 supplementation trials have been disappointing with regard to inflammatory marker outcomes. Omega-3 supplementation studies may benefit from the use of an in vivo induced inflammatory challenge similar to those utilized in cell- and animal-based model systems. The low-dose human endotoxemia model (0.6 ng lipopolysaccharide per kg body weight) evokes a moderate systemic inflammatory response that is reflective of low-grade inflammation in cardiometabolic disease and provides a safe and broadly applicable model for studying novel therapeutics and dietary interventions.

The objective of the first study was to evaluate whether incorporating 40 g of freeze dried strawberry powder into a high fat meal altered postprandial vascular function or attenuated triglyceride, glucose, and insulin responses. Overweight but otherwise healthy adults (n = 30) consumed a control and strawberry meal in a randomized crossover design. Testing sessions were separated by ≥1 week for men and ~1 month for women to control for hormonal variations. Blood samples were obtained prior to the meal and 0.5, 1, 2, and 4 hours after the meal was consumed. Central blood pressure and arterial stiffness indices were measured at baseline and 2 and 4 hours post-meal, using pulse waveform analysis. There were no significant differences between the strawberry and control meals for any vascular function indices or metabolic responses. Consumption of either meal resulted in a significant decrease in the augmentation index (p < 0.002) and significant increases in serum triglycerides, insulin, and glucose compared to baseline (p < 0.001). Longer-term supplementation rather than a single acute dose of strawberries may be needed to improve CVD risk factors.

The second study investigated the effects of a pharmaceutical dose (3.4 g/d) of EPA+DHA on inflammatory responses to low-dose endotoxemia. This was a placebo-controlled, double-blind, randomized, crossover trial (8 wk treatment, 8 wk washout) comparing 3.4 g/d EPA+DHA to an olive oil control in 20 healthy young men. Low-dose endotoxemia produced a robust inflammatory response with consistent elevations in C-reactive protein, interleukin-6, and tumor necrosis factor-α. There was no difference in the peak concentration of any inflammatory markers following EPA+DHA supplementation compared to the
control (p > 0.05), despite significant increases in erythrocyte omega-3 fatty acid content. EPA+DHA supplementation reduced diastolic blood pressure (-2.5 mm Hg) compared to the placebo, and a produced a marginally significant reduction in triglycerides (-14.5 mg/dL) relative to baseline. Although EPA+DHA supplementation did not alter inflammatory responses to induced low-dose endotoxemia, omega-3 fatty acids may exert beneficial effects on inflammation via mechanisms other than cytokine production.

Finally, we analyzed the effect of EPA+DHA supplementation on acute oxidative stress responses in a model of induced inflammation (intravenous 0.6 ng/kg purified lipopolysaccharide [LPS], or endotoxin challenge) in two study populations. Urine concentrations of F_2-isoprostanes (F_2-IsoP), the F_2-isoprostane metabolite 2,3-dinor-5,6-dihydro-15-F_2t-IsoP (F_2t-IsoM), and F_3-isoprostanes (F_3-IsoP) were measured following low-dose endotoxin challenge. In Study 1, healthy young adults (n = 17 [10M]) were supplemented with 0, 300, 600, 900, or 1800 mg/d EPA+DHA for 5 months before undergoing a low-dose endotoxin challenge. In Study 2, healthy young men (n = 20) were supplemented for 8 weeks with 3.4 g/d EPA+DHA and an olive oil placebo in a crossover design, with low-dose endotoxin challenges following both supplementation periods. In Study 1, supplementation with omega-3 fatty acids (600-1800 mg/d) for 5 months attenuated total urinary isoprostane production following low-dose endotoxin challenge in a model that also included body mass index, despite elevated F_3-IsoP concentrations. In Study 2, eight weeks of 3.4 g/d EPA+DHA supplementation prior to low-dose endotoxin challenge similarly increased F_3-IsoPs but did not alter total isoprostane production. EPA+DHA supplementation increases F_3-IsoP production but may attenuate the total oxidative stress response (sum of isoprostane species) following low-dose endotoxin challenge. More research is needed to clarify whether a reduction in total isoprostane production is contingent on the duration of supplementation and/or the form of omega-3 fatty acids administered.
# TABLE OF CONTENTS

LIST OF FIGURES............................................................................................................................................. x
LIST OF TABLES.................................................................................................................................................. xii
LIST OF SUPPLEMENTAL TABLES.................................................................................................................... xiii
LIST OF SUPPLEMENTAL FIGURES................................................................................................................... xiv

Chapter 1. Introduction ........................................................................................................................................ 1
   1.1 Background and significance .................................................................................................................... 1
   1.2 Premise of dissertation .............................................................................................................................. 2
   1.3 Emerging CVD risk factors used in clinical nutrition research .............................................................. 2
      1.3.1 Postprandial dysmetabolism .............................................................................................................. 3
      1.3.2 Central blood pressure and arterial stiffness ...................................................................................... 3
      1.3.3 Markers of oxidative stress ................................................................................................................ 3
      1.3.4 Markers of inflammation .................................................................................................................. 4
   1.4 Study designs to investigate nutritional interventions for cardiovascular risk ..................................... 4
      1.4.1 Provision of nutritional interventions in the form of a supplement ............................................... 5
      1.4.2 Challenge models for nutrition research .......................................................................................... 5
         1.4.2.1 Oral fat challenge model .............................................................................................................. 6
         1.4.2.2 Low-dose human endotoxemia model of acute inflammation ................................................... 6
   1.5 Conclusion ................................................................................................................................................ 6

Chapter 2. Review of the literature .................................................................................................................... 7
   2.1 The roles of inflammation and oxidative stress in CVD ........................................................................ 7
      2.1.1 Markers of inflammation used in clinical research ........................................................................ 8
         2.1.1.1 Pro-inflammatory cytokines ....................................................................................................... 8
         2.1.1.2 C-reactive protein (CRP) .......................................................................................................... 8
      2.1.2 Markers of oxidative stress used in clinical research ...................................................................... 9
         2.1.2.1 Malondialdehyde (MDA) ......................................................................................................... 9
         2.1.2.2 Oxidized low density lipoprotein-cholesterol (ox-LDL) ......................................................... 10
         2.1.2.3 Isoprostanes ............................................................................................................................ 10
   2.2 Plant-based bioactives and cardiovascular disease risk factors ............................................................ 12
      2.2.1 Central blood pressure and arterial stiffness in clinical nutrition research ................................... 14
         2.2.1.1 Pulse wave analysis (PWA) ...................................................................................................... 15
Chapter 3. Incorporating freeze dried strawberry powder into a high fat meal does not alter postprandial vascular function or blood markers of cardiovascular risk. .............................. 47

3.1 Abstract .................................................................................................................. 47

3.2 Introduction .............................................................................................................. 48

3.3 Subjects and Methods ............................................................................................ 48

3.3.1 Study population .................................................................................................. 48

3.3.2 Participant recruitment ......................................................................................... 49

3.3.3 Study design and intervention ............................................................................... 50

3.3.4 Blood sample collection and assay methods ....................................................... 51

3.3.4.1 Biomarkers of oxidative stress ........................................................................ 52

3.3.4.2 Pancreatic lipase inhibition ............................................................................. 52

3.3.5 Vascular function measures ................................................................................. 52
Chapter 4. Omega-3 fatty acid supplementation does not alter inflammatory responses to low-dose endotoxemia in healthy young men despite increases in erythrocyte omega-3 fatty acid content........65

4.1 Abstract.................................................................65

4.2 Introduction........................................................................66

4.3 Subjects and Methods ..........................................................66

4.3.1 Study population...............................................................66

4.3.2 Participant Recruitment.......................................................67

4.3.3 Study design and intervention...............................................68

4.3.3.1 Low-dose endotoxin challenge ...........................................70

4.3.4 Blood sample collection and assay methods..........................71

4.3.4.1 Serum measures............................................................71

4.3.4.2 Red blood cell fatty acids..................................................71

4.3.4.3 Inflammatory cytokines......................................................72

4.3.4.4 Complete blood count and coagulation parameters...............72

4.3.5 Statistical Analyses............................................................72

4.4 Results.............................................................................72

4.4.1 Effect of EPA+DHA supplementation on blood pressure, lipids/lipoproteins, markers of inflammation, and leukocytes............................................................73

4.4.2 Effect of EPA+DHA supplementation on red blood cell (RBC) fatty acid composition ....74

4.4.3 Effect of EPA+DHA supplementation on inflammatory responses to low-dose endotoxin challenge ........................................................................................................78

4.4.3.1 C-reactive protein (CRP)......................................................78
6.2.2 Future directions

6.3 Conclusions

Bibliography
LIST OF FIGURES

Figure 2-1. Representative isoprostane compounds typically assessed in clinical nutrition research...... 11
Figure 2-4. Cytokine production following intravenous administration of LPS. Source: Lowry SF. Human endotoxemia: a model for mechanistic insight and therapeutic targeting. Shock 2005:24 Suppl 1:94-100 [45]. .................................................................................. 43
Figure 2-5. TNF-a (A), IL-6 (B), white blood cell (C), and CRP (D) production due to low-dose endotoxemia. Source: Mehta NN, et al. A human model of inflammatory cardio-metabolic dysfunction; a double blind placebo-controlled crossover trial. J Transl Med 2012;10:124.......................................................... 44
Figure 3-1. Schematic of participant recruitment and reasons for exclusion. ........................................ 50
Figure 3-2. Changes in vascular function indices following consumption of the control and freeze dried strawberry test meals. ........................................................................................................ 55
Figure 3-3. Change in triglycerides, glucose, and insulin following consumption of the control and freeze dried strawberry test meals........................................................................................................ 56
Figure 3-4. Peak triglyceride, glucose, and insulin responses to the strawberry and control meal for each participant ..................................................................................................................... 58
Figure 3-5. Postprandial triglyceride elevations in three participants who displayed an attenuated triglyceride response to the strawberry meal. ............................................................... 59
Figure 3-6. Postprandial insulin (left) and glucose (right) concentrations of select participants who exhibited attenuated responses to the strawberry meal. .................................................. 60
Figure 4-1. Schematic of subject recruitment and reasons for exclusion. ............................................... 68
Figure 4-2. Schematic of study procedures. ............................................................................................... 69
Figure 4-3. Schematic of low-dose endotoxin challenge visits. ................................................................. 70
Figure 4-4. Mean (±SEM) changes in red blood cell (RBC) long-chain omega-3 fatty acids and the Omega-3 Index.................................................. 75
Figure 4-5. Effect of supplementation order on the change in red blood cell (RBC) docosahexaenoic acid (DHA) values ................................................................................................................. 77
Figure 4-6. Effect of supplementation order on the change in the Omega-3 Index ....................................... 78
Figure 4-7. CRP response to low-dose endotoxin challenge for all twenty participants .......................... 79
Figure 4-8. Time course of C-reactive protein (CRP) production in response to low-dose endotoxemia.. 80
Figure 4-9. Peak 24-hour CRP (mg/L) responses to low-dose endotoxemia following placebo and EPA+DHA supplementation for each participant. .................................................................................................................................................................................. 81
Figure 4-10. Time course of TNF-α production in response to low-dose endotoxemia. .......................................................... 82
Figure 4-11. Time course of IL-6 production in response to low-dose endotoxemia...................................................... 83
Figure 5-1. Study design schematic for Study 1 (A) and Study 2 (B). .......................................................................................... 91
Figure 5-2. Effect of high versus low dose EPA+DHA supplementation on F3-isoprostanes. ............................ 95
Figure 5-3. Effect of EPA+DHA supplementation on F3-isoprostanes ................................................................. 95
Figure 5-4. Time course of F3-isoprostane (F3-IsoP) production in response to low-dose endotoxemia. 96
Figure 5-5. Interaction between EPA+DHA dose and BMI on total IsoP production .................................................. 97
Figure 5-6. Time course of F2-isoprostane (F2-IsoP; panel A), F2-isoprostane metabolite (F2-IsoP-M; panel B), and total isoprostane production (F2-IsoP + F2-IsoP-M + F3-IsoP; panel C) in response to low-dose endotoxin challenge .................................................................................................................. 98
## LIST OF TABLES

Table 2-1. Vascular function and arterial stiffness indices measured by the Sphygmocor System........ 16
Table 2-2. Studies evaluating the acute effects of a plant-based bioactive dietary intervention on central (aortic) pressures, augmentation index (Alx), and pulse wave velocity (PWV). .............................................. 19
Table 2-3. Studies evaluating the effects of a single acute dose of a dietary intervention on postprandial responses to an energy-dense, high-fat and/or high-carbohydrate meal.......................................................... 22
Table 2-4. Studies evaluating the effect of chronic strawberry supplementation on cardiovascular disease risk factors.................................................................................................................................................. 29
Table 2-5. Studies investigating self-reported dietary intake of fish and/or EPA+DHA and associations with inflammatory markers. ............................................................................................................................................................................. 36
Table 2-6. Studies evaluating effect of omega-3 fatty acid supplementation on isoprostane production. ........................................................................................................................................................................ 39
Table 3-1. Nutrient composition of meals. ........................................................................................................... 51
Table 3-2. Participant characteristics at screening (n = 30). ................................................................................. 54
Table 3-3. Baseline characteristics of participants at study visits (n = 30; 17 M, 13 F). ................................. 54
Table 4-1. Fatty acid composition (mg per 4 g daily dose) of the placebo and EPA+DHA intervention.... 69
Table 4-2. Pre-supplementation characteristics of participants who completed the study (n = 20). ........ 73
Table 4-3. Participant characteristics following supplementation (n = 20). ......................................................... 74
Table 4-4. Effect of omega-3 supplementation on red blood cell (RBC) membrane long-chain omega-3 fatty acid content. ........................................................................................................................................................................ 76
Table 5-1. Study design and characteristics of participants who completed Study 1 and Study 2. ........ 92
Table 5-2. Omega-3 fatty acid content (mg/d) of each supplement dose in Study 1 and Study 2............. 93
Table 6-1. Studies evaluating postprandial effects of meal consumption on central (aortic) pressures, augmentation index (Alx), and pulse wave velocity (PWV). ................................................................. 108
LIST OF SUPPLEMENTAL TABLES

Supplemental Table 3-1. Nutrient composition of freeze dried strawberry and control powders........... 63
Supplemental Table 3-2. Phytochemical content of freeze dried strawberry powder. ................................. 64
Supplemental Table 4-1. Nutrient composition of meals and snacks provided during low-dose endotoxin challenge. ............................................................................................................................................... 87
Supplemental Table 4-2. Liver enzymes, general chemistry panel measures, complete blood count, and coagulation parameters following supplementation. ......................................................................................... 88
LIST OF SUPPLEMENTAL FIGURES

Supplemental Figure 5-1. Histogram of total urinary isoprostanes in Study 1. ......................................................... 101

Supplemental Figure 5-2. Histogram of leverage values from the multivariate regression model of all participants in Study 1. ........................................................................................................... 102

Supplemental Figure 5-3. Effect of BMI on Total IsoP production by sex. ................................................................. 103

Supplemental Figure 5-4. Effect of EPA+DHA dose on Total IsoP production by sex................................................. 103

Supplemental Figure 5-5. Effect of BMI on Total IsoP production by sex. ................................................................. 104
Chapter 1. Introduction

1.1 Background and significance
Cardiovascular diseases (CVD) remain the leading cause of death in the United States [1] and worldwide [2], despite many advances in pharmacological and surgical treatment strategies. In the United States alone, the direct medical costs of CVD are projected to reach over $800 billion by 2030, with indirect costs from lost productivity adding a further $276 billion [3]. Thus, there is a need for additional strategies that can augment current standard-of-care pharmaceutical interventions and help to reduce CVD risk factors. Established intermediate CVD risk factors are characterized as non-modifiable (e.g., age, ethnicity, sex, and family history) or modifiable [4]. Modifiable risk factors can be altered by dietary and/or lifestyle changes and include: elevated total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), and triglycerides (TG), reduced high density lipoprotein-cholesterol (HDL-C), hypertension, diabetes mellitus, and overweight/obesity [5, 6]. This chapter briefly introduces considerations for nutritional interventions for CVD risk reduction and the premise of this dissertation.

Nutritional strategies are a fundamental component of CVD risk management [7-9], and in some instances, may be preferable to pharmaceutical strategies with regard to cost and potential side effects. Except in the case of food allergies and/or intolerance, the majority of nutritional strategies can be adopted with minimal risk. Conversely, pharmaceutical therapies are much more likely to cause side effects that reduce compliance or necessitate discontinuation. For instance, despite the well-established benefits of statin therapy for lowering low density lipoprotein-cholesterol (LDL-C) and overall CVD risk reduction, long-term adherence with statin regimens is poor and tends to decrease with time [10]. Although financial barriers can exist for nutritional strategies (e.g. the cost of fresh produce, oily fish, or fish oil supplements in low-income populations), costs are likely to be similar or higher for many medical therapies.

Great progress has been made in developing dietary recommendation and nutritional strategies to promote health and reduce the risk of chronic disease. The 2015 Dietary Guidelines for Americans recommends: an emphasis on vegetables and fruits, whole grains, fat-free or low-fat dairy, seafood, lean meat and poultry, eggs, beans, soy, and unsalted nuts and seeds; limiting refined grains, solid fats, and added sugars; reducing sodium intake; and reducing saturated fat (SFA) intake to < 10% of total calories, replacing it with unsaturated fats [11].

Dietary patterns that reduce CVD risk factors, as well as morbidity and mortality, have also been identified. For instance, the 2013 American Heart Association/American College of Cardiology (AHA/ACC) Guideline on Lifestyle Management to Reduce Cardiovascular Risk recommended the Dietary Approaches to Stop Hypertension (DASH) diet as an effective method of reducing blood pressure and LDL-C [12]. Other dietary patterns, such as the Mediterranean dietary pattern, have also been shown to reduce many CVD risk factors, as well as morbidity and mortality, in clinical intervention studies [13]. Although these dietary patterns have unique features, both emphasize nutrient-dense foods such as vegetables, fruits, whole grains, seafood, eggs, low-fat dairy, nuts, lean meats, and poultry while limiting saturated fat, trans fat, added sugars, and refined grains [13, 14]. It is likely that offering multiple evidence-based dietary pattern options will help individuals maintain a healthy diet and achieve maximal CVD risk reduction by allowing them choose a dietary pattern that more closely reflects their cultural and personal preferences.
However, there are remaining limitations in current dietary guidelines and nutritional strategies for CVD risk reduction. For instance, implementation of dietary guidelines remains problematic. Saturated fat consumption in the United States continues to remain greater than 10% of total calories, despite decades of recommendations to limit saturated fat intake [15]. Consumer misinterpretation of guidelines can also result in unintended consequences, as in the case of low-fat diet recommendations leading to greater refined carbohydrate consumption and subsequent negative health outcomes. Certain dietary patterns may also be considered overly restrictive by some individuals, such as the low-fat nature of the DASH diet, making long-term compliance difficult. Furthermore, nutritional strategies should also be designed to address emerging CVD risk factors (e.g., oxidative stress and inflammation) in addition to traditional blood pressure and LDL-C targets [16-19].

While the combination of foods comprising an overall dietary pattern likely confers synergistic and cumulative cardioprotective effects, it is also useful to determine the contribution of individual foods/food groups and the dietary bioactives that they provide. Two of the dietary bioactives with the strongest evidence base supporting their consumption are: 1) phytochemicals in fruits and vegetables and 2) long-chain omega-3 fatty acids in oily fish. Both of these dietary components are remarkably lacking in the average American diet despite strong evidence for their beneficial effects. The evidence base for recommending increased phytochemical and omega-3 fatty acid intake is reviewed further in Chapter 2. Despite relatively strong evidence that increasing the intake of plant-derived phytochemicals and omega-3 fatty acids is beneficial for preventing and/or reducing CVD risk, additional research is needed to better define the amounts and dietary sources that should be recommended, target populations that would receive the most benefit, mechanisms of action, and the strength of effects. Addressing these limitations will aid in the development of more precise, evidence-based dietary recommendations.

1.2 Premise of dissertation

Therefore, the purpose of this dissertation research was to investigate the effects of plant-derived bioactives and long-chain omega-3 fatty acids. In either case, a dietary intervention in the form of a whole food (i.e., strawberries) or a whole food extract (i.e., fish oil) was provided as a supplement to the background diet. This type of research design will improve our understanding of which CVD risk factors can be modified with these dietary bioactives within the context of the average American diet and lifestyle. These results will aid in the development of straightforward public health messages. The plant-based bioactive intervention was studied using a freeze dried strawberry powder that was incorporated into a meal. The omega-3 fatty acid intervention was studied using a prescription fish oil concentrate. Although these study designs differed in many aspects, the underlying premise of both interventions was to improve intermediate CVD risk factors by adding beneficial dietary components to a suboptimal Westernized dietary pattern.

1.3 Emerging CVD risk factors used in clinical nutrition research

In addition to the traditional established CVD risk factors, greater recognition has recently been given to the role of emerging risk factors, such as inflammation, oxidative stress, and metabolic derangements (i.e., elevations in insulin and glucose, or altered lipid/lipoprotein concentrations) that are not traditionally assessed in a standard fasting measurement.
1.3.1 Postprandial dysmetabolism
The term postprandial refers to the time period following the consumption of a meal. Although the majority of clinical assessments for cardiovascular risk are performed specifically in the fasting state, humans spend the majority of their waking hours in a postprandial state [20]. Transient exaggerated elevations in blood glucose and triglyceride concentrations caused by the consumption of a high-calorie meal composed of refined carbohydrates and saturated fat are termed “postprandial dysmetabolism” [21]. Evidence from both prospective observational studies and clinical trials has demonstrated that postprandial metabolic impairments are a major, independent risk factor for CVD. For instance, in the Diabetes Intervention Study, postprandial blood glucose predicted myocardial infarction and atherosclerosis, whereas fasting blood glucose did not [22]. Impaired glucose tolerance (IGT) is now a recognized feature of pre-diabetes and risk factor for CVD, and a standardized oral glucose tolerance test (OGTT) is used diagnostically [20]. Hyperlipidemia, as assessed by non-fasting triglyceride concentrations, has also been demonstrated to predict CVD risk in numerous prospective case-control studies [23]. Therefore, identifying individuals who have normal fasting triglycerides but impaired clearance of postprandial fat may provide analogous predictive value as testing for impaired glucose tolerance. Postprandial dysmetabolism can be modified by diet and lifestyle changes. Specific foods and spices that are rich in bioactives, such as cinnamon and nuts, have been shown to attenuate postprandial glucose responses [24-26]. Postprandial triglycerides, glucose, and insulin were evaluated in the study of freeze dried strawberry powder (Chapter 3).

1.3.2 Central blood pressure and arterial stiffness
Vascular health is recognized as an important CVD risk marker, as cardiovascular events are largely driven by atherosclerotic cardiovascular disease. Resting brachial blood pressure is the only measurement that is currently used diagnostically, but many other testing modalities are commonly used in the research setting to evaluate whether interventions can improve vascular health. These methods measure different aspects of both the large conduit arteries and the microvasculature as indicators of cardiac vascular health. For example, flow mediated dilation (FMD), a non-invasive index of endothelial health, has been widely used in nutrition research, and many bioactive-rich foods and beverages have been shown to improve FMD, including cocoa and chocolate [27-29], tea [30, 31], and soy isoflavones [32]. However, the accuracy and reproducibility of results derived from many of these methods are highly dependent on the skill and experience of the person obtaining and analyzing the measurement. Newer modalities, such as the Endopat and SphygmoCor systems, have been developed that are less operator dependent. This may increase reproducibility and provide more opportunity for between-study comparison. The SphygmoCor System (AtCor Medical) is one of the most widely used methods for non-invasive measurement of central blood pressure and measures of arterial stiffness [33]. Central blood pressure characteristics and indices of arterial stiffness derived from the SphygmoCor System were the primary outcomes for the study of freeze dried strawberry powder (Chapter 3). The Pulse Wave Analysis (PWA) and Pulse Wave Velocity (PWV) components of the SphygmoCor System, as well as previous studies investigating plant-based bioactives and arterial stiffness outcomes are discussed in more detail in Chapter 2.

1.3.3 Markers of oxidative stress
Oxidative stress is a key component in vascular dysfunction and the etiology of cardiovascular disease [34]. Reactive oxygen species (ROS) are products of normal cellular metabolism and have important biological functions in regulating redox-sensitive signal transduction pathways. Typically, they are produced in small quantities, and are safely regulated and neutralized by the endogenous antioxidant
defense system, which includes both enzymatic (e.g., glutathione peroxidase and glutathione reductase) and non-enzymatic antioxidants (e.g., glutathione, vitamins E and C, and beta-carotene). However, when the rate of ROS production exceeds the neutralizing capacity of antioxidants, oxidative stress occurs. Under these conditions, ROS can damage macromolecules such as lipids and proteins, with negative consequences for cell structure and function [34, 35]. Polyunsaturated fatty acids are primary targets for free radicals. Numerous byproducts of lipid peroxidation can be measured in plasma and urine to determine the relative amount of oxidative stress that an individual is experiencing, including oxidized LDL (ox-LDL), malondialdehyde (MDA), and isoprostanes. These concepts are discussed in greater depth in Chapter 2. Plasma ox-LDL and MDA were measured in the study of freeze-dried strawberry powder (Chapter 3), while urinary isoprostanes were analyzed in the study of omega-3 fatty acids (Chapter 5). Further discussion of the relationship between oxidative stress and cardiovascular disease, as well as a brief review of commonly used markers of oxidative stress, is provided in Chapter 2.

1.3.4 Markers of inflammation

Acute inflammation is a protective response to injury or infection that involves complex interactions between numerous cell types and inflammatory mediators (e.g., cytokines and eicosanoids) [36]. It is characterized by increased blood flow and membrane permeability that allows leukocytes to migrate from the bloodstream to the site of inflammation, where they can respond to the pathogens and/or affected cells. This process is responsible for the cardinal signs of inflammation: edema, redness, heat, pain, and loss of function. Because these cellular activities can also damage host tissues, they are self-regulated by negative feedback mechanisms that quickly resolve or terminate the inflammatory cascade. If these regulatory mechanisms are impaired, the inflammatory response is sustained and can develop into chronic inflammation, with substantial injury to host tissues. The systemic consequences of this excessive, unresolved inflammation are demonstrated by its central role in the etiology of numerous diseases, including cardiovascular diseases, obesity, cancer, inflammatory bowel diseases, rheumatoid arthritis, and asthma [36]. Many of the proteins that regulate the immune response can be used as non-specific markers of inflammation, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP) [37]. These markers are discussed in more detail in Chapter 2. Circulating concentrations of these inflammatory markers following low-dose endotoxemia were the primary endpoint for the omega-3 fatty acid intervention discussed in Chapter 4.

1.4 Study designs to investigate nutritional interventions for cardiovascular risk

Nutritional interventions can be designed and evaluated by using epidemiological analyses, placebo-controlled clinical trials, and mechanistic studies. These different but complimentary approaches each add unique elements to our understanding of nutritional strategies for reducing chronic disease risk. Epidemiological studies have provided important evidence demonstrating the benefit of dietary patterns and specific food groups. However, this type of data is limited by potential confounding from other lifestyle factors, and these types of studies are unable to demonstrate causation. Conversely, cell and animal models provide a highly controlled study system that is less prone to confounding from other variables. These types of studies are crucial for gaining a mechanistic understanding of nutritional interventions, but their findings may not be applicable in a clinical setting. Controlled clinical trials with hard endpoints (i.e., mortality or disease incidence) have traditionally been the gold standard for demonstrating causation between an intervention and clinical improvements. However, these types of studies are often very expensive and require longer durations for events to occur. Additionally, they are not necessarily well-suited for nutrition research as they are traditionally intended to test the effects (and
side effects) of pharmaceutical interventions, which are typically much stronger than those of nutritional interventions.

The limitations and discrepancies of these study designs can be bridged by the use of randomized controlled trials that employ intermediate risk factors as outcomes. For instance, epidemiological findings can be strengthened by demonstrating a causal effect of the intervention on risk factors and provide evidence that other confounding lifestyle factors were not responsible for the observed benefits. Demonstrating effects on intermediate risk factors can also provide potential mechanisms for results found in hard endpoint trials. These studies further offer a means of translating mechanistic results from *in vitro* cell and/or animal models to clinical outcomes. Taken as a whole, these study designs all provide valuable information for the development of precise, evidence-based dietary recommendations.

1.4.1 Provision of nutritional interventions in the form of a supplement

One of the approaches used in randomized controlled trials is to provide the nutritional intervention as a supplement to the background diet. Although the goals of dietary recommendations are to improve the overall intake of the general public, comprehensive dietary changes are often very difficult to achieve and maintain. Controlled feeding studies provide a unique means of isolating and testing the effects of specific nutrient profiles and/or particular foods within the overall diet. However, under “real world” conditions, many people do not elect to adopt or are unable to maintain diets with such a rigid structure. For instance, adherence to intervention diets tends to deteriorate under free-living conditions when less structure is provided by researchers/dieticians [38]. It is difficult to fully achieve prescribed dietary intake goals of intensive behavioral modification programs even when participants are provided with frequent nutrition counseling [39]. Conversely, a large percentage of U.S. adults report current daily supplement use [40, 41]. Therefore, a whole food-based intervention that is designed to supplement or replace a small component of the current background diet may be more easily translated into effective public health recommendations.

1.4.2 Challenge models for nutrition research

Most intervention research focuses on modifying CVD risk factors that are measured in a fasted, resting state. For example, guidelines call for measuring blood lipids following an overnight fast, and blood pressure following a 5 minute rest period. This approach seeks to control the variability that an individual experiences throughout the day. Measurements performed in the resting state provide valuable information about an individual’s current physiological status and quantitatively evaluate the influence of environmental or lifestyle factors (e.g., smoking, obesity, age, physical inactivity, genetics, etc.). However, these measurements provide little insight into how an individual’s system responds when it is stressed or otherwise physiologically challenged.

Dynamic responses to an acute stressor may provide a more sensitive indication of the effect of nutritional interventions on CVD risk factors [37, 42]. Numerous challenge models have been developed and can be classified by the primary type of stimuli: oral fat or glucose challenge; injection of lipopolysaccharide (LPS); early response to vaccines; tissue damage (e.g., acute exercise or suction blister); or psychological stress [37]. Each of these models has unique advantages, limitations, and applications in which it is most informative. An oral high fat challenge (discussed further below) was used in the study of freeze dried strawberry powder presented in Chapter 3. A low-dose endotoxemia model of acute inflammation (discussed further below) was used in the study of omega-3 fatty acids presented in Chapters 4 and 5.
1.4.2.1 Oral fat challenge model
An oral fat challenge induces metabolic stress that is characterized by postprandial spikes in triglycerides, glucose, and insulin. Exaggerated postprandial elevations in triglycerides and glucose following the consumption of a meal are associated with significantly increased CVD risk (discussed further below). It has been proposed that non-fasting (i.e., postprandial) triglyceride concentrations are more predictive of CVD risk than a single fasting triglyceride measurement [23]. Thus, this model provides the opportunity to investigate whether a nutritional intervention can reduce CVD risk by attenuating post-meal elevations in triglycerides and glucose. Currently, there is no standardized methodology for the design and implementation of an oral fat challenge, which creates some difficulty for comparing results among studies that have used somewhat different oral fat challenges. However, it has been suggested that a standardized protocol should include a lipid load of 50-60 g total fat, to be administered to a fasted individual as a breakfast meal with a fatty acid and overall macronutrient composition representative of a typical Westernized meal (40-100 g carbohydrate and 10-20 g protein) [43]. Higher lipid loads are not physiological and are likely to cause unrepresentative postprandial responses due to delayed gastric emptying [43]. A fasting blood sample followed by hourly blood sampling throughout the postprandial period up to 6-8 hours post-test meal are recommended for research purposes. Peak triglyceride concentrations are reached approximately 4 hours following meal consumption; thus, triglyceride concentrations at this time point are most informative with regard to CVD risk [43].

1.4.2.2 Low-dose human endotoxemia model of acute inflammation
Purified Escherichia coli-derived endotoxin, or lipopolysaccharide (LPS), is a major component of the outer membrane of gram-negative bacteria. Intravenous administration of LPS activates the innate immune response and induces characteristic elevations in inflammatory mediators, as well as changes in glucose and lipoprotein metabolism [44, 45]. This model was first developed using higher doses (3-5 ng LPS/kg body weight) to investigate the pathophysiological derangements observed in sepsis. Lower doses (0.2-2 ng LPS/kg body weight) are currently used most commonly to produce a transient model of moderate systemic inflammation [44]. This provides the opportunity to investigate whether nutritional interventions can alter the production of pro-inflammatory or pro-resolving signals; thus providing a means of evaluating a potential nutritional strategy for preventing the development of inflammation. Further discussion of the low-dose human endotoxemia model is provided in Chapter 2.

1.5 Conclusion
The studies presented in this dissertation contribute to the understanding of how nutritional interventions, specifically plant-based bioactives and long-chain omega-3 fatty acids, can modify established and emerging CVD risk factors. The following chapter reviews the existing literature on the effects of plant-based bioactives and omega-3 fatty acids on intermediate cardiovascular risk factors. Chapter 3 presents the postprandial vascular and metabolic effects of incorporating freeze dried strawberry powder into a high-fat meal. In Chapter 4, the effect of a prescription dose omega-3 fatty acid supplementation on red blood cell fatty acids and inflammatory responses to low-dose endotoxemia are presented. Chapter 5 presents the effect of EPA+DHA supplementation on oxidative stress, in terms of urinary isoprostane production, following low-dose endotoxemia. A summary of this dissertation research, limitations, and future directions are reviewed in Chapter 6.
Chapter 2. Review of the literature

This chapter reviews critical evidence informing the design of the studies presented in Chapters 3-5. Section 2.1 briefly reviews the role of inflammation and oxidative stress in CVD, as well as the markers of inflammation and oxidative stress commonly used in nutrition research. Section 2.2 discusses the effects of plant-based bioactives on intermediate CVD risk factors, including vascular health and postprandial responses, with particular focus on strawberries and the existing evidence regarding their use as a nutritional intervention in clinical studies. The effects of omega-3 fatty acids on inflammation and oxidative stress are reviewed in Section 2.3, which also includes a discussion of the human model of endotoxemia and its utility in studying omega-3 fatty acids and inflammatory responses.

2.1 The roles of inflammation and oxidative stress in CVD

Inflammation and oxidative stress are closely linked physiological processes. For instance, oxidative stress results in the activation of redox-sensitive signaling pathways that include nuclear factor kappa B (NFκB) [46], which serves as the general transcription factor for pro-inflammatory cytokines, chemokines, and adhesion molecules. The production of cytokines and adhesion molecules results in the activation of leukocytes and their subsequent infiltration into surrounding tissues. The production of ROS is an inherent function of activated immune cells and a critical component of the innate immune response to an invading pathogen. When activated leukocytes are recruited to the site of infection or tissue damage, the abrupt increase in oxygen utilization by these cells results in a “respiratory burst”, which refers to the enhanced release and accumulation of ROS. These ROS have both anti-microbial properties and secondary messenger functions that enhance the expression of inflammatory mediators [47, 48]. Thus, oxidative stress and inflammation are two components of a self-perpetuating cycle in which both processes can trigger and amplify one another [35].

Excessive, non-resolving inflammation and oxidative stress are both key elements in the pathogenesis of many chronic diseases, particularly atherosclerotic CVD. Atherosclerosis was once considered simply a lipid storage disease; however, it is now understood that low-grade vascular inflammation is a fundamental component in all stages of atherosclerosis [49], and that lipid accumulation and inflammation occur concomitantly in the artery wall. Thus, rather than replacing previous hypotheses for atherogenesis, inflammation provides a mechanistic pathway linking traditional risk factors to modifications in the artery wall that give rise to atherosclerosis, as reviewed by Libby et al. [50]. Products of oxidative stress, such as oxidized LDL (ox-LDL), also play a key role in atherogenesis.

Atherogenesis begins with the formation of a fatty streak, which is a small sub-endothelial deposit of lipid-filled macrophages or foam cells [51]. Under non-inflamed conditions, leukocytes do not adhere to the endothelium and thus remain in circulation. Formation of the fatty streak begins when the vascular wall is damaged by traditional atherosclerotic risk factors (e.g., dyslipidemia, hypertension, smoking, etc.). This results in endothelial dysfunction and the expression of adhesion markers on the surface of endothelial cells that aid in the recruitment of monocytes and T lymphocytes that accumulate in early atherosclerotic plaque [51, 52]. Monocytes bound to the endothelium can then transmigrate into the intima in response to signaling from chemokines, such as monocyte chemoattractant protein-1 (MCP-1) [53]. Once in the arterial intima, monocytes differentiate into macrophages that scavenge and remove damaged cells, such as ox-LDL. The accumulation of ox-LDL particles results in the transformation of macrophages into foam cells [52]. Deposition of these cells in the intima forms a nascent atheroma that is a hallmark of early atherosclerosis. As the inflammatory process continues, these macrophages also
promote the recruitment of smooth muscle cells that contribute to the dense extracellular matrix that is characteristic of a more advanced atherosclerotic lesion. Finally, macrophages also contribute to the rupture of atherosclerotic plaque and its subsequent thrombotic complications. Proteolytic enzymes are released by these macrophages within the atheroma that degrade the collagen in the plaque’s fibrous cap, weakening the cap and making it susceptible to rupture. Circulating markers of inflammation and oxidative stress can thus be measured as an indicator of CVD risk (discussed below).

2.1.1 Markers of inflammation used in clinical research

Many of the proteins that regulate the immune response can be used as non-specific markers of inflammation. The most well-established markers of inflammation in clinical research are tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP) [37].

2.1.1.1 Pro-inflammatory cytokines

TNF-α and IL-6 are both secreted by immune cells in response to an inflammatory trigger and serve numerous functions in the inflammatory response. TNF-α increases vascular permeability to facilitate the migration of neutrophils and monocytes to the site of injury, induces platelet aggregation to contain an infection, and elevates body temperature [54]. IL-6 similarly functions to amplify the localized inflammatory response by stimulating the production of other inflammatory mediators [54]. TNF-α and IL-6 also exert systemic effects when they spillover into circulation from the site of inflammation. For instance, IL-6 stimulates the production of the acute phase protein CRP by the liver [54]. Chronic TNF-α and IL-6 elevations contribute to host tissue destruction and are a characteristic feature of many chronic diseases. However, they tend to fluctuate rapidly and are present in very low concentrations in the absence of an acute inflammatory event. Therefore, their use as inflammatory biomarkers is restricted primarily to research, rather than clinical applications.

2.1.1.2 C-reactive protein (CRP)

CRP assists in the removal of dead or dying cells, as well as some types of bacteria, by binding to those cells and marking them for destruction by macrophages. CRP elevations are caused by both chronic inflammatory conditions and acute injury or infection. CRP values can be increased several hundredfold by acute injury or infection (i.e., viral illness, bacterial infection, and physical trauma), whereas the CRP values that are reflective of low-grade chronic inflammation are much lower. Chronic inflammatory conditions such as inflammatory bowel disease, rheumatoid arthritis, long-term alcoholism, and abdominal obesity can also cause mild to moderate CRP elevations [55].

CRP can also be modified by anti-inflammatory therapies. In individuals with rheumatoid arthritis, the use of anti-TNF biologics has been shown to reduce CRP concentrations and other intermediate CVD risk factors [56-58]. Furthermore, statin therapy has been shown to reduce CRP as well as LDL-C in multiple large clinical trials [59-62]. In the Pravastatin or Atorvastatin Evaluation and Infection Therapy—Thrombolysis in Myocardial Infarction 22 (PROVE-IT—TIMI 22) study, individuals who achieved both the target LDL-C and target CRP concentrations experienced the greatest reduction in recurrent myocardial infarction or coronary death [63].

CRP is the only inflammatory marker recognized by the American Heart Association (AHA) for assessing cardiovascular risk in intermediate-risk individuals [18, 64]. In numerous epidemiological studies, CRP has been shown to be a strong predictor of future CVD events, including myocardial infarction, stroke, peripheral arterial disease, and sudden cardiac death, in apparently healthy men and women [65]. For
instance, in the Physicians’ Health Study and the Women’s Health Initiative, baseline CRP values were significantly higher in men who later experienced a myocardial infarction or stroke [66], and in women who subsequently suffered a CVD event, compared to those who remained free of CVD [67].

Based on the population distribution of CRP, the cut points for CVD risk assessment endorsed by the AHA are < 1.0 mg/L (low risk), 1.0-3.0 mg/L (average risk), and > 3.0 mg/L (high risk) [55]. Because CRP can be transiently elevated by an acute inflammatory trigger, if a CRP value greater than 10 mg/L is obtained, it is recommended that the measurement be repeated and the individual examined for sources of infection or inflammation. Similarly, it is often recommended that the average of two measurements, taken 2 weeks apart, be used to define an individual’s steady-state CRP concentration [55]. Thus, CRP is both a useful biomarker of inflammation and a clinically relevant indicator of cardiovascular risk that provides a reliable measure of underlying systemic inflammation.

2.1.2 Markers of oxidative stress used in clinical research

Lipids, particularly polyunsaturated fatty acids, are a common target of oxidative stress. The non-enzymatic oxidation of PUFA, or lipid peroxidation, can be stimulated by ROS, chelated metal ions (e.g., iron and copper), or fatty acid radicals. This process is comprised of three general steps: initiation, propagation, and termination [68]. These reactions generate a variety of products that can be measured in biological fluids as a measure of oxidative stress, and many have been directly implicated in the pathogenesis of disease. Because of the variety of lipid peroxidation products, one single assay may be insufficient and measurement of several markers likely provides a more accurate indication of oxidative stress and cardiovascular risk [69]. The markers of oxidative stress that are commonly used in nutrition research are discussed further below.

2.1.2.1 Malondialdehyde (MDA)

Malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation [70], and is one of the most routinely used biomarkers of oxidative stress and lipid peroxidation [71, 72]. MDA is also strongly associated with many diseases. For instance, individuals with different forms of cancer [71], type 2 diabetes [73], and congestive heart failure [74] have been shown to have higher amounts of MDA compared to healthy individuals. MDA was also predictive of cardiovascular events in individuals with stable coronary artery disease [75]. The most commonly used method of measuring MDA in biological samples is termed the thiobarbituric acid (TBA) reactive substances (TBARS) colorimetric assay. This assay is based on the reaction between MDA and TBA that generates a red, fluorescent compound that is then quantified by spectrophotometry. The TBARS test allows for simultaneous analysis of multiple samples and rapid quantification. However, there are many limitations in the TBARS assay that should be appreciated [70, 71]. The TBARS assay is inherently non-specific for MDA as TBA readily reacts with other compounds, and non-MDA products can interfere with the determination of true MDA content. Due to this lack of specificity, results may be more accurately expressed as “thiobarbituric reacting substances” rather than MDA. Furthermore, the assay requires high temperature and acidic conditions that may generate additional, artefactual MDA and overestimate results [70]. Based on these limitations, some have questioned the utility of MDA and the TBARS test as quantitative index of lipid peroxidation and suggest that this method offers a somewhat narrow window of insight into the complex process of fatty acid oxidation [72]. Nonetheless, this method remains widely used in clinical studies and can provide valuable information if results are interpreted carefully and used in combination other markers of lipid peroxidation [71, 76].
2.1.2.2 Oxidized low density lipoprotein-cholesterol (ox-LDL)
The term oxidized low density lipoprotein-cholesterol (ox-LDL) refers to an oxidatively modified particle derived from circulating LDL. Oxidation of LDL is a complex process in which both the protein and lipid components of the lipoprotein particle undergo oxidative changes [77]. This process likely occurs once the LDL particle is within the intima of the vessel wall rather than circulating in plasma [78-80], and begins with PUFA on the surface of the phospholipid, quickly spreading to involve the bulk of lipids in the LDL core [78]. Thus, how susceptible an LDL particle is to oxidation depends in part on the fatty acid composition of the LDL particle, which in turn is influenced by dietary factors, particularly antioxidant and fat intake. For instance, modifying dietary fat intake can alter the number of LDL particles present in circulation and dietary antioxidants may modulate pro-oxidant activity [78]. Plasma ox-LDL concentrations are frequently used as a marker of oxidative stress and have been positively associated with many forms of heart disease. For instance, plasma ox-LDL has been found to be markedly elevated in patients with acute myocardial infarction compared to individuals with stable and unstable angina [81]. Furthermore, ox-LDL content in plaque may relate to plaque instability as ox-LDL concentrations have been shown to exhibit temporal elevations following acute coronary events, such as myocardial infarction [82].

2.1.2.3 Isoprostanes
Isoprostanes (IsoPs) are prostaglandin-like molecules formed via non-enzymatic, free radical-induced lipid peroxidation. IsoPs were first discovered in 1990, with the detection of 15-F_{2t}-isoprostane (F_{2t}-IsoP) derived from arachidonic acid (AA) [83]. Additional related compounds formed from EPA and DHA—termed 15-F_{2t}-isoprostane (F_{3t}-IsoP) and 15-F_{4t}-isoprostane (F_{4t}-IsoP), respectively—were subsequently identified [84, 85]. Unlike cyclooxygenase (COX)-derived prostaglandins, IsoPs are initially formed in situ, from fatty acids in the phospholipid backbone of lipids. They are then released as free acids by phospholipase. Free IsoPs circulate in plasma, are metabolized in the kidney, and are excreted in urine. F_{2t}-IsoPs are chemically stable and ubiquitous in the body. Plasma and urine measurements are most common in clinical studies as the collection of these samples is minimally invasive, but they also be analyzed in any tissue, cerebral spinal fluid, exhaled breath condensate, amniotic fluid, and saliva [86]. Assessment of the major F_{2t}-IsoP metabolite 2,3-dinor-5,6-dihydro-15-F_{2t}-isoprostane (15-F_{2t}-IsoP-M) in urine is also recommended as it has been shown to be a similarly sensitive measure of in vivo oxidative stress [87]. The isoprostane compounds typically assessed in clinical research are depicted in Figure 2-1. With regard to their biological functions, the F_{2t}-IsoPs can induce vasoconstriction and platelet aggregation, inhibit angiogenesis, increase mean systemic arterial pressure, and promote atherosclerosis by stimulating monocyte and neutrophil adhesion to endothelial cells [86, 88]. Conversely, F_{3t}-IsoPs derived from EPA are less biologically active and do not exert the same effects on the vasculature or platelet activity [88].
In the 25 years since their discovery, substantial evidence from both animal and clinical studies has demonstrated the reliability of F\textsubscript{2}-IsoPs as a biomarker of endogenous lipid peroxidation [89, 90]. F\textsubscript{2}-IsoPs are elevated in conditions associated with enhanced oxidative stress and risk of CVD, such as smoking [91, 92], type 2 diabetes [93-95], hypercholesterolemia [96], and overweight or obesity [97, 98]. F\textsubscript{2}-IsoPs are also elevated in ischemia/reperfusion and may be predictive of poor post-operative outcomes in surgical settings where increases in oxidative stress due to ischemia/reperfusion are thought to contribute to post-operative complications, such as coronary artery bypass grafting or cardiopulmonary bypass surgery [86].

The relationship between diet and F\textsubscript{2}- IsoP concentrations have been evaluated in multiple studies. In a longitudinal cohort study of over 2500 adults, a dietary pattern high in fruits and vegetables and low in red meat was associated with lower plasma F\textsubscript{2}-IsoPs [99]. With respect to dietary interventions, studies have focused primarily on vitamin E, due to its antioxidant capacity. Results from vitamin E supplementation studies have been inconsistent, and direct comparisons are made difficult by variation in the dose, supplementation duration, biological fluid analyzed (i.e., plasma, urine, etc.), and the IsoP compound analyzed (i.e. F\textsubscript{2}-IsoP, F\textsubscript{2}-IsoP-M, F\textsubscript{3}-IsoP, etc.) among studies [86, 100]. However, daily supplementation with 1600 IU of vitamin E for 8 weeks was found to reduce plasma F\textsubscript{2}-IsoP [101], and lower doses may be effective in populations with elevated oxidative stress, where lipid peroxidation is expected to be higher [102]. There is limited evidence that anthocyanins may similarly reduce F\textsubscript{2}-IsoP concentrations [103, 104].
2.2 Plant-based bioactives and cardiovascular disease risk factors

There is substantial epidemiological evidence that fruit and vegetable intake is associated with health benefits, particularly the prevention of cardiovascular diseases. For instance, in meta-analyses of cohort studies, greater fruit and vegetable intake has been associated with reduced risk for coronary heart disease [105, 106] and stroke [107, 108]. In particular, in a meta-analysis of 13 prospective cohorts comprising over 275,000 individuals, the consumption of more than 5 servings/day of fruits and vegetables was associated with a 17% reduction in coronary heart disease risk [106]. Based on the strength of these associations, dietary guidelines recommend greater fruit and vegetable consumption.

However, the mechanisms underlying these health benefits are not fully understood. In addition to fiber and numerous vitamins and minerals, fruits and vegetables also provide phytosterols—the plant analog of cholesterol—and other phytochemicals (i.e., bioactive non-nutritive compounds found in fruits, vegetables, whole grains, and other plant foods). It is likely that each component of this whole food matrix contributes protective effects, and the health benefits of fruits and vegetables cannot be attributed to a single dietary compound. For instance, soluble fiber has well-established effects on blood pressure, cholesterol, and glycemic control [109]. Similarly, phytosterols are also known for their cholesterol-lowering effects [110-112]. Phytochemicals—which can be classified as carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds [113] (Figure 2-2)—have potent antioxidant activity and can effectively scavenge reactive oxygen species [114] and inhibit lipid peroxidation in vitro [115]. As oxidative stress is a key driver in the development of many chronic diseases, it is likely that phytochemicals also contribute to the health benefits associated with fruit and vegetable intake [116]. Furthermore, many epidemiological studies have found an inverse relationship between CVD and polyphenol intake, as well as the consumption of particular polyphenol-rich foods and beverages [117-121]. This has led to great interest in identifying particular bioactive compounds capable of reducing CVD risk. Although more than 5,000 individual phytochemicals have been identified, it is thought that a large percentage remain uncharacterized.
However, supplementation with an isolated micronutrient such as vitamin E, vitamin C, and/or beta-carotene has been largely unsuccessful in intervention trials [122-125]. In the Heart Outcomes Prevention Evaluation (HOPE) Study, supplementation with vitamin E for 4.5 years had no effect on cardiovascular outcomes in individuals at high risk of cardiovascular events [126]. Similarly, in the Heart Protection Study, supplementation with vitamin E, vitamin C, and beta-carotene had no effect on mortality or incidence of any type of vascular disease [127]. Supplementation with beta-carotene may even increase the risk of death from CVD in smokers and individuals exposed to asbestos [128]. Thus, it is likely that the beneficial effects of fruits and vegetables do not rely on well-known antioxidants (i.e., vitamin E, vitamin C, and beta-carotene), but rather on lesser characterized and/or unidentified bioactives, or a concerted action of multiple compounds.

A whole-food approach focused on the consumption of fruits and vegetables rather than isolated dietary compounds may be more effective for reducing CVD risk. The macro- and micronutrient content of fruits and vegetables is relatively well known, but this does not account for the thousands of phytochemicals that they provide—many of which have yet to be identified or characterized. Outside of the whole food matrix, a purified phytochemical may lose its bioactivity or behave in a different manner. The combination of phytochemicals in a particular fruit or vegetable is critical. For instance, the vitamin C content of an apple has been shown to account for less than 0.4% of the antioxidant capacity of a whole apple [129]. This may explain why a single, purified antioxidant cannot replace the natural combination of bioactives in a whole fruit or vegetable [116]. Furthermore, the consumption of fruits in combination may further enhance antioxidant activity. Liu et al. demonstrated the combination of apple, blueberry, grape, and orange resulted in a synergistic effect on antioxidant activity compared to the any of the fruits alone [113].
Therefore, consuming a variety of nutrient-dense fruits and vegetables will provide numerous bioactive compounds that may have additive and/or synergistic effects and result in greater CVD risk reduction.

CVD risk can be reduced by consuming a diet rich in fruits and vegetables and other nutrient-dense foods. For instance, the DASH Diet and the Mediterranean-style dietary pattern—both of which are diets that emphasize the consumption of nutrient-dense foods such as vegetables, fruits, whole grains, seafood, eggs, low-fat dairy, nuts, and lean meat—can improve many CVD risk factors, including blood pressure and LDL-C [13, 130, 131]. There is also evidence that specific foods rich in dietary bioactives, such as nuts [132-134], legumes [135, 136], omega-3 fatty acid concentrates from oily fish [137], and berries [138] can improve intermediate CVD risk factors. Arterial stiffness and central blood pressure are emerging CVD risk factors that recent evidence suggests may be modifiable with plant-based bioactives. This evidence is discussed further in Section 2.2.1. Strawberries are a particularly good source of phytochemicals and emerging evidence suggests that greater strawberry consumption may improve many CVD risk factors. The phytochemical content of strawberries and the epidemiological and clinical evidence for their cardioprotective effects are discussed below in Section 2.2.3.

2.2.1 Central blood pressure and arterial stiffness in clinical nutrition research

Although resting brachial blood pressure is a key component of risk assessment [139], central or aortic blood pressure may improve risk stratification and better predict CVD outcomes. Conventional cuff sphygmomanometer measurements of brachial systolic and diastolic pressure have traditionally been assumed to accurately reflect the pressure load in large conduit arteries, despite the fact that they are measured in a peripheral limb. Although this assumption largely holds true for diastolic pressure measurements, the arterial pulse is modified as it travels away from the heart to the periphery; thus, systolic pressure and pulse pressure in the brachial vasculature is generally not the same as that in the central aorta. Due to anatomical proximity, the central aortic pulse pressure more closely reflects the pulsatile stress experienced by organs such as the heart, brain, and kidneys than the peripheral pulse pressure [140]. Numerous studies have demonstrated that measurement of central blood pressure provides greater predictive value and may respond differently to an intervention than brachial blood pressure. For instance, in the Strong Heart Study, central pulse pressure was a better predictor of cardiovascular events, vascular hypertrophy, and the extent of atherosclerosis in 3520 individuals than brachial pulse pressure [141]. Similarly, in the Conduit Artery Function Evaluation (CAFÉ) Study, it was shown that different blood pressure-lowering medications can have substantially different effects on central aortic pressure—despite producing similar reductions in brachial pressures—and that this may better predict clinical outcomes, such as total cardiovascular events and the development of renal impairment [142, 143]. The use of central blood pressure to guide hypertension management has also been shown to reduce the need for medication to control brachial pressure and resulted in significantly greater cessation of medication compared to individuals treated according to standard-of-care practices [144]. Diagnostic thresholds for hypertension based on central blood pressure have also been defined and validated [145].

Central pressures can be measured non-invasively from peripheral pressure wave forms by using a Fourier generalized transfer function [33]. These mathematical equations have been derived from studies in which the central ascending aortic waveform is obtained invasively while the peripheral waveform is measured simultaneously [146]. The SphygmoCor System (AtCor Medical) has become one of the most widely used methods for non-invasive measurement of central blood pressure and measures of arterial stiffness [33]. The Pulse Wave Analysis (PWA) and Pulse Wave Velocity (PWV) components of the
SphygmoCor System are described in more detail in the following sections, and the full list of outcome measurements obtained from the SphygmoCor System are explained in Table 2-1.

2.2.1.1 Pulse wave analysis (PWA)
Pulse wave analysis is conducted using measurements obtained from a brachial blood pressure cuff and a generalized transfer function to derive central blood pressure characteristics. As the incident pulse generated by the contraction of the left ventricle travels towards the periphery, part of this wave is reflected back and merges with the incident wave. Thus, the central pulse pressure wave is formed by the combination of the incident wave generated by the left ventricle in systole and the wave that is reflected back from the periphery. In healthy, elastic arteries the reflected wave travels slower and returns later, merging with the incident wave at diastole. As arteries become stiffer, the rate and amplitude of the reflected wave are increased and the reflected wave returns to the heart sooner, merging with the incident wave during systole, prior to aortic closure [147]. This results in an increase in central systolic pressure and greater left ventricular afterload that accelerates left ventricular hypertrophy [146]. This increase in pressure is expressed as the augmentation index (AIx). The augmentation index depends primarily on: 1) the timing of the reflected wave, which is determined by pulse wave velocity and arterial stiffness, and 2) the intensity or amplitude of the reflected wave, which is determined largely by peripheral resistance. Thus, larger AIx values indicate greater wave reflection and/or earlier return of the reflected wave as a result of increased pulse wave velocity due to arterial stiffness. Higher AIx values are associated with coronary artery disease, cardiovascular risk, and other important cardiovascular intermediate endpoints [33].

2.2.1.2 Pulse wave velocity (PWV)
Pulse wave velocity (PWV) is a measure of the time required for a pulse wave to travel from one point to another [146]. The SphygmoCor PWV is performed by simultaneously recording the wave forms at the carotid and femoral arteries. Pulse wave velocity is dependent on the stiffness of the vasculature, with PWV increasing with greater vessel stiffness. PWV was associated with coronary artery plaque burden in a cross-sectional analysis, and has been shown to be a strong predictor of mortality in multiple longitudinal studies [146].
Due to rising appreciation of the role of arterial stiffness in CVD, there has been a rapid increase in the number of devices available to assess arterial stiffness. Although these devices provide similar indices, they are not necessarily interchangeable and differ in many methodological respects [148]. Thus, standardization of measurement techniques for PWV and AIx is crucial. Although the SphygmoCor System is less operator-dependent than some testing modalities (e.g. FMD, which requires a highly trained sonographer), proper training and familiarization with the device is critical for obtaining precise and accurate values, particularly for PWV measurements. Multiple expert consensus reports have been issued to address the methodological issues related to measurements of PWV [148-150]. For instance, it is recommended that measurements be performed in a quiet, temperature-stable room following a minimum of 10-15 minutes of rest in the supine position. Intra-individual measurements should also be performed at the same time of day as arterial stiffness exhibits diurnal variations. Participants should be familiarized with the SphygmoCor devices and techniques prior to any endpoint measurements to reduce the potential for “white coat” effects. Furthermore, it is recommended that at least one measurement be performed prior to the start of data collection to minimize any surprise reactions experienced by the participants [148]. Accuracy of the PWV depends on the precision of the distance measurements between the carotid and femoral arteries, and small inaccuracies may influence the absolute value of the PWV. Distances should be measured as a straight line so as to approximate the true length of the path traveled by the pulse wave from the carotid to the femoral artery. This measurement may be made more difficult in participants with obesity and in women with a large bust size [150], and care should be taken to ensure that body contours do not inflate distance measurements. There also remains debate regarding the most

<table>
<thead>
<tr>
<th>SphygmoCor Measure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central systolic blood pressure (cSBP) [mm Hg]</td>
<td>Estimated from brachial blood pressure waveform using generalized transfer function.</td>
</tr>
<tr>
<td></td>
<td>Pressure experienced by organs during systole.</td>
</tr>
<tr>
<td>Central pulse pressure (PP) [mm Hg]</td>
<td>Difference between cSBP and central DBP.</td>
</tr>
<tr>
<td>Augmentation pressure (AP) [mm Hg]</td>
<td>Difference between the systolic peak and first systolic inflection of the central pressure waveform.</td>
</tr>
<tr>
<td></td>
<td>Arterial stiffness increases AP as the reflected wave arrives earlier in systole.</td>
</tr>
<tr>
<td>Augmentation index @ 75 (AIx75) [%]</td>
<td>Measured as AP over PP and standardized to 75 beats per minute.</td>
</tr>
<tr>
<td></td>
<td>Higher values indicate greater degree of wave reflection and arterial stiffness.</td>
</tr>
<tr>
<td>Pulse wave velocity (PWV) [m/sec]</td>
<td>Speed of the pressure wave as it travels through the arteries.</td>
</tr>
<tr>
<td></td>
<td>Higher PWV indicates greater arterial stiffness and less vascular elasticity.</td>
</tr>
</tbody>
</table>
accurate distance calculation [151]. For instance, the direct carotid-femoral distance has been shown to overestimate the real traveled distance [152], and devices such as the SphygmoCor System instead use a calculated distance that is based on the distance between the arteries and the suprasternal notch. Others recommend adjusting for age or applying a scaling factor to correct for overestimation of path length [151-153]. Because these methodological differences can result in different absolute PWV values, it is important to note the particular methods used when comparing results between studies. The femoral artery may also be difficult to record accurately in participants with obesity, metabolic syndrome, diabetes, and peripheral artery disease [150]. Therefore, it is imperative that these recommendations be implemented when using the SphygmoCor System, and repeated measurements should be taken and combined for analysis to minimize possible errors in measurement.

2.2.1.3 Postprandial arterial stiffness responses and effects of plant-based bioactives
As emerging CVD risk factors, central blood pressure and arterial stiffness indices are now more commonly evaluated in nutrition research and there is evidence that these measures of vascular health are modifiable by dietary interventions. Central blood pressure and indices of arterial stiffness (i.e., augmentation index and pulse wave velocity) measured by the SphygmoCor System were the primary endpoint of the strawberry supplementation study presented in Chapter 3; thus, only studies that included similar measures of central pressure, augmentation index (Alx), and pulse wave velocity (PWV) will be presented in the following discussion.

It is well-established that consumption of a high-fat meal and subsequent postprandial hyperlipidemia causes transient endothelial dysfunction [154], and these impairments may be attenuated by phytochemical-rich dietary interventions [155-157]. Comparatively, relatively few studies have investigated how particular phytochemical-rich foods influence postprandial central blood pressure and indices of arterial stiffness, either when they are consumed alone or in combination with a meal. Detailed characteristics and the results of these studies are presented in Table 2-2. Previous study interventions have been limited to spinach [158, 159], beetroot juice [160], dark chocolate [161], red wine [162-164], and tea [165], but some have shown promising results. For instance, a nitrate-rich beet juice reduced PWV in adults with grade 1 hypertension compared to an equal volume of water [160]. However, the effects of a nitrate-rich meal compared to a low-nitrate control meal are mixed in healthy adults. In the study by Liu et al., there was no effect on PWV or Al of a nitrate-rich meal containing spinach relative to the same meal containing energy-matched rice milk in place of spinach [159]. However, Jovanovski et al. found that a nitrate-rich spinach soup reduced Al compared to a low-nitrate soup made with asparagus [158]. It should be noted that the spinach soup provided in the study by Jovanovski et al. provided much more nitrate than the spinach-containing meal used by Liu et al. (845 mg versus 220 mg) [158, 159]. Flavonoid-rich dark chocolate has also been shown to reduce the Alx in healthy adults compared with sham-eating [161]; however, the effect of a chocolate-containing meal relative to a control meal have not been studied. Beneficial effects on central blood pressure and arterial stiffness have also been found with red wine [162-164]. Dealcoholized red wine also reduced arterial stiffness in the study by Karatzis et al., suggesting that effects could be attributed primarily to the phytochemical content of the wine [163]. However, others have found no effect of dealcoholized red wine [164]. Compared to caffeine alone, smaller increases in Al and PWV were caused by green and black tea, suggesting that the phytochemicals in tea attenuate the acute increase in arterial stiffness caused by caffeine consumption [165]. The vascular effects of incorporating red wine or tea in a meal, relative to a control meal, have not been studied. Nitrate is the only phytochemical that has been tested in this context. Additional research is needed to determine
whether a meal containing phytochemicals provided by foods/beverages other than spinach can alter postprandial vascular function relative to a control meal. Effects on central blood pressure and arterial stiffness may depend on the unique phytochemical profile provided by a particular food or beverage.
Table 2-2. Studies evaluating the acute effects of a plant-based bioactive dietary intervention on central (aortic) pressures, augmentation index (AIX), and pulse wave velocity (PWV).

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study design</th>
<th>Study population</th>
<th>Intervention</th>
<th>Control and/or comparator</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jovanovski, 2015 [158]</td>
<td>Crossover</td>
<td>Healthy adults (n = 27)</td>
<td>High-nitrate spinach soup (845 mg nitrate)</td>
<td>Low-nitrate asparagus soup (0.6 mg nitrate)</td>
<td>High-nitrate soup ↓ AIX at 3 h compared to low-nitrate soup</td>
</tr>
<tr>
<td>Liu, 2013 [159]</td>
<td>Crossover</td>
<td>Healthy adults (n = 26)</td>
<td>250 g cooked spinach (220 mg nitrate) consumed with sandwich comprised of white bread, chicken, and cheese (~478 kcal)</td>
<td>Rice milk (energy-matched; 100 mL) consumed with same meal</td>
<td>No effect on PWV or AIX</td>
</tr>
<tr>
<td>Ghosh, 2013 [160]</td>
<td>Crossover</td>
<td>Adults with grade 1 hypertension (n = 15)</td>
<td>250 mL nitrate-rich beet juice</td>
<td>250 mL water</td>
<td>PWV ↓ at 3 h</td>
</tr>
<tr>
<td>Papamichael, 2008 [162]</td>
<td>Crossover</td>
<td>Healthy adults (n = 15)</td>
<td>Light meal (vegetable soup with 50 g of oil, 2 slices of white bread, and 250 mL wine) • 4 combinations of: green olive oil or refined olive oil, and red wine or white</td>
<td>Separate control group of participants that did not consume any meal (n = 15)</td>
<td>All meal combinations ↓ AIX@75, cSBP, and cDBP Red wine meals resulted in an earlier (1 h vs. 2 and 3) and more prolonged (full 3 h vs. 1 h) ↓ in AIX@75</td>
</tr>
<tr>
<td>Vlachopoulos, 2006 [165]</td>
<td>Crossover</td>
<td>Healthy adults (n = 16)</td>
<td>6 gm black tea in 450 mL hot water</td>
<td>Hot water (control) 175 mg caffeine (comparator)</td>
<td>Relative to control: ↑ in cSBP and cDBP No change in cPP PWV ↑ during first 90 min (0.5 m/s) then rapidly ↓ to baseline (vs. sustained ↑ with caffeine) AIX ↑ 5.0% (greater increase with caffeine)</td>
</tr>
<tr>
<td>Vlachopoulos, 2005 [161]</td>
<td>Crossover</td>
<td>Healthy adults (n = 13)</td>
<td>6 gm green tea in 450 mL hot water</td>
<td>Hot water (control) 125 mg caffeine (comparator)</td>
<td>Relative to control: ↑ in cSBP and cDBP No change in cPP No ↑ in PWV (vs. sustained ↑ with caffeine) AIX ↑ 6.6% (greater increase with caffeine)</td>
</tr>
<tr>
<td>Vlachopoulos, 2005 [161]</td>
<td>Crossover</td>
<td>Healthy adults (n = 17)</td>
<td>100 g flavonoid-rich dark chocolate</td>
<td>Sham-eating (i.e., chewing)</td>
<td>No change in cSBP, cDBP, or cPP AIX ↓ by 7.8% over 3 hours</td>
</tr>
<tr>
<td>Karatzi, 2005 [163]</td>
<td>Crossover</td>
<td>Men with stable CAD (n = 15)</td>
<td>250 mL red wine</td>
<td>250 mL dealcoholized red wine</td>
<td>Dealcoholized red wine ↓ cDBP Red wine ↓ cPP Both ↓ cSBP Both ↓ Alx@75 for 60 min—red wine extended reduction to 90 min No change in PWV</td>
</tr>
<tr>
<td>----------------------</td>
<td>-----------</td>
<td>-----------------------------</td>
<td>----------------</td>
<td>-----------------------------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Mahmud, 2002 [164]</td>
<td>Crossover</td>
<td>Healthy adults (n = 8)</td>
<td>500 mL red wine</td>
<td>500 mL dealcoholized red wine</td>
<td>Only red wine with alcohol ↓ cSBP, cDBP, PWV, and Alx</td>
</tr>
</tbody>
</table>

Abbreviations: Alx, augmentation index; Alx@75, augmentation index standardized to a heart rate of 75 beats per minute; CAD, coronary artery disease; cDBP, central diastolic blood pressure; cPP, central pulse pressure; cSBP, central systolic blood pressure; PWV, pulse wave velocity.
2.2.2 Effects of plant-based bioactives on postprandial dysmetabolism

Postprandial dysmetabolism, which is characterized by excessive spikes in glucose, insulin, and triglycerides, substantially increases CVD risk. Numerous mechanisms have been posited to explain how repeated postprandial impairments create an environment conducive to the development of atherosclerosis. Consumption of calorically dense, easily digestible foods overwhelm the metabolic capabilities of mitochondria, ultimately resulting in greater production of free radicals (i.e., reactive oxygen species) [21]. Subsequent oxidative stress triggers a cascade of atherogenic biochemical events, including inflammation, endothelial dysfunction and vasoconstriction, thrombogenicity, and oxidation of LDL-C [21, 166]. Postprandial triglycerides are determined by the amount and type of dietary fat provided in the meal and the duration of the preceding fast [21, 23], but also vary substantially between individuals [43]. Genetic factors may explain some of the heterogeneity in postprandial responses, but likely have a relatively small influence compared to known modulators, such as body fat percentage, age, sex, and body mass index [43].

Dietary patterns that emphasize phytochemical-rich fruits and vegetables have been proposed as a means of reducing postprandial metabolic impairments [21], but less is known about the postprandial effects of select foods. Studies that have previously evaluated the postprandial effects of nutritional interventions are described in Table 2-3. Promising effects on triglyceride, glucose, and insulin responses, as well as lipid peroxidation, have been reported for many phytochemical-rich interventions, including spices [167-170], tea [171, 172], fruit juice [173], pomegranate extract [174], avocado [175], berries [176-179], and tomato paste [180]; however, some studies have reported null findings [155-157, 181-184] and results have not always been consistent between different studies or for different endpoints. For instance, different types of berry purees have been shown to improve glucose and insulin responses after sucrose and/or carbohydrate consumption [176, 177, 185]. However, others have found no effect of including raspberries and blueberries in a high carbohydrate meal [182]. Studies investigating phytochemical-rich spice blends have also had disparate results. Two studies that used a similar spice blend and high fat meal both demonstrated a reduction in postprandial triglycerides [167, 169]; however, only one of these studies found a beneficial effect on insulin responses [169]. No attenuation of triglycerides, glucose, or insulin was found using a smaller amount of spices (11 g vs 14 g) and a meal containing less fat and calories (440 kcal and 25 g fat versus 1000-1200 kcal and 43-49 g fat)[167, 169], although lipid peroxidation was reduced [168]. It is difficult to directly compare the results from these studies due to the variation in energy, carbohydrate, and fat content of the test meals used. Each of these different dietary interventions also provided a unique profile of dietary phytochemicals and thus, may have had different effects of particular postprandial responses. Effects may also depend on characteristics of the study population, such as age, BMI, lipid profile, and disease history. Furthermore, most of these studies were conducted in relatively small samples, and in many cases, the particular phytochemical-rich intervention has only been tested in a single study. However, the results from these studies generally support the hypothesis that phytochemical-rich dietary interventions have the potential to improve postprandial triglycerides, glucose, insulin, and/or lipid peroxidation.
Table 2-3. Studies evaluating the effects of a single acute dose of a dietary intervention on postprandial responses to an energy-dense, high-fat and/or high-carbohydrate meal.

<table>
<thead>
<tr>
<th>Author, Year</th>
<th>Study Design</th>
<th>Participants</th>
<th>Test Meal</th>
<th>Dietary Intervention¹</th>
<th>Control</th>
<th>Effects on Postprandial Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>McCrea, 2015 [167]</td>
<td>Crossover</td>
<td>Overweight/obese adults with hyperlipidemia (n = 20)</td>
<td>Lunch meal (1000 kcal, 43 g fat, 98 g CHO)</td>
<td>14.5 g spice blend²</td>
<td>Lunch meal with no spices plus placebo capsules containing crystalline methylcellulose</td>
<td>TG ↓ 31% during the 4 hours post meal vs no spice meal No effect on glucose or insulin</td>
</tr>
<tr>
<td>Koutelidakis, 2014 [171]</td>
<td>Parallel arm</td>
<td>Patients with CAD (n = 43)</td>
<td>Bread and butter (~380 kcal, 18 g fat, 40 g CHO)</td>
<td>4.5 g green tea in 330 mL water</td>
<td>Bread and butter with 330 mL water</td>
<td>Tea prevented the ↑ in TG at 3 hours post-meal caused by control meal No effect on glucose</td>
</tr>
<tr>
<td>Peluso, 2014 [173]</td>
<td>Crossover</td>
<td>Healthy overweight adults (n = 14)</td>
<td>High-fat meal (1334 kcal, 82 g fat, 100 g CHO)</td>
<td>500 mL fruit-juice drink³</td>
<td>500 mL placebo drink</td>
<td>↓ TG from 4-8 hours post-meal ↓ TNF-α production at all time points ↓ IL-6 production at 2 hours post-meal</td>
</tr>
<tr>
<td>Joris, 2013 [181]</td>
<td>Crossover</td>
<td>Healthy overweight/obese men (n = 25)</td>
<td>Mixed meal [2 muffins] (1122 kcal, 56.6 g fat, 137 g CHO)</td>
<td>140 mL concentrated beet juice (500 mg dietary nitrate)</td>
<td>140 mL control drink (calorie and CHO matched)</td>
<td>No effect on TG, glucose, or insulin Attenuated impairment in FMD</td>
</tr>
<tr>
<td>Li, 2013 [175]</td>
<td>Crossover</td>
<td>Healthy men (n = 11)</td>
<td>Hamburger cooked with salt (250 g ground beef; 436 kcal, 25 g fat)</td>
<td>½ Hass avocado (68 g)</td>
<td>Hamburger alone</td>
<td>Attenuated ↓ in peripheral arterial tonometry score and ↑ in IL-6 Preserved IkBα in PBMCs</td>
</tr>
<tr>
<td>Li, 2013 [168]</td>
<td>Crossover</td>
<td>Men with type 2 diabetes (n = 18)</td>
<td>Hamburger cooked with salt (250 g ground beef; 436 kcal, 25 g fat)</td>
<td>11.25 g spice mix⁴</td>
<td>Hamburger alone</td>
<td>No effect on TG, glucose, or insulin Urinary MDA ↓ 31% ↑ peripheral arterial tonometry score</td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Participants</td>
<td>Intervention A</td>
<td>Intervention B</td>
<td>Outcome 1</td>
<td>Outcome 2</td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>--------------</td>
<td>----------------</td>
<td>----------------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Torronen, 2013 [177]</td>
<td>Crossover</td>
<td>Healthy women (Study 1: n = 15; Study 2: n = 13; Study 3: n = 20)</td>
<td>White bread or rye bread (50 g CHO)</td>
<td>150 g whole-berry puree Study 1: strawberry, bilberry, or lingonberry Study 2: raspberry, cloudberry, or chokeberry Study 3: mixture of strawberries, bilberries, cranberries, and blackcurrants</td>
<td>Same amount of white or rye bread without berries</td>
<td>Strawberries, bilberries, lingonberries, chokeberries, and berry mixture ↓ insulin response (similar reductions for both white and rye bread) Only strawberries and the berry mixture improved the glycemic profile</td>
</tr>
<tr>
<td>Mathew, 2012 [174]</td>
<td>Crossover</td>
<td>Healthy young men (n = 19)</td>
<td>Muffin (691 kcal, 50 g fat, 56 g CHO)</td>
<td>POMx pomegranate extract consumed with meal or 15 minutes prior</td>
<td>Placebo drink with no pomegranate polyphenols (consumed with meal only)</td>
<td>POMx ↑ TG response when consumed prior to meal Trend towards ↓ SBP</td>
</tr>
<tr>
<td>Burton-Freeman, 2012 [180]</td>
<td>Crossover</td>
<td>Healthy young adults (n = 25)</td>
<td>High-fat meal (850 kcal, 43 g fat, 99 g CHO)</td>
<td>94 g tomato paste (1.5 servings)</td>
<td>Control meal with non-tomato alternative (matched for calories, macro- and micronutrients)</td>
<td>Attenuated ox-LDL and IL-6 production Insulin ↓ 30 and 90 minutes post-meal ↑ TG concentration at 300 minutes (no difference in AUC)</td>
</tr>
<tr>
<td>Torronen, 2012 [185]</td>
<td>Crossover</td>
<td>Healthy adults (n = 12)</td>
<td>35 g sucrose</td>
<td>150 g berry puree (bilberries, blackcurrants, cranberries, and strawberries)</td>
<td>Sucrose alone (35 g in 250 mL water; CHO matched)</td>
<td>Delayed glucose response: ↓ glucose and insulin concentrations at 15 minutes, but ↑ 90 minute concentrations</td>
</tr>
<tr>
<td>Torronen, 2012 [176]</td>
<td>Crossover</td>
<td>Healthy women (n = 20)</td>
<td>35 g sucrose</td>
<td>150 g blackcurrants or lingonberries (whole berries or nectar)</td>
<td>Sucrose alone (35 g in 300 mL water; CHO matched)</td>
<td>↓ glucose and insulin concentrations in first 30 minutes Improved glycemic profile</td>
</tr>
<tr>
<td>Skulas-Ray, 2011 [169]</td>
<td>Crossover</td>
<td>Healthy overweight men (n = 6)</td>
<td>Lunch meal (1200 kcal, 49 g fat, 128 g CHO)</td>
<td>14 g spice blend²</td>
<td>Lunch meal with no spices</td>
<td>Insulin ↓ 21% TG ↓ 31% No effect on glucose response</td>
</tr>
</tbody>
</table>

² Spike mixture containing 9 g dried spices and 5 g dried fruits
<table>
<thead>
<tr>
<th>Authors</th>
<th>Study Design</th>
<th>Participants</th>
<th>Intervention</th>
<th>Outcome Measures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clegg, 2011 [182]</td>
<td>Crossover</td>
<td>Healthy adults (n = 12)</td>
<td>Pancakes (333 kcal, 7 g fat, 50 g CHO) 100 g raspberries and blueberries Pancakes with fructose and sucrose to CHO match berries</td>
<td>No difference in glycemic response</td>
</tr>
<tr>
<td>Burton-Freeman, 2010 [179] and Edirisinghe, 2011 [186]</td>
<td>Crossover</td>
<td>Older hyperlipidemic adults (n = 24)</td>
<td>Breakfast meal (960 kcal, 30 g fat, 135 g CHO) 10 g freeze dried strawberry powder Placebo powder (calorie, macro- and micronutrient matched)</td>
<td>↓ TG, insulin, IL-6, and oxidized LDL</td>
</tr>
<tr>
<td>Li, 2010 [170]</td>
<td>Crossover</td>
<td>Healthy adults (n = 10)</td>
<td>Hamburger cooked with salt (250 g ground beef; 436 kcal, 25 g fat) 11.25 g spice mix</td>
<td>Hamburger alone</td>
</tr>
<tr>
<td>Torronen, 2010 [178]</td>
<td>Crossover</td>
<td>Healthy older adults (n = 12)</td>
<td>35 g sucrose load 150 g berry puree</td>
<td>35 g sucrose load (CHO matched)</td>
</tr>
<tr>
<td>Cortes, 2006 [155]</td>
<td>Crossover</td>
<td>Healthy adults (n = 12) and adults with hypercholesterolemia (n = 12)</td>
<td>High-fat meal with 25 mL olive oil (1200 kcal, 8 g fat, 66 g CHO) 40 g walnuts added to meal</td>
<td>High-fat meal with 25 mL olive oil and no walnuts</td>
</tr>
<tr>
<td>Unno, 2005 [172]</td>
<td>Crossover</td>
<td>Men with hypertriglyceridemia (n = 9)</td>
<td>Bread and butter meal (326 kcal, 19 g fat, 32 g CHO) Tea catechins (224 mg or 674 mg)</td>
<td>10 mg tea catechins</td>
</tr>
<tr>
<td>Esposito, 2003 [157]</td>
<td>Crossover</td>
<td>Healthy adults (n = 25)</td>
<td>High-fat breakfast meal (760 kcal, 50 g fat, 58 g CHO) High-fat breakfast plus 100 g tomatoes, 200 g carrots, and 100 g peppers</td>
<td>High-fat breakfast meal</td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Participants (n)</td>
<td>Treatment 1</td>
<td>Treatment 2</td>
</tr>
<tr>
<td>---------------------</td>
<td>------------</td>
<td>------------------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Kay, 2002 [183]</td>
<td>Crossover</td>
<td>Middle-aged men (n = 8)</td>
<td>High-fat meal (853 kcal, 47 g fat, 75 g CHO)</td>
<td>100 g freeze dried blueberry powder in 500 mL water</td>
</tr>
<tr>
<td>Blanco-Colio, 2000 [184]</td>
<td>Crossover</td>
<td>Healthy adults (n = 16)</td>
<td>Fat-enriched breakfast (602 kcal/m², 38 g/m² fat)</td>
<td>Red wine (moderate dose: 20 g/m² for men, 12 g/m² for women; low dose: 12 g/m² for men, 7.2 g/m² for women)</td>
</tr>
<tr>
<td>Vogel, 2000 [156]</td>
<td>Crossover</td>
<td>Healthy adults (n = 10)</td>
<td>High-fat meal with 50 g extra-virgin olive oil (900 kcal, 50 g fat)</td>
<td>Same meal with vitamins C (1 g) and E (800 IU), or balsamic vinegar (100 mL) and salad</td>
</tr>
</tbody>
</table>

1. Interventions were provided by incorporating them into the same test meal described as the control unless otherwise noted.
2. Included black pepper, cinnamon, cloves, garlic powder, ginger, oregano, paprika, rosemary, and turmeric.
3. Contained apple, red grape, pomegranate, and raspberry juice; citric acid; green tea extract; ascorbic acid; apple polyphenols; and lemon flavonoids.
4. Included cloves, cinnamon, oregano, rosemary, ginger, black pepper, paprika, and garlic powder.
5. Equal portions of bilberries, blackcurrants, cranberries, and strawberries.
2.2.3 Strawberries as a source of plant-based bioactives for reducing CVD risk

Strawberries (Fragaria x ananassa) are one of the most commonly consumed fruits in the United States [187] and are an important source of dietary bioactives [188-190]. They are rich in B-vitamins, vitamin C, vitamin E, potassium, folic acid, carotenoids, fiber, phytosterols, and numerous phenolic compounds. Strawberries were one of the 100 richest sources of dietary polyphenols, and are among a relatively short list of foods and beverages providing more than 1 mg of total polyphenols per serving [191]. The phenolic compounds in strawberries include ellagic acid, ellagitannins, anthocyanins, and the flavonols catechin, quercetin, and kaempferol [192, 193]. Compared to other fruits and vegetables, strawberries consistently rank among the highest contributors to antioxidant capacity [194]. Among 25 commonly consumed fruits in the United States, strawberries were one of the top two contributors to dietary cellular antioxidant activity (along with apples) [195]. Similarly, among commonly available fruits and vegetables in the United Kingdom, strawberries had the highest antioxidant activity per 100 g fresh weight portion sizes [196]. Strawberries also had the highest oxygen radical absorbance capacity compared to twelve other fruits [197], and were shown to effectively scavenge multiple types of free radicals [114]. Vitamin C, followed by ellagitannins and anthocyanins, are the greatest contributors the antioxidant capacity of strawberries [192]. Consumption of strawberries and other types of berries significantly increases serum antioxidant capacity [198, 199], as well as plasma and urine polyphenol concentrations [200, 201]. This provide evidence that strawberry bioactives are bioavailable and can be effectively absorbed to protect against oxidative damage and exert other cardioprotective effects.

2.2.3.1 Preclinical evidence for strawberries and CVD risk reduction

In addition to their antioxidant effects, cell- and animal-based studies have demonstrated the potential of strawberry bioactives to exert effects on vascular function and metabolism. With regard to vascular function, treatment of human endothelial cells with anthocyanidins upregulated endothelial nitric oxide synthase (eNOS), which plays a crucial role in maintaining cardiovascular homeostasis and endothelial function [202]. Similarly, freeze dried strawberry powder can augment endothelium-dependent relaxation in the rabbit aorta that is dependent on eNOS activation [203]. Purified ellagitannins from strawberries have also been shown to inhibit angiotensin I-converting enzyme (ACE) in vitro [204], which could translate to effects on human blood pressure. Strawberry extracts have also demonstrated effects on glucose and lipid metabolism. For instance, in Caco-2 intestinal cells, the anthocyanin pelargonidin-3-O-glucoside inhibited glucose uptake and transport [205]. Strawberry extracts can also inhibit carbohydrate digestive enzymes, including α-amylase and α-glucosidase, likely due to its high ellagitannin content [204, 206, 207]. Inhibition of pancreatic lipase by strawberry extracts [208] indicates that strawberries may also have benefits for lipid metabolism. Furthermore, in mice fed a high-fat diet, treatment with purified anthocyanins from strawberries and blueberries for 90 days prevented the development of dyslipidemia and obesity [209]. Surprisingly, whole berry powders providing an equivalent amount of anthocyanins were not effective in this animal model of obesity [209]. Few studies have used whole berry powders and similar results have not been found in other studies; however, it is possible that an unknown component of the whole berry may counteract or alter the effect of the anthocyanins [209]. As a whole, results from cell and animal studies have identified potential mechanisms by which strawberry bioactives could beneficially influence CVD risk factors in humans.

Although there is substantial epidemiological evidence for the benefit of fruit and vegetable intake, few observational studies have evaluated strawberry consumption in particular. However, in an analysis of 34,489 postmenopausal women in the Iowa Women’s Health Study, strawberries were associated with
reduced CVD mortality [119]. In the Nurses’ Health Study, individuals who consumed over 3 servings/wk of blueberries and strawberries tended to have reduced risk of myocardial infarction compared to those who rarely consumed these fruits, although the relationship did not reach significance [210]. Consuming at least 2 servings of strawberries per week is associated with reduced likelihood of having elevated CRP concentrations. In the Women’s Health Study, women who consumed ≥ 2 servings/week had a borderline significant 14% lower risk of having a CRP concentration ≥ 3 mg/L [211]. Analyses of dietary flavonoid intakes in the Nurses’ Health Study and Health Professionals Follow-Up Study also demonstrated associated cardiovascular benefits of consuming anthocyanins from blueberries and strawberries. Participants in the highest quintile of anthocyanin intake had an 8% lower risk of hypertension compared to those in the lowest quintile [212]. Although these population-based results are limited, they are in accordance with findings from mechanistic studies and provide further support for clinical intervention studies.

2.2.3.2 Clinical studies of strawberries and CVD risk factors
Freeze dried strawberry powder is often used in clinical studies as it has a more consistent phytochemical profile than fresh strawberries and is a well-characterized intervention. The phytochemical content of fresh strawberries can be influenced by multiple factors, including agricultural practices such as the use of compost, growing and storage temperatures, and cultivar differences, and processing methods [194]. As the harvesting season for strawberries is also typically very short, fresh strawberries are often consumed in small quantities and it is more efficient to process large amounts into frozen or dehydrated forms, purees, or juices [213]. Although relatively few studies have compared the effect of different processing methods on antioxidant concentrations in strawberries, freeze drying has generally been shown to preserve antioxidant activity, phenolic compounds, anthocyanins, and ascorbic acid compared to other common dehydration techniques [213, 214]. However, in one study, the total phenolic content was higher in frozen strawberries, followed by freeze dried and air-dried [215]. Particular types of antioxidants may be uniquely affected by different dehydration methods. For instance, freeze drying may significantly reduce lipophilic antioxidant activity, whereas more hydrophilic antioxidant activity is lost by freezing and thawing strawberries [214]. Because freeze dried strawberries are a more concentrated source of antioxidants than fresh strawberries, consumers can obtain the same amount of antioxidants by eating smaller quantities [213]. For instance, 40-50 g (or ~1.5 ounces) of freeze dried strawberry powder is equivalent to approximately one pound of fresh strawberries. Thus, freeze dried strawberries are a viable alternative to fresh strawberries and are often used in clinical nutrition research.

2.2.3.2.1 Long-term strawberry supplementation studies
Strawberries have been used as a nutritional intervention in multiple studies, with mixed results for intermediate CVD risk factors. Detailed results and characteristics of these studies are presented in Table 2-4. It is difficult to directly compare the effects of strawberry supplementation among studies as a variety of study designs, doses, durations, and study populations have been evaluated. The majority of clinical studies investigating the effects of strawberries have provided a daily dose for 3-12 weeks. Fresh, frozen, or freeze dried strawberry (FDS) powder have all been used. With regard to the FDS powder, a 50 g dose has been used most frequently. This is equivalent to ~500 g or 3 cups of fresh strawberries (1 serving of fresh strawberries is ~1 cup) [216]. Similar amounts of fresh strawberries have also been used (4 servings/d [217] and 1 pound/d [218]). The strawberry intervention is typically provided as a beverage or incorporated into different foods. Concerns have been raised about the potential for other foods to interfere with the absorption of strawberry bioactive compounds. Binding between polyphenols and
peptides results in the formation of protein-polyphenol complexes that precipitate out of solution [219]. Thus, it has been posited that this reduces polyphenol bioavailability. In some studies, the consumption of milk with tea or blueberries reduced in vivo plasma antioxidant capacity [220, 221]. However, results are highly discrepant and others have found no effect of milk on the bioavailability of tea catechins [222] or the absorption of tea flavonols [223]. With respect to strawberries, the consumption of cream delayed anthocyanin absorption but did not influence the overall amount absorbed [224]. Nonetheless, based on these concerns of reduced antioxidant capacity, many strawberry supplementation studies have instructed that the strawberry intervention not be consumed with any snack, lunch, or dinner [216, 225-227]. However, cardioprotective effects were found when the strawberry intervention was consumed in a milk-based beverage [179]. Thus, the biological significance of potential losses in strawberry antioxidant activity due to dairy protein binding remains unclear [186].

Previous studies indicate that strawberry supplementation can improve selected CVD risk factors in individuals with elevated CVD risk, including those with obesity, dyslipidemia, metabolic syndrome, and type 2 diabetes. Due to the antioxidant content of strawberries, many studies have evaluated effects on markers of oxidative stress. Jenkins et al. were the first to demonstrate that strawberry supplementation, in combination with a Portfolio Diet, reduced lipid peroxidation in adults with elevated LDL-C [218]. Subsequent studies have similarly found reductions in MDA in healthy adults [228], women with metabolic syndrome [216], adults with type 2 diabetes [226], and adults with abdominal obesity and dyslipidemia [227]. Improvements in the lipid profile have also been found in some studies [217, 225, 227, 228]. In general, clinical studies have not found an effect of strawberry supplementation on adiposity, blood pressure, or fasting glucose or insulin. However, a trend towards a reduction in HbA1C was found in adults with type 2 diabetes following 6 weeks of consuming 50 g/d of FDS powder [226]. Therefore, the effects of strawberries on these outcomes may be modest and difficult to measure, or other dietary interventions may be needed to address these particular CVD risk factors.
Table 2-4. Studies evaluating the effect of chronic strawberry supplementation on cardiovascular disease risk factors.

<table>
<thead>
<tr>
<th>Author, Year</th>
<th>Study design</th>
<th>Study population</th>
<th>Strawberry dose and vehicle</th>
<th>Control</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alvarez-Suarez, 2014 [228]</td>
<td>Pre-post analysis 4 week intervention</td>
<td>Healthy adults (n = 23)</td>
<td>500 g/d fresh strawberries</td>
<td>None</td>
<td>↓ TC, LDL-C, TG, MDA, and urinary isoprostanes relative to baseline</td>
</tr>
<tr>
<td>Basu, 2014 [227]</td>
<td>Parallel arm 12 week intervention</td>
<td>Adults with abdominal adiposity and dyslipidemia (n = 60)</td>
<td>50 g/d (high dose) or 25 g/d (low dose) FDS powder consumed in water-based beverage</td>
<td>Placebo powder (high and low dose) matched for calorie and fiber content</td>
<td>Both doses ↓ MDA Only high dose ↓ TC, LDL-C, and small LDL particles compared to control</td>
</tr>
<tr>
<td>Moazen, 2013 [226]</td>
<td>Parallel arm 6 week intervention</td>
<td>Older adults with type 2 diabetes (n = 36)</td>
<td>50 g/d FDS powder consumed as a water-based beverage</td>
<td>Placebo powder (40 g/d) matched for calories, carbohydrate and fiber content, color, and flavor</td>
<td>↓ CRP and MDA ↓ HbA1C† No change in fasting glucose</td>
</tr>
<tr>
<td>Zunino, 2012 [217]</td>
<td>Crossover controlled feeding study 3 week intervention periods (no washout)</td>
<td>Obese adults (n = 20)*</td>
<td>Average American diet supplemented with 4 servings/d frozen strawberries mixed into milkshakes, yogurt, cream cheese, or water-based beverages</td>
<td>Average American diet with same foods (imitation strawberry flavoring, strawberry Kool-aid, and red food coloring used as controls)</td>
<td>↓ TC and small HDL particles ↑ LDL particle size</td>
</tr>
<tr>
<td>Burton-Freeman, 2010 [179]</td>
<td>Crossover 6 week intervention periods (no washout period)</td>
<td>Older, overweight adults with dyslipidemia (n = 24)</td>
<td>10 g/d FDS powder consumed as a milk-based beverage</td>
<td>Placebo beverage (matched for calorie, macro- and micronutrient, and fiber content)</td>
<td>No effect on fasting lipids or lipid peroxidation</td>
</tr>
<tr>
<td>Basu, 2010 [225]</td>
<td>Parallel arm 8 week intervention</td>
<td>Older, obese adults with metabolic syndrome (n = 27; 2 M)</td>
<td>50 g/d FDS powder consumed as a water-based beverage</td>
<td>Water (matched for daily fluid intake only)</td>
<td>↓ TC, LDL-C, and small LDL particles ↓ VCAM-1 No change in BP, TG, or fasting glucose</td>
</tr>
<tr>
<td>Basu, 2009 [216]</td>
<td>Pre-post analysis</td>
<td>Women with metabolic syndrome (n = 16)</td>
<td>50 g/d FDS powder consumed as a water-based beverage</td>
<td>None</td>
<td>↓ TC and LDL-C ↓ MDA</td>
</tr>
<tr>
<td>Jenkins, 2008 [218]</td>
<td>Crossover 4 week intervention (2 week washout) nested within 5-year Portfolio Diet study</td>
<td>Older adults with elevated LDL-C (n = 28)</td>
<td>1 lb (454 g) per day fresh strawberries (consumed as snacks, desserts, and blended with soy milk as “smoothies”)</td>
<td>Oat bran bread (calorie matched)</td>
<td>Trend towards ↓ ox-LDL No change in BP, TG, or fasting glucose</td>
</tr>
</tbody>
</table>

*All lipid outcomes based on n = 10. Due to carry-over effect, statistical analysis was limited only to intervention period 1 values.
†Change compared to placebo group. Change from baseline non-significant in strawberry group; trend towards an increase from baseline in placebo group. Abbreviations: FDS, freeze dried strawberries.
2.2.3.2.2 Postprandial effects of strawberries

With regard to strawberries, our knowledge of their postprandial effects remains relatively limited. In healthy women, a strawberry puree was shown to attenuate excess insulin production following a carbohydrate load and improve the glycemic profile [177]. The effects of strawberries on postprandial responses to a fat-containing meal have also been studied in a population of older, overweight, hyperlipidemic adults. The meal used in this study was designed to reflect the average American dietary pattern and consisted of a bagel, cream cheese, whole milk, egg, margarine, and cantaloupe (providing approximately 960 kcal, 135 g carbohydrate, and 30 g total fat). Incorporating 10 g of FDS powder into this meal attenuated elevations in triglycerides, insulin, IL-6, and oxidized LDL [179, 186]. It is of note that each of these effects occurred over a unique time course. For instance, elevations in insulin were attenuated as early as 60 minutes following consumption of the meal, while effects on oxidized LDL occurred at 3 hours post-meal [179, 186]. Effects on triglycerides and IL-6 first occurred at later time points (4 and 6 hours post-meal, respectively) [179, 186]. As there was no effect on the glucose response, the reduction in peak insulin production may suggest that the FDS powder improved insulin sensitivity and enabled glucose homeostasis to be achieved with less insulin [186]. Sex-based differences were also apparent in the oxidized LDL response. Whereas there was no significant effect of the FDS powder in women at any time point, the change in oxidized LDL production was significantly reduced in men when the FDS powder beverage was consumed [179]. The effect on triglycerides translated to a 4% decrease in the overall response over 6 hours, and a 5 mg/dL reduction in peak concentrations. The clinical implication of such a change remains unclear. Additional research is needed to confirm that strawberries may have the potential to mitigate the metabolic consequences of consuming large amounts of dietary fat, and further investigate possible that sex-based differences in postprandial responses.

The effect of chronic strawberry consumption on postprandial responses was also evaluated in the same study population. Following 6 weeks of consuming 10 g/d of FDS powder, participants underwent the same test meal protocol. No FDS powder was included in the test meal. Compared to pre-supplementation postprandial responses, elevations in triglycerides, oxidized LDL, IL-1β, and plasminogen activator inhibitor-1 (PAI-1) were attenuated following FDS powder supplementation [179, 229]. The overall IL-6 response was not significantly altered by strawberry supplementation. However, in the placebo group, IL-6 production increased post-meal whereas it tended to remain flat in those supplemented with FDS powder [229]. As in the acute portion of the study, sex-based differences were again found in the oxidized LDL response. However, in this case, the benefits of strawberry supplementation were most apparent in women. Whereas women experienced a significant reduction in postprandial oxidized LDL production following 6 weeks of strawberry supplementation, men exhibited an improved oxidized LDL response whether they received the strawberry intervention or the placebo—suggesting a benefit of simply participating in the study [179]. Although these results have yet to be replicated, this suggests that chronic strawberry consumption may confer postprandial metabolic benefits even when strawberries are not incorporated into the fat-containing meal.

Thus, antioxidant-rich strawberries may confer cardioprotective effects on postprandial responses to an oral carbohydrate and/or fat load, but further research is needed. For instance, current evidence in the context of a fat-containing meal is limited to a population of relatively old, overweight, and dyslipidemic adults; it has yet to be evaluated whether beneficial effects occur in younger adults without established CVD risk factors. Other types of test meals, such as those with higher amounts of fat, have also not been investigated. Furthermore, dose-response effects have also not been evaluated and it remains unclear.
what dose of strawberries is needed to achieve cardioprotective effects. Notably, no studies have examined whether strawberries can influence the changes in central blood pressure and arterial stiffness that are caused by the consumption of a meal.
2.3 Omega-3 fatty acids, inflammation, and oxidative stress

There is a large body of pre-clinical evidence demonstrating the anti-inflammatory potential of omega-3 fatty acids [36], and individuals who report greater fish and/or LC omega-3 FA intake tend to have lower concentrations of inflammatory markers [230-234]. However, results from clinical omega-3 supplementation trials have been disappointing with regard to inflammatory marker outcomes.

2.3.1.1 Mechanisms of omega-3 fatty acids and inflammation

There is substantial mechanistic evidence supporting the anti-inflammatory capacity of EPA and DHA. The influence of fatty acids on inflammatory processes likely depends on the fatty acid composition of cell membrane phospholipids, and may occur through a variety of mechanisms. In individuals consuming a typical Western diet, the phospholipid bilayer of cells involved in the inflammatory response is generally enriched in arachidonic acid. However, these cells can be enriched in EPA and DHA by increasing omega-3 fatty acid intake. This change in the fatty acid composition typically occurs in a time- and dose-dependent manner, and largely by replacing arachidonic acid. This influences the inflammatory response as these fatty acids are the precursors to bioactive lipid mediators that can orchestrate both the initiation and resolution of inflammation. Fatty acids in the phospholipid bilayer are released as free fatty acids by the action of phospholipase A2 (PLA2) enzymes that are activated by inflammatory stimuli. These free fatty acids can then be metabolized by cyclooxygenase and lipoxygenase enzymes to form a variety of hormone-like compounds called eicosanoids, which include prostaglandins, thromboxanes, leukotrienes, and lipoxins. Because arachidonic acid is typically the most prevalent fatty acid in the cell membrane due to its abundance in the Western diet, it is generally the primary substrate for PLA2. Arachidonic acid metabolism results in the formation of 2-series prostaglandins and thromboxanes, as well as 4-series leukotrienes, which are generally considered pro-inflammatory and pro-thrombotic. However, EPA is metabolized into 3-series prostaglandins and thromboxanes, and 5-series leukotrienes. The structure of most EPA-derived eicosanoids renders them less pro-inflammatory than their arachidonic acid-derived counterparts. Therefore, greater EPA content in the cell membrane can shift the production of lipid mediators towards less inflammatory eicosanoids.

Furthermore, EPA and DHA can also be metabolized into novel pro-resolving lipid mediators that actively promote the resolution of inflammation. These EPA- and DHA-derived lipid mediators have been collectively termed specialized pro-resolving mediators (SPMs) and include resolvins, protectins, and maresins [235]. SPMs promote the resolution of inflammation and a return to homeostasis by influencing the function of several cell types [236]. For instance, they limit further neutrophil recruitment to the site of inflammation, stimulate non-phlogistic (non-inflammatory) recruitment of monocytes, promote phagocytosis of cell debris, apoptic cells, and bacteria by macrophages, and ultimately enhance the departure of immune cells from the inflamed site via the lymphatics[236]. EPA and DHA produce the E-series and D-series resolvins, respectively. DHA can also be metabolized into protectins and maresins. The term “maresin” is an abbreviation of the phrase “macrophage mediators in resolving inflammation” as maresins are formed from DHA by macrophage lipoxygenases [237]. The anti-inflammatory effects of SPMs have studied primarily in cell-based studies and animal models of disease [238]. For instance, resolvine E1 (RvE1) has been shown to exhibit anti-inflammatory effects in rodent models of periodontitis, peritonitis, retinopathy, and colitis [239]. Pro-resolution effects of protectins have also been found in rodent models of peritonitis, asthma, ischemia-reperfusion injury, retinopathy, and ischemic stroke [239]. Less is known about the role of SPMs in human physiology. However, measurable levels of SPMs have been found in human plasma. For instance, RvE1 was present in human plasma following acute intake of
EPA with or without aspirin [240]. More clinical research is needed to establish which SPMs are endogenously produced in quantities that are sufficient for pro-resolving bioactivity in humans.

In addition to lipid mediator production, EPA and DHA may exert anti-inflammatory effects by altering the production of pro-inflammatory cytokines and adhesion molecules. These cell-based studies typically measure cytokine or adhesion molecule production by monocytic or endothelial cells that have been stimulated with bacterial endotoxin following omega-3 fatty acid supplementation. These studies have been reviewed extensively by Calder et al. [36]. In brief, EPA and DHA have been shown to inhibit IL-6, TNF-α, and adhesion molecule production in human endothelial cells and monocytes. It has been proposed that these effects may be achieved via changes in transcription factor activity (e.g., inhibition of nuclear factor kappa B [NFκB]).

The anti-inflammatory potential of omega-3 fatty acids has been further demonstrated using the fat-1 transgenic mouse model. In this model, the fat-1 gene from the roundworm C. elegans is transferred into mice. This gene encodes for an omega-3 fatty acid desaturase enzyme that converts omega-6 fatty acids to the omega-3 type, which is absent in most animals. These mice and their wild-type littermates were fed identical diets enriched in omega-6 fatty acids and deficient in omega-3. Unlike their wild-type counterparts, the tissues and organs of the transgenic mice were enriched in omega-3 fatty acids and had lower amounts of omega-6 fatty acids—indicating that the dietary omega-6 had been converted to omega-3 fatty acids [241]. Furthermore, in a model of induced colitis, these transgenic mice lost less body weight and experienced less colon tissue damage [242]. Physiologically meaningful quantities of resolvins and protectins were also identified within the colons of these mice. Comparatively, their wild-type littermates exhibited wasting and profound histologic changes in the colon, with no production of anti-inflammatory lipid mediators. Thus, this animal model indicates that meaningful amounts of resolvins and protectins can be generated in vivo when tissues are enriched with EPA and DHA, and suggests that increasing tissue omega-3 fatty acid content may be beneficial for colitis and other inflammatory diseases. Thus, based on the large body of evidence from cell and animal models, it is biologically plausible that greater EPA and DHA consumption could translate to similar benefits in humans.

### 2.3.1.2 Epidemiological evidence for omega-3 fatty acid intake and inflammatory markers

Greater dietary intake of fish and/or EPA+DHA has been associated with lower concentrations of multiple inflammatory markers in many epidemiological studies [230-234, 243] (Table 2-5). However, correlations have not always been consistent for particular markers of inflammation. For instance, in the earliest study, EPA and DHA were both inversely associated with soluble TNF receptor 1 and 2, but not significantly related to CRP or IL-6 [234]. Conversely, subsequent studies have found that CRP and IL-6 are inversely related to fish consumption [232], as well as EPA+DHA intake [231] and total n-3 intake [233]. In the most recent study, fish was the only food group inversely associated with the change in markers of low-grade inflammation (i.e., CRP, serum amyloid A, IL-6, IL-8, TNF-α, and soluble intercellular adhesion molecule-1) over the 6 year follow-up period [243]. Furthermore, EPA+DHA intake, which was also inversely associated with the change in inflammatory marker concentrations, explained 40% of the inverse association between fish intake and low-grade inflammation [243]. However, fish intake is sporadic in many populations, and thus may not be accurately captured by dietary assessments that rely on the respondent’s memory and may be limited to a narrow window of time (i.e. 24-hour or 3-day recalls). The accuracy of nutrient databases regarding the omega-3 FA content in foods may also be limited. Furthermore, associations in observational studies may be limited by confounding from other dietary...
and/or lifestyle factors, and cannot prove causality. Therefore, although these findings are promising it is difficult to translate these epidemiological relationships to clinical application.
Table 2-5. Studies investigating self-reported dietary intake of fish and/or EPA+DHA and associations with inflammatory markers.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study population</th>
<th>Dietary Assessment</th>
<th>Results</th>
<th>Points of interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>van Bussel, 2011</td>
<td>Healthy adults in Amsterdam (n = 301)</td>
<td>Computer-assisted crosscheck dietary history of previous month</td>
<td>Fish the only food group associated with decreased low-grade inflammation (panel of CRP, SAA, IL-6, IL-8, TNF-α, and sICAM-1) and endothelial dysfunction EPA+DHA also inversely related to low-grade inflammation and endothelial dysfunction</td>
<td>EPA+DHA intake largely explained association for fish consumption No difference between fat and lean fish consumption</td>
</tr>
<tr>
<td>He, 2009</td>
<td>MESA cohort (Multi-Ethnic Study of Atherosclerosis) (n = 5677)</td>
<td>Self-administered FFQ of previous year</td>
<td>LC n-3 PUFA intake inversely associated with IL-6 and matrix metalloproteinase-3 (MMP-3) Non-fried fish consumption was inversely related to CRP and IL-6</td>
<td>Median EPA+DHA intake: 92 mg/d Median non-fried fish consumption: 0.3 servings/wk</td>
</tr>
<tr>
<td>Niu, 2006</td>
<td>Japanese adults (≥ 70 y) (n = 971; 401 men and 570 women)</td>
<td>Self-administered diet-history questionnaire (BDHQ) of previous year</td>
<td>OR of CRP ≥ 1 mg/L decreased across concentrations of total n-3 and EPA+DHA OR of 0.54 for highest EPA+DHA quartile</td>
<td>Only study of a population with high EPA+DHA intake Similar results for individual analysis of EPA and DHA Median EPA+DHA intake: 970 mg/d</td>
</tr>
<tr>
<td>Zampelas, 2005</td>
<td>ATTICA Study (n = 1514 men and 1528 women)</td>
<td>FFQ of previous year</td>
<td>Inverse dose-response relationship for all inflammatory markers Compared to non-fish consumers, those consuming &gt;300 g/wk had lower CRP (-33%), IL-6 (-33%), TNF-α (-21%), SAA (-28%), and WBC counts (-4%)</td>
<td>88% of men and 91% of women reported fish consumption at least once/month Average omega-3 intake was 400 mg/d in men and 520 mg/d in women</td>
</tr>
<tr>
<td>Lopez-Garcia, 2004</td>
<td>Nurses’ Health Study (n = 727)</td>
<td>Semi-quantitative FFQ of previous year</td>
<td>Total n-3 fatty acids inversely related to CRP and IL-6 Highest quintile of total n-3 fatty consumption had 29% lower CRP and 23% lower IL-6 CRP and IL-6 decreased w/ an increase in frequency of fish consumption</td>
<td>Markers of endothelial activation (E-selectin, sICAM-1, sVCAM-1) also inversely associated Median EPA+DHA intake in highest quintile: 450 mg/d (70 mg/d in lowest quintile)</td>
</tr>
<tr>
<td>Pischon, 2003</td>
<td>Health Professionals Follow-Up Study (n = 405) and Nurses’ Health Study (n = 454)</td>
<td>Semi-quantitative FFQ</td>
<td>EPA and DHA intake inversely associated with sTNF-R1 and sTNF-R2 (p=0.03 and p&lt;0.001) Marginally significant inverse association with CRP (p=0.08) No relationship with IL-6</td>
<td>Inverse association with TNF receptors were strongest in high n-6 consumers EPA+DHA intake in the 40-60th percentile (reported as % Energy) was 250 mg/d in men and 121 mg/d in women</td>
</tr>
</tbody>
</table>
2.3.1.3 Clinical studies of omega-3 fatty acids and inflammation

Despite the strong pre-clinical and epidemiological evidence base indicating the efficacy of omega-3 fatty acids for ameliorating inflammation, this has not translated to clinical results [244]. Numerous clinical studies have been conducted to investigate the effect of omega-3 fatty acid supplementation on markers of inflammation. Results have been largely inconsistent, with some studies reporting beneficial effects and other findings being null. These mixed results may be due to heterogeneity in the dose and duration of supplementation, characteristics of the study population, and/or differences in background omega-3 intake. This creates much difficulty in evaluating the efficacy of omega-3 fatty acids as an anti-inflammatory therapy and in determining critical details related to their delivery (e.g., optimal dose and duration, target conditions, and relative efficacy of individual fatty acid species).

Many meta-analyses and systematic reviews have been conducted to evaluate the seemingly contradictory results from clinical supplementation trials, but have been unable to provide firm conclusions due to the inconsistency of findings and variation in study design [245-247]. Most recently, a meta-analysis of 68 randomized controlled trials with a total of 4601 participants was conducted to review the effect of omega-3 fatty supplementation on TNF-α, IL-6, and CRP in healthy individuals, individuals with chronic non-immune disease, and individuals with chronic autoimmune disease [248]. They reported that omega-3 supplementation had a significant lowering effect on TNF-α, IL-6, and CRP in all three types of participants. However, the effect size of these reductions was generally very low. For instance, a significant 0.2 mg/L reduction in CRP was reported across 44 studies in individuals with chronic non-autoimmune disease. The clinical implications of a 0.2 mg/L CRP reduction are unknown and would be difficult to detect in a single clinical trial. Furthermore, these conclusions are limited by the heterogeneity of the studies included in the meta-analysis.

2.3.2 Omega-3 fatty acids and oxidative stress

Despite substantial evidence supporting the health benefits of greater long-chain omega-3 fatty acid intake, there remains a theoretical concern that the incorporation of these fatty acids into the cell membrane may increase lipid peroxidation. This is based largely on in vitro observations that the susceptibility of fatty acids to oxidation is proportional to their degree of unsaturation [249]. However, the oxidative conditions that are used to determine oxidative susceptibility in vitro may not be relevant under more biologically complex in vivo conditions. Additional factors, such as the position of double bonds, may also influence the generation of oxidative products [250]. Thus, a higher degree of unsaturation may not necessarily equate with greater susceptibility to oxidation [251]. The in vivo results from human and animal studies relating to the effects of omega-3 fatty acids on lipid peroxidation are contradictory [249]. These inconsistencies may be related to differences in study design (e.g., study population characteristics, dose and duration of supplementation, etc.), but have primarily been attributed to inconsistencies and limitations in the indirect and/or non-specific assays used to assess lipid peroxidation (i.e., oxidized LDL and TBARS assays) [252].

Numerous studies have evaluated the effect of omega-3 fatty acids on isoprostane production—a reliable in vivo marker of oxidative stress—to address the concern that the cardioprotective benefits of omega-3 fatty acid intake could be mitigated by enhanced lipid peroxidation (Table 2-6). None have found an increase in F2-Isos following omega-3 fatty acid supplementation [253-263]. Many have found that omega-3 fatty acid supplementation decreased F2-Isos [257, 258, 260, 262, 263] and/or increased the production of F3-Isos [254]. A reduction in F2-Isos due to omega-3 fatty acid intake was first demonstrated by Mori et al. in 1999 [263]. In this study, 8 weeks of consuming one daily fish meal
(providing ~3.6 g/d of EPA+DHA) in combination with a low-fat diet reduced urinary F$_2$-IsoPs by 20% in sedentary dyslipidemic men with type 2 diabetes [263]. Subsequent studies have shown similar effects on both plasma and urine F$_2$-IsoP concentrations in different study populations, with varying doses and supplementation duration. For instance, plasma F$_2$-IsoPs were also reduced by omega-3 supplementation in healthy men and women when the background diet was high in saturated fat or monounsaturated fat [258]. Notably, cyclooxygenase-mediated lipid peroxidation was not altered by omega-3 supplementation in this study [258]. Maternal fish oil supplementation during pregnancy also reduced neonatal F$_2$IsoPs [260]. When consumed individually, EPA and DHA have also been shown to reduce F$_2$-IsoPs in the plasma and urine of overweight, hyperlipidemic men and individuals with type 2 diabetes and treated hypertension following 6 weeks of supplementation [257]. It is important to note that these reductions in F$_2$-IsoPs were unrelated to changes in plasma [257], red blood cell [260], and/or platelet fatty acid content that were caused by omega-3 fatty acid supplementation [263]. Therefore, the decrease in F$_2$-IsoPs most likely reflects a true reduction in oxidative stress and is not simply a result of limited AA substrate availability. Thus, there is substantial evidence that omega-3 fatty acids do not adversely affect oxidative stress in terms of F$_2$-IsoP production and may even have beneficial effects.
Table 2-6. Studies evaluating effect of omega-3 fatty acid supplementation on isoprostane production.

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study population</th>
<th>Study design</th>
<th>Omega-3 fatty acid intervention</th>
<th>Comparator and/or placebo</th>
<th>Duration of supplementation</th>
<th>Biological fluid analyzed</th>
<th>Effect on isoprostanes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skarke, 2015 [253]</td>
<td>Healthy adults (n = 12)</td>
<td>Pre-post</td>
<td>17 g/d EPA+DHA</td>
<td>None</td>
<td>24 days</td>
<td>Urine</td>
<td>No change in F₂-IsoPs, F₃-IsoPs ↑</td>
</tr>
<tr>
<td>Ferguson, 2014 [254]</td>
<td>Healthy adults (n = 60)</td>
<td>Parallel</td>
<td>3.6 g/d EPA+DHA</td>
<td>0.9 g/d EPA+DHA or corn oil placebo</td>
<td>8 weeks</td>
<td>Urine</td>
<td>High dose (3.6 g/d) EPA+DHA supplementation ↑ F₃-IsoPs, No change in F₂-IsoPs</td>
</tr>
<tr>
<td>Ulven, 2011 [255]</td>
<td>Healthy adults (n = 113)</td>
<td>Parallel</td>
<td>Fish oil (864 mg/d EPA+DHA)</td>
<td>Krill oil (543 mg/d EPA+DHA) or no supplement</td>
<td>7 weeks</td>
<td>Plasma</td>
<td>No change in F₂-IsoPs in either group</td>
</tr>
<tr>
<td>Ottestad, 2012 [256]</td>
<td>Healthy adults (n = 54)</td>
<td>Parallel</td>
<td>Fish oil (1.6 g/d EPA+DHA)</td>
<td>Oxidized fish oil (1.6 g/d EPA+DHA) or high-oleic sunflower</td>
<td>7 weeks</td>
<td>Urine</td>
<td>No change in F₂-IsoPs within or between groups</td>
</tr>
<tr>
<td>Mas, 2010 [257, 264]</td>
<td>Study A: overweight, dyslipidemic men (n = 56)</td>
<td>Parallel arm</td>
<td>4 g/d EPA or DHA</td>
<td>4 g/d olive oil placebo</td>
<td>6 weeks</td>
<td>Plasma and urine</td>
<td>EPA ↓ plasma F₂-IsoPs by 24%, DHA ↓ plasma F₂-IsoPs by 14%</td>
</tr>
<tr>
<td></td>
<td>Study B: men and women with type 2 diabetes and treated hypertension (n = 51)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Author, Year</td>
<td>Population/Intervention</td>
<td>Study Design</td>
<td>Intervention Details</td>
<td>Follow-up</td>
<td>Specimen</td>
<td>Findings</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------------</td>
<td>--------------</td>
<td>----------------------</td>
<td>-----------</td>
<td>----------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>Nalsen, 2006 [258]</td>
<td>Healthy adults (n = 162)</td>
<td>Parallel</td>
<td>3.6 g/d omega-3 fatty acids with high SFA or high MUFA background diet</td>
<td>3 months</td>
<td>Plasma</td>
<td>Omega-3 supplementation ↓ F₂-IsoPs irrespective of background diet</td>
<td></td>
</tr>
<tr>
<td>Tholstrup, 2004 [259]</td>
<td>Dyslipidemic men (n = 16)</td>
<td>Crossover</td>
<td>Fish oil-enriched solid test fat (5 g/100 g EPA and DHA, substituted for 80 g of normal dietary fat intake)</td>
<td>3 weeks (3 week washout)</td>
<td>Plasma and urine</td>
<td>No differences in F₂-IsoPs between groups. Trend towards a ↓ in urinary F₂-IsoPs with fish oil test fat after adjustment for urinary excretion rate</td>
<td></td>
</tr>
<tr>
<td>Barden, 2004 [260]</td>
<td>Pregnant women with allergic disease (n=83)</td>
<td>Parallel</td>
<td>4 g/d fish oil (56% DHA, 27.7% EPA)</td>
<td>~20 weeks (from 20 weeks of gestation until delivery)</td>
<td>Urine and cord blood plasma</td>
<td>Maternal fish oil supplementation ↓ F₂-IsoPs in cord plasma. Trend towards a ↓ in urinary F₂-IsoPs</td>
<td></td>
</tr>
<tr>
<td>Stier, 2001 [261]</td>
<td>Pre-term infants (n = 23)</td>
<td>Parallel</td>
<td>Formula enriched with long-chain PUFA (primarily LA, AA, and DHA)</td>
<td>3 weeks</td>
<td>Urine</td>
<td>No differences in F₂-IsoPs among groups</td>
<td></td>
</tr>
<tr>
<td>Higdon, 2000 [262]</td>
<td>Post-menopausal women on HRT (n = 15)</td>
<td>Crossover</td>
<td>3.4 g/d EPA+DHA</td>
<td>5 weeks (7 week washout)</td>
<td>Plasma</td>
<td>Plasma free F₂-IsoPs ↓ 15% after omega-3 supplementation compared to sunflower oil</td>
<td></td>
</tr>
<tr>
<td>Mori, 1999 [263]</td>
<td>Sedentary, dyslipidemic men with type 2 diabetes (n = 49)</td>
<td>Parallel</td>
<td>Low-fat diet with one daily fish meal (~3.6 g/d omega-3 fatty acids)</td>
<td>8 weeks</td>
<td>Urine</td>
<td>Fish diet ↓ urinary F₂-IsoPs by 20%</td>
<td></td>
</tr>
</tbody>
</table>
Less is known about the effect of omega-3 supplementation on F3-IsoPs. The *in vivo* production of F3-IsoPs following EPA+DHA supplementation has been demonstrated in both animal models and clinical trials [84, 253, 254]. However, F3-IsoPs were not detected following 6 weeks of supplementation with 4 g/d EPA or DHA in overweight, hyperlipidemic men or individuals with type 2 diabetes and treated hypertension [257]. It may be that these compounds are undetectable in some populations under basal conditions as many of the animal models in which F3-IsoPs have been analyzed utilized an inflammatory stimuli to induce oxidative stress and increase the formation of isoprostanes [84, 265]. In humans, LPS administration (3 ng/kg) has been shown to induce both F2- and F3-IsoP production [266], with the greatest increase in F3-IsoPs observed 2-8 hours following LPS administration (Figure 2-3) [88]. Very little is known about the effect of lower LPS doses on isoprostane production, and how this may be influenced by omega-3 fatty acid supplementation. It has recently been reported that low-dose LPS administration (0.6 ng/kg; explained further below) following 8 weeks of supplementation with 3.4 g/d EPA+DHA caused no directional change in F2-IsoPs, but increased F3-IsoP production in a time-dependent manner [253]. In this case, F3-IsoP elevations peaked at 4 hours post-LPS injection, and these values substantially decreased by 24 hours post-injection; however, no other measurements were taken in the intervening time periods. It should also be noted that these findings are reported as preliminary results based on a small subset of participants (n = 6) and that no statistical analyses were performed [253]. The effect of omega-3 supplementation on isoprostane production following LPS administration has not been evaluated in other clinical studies. Urinary isoprostane outcomes from our clinical omega-3 supplementation study using a low-dose endotoxin model are presented in Chapter 5.

![Figure 2-3. Synthesis of urinary F3-Isoprostanes in healthy adults following LPS (3 ng/kg) administration (n = 10). Source: Song WL, Novel eicosapentaenoic acid-derived F3-isoprostanes as biomarkers of lipid peroxidation. J Biol Chem 2009;284(35):23636-43 [88].](image-url)
2.3.3 Research on omega-3 fatty acids using human endotoxemia challenge

The lack of consistent clinical findings regarding inflammatory outcomes may be due to inherent limitations in how clinical nutrition studies are typically designed. The anti-inflammatory potential of omega-3 fatty acids is largely based on evidence from cell/animal models that use an inflammatory stimulus—a key component that is lacking in most clinical trials. As the effects of omega-3 fatty acids are likely to be subtle compared to those of anti-inflammatory pharmacological agents, they may be more difficult to detect in the resting physiological state. This may account for the null findings in some clinical studies. Therefore, future omega-3 supplementation studies may benefit from the use of an \textit{in vivo} induced inflammatory challenge model similar to those utilized in cell- and animal-based model systems.

2.3.3.1 Human low-dose endotoxemia model of acute inflammation

A clinical model of inflammation provides the opportunity to investigate whether omega-3 fatty acids can attenuate the response to an inflammatory stimuli. Similar to cell/animal models, this type of study design ensures that the omega-3 fatty acids are in the system prior to the inflammatory challenge and isolates the effects of the intervention to help prove causality. Unlike cell and animal models, human models of induced inflammation provide the added benefit of testing the intervention within the context of human physiology and at physiologically achievable doses of the intervention. The human low-dose (0.6 ng/kg) endotoxin (or lipopolysaccharide, LPS) model likely provides the most useful system for evaluating the anti-inflammatory potential of omega-3 fatty acids as it produces a robust systemic inflammatory response that is consistent between individuals.

Induced endotoxemia has frequently been used as a clinical model of sepsis and as a means of testing the efficacy of anti-inflammatory medications [44]; thus the physiological responses that it produces have been well-characterized [45]. In general, the inflammatory cytokine response to intravenous administration begins with TNF-\(\alpha\) production approximately 45 to 60 minutes following LPS injection. This is quickly followed by IL-6 production, with concentrations of TNF-\(\alpha\) and IL-6 remaining elevated 1-3 hours post-injection. These are then replaced by anti-inflammatory cytokines (e.g., IL-10) as the inflammatory response enters the resolution phase Figure 2-4. Production of the acute phase reactant C-reactive protein (CRP) in the liver occurs much later in the response, with peak elevations occurring approximately 24 hours post-injection. Clinical symptoms (e.g., chills, headache, myalgia, and an increase in body temperature and heart rate) typically begin to occur within 1 hour of administration, with symptoms typically receding in 4-8 hours, in conjunction with the resolution of the inflammatory cytokine response. Higher doses of LPS (3-5 ng/kg) tend to evoke more severe clinical symptoms and inflammatory responses, as well as the production of counter-regulatory hormones (e.g, cortisol and growth hormone) [267], which limits the application of the model for the study of cardiometabolic diseases [268].
The low-dose human endotoxemia model (0.6 ng/kg) evokes a more moderate systemic inflammatory response that is more reflective of low-grade inflammation in cardiometabolic disease. This inflammatory response occurs over a similar time course, but with less extreme elevations in cytokine production compared to those produced by a higher dose of LPS (3 ng/kg) (Figure 2-5). The modest insulin resistance induced by this model is also consistent with that seen in metabolic syndrome and type 2 diabetes [268]. The low-dose model also produces minimal clinical symptoms [268]. Therefore, although this model does not reproduce the chronic pathophysiology of cardiometabolic disease, it acutely approximates the inflammatory and metabolic features of these diseases, and thus provides a safe and broadly applicable model for studying novel therapeutics and dietary interventions.
Our research group has previously conducted two studies using the low-dose endotoxemia model of induced inflammation, and results from these studies informed the design of the current omega-3 fatty acid study presented in Chapter 4. A supplementation study was designed to test the hypothesis that having higher concentrations of omega-3 fatty acids at the time of an inflammatory stimulus will lead to attenuated responses and a more effective resolution of inflammation. Prior to carrying out the supplementation trial, a pilot study was conducted to characterize the low-dose endotoxemia model and build the infrastructure needed to implement the model for future studies. The pilot study was a randomized double-blind, placebo-controlled, crossover study (saline versus 0.6 ng/kg LPS) conducted in six healthy young men. Administration of LPS induced robust cytokine and CRP elevations in these young men (unpublished data) that were consistent with previously reported low-dose endotoxemia responses [268]. Following the pilot study, the larger omega-3 supplementation trial was conducted. This study was a parallel group study in which healthy young men and women who were low fish consumers were supplemented with fish oil (0, 300, 600, 900, or 1800 mg/d) for 5 months prior to undergoing a low-dose endotoxin challenge. Only a subset (n = 19) of the larger study population underwent the low-dose endotoxin challenge and were analyzed for inflammatory responses. Due to the limited sample size, participants were grouped into low (0 and 300 mg/d) and high (600, 900, and 1800 mg/d) dose supplementation groups for analysis. Univariate analyses suggested a trend for a 4 mg/L reduction in peak
CRP elevations in the high dose supplementation group (p = 0.12) (unpublished data). Therefore, to build on these findings, a double-blind crossover study of high dose omega-3 supplementation (3.4 g/d EPA+DHA) using the low-dose endotoxemia model was designed. The use of a crossover design was expected to provide greater statistical power for evaluating effects with a “proof of principle” dose of omega-3 fatty acids. The findings from this study are presented in Chapter 4 and Chapter 5.

2.4 Rationale for dissertation research studies
Nutritional interventions are a key component of CVD risk reduction. Current dietary guidelines for reducing CVD risk emphasize greater intake of nutrient-dense foods (e.g., fruits and vegetables, whole grains, seafood, low-fat dairy, nuts, etc.) in place of refined grains and other foods that are high in saturated fat and added sugars. It is likely that a dietary pattern approach based on whole foods will provide greater cardioprotective effects than focusing solely on macronutrient targets. However, it is also useful to evaluate the contribution of individual foods/food groups and the dietary bioactives that they provide. Two of the dietary bioactives with the strongest evidence base supporting their consumption are: 1) phytochemicals in fruits and vegetables and 2) long-chain omega-3 fatty acids in oily fish. However, additional research is needed to: clarify which CVD risk factors these interventions can modify and the strength of those effects; better define the amounts and dietary sources that should be recommended; and identify target populations that would receive the most benefit. This knowledge would aid in the development of more precise, evidence-based dietary recommendations. Therefore, the purpose of the studies described in the following sections and chapters was to improve our understanding of how supplementation with plant-based bioactives and long-chain omega-3 fatty acids can modify established and emerging CVD risk factors.

2.4.1 Strawberry supplementation study
Strawberries are an important source of dietary bioactives, including fiber, vitamin C, phytosterols, and numerous phenolic compounds. Supplementation with strawberries over the course of weeks or months has previously been shown to improve certain CVD risk factors, such LDL-C and markers of oxidative stress. Previous studies have demonstrated that incorporating bioactive-rich spices into a high-fat meal can attenuate postprandial spikes in triglycerides and insulin, but little is known about whether strawberries can have similar beneficial effects when incorporated in a high-fat meal. One study, in a population of overweight hyperlipidemic adults, found that 10 g of freeze dried strawberry powder modestly attenuated peak triglyceride responses and LDL oxidation following a moderate fat meal. No other studies have evaluated the postprandial effects of strawberries.

2.4.1.1 Specific aims and hypotheses
The objective of this study was to evaluate the effect of incorporating a single dose of freeze dried strawberry powder (40 g) into a high-fat meal (50 g total fat) on postprandial vascular function, triglycerides, glucose, and insulin (compared to a control high-fat meal containing no strawberries). Postprandial vascular function (i.e., central blood pressure, augmentation index, and pulse wave velocity) was the primary endpoint for this study. We hypothesized that incorporating the freeze dried strawberry powder into the high-fat meal would improve these indices of postprandial vascular function compared to the control high-fat meal. Our secondary endpoints were postprandial triglyceride, glucose, and insulin concentrations. We hypothesized that incorporating freeze dried strawberry powder into the meal would attenuate the postprandial spikes in triglycerides, glucose, and insulin that are caused by consuming a high-fat meal. The results of this study are presented in Chapter 3.
2.4.2 Study of omega-3 supplementation and inflammatory responses to low-dose endotoxemia in healthy young men

There is substantial pre-clinical evidence from cell and animal models demonstrating the anti-inflammatory potential of EPA and DHA. However, clinical intervention studies of EPA+DHA supplementation have yielded mixed results for inflammatory outcomes—potentially due to the lack of a controlled inflammatory stimulus similar to those used in pre-clinical models. The low-dose human endotoxemia model (0.6 ng of lipopolysaccharide per kg of body weight) safely produces an acute inflammatory response that is characterized by a consistent time course of inflammatory marker elevations. This simulates the controlled, induced inflammation employed in cell and animal models within the context of human physiology, and provides the opportunity to examine whether omega-3 fatty acids can alter the production of pro-inflammatory signals under conditions of heightened inflammation typically associated with disease.

2.4.2.1 Specific aims and hypotheses

The objective of this study was to evaluate the effect of 3.4 g/d EPA+DHA supplementation versus an olive oil placebo on inflammatory marker responses to low-dose endotoxemia in healthy young men. We hypothesized that EPA+DHA supplementation (3.4 g/d) would reduce peak inflammatory marker concentrations following low-dose endotoxemia compared to placebo supplementation. Our primary endpoint was the peak C-reactive protein (CRP) concentration, which occurs at 24 hours following endotoxin administration. The inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 were also analyzed. The results of this study are presented in Chapter 4.

2.4.3 Study of omega-3 supplementation and the oxidative stress response following low-dose endotoxemia

Isoprostanes (IsoPs) are prostaglandin-like molecules formed via non-enzymatic, free radical-induced lipid peroxidation. F2-isoprostanes (F2-IsoPs) are formed from arachidonic acid and are a reliable marker of oxidative stress. Conversely, F3-isoprostanes (F3-IsoPs) derived from EPA are less biologically active and do not exert the same vasoconstrictive or platelet aggregating effects as F2-IsoPs. There is substantial evidence that omega-3 supplementation does not adversely affect oxidative stress in terms of F2-IsoP production and may have beneficial effects. However, little is known about urinary isoprostane production due to an inflammatory stimulus (e.g., low-dose endotoxemia) and how this response may be influenced by EPA+DHA supplementation.

2.4.3.1 Specific aims and hypotheses

The objective of this study was to evaluate the effect of omega-3 fatty acid supplementation on oxidative stress, measured as urinary isoprostane production, following low-dose endotoxemia. We utilized urine samples from two studies of healthy young adults (Study 1) and healthy young men (Study 2) who were low fish consumers. In exploratory analyses, we also sought to investigate how other factors (e.g., BMI, sex, and age) might influence urinary isoprostane production. We hypothesized that omega-3 supplementation prior to low-dose endotoxemia would increase F3-IsoP production but attenuate the overall oxidative stress response in terms of total isoprostane production. These analyses are presented in Chapter 5.
Chapter 3. Incorporating freeze dried strawberry powder into a high fat meal does not alter postprandial vascular function or blood markers of cardiovascular risk

3.1 Abstract

**Background:** Postprandial dysmetabolism, an exaggerated elevation in serum triglycerides and glucose, increases cardiovascular disease (CVD) risk by inducing oxidative stress, inflammation, and endothelial dysfunction. Phytochemical-rich foods may blunt these effects when they are incorporated into a high-fat calorie-dense meal. Strawberries are a rich source of phytochemicals but little research exists on their postprandial effects.

**Objective:** The purpose of this study was to evaluate effect of adding 40 g of freeze dried strawberry powder (~1 lb. fresh strawberries) to a high-fat (50 g total fat) 1000 kcal meal on postprandial vascular function, as well as triglyceride, glucose, and insulin responses.

**Methods:** Healthy, overweight/obese (mean BMI = 31 ± 0.5 kg/m²) adults (mean age = 28 ± 2 years; 17M, 13F) consumed a control meal and strawberry meal in a randomized crossover design. Testing sessions were separated by ≥1 week for men and ~1 month for women to control for hormonal variations. Blood samples were obtained prior to the meal and 0.5, 1, 2, and 4 hours after the meal was consumed. Central blood pressure and arterial stiffness indices were measured at baseline and 2 and 4 hours post-meal, using pulse waveform analysis.

**Results:** There were no significant differences between the strawberry and control meals for any vascular function indices or metabolic responses. Consumption of either meal resulted in a significant decrease in the augmentation index (p < 0.002) and significant increases in serum triglycerides, insulin, and glucose compared to baseline (p < 0.001).

**Conclusion:** Acute consumption of freeze dried strawberries may be insufficient to ameliorate the postprandial metabolic derangements caused by a high-fat meal. Further research is needed to clarify the lipid and vascular function effects of longer-term freeze dried strawberry consumption.
3.2 Introduction

Postprandial dysmetabolism, which is characterized by exaggerated elevations in glucose, insulin, and triglycerides, substantially increases CVD risk [43, 269]. Large spikes and delayed clearance of triglycerides and glucose increase oxidative stress. This production of free radicals and oxidized low density lipoprotein-cholesterol (LDL-C) [166] damages the vascular endothelium and promotes atherogenesis [269]. Dietary patterns that emphasize phytochemical-rich fruits and vegetables have been proposed as a means of attenuating post-meal spikes in triglycerides and glucose, but less is known about the postprandial effects of individual foods.

Promising results have been found for certain phytochemical-rich foods, but these findings are limited to a relatively small number of studies and may depend on the unique bioactive profile of the intervention. For example, in overweight men, incorporating 14 g of antioxidant-rich spices in a high-fat meal attenuated postprandial insulin and triglyceride responses [270], potentially via inhibition of lipase enzymes. Improvements in the glycemic profile have also been found when berries are provided with a carbohydrate-rich meal [176-178].

Strawberries are a good source of numerous phytochemicals (e.g. ellagic acid, ellagitannins, anthocyanins, and flavonols) [194], and previous research has demonstrated that strawberry consumption can improve multiple CVD risk factors. For instance, consuming 50 g/d of freeze dried strawberry powder for 12 weeks reduced total cholesterol and LDL-C in men and women with abdominal adiposity and elevated lipids [227]. Similar improvements in the lipid profile were found in individuals with metabolic syndrome following 8 weeks of supplementation with 50 g per day [225]. Others have reported beneficial changes in antioxidant status [198, 226] and glycemic control [226], as well as reduced oxidative stress [226, 227, 271] following strawberry supplementation.

However, the effect of strawberries on central blood pressure and arterial stiffness has not been studied. In epidemiologic studies, increased anthocyanin intake, such as that achieved by 1-2 servings of berries per day, has been associated with lower arterial stiffness and central blood pressure in women [272]. Other phytochemical-rich foods, such as soy, have been shown to reduce arterial stiffness [273]. Thus, strawberry phytochemicals may have similar beneficial effects on vascular function; however, intervention studies are needed to confirm epidemiological findings and determine if there is a causal relationship between strawberry intake and decreased arterial stiffness.

Additionally, little research has examined the acute effects of strawberries in a postprandial setting. In a study of 24 overweight hyperlipidemic adults, 10 g of freeze dried strawberry powder modestly attenuated peak triglyceride responses and LDL oxidation following a moderate fat meal [274]. A 40 g dose of freeze dried strawberry powder, which is equivalent to approximately one pound of fresh strawberries, provides a substantial amount of phytochemicals [179] while contributing minimal calories. Larger doses (50 g/d) have also been used effectively in supplementation studies lasting 4-8 weeks [275, 276]. Therefore, this study was designed to investigate effects on postprandial vascular function from incorporating 40 g of freeze dried strawberry powder into a high fat meal. Our secondary aim was to evaluate postprandial triglyceride, glucose, and insulin responses, as well as biomarkers of lipid oxidation.

3.3 Subjects and Methods

3.3.1 Study population

Men and women 20-50 years of age and free of any serious illness were recruited for the study. Other inclusion criteria consisted of a body mass index (BMI) of 28-39 kg/m², resting blood pressure < 160/100
mm Hg, fasting triglycerides < 350 mg/dL, and fasting LDL-C < 160 mg/dL. Exclusion criteria included acute or chronic inflammatory conditions, liver or kidney dysfunction, a history of heart disease, use of tobacco products, intolerance for high fat meals, allergies/sensitivities to wheat or strawberries, and use of non-steroidal anti-inflammatories, immunosuppressants, or medications/supplements for elevated lipids, blood pressure, or glucose.

3.3.2 Participant recruitment
Subjects were recruited via fliers in the community and campus e-mail lists. Potential subjects emailed or called to indicate interest in participating in the study and were then given additional information about the study. If interested, they were asked a series of medical history and lifestyle questions. Of the 235 initial respondents, 121 were screened by telephone. Forty-four met study criteria and completed a clinic screening at the Penn State Clinical Research Center (CRC). After written informed consent was obtained, a blood sample was drawn for a complete blood count and standard chemistry profile (lipid panel, glucose, liver and kidney function) to rule out the presence of illness (autoimmune disease, cancer, and immunodeficiency). Blood pressure was measured according to JNC 7 guidelines [277]. Briefly, after a 5-minute 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. Body weight and height were measured (without shoes and in light clothing) to calculate BMI. Of the 44 individuals who were screened, 34 met eligibility criteria and were enrolled in the study. Four participants withdrew during the study. Thus, data are reported for 30 participants (n = 17 men, n = 13 women) (Figure 3-1). 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.
3.3.3 Study design and intervention
In this randomized, controlled, 2-period crossover study participants completed two postprandial challenges that consisted of single-day visits of approximately 5 hours each. The two test conditions were: 1) a control meal and 2) the control meal with added freeze dried strawberry powder. The Control and Strawberry Meal consisted of two cheese blintzes with whipped cream and strawberry syrup, a hard-boiled egg, and bacon. The meals contained equivalent macro- and micronutrient profiles and differed only in antioxidant content (Table 3-1). Strawberry Meal blintzes contained 40 g of freeze dried strawberry powder mixed into the cheese filling. The nutrient profile and antioxidant content of the strawberry and control powders are provided in Supplemental Table 3-1 and Supplemental Table 3-2, respectively. To maintain blinding and ensure that the taste and appearance of both meals were comparable, Control Meal blintzes were made with a strawberry flavored powder that was devoid of strawberry bioactives (e.g., vitamin C and polyphenolic compounds). Both the freeze dried strawberries and control powder were provided by the California Strawberry Commission. The meal components were weighed in advance and prepared fresh prior to each day of testing.
### Table 3.1. Nutrient composition of meals.

<table>
<thead>
<tr>
<th></th>
<th>Control Meal</th>
<th>Strawberry Meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories (kcal)</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Calories from Fat (kcal)</td>
<td>450</td>
<td>450</td>
</tr>
<tr>
<td>Total Fat (g)</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Saturated fat (g)</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Trans fat (g)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>435</td>
<td>435</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>106</td>
<td>105</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>30</td>
<td>32</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>1390</td>
<td>1320</td>
</tr>
<tr>
<td>Vitamin C (% Daily Value)</td>
<td>2%</td>
<td>380%</td>
</tr>
</tbody>
</table>

Postprandial challenges were separated by a minimum of one week for men. Visits for female participants were scheduled during the first seven days of the menstrual cycle to avoid hormonal effects on vascular measures and were therefore separated by approximately one month. All postprandial challenges were conducted at the Pennsylvania State University CRC according to standardized protocols. Prior to testing visits, participants were instructed to: avoid high antioxidant foods (berries, cocoa/chocolate), strenuous exercise, and alcohol; refrain from taking pain relievers, vitamins, or minerals; and limit their caffeine intake to no more than 1 cup per day for 48 hours. Testing visits were conducted following an overnight fast (no food or drink other than water for 12 hours).

At each postprandial test visit, an intravenous (IV) catheter was established for blood sampling following baseline vascular function testing. A topical skin anesthetic was offered to numb the area prior to catheter insertion. Following baseline blood sample collection, participants were asked to consume the Control or Strawberry Meal within ~15 minutes. No other food or drinks other than water were allowed for the remainder of the testing period. Additional blood samples and vascular function measures were taken at pre-specified time intervals relative to when the meal was finished: blood samples were collected at 30 minutes, 1 hour, 2 hours and 4 hours after the meal; vascular function measures were performed at 2 and 4 hours following the meal. At the end of the 4-hour period, the catheter was discontinued and participants were briefly evaluated for safety before leaving the CRC. This procedure was repeated for the second postprandial visit.

#### 3.3.4 Blood sample collection and assay methods

Blood drawn into lithium heparin and EDTA anticoagulated tubes was immediately centrifuged at 1500 g for 15 minutes. Blood drawn into serum separator tubes was allowed to clot for 30 minutes prior to centrifugation. 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 calculated using the Friedewald equation \[\text{LDL-C} = \text{TC} - (\text{HDL-C} + \text{TG}/5)\]. Postprandial LDL-C was not interpreted since calculated values are not accurate during non-fasting conditions. Insulin was measured by radioimmunoassay (Quest Diagnostics). Glucose was determined by Spectrophotometry procedures (Quest Diagnostics). Serum high-sensitivity C-reactive protein (CRP) was measured by latex-enhanced immunonephelometry (Quest Diagnostics; assay CV < 8%). For other endpoints, aliquots of serum and plasma were stored at -80 °C for batch analysis.
3.3.4.1 Biomarkers of oxidative stress
Malondialdehyde (MDA) was measured in EDTA plasma using the thiobarbituric acid reactive substances (TBARS) assay (Cayman Chemical Co., Ann Arbor, MI). Oxidized LDL was measured in EDTA plasma by enzyme-linked immunosorbent assay (ELISA; Mercodia AB, Uppsala, Sweden).

3.3.4.2 Pancreatic lipase inhibition
Extracts of the freeze-dried strawberry powder were prepared with 10 volumes of acetone:water:acetic acid (80:20:0.1%, v:v:v) overnight. The organic solvent was removed under vacuum and the remaining aqueous solution was freeze-dried. Lipase from porcine pancreas (PL, type II) and 4-nitrophenyl butyrate (4-NPB, 98%) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared in dimethyl-sulfoxide (EMD Chemicals Inc.) and stored at -20°C.

Inhibition of pancreatic lipase (PL) by the freeze-dried strawberry powder was tested by monitoring the cleavage of 4-NPB to release 4-nitrophenol. PL was suspended in water (10 mg/mL) and incubated at 37°C for 5 minutes. The solution was centrifuged for 5 minutes at 664 x g and the supernatant was then used as the enzyme source for subsequent experiments. For each experiment, the PL supernatant was diluted 1:50 in buffer solution (20 mM Tris-HCl, 1.3 mM CaCl₂, pH = 8.0). Freeze-dried strawberry powder extracts (0-200 μg/mL) were combined with PL, and 4-NPB (0.2 mM) was added to start the reaction. Following 10 minutes of incubation at 37°C, absorbance was read at 400 nm. Analyses were performed in triplicate.

3.3.5 Vascular function measures
Vascular function, in terms of central blood pressure and arterial stiffness indices, was assessed using the SphygmoCor System pulse waveform analysis (AtCor Medical, Sydney, Australia). Measures were taken at baseline and 2 and 4 hours after consuming the meal, in a quiet, dimly lit room.

3.3.5.1 Pulse wave analysis (PWA): Central (aortic) blood pressure and augmentation index
Following a 5 minute seated rest, aortic (central) blood pressure and wave reflection characteristics were derived from brachial pressure waveforms using a validated generalized transfer function. At each time point, three PWA measures were taken, following JNC 7 blood pressure guidelines, with 1 minute between each reading. The last 2 PWA results were averaged and used for analysis. The augmentation index (AI) was standardized to a heart rate of 75 beats per minute to correct for the independent inverse effect of heart rate on the pulse wave form [278].

3.3.5.2 Pulse wave velocity (PWV): Arterial stiffness
Aortic stiffness was assessed by carotid-femoral pulse wave velocity (PWV). Carotid and femoral arterial pressure waveforms were measured simultaneously via an applanation tonometry sensor manually held in place above the right common carotid artery and a blood pressure cuff placed on the right femoral artery. At each time point, two PWV measurements were obtained in the supine position, with 1 minute between readings, and averaged for analysis. Distance measurements were taken from the sternal notch to the carotid artery, sternal notch to the top of the femoral cuff, and femoral artery to the top of the femoral cuff. Based on these measurements, the SphygmoCor System automatically calculates PWV as distance over transit time. Transit time between the carotid and femoral pressure waves is determined by the SphygmoCor System using the foot-to-foot method [149].

3.3.6 Statistical Analyses
Descriptive statistics for participants’ screening characteristics were calculated in Minitab (version 17.0; State College, PA). Statistical analyses were performed using SAS (version 9.3; SAS Institute). Change
scores were calculated by subtracting the fasting baseline values for each visit from post-meal values. Outcomes were assessed for normality (PROC UNIVARIATE) and positively skewed variables (skew > 1) were log transformed for analysis. Two participants experienced symptoms of a vasovagal reaction (acute blood pressure decrease and lightheadedness) in response to blood sampling prior to the postprandial SphygmoCor measurements and were excluded from analyses of vascular outcomes. Two participants with acute inflammation (i.e., CRP > 9.5 mg/L) were excluded from all analyses to ensure that the analysis was performed on the target population of healthy overweight adults. One participant with fasting triglyceride (TG) values > 350 mg/dL at visit baseline (prior to consuming the test meal) was excluded from analyses of blood outcomes as he did not meet specified screening criteria (i.e., TG values < 350 mg/dL). The mixed models procedure (PROC MIXED) was used to test the effects of meal, time point, and their interaction on outcome measures. Baseline values were included as covariates. Period effects were also assessed to examine any habituation to testing and were found to be universally non-significant. Selection of model covariance structures was based on optimizing fit statistics (evaluated as lowest Bayesian Information Criterion). Models for postprandial metabolic effects (glucose, insulin, and triglycerides) used a covariance structure with repeated unstructured by time point and compound symmetry by period. Compound symmetry was used to model both period and time point for all vascular endpoints other than arterial pressure [AP] and pulse wave velocity [PWV]. Models for AP and PWV used a covariance structure with repeated unstructured by time point and compound symmetry by period. Compound symmetry was used to model period for markers of oxidative stress (oxidized LDL and MDA), which were measured at a single postprandial time point. Means are reported as least-squares means ± SEM. For all tests, α was set at 0.05.

Exploratory subgroup analyses were also conducted using the mixed models procedure (PROC MIXED) in SAS to evaluate potential interactions between sex, age, BMI, and baseline CRP, TG, and LDL values on postprandial outcomes. Subgroups for age, BMI, CRP, TG, and LDL were created according to the median value for those variables at the visit baseline (prior to meal consumption). Baseline values were included in these models as covariates, and the same covariance structures described above were used.

Additional, unblinded analyses were conducted to identify predictors of participants’ responses, including BMI, sex, and age. Participants were designated as “non-responders” or “responders” based on their response to the strawberry intervention. Individuals who exhibited the hypothesized beneficial effects of the strawberry intervention for glucose, insulin, or triglyceride responses were considered “responders”. Logistic regression was performed in SAS using BMI, sex, and age as main effects.

3.4 Results
Screening and visit baseline characteristics of participants are presented in Table 3-2 and Table 3-3, respectively. There were no significant differences between the baseline characteristics of participants at the Control and Strawberry Meal visits.
### Table 3-2. Participant characteristics at screening (n = 30).1

<table>
<thead>
<tr>
<th></th>
<th>Women (n = 13)</th>
<th>Men (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>28 ± 3 (20-47)</td>
<td>28 ± 2 (20-49)</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.65 ± 0.02 (1.55-1.73)</td>
<td>1.78 ± 0.02 (1.69-1.93)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>85 ± 3.0 (74-111)</td>
<td>99 ± 2.2 (84-114)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>31.4 ± 0.8 (28-37.9)</td>
<td>31.3 ± 0.6 (28.5-36.5)</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>113 ± 3 (97-135)</td>
<td>123 ± 2 (109-145)</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>77 ± 2 (68-94)</td>
<td>82 ± 1 (68-89)</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>86 ± 2 (77-91)</td>
<td>92 ± 1.0 (83-98)</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>161 ± 10 (117-228)</td>
<td>165 ± 7 (110-222)</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>55 ± 2 (44-69)</td>
<td>42 ± 2 (28-57)</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>88 ± 9 (39-158)</td>
<td>97 ± 6 (48-147)</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>93 ± 16 (33-186)</td>
<td>131 ± 17 (35-339)</td>
</tr>
</tbody>
</table>

1All values are means ± SEM. Ranges are provided in parentheses. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides.

### Table 3-3. Baseline characteristics of participants at study visits (n = 30; 17 M, 13 F).1

<table>
<thead>
<tr>
<th></th>
<th>Strawberry Meal</th>
<th>Control Meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>93.5 ± 2.1</td>
<td>93.1 ± 2.2</td>
</tr>
<tr>
<td>BMI (m/kg²)</td>
<td>31.4 ± 0.5</td>
<td>31.3 ± 0.5</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>2.8 ± 0.7</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>Insulin (µIU/mL)*</td>
<td>6.1 ± 0.9</td>
<td>5.1 ± 0.5</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>88 ± 1</td>
<td>87 ± 1</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>160 ± 5.5</td>
<td>158 ± 5.4</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>45 ± 2</td>
<td>43 ± 2</td>
</tr>
<tr>
<td>LDL-C (mg/dL)†</td>
<td>95 ± 5</td>
<td>94 ± 5</td>
</tr>
<tr>
<td>TC:HDL ratio</td>
<td>3.8 ± 0.2</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>102 ± 14</td>
<td>102 ± 10</td>
</tr>
<tr>
<td>Central SBP</td>
<td>106 ± 2</td>
<td>105 ± 2</td>
</tr>
<tr>
<td>Pulse Pressure</td>
<td>32 ± 1</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>Augmentation Pressure</td>
<td>6.6 ± 0.6</td>
<td>6.7 ± 0.7</td>
</tr>
<tr>
<td>Augmentation Index @ 75</td>
<td>17 ± 1.8</td>
<td>17 ± 2.1</td>
</tr>
<tr>
<td>Pulse Wave Velocity</td>
<td>6.2 ± 0.2</td>
<td>6.1 ± 0.2</td>
</tr>
</tbody>
</table>

1All values are reported at mean ± SEM. * indicates n = 28; insulin values could not be measured for two participants due to sample hemolysis. † indicates n = 29; LDL-C could not be calculated using the Friedewald equation due to the triglyceride concentration being > 400 mg/dL. Abbreviations: BMI, body mass index; CRP, C-reactive protein; TC, total cholesterol; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides; SBP, systolic blood pressure.
3.4.1 Vascular function
Meal consumption significantly altered certain indices of vascular function, but these effects were not significantly different between the strawberry and control meals (Figure 3-2). Augmentation pressure (AP) and augmentation index (AI) were both significantly lower than baseline at 2 and 4 hours after meal consumption (Figure 3-2). For pulse wave velocity (PWV) and central pulse pressure (PP), values at 2 and 4 hours were significantly different from one another, but not compared to baseline (Figure 3-2). PWV values were greater at the 4 time point compared to 2 hours, whereas central PP values were lower at the 4 time point compared to 2 hours. There were no significant changes in central systolic blood pressure following either meal.

![](image)

**Figure 3-2. Changes in vascular function indices following consumption of the control and freeze dried strawberry test meals.** Times annotated with different letters were significantly different from one another, while * indicates a significant difference from baseline. AP, augmentation pressure; central PP, central pulse pressure; AI, augmentation index; PWV, pulse wave velocity.

3.4.2 Effects on postprandial metabolism
Serum triglycerides, insulin, and glucose significantly increased from baseline following the consumption of both meals (Figure 3-3). There were no significant differences between the two meals. The triglyceride response was highest at 4 hours post meal, while insulin and glucose responses peaked 30 minutes post meal and decreased thereafter.
Figure 3-3. Change in triglycerides, glucose, and insulin following consumption of the control and freeze dried strawberry test meals. All time points were significantly different from baseline. Times annotated with different letters were significantly different from one another (p < 0.05). TG, triglycerides.

3.4.2.1 Exploratory analyses
3.4.2.1.1 Subgroup analyses
Exploratory subgroup analyses identified age, sex, and baseline triglyceride, CRP, and LDL-C values as potential factors influencing postprandial responses. For postprandial triglyceride responses, there was a significant meal by time point by age interaction (p = 0.03), a significant meal by time point by sex interaction (p = 0.03), and a trend for a meal by time point by triglyceride interaction (p = 0.07). There was also a trend for a meal by sex interaction (p = 0.08) and a meal by LDL-C interaction (p = 0.1) for central SBP. However, no post-hoc comparisons were significant after applying a Bonferroni correction for multiple comparisons. Compared to male participants, female participants experienced a greater increase in postprandial insulin (p = 0.05). There was a trend towards the same relationship for postprandial glucose (p = 0.1), and a trend for female participants to experience a decrease in PP compared to men, who experienced an increase (p = 0.1). Participants with baseline LDL-C less than 95 mg/dL experienced a greater reduction in AP (p = 0.02) and a decrease in central PP (p = 0.04) compared to participants with higher baseline LDL-C who experienced less of a decrease in AP and an increase in central PP. There was a trend towards the same relationship for AIx75 and PWV (p = 0.09 and p = 0.08, respectively). Younger participants also exhibited greater reductions in AP (p = 0.002) and AIx75 (p = 0.03) compared to older participants. There was also a trend for participants with higher baseline CRP values to experience greater increases in insulin compared to individuals with lower baseline CRP values (p = 0.06).
3.4.2.1.2 Predictors of postprandial metabolic responses

Hypothesis-generating subset analyses identified age and sex as potential predictors of the effect of the freeze-dried strawberry powder intervention on triglyceride, glucose, and insulin responses. Older age increased the odds of an individual having an attenuated triglyceride response with the strawberry intervention (odds ratio [OR]: 1.16; p < 0.05). However, for glucose responses, older individuals were marginally less likely to respond to the strawberry intervention than younger individuals (OR: 0.88; p = 0.0636). This negative effect of age was also found for insulin responses, although it did not reach significance (OR: 0.87; p = 0.0975). Men were also significantly more likely to respond to the strawberry intervention with attenuated glucose responses (OR: 2.22; p = 0.0264).

3.4.2.1.3 Individual postprandial responses

There was a wide range in triglyceride, glucose, and insulin responses to the strawberry meal compared to the control meal among all study participants. Each participant’s peak triglyceride, glucose, and insulin response to both meals are presented in Figure 3-4. In the entire study population, there was no clear trend towards a lower peak response following the strawberry meal for triglycerides, glucose, or insulin.
Figure 3-4. Peak triglyceride, glucose, and insulin responses to the strawberry and control meal for each participant. Each line represents a different participant.

However, some individuals did exhibit attenuated postprandial metabolic spikes following the strawberry meal. The postprandial triglyceride concentrations of three representative participants who had a lower peak triglyceride response to the strawberry meal are presented in Figure 3-5.
Figure 3-5. Postprandial triglyceride elevations in three participants who displayed an attenuated triglyceride response to the strawberry meal.

The postprandial insulin and glucose concentrations of participants who had a lower peak response to the strawberry meal are presented in Figure 3-6. There were no obvious distinguishing factors between these participants and participants who did not have attenuated triglyceride responses, such as age, BMI, dyslipidemia, or inflammation.
3.4.3 Effects on markers of oxidative stress
There was no significant postprandial change from baseline in MDA or ox-LDL, and no significant difference between the response to strawberry meal versus the control meal (data not shown).

3.5 Discussion
The purpose of this study was to evaluate the postprandial effects of incorporating freeze dried strawberry powder into a high-fat meal. Consumption of the high-fat test meals produced substantial postprandial elevations in triglycerides, glucose, and insulin, but there was no attenuating effect of including 40 g of freeze dried strawberries. Incorporating freeze dried strawberry powder also did not
alter postprandial vascular outcomes or markers of oxidative stress. This is in contrast to the significant reduction in postprandial triglycerides, insulin, and oxidized LDL reported by Burton-Freeman et al. which, to our knowledge, is the only other postprandial study of acute strawberry consumption in conjunction with a fat-containing meal [186, 274]. It is unclear why similar benefits were not achieved in our study, which utilized a higher dose of freeze dried strawberries (40 g versus 10 g) and higher fat meal (50 g versus 30 g). Torronen et al. also evaluated the postprandial effects strawberries and found that consuming 150 g of strawberry puree with white bread significantly attenuated both maximum insulin production and reduced insulin AUC [177]; however, this study was designed to investigate the effects of strawberries on carbohydrate metabolism and the meal contained negligible amounts of fat. The effects of different doses of freeze dried strawberries, in conjunction with varying amounts of dietary fat, have yet to be evaluated or reproduced by other studies.

The postprandial reductions in central pressures and measures of arterial stiffness that we observed are consistent with previous studies of postprandial vascular function. Various types of meals have been shown to produce postprandial improvements in arterial stiffness, including high-fat meals [279-282]. It has been proposed that these reductions in stiffness are due to the vasodilatory effect of insulin, which is elevated following meal consumption [280, 283]. Although we found no effect of freeze dried strawberry powder on postprandial vascular outcomes, certain phytochemical-rich foods have been shown to produce greater improvements in vascular function such as dark chocolate [161] and green and black tea [165]. Therefore, these effects may [159] be dependent on the unique phytochemical profile of the dietary intervention; however, relatively few dietary interventions have been evaluated for their effects on postprandial vascular function.

Multiple studies have investigated the effects of longer-term strawberry supplementation, but results have been mixed for metabolic outcomes. A triglyceride-lowering effect was reported for one month of 500 g/d of strawberry consumption [228]. However, this reduction was relative to baseline rather than a control group and no effect on fasting triglycerides has been reported in other long-term supplementation studies [216, 225, 227, 228, 274, 284]. Our finding of no change in glucose is consistent with previous postprandial [186] and long-term supplementation studies [216, 225-227, 271], but a trend for HbA1c reduction was found following consumption of 50 g/d of freeze dried strawberry powder for 6 weeks [226]. Although we found no postprandial effects on markers of oxidative stress, strawberry supplementation for 1 to 2 months has been shown to significantly reduce MDA [216, 227, 228], TBARS [271], and urinary isoprostanes [228]. Therefore, sustained strawberry consumption may be necessary to reduce markers of oxidative stress.

Little research has investigated potential dose response effects of strawberries, and the amount required to optimize effects on clinical outcome measures is unknown. A 10 g portion of freeze dried strawberry powder is typically equivalent to approximately 133 g of fresh strawberries (personal communication, California Strawberry Commission). The two previous postprandial studies of strawberries employed 10 g of freeze dried strawberry powder and 150 g frozen strawberries, respectively [177, 274]. Doses used in longer-term supplementation studies have ranged from 20 - 50 g/d of freeze dried powder [216, 225, 227, 284] or one pound (454 g) to 500 g/d of fresh strawberries [228, 271]. Our 40 g dose of freeze dried strawberries is comparable to that used in studies of 4-8 weeks of supplementation, which demonstrated beneficial effects on blood lipids, glycemic responses, and oxidative stress. This dose of strawberries provides a substantial amount of bioactive compounds, including ellagic acid, anthocyanins, quercetin, catechin, and ascorbic acid (Supplemental Table 3-2). Therefore, it is unlikely that our null findings are due to the dose of freeze dried strawberries provided.
Previous clinical studies of strawberries have used a variety of methods for delivering the intervention. The majority of studies have provided the strawberry intervention as a beverage [198, 216, 225-227, 274]. However, fresh strawberries [228, 271], a strawberry puree [177], or freeze dried strawberry powder mixed into milkshakes, yogurt, or cream cheese [284] have also been used. It has been suggested that polyphenol absorption may be inhibited by the formation of bonds with dairy proteins [186, 227]; thus, some studies have required that the strawberry intervention be consumed between meals to prevent any potential interference with the absorption of strawberry bioactives [227]. However, beneficial effects were reported by Burton-Freeman et al. [274] when the strawberry intervention was provided in a milk-based beverage. Heat may also degrade the strawberry bioactives. In the present study, the strawberry powder was not exposed to any heat during the preparation of study foods. Therefore, our method of delivering the strawberry intervention was consistent with previous clinical studies and is unlikely to be the cause of our disparate findings.

Particular characteristics of the study population are more likely to have influenced our results. On average, our participants were substantially younger and did not have elevated total cholesterol or LDL-C compared to the population studied by Burton-Freeman et al. [274]. Whereas the average age of our participants was 28 years, the average age of the population studied by Burton-Freeman et al. was nearly 51 years [274]. Similarly, average total cholesterol and LDL-C in our population was 163 mg/dL and 93 mg/dL, respectively, compared to 210 mg/dL and 142 mg/dL [274]. Long-term strawberry supplementation studies have also typically been conducted in individuals with established diseases, such as metabolic syndrome [216, 225] or type 2 diabetes [226], or in older individuals who are dyslipidemic [227, 271] or more overweight [284]. Therefore, it is possible that the beneficial effects of the strawberry bioactives are more apparent in a metabolic system that is already under more stress. Our exploratory subgroup analyses also indicate that the age and sex of participants, as well as baseline TG, LDL-C, and CRP values, may influence whether individuals respond to a freeze dried strawberry powder intervention. However, these are preliminary, hypothesis-generating analyses and need to be replicated in future studies.

Although few studies have investigated the acute effects of strawberries, postprandial studies have been conducted with other foods high in bioactive compounds. Similar to our results, studies of a pomegranate drink [174], processed tomato product [180], beetroot juice [181], and raspberry/blueberry puree [182] did not find significant reductions in postprandial lipemia or glycemic responses. Conversely, Skulas-Ray et al. found that the addition of a 14 g high antioxidant spice blend to a high fat meal significantly reduced postprandial insulin and triglycerides [270]. We speculate that outcomes may be influenced by the relative hydrophilicity/lipophilicity of the antioxidant compounds provided by the respective interventions. The total antioxidant capacity of strawberries (and other fruits/vegetables) is comprised mainly from hydrophilic antioxidant compounds, whereas spices like turmeric and cinnamon provide a much greater proportion of lipophilic antioxidants [285]. Furthermore, the spice blend used by Skulas-Ray et al. exhibited potent pancreatic lipase inhibition in vitro (unpublished data), whereas the freeze dried strawberry powder used in the present study had no inhibitory effect (data not shown). Therefore, the discrepancy in the effect on postprandial triglycerides may potentially be due to differences in the inhibitory capacity of the respective intervention compounds.

3.5.1.1 Strengths and limitations
The characteristics of our participants were consistent with those of the general population and the strawberry intervention was provided as part of a meal, potentially making it more reflective of dietary habits. The strawberry and control meals were also well-matched for macro- and micronutrients. Multiple endpoints, all of which are important risk factors for cardiovascular disease and other chronic diseases,
were assessed. Hormonal effects on vascular function were controlled for by ensuring that female participants were tested within the first seven days of their menstrual cycle. Although the study meals induced a robust triglyceride response, it is possible that the insulin and glucose response was insufficient to identify a significant effect of the strawberry intervention on these outcomes. As previous studies indicate that the strawberries may have more of an effect on glycemic control, it is possible that a higher glycemic meal may have demonstrated a significant effect of the strawberry intervention on insulin/glucose responses. Although our sample size was consistent with previous studies and the cross-over nature of our study design allowed participants to act as their own controls, it is possible that our study was underpowered to detect a significant effect. However, longer-term supplementation rather than a single acute dose may also be required to alter these metabolic outcomes.

3.5.1.2 Conclusions
We found that the incorporation of 40 g of freeze dried strawberry powder into a high fat meal did not alter postprandial changes in vascular function or attenuate postprandial elevations in triglycerides, glucose, or insulin. Although this is in contrast to the beneficial effects of strawberries previously reported, the evidence remains limited. Few studies have investigated the acute effects of strawberries, and effects of longer-term strawberry supplementation have been mixed. Additional long-term supplementation studies are needed to clarify the effect of strawberries on metabolic outcomes and determine the optimal dose and meal type in which they are most effective.

3.6 Supplementary tables

**Supplemental Table 3-1. Nutrient composition of freeze dried strawberry and control powders.**

<table>
<thead>
<tr>
<th></th>
<th>Freeze dried strawberry powder¹</th>
<th>Control powder¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories (g)</td>
<td>138</td>
<td>144</td>
</tr>
<tr>
<td>Carbohydrates (g)</td>
<td>31.2</td>
<td>31.7</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>2.57</td>
<td>0.152</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>0.26</td>
<td>0.157</td>
</tr>
<tr>
<td>Total Dietary Fiber (g)</td>
<td>6.48</td>
<td>3.75</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>229</td>
<td>0.196</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>0.72</td>
<td>0.12</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>65.2</td>
<td>0.314</td>
</tr>
<tr>
<td>Thiamine (B₁) (mg)</td>
<td>0.11</td>
<td>0.012</td>
</tr>
<tr>
<td>Riboflavin (B₂) (mg)</td>
<td>0.29</td>
<td>0.044</td>
</tr>
<tr>
<td>Niacin (B₃) (mg)</td>
<td>1.41</td>
<td>0.0424</td>
</tr>
<tr>
<td>Pantothenic Acid (B₅) (mg)</td>
<td>1.43</td>
<td>0.008</td>
</tr>
<tr>
<td>Pyridoxine (B₆) (mg)</td>
<td>0.37</td>
<td>0.008</td>
</tr>
<tr>
<td>Folic Acid (µg)</td>
<td>399</td>
<td>6.32</td>
</tr>
</tbody>
</table>

¹Amounts per 40 g of powder.
## Supplemental Table 3-2. Phytochemical content of freeze dried strawberry powder.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount per 40 g</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ORAC</strong></td>
<td>15760 µmol TE</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.16 mg</td>
</tr>
<tr>
<td>3,4-dihydrobenzoic acid</td>
<td>0.06 mg</td>
</tr>
<tr>
<td>Procyanidin B1</td>
<td>12.25 mg</td>
</tr>
<tr>
<td>(+)-catechin</td>
<td>10.02 mg</td>
</tr>
<tr>
<td>Cyanidin-3-glucoside</td>
<td>4.65 mg</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>0.01 mg</td>
</tr>
<tr>
<td>Pelargonidin-3-glucoside</td>
<td>158.76 mg</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>0.10 mg</td>
</tr>
<tr>
<td>2-hydroxycinnamic acid</td>
<td>0.08 mg</td>
</tr>
<tr>
<td>Rutin (quercetin-rutinoside)</td>
<td>1.34 mg</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>5.04 mg</td>
</tr>
<tr>
<td>Isoquercetin (quercetin-glucoside)</td>
<td>2.65 mg</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>0.16 mg</td>
</tr>
<tr>
<td>Tiliroside (kaempferol-3-glucoside-6”-p-</td>
<td>0.30 mg</td>
</tr>
<tr>
<td>coumaroyl)</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.58 mg</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>0.14 mg</td>
</tr>
</tbody>
</table>

ORAC = oxygen radical antioxidant capacity; TE = Trolox equivalents.
Chapter 4. Omega-3 fatty acid supplementation does not alter inflammatory responses to low-dose endotoxemia in healthy young men despite increases in erythrocyte omega-3 fatty acid content

4.1 Abstract

Background: Pre-clinical evidence of the anti-inflammatory effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has not translated to consistent results on inflammatory outcomes in clinical intervention studies—potentially due to the lack of a controlled inflammatory stimulus, which is a key component in cell and animal models. The low-dose endotoxemia model can be used to induce a consistent and well-characterized acute inflammatory response in the context of human physiology.

Objective: The objective of this study was to evaluate the effects of a pharmaceutical dose (3.4 g/d) of EPA+DHA on inflammatory responses to induced inflammation (intravenous 0.6 ng/kg purified lipopolysaccharide [LPS], or endotoxin challenge) in healthy young men.

Design: This was a placebo-controlled, double-blind, randomized, crossover trial (8 wk treatment, 8 wk washout) comparing 3.4 g/d EPA+DHA to an olive oil control in healthy young men (n = 20), using a low-dose endotoxin challenge.

Results: The low-dose endotoxin challenge produced a robust inflammatory response with consistent elevations in C-reactive protein, interleukin-6, and tumor necrosis factor-α. There was no difference in the peak concentration of any inflammatory markers following EPA+DHA supplementation compared to the control (p > 0.05), despite significant increases in erythrocyte omega-3 fatty acid content. EPA+DHA supplementation reduced diastolic blood pressure (-2.5 mm Hg) compared to the placebo, and a produced a marginally significant reduction in triglycerides (-14.5 mg/dL) relative to baseline.

Conclusions: EPA+DHA supplementation did not alter inflammatory responses to induced low-dose endotoxemia; however, omega-3 fatty acids may exert beneficial effects on inflammation via mechanisms other than cytokine production.
4.2 Introduction
Excessive or non-resolving inflammation plays a central role in numerous chronic diseases, including cardiovascular disease (CVD), autoimmune disorders, cancer, and neurodegenerative diseases [36]. Current standard-of-care therapies for these inflammatory diseases are often very costly and/or confer substantial side effects. The long-chain (LC) omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been proposed as a means of preventing the development of chronic inflammation; however, results on inflammatory outcomes in clinical intervention studies have been disappointing [244, 245].

Cell and animal models that employ a model of induced-inflammation have generated substantial pre-clinical evidence supporting the anti-inflammatory potential of EPA and DHA. In cell-based studies, pro-inflammatory cytokine responses to a stimulus such as endotoxin (or lipopolysaccharide, LPS) are attenuated by EPA and/or DHA [36, 286]. Similarly, animal disease models that utilize LPS have also shown reductions in the intensity of inflammatory responses with EPA and/or DHA [287]. These pre-clinical models have identified numerous mechanisms by which EPA and DHA can reduce inflammation [288], including shifting lipid mediator production towards less inflammatory eicosanoids and increasing production of specialized pro-resolving mediators (SPMs) that are formed from LC omega-3 fatty acids [36, 288]. SPMs are produced in response to inflammatory stress and actively promote the resolution of inflammation [289, 290].

However, clinical intervention studies examining the effects of EPA+DHA supplementation on inflammation have yielded mixed results [244, 245]—potentially due to the lack of a controlled inflammatory stimulus similar to that used in pre-clinical models. The low-dose intravenous human endotoxemia model (0.6 ng LPS per kg body weight) safely produces an acute inflammatory response that is characterized by a consistent time course of inflammatory marker elevations [45, 291, 292]. This simulates the controlled, induced inflammation employed in cell and animal models within the context of human physiology. Furthermore, this provides the opportunity to examine whether LC omega-3 fatty acid supplementation can alter the production of pro-inflammatory signals and SPMs under conditions of heightened inflammation typically associated with disease.

This study evaluated the effects of eight weeks of supplementation with 3.4 g/d of EPA+DHA on inflammatory responses to low-dose endotoxemia in 20 healthy young men, using a randomized, placebo-controlled, double-blinded crossover design. Effects of EPA+DHA supplementation on erythrocyte fatty acid content and other CVD risk factors were also analyzed. We hypothesized that 3.4 g/d of EPA+DHA supplementation would reduce peak inflammatory responses to low-dose endotoxemia, as measured by C-reactive protein (CRP), compared to an olive oil placebo.

4.3 Subjects and Methods
4.3.1 Study population
Men who were 20-45 years of age, consumed less than two servings of oily fish per week, and did not have any serious illness were recruited for the study. Females were not included due to the potential for the menstrual cycle phase to influence results. Other inclusion criteria consisted of a body mass index (BMI) of 20-30 kg/m², resting heart rate > 55 bpm, and fasting LDL-C < 160 mg/dL. Exclusion criteria included: history of vasovagal reactions or unprovoked fainting; elevated blood pressure (BP > 159/99); acute or chronic inflammatory conditions; liver or kidney dysfunction; a history of heart disease or diabetes; use of tobacco products; allergies/sensitivities to fish; history of liver disease or abnormal liver
function enzymes; elevated prothrombin time international normalized ratio (INR); and use of non-steroidal anti-inflammatories, immunosuppressants, or medications/supplements for elevated lipids, blood pressure, or glucose.

4.3.2 Participant Recruitment

Subjects were recruited via fliers in the community, campus e-mail lists, and a university research website. Potential subjects emailed or called to indicate interest in participating in the study and were then given additional information about the study. If interested, they were asked a series of medical history and lifestyle questions to screen for eligibility. Of the 155 initial respondents who provided contact information, 71 elected to complete the initial screening questions (Figure 4-1). Thirty-five of these volunteers remained eligible following this screening process and scheduled a screening appointment at the Penn State Clinical Research Center (CRC) to verify eligibility. After written informed consent was obtained, a blood sample was drawn for a complete blood count and standard chemistry profile (lipid panel, glucose, liver and kidney function) to rule out the presence of illness (autoimmune disease, cancer, and immunodeficiency). Blood pressure was measured according to JNC 7 guidelines [277]. Briefly, after a 5-minute 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. Body weight and height were measured (without shoes and in light clothing) to calculate BMI. A CRC clinician reviewed participants’ medical histories and conducted a brief physical examination to verify the eligibility of participants. Of the 35 individuals who were screened, 21 met eligibility criteria and were enrolled in the study. One participant withdrew from the study due to scheduling difficulties prior to completing the first supplementation period and undergoing a low-dose endotoxin challenge. Thus, data are reported for 20 participants (Figure 4-1). 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.
4.3.3 Study design and intervention

This was a randomized, double-blind, 2-period crossover, placebo-controlled study with 8 week treatment periods and an 8 week washout period (Figure 4-2). Treatment periods were extended by up to 4 weeks (12 weeks total) in the case of illness, injury, or scheduling difficulties. Treatment was provided as 4 identical capsules per day during both periods. All capsules were provided by Pronova Biopharma (Oslo, Norway) (Lovaza and identical olive oil placebo). Each 1 gram capsule of omega-3 fatty acid ethyl esters contains ≈465 mg EPA and 375 mg DHA (ratio 1.2:1). We performed an independent analysis of one sample of active and placebo capsules (Table 4-1; methods previously described, [293]). Analysis of the EPA+DHA capsules verified that they contained 50% EPA (20:5n-3), 41% DHA (22:6n-3), 3% DPA (22:5n-3), and small amounts of other fatty acids. The olive oil placebo contained 80% oleic acid (18:1n-9), 10% palmitic acid (16:0), 3% stearic acid (18:0), and smaller amounts of other fatty acids. The total amount of fatty acids provided per day by these treatment regimens, as determined by independent analysis, is summarized in Table 4-1.

Figure 4-1. Schematic of subject recruitment and reasons for exclusion.
Figure 4-2. Schematic of study procedures.

Table 4-1. Fatty acid composition (mg per 4 g daily dose) of the placebo and EPA+DHA intervention.\(^1\)

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Placebo (mg/d)</th>
<th>EPA+DHA (mg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic, 16:0</td>
<td>410</td>
<td>2</td>
</tr>
<tr>
<td>Stearic, 18:0</td>
<td>114</td>
<td>3</td>
</tr>
<tr>
<td>Oleic, 18:1n-9</td>
<td>3164</td>
<td>12</td>
</tr>
<tr>
<td>Linoleic, 18:2n-6</td>
<td>170</td>
<td>10</td>
</tr>
<tr>
<td>Eicosadienoic, 20:2n-6</td>
<td>1</td>
<td>53</td>
</tr>
<tr>
<td>Arachidonic, 20:4n-6</td>
<td>1</td>
<td>81</td>
</tr>
<tr>
<td>Eicosapentaenoic, 20:5n-3</td>
<td>0</td>
<td>1988</td>
</tr>
<tr>
<td>Docosapentaenoic (n-6), 22:5n-6</td>
<td>0</td>
<td>43</td>
</tr>
<tr>
<td>Docosapentaenoic (n-3), 22:5n-3</td>
<td>0</td>
<td>109</td>
</tr>
<tr>
<td>Docosahexaenoic, 22:6n-3</td>
<td>0</td>
<td>1608</td>
</tr>
</tbody>
</table>

\(^1\)Values were calculated from independent analysis of fatty acid composition of a sample of active and placebo capsules; only fatty acids that were ≥1% of total fatty acids for either active treatment or placebo capsules are shown.

During the 2 treatment periods, participants received 0 g/d EPA+DHA (olive oil placebo) or 3.4 g/d EPA+DHA, in random order. Treatments were matched to coded alphanumeric identifiers so that the researchers and participants were blinded to treatment assignment. The code break was maintained in a sealed envelope that was available only to the CRC study clinical and principal investigator (GLJ). The envelope was unsealed after analysis of primary outcomes. Participants were instructed to maintain their weight and activity level throughout the course of the study, avoid fish-oil or flax-based supplements, and to consume no more than one meal of oily fish (including salmon, tuna, mackerel, and herring) per week. Participants were contacted after 2 weeks of starting each supplementation period to assess compliance and discuss any difficulties with taking the capsules. At the midpoint of each supplementation period (~4 weeks), participants reported to the CRC to return their empty bottles and any remaining capsules, and to receive new supplies for the remainder of the supplementation period.
4.3.3.1 Low-dose endotoxin challenge

Following each 8 week supplementation period, participants underwent a low-dose (0.6 ng lipopolysaccharide/kg body weight) endotoxin challenge. All testing visits were conducted at the Pennsylvania State University CRC according to standardized protocols. These visits consisted of an approximately 10 hour test day. Participants were contacted within 10 days prior to each endotoxin challenge to verify that their health had not changed and that they had not experienced any recent injuries or illnesses. Endotoxin challenge visits were postponed for a minimum of 1 week if participants reported any recent vaccinations, medical/dental procedures, or symptoms of acute infection (e.g., cold or flu symptoms). Prior to endotoxin challenge test days, participants were instructed to avoid alcohol, strenuous exercise, and anti-inflammatory medications for 48 hours.

At each endotoxin challenge test day, participants reported to the CRC at approximately 6:30 am, after a 12 hour fast (no food or drink other than water) (Figure 4-3). Body weight was measured in light clothing using a calibrated, digital scale. Baseline vital signs (blood pressure, heart rate, and body temperature) were taken and two intravenous (IV) catheters were established to administer sterile saline and facilitate blood sampling. In some cases, only one IV catheter could be established and this was used for both administration of fluids and blood sampling. Following IV administration of a 500 mL bolus of sterile saline, baseline blood and urine samples were obtained. A sterile solution of protein-free endotoxin was then injected at a dose of 0.6 ng/kg body weight over a 1 minute period. IV saline was provided continuously for the duration of the test day. Participants were provided with standardized breakfast and lunch meals (Supplemental Table 4-1) thirty minutes and four hours after endotoxin administration, respectively. Blood samples were obtained via the IV catheter at 1, 2, 4, and 8 hours post-injection, and by venipuncture for the 24, 48, 72, and 168 hour time points. Urine was collected for the duration of the test day, in two aggregate batches comprising the first four hours and second four hours following endotoxin injection. Mood, sleep, and physical activity questionnaires were administered to participants at the end of the test day.

![Figure 4-3. Schematic of low-dose endotoxin challenge visits.](image)

Participant vitals (blood pressure, heart rate, and body temperature) were monitored for the duration of the test day. Specifically, blood pressure and heart rate were measured every 15 minutes for the first four hours following endotoxin administration, and every 30 minutes thereafter. Body temperature was monitored every 30 minutes for the entirety of the test day. Mild clinical symptoms including headache, muscle aches, chills, nausea, modest elevations in heart rate and body temperature, and modest...
reductions in blood pressure were expected to occur. Symptom onset typically occurs approximately 30 minutes following endotoxin injection and continues for the next 3-4 hours, with symptoms generally subsiding 6-8 hours post-injection. In the case of more severe symptoms, Alert Criteria for body temperature, heart rate, and systolic blood pressure were outlined in the study Safety Plan, along with corresponding medical response procedures.

Following the collection of the 8 hour blood sample, IV catheters were discontinued and participants were briefly evaluated for safety by a nurse before being allowed to leave the CRC at approximately 4:30 pm. Participants were asked not to take any anti-inflammatory medication and were advised to limit any strenuous activity if they continued to experience lingering symptoms. A study team member (CKR) contacted participants approximately 12 hours post endotoxin administration (i.e., 8:00 pm) for a brief symptom assessment. Participants returned to the CRC for follow-up blood draws at 24, 48, 72, and 168 hours post endotoxin injection. Participants were not required to fast or avoid alcohol or strenuous exercise in preparation for these follow up visits.

4.3.4 Blood sample collection and assay methods
Blood samples were collected at baseline (prior to supplementation), at each midpoint visit, at the end of treatment periods, and at designated times following endotoxin administration. Baseline and end of treatment period samples were collected in the fasting state (12 hours with nothing but water and no vigorous exercise, and 48 hours without alcohol). A general health profile was obtained with fresh serum samples to monitor liver enzyme function (Chem 24 panel; Quest Diagnostics, Pittsburgh, PA). Blood drawn into lithium heparin and EDTA anticoagulated tubes was immediately centrifuged at 1500 g for 15 minutes. Blood drawn into serum separator tubes was allowed to clot for 30 minutes prior to centrifugation. Except for endpoints that required unfrozen samples, aliquots were stored at -80 °C for batch analysis.

4.3.4.1 Serum measures
Whole blood was drawn into serum separator tubes, allowed to clot for 30 minutes, and centrifuged. Liver enzyme concentrations were measured as part of a general chemistry profile. 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 calculated using the Friedewald equation \[\text{LDL-C} = \text{TC} - (\text{HDL-C} + \text{TG}/5)\]. Glucose was determined by an immobilized enzyme biosensor using the YSI 2300 STAT Plus Glucose and Lactate Analyzer (Yellow Springs Instruments, Yellow Springs, OH). Insulin was measured by radioimmunoassay using \(^{125}\text{I}\)-labeled human insulin and a human insulin antiserum (Quest Diagnostics). High-sensitivity C-reactive protein (CRP) was measured by latex-enhanced immunonephelometry (Quest Diagnostics; assay CV < 8%).

4.3.4.2 Red blood cell fatty acids
Red blood cells (RBC) were collected following separation from plasma by centrifugation and frozen at –80 °C prior to analysis. As previously described, RBC FA composition was analyzed by gas chromatography with flame ionization detection [294]. Briefly, lipids were extracted, methylated to form fatty acid methyl esters (FAMEs), and analyzed by gas chromatography on a GC2010 (Shimadzu Corporation, Columbia, MD, USA) equipped with a 100-m SP-2560 column (Supelco, Bellefonte, PA, USA). Fatty acids were identified by comparison with a standard mixture of FAs characteristic of RBCs (GLC 727; Nu-Check Prep, Waterville, MN). FAME composition was expressed as a percentage of total identified FAMEs (CV < 3.7%). The omega-3 index is reported as the sum of EPA and DHA.
4.3.4.3 **Inflammatory cytokines**
Plasma concentrations of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured using high-sensitivity enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN) in duplicate (average intra-assay CV < 10%).

4.3.4.4 **Complete blood count and coagulation parameters**
A complete blood count was obtained from whole blood collected in an EDTA anticoagulated vacutainer (CBC; Quest Diagnostics). The prothrombin time international normalized ratio (INR) was measured from blood drawn into a 3.2% sodium citrate tube using photo-optical clot detection, and was used to ensure that participants had no blood clotting abnormalities (Quest Diagnostics).

4.3.5 **Statistical Analyses**
Descriptive statistics for participants’ baseline and endpoint values were calculated in Minitab (version 17.0; State College, PA). Statistical analyses were performed using SAS (version 9.3; SAS Institute, Cary, NC). Outcome variables were checked for normality and positively skewed variables were logarithmically transformed. For end of treatment values, the mixed models procedure (PROC MIXED) in SAS was used to test the effects of treatment, period, and treatment by period interactions for each outcome. Subject was treated as a random effect and the remaining factors were fixed effects. When period effects were significant, they were retained in the final model of treatment effects. Change scores for end of treatment values were calculated by subtracting study-entry baseline values from each post-supplementation measure. For repeated inflammatory response measures following endotoxin injection, the mixed models procedure (PROC MIXED) in SAS was used to test the effects of treatment, period, time, and treatment by time interactions. Outcomes were modeled as doubly repeated measures with unstructured and compound symmetry variance structures for time and treatment, respectively. Change scores for these inflammatory response outcomes were calculated by subtracting pre-injection baseline values from each time point. Values that were measured in duplicate (i.e. plasma cytokine assay duplicates) were averaged prior to analysis. Means are reported as least squares mean ± SEM unless otherwise specified.

4.4 **Results**
Twenty participants completed the study and underwent both low-dose endotoxin challenges. In general, participants were young, healthy, and normotensive (Table 4-2). The majority of the study population was Caucasian (n = 12), but included six participants of Asian descent and two African-Americans. Compliance, based on capsule logs and returned capsules, was excellent (mean ± SEM, 94% ± 1.5).
Table 4-2. Pre-supplementation characteristics of participants who completed the study (n = 20).<sup>1</sup>

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (y)</strong></td>
<td>27 ± 1 (20-40)</td>
</tr>
<tr>
<td><strong>Height (m)</strong></td>
<td>1.81 ± 0.02 (1.70-1.94)</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td>82.3 ± 2.8 (62.3-111)</td>
</tr>
<tr>
<td><strong>BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</strong></td>
<td>25.2 ± 0.5 (21.2-29.6)</td>
</tr>
<tr>
<td><strong>SBP (mm Hg)</strong></td>
<td>118 ± 2 (103-138)</td>
</tr>
<tr>
<td><strong>DBP (mm Hg)</strong></td>
<td>77 ± 1 (68-89)</td>
</tr>
<tr>
<td><strong>Lipids and lipoproteins</strong></td>
<td></td>
</tr>
<tr>
<td><strong>TC (mg/dL)</strong></td>
<td>163 ± 6 (121-212)</td>
</tr>
<tr>
<td><strong>HDL-C (mg/dL)</strong></td>
<td>50 ± 2 (30-74)</td>
</tr>
<tr>
<td><strong>LDL-C (mg/dL)</strong></td>
<td>94 ± 4 (66-123)</td>
</tr>
<tr>
<td><strong>TG (mg/dL)</strong></td>
<td>89 ± 12 (43-248)</td>
</tr>
<tr>
<td><strong>Glucose metabolism</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Insulin (μIU/mL)</strong></td>
<td>4.5± 0.5 (1.9-9)</td>
</tr>
<tr>
<td><strong>Glucose (mg/dL)</strong></td>
<td>91 ± 1 (79-102)</td>
</tr>
<tr>
<td><strong>Inflammatory markers</strong></td>
<td></td>
</tr>
<tr>
<td><strong>IL-6 (pg/mL)</strong></td>
<td>0.97 ± 0.11 (0.48-2.45)</td>
</tr>
<tr>
<td><strong>TNF-α (pg/mL)</strong></td>
<td>1.03 ± 0.07 (0.58-1.78)</td>
</tr>
<tr>
<td><strong>CRP (mg/L)</strong></td>
<td>1.0 ± 0.3 (&lt;0.2-5.6)</td>
</tr>
<tr>
<td><strong>Erythrocyte fatty acid content (% of total fatty acids)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Eicosapentaenoic acid (20:5n3)</strong></td>
<td>0.35 ± 0.02 (0.20-0.55)</td>
</tr>
<tr>
<td><strong>Docosapentaenoic acid (22:5n3)</strong></td>
<td>2.57 ± 0.1 (1.91-3.64)</td>
</tr>
<tr>
<td><strong>Docosahexaenoic acid (22:6n3)</strong></td>
<td>4.08 ± 0.16 (2.99-5.34)</td>
</tr>
<tr>
<td><strong>Omega-3 Index (EPA+DHA)</strong></td>
<td>4.43 ± 0.17 (3.37-5.86)</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values were calculated in Minitab and are presented as means ± SEM, with ranges provided in parentheses. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; TG, triglycerides; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; CRP, C-reactive protein.

4.4.1 Effect of EPA+DHA supplementation on blood pressure, lipids/lipoproteins, markers of inflammation, and leukocytes

Post-supplementation values for anthropometric characteristics, blood pressure, lipids/lipoproteins, glucose metabolism, and inflammatory markers are presented in Table 4-3. Supplementation with 3.4 g/d EPA+DHA significantly reduced diastolic blood pressure by 3.75 mm Hg relative to the placebo (p = 0.007). There was no significant treatment effect for other outcomes. There was a trend for a 22% reduction from baseline in fasting triglycerides following EPA+DHA supplementation (p = 0.09). Insulin, glucose, total cholesterol, HDL-C, LDL-C, and non-HDL-C were all significantly reduced compared to study entry baseline, regardless of treatment (Table 4-3). A significant treat by visit effect was found for fasting insulin (p = 0.02), although post-hoc comparisons were no longer significant after applying a Bonferroni correction for multiple comparisons. No effects were observed for fasting glucose values.
Table 4.3. Participant characteristics following supplementation (n = 20).1

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>3.4 g/d EPA+DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value</td>
<td>Δ from baseline</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>82.6 ± 3.05</td>
<td>0.28 ± 0.53</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.1 ± 0.6</td>
<td>-0.12 ± 0.16</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>115 ± 1.66</td>
<td>-2.7 ± 1.9</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>78 ± 1.51</td>
<td>1 ± 1.5 a</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>0.97 (0.79-1.19)</td>
<td>0.11 ± 0.10</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>1.02 ± 0.09</td>
<td>-0.003 ± 0.07</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>0.4 (0.3-0.6)</td>
<td>-0.4 ± 0.3</td>
</tr>
<tr>
<td>Insulin (μU/mL)</td>
<td>2.3 (1.8-2.9)</td>
<td>-2.1 ± 0.42*</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>87.6 ± 1.28</td>
<td>-3.55 ± 1.40*</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>144 ± 5.81</td>
<td>-18.4 ± 3.56*</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>45 ± 2.4</td>
<td>-5.35 ± 1.48*</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>75.8 (58.9-97.7)</td>
<td>-0.5 ± 7.4</td>
</tr>
<tr>
<td>Non-HDL (mg/dL)</td>
<td>99.4 ± 5.51</td>
<td>-13.3 ± 2.96*</td>
</tr>
</tbody>
</table>

1Values are presented as means ± SEM. Values that were not normally distributed are presented as geometric means with the 95% confidence interval in parentheses. Values for insulin are based on n = 19.

Different letters within a row indicate a significant difference between treatment groups.

*indicates a significant change from study entry baseline values (p < 0.05).

†indicates a trend towards a significant difference between treatments (p < 0.1).

‡indicates a trend towards a significant change from study entry baseline values (p < 0.1).

There were no significant effects of EPA+DHA supplementation on liver enzymes, kidney function, or CBC values relative to the placebo (Supplemental Table 4-2). Many outcomes were significantly different from baseline values following both treatment periods (Supplemental Table 4-2). Small, statistically significant period effects were also found for multiple outcomes (Supplemental Table 4-2). A significant treat by visit effect was found for percent eosinophils (p = 0.04) and the mean corpuscular hemoglobin concentration (MCHC; p = 0.03), but no post-hoc comparisons were significant after a Bonferroni correction for multiple comparisons.

4.4.2 Effect of EPA+DHA supplementation on red blood cell (RBC) fatty acid composition

EPA+DHA supplementation significantly increased the percentage of long-chain omega-3 fatty acids in the erythrocyte membrane (Figure 4-4). Following 8 weeks of EPA+DHA supplementation, the percentage of eicosapentaenoic acid (EPA) in the red blood cell (RBC) membrane increased 6-fold relative to study entry baseline values (Table 4-4). Omega-3 docosapentaenoic acid (DPA) also increased by 35% from study entry baseline values following EPA+DHA supplementation (Table 4-4). Comparatively, no significant changes in RBC EPA and DPA occurred following the placebo supplementation period (Figure 4-4, Table 4-4). RBC DPA also exhibited a significant period effect, with higher values following the second supplementation period (2.9% for period 1 versus 3.2% for period 2; Table 4-4). The percentage of the shorter-chain omega-3 fatty acid α-linolenic acid (ALA) in the RBC membrane was not altered by EPA+DHA supplementation.
Figure 4-4. Mean (±SEM) changes in red blood cell (RBC) long-chain omega-3 fatty acids and the Omega-3 Index. Change scores were calculated as the end of treatment values minus the baseline value, and were compared using the MIXED procedure (SAS version 9.2). Different lowercase letters within outcomes indicate significant differences between treatment groups, $p < 0.05$. * indicates a significant change from study entry baseline. † indicates significant treat by visit effect of supplementation.
Table 4-4. Effect of omega-3 supplementation on red blood cell (RBC) membrane long-chain omega-3 fatty acid content.1

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Common Name</th>
<th>Baseline</th>
<th>Placebo (0 g/d EPA+DHA)</th>
<th>3.4 g/d EPA+DHA</th>
<th>P value2</th>
<th>Treatment</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>Myristic</td>
<td>0.27 ± 0.02</td>
<td>0.28 ± 0.01</td>
<td>0.28 ± 0.01</td>
<td>0.7725</td>
<td>0.8033</td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>Palmitic</td>
<td>21.12 ± 0.17</td>
<td>21.21 ± 0.19</td>
<td>21.40 ± 0.19</td>
<td>0.4185</td>
<td>0.2914</td>
<td></td>
</tr>
<tr>
<td>C18:0</td>
<td>Stearic</td>
<td>17.55 ± 0.09</td>
<td>17.95 ± 0.11*</td>
<td>18.01 ± 0.11*</td>
<td>0.5872</td>
<td>0.131</td>
<td></td>
</tr>
<tr>
<td>C20:0</td>
<td>Arachidic3</td>
<td>0.15 (0.14-0.17)</td>
<td>0.16 (0.13-0.19)</td>
<td>0.15 (0.12-0.18)</td>
<td>0.6033</td>
<td>0.041</td>
<td></td>
</tr>
<tr>
<td>C22:0</td>
<td>Behenic4</td>
<td>0.23 ± 0.01</td>
<td>0.16 ± 0.01*</td>
<td>0.16 ± 0.01*</td>
<td>0.7976</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>C24:0</td>
<td>Lignoceric3</td>
<td>0.5 (0.43-0.58)</td>
<td>0.38 (0.34-0.42)*</td>
<td>0.38 (0.34-0.42)*</td>
<td>0.9272</td>
<td>0.0035</td>
<td></td>
</tr>
<tr>
<td>Monounsaturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1n7</td>
<td>Palmitoleic</td>
<td>0.16 (0.13-0.19)</td>
<td>0.15 (0.13-0.18)</td>
<td>0.13 (0.11-0.15)*</td>
<td>0.0052</td>
<td>0.1769</td>
<td></td>
</tr>
<tr>
<td>C18:1n9</td>
<td>Oleic†</td>
<td>13.82 ± 0.24</td>
<td>13.91 ± 0.23</td>
<td>13.51 ± 0.23*</td>
<td>0.0158</td>
<td>0.0034</td>
<td></td>
</tr>
<tr>
<td>C20:1n9</td>
<td>Eicosenoic</td>
<td>0.24 (0.23-0.26)</td>
<td>0.35 (0.24-0.32)*</td>
<td>0.24 (0.22-0.27)</td>
<td>0.1697</td>
<td>0.0729</td>
<td></td>
</tr>
<tr>
<td>C24:1n9</td>
<td>Nervonic3</td>
<td>0.39 (0.32-0.48)</td>
<td>0.30 (0.27-0.33)*</td>
<td>0.28 (0.25-0.32)*</td>
<td>0.3654</td>
<td>0.0466</td>
<td></td>
</tr>
<tr>
<td>Trans fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1n7t</td>
<td>Palmitelaidic</td>
<td>0.10 ± 0.01</td>
<td>0.11 ± 0.01*</td>
<td>0.11 ± 0.01</td>
<td>0.8953</td>
<td>0.8621</td>
<td></td>
</tr>
<tr>
<td>C18:1t</td>
<td>Elaidic†</td>
<td>0.65 (0.6-0.72)</td>
<td>0.67 (0.60-0.74)</td>
<td>0.57 (0.52-0.62)*</td>
<td>0.0005</td>
<td>0.0051</td>
<td></td>
</tr>
<tr>
<td>C18:2n6t</td>
<td>Linoleicd4</td>
<td>0.23 ± 0.02</td>
<td>0.25 ± 0.01*</td>
<td>0.22 ± 0.01</td>
<td>0.0591</td>
<td>0.0097</td>
<td></td>
</tr>
<tr>
<td>n-6 Polyunsaturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:2</td>
<td>Linoleic</td>
<td>12.46 ± 0.27</td>
<td>11.96 ± 0.31*</td>
<td>10.97 ± 0.31*</td>
<td>0.0004</td>
<td>0.6248</td>
<td></td>
</tr>
<tr>
<td>C18:3</td>
<td>γ-linolenic</td>
<td>0.05 ± 0.003</td>
<td>0.06 ± 0.0004</td>
<td>0.05 ± 0.0004</td>
<td>0.0792</td>
<td>0.4568</td>
<td></td>
</tr>
<tr>
<td>C20:2</td>
<td>Eicosadienoic1</td>
<td>0.32 ± 0.02</td>
<td>0.29 ± 0.01*</td>
<td>0.27 ± 0.01*</td>
<td>0.3255</td>
<td>0.0208</td>
<td></td>
</tr>
<tr>
<td>C20:3</td>
<td>Dihomo-γ-linolenic</td>
<td>1.94 (1.72-2.19)</td>
<td>1.86 (1.65-2.09)</td>
<td>1.45 (1.29-1.62)*</td>
<td>&lt;0.0001</td>
<td>0.7516</td>
<td></td>
</tr>
<tr>
<td>C20:4</td>
<td>Arachidonic</td>
<td>17.38 ± 0.33</td>
<td>17.41 ± 0.35</td>
<td>15.54 ± 0.35*</td>
<td>&lt;0.0001</td>
<td>0.4335</td>
<td></td>
</tr>
<tr>
<td>C22:4</td>
<td>Docosatetraenoic</td>
<td>4.31 ± 0.15</td>
<td>4.18 ± 0.12</td>
<td>3.25 ± 0.12*</td>
<td>&lt;0.0001</td>
<td>0.5294</td>
<td></td>
</tr>
<tr>
<td>C22:5</td>
<td>Docosapentaenoic3</td>
<td>0.83 ± 0.04</td>
<td>0.71 ± 0.03*</td>
<td>0.55 ± 0.03*</td>
<td>&lt;0.0001</td>
<td>0.0403</td>
<td></td>
</tr>
<tr>
<td>n-3 Polyunsaturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:3</td>
<td>α-linolenic</td>
<td>0.13 (0.11-0.15)</td>
<td>0.11 (0.10-0.13)</td>
<td>0.10 (0.09-0.11)*</td>
<td>0.1458</td>
<td>0.5474</td>
<td></td>
</tr>
<tr>
<td>C20:5</td>
<td>Eicosapentaenoic</td>
<td>0.35 ± 0.02</td>
<td>0.41 ± 0.09</td>
<td>2.23 ± 0.09*</td>
<td>&lt;0.0001</td>
<td>0.2008</td>
<td></td>
</tr>
<tr>
<td>C22:5</td>
<td>Docosapentaenoic4</td>
<td>2.57 ± 0.10</td>
<td>2.62 ± 0.09</td>
<td>3.47 ± 0.09*</td>
<td>&lt;0.0001</td>
<td>0.0004</td>
<td></td>
</tr>
<tr>
<td>C22:6</td>
<td>Docosahexaenoic†</td>
<td>4.08 ± 0.16</td>
<td>4.46 ± 0.11*</td>
<td>6.61 ± 0.11*</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Omega-3 Index†</td>
<td></td>
<td>4.43 ± 0.17</td>
<td>4.86 ± 0.18*</td>
<td>8.83 ± 0.18*</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>

1All values are expressed as a % of total fatty acids in the red blood cell membrane. Baseline values are expressed as unadjusted means ± SEM. Endpoint values are expressed as least-square means ± SEM. Variables that exhibited non-normal distribution are expressed as geometric means with the 95% confidence interval in parentheses.

2P values are for the main effects of treatment and period based on the MIXED procedure, with both fixed effects in the model when period effects were significant. When period was nonsignificant, it was removed from the model to determine treatment effects.

3Period 2 values were significantly lower than period 1 values.

4Period 2 values were significantly greater than period 1 values.

*Indicates a significant change from baseline.

†Indicates a significant treatment by period effect of supplementation.
A potential carryover effect was observed for docosahexaenoic acid (DHA; treatment*period p = 0.0157) and the change in the Omega-3 Index (treatment*period p = 0.0065). This suggests incomplete washout between the supplementation periods and indicates that treatment order influenced these outcomes. Thus, significant main effects for treatment and period should not be interpreted across both study periods. Whereas EPA+DHA supplementation significantly increased RBC DHA by a similar amount whether it was received in the 1st or 2nd supplementation period, RBC DHA values following placebo supplementation differed according to treatment order (Figure 4-5). When the placebo was received second (following EPA+DHA supplementation in the 1st supplementation period), RBC DHA values were significantly higher than those observed when the placebo was received first (Figure 4-5). The same pattern was observed for the change in the Omega-3 Index (Figure 4-6).

![Figure 4-5. Effect of supplementation order on the change in red blood cell (RBC) docosahexaenoic acid (DHA) values. * indicates a significant difference between groups (p < 0.0001).](image)

The increase in long-chain omega-3 fatty acids with EPA+DHA supplementation was accompanied by a concomitant decrease in the percentage of omega-6 fatty acids in the RBC membrane (Table 4-4). The monounsaturated fatty acids palmitoleic and oleic acid were also significantly reduced following EPA+DHA supplementation compared to placebo. There was no effect of EPA+DHA supplementation on the percentage of saturated fatty acids in the RBC membrane. Period effects were found for omega-6 DPA, oleic acid, arachidic, behenic, lignoceric, and linoleaidic acid. All of these fatty acids were lower following the 2nd supplementation period except linoleaidic acid. There was also a significant treatment by period interaction for elaidic acid (treatment*period p = 0.05). In post-hoc analyses, RBC elaidic acid content was significantly higher when the placebo treatment was received in the first period compared to when the placebo was received second, and it was also higher following EPA+DHA treatment relative to placebo for both periods 1 and 2 (unadjusted p < 0.008).
4.4.3 Effect of EPA+DHA supplementation on inflammatory responses to low-dose endotoxin challenge

No serious adverse events occurred in response to low-dose endotoxemia. Participants experienced mild flu-like symptoms (e.g., headache, muscle aches, chills, and fatigue) and minor elevations in body temperature and heart rate, as expected. Symptom on-set typically occurred 1.5-4 hours post endotoxin injection, diminished throughout the remainder of the test day, and had fully resolved within 24 hours.

4.4.3.1 C-reactive protein (CRP)

All participants exhibited the expected CRP response to low-dose endotoxin challenge (Figure 4-7). The CRP elevation for one participant following the 72 hour time point was presumable due to the onset of a cold deemed unrelated to study procedures.

CRP values began to increase at 8 hours post-injection, with peak CRP elevations occurring at 24-hours post-injection (Figure 4-8). Values returned to near-baseline concentrations by 7 days post-injection (Figure 4-8). There was no difference in the peak CRP response to low-dose endotoxemia following EPA+DHA supplementation compared to the placebo (Figure 4-8). This is further demonstrated by visual comparison of peak CRP responses within individual participants following each treatment (Figure 4-9). Similar numbers of participants exhibited lower peak CRP values, higher peak CRP values, or no change in peak CRP values following EPA+DHA supplementation compared to placebo (Figure 4-9).
Figure 4-7. CRP response to low-dose endotoxin challenge for all twenty participants. Each participant’s CRP response to low-dose endotoxemia following 8 weeks of supplementation with the placebo and 3.4 g/d EPA+DHA is graphed as individual line.
Figure 4-8. Time course of C-reactive protein (CRP) production in response to low-dose endotoxemia. Data are presented as unadjusted means ± SEM calculated in Minitab. Tests for significant treatment and time point effects for change from pre-LPS value were performed using the mixed models procedure in SAS with log-transformed values. *indicates significant change from baseline for the time point.
Figure 4-9. Peak 24-hour CRP (mg/L) responses to low-dose endotoxemia following placebo and EPA+DHA supplementation for each participant.

4.4.3.2 Tumor necrosis factor (TNF)-α
Low-dose endotoxemia significantly increased TNF-α production at 1, 2, and 4 hours post-injection, with peak elevations at 2 hours (Figure 4-10). There was no effect of EPA+DHA supplementation on TNF-α responses to low-dose endotoxemia compared to the placebo (Figure 4-10).
Figure 4-10. Time course of TNF-α production in response to low-dose endotoxemia. Values are presented as unadjusted means ± SEM calculated in Minitab. Tests for significant treatment and time point effects for change from pre-LPS value were performed using the mixed models procedure in SAS with log-transformed values. *indicates significant change from baseline for the time point.

4.4.3.3 Interleukin (IL)-6
IL-6 was significantly increased by low-dose endotoxemia at 2 and 4 hours post-injection, with peak values at 2 hours (Figure 4-11). There was no significant difference in IL-6 production due to low-dose endotoxemia following EPA+DHA supplementation compared to placebo for any time point (Figure 4-11).
Due to the potential carryover effect for RBC DHA and the change in the Omega-3 Index, additional analyses were performed to determine whether this influenced inflammatory marker outcomes. Analyses using only period 1 values produced the same null finding for CRP (treatment $p = 0.5$), TNF-$\alpha$ (treatment $p = 0.2$), and IL-6 (treatment $p = 0.3$). The order of supplementation was also non-significant in all models ($p > 0.2$).

### 4.5 Discussion

This study investigated the effect of 3.4 g/d of EPA+DHA supplementation on acute inflammatory responses to low-dose induced endotoxemia in healthy young men. Following eight weeks of supplementation, red blood cell (RBC) omega-3 fatty acid content was substantially greater, confirming participant compliance with the study intervention and appropriate changes in the fatty acid composition of cell membranes. However, this did not translate to any effects on inflammatory responses to low-dose endotoxemia. Peak elevations in CRP, IL-6 and TNF-$\alpha$ following low-dose endotoxemia were not reduced following eight weeks of supplementation with 3.4 g/d of EPA+DHA compared to inflammatory responses after placebo supplementation. With regard to other post-supplementation effects, EPA+DHA lowered diastolic blood pressure (-3.75 mm Hg) compared to the placebo, and a 22% reduction in fasting
triglycerides from baseline was marginally significant. Both placebo and EPA+DHA supplementation reduced other fasting measures of lipids/lipoproteins and glucose metabolism relative to study-entry values, but these changes were not significantly different between treatment groups.

The magnitude and time course of cytokine and CRP elevations that were observed following endotoxin administration are in accordance with previous studies that have utilized a human model of endotoxemia [45, 268] and confirm that participants experienced a robust, resolving inflammatory response. Although tolerance to endotoxin has been shown to develop following high-dose (2 ng/kg) administration on five consecutive days [295], we saw no indication of habituation when two endotoxin challenges were performed 16 weeks apart. Although there were differences in the severity of clinical symptoms and the production of inflammatory markers between individuals, inflammatory responses were consistent within each participant. For instance, mean peak CRP concentrations at 24-hours post-injection were 17.9 ± 1.7 and 18.5 ± 2.0 in period 1 and period 2, respectively.

Effects of EPA+DHA supplementation on active inflammatory processes, including induced endotoxemia, have varied among previous studies. In sepsis, acute respiratory distress syndrome, and post-operative recovery, including omega-3 fatty acids in parenteral nutrition has been shown to improve clinical outcomes by preserving immune function while diminishing the extent of the inflammatory response [296]. Similarly, body temperature, TNF-α, and neuroendocrine responses to induced endotoxemia were blunted by intravenous administration of a lipid emulsion enriched with EPA+DHA in healthy young adults [297, 298]. However, while intravenous infusion results in much more rapid incorporation of omega-3 fatty acids into cell membranes than oral administration, it has limited application for the broader population and dietary intake recommendations. Providing EPA+DHA as a daily oral dose has been shown to be similarly effective in reducing body temperature [254, 299] and neuroendocrine stress hormone production [299] due to endotoxemia. For instance, 3-4 weeks of fish oil supplementation (1.8 g/d EPA+DHA provided as triglycerides from fish oil) blunted temperature and neuroendocrine stress responses to endotoxemia (2 ng/kg) in young men [299]. Ferguson et al. also reported a significant effect of 3.4 g/d EPA+DHA ethyl esters on body temperature following a lower dose of LPS (0.2 ng/kg) in healthy adults [254]. However, similar to our own results, there was no effect of supplementation on cytokine and acute phase protein production in either study [254, 299]. Therefore, the form in which EPA and DHA are provided may influence anti-inflammatory effects. Additional research is needed to evaluate whether oral administration of EPA and DHA can achieve clinically relevant effects on induced inflammatory responses.

Our results may also have important implications for the design of future studies of EPA+DHA supplementation. The average lifespan of an RBC is ~120 days. Although endpoint measures were separated by ≥ 16 weeks (8 week treatment + 8 week washout), we observed a significant treatment by period effect indicating likely carryover of RBC DHA content for participants who received EPA+DHA during the first treatment period. Therefore, this washout period did not provide sufficient time for complete turnover of RBC DHA content. Whereas EPA has been posited to be a more dynamic and readily available pool of long-chain omega-3 fatty acids, DHA stores in cells and tissues appear to require greater time for enrichment and depletion [300, 301]. Our results support these proposed differences in EPA and DHA metabolism as the test for carryover effect in our study was significant only for RBC DHA and not RBC EPA content. The potential carryover effect observed for RBC DHA was not responsible for the lack of anti-inflammatory effects in the current study. However, it indicates that longer periods of supplementation may achieve greater changes in cell and tissue reserves of DHA, and that some benefits of increased omega-3 fatty acid consumption may be present for several months after supplementation has been
discontinued. This should be taken into consideration with regard to the prevention and/or treatment of diseases related to omega-3 fatty acid intake and when designing future crossover EPA+DHA supplementation studies.

With respect to other study outcomes, diastolic blood pressure was significantly reduced by EPA+DHA supplementation compared to the placebo. The potential of EPA+DHA supplementation to reduce systolic and/or diastolic blood pressure is supported by multiple meta-analyses [302, 303]. However, it should be noted that our result is based upon a single blood pressure measurement taken prior to endotoxin administration, which is not in accordance with JNC 7 guidelines for blood pressure assessment [277]. A trend towards a reduction in triglycerides was also found, and the triglyceride-lowering effects of EPA+DHA are well-established [304]. We were surprised by the period effects found for RBC fatty acids, blood count values, and laboratory outcomes in the general chemistry panel. These effects may relate to the volume of blood that was sampled during the first period endpoint testing despite there being at least 16 weeks between endpoint blood draws. The clinical relevance of these changes was not apparent. Significant reductions from baseline for glucose, insulin, and lipids/lipoproteins following both supplementation periods were also unexpected, as participants were healthy at study entry and expected to maintain their diet and levels of physical activity. Treatment by period effects were found for insulin and a small number of CBC values, although no comparisons were significant in post-hoc analyses corrected for multiple comparisons. It is likely that these effects are the result of Type I error due to the number of endpoints that were analyzed, rather than a meaningful effect of EPA+DHA supplementation.

Although cell/animal models have demonstrated the anti-inflammatory potential of omega-3 fatty acids [36], these results have not translated into consistent effects on inflammatory outcomes in clinical studies of EPA+DHA supplementation [244, 245]. Mixed results may be due to heterogeneity in the dose and duration of supplementation, characteristics of the study population, and/or differences in background omega-3 intake. However, a key component of cell/animal models that is lacking in most clinical trials is the use of an inflammatory stimulus. As the effects of omega-3 fatty acids are likely to be subtle compared to those of anti-inflammatory pharmacological agents, they may be more difficult to detect in the resting physiological state and this may account for null findings in some clinical studies. Omega-3 fatty acids may also act on other components of the inflammatory response, such as oxidative stress or the resolution of inflammation, while production of acute phase proteins is preserved. For example, some previous studies have found no effect of daily EPA+DHA supplementation on cytokine production due to induced endotoxemia, but significant attenuation of body temperature [254] and stress hormone responses [299]. Importantly, adverse immunosuppressive effects have not been found in clinical studies [296]. Urinary isoprostanes formed from arachidonic acid, which are considered a gold standard marker of oxidative stress, have also been shown to be reduced by omega-3 supplementation in many studies [257, 258, 263]. The production of specialized pro-resolving mediators (SPMs) has also been proposed as a mechanism by which omega-3 fatty acids may help to prevent the development of chronic inflammation. One study has evaluated SPMs in human plasma following EPA+DHA supplementation and induced endotoxemia, and found no clear pattern of bioactive lipid mediator production [253]. Clearly, much remains to be understood regarding SPM production in humans and how this influences the inflammatory response. Thus, additional research is needed to explore other pathways by which omega-3 fatty acids may influence the inflammatory response.
4.5.1 Study strengths and limitations

The crossover design of this study allowed us to evaluate the effect of EPA+DHA supplementation on inflammatory responses to low-dose endotoxemia compared to placebo supplementation within the same participant. This decreases potential sources of error by minimizing the influence of between-subject variability when analyzing treatment effects. Moreover, all twenty participants completed both low-dose endotoxemia challenges and reported good compliance with the study intervention, which was verified by consistent increases in RBC EPA. Endotoxin preparation and all clinical procedures were performed with precision and consistency by the same personnel, minimizing the potential for variation in inflammatory responses due to differences in preparation techniques. All baseline CRP values prior to endotoxemia challenges were < 3 mg/L, which indicates that participants were not experiencing an acute inflammatory event unrelated to study procedures. This reduced the potential for confounding from external factors and enhanced our ability to specifically evaluate the induced response to a controlled stimulus. Recruitment was based on the criterion of low omega-3 fatty acid consumption, and baseline RBC EPA+DHA content of our participants was representative of the average American population that consumes very little oily fish. However, our moderately small study population consisted entirely of healthy men, most of whom were relatively young and Caucasian, which may have limited our ability to detect effects that may be present in women [305, 306], different ethnic groups [306], older people, and/or those with chronic health conditions. Future studies employing a similar design should be conducted in women and other ethnicities. With regard to other population characteristics, the use of experimental endotoxemia in humans is currently restricted to relatively young (<45 years), healthy, non-obese, and non-smoking individuals by the United States Food and Drug Administration. Dietary intake was not monitored, but participants were instructed to limit their consumption of oily fish and avoid flax- or fish oil-based supplements. The lack of change in RBC EPA during the placebo period provides further confirmation that participants did alter their dietary habits. A pharmaceutical grade preparation of EPA+DHA was used to minimize variation in the composition of study supplements and maximize the dose of EPA+DHA provided by four one-gram capsules. The 3.4 g/d dose is clinically relevant and corresponds to the dose clinically recommended for triglyceride-reduction in individuals with hypertriglyceridemia. Although the duration of the washout period was insufficient to prevent likely carryover effects of EPA+DHA supplementation on RBC DHA values this was not responsible for the lack of effect on inflammatory responses to low-dose endotoxemia since responses were consistent between period 1 and period 2 regardless of the order in which participants received EPA+DHA or placebo.

4.5.2 Conclusions

Supplementation with 3.4 g/d of EPA+DHA for eight weeks did not alter acute cytokine and CRP responses to low-dose endotoxemia in healthy young men, despite the enrichment of erythrocytes with LC omega-3 fatty acids. The time course of CRP and cytokine elevations during the inflammatory response was highly consistent between and within participants following both supplementation periods, supporting the utility of this inflammatory stimulus in modelling innate immune responses. The lack of effect on inflammatory responses to low-dose endotoxemia may reflect that omega-3 fatty acids are not immunosuppressive, and that beneficial effects might instead be exerted on aspects of the inflammatory response beyond cytokine and CRP production. Future studies are needed to determine whether women and other populations at risk of excessive inflammation experience attenuated inflammatory responses following EPA+DHA supplementation.
## 4.6 Supplemental Tables and Figures

Supplemental Table 4-1. Nutrient composition of meals and snacks provided during low-dose endotoxin challenge.

<table>
<thead>
<tr>
<th></th>
<th>Energy (kcal)</th>
<th>Fat (g)</th>
<th>CHO (g)</th>
<th>Protein (g)</th>
<th>Sodium (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BREAKFAST</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sandwich - Croissant</td>
<td>290</td>
<td>16</td>
<td>27</td>
<td>10</td>
<td>470</td>
</tr>
<tr>
<td>Sandwich - Croissant</td>
<td>290</td>
<td>16</td>
<td>27</td>
<td>10</td>
<td>470</td>
</tr>
<tr>
<td>Hash brown patty</td>
<td>130</td>
<td>7</td>
<td>15</td>
<td>1</td>
<td>300</td>
</tr>
<tr>
<td>Apple Juice</td>
<td>120</td>
<td>0</td>
<td>29</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td><strong>830</strong></td>
<td><strong>39</strong></td>
<td><strong>98</strong></td>
<td><strong>21</strong></td>
<td><strong>1250</strong></td>
</tr>
<tr>
<td><strong>LUNCH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mac &amp; Cheese</td>
<td>260</td>
<td>6</td>
<td>39</td>
<td>10</td>
<td>760</td>
</tr>
<tr>
<td>Lays Chips (1 package)</td>
<td>160</td>
<td>10</td>
<td>15</td>
<td>2</td>
<td>170</td>
</tr>
<tr>
<td>Apple Juice</td>
<td>120</td>
<td>0</td>
<td>29</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Ice cream cup</td>
<td>410</td>
<td>22</td>
<td>46</td>
<td>9</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td><strong>950</strong></td>
<td><strong>38</strong></td>
<td><strong>129</strong></td>
<td><strong>21</strong></td>
<td><strong>1130</strong></td>
</tr>
<tr>
<td><strong>End of day Snack</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oreo Cookie Snack Pack</td>
<td>270</td>
<td>11</td>
<td>41</td>
<td>2</td>
<td>240</td>
</tr>
<tr>
<td>Chips Ahoy! Minis</td>
<td>140</td>
<td>7</td>
<td>19</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Aple Juice</td>
<td>120</td>
<td>0</td>
<td>29</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td><strong>530</strong></td>
<td><strong>18</strong></td>
<td><strong>89</strong></td>
<td><strong>3</strong></td>
<td><strong>350</strong></td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>2310</strong></td>
<td><strong>95</strong></td>
<td><strong>316</strong></td>
<td><strong>45</strong></td>
<td><strong>2730</strong></td>
</tr>
</tbody>
</table>

~kcal

<table>
<thead>
<tr>
<th>% total kcal</th>
</tr>
</thead>
<tbody>
<tr>
<td>37%</td>
</tr>
<tr>
<td>55%</td>
</tr>
<tr>
<td>8%</td>
</tr>
</tbody>
</table>
Supplemental Table 4-2. Liver enzymes, general chemistry panel measures, complete blood count, and coagulation parameters following supplementation.

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>EPA+DHA</th>
<th>Period p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value</td>
<td>Δ from baseline</td>
<td>Value</td>
</tr>
<tr>
<td>ALP</td>
<td>51.4 ± 2.36</td>
<td>-8.55 ± 1.80†</td>
<td>47.5 ± 2.36*</td>
</tr>
<tr>
<td>AST</td>
<td>19.0 (16.2-22.3)</td>
<td>-1.75 ± 1.84</td>
<td>19.3 (16.4-22.6)</td>
</tr>
<tr>
<td>ALT</td>
<td>16.4 (12.9-21.0)</td>
<td>-0.05 ± 2.04</td>
<td>15.9 (12.6-20.0)</td>
</tr>
<tr>
<td>LD</td>
<td>141 ± 6.16</td>
<td>-8.80 ± 7.41</td>
<td>143 ± 6.16</td>
</tr>
<tr>
<td>Urea nitrogen</td>
<td>13.9 ± 0.75</td>
<td>-0.55 ± 0.78</td>
<td>13.9 ± 0.75</td>
</tr>
<tr>
<td>Creatinine‡</td>
<td>0.97 ± 0.03</td>
<td>-0.06 ± 0.02†</td>
<td>0.97 ± 0.03</td>
</tr>
<tr>
<td>BUN:creatinine</td>
<td>14.5 ± 0.93</td>
<td>0.58 ± 0.81</td>
<td>14.4 ± 0.93</td>
</tr>
<tr>
<td>Protein</td>
<td>6.28 ± 0.06</td>
<td>-0.74 ± 0.06†</td>
<td>6.36 ± 0.06</td>
</tr>
<tr>
<td>Iron⁺</td>
<td>97.2 ± 6.08</td>
<td>-15.7 ± 8.41‡</td>
<td>98.1 ± 6.08</td>
</tr>
<tr>
<td>Albumin¹</td>
<td>4.09 ± 0.04</td>
<td>-0.57 ± 0.04†</td>
<td>4.12 ± 0.04</td>
</tr>
<tr>
<td>Globulin²</td>
<td>2.20 ± 0.06</td>
<td>-0.22 ± 0.03†</td>
<td>2.25 ± 0.06</td>
</tr>
<tr>
<td>A:G ratio†</td>
<td>1.90 ± 0.06</td>
<td>-0.08 ± 0.04‡</td>
<td>1.87 ± 0.06</td>
</tr>
<tr>
<td>Prothrombin time</td>
<td>1.06 ± 0.01</td>
<td>-0.01 ± 0.01</td>
<td>1.08 ± 0.01</td>
</tr>
<tr>
<td>INR</td>
<td>5.06 ± 0.30</td>
<td>-0.55 ± 0.21†</td>
<td>5.09 ± 0.30</td>
</tr>
<tr>
<td>WBC</td>
<td>4.56 ± 0.06</td>
<td>-0.35 ± 0.04†</td>
<td>4.54 ± 0.06</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>13.7 ± 0.13</td>
<td>-1.05 ± 0.11†</td>
<td>13.6 ± 0.13</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>40.4 ± 0.41</td>
<td>-3.1 ± 0.39†</td>
<td>40.1 ± 0.41</td>
</tr>
<tr>
<td>MCH</td>
<td>88.6 ± 0.61</td>
<td>-0.12 ± 0.26</td>
<td>88.4 ± 0.61</td>
</tr>
<tr>
<td>MCHC</td>
<td>30.1 ± 0.24</td>
<td>0.05 ± 0.11</td>
<td>30.0 ± 0.24</td>
</tr>
<tr>
<td>RDW</td>
<td>34.0 ± 0.13</td>
<td>0.07 ± 0.14</td>
<td>33.9 ± 0.13</td>
</tr>
<tr>
<td>Platelet count¹</td>
<td>193 ± 7.70</td>
<td>-17.5 ± 4.46†</td>
<td>194 ± 7.70</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>2543 ± 192</td>
<td>-278 ± 92.3†</td>
<td>2465 ± 192</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1977 ± 124</td>
<td>-7.0 ± 96.4</td>
<td>2054 ± 124</td>
</tr>
<tr>
<td>Monocytes</td>
<td>371 ± 27.1</td>
<td>-54.5 ± 28.3‡</td>
<td>383 ± 27.1</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>122 (88.5-167)</td>
<td>-17.3 ± 15.1</td>
<td>111 (75.2-164)</td>
</tr>
<tr>
<td>Basophils</td>
<td>24.0 ± 2.61</td>
<td>1.0 ± 2.67</td>
<td>23.5 ± 2.61</td>
</tr>
<tr>
<td>Neutrophils %</td>
<td>49.3 ± 1.54</td>
<td>-2.75 ± 1.23†</td>
<td>48.3 ± 1.54</td>
</tr>
<tr>
<td>Lymphocytes %</td>
<td>39.9 ± 1.65</td>
<td>3.45 ± 1.07†</td>
<td>40.8 ± 1.65</td>
</tr>
<tr>
<td>Monocytes %</td>
<td>7.35 ± 0.38</td>
<td>-0.3 ± 0.48</td>
<td>7.60 ± 0.38</td>
</tr>
<tr>
<td>Eosinophils %</td>
<td>2.95 ± 0.43</td>
<td>-0.35 ± 0.44</td>
<td>2.90 ± 0.43</td>
</tr>
<tr>
<td>Basophils %</td>
<td>0.2 ± 0.10</td>
<td>-0.15 ± 0.15</td>
<td>0.3 ± 0.10</td>
</tr>
</tbody>
</table>

Abbreviations: ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine transaminase; LD, lactate dehydrogenase; BUN, blood urea nitrogen; INR, international normalized ratio; WBC, white blood cell; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width.

*indicates a trend towards a significant treatment effect (p = 0.06).
†indicates a significant change from baseline.
‡indicates a trend towards significant change from baseline (0.05 < p < 0.1).
§indicates a significant trend by visit effect (p < 0.05).

1Period 2 values were significantly lower than period 1 values.
2Period 2 values were significantly higher than period 1 values.
Chapter 5. Omega-3 fatty acid supplementation reduces total urinary isoprostane production in healthy young adults following low-dose endotoxemia despite increases in EPA-derived isoprostanes

5.1 Abstract

Background: Increased eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) intake is associated with prevention of chronic diseases involving oxidative stress. However, clinical trials have produced mixed results, and the mechanisms underlying the role of EPA+DHA in the development or attenuation of oxidative stress remain incompletely understood.

Objective: The objective of this analysis was to evaluate the effect of EPA+DHA supplementation on acute oxidative stress responses in a model of induced inflammation (intravenous 0.6 ng/kg purified lipopolysaccharide [LPS], or endotoxin challenge) in two study populations.

Design: Urine concentrations of F2-isoprostanes (F2-IsoP), the F2-isoprostane metabolite 2,3-dinor-5,6-dihydro-15-F21-IsoP (F2-IsoM), and F3-isoprostanes (F3-IsoP) were measured following low-dose endotoxin challenge. In Study 1, healthy young adults (n = 17 [10M]) were supplemented with 0, 300, 600, 900, or 1800 mg/d EPA+DHA for 5 months before undergoing a low-dose endotoxin challenge. In Study 2, healthy young men (n=20) were supplemented for 8 weeks with 3.4 g/d EPA+DHA and an olive placebo in a crossover design, with low-dose endotoxin challenges following both supplementation periods.

Results: In Study 1, supplementation with omega-3 fatty acids (600-1800 mg/d) for 5 months attenuated total urinary isoprostane production following low-dose endotoxin challenge in a model that also included body mass index, despite elevated F3-IsoP concentrations. In Study 2, eight weeks of 3.4 g/d EPA+DHA supplementation prior to low-dose endotoxin challenge similarly increased F3-IsoPs but did not alter total isoprostane production.

Conclusion: EPA+DHA supplementation increased F3-IsoP production following low-dose endotoxin challenge, but may attenuate the total oxidative stress response (sum of isoprostane species) to an acute inflammatory challenge. However, more research is needed to clarify if a reduction in total isoprostane production is contingent on the duration of supplementation and/or the form of omega-3 fatty acids administered.
5.2 Introduction

Oxidative stress is closely inter-linked with an inappropriate inflammatory response and is also a common underpinning of many chronic diseases, including cardiovascular disease (CVD) [34]. Oxidative stress is characterized by an imbalance in the production of pro-oxidant reactive oxygen species (ROS) or free radicals and the body's natural antioxidant defenses. The long-chain omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been associated with reduced CVD risk, and they have been shown to have anti-inflammatory effects in pre-clinical models. However, polyunsaturated fatty acids can be a target of free radical insult during oxidative stress, and their role in the development or attenuation of oxidative stress is not fully understood.

Products of lipid peroxidation, such as isoprostanes, can be measured in biological fluids as a measure of oxidative stress. Isoprostanes (IsoPs) are prostaglandin-like molecules formed via non-enzymatic, free radical-induced lipid peroxidation. F₂-isoprostanes (F₂-IsoPs) are formed from arachidonic acid and can induce vasoconstriction and platelet aggregation, inhibit angiogenesis, increase mean systemic arterial pressure, and promote atherosclerosis by stimulating monocyte and neutrophil adhesion to endothelial cells [86, 88]. They are recognized as a reliable marker of endogenous lipid peroxidation [89, 90] and are elevated in conditions associated with enhanced oxidative stress and risk of CVD, such as smoking [91, 92], type 2 diabetes [93-95], hypercholesterolemia [96], and overweight or obesity [97, 98]. F₂-IsoP metabolites (F₂-IsoP-M) formed in the liver have also been shown to be important biomarkers of oxidative stress in age- and obesity-related diseases [87, 307, 308]. F₂-IsoP-Ms may be a more sensitive marker of endogenous oxidation, and assessment of these compounds can provide additional information beyond that afforded by measurement of F₂-IsoP alone [87]. Conversely, F₃-isoprostanes (F₃-IsoPs) derived from EPA are less biologically active and do not exert the same effects on the vasculature or platelet activity as F₂-IsoPs [88].

However, there remains a theoretical concern that greater intake of omega-3 fatty acids and their subsequent incorporation into cell membranes may increase lipid peroxidation. Numerous studies have been conducted to evaluate the effect of omega-3 fatty acid supplementation on isoprostane production. None have found that EPA+DHA supplementation increases F₂-IsoPs, and many have found reductions in F₂-IsoPs and/or increased production of F₃-IsoPs [254, 255, 257, 258, 262, 263]. Reductions in F₂-IsoPs were also unrelated to changes in plasma [257], red blood cell [260], and/or platelet fatty acid content that were caused by omega-3 fatty acid supplementation [263]—indicating the decrease in F₂-IsoPs most likely reflects a true reduction in oxidation and is not simply a result of limited arachidonic acid substrate availability. Thus, there is substantial evidence to support that omega-3 fatty acids do not adversely affect oxidative stress in terms of F₂-IsoP production and may have beneficial effects.

Less is known about the oxidative stress response and urinary isoprostane production due to an inflammatory stimulus and how this may be influenced by EPA+DHA supplementation. Administration of LPS (3 ng/kg) has previously been shown to induce both F₂- and F₃-IsoP production in healthy adults [88, 266]. However, no other clinical studies have reported the effects of LPS administration on isoprostane production. Furthermore, the effect of omega-3 fatty acid supplementation on isoprostane production in response to low-dose endotoxemia has also not yet been reported. Based on the effects of omega-3 supplementation on F₂- and F₃-IsoPs in the resting physiological state, we hypothesized that omega-3 supplementation prior to a low-dose endotoxin challenge would increase F₃-IsoP production but attenuate the overall oxidative stress response in terms of total isoprostane production. If omega-3 fatty
acids can reduce the oxidative stress response to an inflammatory stimuli, it is feasible that this may, in part, contribute to the beneficial health outcomes associated with EPA and DHA. To evaluate the effect of omega-3 fatty acid supplementation on urinary isoprostane production following an acute inflammatory challenge, we utilized urine samples from two studies that enrolled healthy young adults (Study 1; n = 17) and healthy young men (Study 2; n = 20).

5.3 Methods
5.3.1 Participant population and recruitment

5.3.1.1 Experimental overview
Detailed experimental methods for both studies have been previously described ([309] and Chapter 4.3). Both studies were conducted at the Pennsylvania State University Clinical Research Center (CRC), and were approved annually by the Pennsylvania State University Institutional Review Board.

5.3.1.2 Study 1
In Study 1 (Figure 5-1(A), Table 5-1), healthy young adults (20-45 years of age, BMI 20-30 kg/m²) reporting no or low habitual oily fish consumption (< 4 meals per month) and not taking omega-3 fatty acid supplements were randomly assigned to one of five doses of daily fish oil supplements (0, 300, 600, 900, 1800 mg EPA+DHA; soybean oil placebo) for approximately 5 months (Nordic Naturals, Watsonville, CA, USA). A total of 125 participants were enrolled and 116 completed the study. A subset of 17 participants who underwent a low-dose endotoxin challenge (described further below) following supplementation were analyzed.

![Figure 5-1: Study design schematic for Study 1 (A) and Study 2 (B).]
Table 5-1. Study design and characteristics of participants who completed Study 1 and Study 2.1

<table>
<thead>
<tr>
<th></th>
<th>Study 1</th>
<th>Study 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Study design</strong></td>
<td>Parallel</td>
<td>Crossover</td>
</tr>
<tr>
<td><strong>Duration of</strong></td>
<td>20 weeks</td>
<td>8 weeks</td>
</tr>
<tr>
<td><strong>supplementation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total sample size</strong></td>
<td>17 (10 M; 7 F)</td>
<td>20 M</td>
</tr>
<tr>
<td><strong>Age (y)</strong></td>
<td>25 ± 2 (20-44)</td>
<td>27 ± 1 (20–40)</td>
</tr>
<tr>
<td><strong>Supplement Group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Low Dose</strong></td>
<td>(n=7; 3 M)</td>
<td></td>
</tr>
<tr>
<td><strong>High Dose</strong></td>
<td>(n=10; 7 M)</td>
<td></td>
</tr>
<tr>
<td><strong>Placebo</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>24.7 ± 0.8 (22.6-28.5)</td>
<td>24.2 ± 0.8 (21.2-27.9)</td>
</tr>
<tr>
<td></td>
<td>25.1 ± 0.6 (20.7-29.3)</td>
<td>25.1 ± 0.6 (19.5-30.4)</td>
</tr>
<tr>
<td><strong>CRP (mg/L)</strong></td>
<td>1.1 ± 0.4 (0.2-3.0)</td>
<td>0.5 ± 0.1 (0.2-1.3)</td>
</tr>
<tr>
<td></td>
<td>0.6 ± 0.1 (&lt;0.2-2.5)</td>
<td>0.5 ± 0.1 (&lt;0.2-1.3)</td>
</tr>
<tr>
<td><strong>Erythrocyte fatty acid content</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eicosapentaenoic acid</td>
<td>0.6 ± 0.09 (0.38-0.98)</td>
<td>1.3 ± 0.14 (0.8-2.32)</td>
</tr>
<tr>
<td>(20:5n3)</td>
<td>0.41 ± 0.03 (0.22-0.66)</td>
<td>2.23 ±0.12 (1.23-3.39)</td>
</tr>
<tr>
<td>Docosapentaenoic acid</td>
<td>2.72 ± 0.2 (1.68-3.49)</td>
<td>3.35 ± 0.17 (2.45-4.24)</td>
</tr>
<tr>
<td>(22:5n3)</td>
<td>2.62 ± 0.1 (1.72-3.57)</td>
<td>3.47 ± 0.09 (2.79-4.47)</td>
</tr>
<tr>
<td>Docosahexaenoic acid</td>
<td>4.67 ± 0.53 (2.67-6.94)</td>
<td>5.51 ± 0.36 (3.72-7.65)</td>
</tr>
<tr>
<td>(22:6n3)</td>
<td>4.46 ± 0.18 (3.2-6.02)</td>
<td>6.61 ± (5.7-7.67)</td>
</tr>
<tr>
<td>Omega-3 Index (EPA+DHA)</td>
<td>5.31 ± 0.61 (3.06-7.92)</td>
<td>6.78 ± 0.47 (4.60-9.98)</td>
</tr>
<tr>
<td></td>
<td>4.86 ± 0.21 (3.49-6.64)</td>
<td>8.83 ± 0.19 (6.93-10.6)</td>
</tr>
</tbody>
</table>

1Values represent participant characteristics following supplementation, directly prior to low-dose endotoxin challenge, and are presented as means ± SEM with ranges in parentheses.
2The low dose group received 0 or 300 mg/d of EPA+DHA.
3The high dose group received 600, 900, or 1800 mg/d of EPA+DHA.

5.3.1.3 Study 2

In Study 2 (Figure 5-1(B), Table 5-1), healthy young men (20-45 years of age, BMI 20-30 kg/m²) who reported low habitual oily fish consumption (< 2 servings/week) were enrolled in a 2-period randomized crossover study investigating the effects of 3400 mg/d EPA+DHA to an olive oil placebo. Supplementation periods were 8 weeks long and were separated by an 8 week wash out period. Following each supplementation period, participants underwent a low-dose endotoxin challenge (described further below). Twenty-one participants were randomized to treatment groups. One participant withdrew from the study prior to completing the first supplementation period. The remaining twenty participants completed both supplementation periods and low-dose endotoxin challenges.

The omega-3 fatty acid content provided by each supplement in Study 1 and Study 2, as determined by independent analysis, is provided in Table 5-2.
Table 5-2. Omega-3 fatty acid content (mg/d) of each supplement dose in Study 1 and Study 2.\(^1\)

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>STUDY 1 Dose (mg/d)</th>
<th>STUDY 2 Dose (mg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eicosapentaenoic acid (EPA)</td>
<td>0 300 600 900 1800</td>
<td>0 3400</td>
</tr>
<tr>
<td>Docosapentaenoic acid (DPA n-3)</td>
<td>9 191 374 556 1103</td>
<td>&lt;1 1988</td>
</tr>
<tr>
<td>Docosahexaenoic acid (DHA)</td>
<td>1 20 40 59 118</td>
<td>&lt;1 109</td>
</tr>
<tr>
<td>Total EPA + DHA</td>
<td>6 121 237 352 698</td>
<td>&lt;1 1608</td>
</tr>
<tr>
<td>Total EPA + DPA + DHA</td>
<td>15 312 611 908 1801</td>
<td>&lt;1 3596</td>
</tr>
</tbody>
</table>

\(^1\)Values were calculated by independent analysis of fatty acid composition of a sample of active and placebo capsules.

5.3.1.4 Low-dose endotoxin challenge
The same standardized protocol was used to perform the low-dose endotoxin challenges at the end of each supplementation period in Study 1 and Study 2. Briefly, participants were instructed to avoid alcohol and strenuous exercise for 48 hours and fast for 12 hours (no food or drink other than water) prior to their appointment. Endotoxin challenges were postponed for a minimum of 1 week if participants reported a recent change in medical history, medical or dental procedures, physical injury, or cold symptoms. When participants arrived at the CRC, weight, baseline vital signs (blood pressure, heart rate, and body temperature), and a baseline urine sample were obtained. Two intravenous catheters were then established for saline administration and blood sampling. Following a 500 mL bolus of sterile saline, a sterile solution of protein-free endotoxin (or lipopolysaccharide; LPS) was injected at a dose of 0.6 ng/kg body weight over a 1 minute period. IV saline was administered continuously for the duration of the 8 hour test day. Participants were provided with standardized breakfast and lunch meals approximately thirty minutes and four hours after endotoxin administration, respectively. In the crossover study (Study 2) all participants were offered the same breakfast and lunch meal during their first endotoxin challenge. For the second endotoxin challenge, each participant was provided with the same breakfast and lunch foods that they consumed during the first endotoxin challenge. If a participant requested additional portions or declined particular foods during their first endotoxin challenge, that was replicated for their second visit. In the parallel arm study (Study 1), all participants received the same breakfast and lunch meals. Participant vitals (blood pressure, heart rate, and body temperature) were monitored for the duration of the 8 hour visit by CRC clinicians. Following the collection of the 8 hour samples, IV catheters were discontinued and participants were briefly evaluated for safety before being allowed to leave the CRC.

5.3.2 Sample collection and assay methods
Both studies utilized similar methods for urine sample collection and measurement of urinary isoprostanes (IsoP). In Study 1, urine was collected in aggregate, following LPS administration, for the duration of the 8 hour endotoxin challenge visit. In Study 2, a single urine sample was obtained from participants prior to LPS administration, and urine was collected in two aggregate batches that comprised the 1\(^{st}\) four hours after LPS injection and the 2\(^{nd}\) four hours after LPS injection. Urine collections were kept chilled by storing them on ice prior to aliquoting. Aliquots were stored at \(-80^\circ\text{C}\) until analysis. F\(_2\)-isoprostanes (F\(_2\)-IsoP), the F\(_2\)-isoprostane metabolite 2,3-dinor-5,6-dihydro-15-F\(_{2t}\)-IsoP (F\(_{2t}\)-IsoM), and F\(_3\)-
isoprostanes (F3-IsoP) were measured in the Eicosanoid Core Laboratory at Vanderbilt University using negative ion chemical ionization gas chromatography/mass spectrometry (GC/MS) with isotopically labeled internal standards.

5.3.3 Statistical Analyses
Analyses were performed on F2-IsoP, F2-IsoM, F3-IsoP, and total IsoP (F2-IsoP + F2-IsoM + F3-IsoP). Due to sample size limitations in Study 1, analyses were performed using a dichotomous variable of low dose (0 and 300 mg/d EPA+DHA, n = 7) and high dose (600, 900, and 1800 mg/d, n = 10). A total of 24 participants underwent a low-dose endotoxin challenge following supplementation; of those 24, inflammatory and oxidative stress biomarker data from 19 participants was suitable for analysis. Reasons for excluding participants from analysis include suspected protocol deviation (markedly lower inflammatory response, n = 2), acute use of pain reliever (n = 2), and acutely elevated baseline CRP (n = 1). These decisions to exclude participants were made while blinded, prior to statistical analysis. Following initial statistical analysis, two participants were found to have total urinary isoprostane values that were more than two standard deviations beyond the mean (Supplemental Figure 5-1). These two participants were deemed outliers and excluded from the final analysis; thus, the final analysis is based on data from 17 participants. Regression modeling for Study 1 was performed in Minitab (version 17; Minitab, State College, PA). The Assistant Function was used to select between linear, quadratic, or cubic univariate regression models. Potential multivariate models were identified using the best subsets procedure. Model selection was based on adjusted R-squared values and the Mallow’s Cp fit statistic. Regression modeling was also performed on the full sample (n = 19), and results from these analyses are presented as supplemental material in Section 5.6. In these regression models, the two participants who were deemed outliers had large residuals and were not well fit by the models. For Study 2, which was a crossover design, the mixed models procedure (PROC MIXED) in SAS (version 9.3; SAS Institute; Cary, NC) was used to test the effects of treatment, period, and treatment by period interactions. Outcome variables were checked for normality and positively skewed variables were logarithmically transformed prior to analysis. Subject was treated as a random effect and the remaining factors were fixed effects. Period by treatment interactions were universally non-significant and were removed from the final model of treatment effects. Means are reported as least squares means ± SEM.

5.4 Results
5.4.1 Omega-3 fatty acid supplementation increased F3-isoprostane production
In Study 1, univariate regression using the dichotomous variable of high dose versus low dose demonstrated that high dose EPA+DHA supplementation resulted in a 0.12 ng/mg creatinine increase in urinary F3-isoprostanes (F3-IsoPs) in the 8 hours following endotoxin challenge (Figure 5-2) (p = 0.05).
Performing the regression analysis with each of the five EPA+DHA dose categories demonstrated similar results, with each 1 g/d increase in EPA+DHA resulting in a 0.17 ng/mg creatinine increase in F3-IsoPs (Figure 5-3) (p = 0.02).

In Study 2, EPA+DHA supplementation significantly increased F3-IsoP compared to the placebo, prior to and following LPS administration (Figure 5-4). Low-dose endotoxemia significantly increased F3-IsoP production at 8 hours regardless of treatment (p < 0.0001 for 8 hr vs. pre-LPS). The concentration of urinary F3-IsoP was greater with EPA+DHA supplementation at all time points (treatment p = < 0.001).
5.4.2 Effects of omega-3 supplementation on total isoprostane production ($F_2$-IsoP + $F_2$-IsoM + $F_3$-IsoP)

Five months of supplementation with 600-1800 mg/d EPA+DHA (Study 1) decreased total IsoP ($F_2$-IsoP + $F_2$-IsoP-M + $F_3$-IsoP) production despite an increase in $F_3$-IsoP. The multivariate model that best predicted total IsoP included the dose of EPA+DHA and BMI (model $p < 0.0001$, model adjusted $R^2$ = 72%). High dose EPA+DHA supplementation (600-1800 mg/d) decreased total IsoP production by 1.11 ng/mg creatinine ($p < 0.0001$). In this multivariate model, a significant 0.11 ng/mg creatinine decrease in total IsoP production also occurred for every 1 unit increase in BMI ($p = 0.02$). In univariate analysis, EPA+DHA dose alone explained 61% of the variation in total IsoPs, and high dose EPA+DHA reduced total IsoP production by 1.05 ng/mg creatinine ($p < 0.0001$). BMI was not a significant predictor of total IsoP production in univariate analysis ($p = 0.3$). When an interaction term was added to the model, the interaction between dose and BMI was a significant predictor of total IsoP production ($p = 0.03$). In a model of the interaction between EPA+DHA dose and BMI, participants who received high dose EPA+DHA supplementation experienced an additional 0.05 ng/mg creatinine decrease in total IsoP production for every 1 unit increase in BMI (Figure 5-5). Participants who received the low dose had an average total IsoP concentration of 3.3 ng/mg creatinine, regardless of BMI (Figure 5-5). Thus, participants who received high dose EPA+DHA and who had the highest BMIs had the lowest total IsoP, whereas participants who received low dose EPA+DHA had the highest concentrations of total IsoP whether their BMI was relatively high or low.
In Study 2, there was no reduction in total IsoP production following omega-3 supplementation. In this population of healthy young men, \( F_2\)-IsoP, \( F_2\)-IsoP-M, and total IsoP were significantly higher at 8 hours following LPS administration compared to pre-LPS values, but there was no significant difference between treatments (Figure 5-6). A potential carryover effect of EPA+DHA supplementation was found for RBC DHA (treatment by period \( p = 0.02 \)) and the change in the Omega-3 Index (treatment by period \( p = 0.007 \)) in Study 2, indicating that treatment order influenced these RBC fatty acid concentrations. Analysis of isoprostane data from period 1 only produced the same null findings for the effect of EPA+DHA supplementation on \( F_2\)-IsoP-M (\( p = 0.6 \)) and total IsoP (\( p = 0.6 \)). There was a trend towards a significant reduction in period 1 \( F_2\)-IsoP values with EPA+DHA supplementation, from 2.73 ng/mg creatinine following placebo supplementation to 2.08 ng/mg creatinine following EPA+DHA supplementation (\( p = 0.06 \)).
5.5 Discussion

To improve our understanding of the effect of EPA+DHA supplementation on the oxidative stress response to an acute inflammatory challenge, we performed secondary analyses of two omega-3 supplementation studies that employed an induced low-dose endotoxin challenge model. In both studies, low-dose endotoxemia caused a robust oxidative stress response with significant increases in the production of all urinary isoprostane compounds over the 8 hours following LPS injection, which is in accordance with previous studies [88, 266]. In a small sample of healthy young adults (Study 1), in a model controlling for BMI, higher doses of EPA+DHA supplementation (600-1800 mg/d) were predictive of attenuated oxidative stress (in terms of total isoprostane production) during acute inflammation, despite producing elevated levels of F3-isoprostranes (F3-IsoPs). In the study of healthy young men (Study 2), EPA+DHA supplementation similarly increased F3-IsoPs, but did not reduce total isoprostane production following low-dose endotoxemia.
Previous clinical trials have consistently demonstrated that \(F_2\)-isoprostane (\(F_2\)-IsoP) production is not increased by EPA+DHA supplementation [254-263], but less is known about urinary isoprostane production due to an acute inflammatory challenge. Two previous clinical studies have investigated urinary isoprostane production due to intravenous LPS administration [88, 266]. In these healthy non-smoking adults, LPS administration (3 or 4 ng LPS per kg body weight) produced significant increases in both \(F_2\)- and \(F_3\)-IsoP production 2-8 hours post-injection, with values returning to baseline concentrations in conjunction with the resolution of clinical symptoms and inflammatory cytokine production [88, 266]. Urinary isoprostane production following administration of lower doses of LPS has not previously been evaluated. In the current study populations, low-dose LPS administration (0.6 ng/kg) resulted in significant increases in urinary isoprostane production but the magnitude of this response was lower compared to that previously reported by studies using higher dose LPS administration.

To date, there has been little investigation into how the oxidative stress response and urinary isoprostane production following induced endotoxemia may be altered by EPA+DHA supplementation. Skarke et al. recently reported that low-dose endotoxemia (0.6 ng/kg) increased \(F_2\)-IsoPs but had no effect on \(F_2\)-IsoPs in healthy adults (\(n = 6\)) supplemented with 3.4 g/d EPA+DHA for 6-8 weeks [253]. However, it should be noted that these were exploratory findings based on raw data values with no statistical tests of significance. Furthermore, these changes in urinary isoprostanes are relative to pre-LPS values and not compared to a control group of individuals who did not receive EPA+DHA supplementation. To our knowledge, effects of EPA+DHA supplementation on isoprostane production due to low-dose endotoxemia have not otherwise been reported.

The discrepancy in results between Study 1 and Study 2 may be due to differences in study design (e.g., dosing, duration, and study population). Both studies used the same low-dose endotoxin challenge protocol and enrolled healthy individuals of a similar age and BMI; however, Study 1 included females while Study 2 was limited to men. We also speculate that differences in the formulation of omega-3 fatty acids and/or the duration of supplementation may be responsible for these disparities. In Study 1, omega-3 fatty acids were provided as fish body oils (i.e., triglycerides) whereas a concentrated form of EPA and DHA ethyl esters were used in Study 2. There is much debate regarding the relative bioavailability of different EPA+DHA formulations. Some have suggested that ethyl esters are less readily absorbed, but others have reported similar rates of bioavailability [310, 311]. Supplementation with EPA+DHA ethyl esters also consistently increases the omega-3 fatty acid content of RBCs [312, 313], and effectively lowers triglycerides [137, 314, 315]. Furthermore, ethyl ester fish oil concentrates are typically considered the gold standard for EPA+DHA supplementation as higher doses are easier to achieve. Although we consider it unlikely, it is possible that some aspect of the refining process may unintentionally cause undesirable alterations of the fatty acids that have not yet been identified, or that other components of fish body oils that are removed as “impurities” have beneficial effects. Direct comparisons of the physiological effects of the different formulations are lacking.

The duration of supplementation was much longer in Study 1 compared to Study 2 (5 months versus 8 weeks). An 8 week supplementation period was sufficient for significant enrichment of red blood cells with omega-3 fatty acids to occur (Chapter 4). The RBC omega-3 concentrations achieved with 3400 mg/d EPA+DHA supplementation in Study 2 were similar to the values exhibited by Study 1 participants who received 600-1800 mg/d EPA+DHA supplementation. Although there was a potential carryover effect for RBC DHA in Study 2, it is unlikely that this is responsible for the lack of effect of EPA+DHA supplementation on total IsoP. Considering period 1 data only, there was a trend towards a reduction in \(F_2\)-IsoP, but no
significant treatment effect for F₂-IsoP-M or total IsoP. Thus, the shorter duration of supplementation or the form of the omega-3 fatty acids provided in Study 2 may have influenced how the EPA and DHA were metabolized and may help to explain the lack of effect on total urinary isoprostane production following low-dose endotoxemia.

Additional research is needed to further our understanding of whether EPA+DHA supplementation can exert a beneficial effect on the oxidative stress response invoked by an inflammatory stimulus. Results from the two studies presented here suggest that the duration of supplementation and/or the form of omega-3 fatty acids may be important factors in determining the effect of low-dose endotoxemia on isoprostane outcomes. Certain participant characteristics may also influence urinary isoprostane production following an acute inflammatory challenge and should be investigated. For instance, we found an inverse relationship between BMI and total IsoP production in Study 1 participants receiving high dose EPA+DHA supplementation. This is surprising given that higher F₂-IsoP and F₂-IsoP-M concentrations are typically associated with overweight/obesity [97, 98, 308]. However, the participants in Study 1 were generally healthy young adults with an average BMI of 25 kg/m². In this study population, it is possible that a higher BMI may be due to greater muscle mass rather than being an indication of excess body weight and higher chronic disease risk. Women also tend to have higher F₂-IsoP concentrations than men [97], and concentrations of F₂-IsoP and F₂-IsoP-M have been shown to increase with age [308, 316]. Although isoprostane production did not differ by sex or age in the current analysis, this may be due to sample size limitations and the relatively young age of participants. It is also possible that other factors, such as a participant’s fasting lipid profile, CRP, level of physical activity, and sleep quality may also influence oxidative stress and urinary isoprostane production. These potential relationships warrant further exploration in future studies.

5.5.1 Study strengths and limitations

Detailed descriptions of the respective strengths and limitations of Study 1 and Study 2 have previously been discussed ([309] and Chapter 4). For the context of the current analysis, both studies used well-characterized omega-3 fatty acid supplements and compliance rates were very high. Participants for both studies were also recruited according to strict inclusion/exclusion criteria and were required to be low oily fish consumers. Moreover, the same low-dose endotoxin challenge procedures were used in both studies, minimizing the potential for variation in inflammatory and oxidative stress responses due to differences in preparation techniques. The food provided during these visits was also highly controlled within each study. All Study 1 participants received similar meals and participants in Study 2 received the same meals at each visit. However, both studies consisted of relatively small sample sizes. Furthermore, the Study 1 population included both men and women and was generally Caucasian, whereas Study 2 was composed exclusively of men but included more Asian and African American individuals. The two studies also differed in design, with Study 1 being a parallel group study in which fish body oils were provided for 5 months, whereas Study 2 was a crossover study that provided EPA+DHA ethyl esters for 8 weeks. Combined with the divergent results from the two studies, this suggests that potential duration-response effects and differences between omega-3 fatty acid formulations may exist; however, because both factors differed in the two studies, we cannot determine which of these may have influenced our findings. Additional studies are needed to further investigate the effects of these factors individually. Although these are preliminary hypothesis-generating results, they warrant further exploration in future omega-3 supplementation studies. It is unlikely that the potential carryover effect of EPA+DHA supplementation on RBC DHA in Study 2 was responsible for the lack of effect on total IsoP production and accounts for the
disparate findings in Study 1 and Study 2. These analyses were strengthened by the robust inflammatory and oxidative stress responses that were achieved in both Study 1 and Study 2. Both studies also utilized urinary isoprostane analysis, a recognized marker of in vivo oxidative stress.

5.5.2 Conclusions
Supplementing healthy young adults with omega-3 fatty acids (600-1800 mg/d) for 5 months prior to inducing low-dose endotoxemia significantly attenuated oxidative stress responses in terms of total urinary isoprostane production, despite elevations in F₃-isoprostanes. Eight weeks of supplementation with 3.4 g/d EPA+DHA similarly increased F₃-isoprostane production following low-dose endotoxemia in healthy young men, but did not alter total isoprostane production. These disparities may be due to differences in the duration of supplementation and/or the form of omega-3 fatty acids administered as supplements. Further research is needed to investigate these hypothesis-generating results and determine whether omega-3 fatty acid supplementation can attenuate oxidative stress responses to an acute inflammatory challenge.

5.6 Supplemental Results and Discussion
Visual evaluation of the distribution of total isoprostane values in this study population demonstrated that two participants had values that did not fit a normal distribution (Supplemental Figure 5-1). The total IsoP concentration of these two participants were more than two standard deviations beyond the mean value (4.5 and 0.68 ng/mg creatinine, respectively), and were excluded from the final analysis.

Regression modeling was also performed using the entire Study 1 sample population, including the two outliers (n = 19), and is presented below. These analyses produced similar findings compared to the results from the final model; however, the two participants deemed outliers were influential in the regression model, and BMI and sex became stronger predictors of total IsoP production. One of the excluded
participants, who was a healthy 25-year old woman, had the lowest BMI (BMI = \(21.1\text{ kg/m}^2\)), received high dose EPA+DHA supplementation, and had the highest total IsoP concentration. Conversely, the other excluded participant was a 41-year old man with elevated cholesterol. This participant also had the highest BMI (BMI = \(29.8\text{ kg/m}^2\)), received low dose EPA+DHA supplementation, and had the lowest total IsoP concentration. Based on these characteristics, these two participants can be easily identified as the extreme values in the following figures. There were no other remarkable features about these two participants that might explain their total IsoP concentrations. It is possible that the extremely low total IsoP concentration may be due to measurement error.

The multivariate model that best predicted total IsoP \((F_2\text{-IsoP} + F_2\text{-IsoP-M} + F_3\text{-IsoP})\) production included the dose of EPA+DHA, BMI, and sex (model \(p = 0.002\), model adjust R-squared = 62%). High dose EPA+DHA supplementation (600-1800 mg/d) decreased total IsoP production by 0.7 ng/mg creatinine \((p = 0.03)\). A significant 0.2 ng/mg creatinine decrease in total IsoP production was also found for every 1 unit increase in BMI \((p = 0.003)\). In the multivariate regression model, the two participants who were deemed outliers had leverage values of and 0.22 and 0.32, respectively. A histogram of the leverage values for all 19 participants is presented in Supplemental Figure 5-2.

![Histogram of leverage values from the multivariate regression model of all participants in Study 1.](image)

**Supplemental Figure 5-2.** Histogram of leverage values from the multivariate regression model of all participants in Study 1.

BMI was also a significant predictor of total IsoP production in a univariate model \((p = 0.015\), adjusted R-squared 26.2\%) (Supplemental Figure 5-3).
In the multivariate model, being male tended to decrease total IsoP production by 0.6 ng/mg creatinine ($p = 0.07$). The effect of EPA+DHA dose and BMI were also driven primarily by male participants (Supplemental Figure 5-4 and Supplemental Figure 5-5).
Supplemental Figure 5-5. Effect of BMI on Total IsoP production by sex.
Chapter 6. Summary and Future Directions

The studies presented in this dissertation were designed to investigate the effects of nutritional interventions on cardiovascular disease risk factors. Specifically, these studies evaluated (1) the effects of freeze dried strawberry powder on postprandial vascular function, glucose and lipid metabolism, and oxidative stress following consumption of a high-fat meal; (2) the effects of omega-3 fatty acid supplementation on erythrocyte fatty acids and inflammatory marker production following low-dose endotoxemia; and (3) the effects of omega-3 fatty acid supplementation on oxidative stress responses to low-dose endotoxemia in two study populations. This chapter aims to summarize the results of these research studies and propose additional avenues for future research based on these findings.

6.1 Plant-based bioactives

6.1.1 Summary of strawberry supplementation study

The results of our clinical postprandial supplementation study of freeze dried strawberries were presented in Chapter 3. Incorporation of 40 g of freeze dried strawberry powder into a high fat meal had no effect on any of the postprandial outcomes measured relative to the control meal, including: central blood pressure and indices of arterial stiffness; glucose, insulin, and lipid metabolism; and plasma markers of oxidative stress. This study design was based on previous findings that phytochemical-rich foods, such as strawberries, could attenuate postprandial dysmetabolism [179]. All of the analyses specified for this study have been carried out. At this time, there are no additional analyses planned for the remaining samples from this study. Despite the lack of significant postprandial findings, these results provide insight for the design of future supplementation studies of strawberries and other phytochemical-rich foods.

6.1.2 Future directions

6.1.2.1 Postprandial studies

Multiple studies have evaluated the postprandial effects of particular phytochemical-rich interventions that are provided in combination with meal, but results are inconsistent. It is likely that there is considerable inter- and intra-individual variability in the postprandial response to a meal, and this has yet to be fully quantified. Additional studies are needed to determine whether an individual’s response to the same meal is consistent over multiple occasions, and how much that response varies among a group of individuals. Furthermore, comparisons between different postprandial studies are particularly difficult due to the lack of standardized methodology for evaluating postprandial lipids responses. For instance, there is currently no standardized protocol for the design and implementation of an oral fat challenge; thus, the form and/or quantity of fat provided tends to vary among different studies. In some studies, the fat is provided as a single oral load, such as dairy cream [317]. However, it is also common to provide the fat in a meal, with the entire meal providing a specified amount of fat. With regard to high-fat meals, there also tends to be additional variation between studies in other aspects of the meal such as total calories, type/source of fat, and carbohydrate content. Furthermore, normative values for postprandial triglycerides are not well-defined [21]. Thus, future research would benefit from the development of a standard oral fat challenge model, with clinical reference standards that can be used to define risk and therapeutic strategies.

Future postprandial strawberry supplementation studies should also be conducted using a test meal with a different nutrient profile. The test meal used in the current study was designed to have a high fat content, in order to achieve a robust postprandial triglyceride response. A high-fat meal is commonly used
in postprandial nutrition research and many studies have demonstrated attenuated triglyceride responses when a phytochemical-rich intervention is provided with the meal [169, 179]. However, the majority of postprandial studies of berries have focused on the glycemic response to a sucrose load or a high-carbohydrate meal. These studies indicate that including different types of berries with a carbohydrate load can improve glucose and insulin responses [176-178]. Therefore, future strawberry research should use a similar postprandial study design with high-carbohydrate meal to determine if a single dose of freeze dried strawberry powder can improve glucose and insulin responses.

Impaired lipid absorption, via inhibition of digestive lipase enzymes, is one of the potential mechanisms by which particular phytochemical-rich foods/spices may attenuate exaggerated postprandial triglyceride responses. Previous work by our research group found that the spice blend used by Skulas-Ray et al. [169] exhibited potent pancreatic lipase inhibition in vitro. Conversely, the freeze dried strawberry powder used in the current study demonstrated no inhibitory effect on pancreatic lipase using the same in vitro model. This may explain the null findings of the present study, but previous work has demonstrated that strawberry extracts can inhibit pancreatic lipase [208]. Furthermore, our freeze dried strawberry powder was very similar to that used by Burton-Freeman et al. [179], which modestly attenuated postprandial triglycerides. Pancreatic lipase inhibition assays could be performed with commercially available strawberry powders to evaluate the inhibitory activity of these products and determine whether there is any variation among them. Testing the ability of other phytochemical-rich foods to inhibit digestive lipases would add to our understanding of which foods have the potential to exert the greatest hypolipemic effects when they are consumed in conjunction with a fat-containing meal. We posited that foods with more hydrophilic antioxidant content compared to lipophilic antioxidant content may be less effective inhibitors of lipid absorption, and thus have less potent effects on postprandial triglyceride elevations. Additional in vitro analyses are needed to investigate this hypothesis. These results would help to identify the dietary interventions with the most efficacy in a lab-based setting, and this could then be translated to inform to the design of future clinical trials.

Previous postprandial studies have also demonstrated that the consumption of a meal may transiently improve central blood pressure and reduce arterial stiffness (Table 6-1). For instance, in healthy adults, consumption of a light breakfast (~330 kcal and 10 g fat) significantly reduced central systolic blood pressure, central diastolic blood pressure, and the AIx compared to water alone [279]. A high-fat meal has been shown to produce similar effects [280-282], although some have found no change [318] or an increase in central systolic blood pressure [319]. Reductions in arterial stiffness following a meal may be due to postprandial changes in blood flow that direct blood towards the digestive organs with compensatory reductions in flow to other organs [320]. It has also been proposed that postprandial reductions in arterial stiffness may be due in part to the vasodilatory effects of insulin [280], which is elevated following meal consumption. For instance, in insulin-sensitive postmenopausal women, a greater reduction in the AIx was observed following a high-carbohydrate high-fat meal compared to a low-carbohydrate high-fat meal [280]. Conversely, insulin-resistant postmenopausal women in this study experienced similar AIx responses following both meals, suggesting that postprandial arterial relaxation may, in part, depend on insulin sensitivity [280]. Unfortunately, the authors did not report whether the insulin-resistant individuals had a reduced AIx response following meal consumption compared to insulin-sensitive individuals, and this could not be inferred from the visual representations of the data. A study of healthy men that used the hyperinsulinemic-euglycemic clamp technique provided further evidence that insulin, rather than glucose, is the primary factor influencing postprandial arterial stiffness. In this study,
Alx was significantly reduced within 1 hour of insulin administration [283]. In our strawberry study, vascular function measurements were timed to coincide with the peak polyphenol and triglyceride concentrations, which occur at 2 and 4 hours following meal consumption, respectively. However, peak insulin concentrations tend to occur much earlier (i.e. 30 minutes to 1 hour) in the postprandial period, and insulin concentrations have largely declined by two hours after the meal. Nonetheless, there was a trend towards a correlation between the change in Alx and insulin at two hours (p = 0.1). In future studies, vascular function measurements should also be performed at 30 minutes and 1 hour following meal consumption, when insulin concentrations are highest. This will provide further insight into the relationship between postprandial insulin concentrations and indices of arterial stiffness.

However, it is also possible that insulin may have a paradoxical effect whereby elevated insulin concentrations are acutely beneficial but have long-term deleterious effects on vascular function. Analogously, while acute elevations in insulin improve glucose concentrations, over time, elevated fasting glucose and insulin often occur together. Multiple studies have found an association between greater arterial stiffness and elevated fasting insulin concentrations [321] or insulin insensitivity [322, 323]. This would suggest that although acute, meal-induced elevations in insulin improve vascular function, sustained hyperinsulinemia and resulting insulin resistance could contribute to arterial stiffness. Therefore, postprandial relationships between insulin and vascular function should be interpreted in the context of potential long-term detrimental effects on arterial stiffness. Additional studies are needed to further investigate the effect of insulin sensitivity on postprandial vascular responses.
Table 6-1. Studies evaluating postprandial effects of meal consumption on central (aortic) pressures, augmentation index (AIx), and pulse wave velocity (PWV).

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study design</th>
<th>Study population</th>
<th>Test meal</th>
<th>Control and/or comparator</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taylor, 2014 [324]</td>
<td>Pre-post</td>
<td>Healthy adults (n = 17)</td>
<td>Liquid mixed meal (Ensure; 40% of each participant’s daily REE; mean kcal of 661)</td>
<td>No control</td>
<td>Meal consumption ↓ central pressures at 3 h, ↓ AIx, AIx@75, AP, and PWV at 1 h and 3 h (compared to baseline)</td>
</tr>
<tr>
<td>Lithander, 2013 [319]</td>
<td>Crossover</td>
<td>Healthy men (n = 20)</td>
<td>High-fat meal [strawberry-flavored milkshake] enriched in MUFA (35 g oleic acid from olive oil) (~700 kcal, 56 g fat)</td>
<td>High-fat meal [strawberry-flavored milkshake] enriched in SFA (palmitic acid from dairy cream) (~700 kcal, 56 g fat)</td>
<td>No differential effect of fat type cSBP ↑ from baseline AIx@75 ↓ from baseline PWV ↑ 0.3 m/s from baseline over 4 hours</td>
</tr>
<tr>
<td>Phillips, 2010 [282]</td>
<td>Crossover within parallel arm subgroups</td>
<td>Lean (n = 8), obese (n = 10), and type 2 diabetic (n = 10) men</td>
<td>High-fat meal [bacon and egg muffin, 2 hash browns, caramel-flavored milk drink] (~990 kcal, 57.5 g fat [19.8% SFA], 35 g protein, 83 g CHO)</td>
<td>Water</td>
<td>AIx ↓ following high-fat meal in all 3 groups (unchanged after water control) Smaller ↓ in AIx in obese group AIx ↓ prolonged in men with T2DM Correlation between iAUC AIx and iAUC TG</td>
</tr>
<tr>
<td>Ayer, 2010 [318]</td>
<td>Pre-post with parallel arm subgroups</td>
<td>Obese (n = 11) and normal weight (n = 11) adults</td>
<td>High-fat meal [slice of carrot cake and a milkshake] (1000 kcal, 60 g fat)</td>
<td>No control</td>
<td>No significant changes Trend for ↓ in AIx@75 (p = 0.06) after meal in both groups</td>
</tr>
<tr>
<td>Ahuja, 2009 [279]</td>
<td>Crossover</td>
<td>Healthy adults (n = 35)</td>
<td>Light breakfast [2 slices whole-meal toast, 10 g margarine, 27 g berry jam, and 350 mL water] (~310 kcal)</td>
<td>Water (350 mL, room temperature)</td>
<td>Food consumption ↓ cSBP (7%), cDBP (8%), cPP, AP, and AIx (27%)</td>
</tr>
<tr>
<td>Berry, 2008 [281]</td>
<td>Crossover</td>
<td>Healthy men (n = 17)</td>
<td>Stearic acid-rich meal (shea butter blend) [2 muffins and milkshake, providing ~850 kcal, 50 g fat, 15 g protein, 89 g CHO]</td>
<td>Oleic-acid rich meal (high oleic sunflower oil) [2 muffins and milkshake, providing ~850 kcal, 50 g fat, 15 g protein, 89 g CHO]</td>
<td>Both meals ↓ PWV similarly</td>
</tr>
<tr>
<td>Greenfield, 2007 [280]</td>
<td>Crossover with parallel sub-group analyses</td>
<td>Insulin-sensitive post-menopausal women (n = 7)</td>
<td>High-CHO and high-fat meal (~1385 kcal, 88 g fat, 37 g protein, 104 g CHO)</td>
<td>Low-CHO and high-fat meal (~1084 kcal, 85 g fat, 45 g protein, 23 g CHO)</td>
<td>Alx@75 ↓ in both groups after both meals</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>-------------------------------------------------</td>
<td>-------------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Insulin-resistant post-menopausal women (n = 7)</td>
<td></td>
<td></td>
<td>• Insulin-sensitive: ↓ in Alx@75 greater following high-CHO high-fat meal (vs. low-CHO meal)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Insulin-resistant: same response following both meals</td>
</tr>
</tbody>
</table>

Abbreviations: Alx, augmentation index; Alx@75, augmentation index standardized to a heart rate of 75 beats per minute; AP, augmentation pressure; cDBP, central diastolic blood pressure; CHO, carbohydrate; cPP, central pulse pressure; cSBP, central systolic blood pressure; iAUC, incremental area under the curve; MUFA, monounsaturated fatty acid; PWV, pulse wave velocity; REE, resting energy expenditure; SFA, saturated fatty acid; T2DM, type 2 diabetes mellitus; TG, triglycerides.

1Increase in PWV no longer significant after adjusting for the increase in mean arterial pressure.
6.1.2.2 Strawberry supplementation studies

Additional research is needed to determine whether including a phytochemical-rich intervention with a meal can provide additional benefits for postprandial vascular function. Although we detected no effect of strawberries on postprandial arterial stiffness, a spinach-containing meal has been shown to decrease the AIx at three hours compared to a control meal [158]. To our knowledge, no other dietary bioactives have been tested for their effects on postprandial arterial stiffness using a study design in which the intervention is provided in conjunction with a meal and compared to the same control meal. It is possible that the vascular effects of different foods may depend on the unique phytochemical profile of each food. For instance, the vascular benefits of the spinach meal were attributed to nitrate, and strawberries contain much less nitrate compared to spinach [325-327]. Therefore, additional studies of postprandial vascular function are needed in which a phytochemical-rich food is incorporated into the test meal and compared to a matched control meal.

Longer periods of strawberry supplementation, rather than a single dose, may also be needed for beneficial effects on CVD risk factors. Previous studies indicate that regular strawberry consumption can improve lipids/lipoproteins and measures of oxidative stress, but additional research is needed to clarify these effects. For instance, daily consumption of strawberries for weeks to months has been shown to improve LDL-C; however, the degree of LDL-lowering achieved with different doses of strawberry consumption remains unclear. For instance, reductions in LDL-C have been demonstrated with doses of 10-50 g/d [216, 225, 227, 284]. However, a 10 g/d dose of FDS powder provided for 6 weeks had no effect on fasting lipids in overweight hyperlipidemic adults [179]. Furthermore, little research has examined dose-response effects. One parallel arm study of adults with abdominal adiposity and dyslipidemia compared 12 weeks of supplementation with 25 g/d to 50 g/d, and found that only participants in the 50 g/d group experienced significant reductions in total cholesterol and LDL-C compared to controls [227]. Similar dose-response questions also remain for oxidative stress outcomes. One to two months of strawberry supplementation has previously been demonstrated to significantly reduce MDA [216, 227, 228], TBARS [271], and urinary isoprostanes [228]. However, these effects have been achieved primarily with higher doses, such as 50 g/d of FDS powder [216, 227] or similar amounts of fresh strawberries [218, 228]. No effect on lipid peroxidation was found following 6 weeks of supplementation with 10 g/d of FDS powder [179]. In the parallel arm dose-response study, both the 25 g/d and 50 g/d dose of FDS powder significantly reduced MDA compared to controls [227]. No crossover dose-response studies have been published, and further research is needed to determine the amount of strawberries required to optimize effects on clinical outcomes.

Little is known about the effect of dietary plant-based bioactive interventions on arterial stiffness indices such as AIx and PWV following longer-term supplementation. The majority of studies have used soy or soy-based isoflavones, but other interventions have also been used, including garlic [328], spinach [329] and other dark leafy greens [158], grapefruit juice [330], blueberries [331, 332], tart cherry juice [333], and cocoa [334]. A systematic analysis of dietary interventions on arterial stiffness that was conducted in 2010 concluded that soy isoflavones were the only plant-based dietary intervention that effectively reduced arterial stiffness [273]. This conclusion was based on five studies, three of which reported a reduction in PWV [335-337]. However, it should be noted that one of these studies reported femoral-dorsalis PWV [335] rather than the gold standard carotid-femoral PWV measurement [148, 151]. With respect to studies that have been conducted since this review, some have found beneficial effects on AIx and/or PWV, but results have been mixed. Most interventions have been tested in a single study and
results remain insufficient to draw a firm conclusion regarding particular foods. Notably, no studies have evaluated the effect of longer term strawberry supplementation on these indices of arterial stiffness. Observational evidence suggests there may be a protective relationship between berry consumption and vascular function, but intervention studies are needed to determine if a causal relationship exists.

6.2 Omega-3 fatty acids

6.2.1 Summary of omega-3 supplementation studies

The results of our clinical study investigating the effects of 3.4 g/d EPA+DHA supplementation in a model of induced low-dose endotoxemia are presented in Chapters 4 and 5. Eight weeks of supplementation resulted in significant incorporation of omega-3 fatty acids in erythrocytes, but did not alter inflammatory cytokine production or oxidative stress responses following low-dose endotoxemia in healthy young men. Although there is a large body of pre-clinical evidence to indicate that omega-3 fatty acids may have beneficial effects on the inflammatory response, these findings have yet to translate to consistent results in clinical studies. Further research is needed to determine the mechanisms by which omega-3 fatty acids may improve the immune response to an inflammatory challenge. The potential carryover effect observed for RBC DHA and the Omega-3 Index indicates that future omega-3 supplementation studies with a crossover design should employ longer washout periods (> 8 weeks) in between supplementation periods.

6.2.2 Future directions

With regard to the existing samples, lipid mediator analyses are currently ongoing. These results may offer greater insight into the role of EPA and DHA in the resolution phase of inflammation. Plasma samples are being analyzed by the Wayne State University Lipidomics Core Facility using a LC/MS-based targeted lipidomics approach. A broad spectrum of EPA and DHA metabolites, including specialized pro-resolving mediators (SPMs), can be analyzed by these methods. The role of SPMs in the resolution of inflammation has been clearly demonstrated in both cell- and animal-based studies. However, little is known about the role of SPMs in human physiology. Some studies have detected resolvins and protectins in human plasma following EPA+DHA supplementation, but results are mixed [238]. It remains to be determined whether SPMs are produced endogenously in human plasma in quantifiable amounts, and if so, whether these quantities are sufficient to exert similar pro-resolving actions as those observed in cell and animal studies that typically use much higher doses. Thus, if EPA+DHA supplementation increases the production of SPMs following low-dose endotoxemia, this may be a mechanism by which omega-3 fatty acids could exert anti-inflammatory effects.

Plasma cortisol concentrations following low-dose endotoxemia could also be measured. Relatively little is known about the stress response to induced endotoxemia and whether it is modified by EPA+DHA supplementation. In a previous study, cortisol production following a higher dose of LPS (2 ng/kg) was unchanged by intravenous administration of a lipid emulsion enriched with EPA+DHA [298]. However, in a study of healthy young men, 3-4 weeks of oral supplementation with 1.8 g/d of EPA+DHA significantly reduced peak cortisol production by 30-40% following 2 ng/kg LPS administration [299]. Furthermore, this reduction in cortisol was found in the absence of any significant changes in inflammatory markers [299]. The cortisol response to low-dose endotoxemia, and the effect of EPA+DHA supplementation, has yet to be evaluated. However, based on the findings of Michaeli et al. [299], it is possible that the higher of dose of EPA+DHA used in the current study may have similarly reduced cortisol production with no alteration in acute cytokine and CRP production. If EPA+DHA supplementation can modulate cortisol production in response to an inflammatory stimulus, this would suggest that an attenuated stress response may be a
mechanism by which omega-3 fatty acids influence inflammation. Mood, psychosocial, and lifestyle questionnaires were also completed by participants following both low-dose endotoxin challenges and will be analyzed in the future. In a previous omega-3 supplementation study conducted by our research group, regression analyses identified subjective sleep quality during the past week as the best predictor of peak CRP responses following low-dose endotoxemia (unpublished data). Certain physical activity parameters, such as days of walking and days of moderate physical activity, were also significant predictors of peak CRP responses (unpublished data). Analysis of the questionnaire responses from the current study may provide valuable insight into predictors of inflammatory and/or oxidative stress responses. The current study was conducted in a similar population of healthy young men; thus, if similar sleep and/or physical activity parameters are significant predictors of peak CRP and/or cytokine concentrations, this would provide further support for their potential role in inflammatory responses. The psychosocial questionnaires may also provide valuable insight into stress responses, and correlations between self-reported responses and plasma cortisol concentrations should be analyzed.

Additional studies of omega-3 fatty acids using a human model of induced low-dose endotoxemia may also be warranted. Although we and others [254] have found no attenuation of inflammatory responses to low-dose endotoxemia following oral EPA+DHA supplementation, beneficial effects have been found with intravenous administration of a lipid emulsion enriched with EPA+DHA [297, 298]. Thus, the mode of omega-3 fatty acid delivery may be a key factor in modulating inflammatory responses. The current study was also conducted exclusively in healthy young men. Women generally exhibit a more pronounced inflammatory response to infection compared to men [338, 339], but sex differences in studies of induced endotoxemia have been less consistent [305, 306]. It has also been suggested that certain ethnicities may experience more robust inflammatory responses [306], but current evidence is limited. Therefore, additional studies of women and other populations at-risk of excessive inflammation, as well as other ethnicities, are warranted. However, current United States Food and Drug Administration regulations limit the use of experimental endotoxemia to non-smoking individuals who are relatively young (< 45 years), healthy, and non-obese. The 3.4 g/d dose used in our study is the pharmacological dose used to treat very high triglycerides, but it is possible that a higher dose may be needed to exert effects on the inflammatory response. However, future studies will need to consider the greater likelihood of potential side effects when using higher doses of fish oil. Furthermore, higher doses may be more difficult to translate into public health messages. The duration of supplementation may also be a key factor in determining whether inflammatory outcomes are modified. For instance, in the previous omega-3 supplementation study conducted by our research group, a 5 month supplementation period was used. In univariate analyses, there was a trend for a reduction in the peak CRP response to low-dose endotoxemia in the high-dose (600, 900, and 1800 mg/d EPA+DHA) supplementation group compared to the low-dose group (0 and 300 mg/d EPA+DHA) (p = 0.12; unpublished data). In this previous study, EPA+DHA was also provided in the form of natural fish body oils whereas the current study used EPA and DHA ethyl esters. There has been much debate regarding potential differences in the bioavailability of these different EPA+DHA formulations [310, 311, 340, 341]. Supplementation with EPA+DHA ethyl esters is typically considered the gold standard for fish oil supplementation, and consistently increases the omega-3 fatty acid content of RBCs [312, 313] and lowers triglycerides [137, 314, 315]. However, it is possible that some aspect of the refining process may unintentionally cause undesirable alterations of the fatty acids that have not yet been identified, or that other components of fish body oils that are removed as “impurities” have beneficial effects. If so, this could influence effects on inflammatory and oxidative stress responses. Direct comparisons of the physiological effects of the different formulations are lacking and additional studies
are needed to compare the effects of fish body oils and ethyl esters on inflammatory responses to low-dose endotoxemia.

Due to the lack of consistent effects on inflammatory markers in clinical trials of omega-3 fatty acids, many other mechanisms have been investigated as potential physiological pathways by which EPA and DHA may influence inflammation, such as oxidative stress. Numerous studies have demonstrated that omega-3 fatty acid supplementation does not increase the oxidative stress, as measured by F$_2$-IsoPs [254, 255], and in some cases, may reduce F$_2$-IsoP production [257, 258, 262, 263]. Previous analyses have found no correlation between reductions in F$_2$-IsoPs and changes in plasma [257] or RBC [260, 263] fatty acid content caused by omega-3 supplementation. Thus, as isoprostanes are formed non-enzymatically while the fatty acid is still attached to phospholipid membrane [86], the mechanism by which omega-3 fatty acids reduce oxidative stress and F$_2$-IsoP production remains unclear. It has been hypothesized that the putative anti-inflammatory actions of omega-3 fatty acids may, in part, be responsible. For instance, activated leukocytes release ROS as part of the immune response to an infection; thus, if omega-3 fatty acids can help to regulate this pro-inflammatory response, this could prevent over-production of ROS and oxidative stress. Furthermore, in individuals with type 2 diabetes and treated hypertension, the reduction in F$_2$-IsoPs that was found following 6 weeks of supplementation with 4 g/d of EPA or DHA was associated with the change in TNF-α [264]. Results reported in Chapter 5 also suggest that certain participant characteristics (e.g., BMI, age, sex, CRP, fasting lipid/lipoprotein concentrations, level of physical activity, sleep quality, etc.) may also influence oxidative stress responses and urinary isoprostane production. Epidemiological studies have demonstrated an association between traditional CVD risk factors, such as BMI and hypercholesterolemia, and elevated F$_2$-IsoP concentrations and these findings warrant further exploration in future studies utilizing the low-dose endotoxemia model. Therefore, additional research is needed to better understand the mechanism by which omega-3 fatty acids reduce oxidative stress and determine whether this is linked to inflammatory processes.

Future studies of omega-3 supplementation using the low-dose endotoxin model should also include measurements of arterial stiffness and heart rate variability following LPS-injection. To our knowledge, arterial stiffness responses to low-dose endotoxemia have not been evaluated. However, higher plasma and RBC omega-3 content has been associated with lower PWV [342, 343], and omega-3 supplementation has been shown to reduce arterial stiffness [273], particularly in older adults [344]. Beneficial effects have not generally been found in younger adults [344], who tend to have relatively more elastic arteries. It is likely that an acute inflammatory stimulus would cause transient increases in arterial stiffness in healthy young adults, and this would provide the opportunity to evaluate whether omega-3 fatty acids can modulate these changes in the vasculature. Furthermore, heart rate variability—a measure of autonomic influence on cardiac function that has been associated with CVD risk [345, 346]—may also be improved by omega-3 fatty acid supplementation [347]. This has been proposed as one of the potential mechanisms by which omega-3 fatty acids protect against arrhythmias [348, 349]. Reduced heart rate variability at rest that persists following an acute psychological stressor is associated with increased risk of hypertension [350, 351] and greater coronary artery calcification [352]. EPA+DHA supplementation may improve heart rate variability following an acute stressor. For instance, Sauder et al. demonstrated that 8 weeks of supplementation with 3.4 g/d EPA+DHA improved heart rate variability during an acute psychological stressor [353]. A decrease in heart rate variability is a key component of the response to endotoxemia [354, 355]. In a parallel arm study of 16 healthy men (8 per group), intravenous administration of a lipid emulsion enriched in omega-3 fatty acids did not significantly alter heart rate variability responses to LPS
administration (2 ng/kg) [297]. However, no other studies of omega-3 supplementation and induced endotoxemia have reported effects on heart rate variability. Thus, based on previous findings for resting heart rate variability and the study of induced stress responses by Sauder et al., further assessment of the effect of omega-3 supplementation on heart rate variability reductions following low-dose endotoxemia are warranted.

Although we found no effect of omega-3 fatty acid supplementation, low-dose administration of LPS induced a robust, resolving inflammatory and oxidative response. This model of induced inflammation is highly controlled, well-characterized, and produces consistent elevations in inflammatory markers among participants. This is in contrast to other established models of inflammation (e.g., suction blisters, strenuous exercise, and psychological stress), which typically produce more variable responses between individuals [37]. We also saw no evidence of habituation with the administration of two doses over the course of at least 16 weeks. This provides further support for the utility of the low-dose endotoxemia model in evaluating induced inflammatory responses. This model could be used to test other dietary bioactive interventions for which there is pre-clinical evidence that indicates potential effects on inflammatory responses.

Future research should also examine the effects of individual long-chain omega-3 fatty acids on inflammatory markers. Previous research indicates that EPA and DHA are metabolized differently, and this may influence how these fatty acids modify CVD risk factors, such as inflammation. For instance, plasma EPA appears to be a more dynamic and readily available pool of long-chain omega-3 fatty acids that increases and decreases more quickly than DHA [10,42,43]. There is also likely cell/tissue specificity for omega-3 fatty acid storage as DHA is enriched in myocardial and neuronal membranes [50] and the EPA content of RBCs is lower than that of DHA [18,26,43,48]. EPA and DHA also have differential effects on CVD risk factors, such as HDL and LDL particle characteristics [356-358]. With regard to inflammatory markers, EPA and DHA have been shown to have specific effects on particular pro-inflammatory cytokines in cell-based models [36]. Thus, it is likely that EPA and DHA may have differential effects on inflammatory markers in a clinical setting. If EPA or DHA is more effective for reducing a particular inflammatory marker, this would have important implications for clinical outcomes and the development of more precise therapies and dietary guidelines for inflammatory related diseases.

6.3 Conclusions
These studies were designed to investigate dietary bioactives and CVD risk factors, specifically: 1) the postprandial effects of a single dose of freeze dried strawberries on arterial stiffness and postprandial dysmetabolism following a high-fat meal, and 2) the effects of omega-3 fatty acids on inflammatory and oxidative stress responses to a low-dose endotoxin challenge. Neither the strawberry intervention nor the omega-3 fatty acid intervention demonstrated a significant effect on the specified intermediate CVD risk factors. However, these null findings further our understanding of these interventions and will aid in the development of future clinical intervention studies of dietary bioactives.
Bibliography


118


VITA

Chesney K. Richter

EDUCATION

The Pennsylvania State University
   Dual-title Ph.D., Nutritional Sciences and Clinical & Translational Sciences 2016

Juniata College
   B.S., Biology 2013

PUBLICATIONS


Skulas-Ray A, Flock M, Richter C, Harris W, West S, Kris-Etherton P. Red Blood Cell Docosapentaenoic Acid (DPA n-3) is Inversely Associated with Triglycerides and C-reactive Protein (CRP) in Healthy Adults and Dose-Dependently Increases Following n-3 Fatty Acid Supplementation. *Nutrients* 2015;7(8):5291.


Richter CK, Skulas-Ray AC, Lambert JD, Proctor DN, Kris-Etherton PM. Incorporating freeze dried strawberry powder into a high fat meal does not alter postprandial vascular function or blood markers of cardiovascular risk. In preparation.

AWARDS

**University Graduate Fellowship**, The Pennsylvania State University 2013-2014

**NIH Clinical and Translational Sciences Fellowship**, The Pennsylvania State University 2014-2016

**Grace M. Henderson Graduate Scholarship**, The Pennsylvania State University 2015