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OMEGA-3 POLYUNSATURATED FATTY ACIDS: IMPACT ON OXIDATIVE STRESS, ANTIOXIDANT RESPONSES AND VASCULAR FUNCTION

BY

KAYLA R. ZEHR

B.S., Biology-Chemistry, Manchester University, 2012

DISSERTATION

Submitted in Partial Fulfillment of Requirements for the Degree of

DOCTOR OF PHILOSOPHY
BIOMEDICAL SCIENCES

The University of New Mexico
Albuquerque, New Mexico

MAY, 2018

DEDICATION

I would like to dedicate this work to my parents, Sam and Mary Lou Zehr. Thank you for instilling in me the constant drive to do what it takes to accomplish my goals, and to do it whole-heartedly and with integrity. Where there is a will, there is a way, and we did it! Your love, guidance and support has meant everything to me.

I would also like to dedicate this work to my significant other, Cory LaMothe, who has continued to be my rock the past 5 years. Through the many difficulties I have faced, you have always been there to support and encourage me, even in the darkest of moments. I cannot express in words how much your support and love has meant to me.

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Omega-3 Polyunsaturated Fatty Acids: Impact on Oxidative Stress, Antioxidant Responses and Vascular Function

By

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ABSTRACT

Cardiovascular disease (CVD) is the leading global cause of death, and cigarette smoking (CS) is a major risk factor for CVD. Although there are smoking cessation programs that include a combination of pharmacological and behavior treatments, failure to curb behavioral habits and pharmacological addiction leads to low cessation success rates. Thus, developing a treatment to decrease CVD risk is critical. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) decrease oxidative stress and have been shown to restore endothelial function, an indicator of CV health, in cigarette smokers. Metabolites of n-3 PUFAs have been shown to have CV-protective effects, but the mechanism by which n-3 PUFAs or their metabolites restore endothelial function and increase antioxidant capacity has not been fully elucidated.

The goal of this project is to determine the antioxidant mechanism by which n-3 PUFAs or their metabolites increase antioxidant responses. The long term goals of this project are to investigate the relationships between n-3 PUFAs, their metabolites and antioxidant markers, oxidative stress and vascular function.

Previous studies have shown increases in Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) expression with n-3 PUFA eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA). In order to investigate the specific mechanism through which n-3 PUFAs

activate Nrf2, we treated an antioxidant response element (ARE) luciferase plasmid stably-transfected hepatocyte cell line with EPA and DHA, their epoxide metabolites, and cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 metabolism inhibitors. We made the observations that only DHA activated Nrf2, and that inhibiting COX, LOX or P450 pathways further increased Nrf2 activation. Upon further investigation, we discovered oxidation products in our DHA stock that were not present in newer stocks of DHA, and when assessed, these oxidation products included ketones. Only one ketone, 17-oxo-4(Z),7(Z),10(Z),13(Z),15(E),19(Z)-DHA (17-oxo-DHA), is commercially available; we found that 17-oxo-DHA activates Nrf2, increases Nrf2 localization to the nucleus in human umbilical vein endothelial cells (HUVECs), and increased downstream antioxidant genes including heme-oxegenase-1 (Hmox1), glutamate cysteine ligase catalytic subunit (Gclc), and NADPH dehydrogenase quinone 1 (Nqo1). These results suggest that DHA oxidation increases antioxidant responses.

In order to investigate the relationship between vascular function, n-3 PUFAs and their metabolites and antioxidant markers, we conducted flow-mediated dilation in subjects between 19 and 50 yrs old. We found that an omega-3 index (RBC% eicosapentaenoic plus docosahexaenoic acids [EPA+DHA]) $\geq$ 4.5% was associated with higher FMD, but not lower oxidative stress. Conjugated linoleic acid (CA), palmitic acid (PA), and the ratio of linoleic acid diol-to-epoxide metabolites were predictive of MDA; 14,15-epoxyeicosatetraenoic acid (EET) and 10,17-dihydroxydocosahexaenoic acid (DiHDHA) were predictive of 8-epi-$PGF_{2\alpha}$; and oleic acid (OA), 16,17-dihydroxydocosapentaenoic acid (DiHDPA), and eicosenoic acid (EA) were predictive of

FMD. These results suggest that n-3 PUFAs may be vasoprotective in young healthy smokers, but other dietary fatty acids may also be beneficial.

These studies contribute to the understanding of antioxidant mechanisms of n-3 PUFAs, in particular DHA, and how DHA may protect against oxidative stress present in CS-induced CVD. Furthermore, these findings contribute to the development of therapeutic treatment to decrease CVD risk in smokers.

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Introduction

Cigarette smoke in cardiovascular disease

Global and national impact

Cardiovascular disease (CVD) is the leading global cause of death, contributing to over 17 million deaths annually. The broad term of CVD refers to disorders of the heart and blood vessels, including hypertension, stroke, heart attack, peripheral artery disease, heart failure and rheumatic heart disease. Risk factors for CVD include high blood pressure, diabetes, high cholesterol, and having a family history of CVD (Fryar, Chen, and Li 2012), and there are some risk factors for CVD that come with lifestyle choices such as harmful use of alcohol, physical inactivity and tobacco use (World Health Organization 2014; Roth et al. 2015). Cigarette smoke (CS) is a risk factor for increased mortality from ischemic stroke, peripheral artery disease, coronary heart disease, atherosclerosis and other chronic diseases (Mozaffarian et al. 2014; Ockene and Miller 1997; US Department of Health and Human Services 2014). Vascular remodeling occurs in smokers, causing the intimal layer of the vessels to thicken, creating a prothrombotic environment with increased plaque formation and extracellular matrix degradation. This process stiffens the vessels and decreases vascular reactivity, thereby contributing to CVD development (Messner and Bernhard 2014).

Globally, tobacco use is responsible for 6 million deaths per year, which includes 600,000 deaths due to second-hand smoke, and is projected to cause over 8 million deaths by 2030 (World Health Organization 2011). While overall tobacco use is decreasing, smoking has increased in males living in Eastern and Southeastern Asia, males and

females in the Eastern Mediterranean region, and males living in Western and Southern coasts of Africa (World Health Organization 2015). In the United States, CS is the second leading cause of death; it is responsible for over 480,000 deaths every year, which includes over 41,000 deaths resulting from secondhand smoke exposure.

Limitations of smoking cessation therapy

Former smokers have reduced risk of CVD following cessation (Vollset, Tverdal, and Gjessing 2006; Goldenberg et al. 2003). Cessation programs include a combination of pharmacological and behavioral treatments; behavioral treatments include telephone quit lines and face to face counseling with a health care provider (Rumberger, Hollenbeak, and Kline 2010). Pharmacological treatments include the antidepressant Bupropion and the nicotinic receptor partial agonist Varenicline to lessen cravings and withdrawal symptoms (Wilkes 2008; Fagerstrom and Hughes 2008). Pharmacological treatment also includes nicotine replacement therapy (patches, lozenges or gum). Unfortunately, these pharmaceutical options have negative side effects: Bupropion and Varenicline can cause anxiety, depression and negative CV events such as irregular heartbeat and hypertension, while nicotine replacement therapy can cause sinus cavity irritation.

While electronic cigarettes were originally advertised as a possible path to cessation, new studies have shown that sales of electronic cigarettes have rapidly increased since 2007 and that a greater percentage of young people are using them. Over two million high school students and 620,000 middle school students were found to have used an electronic cigarette at least once in 2015 (US Surgeon General 2016). Thus,

electronic cigarettes are not consistent in increasing cessation. Furthermore, the liquid used in electronic cigarettes includes propylene glycol and glycerin that become oxidized and can cause increased ROS products (Lerner et al. 2015). In one study of 700 participants were randomly assigned to a group that gradually reduced their smoking habits over two weeks, or a group that quit abruptly; the group assigned to quit abruptly was significantly more successful at smoking cessation compared to the group that gradually reduced their smoking over time. Thus the most ideal way to quit smoking may be to quit abruptly with no preparation (Lindson-Hawley et al. 2016).

Among current smokers, 70% have expressed that they want to quit, and 40% attempt to quit every year; however, only 4-7% of smokers are successful in the endeavor of quitting for at least one year (World Health Organization, 2008; Fiore et al. 2008). It is difficult for smokers to quit, as nicotine is pharmacologically addictive (Office of Public Affairs 1998). Nicotine mimics acetylcholine by increasing dopamine efflux and exciting dopaminergic neurons, which causes a positive feedback loop by stimulating the reward circuit in the brain (Koob and Nestler 1997). People also have reinforcing social rituals surrounding CS, which makes it difficult to curb behavior; there are also public social pressures—cigarette advertising expenses totaled $8.49 billion in 2014 (Federal Trade Commission 2015). Smoking-related illness in the United States costs over $300 billion each year (US Department of Health and Human Services 2014), thus finding pharmacological treatment is vital to lowering CVD risk in smokers.

Cigarette smoke composition

The complex mixture of CS contains over 7,000 carcinogenic and toxic chemicals (US Department of Health and Human Services 2010). These chemicals are absorbed in varying amounts and affect the smoker and surrounding people in different ways. Mainstream CS is smoke that is directly inhaled by the smoker, secondhand CS is smoke from the lighted end of the cigarette and the exhaled mainstream smoke, and thirdhand smoke is residual chemicals left on indoor surfaces by CS. These chemicals are produced from combustion of the compounds in the tobacco plant leaves, pesticides and fertilizers used on the tobacco plants and the cigarette paper that encases the tobacco. While some compounds, such as nicotine, are naturally-occurring, other compounds, such as ammonia, are added to the tobacco leaves by tobacco companies to increase the shelf life and improve nicotine bioavailability (such as glycerol and glycol) (Klus, Scherer, and Müller 2012).

Mainstream CS is divided into the total particulate matter or solid phase, and the gas phase. Total particulate matter excludes all vapors and gases of CS, and can be trapped in a fiber filter that traps smoke particles larger than 0.3 μM. These components that pass through the filter constitutes the gas phase (Hatzinikolaou et al. 2006). Total particulate matter, also commonly known as tobacco residue or tar, contains carcinogenic and mutagenic compounds such as polycyclic aromatic hydrocarbons (PAHs); thus, the total particulate matter phase is associated with multiple mouth, throat, esophageal and lung cancers (Calafat 2004; U.S. Department of Health and Human Services 2010). These PAHs in CS can also be metabolized by cytochrome P450s into reactive radical cations, oxides and quinones (Shimada 2006). The gas phase of CS consists primarily of

nitrogen oxide, ammonia, hydrogen sulfide, oxygen, nitrogen and carbon oxides; the gas phase also consists of volatile organic compounds, such as ketones, single-ring aromatics and heterocyclic compounds, which are associated with cytotoxicity and carcinogenicity (Hatzinikolaou et al. 2006).

Tobacco leaves have a high concentration of alkaloids, one of which is nicotine (U.S. Department of Health and Human Services 2010). Nicotine has been previously associated with reduced vasodilator nitric oxide (NO) bioavailability, and this may contribute to endothelial dysfunction (Ambrose and Barua 2004). Furthermore, nicotine also effects blood coagulation through increased platelet aggregation and blood viscosity, which contributes to the development of arteriosclerosis (Ramsdale et al. 1985). There are also microbes that grow on the tobacco plant, which include fungi such as yeast and mold, Gram positive and Gram negative bacteria, bacterial and fungal spores, exotoxins and endotoxins. This microbial element on tobacco plants are associated with increased lung inflammation; other possible effects of these bacteria are still being studied (Pauly and Paszkiewicz 2011). Other harmful chemicals in CS include heavy metals, such as arsenic, cadmium, lead, copper, chromium, nickel, vanadium and zinc from pesticide sprays and fertilizers that become incorporated into the plant itself and are released when the plant is smoked (Bozhinova 2016).

Cigarette smoke vascular endothelial effects

Endothelial dysfunction & flow-mediated dilation

Endothelial cells line the interior surface of the intima of all vessels and regulate vascular tone through production of vasoconstrictive factors such as thromboxane or

angiotensin II, and vasodilatory factors such as NO, prostacyclin or endothelium-derived hyperpolarizing factor (Coppola et al. 2008). Hemodynamic shear stress, the frictional force of the blood on the endothelial layer, alters intracellular events by stimulating membrane-bound surface receptors on the endothelial cells that cause downstream signaling cascades in the endothelium. For example, thromboxane A2 binds the thromboxane receptor and angiotensin II binds to the angiotensin receptor to cause vasoconstriction; bradykinin binds to the bradykinin receptor that produces NO to cause vasodilation (Coppola et al. 2008; Tang and Vanhoutte 2010). These signaling vasoconstrictors and dilators bind to cell receptors on the vascular smooth muscle where the smooth muscle cells respond by relaxing or contracting, altering the diameter of the vessel.

Endothelial dysfunction occurs as a result of a switch from vasodilatory and anti-inflammatory factors to constrictive and pro-inflammatory factors. Although underlying mechanisms are still being studied, the loss of the primary vasodilator NO is a hallmark of endothelial dysfunction, and thus is a precursor to many CVD (Cai and Harrison 2000; Cannon 1998). Other effects commonly associated with endothelial dysfunction include vascular smooth muscle growth in the vessel wall, increased adhesion molecule expression, platelet aggregation and increased oxidative stress (Félétou and Vanhoutte 2006; Vanhoutte et al. 2009).

Flow-mediated dilation (FMD) is the non-invasive, gold standard for measuring endothelial function in the clinic, and can be used as a predictor of CV events in healthy individuals (Rossi et al. 2008; Shechter et al. 2009). To assess FMD, the baseline brachial artery diameter is recorded via ultrasound and a blood pressure cuff is placed

around the forearm and inflated for 5 minutes, restricting blood flow. The cuff is then deflated, causing a rapid increase in blood flow through the brachial artery, known as reactive hyperemia, and the vessel diameter is continually recorded for 2 minutes post-cuff deflation. This shear stress-induced production of endothelial-derived vasodilatory factors, primarily NO, causes the brachial artery to dilate. As a result, FMD is expressed as the percentage increase in artery diameter relative to baseline diameter (Raitakari and Celermajer 2000).

Prospective studies have shown that FMD is an independent predictor of CV events, such as heart attack or stroke, in individuals without clinical CVD or at low CVD risk. A prospective study of 2,264 women (54 ± 6 years) free of clinical CVD demonstrated that FMD is significantly associated with CV events in a 4 year follow-up and is independent of other classic risk factors (Rossi et al. 2008). Similarly, a prospective study of 435 individuals (54 ± 12 years) without apparent coronary heart disease show that FMD is the best independent predictor of future CV events (Shechter et al. 2009). It has been shown in numerous studies that FMD is significantly impaired in smokers (Neunteufl et al. 2000; Ozaki et al. 2010; Wiest et al. 2015); however, the mechanism of how CS induces endothelial dysfunction, impairing FMD, is not fully understood. There is evidence that CS is responsible for increases in oxidative stress, decreases in NO production and decreases in the enzyme NO synthase (NOS) activity (Barua et al. 2003; Csiszar 2009)

Nitric oxide

Nitric oxide is an endothelium-dependent vasodilator of the vascular smooth muscle and is produced through NOS activity. Caveolae are specialized lipid rafts on the

surface of endothelial cells that are lined with caveolae-oriented protein, caveolin-1 (Cav-1). Endothelial NOS (eNOS), a dimeric enzyme containing a reductase and an oxidase domain, is responsible for NO production. The reductase domain contains binding sites for flavin mononucleotide (FMN), nicotinamide adenine dinucleotide phosphate (NADPH) and flavin adenine dinucleotide (FAD); the oxidase domain contains binding sites for zinc, tetrahydrobiopterin (BH_4), a heme group and L-arginine. Endothelial NOS is associated with the caveolae by being directly bound to Cav-1 under homeostatic conditions with no stimulation; with agonist activation, there is an intracellular influx of calcium that binds to calmodulin (CaM), and the calcium/CaM complex binds to the reductase domain of eNOS, causing a dissociation of eNOS from Cav-1. This is followed by heat shock protein 90 (HSP90) binding to eNOS and protein kinase B (Akt) phosphorylation of eNOS residue Ser-1177 (Balligand, Feron, and Dessy 2009). L-arginine and molecular oxygen to bind to the heme group of eNOS in the oxidase domain and BH_4 also binds to the oxidase domain, which allows for the production of L-citrulline and NO. The cycle is completed with Cav-1 binding to eNOS again (Fig. 1.1) (Razani, Woodman, and Lisanti 2002; Alderton, Cooper, and Knowles 2001; Qian and Fulton 2013).

However, in the absence of BH_4, eNOS changes its confirmation from a dimeric form to a monomeric form. Increased oxidative stress oxidizes BH_4 to BH_2, causing eNOS uncoupling; instead of producing NO, uncoupled eNOS produces superoxide (O_2^-). Decreased BH_4 bioavailability and eNOS uncoupling is associated with endothelial dysfunction (Verhaar 2004), and BH_4 has also been shown to be a major target for

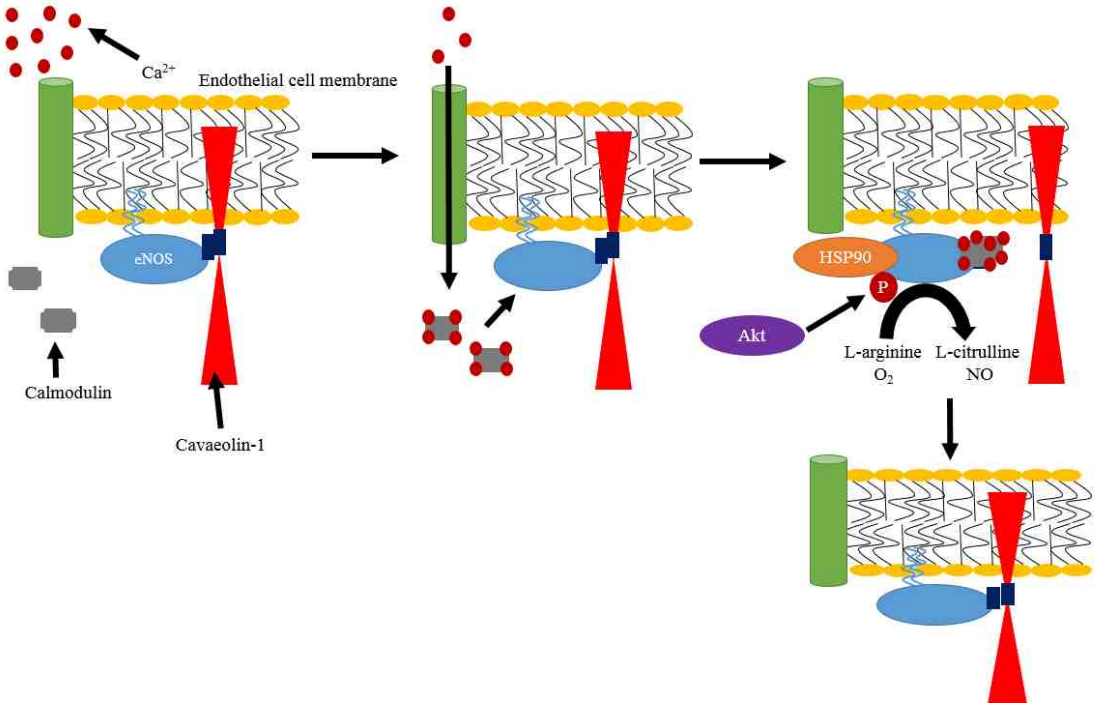

Figure 1.1. Nitric oxide production in caveolae of vascular endothelial cells. eNOS is directly bound to the Cav-1 in caveolae under homeostatic conditions with no stimulation; with agonist activation, there is an intracellular influx of calcium that binds to CaM, and the calcium/CaM complex binds to eNOS, causing a disassociation from Cav-1. eNOS also binds to HSP90 and Akt phosphorylation occurs of residue Ser-1177. L-arginine and molecular oxygen to bind to eNOS, which produces L-citrulline and NO. After returning to baseline, this cycle is completed with Cav-1 binding to eNOS. (Abbreviations: eNOS: Endothelial nitric oxide synthase; NO: Nitric oxide; Cav-1: Calveolin-1; CaM: Calmodulin; HSP90: Heat shock protein 90; Akt: Protein kinase B)

oxidation by peroxynitrite (Kuzkaya et al. 2003), which is an oxidant commonly found in CS. A previous study has shown that BH_4 administration improves endothelial-dependent dilation in smokers (Heitzer et al. 2000), suggesting that smokers may have increased uncoupled eNOS due to increased oxidative stress in smokers.

Oxidative stress

Oxidative stress is associated with an imbalance between increased reactive oxygen species (ROS) such as singlet oxygen, superoxide, peroxide and hydroxyl radicals, and decreased antioxidant levels (Rahal et al. 2014). Increased oxidative stress is associated with various CV events and diseases including atherosclerosis, myocardial infarction and hypertension (Harrison et al. 2003; Ramond et al. 2013; Touyz and Briones 2011). Multiple studies have shown that oxidative stress is significantly increased in individuals who smoke (Cai and Harrison 2000; Neunteufl et al. 2000; Ozaki et al. 2010), and CS contains oxidants such as peroxynitrite, semiquinone radicals and hydrogen peroxide (Pryor and Stone 1993). More specifically, lipid peroxidation products as measured by thiobarbituric acid reactive substances (TBARS) assay, and 8-isoprostane (8-epi-$PGF2_{\alpha}$) are increased in plasma of smokers; these markers are accurate indices of oxidative stress (Lee et al. 2012; Morrow et al. 1995; Seet et al. 2011). Increased ROS can contribute to endothelial dysfunction not only by reducing NO, but by also inhibiting K_{ATP} channels via *S*-glutathionylation (Serizawa et al. 2011; Jerkic, Sotov, and Letarte 2012; Y. Yang et al. 2010; Ozcan et al. 2002). Because reduction of vasodilators leads to endothelial dysfunction and this dysfunction is a precursor to CVD,

finding a way to decrease oxidative stress in cigarette smokers is critical to decreasing CVD risk.

Omega-3 polyunsaturated fatty acids in cardiovascular disease

Structure and types of omega-3 polyunsaturated fatty acids

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) are fatty acids that have a carboxylic acid group at the alpha end with multiple *cis* bonds in the fatty acid tail; the last *cis* double bond is located at the third carbon from the terminal methyl (omega) end of their fatty acid tail. Fatty acids are often listed by their lipid numbers, by which the number of carbon atoms is followed by the number of double bonds, separated by a colon (i.e. 18:3). There are three n-3 PUFAs in particular that are outlined in the literature as having CV-protective benefits: α-linolenic acid (ALA, 18:3), eicosapentaenoic acid (EPA, 20:5), and docosahexaenoic acid (DHA, 22:6). The metabolic precursor to EPA and DHA is ALA (Mori 2017).

Food sources of omega-3 polyunsaturated fatty acids

Omega-3 polyunsaturated fatty acids are essential fatty acids since humans cannot produce n-3 PUFAs endogenously, and they must be consumed from diet. For example, ALA is found in flax and chia seeds, walnuts and soybean oil, while EPA and DHA are found in fish, shellfish, krill oil and microalgae (Betz, Blackman, and Levine 2010). Fish do not endogenously produce n-3 PUFAs, as n-3 PUFAs are naturally produced in microalgae. As fish consume phytoplankton that consume microalgae, n-3 PUFAs are metabolized and stored in the fat and tissues of the fish (Betz, Blackman, and Levine 2010). It is generally accepted that the fattier the fish, the higher the n-3 PUFA content;

thus, farmed fish are typically higher in n-3 PUFA content than wild fish, but the DHA/EPA ratio in the majority of wild fish are higher than farmed fish (Hossain 2011). The American Heart Association recommends eating 3.5 ounces cooked or ¾ cup flaked fatty fish twice per week; fatty fish with higher EPA and DHA content include salmon, tuna, trout, herring and mackerel (Kris-Etherton 2002).

Omega-3 and omega-6 polyunsaturated fatty acid metabolism

Omega-3 polyunsaturated fatty acids are first incorporated into the plasma membrane of the cell, where fatty acid tails are cleaved from second carbon group of glycerol in the membrane by phospholipase A2 (PLA2) (Figure 1.2). Following this cleavage, ALA is metabolized by Δ6 desaturase, elongase and Δ5 desaturase, which produces EPA. Next, EPA is metabolized by two elongase enzymes, Δ6 desaturase and undergoes peroxisomal oxidation before producing DHA (Arterburn, Hall, and Oken 2006). The conversion efficiency of dietary ALA to EPA is less than 10%, and the conversion efficiency of ALA to DHA is approximately 4%, highlighting that it is necessary to receive EPA and DHA directly from diet (Williams and Burdge 2006; Arterburn, Hall, and Oken 2006). Each of these n-3 PUFAs, ALA, EPA and DHA, can be metabolized by cytochrome P450s into epoxides, cyclooxygenase-1 and -2 (COX-1 and -2) or lipoxygenase-5, -12 and -15 (LOX-5, -12 and -15) into prostaglandins, leukotrienes, thromboxanes, resolvins and protectins (Figure 1.3) (Dyall 2015).

Omega 6 (n-6) PUFAs such as linoleic acid (LA) are metabolized by the same enzymes as n-3 PUFAs, and they compete for enzymes such as Δ6 desaturase, P450, COX or LOX metabolism with n-3 PUFAs. These n-6 PUFAs are found in corn,

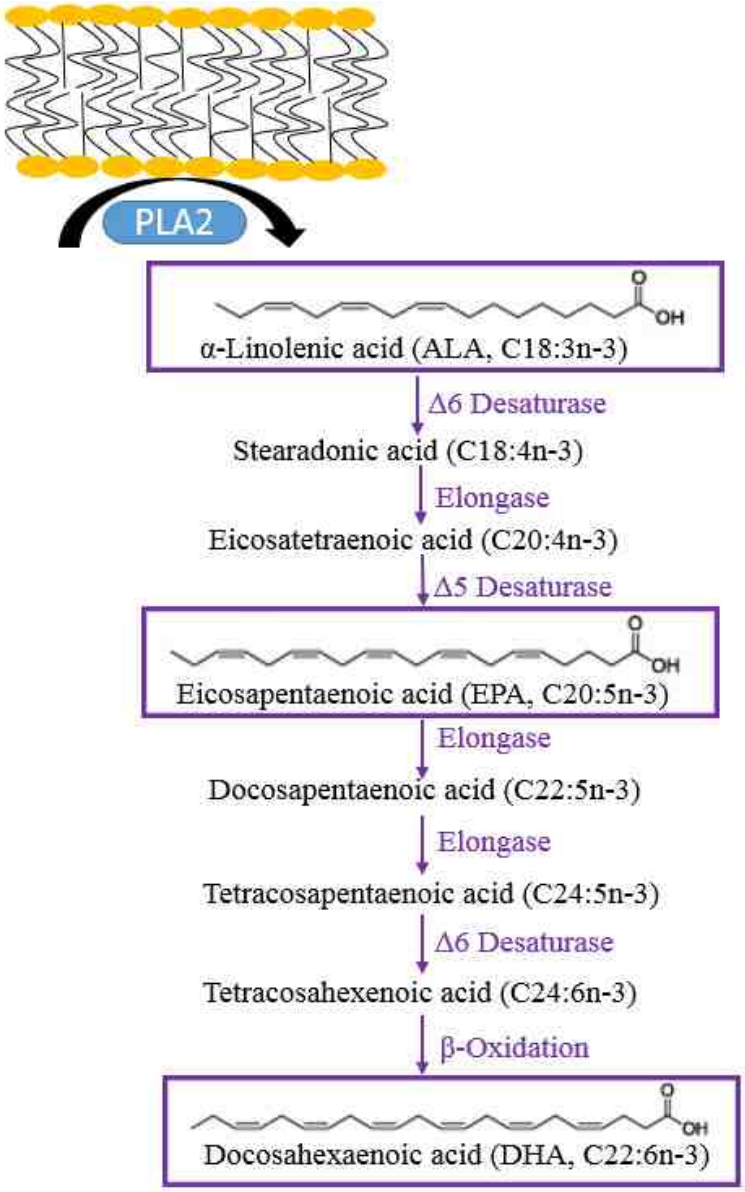

Figure 1.2. Omega-3 polyunsaturated fatty acid metabolism. ALA is cleaved from the fatty acids of the plasma membrane by enzyme PLA2; a double bond is added to ALA by Δ6 desaturase, metabolizing it into stearadonic acid, which is then elongated by elongase into eicosatetraenoic acid. Another double bond is added to eicosatetraenoic acid by Δ5 desaturase, producing EPA. EPA is elongated into docosapentaenoic acid and then tetracosapentaenoic acid via elongase metabolism. One more double bond is added by Δ6 desaturase, producing tetracosahexenoic acid, and then undergoes beta-oxidation, resulting in the production of DHA. (Abbreviations: PLA2: Phospholipase A2; ALA: α-linolenic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid)

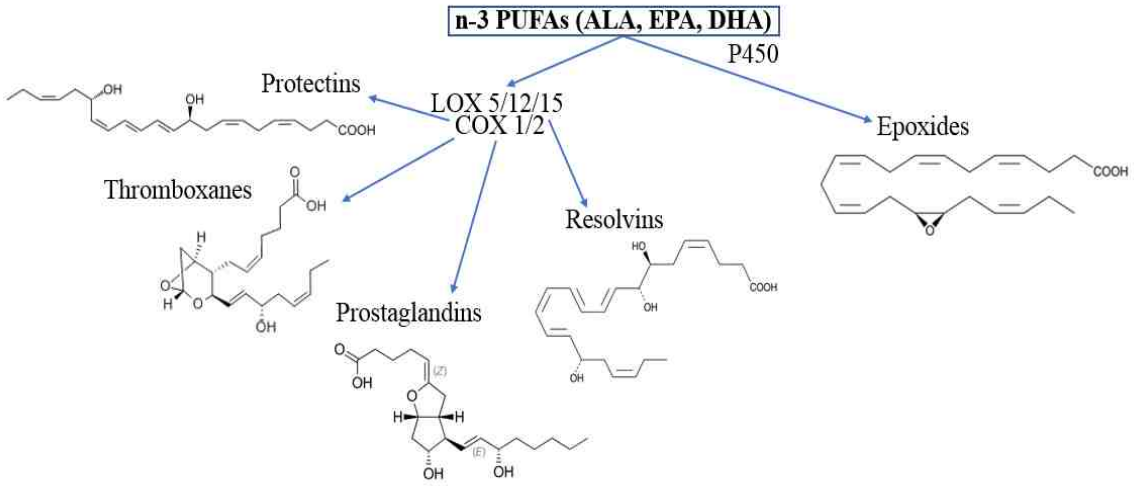

Figure 1.3. DHA, COX, LOX and P450 metabolism. DHA can be metabolized by 5, 12, or 15-LOX and COX-1 or -2 to produce resolvins and protectin metabolites; P450 produces epoxide metabolites. (Abbreviations: DHA: Docosahexaenoic acid; LOX: Lipoxygenase; COX: Cyclooxygenase; P450: Cytochrome P450)

safflower and sunflower oils, and although the Western dietary fats contain higher percentage of LA, Δ6 desaturase has increased affinity for ALA (Tu et al. 2010). Compared to n-3 PUFAs, n-6 PUFAs have been shown to decrease vascular eNOS activation and reduce acetylcholine-mediated vasodilation in isolated mesenteric arterioles (Agbor et al. 2012). Furthermore, a higher n-6:n-3 PUFA dietary ratio, as found in the United States (approximately 15:1), is associated with increased risk for CV disease, obesity, cancer and inflammation. A lower n-6:n-3 PUFA dietary ratio, commonly found in countries such as Japan (approximately 4:1) where fish dietary-intake is higher, confers lower risk for inflammatory-oriented diseases (Patterson et al. 2012; Sugano, Michihiro; Hirahara 2000).

Omega-3 polyunsaturated fatty acid cardiovascular disease risk impacts

Omega-3 polyunsaturated fatty acids prevent atherosclerotic disease development in humans based on multiple epidemiological studies and clinical trials. Positive CV effects of n-3 PUFAs were first observed in the Greenland Inuit, because of their increased intake of fish and decreased risk for CV events, decreased serum cholesterol and platelet aggregation (Bang, Dyerberg, and Hjorne 1976; Dyerberg et al. 1978). Following this work, one epidemiology study observed inverse associations with ALA and n-3 PUFAs in fish oil and coronary heart disease and mortality in almost 13,000 men (Dolecek 1992). Another epidemiological study of over 2,000 men confirmed the inverse association between fish consumption and death from coronary heart disease, as well as myocardial infarction (Daviglus 1997). Later clinical trials revealed that n-3 PUFAs slowed the progression of coronary atherosclerosis and increased stability of atherosclerotic plaques (von Schacky 1987; Thies et al. 2003). Because risk factors for

endothelial dysfunction include classic risk factors for CV disease, a few of which include hyperlipidemia, obesity and CS (Versari et al. 2009), n-3 PUFAs would be considered as primary prevention since the consumption of n-3 PUFAs decreases the incidence of CVD as individuals age.

Hyperlipidemia

Among nine studies enrolling hyperlipidemic subjects that were recently reviewed (Zehr and Walker 2017), eight studies showed that n-3 PUFA supplementation, including formulations (EPA+DHA, EPA alone, DHA alone, and ALA alone), improved endothelial dysfunction as assessed by FMD and eight studies showed n-3 PUFA supplementation also reduced triglycerides. The reasons why one study failed to observe an improvement in FMD is not clear (Skulas-Ray et al. 2011). The study was a randomized, double-blind, placebo-controlled, crossover design with six weeks washout between treatment arms. The investigators used a high dose EPA+DHA for an eight-week duration and observed a significant decrease in triglycerides. However, while the enrolled group size was similar to other studies (n=26), the age range was large and disproportionately male. It has been reported that the age-related decline in endothelial function occurs significantly earlier in men (41 yo) than in women (58 yo) (David S. Celermajer et al. 1994). Thus, it is possible that the age and sex characteristics of the enrolled cohort resulted in the inability of n-3 PUFA supplementation to improve FMD. Taken together these results showed that n-3 PUFA supplementation in hyperlipidemic individuals can improve endothelial dysfunction, particularly as assessed by FMD, and this improvement can occur even in the absence of a reduction in triglycerides.

Elevated body mass index

Elevated BMI has been shown to be a risk factor for endothelial dysfunction (Williams et al. 2005; Oflaz 2003) and for atherosclerosis (Hubert et al. 1983). One study recruited individuals that were overweight or obese (BMI > 25 kg/m^2) and had one or more CV risk factors, including hypertension (140/90-160/100 mm Hg), increased plasma triglycerides (> 142 mg/dL) or elevated total cholesterol (> 212 mg/dL) (Hill et al. 2009). Flow-mediated dilation was assessed in individuals with elevated BMI and were divided into 4 groups. Two groups consumed EPA+DHA, two groups consumed placebo for 12 weeks; one n-3 PUFA group and one placebo group were required to run or walk 3 times/week for 45 minutes. Individuals taking the n-3 PUFA supplement exhibited a significant time-dependent improvement in FMD, compared to placebo (treatment x time interaction), and a significantly higher FMD, compared to the placebo group, after 12 weeks. There was no effect of exercise status on this outcome. The n-3 PUFA supplement also significantly reduced triglycerides at both 6 and 12 weeks, compared to placebo. Taken together, this result showed that n-3 PUFA supplementation in individuals with elevated BMI can improve endothelial dysfunction as measured by FMD and lower triglyceride levels independently of exercise.

Cigarette smoke

In one study, n-3 PUFAs were shown to improve FMD and reduce triglycerides in a double-blind, crossover, placebo-controlled study in current cigarette smokers (> 20 cigarettes per day for > 5 years, n=20), lacking any other classic risk factors of CV disease (Siasos et al. 2013). A baseline FMD was measured before randomly assigning

each subject to receive placebo (composition not reported) or 2 g/d of n-3 PUFAs (84% EPA+DHA, consumed daily dose 1.7 g/d EPA+DHA) for 12 weeks followed by 4 week washout and crossover to the other treatment arm. Flow-mediated dilation was measured again after 4 and 12 wks. The n-3 PUFA supplement non-significantly increased FMD after 4 weeks, compared to baseline, (baseline: 7.27 ± 2.56%, 4 weeks n-3 PUFA: 8.53 ± 3.55%), but significantly improved it after 12 weeks (9.98 ± 5.30%, $P < 0.05$), compared to baseline. In contrast, there were no significant changes in FMD when the smokers consumed the placebo (baseline: 6.92 ± 1.87%, 4 weeks: 6.71 ± 1.58%, 12 weeks: 6.68 ± 1.4%, $P > 0.05$). Additionally, compared to placebo, individuals taking the n-3 PUFA supplement exhibited significantly higher FMD at both 4 weeks ($P < 0.05$) and 12 weeks ($P < 0.001$). These data show that 1.7 g/d EPA+DHA for 12 weeks improved FMD and reduced triglycerides in otherwise young, healthy smokers.

Omega-3 polyunsaturated fatty acid vascular-protective mechanisms in smokers

Nitric oxide

The mechanisms underlying the endothelial function improvement by n-3 PUFA supplementation in human subjects have not been fully elucidated. However, numerous studies suggest that n-3 PUFAs may improve endothelial function by increasing NO levels (Ander et al. 2003). For example, EPA increased NO in endothelial cells *in situ* and stimulated endothelial and NO-dependent dilation in bovine coronary arteries *ex vivo* (Omura et al. 2001). Furthermore, both EPA and DHA activate endothelial nitric oxide synthase (eNOS) in cultured human endothelial cells (Omura et al. 2001; Stebbins et al. 2008) and dietary n-3 PUFAs significantly increase eNOS activation in the mouse aorta (Agbor et al. 2014). It has been shown that DHA also increases NO by increasing

interleukin-1β-induced inducible NOS mRNA through activation of p44/42 mitogen-activated protein kinase signaling (Hirafuji et al. 2002). This allows for increased extracellular calcium release from vascular smooth muscle cells, which improves vasoreactivity (Hirafuji et al. 2003). *In vivo*, dietary n-3 PUFA supplementation normalizes endothelial dysfunction in mouse mesenteric arterioles that is induced by cigarette smoke exposure (Wiest, Walsh-Wilcox, and Walker 2017). Notably, however, the improvement in mesenteric arteriolar FMD is mediated by an increase in NO-independent dilation, suggesting that n-3 PUFAs can also increase the expression and/or activity of other endothelial-derived vasodilators, in addition to NO. This mechanism may account for differences in the vasodilatory benefits of n-3 PUFA supplementation between microvascular arterioles versus conduit arteries.

Antioxidant and Nuclear factor (erythroid-derived 2)-like 2 activity

Antioxidants and n-3 PUFAs may be able to reduce cigarette smoke-induced oxidative stress and improve FMD. In mouse tissues enriched with n-3 PUFAs, hydrogen peroxide production is decreased (Hagopian et al. 2010). Cardiomyocytes treated simultaneously with ROS-inducing drug doxorubicin and EPA or DHA have decreased levels of ROS, compared to untreated cells (Hsu, Chen, and Chen 2014). In humans, one study showed that there was a positive correlation between n-3 PUFAs in red blood cells and total plasma antioxidant capacity (Thorlaksdottir et al. 2006).

One of the mechanisms by which n-3 PUFAs mediate their antioxidant benefits may be through Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activation. In the cytosol, Nrf2 is sequestered in the cytosol by Kelch like-ECH-associated protein 1

(Keap1) and cullin3 through binding the DLG and EGTE domains of Nrf2; under homeostatic conditions, Keap1 facilitates the ubiquitination and proteasomal degradation of Nrf2 (Figure 1.4). When there is increased oxidative stress, cysteine residues on Keap1 are oxidized, allowing a conformational change that releases Nrf2 from its binding to Keap1; Nrf2 then translocates to the nucleus, where it heterodimerizes with Maf proteins and binds to the antioxidant response element (ARE). This causes downstream transcription and translation of multiple antioxidant proteins, such as heme-oxygenase 1 (Hmox1), NAD(P)H quinone dehydrogenase 1 (Nqo1), glutamate-cysteine ligase catalytic subunit (Gclc), superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH). Oxidized EPA and DHA have been shown to destabilize Keap1 and cullin3 binding, thus activating Nrf2 (Gao et al. 2007). Furthermore, Hmox1 is induced in vascular smooth muscle cells treated with DHA (Stulnig et al. 2013), and the anti-inflammatory effects of DHA are dependent on the redox abilities of Nrf2 (Gruber et al. 2015).

Reactive oxygen species

Another possible mechanism underlying the improvement of endothelial function improvement by n-3 PUFAs includes decreasing ROS. Vascular ROS can reduce NO bioavailability and increase endothelial-derived vasoconstrictors, thus impairing endothelial-dependent vasodilation. It has been shown that n-3 PUFAs decrease ROS in doxorubicin-treated cardiomyocytes and in endothelial cells exposed to environmental particulates in vitro (Hsu, Chen, and Chen 2014; Bo et al. 2016). In a mouse model, dietary n-3 PUFA supplementation significantly reduces CS-induced increases in two

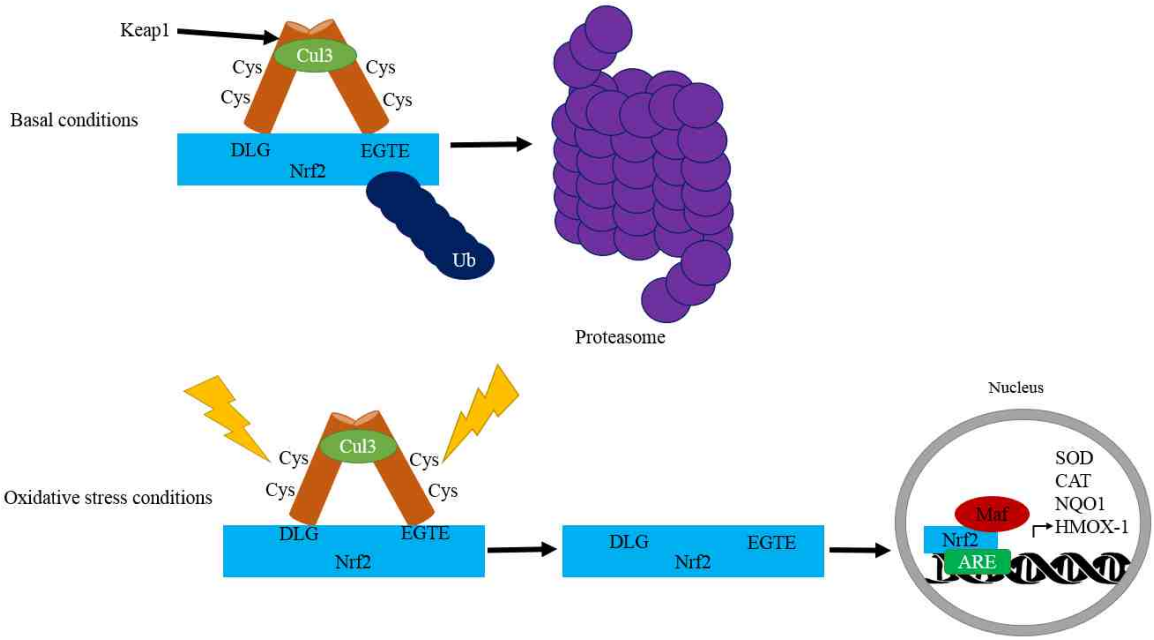

Figure 1.4 Nrf2 signaling. Under basal conditions, Nrf2 is bound to Keap1 through its motifs DLG and EGTE and Nrf2 is targeted to the proteasome via ubiquitination. Under oxidative stress conditions, cysteine residues on Keap1 are oxidized, and Keap1 undergoes conformational change, releasing Nrf2. Nrf2 translocates to the nucleus, binding to and forming a heterodimer with a small Maf protein. This binds to the ARE in the upstream promoter region and increased transcription of antioxidants such as SOD, CAT, Nqo1 and Hmox1. (Abbreviations: Nrf2: Nuclear factor (erythroid-derived 2)-like 2; Keap1: Kelch like ECH associated protein 1; Cys: Cysteine; Cul3: Cullin-3; Ub: Ubiquitin; ARE: Antioxidant response element; Maf: musculoaponeurotic fibrosarcoma oncogene homolog; SOD: Superoxide dismutase; CAT: Catalase; NQO1: NAD(P)H quinone dehydrogenase 1; Hmox1: Heme-oxygenase 1)

markers of oxidative stress, 8-isoprostane and Hmox1 mRNA (Wiest, Walsh-Wilcox, and Walker 2017). In a menopausal rat model, n-3 PUFA supplementation is associated with decreased ROS production through modulation of NADPH oxidase and inducible NOS (Cappellari et al. 2013). In one clinical study, n-3 PUFA intake is positively correlated with in total antioxidant capacity, but unfortunately endothelial function was not assessed in these participants (Thorlaksdottir et al. 2006).

Endothelial dysfunction and inflammation

Endothelial function also is impaired by endothelial activation, inflammation, and hypertension, which are all risk factors for the development of atherosclerosis. Studies show that n-3 PUFAs can inhibit endothelial activation, and are anti-inflammatory and antihypertensive (Calder 2006; Hadi, Carr, and Al Suwaidi 2005). Endothelial activation is associated with increases in surface expression of adhesion molecules which promotes leukocyte adhesion and inflammation, and both EPA and DHA reduce adhesion molecule expression and leukocyte adhesion to endothelial cells *in vitro* (De Caterina et al. 1994; Huang, Sheu, and Chiang 2015; Elsen, Garssen, and Willemsen 2012). Clinical studies also show that n-3 PUFA supplementation reduces monocyte activation, markers of inflammation and hypertension, all of which can contribute to endothelial dysfunction (Y. Yang, Lu, and Chen 2012; Ellulu et al. 2016; Miller, Van Elswyk, and Alexander 2014).

Metabolites and docosahexaenoic acid-specific effects

Metabolites of n-3 PUFAs have anti-inflammatory properties; EPA and DHA COX- and LOX-induced metabolites have anti-inflammatory properties in comparison to

AA COX and LOX metabolites, which have high pro-inflammatory potential (Calder 2010). Specifically, n-3 PUFA COX and LOX metabolites have been associated with decreased inflammation in CVD (Gonzalez-Periz et al. 2009; Capó et al. 2017); some of these properties are due, in part, to receptor-mediated decreases in cytokines. Another possible mechanism includes aspirin-triggered production of various metabolites that reduce inflammation through blocking cytokines and regulation of neutrophils (Serhan et al. 2004). Cytochrome P450-driven metabolism of n-3 PUFAs produce epoxide metabolites. Soluble epoxide hydrolase (sEH) metabolizes epoxides into diols; sEH inhibition has been shown to decrease in inflammation in rats exposed to cigarette smoke (Schmelzer et al. 2005; Smith et al. 2005). More specifically, DHA epoxides have also been associated with lowered blood pressure in angiotensin-II-dependent hypertension and sEH inhibition has shown to decrease angiotensin-II dependent hypertension (Ulu et al. 2014; Ulu et al. 2013). However, the mechanism by which P450-produced epoxide metabolites, specifically DHA epoxides, carry out these cardiovascular-protective effects have not been fully elucidated.

While the above examples consist of enzymatically-controlled oxidation, non-enzymatic oxidation can frequently occur due to ROS because of the unsaturated nature of PUFAs (Anderson and Taylor 2012). Of the three primary n-3 PUFAs presented, DHA is more likely to undergo lipid peroxidation because of its chemical structure (Siddiqui, Harvey, and Stillwell 2008). Compounds such as 4-hydroxyhexenal (4-HHE), a lipid peroxidation product of DHA, is shown to activate Nrf2; these effects are not observed with EPA treatment (Ishikado et al. 2013), and lipid peroxidation levels have been shown to increase alongside increases of anti-inflammatory and antioxidant

enzymes in the heart following n-3 PUFA supplementation (Allard et al. 1997; Foulon and Richard 1999). Taken together, these results suggest that both non-enzymatic and enzymatically-controlled oxidation of n-3 PUFAs, specifically DHA, stimulates their vascular-protective effects.

Cigarette smoking effects on omega-3 polyunsaturated fatty acid levels

It is shown that smokers have decreased serum EPA and DHA (Block, Harris, and Pottala 2008). According to several *in vitro* studies, this decrease in EPA and DHA in smokers is due, in part, to the enzyme inhibition responsible for the conversion of ALA to EPA and DHA (Risé et al. 2009; Ghezzi et al. 2007; Marangoni et al. 2004). In one placebo-controlled, double-blind study of 20 smokers, intervention with n-3 PUFA supplementation significantly increased serum omega-3 index as well as FMD in 4 weeks (Siasos et al. 2013). Thus, supplementing with n-3 PUFAs may decrease overall CV risk, and studying low n-3 PUFA concentration effects in smokers may highlight specific vascular-protective mechanisms by which n-3 PUFAs carry out in humans.

Rationale for research

Worldwide, CVD is the leading cause of death, killing over 17 million people every year, and cigarette smoking is a major risk factor for CVD (Roth et al. 2015). Tobacco use is projected to cause more than 8 million deaths annually by 2030 worldwide, and CS currently causes over 480,000 deaths per year (World Health Organization 2011; US Department of Health and Human Services 2014). Of those who attempt to quit smoking, less than 10% are successful in quitting; thus, finding a therapeutic solution to help prevent CS-induced CVD is critical (Malarcher et al. 2011). A precursor to CS-induced CVD, endothelial dysfunction, is characterized by a switch from vasodilatory and anti-inflammatory factors to constrictive and pro-inflammatory factors and can be detected by FMD. Smokers are shown to have reduced n-3 PUFA levels; n-3 PUFA supplementation reverses endothelial dysfunction in young healthy smokers (Siasos et al. 2013). Endothelial dysfunction in smokers is partially mediated through increased ROS, leading to oxidative stress; n-3 PUFAs, specifically DHA, are shown to increase Nrf2 activity and downstream antioxidant gene expression (Gao et al. 2007; Stulnig et al. 2013; Gruber et al. 2015). Cytochrome P450-driven metabolism of n-3 PUFAs produce epoxide metabolites, which are shown to be vascular-protective (Ulu et al. 2014). However, the mechanism through which n-3 PUFAs prevent and reverse endothelial dysfunction in smokers or increase antioxidant mechanisms are not fully understood. Our objective is to determine the relationship between Nrf2 activity, oxidative stress markers, antioxidant levels, CVD risk and PUFAs in smokers and to determine the mechanism by which they contribute to CS-induced vascular dysfunction.

Central hypothesis Increased n-3 PUFAs and their metabolites activate Nrf2 and downstream antioxidants, specifically in CS-induced oxidative stress.

Aim 1: Elucidate the mechanism by which n-3 PUFAs, specifically DHA, mediates antioxidant responses.

Rationale: The mechanism by which n-3 PUFAs are associated with increased total antioxidant capacity and decreased oxidative stress has not been fully elucidated.

Approach: Our working hypothesis is that antioxidant effects of the omega-3 polyunsaturated fatty acid DHA are mediated by DHA metabolites via Nrf2 activation and induction of antioxidant gene expression. We assessed Nrf2 activity via treating a Hep G2 cell line that is stably transfected with a firefly luciferase gene under the control of ARE with DHA and COX, LOX or P450 inhibitors. We measured our DHA stock by LC-MS, and demonstrated increased oxidized products, including ketones. Using one commercially available ketone of DHA, 17-oxo-DHA, we assessed its Nrf2 activation in the Hep G2 reporter cell line and also assessed the ability of 17-oxo-DHA to shift the localization of Nrf2 to the nucleus in human umbilical vein endothelial cells (HUVECs) using immunofluorescence. Downstream Nrf2 target genes, Hmox1, Gclc and Nqo1 mRNA were measured in HUVECs treated with 17-oxo-DHA at 3, 6 or 12-hour time points. We used a one-way ANOVA to compare Nrf2 activity between cells treated with DHA alone and cells treated with DHA and COX, LOX or P450 inhibitors. Two-way ANOVA was used to compare a dose response of 17-oxo-DHA-treated cells to a ketone of AA in Hep G2 reporter cells; a two-way ANOVA was used to compare Hmox1, Gclc

and Nqo1 mRNA levels at different time points between cells treated with the positive control L-sulforaphane, vehicle or 17-oxo-DHA.

Aim 2: Determine the degree to which n-3 PUFA epoxide metabolites are associated with endothelial dysfunction and oxidative stress in cigarette smokers.

Rationale: Smoking is a risk factor for CV disease, yet despite numerous established programs to help smokers quit, the majority of smokers have difficulty quitting. Tobacco-related health care costs have exceeded $130 billion per year; thus, it is crucial to establish therapeutic approaches and treatments to reduce vascular damage and decrease the risk for CV disease in smokers.

Approach: Our working hypothesis is that higher levels of n-3 PUFA epoxides will be associated with lower levels of oxidative stress and better flow-mediated dilation in current smokers. To test this hypothesis, we recruited healthy, young (19-50 yr. old) current (n=67) smokers. We assessed FMD and other CVD risk factors and calculated the 10 year risk of developing CVD (Framingham risk score). Both n-3 and n-6 PUFAs and their metabolites were measured by LC-MS/MS. We used univariate and multivariate regression analysis to assess correlation between PUFAs and their metabolites, FMD, antioxidant markers and oxidative stress.

CHAPTER II

Docosahexaenoic acid oxidation products, including ketone 17-oxo-4(Z),7(Z),10(Z),13(Z),15(E),19(Z)-DHA, activate Nuclear factor (erythroid-derived 2)-like 2 and downstream antioxidant genes

Kayla R. Zehr and Mary K. Walker.

ABSTRACT

Evidence suggests that omega-3 polyunsaturated fatty acids (n-3 PUFAs) can improve impaired vascular function, and n-3 PUFA epoxides have been shown to decrease blood pressure. Furthermore, n-3 PUFA supplementation has also been associated with increased total antioxidant capacity, but the mechanism between the positive cardiovascular benefits of these epoxide metabolites and increased antioxidant capacity are not fully understood. We hypothesized that n-3 PUFA eicosapentaenoic acid and docosahexaenoic acid (EPA and DHA) epoxide metabolites were responsible for vascular protective effects through activation of Nrf2 and increased antioxidant gene expression. We measured Nrf2 activation in a stably transfected antioxidant response element (ARE) reporter hepatocyte (Hep G2) cell line, assessed Nrf2 localization to the nucleus using immunofluorescence and measured downstream antioxidant gene expression of hemeoxygenase-1 (Hmox1), glutamate cysteine ligase catalytic subunit (Gclc), and NAD(P)H quinone dehydrogenase 1 (Nqo1). We found that oxidation products of DHA were responsible for a significant increase in Nrf2 activation compared to epoxides or EPA ($P<0.05$). Among the oxidation products produced, ketones displayed increased expression, and so the only commercially-available DHA ketone, 17-oxo-4(Z),7(Z),10(Z),13(Z),15(E),19(Z)-DHA (17-oxo-DHA), was used and found to increase Nrf2 activity and also shift Nrf2 localization to the nucleus in human umbilical vein endothelial cells (HUVECs). Furthermore, there was a significant increase in Hmox1, Gclc and Nqo1 gene expression compared to the vehicle and positive control ($P<0.05$).

INTRODUCTION

Cardiovascular disease is the leading cause of death worldwide, leading to over 17 million deaths every year (Roth et al. 2015). A precursor to CS-induced CVD is endothelial dysfunction, which is characterized by a switch from vasodilatory and anti-inflammatory factors to constrictive and pro-inflammatory factors. Although underlying mechanisms are still being studied, the loss of the primary vasodilator, nitric oxide (NO), is a hallmark of endothelial dysfunction, and thus a precursor to CVD (Cai and Harrison 2000; Cannon 1998). Omega-3 polyunsaturated fatty acids (n-3 PUFAs) are fatty acids commonly found in fish and shell fish oil. Two n-3 PUFAs shown to have vascular protective effects are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and n-3 PUFA supplementation has been shown to improve endothelial function in those with risk factors for CV disease (Zehr and Walker 2017).

Antioxidants may be able to reduce oxidative stress and improve endothelial function. In mouse tissues enriched with n-3 PUFAs, hydrogen peroxide production is decreased (Hagopian et al. 2010). Cardiomyocytes treated simultaneously with ROS-inducing drug doxorubicin and eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) have decreased levels of ROS, compared to untreated cells (Hsu, Chen, and Chen 2014). In humans, one study showed that there was a positive correlation between n-3 PUFAs in red blood cells and total plasma antioxidant capacity (Thorlaksdottir et al. 2006).

One of the mechanisms by which n-3 PUFAs mediate their antioxidant benefits may be through Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activation; Nrf2 is negatively regulated through ubiquitination by Kelch like-ECH-associated protein 1

(keap1) and cullin3. Oxidized n-3 PUFAs, including EPA and DHA, have been shown to destabilize keap1 and cullin3 binding, thus activating Nrf2 (Gao et al. 2007). Furthermore, heme oxygenase (decycling) 1 (Hmox1), a downstream antioxidant target of Nrf2, is induced in vascular smooth muscle cells treated with DHA (Stulnig et al. 2013), and the anti-inflammatory effects of DHA are dependent on the redox abilities of Nrf2 (Gruber et al. 2015). Furthermore, EPA and DHA can be further metabolized by cytochrome P450s into their respective epoxide metabolites, 17,18-epoxyeicosatetraenoic acid (EEQ) and 19,20-epoxydocosahexaenoic acid (EDP), respectively. Soluble epoxide hydrolase (sEH) metabolizes these epoxides into their corresponding diol metabolites. These epoxide metabolites have been previously shown to decrease blood pressure in hypertensive model, and that sEH decreases these positive effects (Ulu et al. 2013; Ulu et al. 2014).

However, evidence of n-3 PUFAs directly increasing antioxidant capacity through their epoxide metabolites has not been shown, and to our knowledge, no one has assessed this possible relationship. Thus, we hypothesized that n-3 PUFA epoxide metabolites are responsible for vascular protective effects through activation of Nrf2 and downstream antioxidant gene activation.

2. MATERIALS AND METHODS

2.1. *Cell culture.*

Stably transfected antioxidant response element (ARE) reporter hepatocyte (HepG2-ARE) cell line (BPS Bioscience) was maintained using Minimum Essential Medium with Earle's Balanced Salts (MEM/EBSS, Hyclone) supplemented with 10%

fetal bovine serum (FBS) (Gibco), 1% non-essential amino acids (Hyclone), 1 mN Na-pyruvate (Hyclone), 1% Penicillin/Streptomycin (Hyclone) and 0.6 mg/mL of G-418 (Gibco). Human umbilical vein endothelial cells (HUVECs) (Lonza) were maintained using Endothelial basal medium-2 with endothelial growth medium-2 BulletKit (Lonza) with 10% FBS (Gibco). All cells were used at passages ≤10 and maintained in a humidified 37^{o}C incubator at 5% CO_2.

2.2. *Chemicals*.

Fatty acids—1 μM L-Sulforaphane (Cayman), old DHA (~1 year old) and new DHA stock from Commercial Supplier 1 (range of doses, Cayman), new DHA stock Commercial Supplier 2 (range of doses, Sigma), 30 μM eicosapentaenoic acid (EPA, Cayman), 5 & 10 μM 4-hydroxyhexenal (4-HHE, Cayman), 1 μM 17,18-epoxyeicosatetraenoic (EEQ, Cayman) acid, 1 μM 19,20-epoxydocosapentaenoic (EDP, Cayman) acid, 17- 17-oxo-4(Z),7(Z),10(Z),13(Z),15(E),19(Z)-DHA (range of doses, 17-oxo-DHA, Cayman), 15-oxo-5Z,8Z,11Z,13E-eicosatetraenoic acid (range of doses, 15-oxo-ETE, Cayman).

DHA metabolism inhibitors—1 μM NS398 (Cayman), 1 μM indomethacin (Indo, Sigma), 20 μM baicalein (Baic, Sigma), 5 μM MK886 (Cayman), 1 μM 2-[1-Thienyl]ethyl 3,4-dihydroxybenzylidenecyanoacetate (2-TEDC, Tocris), 10 μM SKF525 (Cayman) and 150 μM 1-aminobenzotriazole (1-ABT, Sigma).

2.3. *Nrf2 activity*.

The ONE-Step Luciferase Assay System (BPS bioscience) was used to assess Nrf2 activity. Next, HepG2-ARE cells were seeded in 96-well plates at 75,000 cells per well and simultaneously treated in triplicate in complete MEM/EBSS noted above, excluding G-418. Cells were incubated for 24 hours; luciferase reagent buffer and substrate were added directly to the cells and media, covered and rocked for 15 minutes at room temperature. Luminescence was read using a microplate reader; background luminescence (growth media only) was subtracted from each well, each experimental well was divided by the vehicle control and each triplicate set of treatment averaged to determine Nrf2 fold induction.

2.4. *LC-MS*.

Fatty acid samples were dissolved in ethanol and analyzed using HPLC simultaneous coupled with a diode array UV-detector and a single quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). The mass spectrometer was equipped with an electrospray ionization source operated in negative mode and full scan spectra m/z 100 – 1000 Da were registered. The stationary phase was a Phenomenex Kinetex C18 150 x 2.1 mm, 2.6 µm; acetonitrile and 0.05 % acetic acid were combined in water to use as the mobile phase. The gradient started at 5% Acetonitrile and was increased to 95 %; acetonitrile was used during ten minutes and held until the 14 minute time point. The injection volume was 1 µL. The following mass traces were extracted from full scan chromatogram using Agilent Chemstation software: DHA: -327; DHA+O: -343; DHA+O - 2H: -341; DHA+2O: -359.

2.5. *Cell viability*.

Cell viability was assessed by measuring lactate dehydrogenase (LDH) release using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Briefly, cells were seeded in 96-well plates at 75,000 cells per well and simultaneously treated in triplicate in their respective growth mediums excluding G-418. Cells were incubated for 24 hours. A volume of 10 µL 10x lysis solution was added into each positive control wells and incubated for 45 minutes, and the plate shaken for 1 minute. Next, 50 µL of media from each well was taken and placed into a fresh 96-well plate, combined with 50 µL substrate mix and lysis solution, covered and incubated for 30 minutes at room temperature. Last, 50 µL stop solution was added and absorbance read at 490 nm using a microplate reader. Background absorbance (growth media only) was subtracted from each well, and each experimental well was divided by the positive control, each treatment triplicate averaged and multiplied by 100 and subtracted from 100 to determine percent cell viability.

2.6 *Immunofluorescence*.

Human umbilical vein endothelial cells were seeded in a 12-well plate with cover slips and 1.5 ml media per well. At 50% confluency, cells were treated with 17-oxo-DHA or vehicle for 3 hours and fixed with cold methanol for 10 minutes at 4^{o}C. Wells were rinsed three times with 1X pH 7.4 PBS (Gibco), 10 minutes per rinse. Cells were permeabilized for 10 minutes in 0.1% Triton X-100 (Sigma) in PBS (PBST), rinsed three times with PBS and blocked with PBS containing 2% BSA (Sigma), 2% Horse serum (Gibco), 2% goat serum (Gibco) and 0.1% Triton X-100 for 1 hr at room temperature.

Cells were incubated with primary antibody, (Abcam) was diluted 1:50 in block and incubated overnight at 4^{o}C. Cells were rinsed with three times with PBST, 10 minutes per rinse, and incubated with secondary antibody (1:1000 dilution) in block and rocked at room temperature for 1.5 hrs. Cells were rinsed three times with PBST, 10 minutes each rinse. Vectashield with DAPI (Vector Laboratories, Inc.) was dropped onto glass slides, and cover slips were fixed onto glass slides with sealant. Cells were imaged using Zeiss LSM 800 Airyscan Confocal microscope.

2.7 *mRNA expression of Hmox1, Nqo1 and Gclc.*

Total RNA was isolated from HUVECs treated with 17-oxo-DHA or vehicle for 3, 6 or 12 hours with RNeasy Mini Kit (Qiagen, GmbH, Germany). Next, cDNA was synthesized using iScript Supermix Kit (Bio-Rad Laboratories, Hercules, CA) with the 5x iScript Reverse Transcription Supermix and 250 ng/µL RNA. Polymerase chain reaction amplification was performed using a CFX96 Real Time Connect (Bio-Rad Laboratories) with a reaction mixture comprised of SYBR Green Supermix (Bio-Rad Laboratories) with sense and antisense primers (Appendix A, Table 5.1, Sigma) and 250 pg cDNA/µL. Cycle threshold data for both target and reference genes were used to calculate mean normalized expression.

2.6. *Statistics.*

Values are expressed as the mean ± standard error and analyzed using SigmaStat 3.5 and SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA). Data were analyzed via one or two-way ANOVA, and were transformed in cases of failed normality or variance tests.

3. RESULTS

3.1 *DHA, not EPA, increases Nrf2 activity*

First, HepG2-ARE cells were treated with fatty acid parent compounds EPA and DHA, their epoxide metabolites 17,18-EEQ and 19,20-EDP, and non-enzymatic DHA peroxidation product 4-HHE. Only DHA significantly increased Nrf2 activity close to the activity of the positive control, LS ($P<0.05$, Figure 2.1A). Furthermore, DHA also increased Nrf2 activity in a dose-dependent fashion, with the highest activity noted at a 30 μM dose (Figure 2.1B). None of these treatments had a significant decrease in cell viability (Appendix A, Figure 5.1).

3.2 *Inhibition of DHA metabolism does not decrease its Nrf2 activity*

In this experiment, HepG2-ARE cells were treated with the fatty acid DHA combined with COX inhibitors (COX-2: NS398 and COX-1/2: Indo), LOX inhibitors (5, 10, 12-LOX: Baic, 5-LOX: MK886, 5, 10, 12-LOX: 2-TEDC) and P450 inhibitors (global P450: SKF525, 1-ABT), with the goal of inhibiting the primary routes of metabolism for DHA. Interestingly, Nrf2 activity increased when cells were treated with DHA combined with metabolism inhibitors, and in some cases these increases were significantly increased above inhibitors alone or compared to DHA alone ($P<0.05$, Figure 2.2A, B, C). None of these treatments significantly decreased cell viability (Appendix A, Figure 5.2A, B, C). When HepG2-ARE cells were treated with three pathway inhibitors (MK886, Indo and 1-ABT) and DHA simultaneously, there was a significant reduction in Nrf2 activity, however, there was also a significant decrease in cell viability with these treatments (data not shown). These results suggest that certain DHA metabolites are not responsible for Nrf2 activity, but do not rule out the possibility

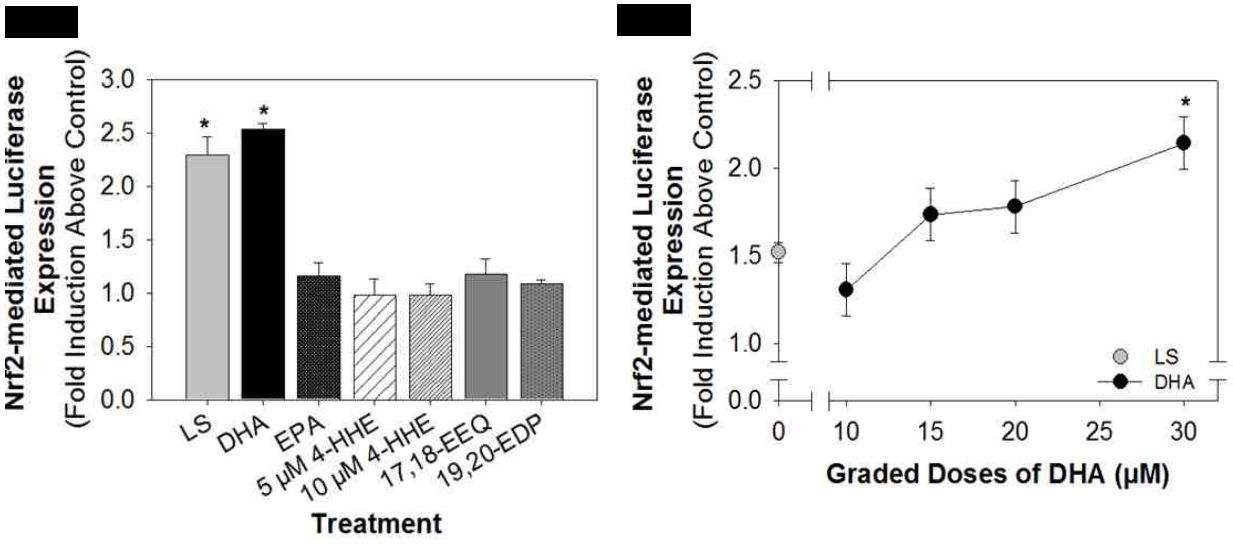

Figure 2.1. Nrf2-mediated luciferase expression in HepG2-ARE cells treated with n-3 PUFAs, 4-HHE and epoxides. Cells were treated with A) n-3 PUFAs DHA and EPA, positive control LS, DHA non-enzymatic oxidation product 4- HHE, and epoxide metabolites of EPA, 17,18-EEQ and DHA 19,20-EDP; and B) graded doses of DHA. Data were analyzed by one-way ANOVA. *P<0.05 compared to EPA, 4-HHE & epoxides (A), *P<0.05 compared to 10 µM DHA (B); n=3 for all treatments. (Abbreviations: Nrf2: Nuclear factor (erythroid-derived 2)-like 2; HepG2-ARE: HepatocyteG2-antioxidant response element; n-3 PUFAs: Omega-3 polyunsaturated fatty acids; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; LS: L-sulphoraphane; 4-HHE: 4-hydroxyhexenal; 17,18-EEQ: 17,18- epoxyeicosatetraenoic acid; 19,20-EDP: 19,20- epoxydocosahexaenoic acid)

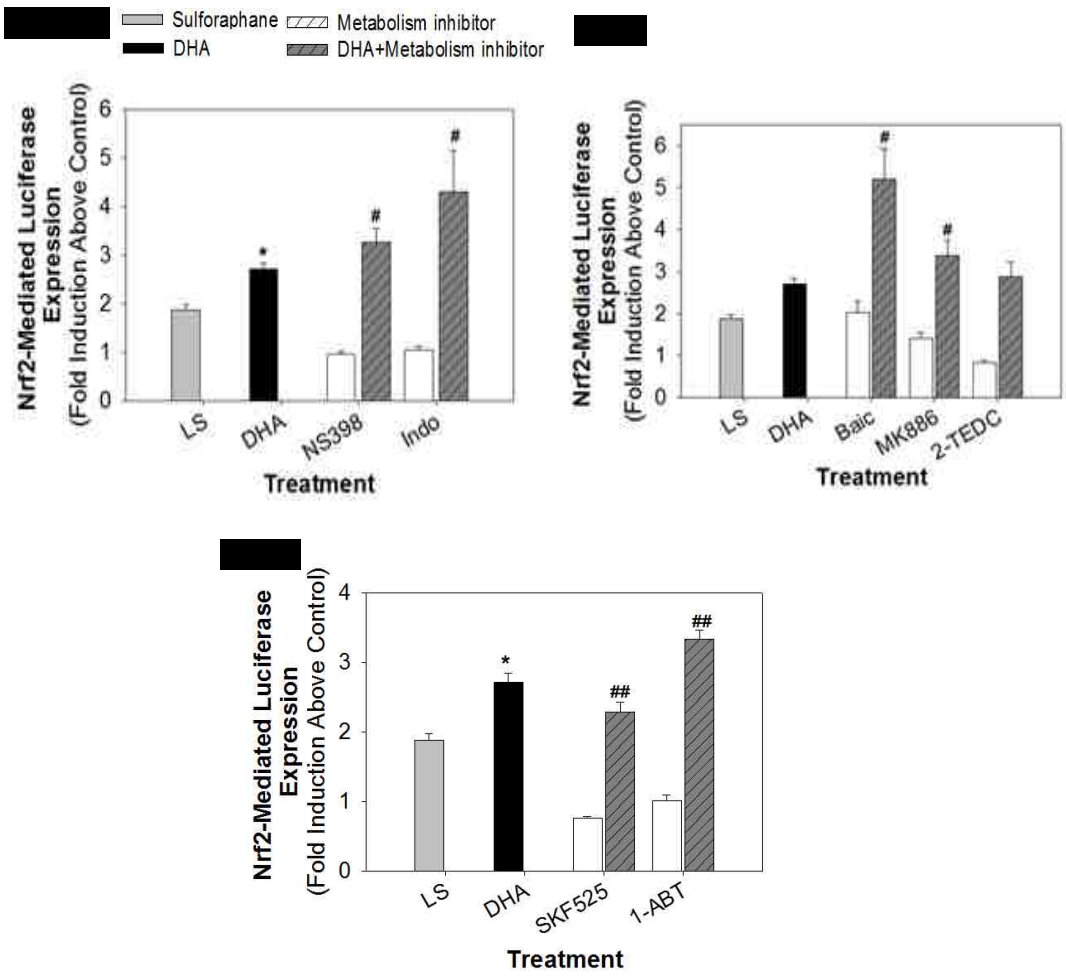

Figure 2.2. Nrf2-mediated luciferase expression HepG2-ARE cells treated with A) COX, B) LOX and C) P450 inhibitors with and without DHA. Data were analyzed by one-way ANOVA on ranks for COX and LOX analysis and standard one-way ANOVA for P450 analysis. *P<0.05 compared to NS398, Indo, SKF525 & 1-ABT alone; #P<0.05 vs respective inhibitor alone; ##P<0.05 vs DHA; n=3 for 1-ABT & 2-TEDC +/-DHA, n=7 for all other treatments. (Abbreviations: Nrf2: Nuclear factor (erythroid-derived 2)-like 2; HepG2-ARE: HepatocyteG2-antioxidant response element; DHA: Docosahexaenoic acid; LS: L-sulphoraphane; COX: Cyclooxygenase; LOX: Lipoxygenase; P450: Cytochrome P450; NS398: N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide; Indo: Indomethacin; Baic: Baicalein; MK886: 1-[(4-chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]-α,α-dimethyl-5-(1-methylethyl)-1H-indole-2-propanoic acid; 2-TEDC: 2-(1-Thienyl)ethyl 3,4-dihydroxybenzylidenecyanoacetate; SKF525: β-diethylaminoethyl diphenylpropylacetate hydrochloride; 1-ABT: 1-aminobenzotriazole)

of the formation of other enzymatic or non-enzymatic DHA metabolism leading to Nrf2 activation.

3.3. *DHA auto-oxidation responsible for Nrf2 activity.*

In this experiment, HepG2-ARE cells were treated with new DHA stock from the same supplier as well as a new DHA stock from a second commercial supplier. Neither of these two new stocks of DHA activated Nrf2, compared to the older stock of DHA used in previous experiments or to the positive control (Figure 2.3). These data show that there were unknown components in the old stock of DHA responsible for Nrf2 activation.

As previously noted, 4-HHE is a non-enzymatic peroxidation product of DHA that has been reported to activate Nrf2. Mass spectrometry was used to assess the composition of a fresh stock of 4-HHE and to assess whether our fatty acid stocks contained 4-HHE. Our stock of EPA, new stock of DHA from commercial supplier 1 and 2, and old DHA stock from commercial supplier 1 did not contain 4-HHE (Figure 2.4). Thus, 4-HHE was not responsible for Nrf2 activation in our cell culture model. LC-MS was performed on our stock of EPA, new stock of DHA from commercial supplier 1 and 2, and old DHA stock from commercial supplier 1 to assess oxidation products. While each of the LC-MS chromatograms revealed oxidation products, (single pink peak = compound tested; oxidation products: blue = ketone, red peaks = peroxy compounds, green peaks = alcohol), the old DHA stock from commercial supplier 1 revealed oxidation peaks. These oxidation peaks included ketone peaks that reached 3-800,000 au, peroxy peaks that reached 3-700,000 au, and alcohol peaks that reach 25-

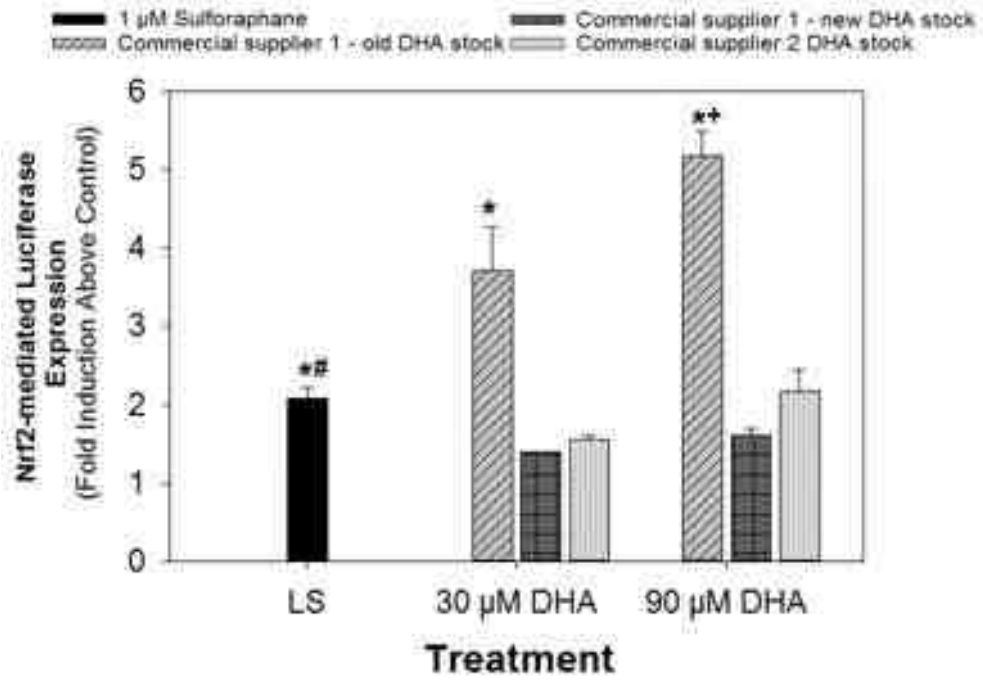

Figure 2.3. Nrf2-mediated luciferase expression HepG2-ARE cells treated with old and New DHA stocks. Cells were treated with LS, or 30 or 90 µM of the 1-yr old DHA stock, new DHA stock from commercial supplier 1 and new DHA stock from commercial supplier 2. Responses to DHA stocks were compared within a concentration via one way ANOVA; ANOVA was executed on ranks for comparing 30 µM DHA samples. T-test was used to compare LS to each treatment group; T-test was executed using rank sum test for comparing LS to 30 µM commercial supplier 1 old DHA stock. n=6 for LS and commercial supplier 2; n=3 for rest of treatments. *$P<0.05$ compared to 30 µM commercial supplier 1 new, $^{+}P<0.05$ compared to commercial supplier 2, #$P<0.05$ compared to 30 µM commercial supplier 2. (Abbreviations: Nrf2: Nuclear factor (erythroid-derived 2)-like 2; HepG2-ARE: HepatocyteG2-antioxidant response element; DHA: Docosahexaenoic acid; LS: L-sulphoraphane)

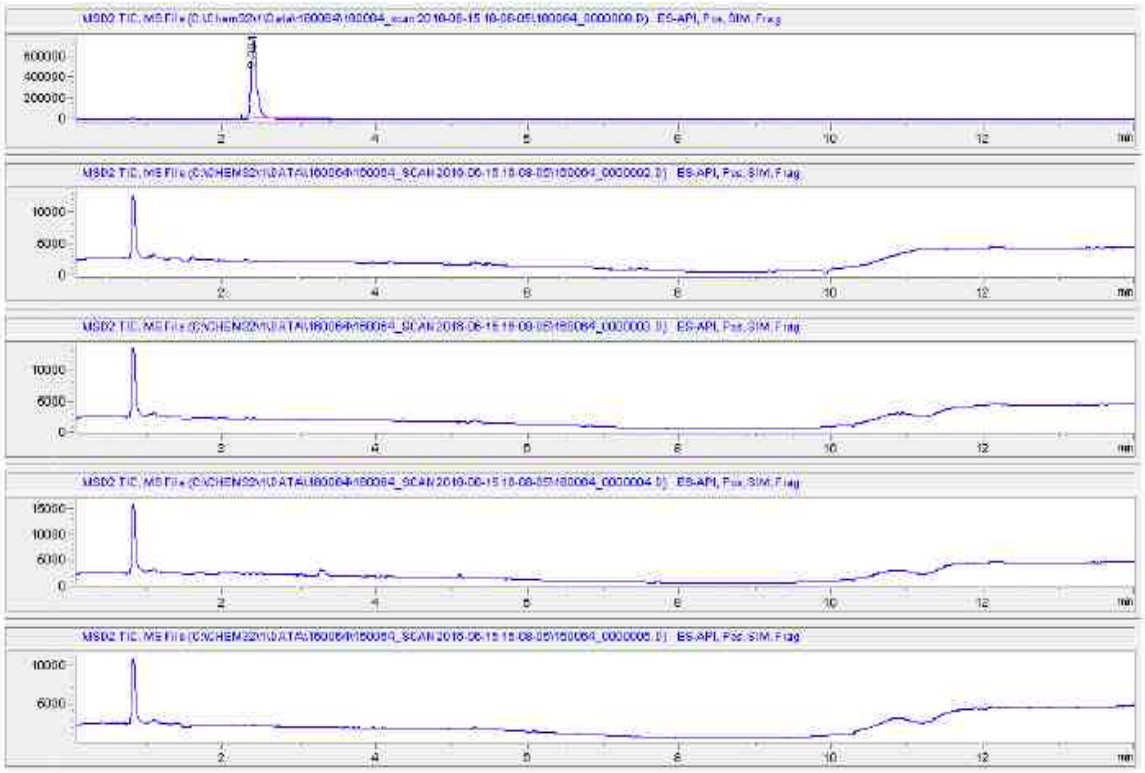

Figure 2.4. Mass spec traces of 4-HHE in fatty acids compared to 4-HHE stock. Mass spec of 4-HHE from 4-HHE stock of commercial supplier 1 in top panel, and 4-HHE traces in EPA from commercial supplier 1 in second panel from top, DHA from commercial supplier 2 in the third panel from the top, 1-yr old DHA stock from commercial supplier 1 in fourth panel from top, and new DHA stock from commercial supplier 1 in the bottom panel. (Abbreviations: 4-HHE: 4-hydroxyhexenal; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; LS)

50,000 au; while ketone and peroxy peaks were similar to the ketone peaks, there was one distinguished ketone peak at approximately 9 minutes that was not observed in the peroxy peaks (Figure 2.5-2.8).

The oxidation products of the old DHA stock from commercial supplier 1 were separated into different fractions, and each fraction was assessed for its ability to activate Nrf2 in our HepG2-ARE cells. Fractions A, B and C contained different groups of oxidation products, while fraction D contained all of the oxidation products together (Figure 2.9A). Only fraction D, containing all of the oxidation products activated Nrf2 (Figure 2.9B), while fractions A, B and C failed to activate Nrf2. These results suggest that multiple products may be necessary to activate Nrf2 or that the concentration of oxidation products was sufficiently high only in fraction D to activate Nrf2 in this system.

A previous study had identified a ketone metabolite of arachidonic acid as having the ability to activate Nrf2, 15-oxo-ETE (Y. Yang et al. 2016). In order to screen if oxo-DHA metabolites could be responsible for Nrf2 activation, we took two approaches. First, we began to develop an enzymatic assay to synthesize oxo-DHA compounds from their precursors. Second, we obtained the only oxo-DHA ketone compound that was commercially available, 17-oxo-DHA, and then we compared the ability of 15-oxo-ETE and 17-oxo-DHA to activate Nrf2 in HepG2-ARE culture model. Interestingly, 17-oxo-DHA increased Nrf2 activity significantly in a dose-dependent and treatment-dependent manner ($P=0.003$) and treatment effect ($P=0.039$) in a two-way ANOVA analysis, and significantly increased in Nrf2 activation at the 10 μM concentration, compared to 15-

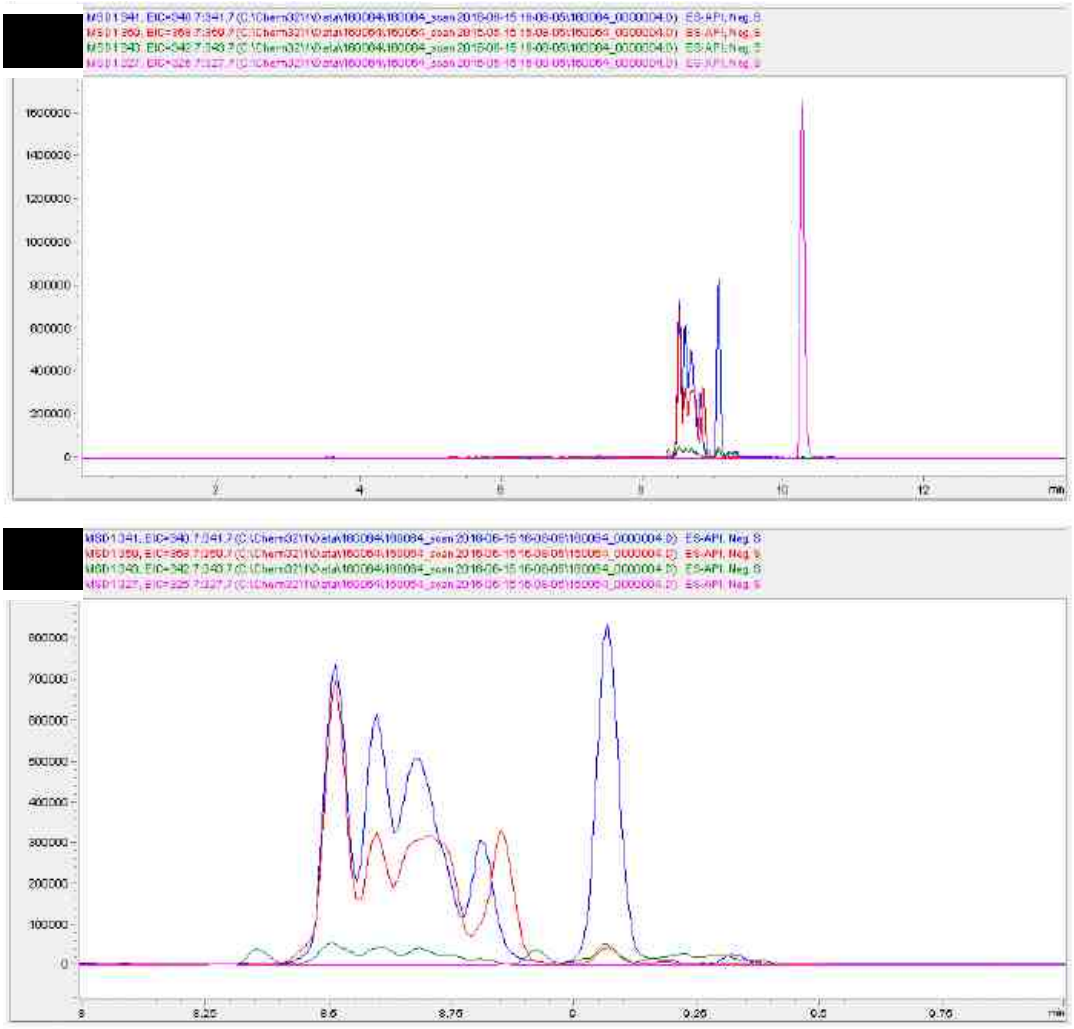

Figure 2.5. Liquid chromatography-mass spec chromatograms of 1-yr old DHA stock from commercial supplier 1. A. Total DHA stock, single pink peak=DHA, blue, red and green peaks=products of oxidation; B. Zoomed-in image of oxidation products; blue = ketone, red peaks = peroxy compounds, green peaks = alcohol. (Abbreviations: DHA: Docosahexaenoic acid)

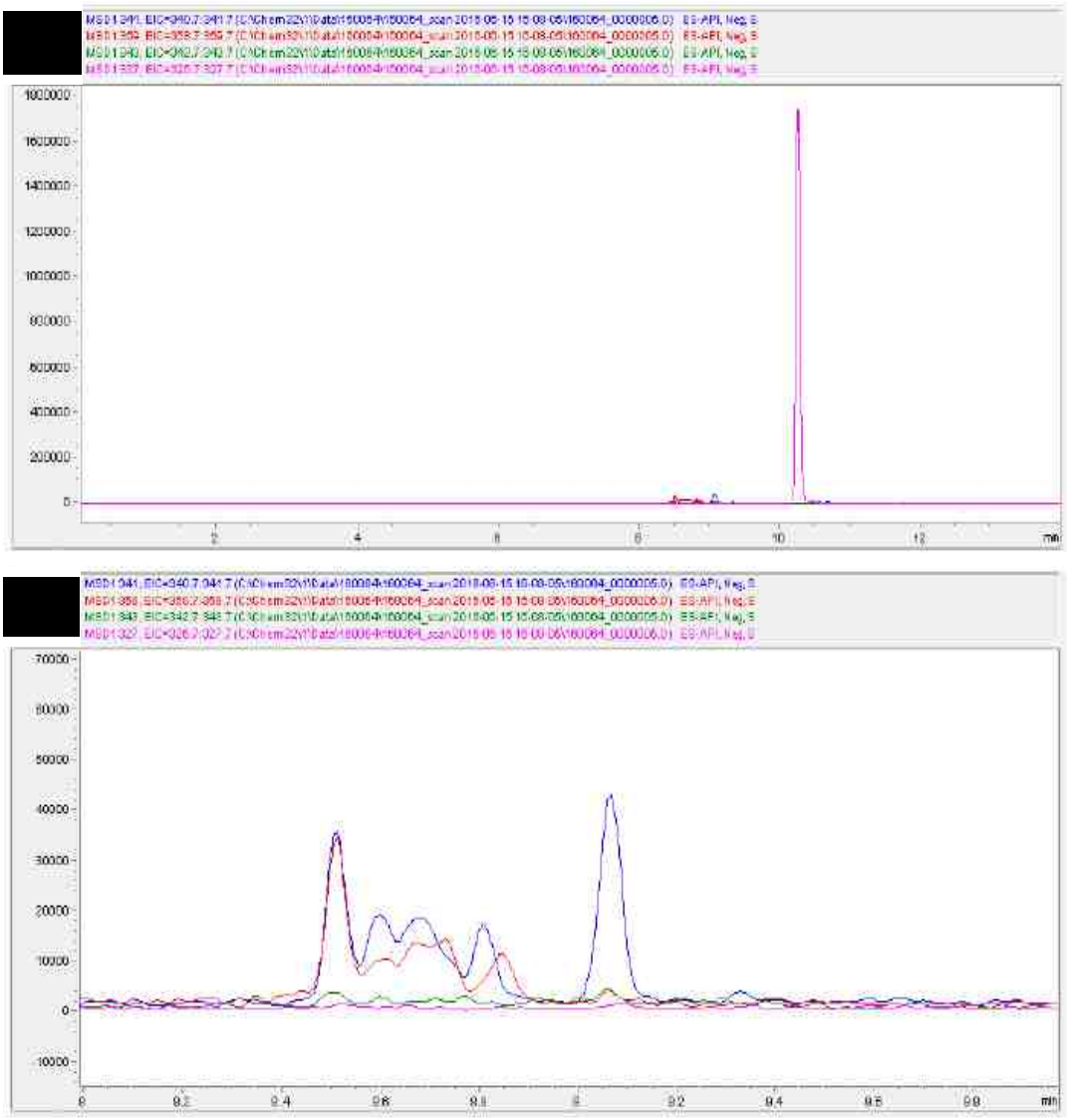

Figure 2.6. Liquid chromatography-mass spec chromatograms of DHA stock from commercial supplier 1. A. Total DHA stock, single pink peak=DHA, blue, red and green peaks=products of oxidation; B. Zoomed-in image of oxidation products; blue = ketone, red peaks = peroxy compounds, green peaks = alcohol.
(Abbreviations: DHA: Docosahexaenoic acid)

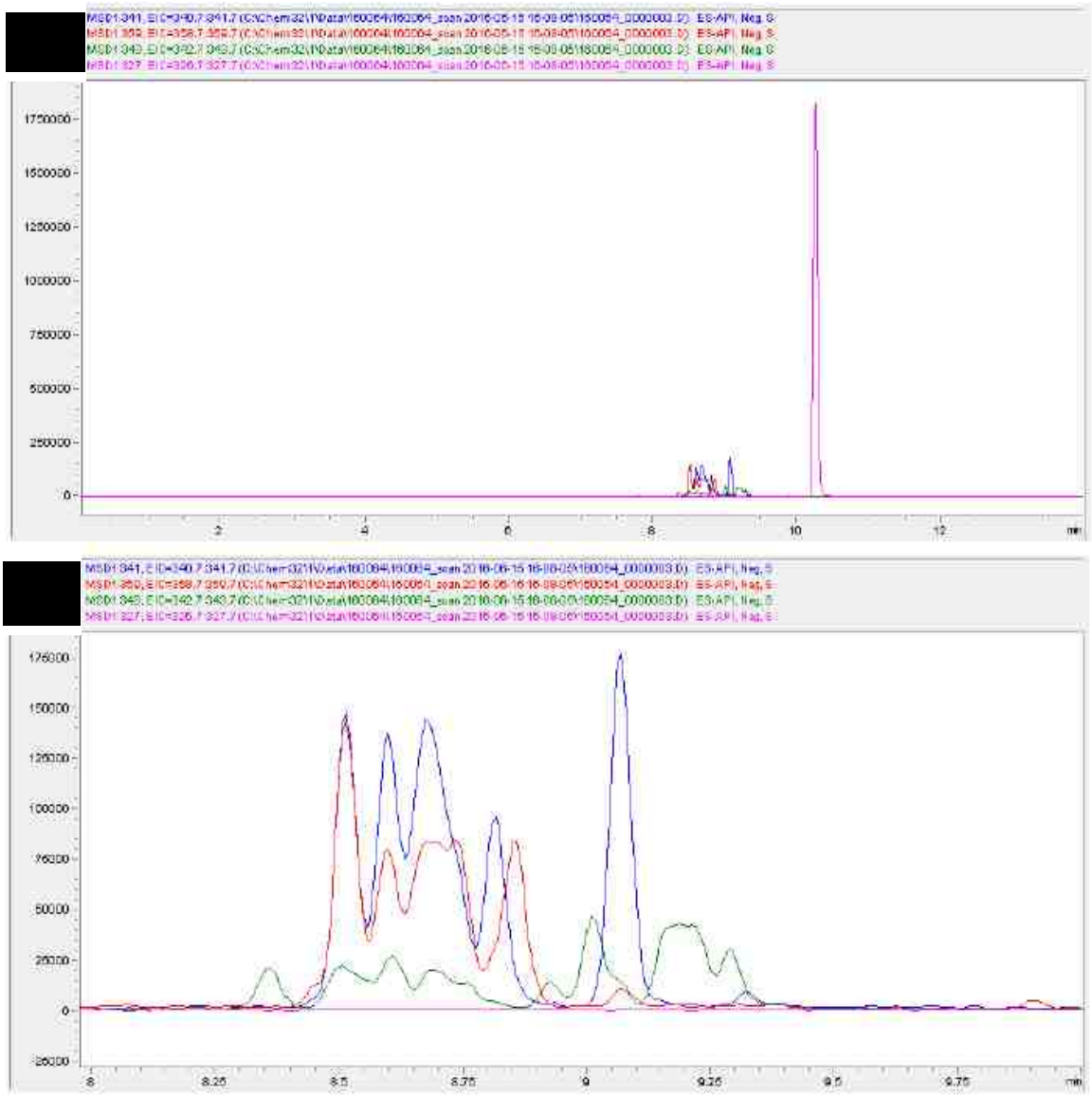

Figure 2.7. Liquid chromatography-mass spec chromatograms of DHA stock from commercial supplier 2. A. Total DHA stock, single pink peak=DHA, blue, red and green peaks=products of oxidation; B. Zoomed-in image of oxidation products; blue = ketone, red peaks = peroxy compounds, green peaks = alcohol. (Abbreviations: DHA: Docosahexaenoic acid)

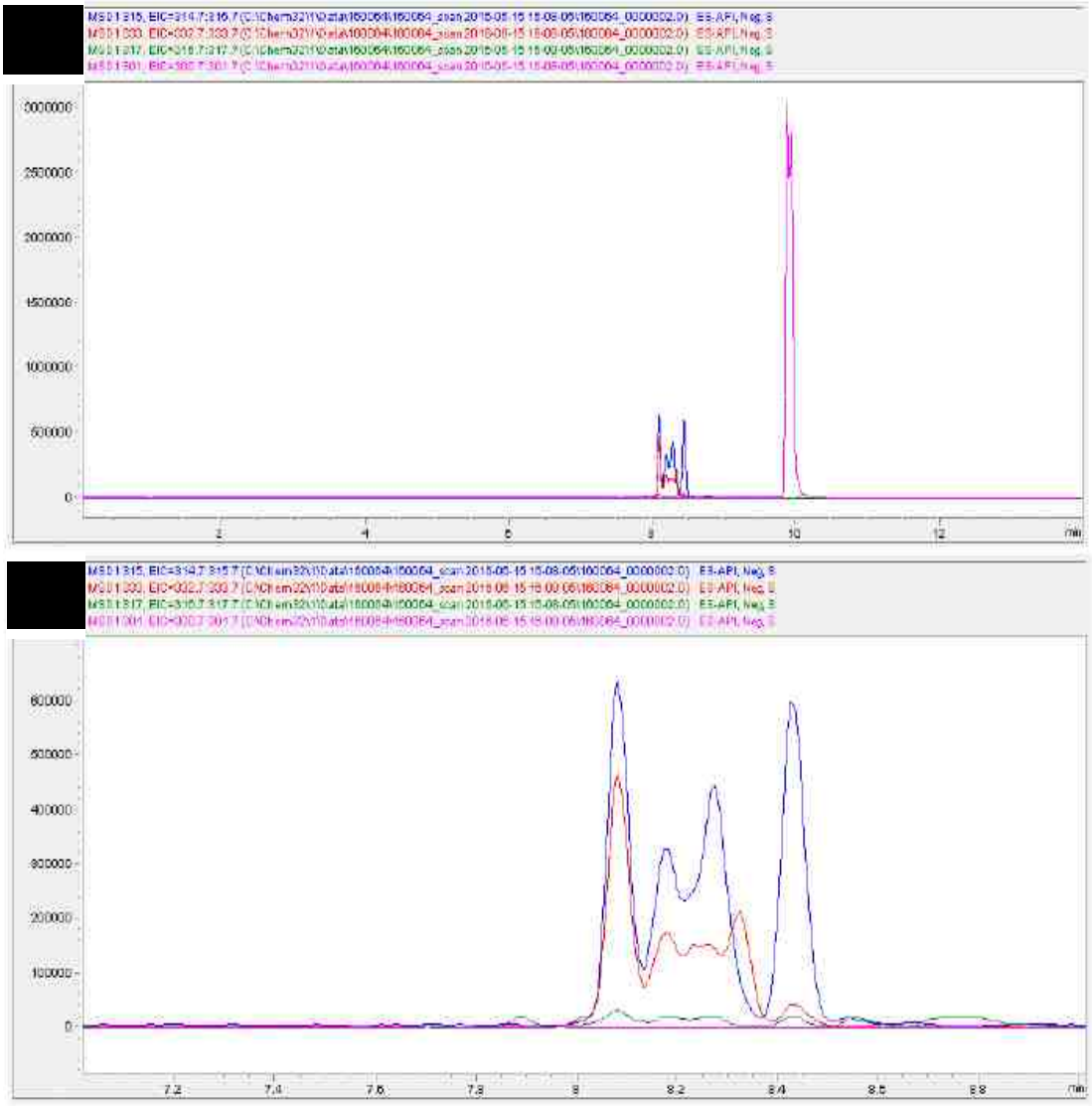

Figure 2.8. Liquid chromatography-mass spec chromatograms of EPA stock from commercial supplier 1. A. Total EPA stock, single pink peak=EPA, blue, red and green peaks=products of oxidation; B. Zoomed-in image of oxidation products; blue = ketone, red peaks = peroxy compounds, green peaks = alcohol. (Abbreviations: EPA: eicosapentaenoic acid**)**

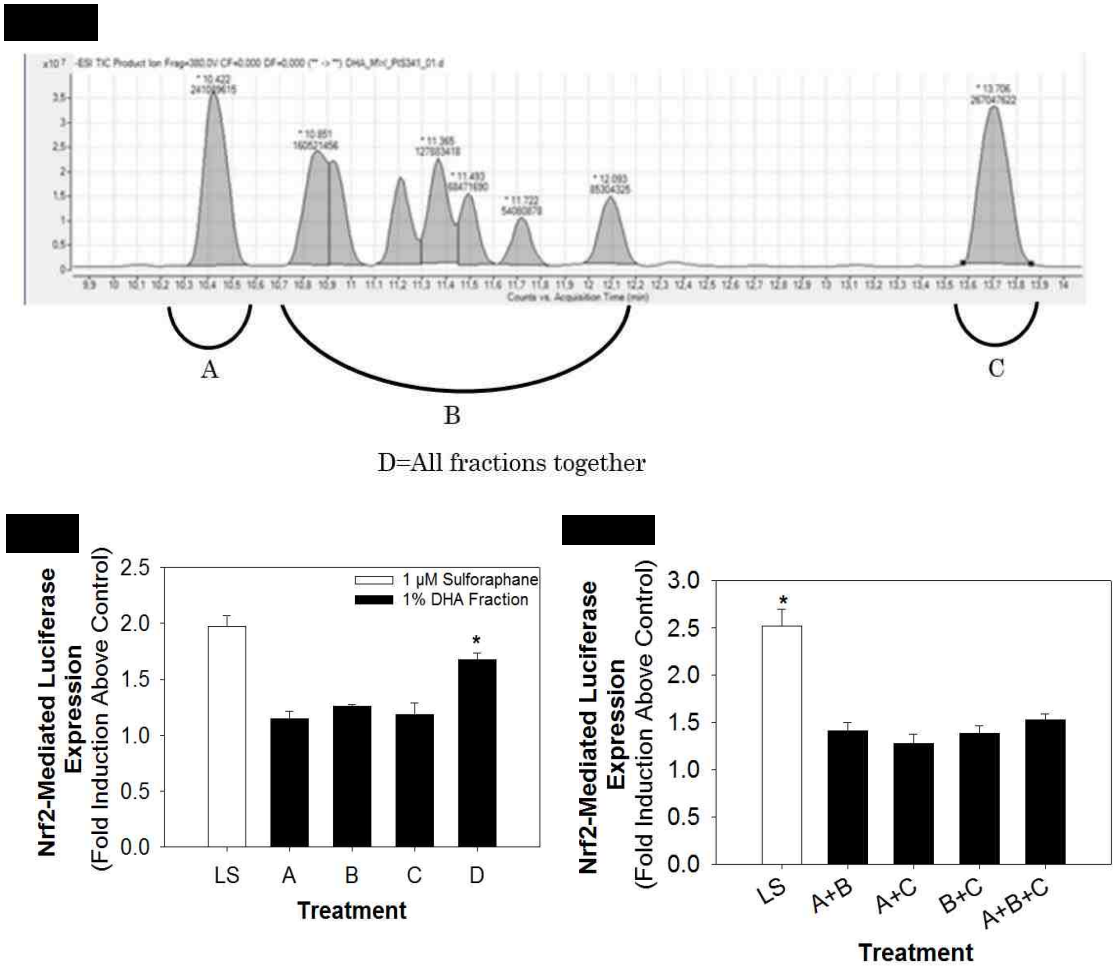

Figure 2.9. Nrf2-mediated luciferase expression HepG2-ARE cells treated with LS and four different fractions of old DHA stock. A. Oxidation products present in individual fractions of 1-yr old DHA stock from commercial supplier 1; B. Nrf2 activation by individual fractions of 1-yr old DHA stock from commercial supplier 1 and LS, n=3 for all treatments; C. Nrf2 activation from combined fractions of old DHA stock from commercial supplier 1 and LS; when 2 fractions were combined, the total vehicle volume remained the same, but the amount of each fraction added was one-half of that added in panel B; when 3 fractions were combined, the amount of each fraction added was one-third of that added in panel B, n=10 for all treatments. Data were analyzed via one-way ANOVA. In panel A, D (which was combined fractions of A, B and C) was significantly higher than fractions A, B or C alone (*P<0.05), but not significantly different than LS; combined fractions in panel B were not significantly different from each other, and LS treatment significantly increased Nrf2 activity compared to all other fraction combinations (*P<0.05). (Abbreviations: Nrf2: Nuclear factor (erythroid-derived 2)-like 2; HepG2-ARE: HepatocyteG2-antioxidant response element; LS: L-sulphoraphane DHA: Docosahexaenoic acid)

oxo-ETE (Figure 2.10). Thus, a ketone product of DHA, 17-oxo-DHA, increased Nrf2 activity. Future studies with 14- and 16-oxo-DHA will be conducted when sufficient amounts of materials are generated and purified.

3.4 *17-oxo-DHA increased Nrf2 localization to the nucleus and downstream Nrf2-activated genes in HUVECs*

To assess if 17-oxo-DHA could activate Nrf2 in the vasculature, we assessed Nrf2 localization and expression of Nrf2-regulated genes in HUVECs. Thus, Nrf2 immunofluorescence was assessed in HUVECs treated with positive control LS, 17-oxo-DHA or vehicle for 3 hours. Compared to vehicle, cells treated with 17-oxo-DHA exhibited increased Nrf2 nuclear localization which appeared similar to the response to LS (Figure 2.11). Under these same treatments for 3, 6 or 12 hours, mRNA expression of Nrf2-regulated genes Hmox1, Nqo1 and Gclc were significantly increased by 17-oxo-DHA, compared to vehicle (Figure 2.12). We found that 17-oxo-DHA significantly increased Gclc mRNA expression by 1.5 to almost 2.5-fold, and was significantly increased compared to LS and vehicle, although at the 12-hr time point, LS significantly increased Gclc mRNA expression to 1.5 fold compared to vehicle ($P<0.05$, Figure 2.12A). Furthermore, 17-oxo-DHA significantly increased Nqo1 mRNA expression by 2 to 6-fold over vehicle, and LS had a similar level of induction (2 to 4-fold induction over vehicle), although the 12-hr time point showed a significant increase in Nqo1 mRNA expression with 17-oxo-DHA treatment compared to vehicle and LS ($P<0.05$, Figure 2.12B). Finally, 17-oxo-DHA increased Hmox1 mRNA expression 50-fold over vehicle at 3 hrs, approximately 175-fold over vehicle at 6 hrs, and decreased to 50-fold over vehicle at 12 hrs ($P<0.05$), and LS also increased Hmox1 mRNA expression compared to

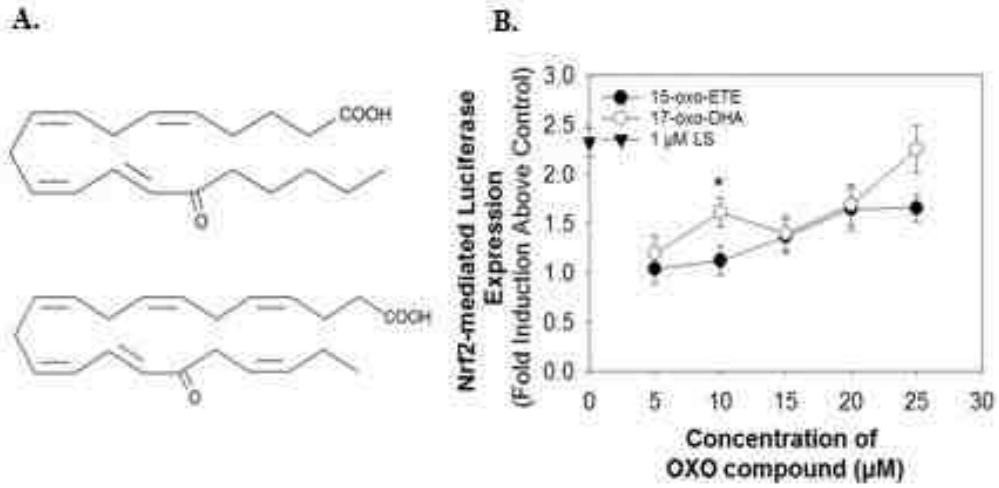

Figure 2.10. Structure of 15-oxo-ETE and 17-oxo-DHA and their Nrf2-mediated luciferase expression in HepG2-ARE cells. A. Structures of 15-oxo-ETE (top) and 17-oxo-DHA (bottom), B. HepG2-ARE cells treated with increasing doses of 17-oxo-DHA and 15-oxo-EET. Data were rank-transformed and analyzed via two-way ANOVA between dose and treatment, and there was a significant dose effect (P=0.003) and treatment effect (P=0.039); n=5 for positive control LS, n=3 for all other treatments. *P<0.05 vs 10 µM 15-oxo-ETE, and *P<0.05 significant compound effect differences between 15-oxo-ETE and 17-oxo-DHA. (Abbreviations: 15-oxo-ETE: 15-oxo-5Z,8Z,11Z,13E-eicosatetraenoic acid; 17-oxo-DHA: 17-oxo-4(Z),7(Z),10(Z),13(Z),15(E),19(Z)-DHA Nrf2: Nuclear factor (erythroid-derived 2)-like 2; HepG2-ARE: HepatocyteG2-antioxidant response element; LS: L-sulphoraphane)

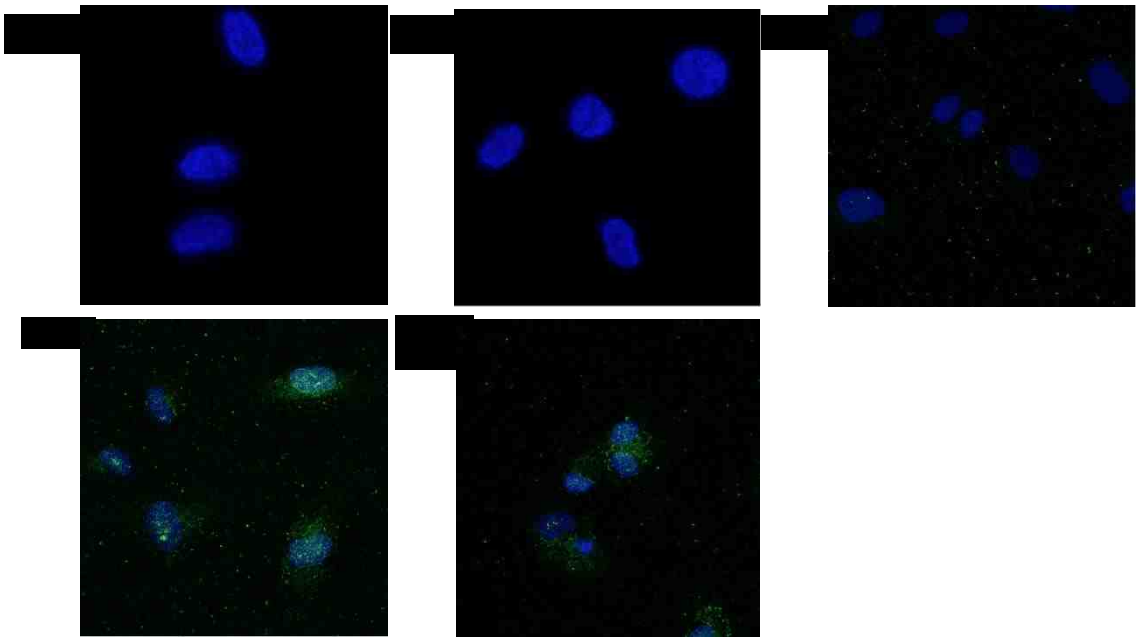

Figure 2.11. Fluorescent images of HUVECs after 3 hours of 17-oxo-DHA, nuclei shown with DAPI staining (blue), Nrf2 shown with FITC staining (green). A. Primary antibody only (negative control), B. Secondary antibody only (negative control), C. Vehicle (negative control), D. 1 µm LS, positive control, E. 25 µM 17-oxo-DHA. (Abbreviations: HUVECs: Human umbilical vein endothelial cells; 17-oxo-DHA: 17-oxo-4(Z),7(Z),10(Z),13(Z),15(E),19(Z)-DHA; DAPI: 4',6-diamidino-2-phenylindole; Nrf2: Nuclear factor (erythroid-derived 2)-like 2; FITC: Fluorescein isothiocyanate; LS: L-sulphoraphane)

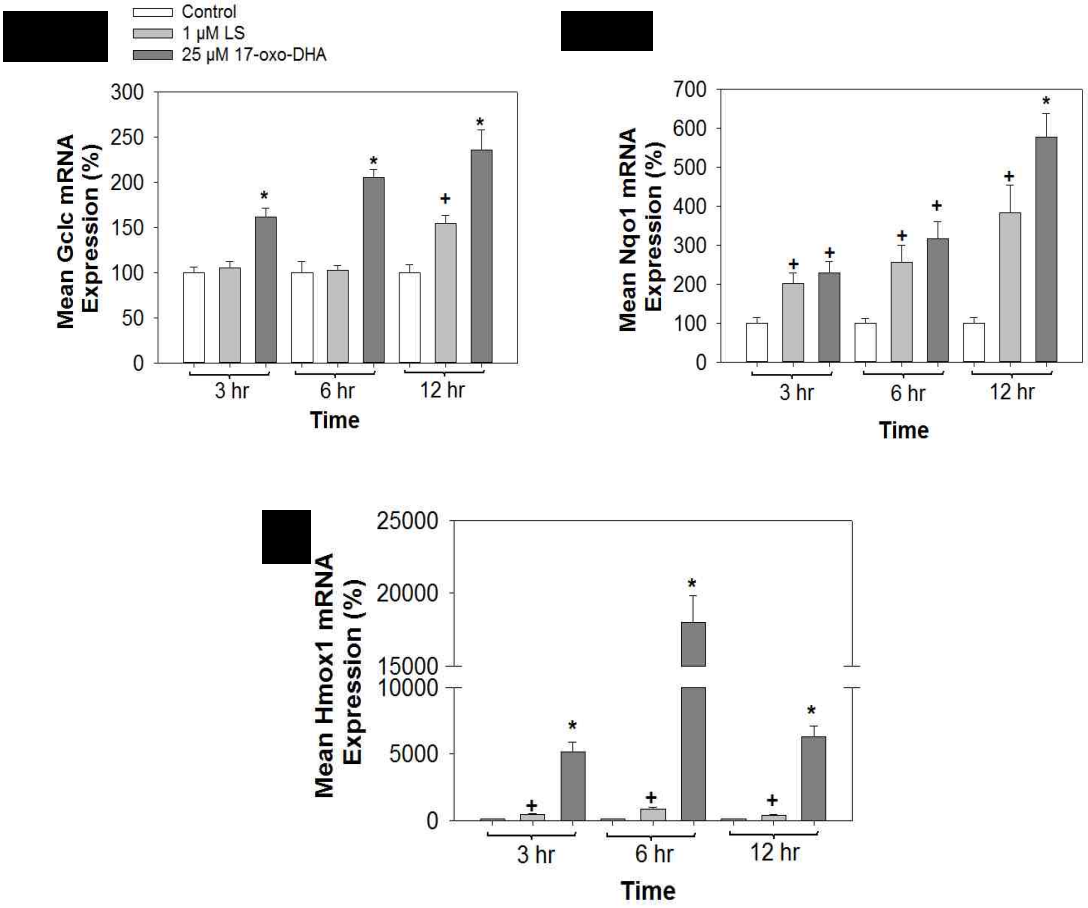

Figure 2.12. mRNA expression of Gclc, Nqo1 and Hmox1 following 3, 6 and 12-hour treatment of vehicle (negative control), LS (positive control) and 17-oxo-DHA in HUVECs. Data were analyzed by two-way ANOVA within each gene; standard one-way was used for Gclc; data were transformed to log10 for Nqo1 and rank transformed for Hmox1. *P<0.05 vs LS and vehicle control, +P=0.05 vs vehicle control; n=6 for each treatment. (Abbreviations: Gclc: Glutamate-cysteine ligase catalytic subunit; Nqo1: NAD(P)H quinone dehydrogenase 1; Hmox1: Heme-oxygenase-1; LS: L-sulphoraphane; 17-oxo-DHA: 17-oxo-4(Z),7(Z),10(Z),13(Z),15(E),19(Z)-DHA; HUVECs: Human umbilical vein endothelial cells)

vehicle at all three time points ($P<0.05$, Figure 2.12C). These data show Nrf2 localization as well as its downstream-regulated genes were increased with 17-oxo-DHA.

DISCUSSION AND CONCLUSIONS

This study observed that oxidation products, and in particular ketones, of DHA were responsible for Nrf2 activity, not necessarily DHA by itself in our cell culture model. We originally hypothesized that EPA and DHA epoxides would activate Nrf2 expression and downstream antioxidant genes based on their electrophilic nature and on our earlier findings that dietary n-3 PUFA supplementation reduced oxidative stress and increased antioxidant genes in mice exposed to cigarette smoke (Wiest, Walsh-Wilcox, and Walker 2017). However, data from the HepG2-ARE cell culture model showed that neither 17,18-EEQ or 19,20-EDP activated Nrf2, and that of the parent fatty acids, DHA, but not EPA, activated Nrf2, which is consistent with previous literature (Stulnig et al. 2013). Furthermore, adding inhibitors to prevent DHA metabolism failed to decrease Nrf2 activation, but instead further activated Nrf2. When a new DHA stock was purchased from the same supplier (commercial supplier 1), Nrf2 was not activated, revealing that something in the old DHA stock was activating Nrf2. After investigating, we discovered higher levels of oxidation products, and in particular, ketones present in the old DHA stock from commercial supplier 1. We found that one DHA ketone obtained commercially, 17-oxo-DHA, increased Nrf2 activation in the HepG2 cell culture model, and stimulated Nrf2 localization to the nucleus and increased downstream antioxidant gene expression in HUVECs.

Non-enzymatic oxidation products of DHA increase Nrf2 expression. Although 17-oxo-DHA is enzymatically produced following COX metabolism of DHA to mono-hydroxy products and subsequent metabolism by 15-prostaglandin dehydrogenase (15-PGDH) to oxo-DHA products (Cipollina et al. 2016), these ketones in our old DHA stock from commercial supplier 1 were non-enzymatically produced. Non-enzymatic oxidation of n-3 PUFAs, and in particular DHA, has already been shown to activate Nrf2 (Ishikado et al. 2013), but this specific example highlighted the DHA peroxy-metabolite 4-HHE. However, 4-HHE did not activate Nrf2 in our HepG2 model and the reason for this discrepancy is not known.

Interestingly, treating our HepG2-ARE cells with DHA and inhibitors of its metabolism further increased Nrf2 activation, showing that preservation of DHA oxidation products were needed to activate Nrf2 in our cell culture model. One COX inhibitor used here, Indo, and other non-steroidal anti-inflammatory drugs have been shown to stimulate 15-PGDH activity, which might lead to increased endogenous production of oxo-DHA metabolites (Tai, Chi, and Tong 2011). When we discovered oxidation products in our old DHA stock from commercial supplier 1, the old DHA stock from commercial supplier 1 revealed oxidation peaks that reached 800,000 au which was higher than all of our other tested stocks. Furthermore, there was also a ketone peak present in all three DHA stocks at approximately 9 minutes that was not present in our EPA stock, and this was one characteristic that did not match the peroxy-oxidation products, which is why we focused on ketones.

To conduct an initial screen to determine if DHA ketones could be responsible for the Nrf2 activation, we obtained the only commercially available DHA ketone, 17-oxo-

DHA. In previous studies, 17-oxo-DHA has been shown to significantly increase anti-inflammatory and antioxidant effects (Cipollina et al. 2014; Cipollina et al. 2016). Additionally, a previous study has shown that 17-oxo-DHA stimulates Nrf2 nuclear translocation and increased protein expression of Hmox1 and Nqo1 in macrophages (Groeger et al. 2011). Furthermore, the activation of Hmox1 and glutathione (GSH) has been shown in bronchial epithelial cells (Ceriello et al. 2014), but to the best of our knowledge, this has yet to be shown with reference to CV endothelial cells or increased Gclc expression.

One ketone of the omega-6 (n-6) PUFA arachidonic acid, 15-oxo-ETE, is structurally similar to 17-oxo-DHA, and is produced by the same metabolism as 17-oxo-DHA—15-HETE undergoes COX metabolism and subsequent metabolism by 15-PGDH (Powell and Rokach 2015). In previous studies, 15-oxoETE has been found to decrease endothelial cell proliferation (Wei et al. 2009), activate peroxisome proliferator-activated receptor γ, activate Nrf2 and decrease nuclear factor kappa-light-chain-enhancer of activated B cells (Shiraki et al. 2005; Y. Yang et al. 2016), suggesting 15-oxo-ETE may have antiangiogenic, antioxidant and anti-inflammatory properties. With increased n-3:n-6 PUFA ratio in the tissues, there is greater presence of n-3 PUFA monohydroxy metabolites compared to n-6 PUFA monohydroxy metabolites (Chiu et al. 2017), suggesting that there may be increased n-3 PUFA ketone metabolites such as 17-oxo-DHA. Considering the numerous CV-protective effects of n-3 PUFAs (Lavie et al. 2009), the effects of these DHA oxidation products remain largely understudied, but may have similar effects to 15-oxo-ETE.

This study contains several caveats. The first caveat includes that the individual fractions of the older stock of DHA did not activate Nrf2. While the reason for this is not fully understood, it may be due to individual fractions degrading and not having the effect they do when they are combined with other fractions. This highlights the instability of these compounds and that further observation must be carried out to investigate the interaction between these metabolites (Metherel and Stark 2016). Furthermore, the only commercially available ketone product of DHA is 17-oxo-DHA; assessing more DHA ketones in our cell culture model would allow us to observe if particular ketones activate Nrf2 more than other ketones.

Thus, future directions of our cell culture study involve synthesizing and purifying additional oxo-DHA ketones, and treating cells with these ketones to assess Nrf2 activity. Another product of DHA non-enzymatic oxidation that appeared in the LC-MS chromatograms of our old DHA stock from commercial supplier 1, peroxy-metabolites, were not further assess in our cell culture model, and could be assessed for future investigation. Furthermore, precise determination of compounds present in the old stock of DHA from commercial supplier 1 could not be completed due to technical limitations. Particular compounds may be assessed in future studies to assess their ability to activate Nrf2 and increase expression of downstream antioxidant gene expression. One DHA peroxy-oxidation product, 4-HHE, was tested in our cell culture system and did not show any Nrf2 activation. However, we could broaden future analysis to include more DHA peroxy-oxidation products. In the future, we will be assessing other DHA ketone and peroxy-oxidation products.

This study highlights the relevance and importance of fatty acid storage, which has also been previously noted (Metherel and Stark 2016). In conclusion, non-enzymatically-controlled oxidation products, specifically ketones of DHA, show promise in further understanding the vascular-protective and antioxidant mechanisms of n-3 PUFAs.

CHAPTER III

Association of Omega-3 Polyunsaturated Fatty Acids and Their Metabolites with Oxidative Stress and Vascular Function in Cigarette Smokers

Zehr, K.R., Ring, K., Langsfeld, M., Rothe, M., Schunck, W.-H., Abrums, A., Naila, T., Elmaoued, A.A., Myers, O.B., Anderson, J.R., and Walker, Mary K. Association of Omega-3 Polyunsaturated Fatty Acids and Their Metabolites with Oxidative Stress and Vascular Function in Cigarette Smokers.

Submitted to *Prostaglandins, Leukotrienes and Essential Fatty Acids*

ABSTRACT

Evidence suggests that omega-3 polyunsaturated fatty acids (n-3 PUFAs) can reduce oxidative stress and improve impaired vascular function, events that often precede clinical cardiovascular disease. We enrolled 67 current smokers (37±9 yrs) lacking cardiovascular disease and hypothesized that n-3 PUFAs would associate negatively with oxidative stress (malondialdehyde [MDA] and 8-epi-prostaglandin F2α [8-epi-PGF2α]) and positively with flow-mediated dilation (FMD). We found that an omega-3 index (RBC% eicosapentaenoic plus docosahexaenoic acids [EPA+DHA]) ≥ 4.5% was associated with higher FMD, but not lower oxidative stress. Multivariate regression modeling revealed that conjugated linoleic acid, palmitic acid, and the ratio of linoleic acid diol-to-epoxide metabolites were predictive of MDA (R=0.652); 14,15-epoxyeicosatetraenoic acid and 10,17-dihydroxydocosahexaenoic acid were predictive of 8-epi-$PGF_{2\alpha}$, (R=0.416); and oleic acid, 16,17-dihydroxydocosapentaenoic acid, and eicosenoic acid were predictive of FMD (R=0.553). These results suggest that n-3 PUFAs may be vasoprotective in young healthy smokers, but other dietary fatty acids may also be beneficial.

INTRODUCTION

Cardiovascular disease (CVD) is the leading global cause of death, causing over 17 million deaths annually, and cigarette smoke (CS) is a major risk factor for CVD (Roth et al. 2015). Tobacco use is the second-leading cause of death in the United States, and is a risk factor for increased mortality from ischemic stroke, peripheral artery disease, coronary heart disease, atherosclerosis, and other chronic diseases (Mozaffarian et al. 2014; Ockene and Miller 1997). Vascular remodeling takes place in smokers, causing

the intimal layer of the vessels to thicken, creating a prothrombotic environment with increased plaque formation and extracellular matrix degradation. This stiffens the vessels and decreases vascular reactivity, setting the stage for CVD (Messner and Bernhard 2014).

A precursor to CS-induced CVD is endothelial dysfunction, which is characterized by a switch from vasodilatory and anti-inflammatory factors to constrictive and pro-inflammatory factors. Although underlying mechanisms are still being studied, the loss of the primary vasodilator, nitric oxide (NO), is a hallmark of endothelial dysfunction, and thus a precursor to many CV diseases (Cai and Harrison 2000; Cannon 1998). Flow-mediated dilation (FMD) is the gold standard for measuring endothelial function in patients, and can be used as a predictor of future CV events in healthy individuals (Rossi et al. 2008; Shechter et al. 2009). It has been shown in numerous studies that FMD is significantly impaired in smokers (Neunteufl et al. 2000; Ozaki et al. 2010; Wiest et al. 2015).

FMD is also impaired in individuals who have significantly increased oxidative stress (Cai and Harrison 2000; Neunteufl et al. 2000; Ozaki et al. 2010). Oxidants in CS include peroxynitrite, semiquinone radicals and hydrogen peroxide (Pryor and Stone 1993). There are also polycyclic aromatic hydrocarbons in CS which can be metabolized by cytochrome P450s into reactive radical cations, oxides and quinones (Shimada 2006). Increased reactive oxygen species (ROS) can contribute to endothelial dysfunction by reducing NO and inhibiting ATP-sensitive potassium channels (Serizawa et al. 2011; Jerkic, Sotov, and Letarte 2012; Y. Yang et al. 2010; Ozcan et al. 2002), thus impairing FMD.

Antioxidants and omega-3 polyunsaturated fatty acids (n-3 PUFAs), may be able to reduce CS-induced oxidative stress and improve FMD. In mouse tissues enriched with n-3 PUFAs, hydrogen peroxide production is decreased (Hagopian et al. 2010). Cardiomyocytes treated simultaneously with ROS-inducing drug doxorubicin and eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) have decreased levels of ROS, compared to untreated cells (Hsu, Chen, and Chen 2014). In humans, one study showed that there was a positive correlation between n-3 PUFAs in red blood cells and total plasma antioxidant capacity (Thorlaksdottir et al. 2006) and a recent review suggests that n-3 PUFAs can improve FMD in humans with classic risk factors for atherosclerosis (Zehr and Walker 2017). One of the mechanisms by which n-3 PUFAs mediate their antioxidant benefits may be through Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activation. Nrf2 is negatively regulated through ubiquitination by Kelch like-ECH-associated protein 1 (Keap1) and cullin3. Oxidized n-3 PUFAs, including EPA and DHA, have been shown to destabilize Keap1 and cullin3 binding, thus activating Nrf2 (Gao et al. 2007). Furthermore, heme-oxygenase 1 (Hmox1), a downstream antioxidant target of Nrf2, is induced in vascular smooth muscle cells treated with DHA (Stulnig et al. 2013), and the anti-inflammatory effects of DHA are dependent on the redox abilities of Nrf2 (Gruber et al. 2015). However, evidence of n-3 PUFAs increasing FMD through antioxidant means is limited.

To our knowledge, no one has assessed the relationship between antioxidant markers, n-3 PUFAs, FMD and oxidative stress markers in smokers lacking other CVD risk factors. More specifically, the mechanism by which n-3 PUFAs increase antioxidant capacity and reverse endothelial function has also not been fully elucidated. Our

objective was to identify dietary fatty acids that may have the potential to reduce oxidative stress markers and improve FMD in current cigarette smokers. We hypothesized that n-3 PUFAs and their metabolites would exhibit the strongest associations with oxidative stress and vascular dysfunction in young current cigarette smokers.

MATERIALS AND METHODS

Study subjects

We recruited male and female current smokers of various ethnicities and races (*n* = 67, Appendix B, Table 5.2) between the ages of 19-50 years. Current smokers were defined as having smoked > 0.5 pk/d for at least the past year. We excluded those subjects that were pregnant, had diagnosed hypertension or diabetes, and those who had a history of chronic kidney disease, ischemic heart disease, stroke, or heart failure. This study was approved by the University of New Mexico Institutional Review Board (HRRC: 15-033) and written and oral informed consent was obtained from all participants.

Brachial artery FMD

Subjects were asked to fast and refrain from smoking or drinking caffeinated beverages for 10 h prior to their appointment for assessment of brachial artery FMD. The FMD assessment was conducted between 0700-1000 in the same room at 70-72 ^{o}F and was performed by the same Registered Vascular Technologist. Blood pressure was measured in both arms, and then subjects were placed in a supine position where they

rested quietly for 15 min with the lights dimmed. Blood volume velocity (cm^3/s) and a longitudinal image of the brachial artery was recorded using a 15-7 linear array transducer in the arm with the highest systolic blood pressure. The ECG was monitored continuously to allow for post-exam image analysis during the same phase of the cardiac cycle. Reactive hyperemia was induced by inflating a blood pressure cuff on the upper forearm to 50 mmHg above the subject's systolic blood pressure. The cuff was inflated for 5 min and continuous scanning of the brachial artery was performed from 30 s prior to cuff release until 2 min after cuff release. Longitudinal view diameter measurements were recorded and the peak change in brachial artery diameter from baseline to the point of greatest diameter during the hyperemic phase was used to determine percent FMD. Brachial artery diameter measurements were conducted by a vascular surgeon using de-identified images.

Blood collection

Following the FMD procedure, whole blood SST samples were taken to measure HbA1c and lipids, using a point-of-care instrument. Additionally, whole blood EDTA samples were collected for RBC and plasma analysis of fatty acids and PUFA metabolites, respectively, while whole blood heparin samples were collected for analysis of plasma and RBC antioxidant markers, and oxidative stress markers. Urine samples were collected to measure cotinine (Abnova, Taiwan).

Analysis of RBC fatty acids and plasma PUFA metabolites

Twenty-three saturated, mono- and poly-unsaturated fatty acids were measured in packed RBCs (OmegaQuant, Sioux Falls, SD). Both n-3 and n-6 PUFA metabolites were measured in plasma by LC-MS/MS as described previously (Arnold et al. 2010). Briefly, 200 µL plasma was mixed with internal standards of 20-hydroxyeicosatetraenoic acid (HETE)-d6, 14,15-epoxyeicosatrienoic acid (EET)-d8, 14,15-dihydroxyeicosatrienoic acid (DHET)-d11, and 15-HETE-d8 (Cayman Chemical, Ann Arbor, MI, USA) in methanol. Each reaction underwent alkaline hydrolysis followed by neutralization to pH 6.0 with acetic acid. Solid phase extraction was performed using Varian Bond Elut Certify II columns, and HPLC measurements were performed using an Agilent 1200 HPLC system equipped with a Phenomenex Kinetex-C18 column. The HPLC was coupled with an Alilent 6460 triplequad mass spectrometer with an electrospray ionization source.

Analysis of oxidative stress markers and antioxidant enzymes

Oxidative stress was assessed from plasma via malondialdehyde (MDA) as measured via thiobarbituric acid reactive substances (TBARS) assay and 8-epi prostaglandin F2α (8-epi-$PGF_{2\alpha}$) via ELISA (Cayman Chemical). Activities of the antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), were assessed in plasma, while reduced and oxidized glutathione (GSH and GSSG) concentrations were measured in RBC lysates (Cayman Chemical).

Statistical analysis

We used SigmaPlot 13.0 (Systat Software Inc., San Jose, CA) for all statistical analyses. We hypothesized that n-3 PUFAs and their metabolites would positively correlate with antioxidant markers and FMD, and negatively correlate with indices of oxidative stress. Thus, our objective was to have adequate power to detect a relationship in multivariate regression analysis. We estimated needing a sample size of 94 to achieve 80% power to detect a correlation ($r \geq 0.35$) with three predictors and alpha=0.05. The detectable effect size with n= 67 and 80% power increased to $r \geq 0.38$.

Data are expressed as mean ± standard deviation (SD) unless labeled otherwise. We conducted correlation analysis of MDA, 8-epi-$PGF_{2\alpha}$, and FMD versus (a) each other, (b) antioxidant markers, including SOD, CAT, and GSH/GSSG, (c) individual clinical characteristics, including age, urinary cotinine, total cholesterol, high density lipoprotein, low density lipoprotein, systolic and diastolic blood pressure, triglycerides, HbA1c, and Framingham risk score, (d) individual RBC fatty acids (%), and (e) individual plasma PUFA metabolites (ng/mL), using a Spearman correlation test. We further conducted linear regression, multivariate regression, and forward stepwise selection multivariate regression analyses with those variables that were significant ($P < 0.05$) from Spearman analysis. We avoided backwards variable selection because <10 observations per candidate variable were available to estimate coefficients at the start of the procedure. Finally, PUFA metabolites were grouped based on their parent fatty acid (i.e. linoleic (LA), arachidonic (AA), EPA or DHA), their structure (i.e. epoxide, monohydroxy or dihydroxy), and/or the manner in which they are generated (i.e. cytochrome P450, lipoxygenase, cyclooxygenase, or autoxidation; Appendix B, Table

5.3). These groups of metabolites were used in forward selection multivariate analysis to determine their ability to predict plasma MDA, 8-epi-$PGF_{2\alpha}$, and FMD. Non-Gaussian distributions were normalized using $\log_{10}$ or rank transformation. $P < 0.05$ was considered statistically significant.

RESULTS

Subject characteristics

The cigarette smokers enrolled in this study were from different ethnicity and races (Appendix B, Table 5.2) and in good CV health, failing to exhibit any significant changes in classic CV disease risk factors, including blood pressure, triglycerides, and low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol (Table 3.1). The Framingham risk score, an estimate of the 10-year risk of developing coronary heart disease (Wilson et al. 1998) was calculated with a mean score of 2.4%, which represented a low risk of developing heart disease or having a heart attack in the next ten years.

Lack of association between oxidative stress markers and FMD

We assessed two oxidative stress markers in plasma, MDA and 8-epi-$PGF_{2\alpha}$, and an index of vascular function, FMD (Figure 3.1). The values were similar to those reported previously in smokers (MDA, 2.59 ± 1.08 μM; 8-epi-PGF2α, 43.98 ± 26.69 pg/mL; and FMD, 7.55 ± 5.50%) (Wiest et al. 2015; Liu et al. 1998; Kato et al. 2006). There was no significant relationship between MDA and 8-epi-$PGF_{2\alpha}$ in a Spearman correlation ($r = 0.049$, $P = 0.698$), nor did either marker correlate with indices of the degree of smoking, including pack years or urinary cotinine (Appendix B, Table 5.7 and

Table 3.1. Characteristics and clinical parameters of cigarette-smoking participants.

Variable	Mean ± SD	n
Age (years)	37 ± 9	67
Male/Female, *n*	36/31	67
Framingham risk score (%)[a]	2.4 ± 3.3 (Range: <1-16)	60
Pack years	8.69 ± 7.71	67
Urinary Cotinine (ng/mL)	5658.61 ± 12423.50	66
Height (cm)	170.7 ± 9.1	67
Weight (kg)	74.4 ± 16.4	67
BMI (%)	25.5 ± 5.2	67
Heart rate (b/min)	70 ± 11	67
Systolic BP (mmHg)	111 ± 12	67
Diastolic BP (mmHg)	72 ± 10	67
Total cholesterol (mg/dL)	166 ± 36	65
LDL (mg/dL)	95 ± 34	65
HDL (mg/dL)	52 ± 12	65
Triglycerides (mg/dL)	99 ± 48	65
HbA1c (%)	5.3 ± 0.4	64

[a]Estimate of the 10-year risk of developing coronary heart disease taking into account age, sex, total cholesterol, HDL cholesterol, blood pressure, treatment for hypertension, and smoking. A value <10% is considered low risk. We were unable to use the new atherosclerotic cardiovascular disease risk calculator which requires age > 40.

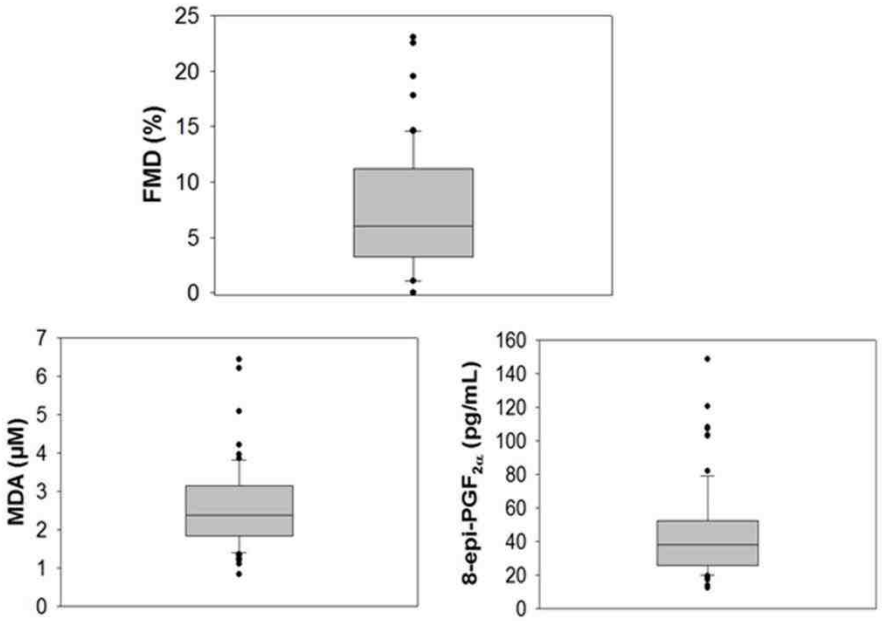

Figure 3.1. Median and 10th, 25th, 75th and 90th percentiles of oxidative stress markers, MDA and 8-epi-$PGF_{2\alpha}$, and an index of vascular function, FMD, in current cigarette smokers. (Abbreviations: MDA: Malondialdehyde; 8-epi-$PGF_{2\alpha}$: 8-isoprostane; FMD: Flow-mediated dilation)

5.9). Similarly, there was no correlation between either oxidative stress marker and FMD (Appendix B, Table 5.11).

RBC PUFAs, oxidative stress and FMD

We assessed n-3 and n-6 PUFAs from RBCs, and calculated the omega-3 index (EPA + DHA), the ratio of AA to its precursor LA, and the ratio of EPA + DHA + docosapentaenoic acid (DPA) to their precursor, alpha-linolenic acid (ALA) (Appendix B, Table 5.5). The omega-3 index ranged from 1.8 to 5.2% (median, 3.0%; mean ± SD, 3.24 ± 0.87%). There was no significant relationship between individual n-3 PUFAs or the omega-3 index with MDA, 8-epi-$PGF_{2\alpha}$, or FMD. When the omega-3 index values were divided into quintiles, individuals in the highest quintile (omega-3 index ≥ 4.5%) had significantly higher FMD (11.9 ± 6.6%), compared to all individuals < 4.5% (7.0 ± 5.3%; $P < 0.02$; Figure 3.2). In contrast, there were no differences in MDA or 8-epi-$PGF_{2\alpha}$ among individuals with different levels of omega-3 index (data not shown). Further, there was no correlation between n-6 PUFAs, the AA/LA ratio, or the (EPA + DHA + DPA)/ALA ratio with MDA, 8-epi-$PGF_{2\alpha}$, and FMD.

Ratios of PUFA diol/epoxide metabolites as indices of soluble epoxide hydrolase activity and their association with oxidative stress and FMD

We assessed PUFA metabolites in plasma (Appendix B, Table 5.6). Soluble epoxide hydrolase converts PUFA epoxides, including epoxy -octadecenoic acid (EpOME), -eicosatrienoic acid (EET), -eicosatetraenoic acid (EEQ), and - acid (DiHOME), - eicosatrienoic acid (DHET), eicosatetraenoic acid (DiHETE), and

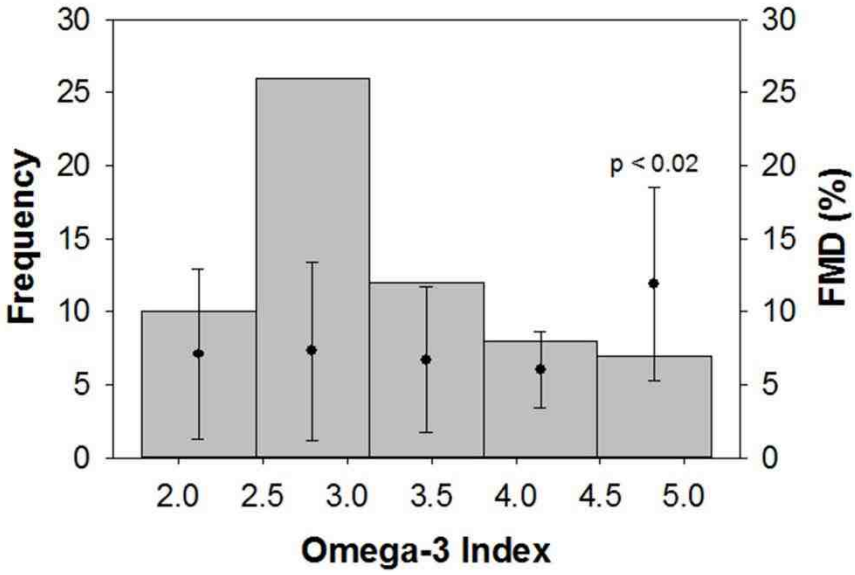

Figure 3.2. Histogram of omega-3 index in quintiles (bars) and mean ± SD of FMD for individuals in each quintile (solid circle and vertical line). (Abbreviations: FMD: Flow-mediated dilation)

-docosapentaenoic acid (EDP), into their corresponding diols, dihydroxy -octadecenoic docosapentaenoic acid (DiHDPA), respectively. The ratio of the sum of the diol products to the sum of the epoxide substrates is used as an index of soluble epoxide hydrolase activity. Interestingly, MDA was significantly correlated with ΣDiHOME/ΣEpOME and ΣDiHDPA/ΣEDP, and tended to be correlated with ΣDHET/ΣEET and ΣDiHETE/ΣEEQ (Table 3.2). In contrast, neither 8-epi-$PGF_{2\alpha}$ nor FMD correlated with these diol/epoxide ratios.

Development of a predictive model for the oxidative stress marker, MDA

Using Spearman correlation analysis, we found significant associations between MDA and plasma CAT, HDL cholesterol, three saturated fatty acids; palmitic acid (C16:0), arachidic acid (C20:0), behenic acid (C22:0); and one trans-fatty acid, conjugated linoleic acid (CLA, C18:2n6t). We found that MDA was also significantly associated with four PUFA metabolites; 13-hydroxyoctadecadienoic acid (HODE), 9,10- and 12,13-EpOME, and 14,15-DiHETE; and with two PUFA diol/epoxide ratios noted above, ΣDiHOME/ΣEpOME and ΣDiHDPA/ΣEDP (Table 3.3). No other antioxidant markers (Appendix B, Table 5.4), clinical characteristics, fatty acids or metabolites significantly correlated with MDA (Appendix B, Table 5.7). Multivariate regression analysis, using all variables that were statistically significant in Spearman correlation analysis, showed C18:2n6t and ΣDiHOME/ΣEpOME significantly predicted MDA.

All variables that exhibited a significant association with MDA by Spearman correlation were further analyzed using linear regression. Catalase, C16:0, C18:2n6t, C20:0, 13-HODE, 12,13-EpOME, 14,15-DiHETE, and ΣDiHOME/ΣEpOME

Table 3.2. Correlation coefficients of MDA with PUFA diol/epoxide ratios, reflecting soluble epoxide hydrolase activity, using Spearman analysis.

Parent PUFA	Diol/Epoxide	Spearman correlation with MDA		
		r	P	n
LA	ΣDiHOME/ΣEpOME	0.337	**0.008**	62
AA	ΣDHET/ΣEET	0.223	0.080	62
EPA	ΣDiHETE/ΣEEQ	0.230	0.072	62
DHA	ΣDiHDPA/ΣEDP	0.267	**0.036**	62

Abbreviations: LA: Linoleic acid; AA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; DiHOME: Dihydroxyoctadecenoic acid; EpOME: Epoxyoctadecenoic acid; DHET: Dihydroxyeicosatrienoic acid; EET: Epoxyeicosatrienoic acid; DiHETE: Dihydroxyeicosatetraenoic acid; EEQ: Epoxyeicosatetraenoic acid; DiHDPA: Dihydroxydocosapentaenoic acid; EDP: Epoxydocosapentaenoic acid.

Table 3.3. Significant associations of MDA with individual antioxidant measurements, clinical characteristics, RBC fatty acids and plasma PUFA metabolites using Spearman correlation analysis ($P < 0.05$), and multivariate regression analysis using the variables that significantly correlated with MDA in Spearman analysis. MDA and all independent variables were log10 transformed for multivariate analysis.

Variable	Spearman correlation			Multivariate analysis			
	r	P	n	β ± SE	R	P	n
CAT	0.365	0.003	65	0.118 ± 0.073		0.115	
HDL	0.286	0.021	65	0.301 ± 0.171		0.084	
C16:0	0.321	0.010	63	0.046 ± 0.020		0.087	
C18:2n6t	-0.568	<0.001	63	-0.438 ± 0.137		**0.002**	
C20:0	-0.254	0.044	63	0.433 ± 0.408		0.294	
C22:0	-0.282	0.025	63	-0.172 ± 0.195	0.725	0.381	62
13-HODE	-0.323	0.011	62	-0.032 ± 0.189		0.864	
9,10-EpOME	-0.290	0.023	62	0.112 ± 0.427		0.794	
12,13-EpOME	-0.360	0.004	62	-0.100 ± 0.363		0.783	
14,15-DiHETE	0.268	0.035	62	0.143 ± 0.131		0.280	
ΣDiHOME/ΣEpOME	0.337	0.008	62	0.349 ± 0.147		**0.022**	
ΣDiHDPA/ΣEDP	0.267	0.036	62	-0.178 ± 0.182		0.335	

Abbreviations: CAT: Catalase; HDL: High-density lipoprotein; C16:0: Palmitic acid; C18:2n6t: Conjugated linoleic acid; C20:0: Arachidic acid; C22:0: Behenic acid; HODE: Hydroxyoctadecadienoic acid; EpOME: Epoxyoctadecenoic acid; DiHETE: Dihydroxyeicosatetraenoic acid; DiHOME: Dihydroxyoctadecenoic acid; DiHDPA: Dihydroxydocosapentaenoic acid; EDP: Epoxydocosapentaenoic acid.

significantly correlated as independent variables with MDA, while HDL significantly correlated as the dependent variable with MDA (Appendix B, Table 5.8). Of these results, C16:0, C18:2n6t, and ΣDiHOME/ΣEpOME exhibited the smallest P-values and largest effect sizes in their association with MDA (Figure 3.3).

In order to develop a model that included those variables that were most predictive of MDA, forward stepwise multivariate regression analysis was conducted using those variables that exhibited significant associations in Spearman correlation analysis. We found C16:0, C18:2n6t and ΣDiHOME/ΣEpOME were significantly associated with MDA (Table 3.4).

To determine if there was one group of PUFA metabolites that were more strongly predictive of MDA, we combined PUFA metabolites into groups based on their parent PUFA, structure, and manner of formation (Appendix B, Table 5.3), and ran forward selection multivariate analysis on the metabolites within each group. This analysis failed to identify any additional metabolites that correlated with MDA beyond those previously identified in Spearman analysis (i.e. 14,15-DiHETE and 12,13-EpOME).

Development of a predictive model for the oxidative stress marker, 8-epi-$PGF_{2\alpha}$

We conducted similar analyses with another marker of oxidative stress, 8-epi-$PGF_{2\alpha}$. Using Spearman correlation analysis, we found significant associations between plasma 8-epi-$PGF_{2\alpha}$ and total cholesterol, C18:2n6t, and six PUFA metabolites; 5,6-, 8,9-, 11,12-, and 14,15-EET; 5,6-DHET and 10,17-dihydroxydocosahexaenoic acid (Table 3.5). We failed to find any associations between antioxidants, other clinical

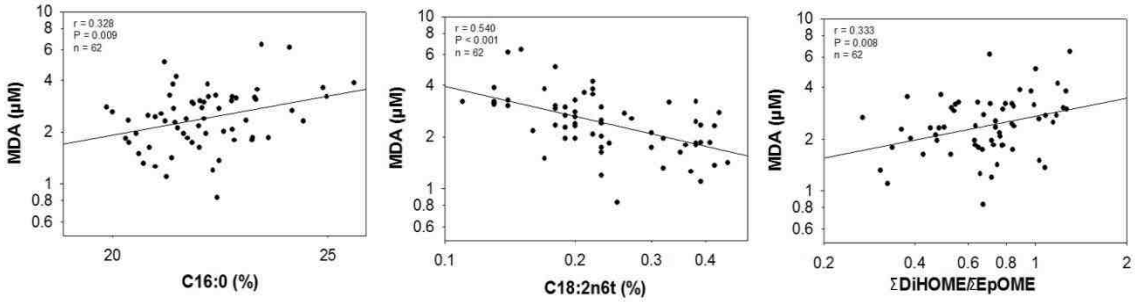

Figure 3.3. Linear regression of log10 transformed RBC fatty acids, C16:0 and C18:2n6t, and the LA diol/epoxide ratio (ΣDiHOME/ΣEpOME) versus log10 transformed oxidative stress marker, MDA. (Abbreviations: MDA: Malondialdehyde; C16:0: Palmitic acid; C18:2n6t: Conjugated linoleic acid; DiHOME: Dihydroxyoctadecenoic acid; EpOME: Epoxyoctadecenoic acid)

Table 3.4. Significant associations of RBC fatty acids with MDA identified by forward selection multivariate regression, using those variables that were significant in Spearman correlation ($P < 0.05$). MDA and all independent variables were log10 transformed.

Variables that predict	**Forward Stepwise Selection Multivariate Analysis**			
MDA	**β ± SE**	**R**	**P**	**n**
C16:0	2.232 ± 0.751		0.004	
C18:2n6t	-0.455 ±0.112	0.652	<0.001	62
ΣDiHOME/ΣEpOME	0.283 ± 0.108		0.011	

Abbreviations: C16:0: Palmitic acid; C18:2n6t: Conjugated linoleic acid; DiHOME: Dihydroxyoctadecenoic acid; EpOME: Epoxyoctadecenoic acid.

Table 3.5. Significant associations of 8-epi-$PGF_{2\alpha}$ with antioxidant measurements, clinical characteristics, RBC fatty acids and plasma PUFA metabolites, using Spearman correlation analysis ($P < 0.05$). Multivariate regression analysis was conducted using all variables that significantly correlated with 8-epi-$PGF_{2\alpha}$ in Spearman analysis. 8-epi-$PGF_{2\alpha}$ and all independent variables were log10 transformed for multivariate analysis.

Variable	Spearman Correlation			Multivariate Analysis			
	R	P	n	β ± SE	R	P	n
Total cholesterol	0.257	0.039	65	0.236 ± 0.351		0.504	
C18:2n6t	0.249	0.05	63	0.273 ± 0.202		0.182	
5,6-EET	0.277	0.029	62	-0.126 ± 0.781		0.872	
8,9-EET	0.270	0.034	62	-0.100 ± 0.345	0.490	0.772	62
11,12-EET	0.307	0.016	62	-0.578 ± 0.952		0.546	
14,15-EET	0.354	0.005	62	1.005 ± 1.043		0.340	
5,6-DHET	0.277	0.029	62	0.142 ± 0.306		0.644	
10,17-DiHDHA	0.355	0.005	62	0.128 ± 0.0794		0.113	

Abbreviations: 18:2n6t: Conjugated linoleic acid; EET: Epoxyeicosatrienoic acid; DHET: Dihydroxyeicosatrienoic acid; DiHDHA: Dihydroxydocosahexaenoic acid.

characteristics, other fatty acids or PUFA metabolites and 8-epi-$PGF_{2\alpha}$ (Appendix B, Table 5.9). Multivariate regression analysis, using all variables that were significant from the Spearman correlation analysis, failed to identify any single variable that significantly predicted 8-epi-$PGF_{2\alpha.}$

In the linear regression analysis, C18:2n6t, 11,12-EET, 14,15-EET, 5,6-DHET and 10,17-DiHDHA significantly correlated as independent variables with 8-epi-$PGF_{2\alpha}$ (Appendix B, Table 5.10). Of these results, 14,15-EET and 10,17-DiHDHA had the smallest P-values and 14,15-EET had the greatest effect size in their association with 8-epi-$PGF_{2\alpha}$ (Figure 3.4). In order to develop a model that included those variables that were most predictive of 8-epi-$PGF_{2\alpha}$, forward selection multivariate regression analysis was conducted, using those variables that exhibited significant associations in Spearman correlation analysis. We found that 14,15-EET and 10,17-DiHDHA were significantly associated with 8-epi-$PGF_{2\alpha}$ (Table 3.6).

To determine if there was one group of PUFA metabolites that were more strongly predictive of 8-epi-$PGF_{2\alpha}$, we also ran forward selection multivariate analysis on the metabolites within each group. In addition to 14,15-EET and 5,6-DHET that had been identified in Spearman analysis, 11-HETE was identified in the metabolite grouping of HETEs, while 11-HETE and 20-hydroxydocosahexaenoic acid (HDHA) were identified from the metabolite grouping of autoxidation products (Table 3.7).

Development of a predictive model for FMD

Lastly, we conducted similar analysis for FMD. Using Spearman correlation analysis, we found significant associations between FMD and three fatty acids; oleic acid

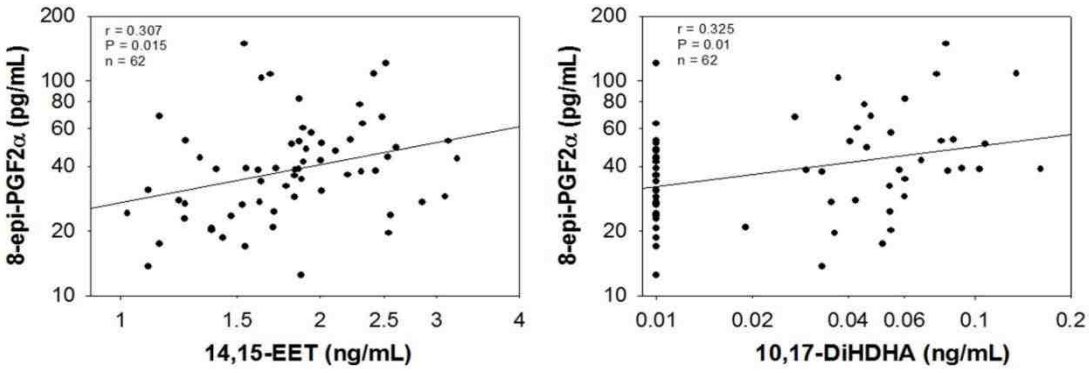

Figure 3.4. Linear regression of PUFA metabolites, 14,15-EET and 10,17-DiHDHA, with oxidative stress marker, 8-epi-$PGF_{2\alpha}$. 8-epi-$PGF_{2\alpha}$ and all independent variables were log10 transformed. (Abbreviations: 8-epi-$PGF_{2\alpha}$: 8-isoprostane; EET: Epoxyeicosatetrienoic acid; DiHDHA: Dihydroxydocosahexaenoic acid)

Table 3.6. Significant associations of fatty acids with 8-epi-$PGF_{2\alpha}$ identified by forward selection multivariate regression, using those variables that were significant in Spearman correlation ($P < 0.05$). 8-epi-$PGF_{2\alpha}$ and all independent variables were log10 transformed.

Metabolites that predict 8-epi-$PGF_{2\alpha}$	**Forward Stepwise Selection Multivariate Analysis**			
	β ± SE	**R**	**P**	**n**
14,15-EET	0.501 ± 0.229	0.416	0.033	62
10,17-DiHDHA	0.163 ± 0.069		0.021	

Abbreviations: EET: Epoxyeicosatrienoic acid; DHET: Dihydroxyeicosatrienoic acid; DiHDHA: Dihydroxydocosahexaenoic acid.

Table 3.7. Significant associations of plasma PUFA metabolites with 8-epi-$PGF_{2\alpha}$ identified by forward selection multivariate regression, using metabolites within specific PUFA metabolite groups (Appendix B, Table 5.3). 8-epi-$PGF_{2\alpha}$ and all independent variables were log10 transformed.

Metabolites that predict 8-epi-$PGF_{2\alpha}$	**Forward Stepwise Selection Multivariate Analysis**			
	β ± SE	**R**	**P**	**n**
11-HETE	0.415 ± 0.193	0.267	0.036	62
11-HETE	0.922 ± 0.287	0.386	0.002	62
20-HDHA	-0.505 ± 0.218		0.024	

Abbreviations: HETE: Hydroxyeicosatetraenoic acid; HDHA: Hydroxydocosahexaenoic acid.

(C18:1n9), eicosenoic acid (C20:1n9), and adrenic acid (C22:4n6), and one DHA metabolite, 16,17-DiHDPA (Table 3.8). We failed to find any associations between antioxidants, clinical characteristics, other fatty acids or PUFA metabolites and FMD (Appendix B, Table 5.11). Additionally, all four fatty acids or metabolites identified from Spearman correlation also were significantly associated with FMD in linear regression analysis with FMD as the dependent variable (Figure 3.5, Appendix B, Table 5.12). Multivariate regression analysis as well as forward selection multivariate regression analysis, using all variables that were significant from the Spearman correlation analysis, identified C18:1n9, C20:1n9, and 16,17-DiHPDA as highly predictive of FMD (Table 3.8 and 3.9). Multivariate regression analysis using forward selection and PUFA metabolites groups failed to identify any additional metabolites that correlated with FMD beyond those identified in Spearman analysis.

DISCUSSION AND CONCLUSIONS

This study assessed for the first-time the relationships between RBC fatty acids and plasma PUFA metabolites, and oxidative stress and vascular function in young healthy cigarette smokers. We focused on smokers lacking clinical CVD in order to identify potential dietary fatty acids that might provide antioxidant and vasoprotective action in the face of considerable oxidative stress. We found that only the highest levels of n-3 PUFAs were associated with improved FMD, but showed no association with oxidative stress. In contrast, CLA (C18:2n6t) and palmitic acid (C16:0) in RBCs, and PUFA diol/epoxide metabolites, reflective of sEH activity, were the most predictive

Table 3.8. Significant associations (P<0.05) of FMD with antioxidant measurements, clinical characteristics, fatty acid parent compounds and their metabolites as correlated using Spearman analysis. Multivariate analysis was conducted using all variables that significantly correlated with FMD in Spearman analysis. FMD was rank transformed and all independent variables were log10 transformed for multivariate analysis.

Variable	Spearman Correlation			Multivariate Analysis			
	r	P	n	β ± SE	R	P	n
18:1n9	0.302	0.024	56	0.457 ± 0.140		**0.002**	
20:1n9	-0.290	0.030	56	-0.302 ± 0.130	0.557	**0.024**	55
22:4n6	-0.267	0.047	56	0.085 ± 0.144		0.555	
16,17-DiHDPA	0.306	0.023	55	0.347 ± 0.124		**0.007**	

Abbreviations: 18:1n9: Oleic acid; 20:1n9: Eicosanoic acid; 22:4n6: Adrenic acid; DiHDPA: Dihydroxydocosapentaenoic acid.

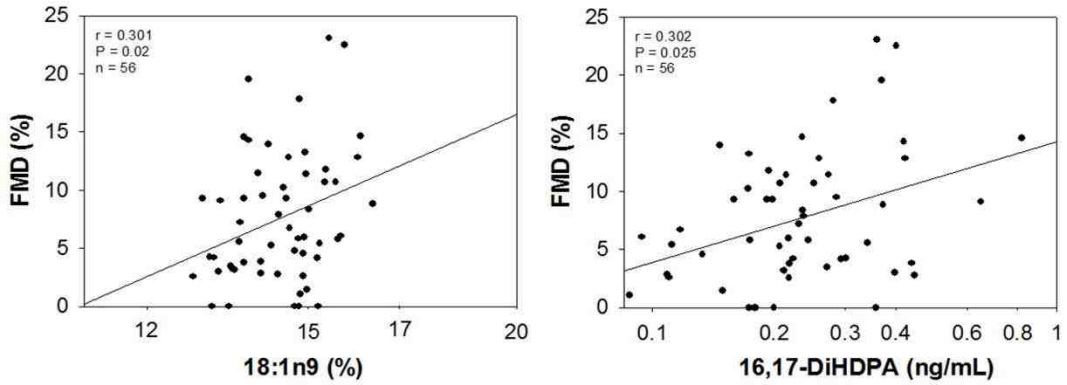

Figure 3.5. Linear regression of log10 transformed RBC fatty acid, C18:1n9, and plasma PUFA metabolite, 16,17-DiHDPA, versus FMD. (Abbreviations: 18:1n9: Oleic acid; DiHDPA: Dihydroxydocosapentaenoic acid)

Table 3.9. Significant associations of fatty acids and PUFA metabolites with FMD identified by forward stepwise selection multivariate analysis, using those variables that were significant in Spearman correlation ($P < 0.05$). FMD was rank transformed and all independent variables were log10 transformed.

Metabolites that predict	**Forward Stepwise Selection Multivariate Analysis**			
FMD	**β ± SE**	**R**	**P**	**n**
18:1n9	0.416 ± 0.121		0.001	
20:1n9	0.270 ± 0.117	0.553	0.025	55
16,17-DiHDPA	0.327 ± 0.119		0.008	

Abbreviations: 18:1n9: Oleic acid; 20:1n9: Eicosanoic acid; DiHDPA: Dihydroxydocosapentaenoic acid.

factors of plasma MDA in multivariate regression. For 8-epi-$PGF_{2\alpha}$, the AA P450 metabolite, 14,15-EET, and the dihydroxy DHA metabolite, 10,17-DiHDHA, were the most predictive; while for FMD two n-9 fatty acids, oleic (18:1n9) and eicosenic acid (20:1n9), and one DHA metabolite, 16,17-DiHPDA were the most predictive. These multivariate regression models exhibited moderate-to-strong relationships, based on correlation coefficients R = 0.416 – 0.652, and identified novel associations that may provide insight into potentially antioxidant and vasoprotective dietary fatty acids. Based on our earlier findings that n-3 PUFAs improve FMD and reduce oxidative stress (Wiest et al. 2015; Wiest, Walsh-Wilcox, and Walker 2017), we hypothesized that n-3 PUFAs and their metabolites would be most predictive of MDA, 8-epi-PGF2α, and FMD. However, one surprising observation of our study was that this cohort of smokers had very low n-3 PUFAs, compared to a U.S. reference range. The distribution of omega-3 index for our cohort was 2.5%, 3.0% and 3.7% for the 25^{th}, 50^{th}, and 75^{th} percentiles, respectively. In comparison, the omega-3 index for a U.S. reference group from the Los Angeles area was 2.5%, 3.7%, and 5.7% for the 25^{th}, 50^{th}, and 75^{th} percentiles, respectively (Superko et al. 2014). More than 40% of our cohort was Hispanic and previous studies have shown that Hispanics are more likely to have n-3 PUFAs below those recommended for CV health by the American Heart Association and Dietary Guidelines Advisory Committee of the U.S. Department of Health and Human Services (Murphy et al. 2015). Notably, 77% of our cohort of smokers had an omega-3 index < 4%, which is considered to be at high risk for coronary heart disease mortality (WS Harris 2008). Although this cohort of cigarette smokers lacked classic risk factors for CVD and had a low calculated Framingham risk score, the very low levels of n-3 PUFAs

may represent an overlooked risk for CVD. Nonetheless, although the omega-3 index was low in our participants, individuals with the highest concentrations (≥ 4.5%) had significantly higher FMD values, supporting our original hypothesis.

Although the two markers of oxidative stress, MDA and 8-epi-PGF2α, and the marker of vascular function, FMD, did not correlate with each other, we did identify a number of fatty acids and PUFA metabolites that were predictive of these individual endpoints. One of our most striking observations was that plasma MDA negatively correlated with the percentage of CLA in RBCs ($P < 0.001$), being the most predictive factor in single correlation analysis and one of three factors that remained in multivariate regression modeling, suggesting that higher levels of CLA are associated with an antioxidant effect in current cigarette smokers. The form of CLA predominately found in food occurs as various isomers of linoleic acid with the *cis*-9, *trans*-11 isomer products (Jun Ho Kim et al. 2016). The antioxidant potential of CLA has been demonstrated in a number of animal and cell culture studies and the antioxidant mechanism likely involves activation of Nrf2. Treatment of mammary epithelial cells with CLA reduces MDA levels and protects cells from oxidative stress induced by H_2O_2 (Basiricò et al. 2017). *In viv*o, CLA supplementation of mice reduces hepatic MDA concentration (Park, Valacchi, and Lim 2010) as well as increases serum antioxidant capacity, enhances the nuclear translocation of hepatic Nrf2, and increases hepatic enzymatic activity of Nrf2-regulated antioxidant genes, glutathione-*S*-transferase and NAD(P)H:quinone oxidoreductase (Bergamo, Maurano, and Rossi 2007). Supplementation with CLA also significantly protects rats from acrolein-induced oxidative stress, one of the major reactive aldehydes found in cigarette smoke (Aydın, Atlı Şekeroğlu, and Şekeroğlu 2017). In two

randomized, placebo-controlled clinical trials, CLA supplementation significantly reduces serum MDA in patients with atherosclerosis and rheumatoid arthritis, respectively (Aryaeian et al. 2014; Eftekhari et al. 2013). Our results support the potential antioxidant benefit of CLA in young healthy cigarette smokers.

We also found that plasma MDA positively correlated with the percentage of palmitic acid (C16:0) in RBCs ($P < 0.004$) and with the ratio of LA diol-to-epoxide metabolites (ΣDiHOME/ΣEpOME, $P < 0.011$), suggesting that higher levels of palmitic acid and higher levels of sEH activity are associated with a pro-oxidant effect in current cigarette smokers. Palmitic acid is a 16-carbon saturated fatty acid that represented the highest percentage of total fatty acids in RBCs in our study (range: 19.88-25.69%). Dietary saturated fatty acids have been linked to an increased risk of CV disease due to their ability to increase LDL-cholesterol (Sacks et al. 2017). Although evidence is limited, both epidemiology and laboratory studies have associated saturated fatty acids and oxidative stress (Liang et al. 2011; Hua et al. 2015; Kwak, Choi, and Cheon 2017). It has been shown that CS induces sEH expression in mice (Maresh et al. 2005) and sEH-inhibition improves vascular function in obese smokers (L. Yang et al. 2017). Thus, increased sEH activity may contribute to CS-induced oxidative stress.

To our surprise we did not find an association between plasma MDA and 8-epi-$PGF_{2\alpha}$. However, we made the novel observation of positive associations between plasma 8-epi-$PGF_{2\alpha}$ and two PUFA metabolites, 14,15-EET and 10,17-DiHDHA. The metabolite 14,15-EET is a cytochrome P450-derived epoxide of AA, primarily produced by human CYP2J2, that mediates vasodilation, and exhibits anti-inflammatory and antioxidant effects. These EETs can protect against cigarette smoke-induced

inflammation in cultured cells and *in vivo* (Smith et al. 2005; Li et al. 2017), and can lead to activation of the antioxidant transcription factor, Nrf2. It is noteworthy that all EETs individually and significantly correlated with 8-epi-$PGF_{2\alpha}$ in our study, while only 14,15-EET remained significantly associated in multivariate analysis. The positive association of 14,15-EET with a marker of CS-induced oxidative stress is surprising. It is well established that CS induces CYP1A1 expression (Wiest, Walsh-Wilcox, and Walker 2017; James H. Kim et al. 2004). Sustained CYP1A1 induction can increase oxidative stress by enzymatic uncoupling (Shertzer et al. 2004) and can increase AA metabolism to produce 14,15-EET (Schwarz et al. 2004). Thus, it is possible that CYP1A1 induction may provide a mechanistic link for the positive association between cigarette smoke-induced oxidative stress and plasma 14,15-EET concentrations.

Besides CYP1A1, other P450 enzymes are also elevated in smokers, such as CYP1A2 and CYP1B1 (Faber and Fuhr 2004, Kim et al. 2004). However, several P450 enzymes, including CYP1A1, CYP2J2, CYP4A and CYP4F subfamilies, also metabolize PUFAs in humans (Arnold et al. 2010). Because CS can stimulates possibly different CYP activity than n-3 PUFAs, CS-induced CYP1A1 n-3 PUFA metabolism may produce more PUFA products, but this may not be enough to significantly change the metabolic profile of smokers. Furthermore, it has been shown that smokers have decreased n-3 PUFA levels, due to CS disruption of ALA metabolism to EPA and DHA (Risé et al. 2009; Ghezzi et al. 2007; Marangoni et al. 2004). Further study of metabolic profiles of smokers and non-smokers may show comparable alterations in PUFA metabolism in relation to CYP activity.

Another PUFA metabolite that positively associated with 8-epi-$PGF_{2\alpha}$, 10,17-DiHDHA, is a 15-lipoxygenase-derived dihydroxy metabolite of DHA with two unique isomers differing in stereochemistry at carbon-10; 10(S),17(S)-DiHDHA (protectin DX) and 10(R),17(S)-DiHDHA (protectin D1) (Balas et al. 2014). Protectin DX can inhibit cyclooxygenase activity thereby inhibiting platelet aggregation and neutrophil production of ROS, while protectin D1, also termed neuroprotectin D1, is anti-inflammatory and protects cells from oxidative stress. The chemical analyses used in our study could not distinguish between the two isomers; nonetheless, the positive association between 10,17-DiHDHA and 8-epi-$PGF_{2\alpha}$ may reflect an increased synthesis of 10,17-DiDHA in response to inflammation and oxidative stress.

Although we had expected that n-3 PUFAs and the two indices of oxidative stress, MDA and 8-epi-$PGF_{2\alpha}$, would be associated with FMD, instead we found a strong positive association between FMD and the RBC percentage of the monounsaturated fatty acid, oleic acid (18:1n9). Oleic acid is the primary fatty acid found in olive oil, making up 65-75% of total fatty acids. The CV protective benefits of olive oil as well as a Mediterranean diet, which is typically high in olive oil, are ascribed, in part, to oleic acid. Studies have shown that a Mediterranean diet as well as a diet enriched in oleic acid significantly improve FMD (Davis et al. 2017; Ryan et al. 2000; Perdomo et al. 2015). Mechanistically, oleic acid prevents endothelial cell activation and protects against decreases in endothelial NO synthase expression in cultured endothelial cells challenged with an inflammatory stimulus (Schwarz et al. 2004). Thus, higher concentrations of oleic acid being associated with higher FMD would be consistent with oleic acid having vasoprotective effects in this cohort of current cigarette smokers.

Additionally, in the most predictive multivariate regression model of FMD, there also was a negative association with eicosenoic acid (20:1n9; also termed gondoic acid) and a positive association with the dihydroxy metabolite of DHA, 16,17-DiHPDA. Information on eicosenoic acid and CV health is limited to one recent publication that suggests higher concentrations are associated with increased CV mortality in women (Delgado et al. 2017). In our study higher concentrations of eicosenoic acid were associated with more impaired FMD, which would be consistent with an increased risk of CV disease (Rossi et al. 2008; Shechter et al. 2009). In contrast, higher concentrations of 16,17-DiHPDA were associated with higher FMD. The DHA dihydroxy metabolites, derived from sEH, inhibit platelet aggregation (Vanrollins and Murphy 1984) and could help to prevent endothelial cell activation, which occurs with impaired FMD.

Lastly, multivariate regression analysis of PUFA metabolite groups failed to reveal any additional metabolites that were associated with MDA or FMD that had not been identified in the Spearman analysis previously. This approach, however, did identify a novel association between 8-epi-$PGF_{2\alpha}$ and two PUFA metabolites that are generated by autoxidation, 11-HETE and 20-HDHA. Our data show that elevated 8-epi-$PGF_{2\alpha}$ is associated with higher concentrations of 11-HETE and simultaneously lower concentrations of 20-HDHA. Specifically, 11-HETE can be derived from 15-lipoxygenase metabolism of AA, but also is formed via autoxidation and is a marker of lipid peroxidation (Guido, McKenna, and Mathews 1993). Furthermore, elevated levels of plasma 11-HETE are associated with an increased risk of a major adverse CV event in patients who have had an acute myocardial infarction (Zu et al. 2016). Metabolite 20-HDHA is formed by non-enzymatic oxidation of DHA by ROS. While other HDHA

isomers are associated with anti-inflammatory and anti-oxidant activity, a biological function of 20-HDHA has not been reported. Our results are consistent with 11-HETE as a marker of oxidative stress, and may suggest 20-HDHA has a protective role similar to other HDHA isomers.

In summary, these results suggest that n-3 PUFAs and oleic acid may be related to better vascular function in young healthy smokers. Since two well-accepted biomarkers of oxidative stress, MDA and 8-epi-$PGF_{2\alpha}$, did not correlate with each other and that they were differentially associated with specific fatty acids and PUFA metabolites, suggests that they assess unique endpoints of lipid peroxidation in cigarette smokers. Further, given that these oxidative stress markers do not correlate with vascular dysfunction as assessed by FMD, suggests that oxidative stress alone may not be the primary mediator of impaired FMD in this cohort of cigarette smokers. Finally, the cause-and-effect relationships of the novel correlations between fatty acids, oxidative stress, and vascular function identified in these young healthy cigarette smokers will require additional study in order to identify those dietary fatty acids that may help provide protection against the development of CVD.

CHAPTER IV

Summary, conclusions, limitations and future directions

Summary

The goal of this work was to define the mechanism by which omega-3 (n-3) polyunsaturated fatty acids (PUFAs) increase antioxidant responses at the cellular level, thus decreasing endothelial dysfunction in smokers. We developed a cell culture model to assess n-3 PUFA downstream antioxidant signaling, and we assessed one cohort of young, healthy current-smokers with no classic symptoms of CVD. Both of these approaches allowed us to measure antioxidant and oxidative stress relationships with n-3 PUFAs and their metabolites, and one protein of interest was nuclear factor (erythroid-derived 2)-like 2 (Nrf2).

To assess the downstream antioxidant signaling in a cell culture model, we used hepatocytes that contained a stably-transfected antioxidant response element (ARE) luciferase (HepG2-ARE), which is a Nrf2 pathway-responsive reporter vector. We treated these cells with n-3 PUFAs eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6), originally hypothesizing that these n-3 PUFAs or their P450-produced epoxide metabolites, activated Nrf2. Consistent with previous literature, our DHA stock significantly increased Nrf2 activity (Stulnig et al. 2013). We added P450, cyclooxygenase (COX) and lipoxygenase (LOX) inhibitors simultaneously with DHA to see which inhibited pathway would decrease Nrf2 activation, but adding inhibitors further increased Nrf2 activation instead of inhibiting it. After assessing our DHA stock with LC-MS, we found it to be enriched with oxidized DHA, especially ketones; oxidized DHA has been shown to increase Nrf2 activity (Ishikado et al. 2013),

and one ketone of arachidonic acid (AA) has been shown to increase Nrf2 activity (Snyder et al. 2015).

One DHA ketone, 17-keto-4(Z),7(Z),10(Z),13(Z),15(E),19(Z)-DHA (17-oxo-DHA), has been shown to have anti-inflammatory and antioxidant effects (Cipollina et al. 2014). We treated HepG2-ARE cells and human umbilical vein endothelial cells (HUVECs) with 17-oxo-DHA to measure Nrf2 activity, Nrf2 localization to the nucleus, Nrf2 downstream antioxidant gene expression. Fluorescent localization of Nrf2 was increased in the nucleus following 3 hours of 17-oxo-DHA treatment. Downstream antioxidant mRNA, including glutamate-cysteine ligase catalytic subunit (Gclc), NAD(P)H quinone dehydrogenase 1 (Nqo1) and heme-oxygenase 1 (Hmox1), were significantly increased at 3-, 6- and 12-hour exposure to 17-oxo-DHA. These cell culture-based study results suggest that oxidation products of DHA, specifically ketones such as 17-oxo-DHA, increase antioxidant responses, in part, through the Nrf2 pathway. Because these studies take place in a cell culture system, which is an isolated and controlled system, we were interested in measuring n-3 PUFAs and their metabolites in smokers.

This limitation led to a study in a population of 67 smokers. In our population of healthy current-smokers, we measured brachial flow-mediated dilation (FMD) as a primary outcome. We measured RBC n-3 PUFAs, plasma n-3 PUFA metabolites, plasma and RBC antioxidants superoxide dismutase (SOD), catalase (CAT), reduced and oxidized glutathione (GSH/GSSG), and oxidative stress markers malondialdehyde (MDA) and 8-isoprostane (8-epi$PGF_{2\alpha}$). We conducted correlation analysis of MDA, 8-epi$PGF_{2\alpha}$ and FMD versus individual clinical measurements (triglycerides, blood

pressure, BMI, sex, age, urinary cotinine, total cholesterol, high density lipoprotein, low density lipoprotein, systolic and diastolic blood pressure, HbA1c and Framingham risk score), antioxidant markers, RBC n-3 PUFAs and plasma n-3 PUFA metabolites.

We found that an omega-3 index (RBC% eicosapentaenoic plus docosahexaenoic acids [EPA+DHA]) ≥ 4.5% was associated with higher FMD, but not lower oxidative stress. Oleic acid (OA, 18:1n9), 16,17-dihydroxydocosapentaenoic acid (DiHDPA), increased omega-3 index and eicosenoic acid (EA, 20:1) were predictive of FMD. Multivariate regression modeling revealed that conjugated linoleic acid (CLA, C18:2n6), palmitic acid (PA, 16:0), and the ratio of linoleic acid (LA, 18:2n6) diol-to-epoxide metabolites were predictive of MDA; 14,15-epoxyeicosatetraenoic acid (EET) and 10,17-dihydroxydocosahexaenoic acid (DiHDPA) were predictive of 8-epi-PGF2α. In summary, these results suggest that in this population of young healthy smokers, other dietary fatty acids may also be beneficial.

Conclusions, limitations and future directions

One strength of our cell culture study was that it was unbiased, and as we assessed what was in our older DHA stock that may have activated Nrf2, we discovered that epoxides were not responsible for Nrf2 activation as we previously hypothesized. This allowed us to observe enzymatic and non-enzymatic oxidation products, particularly ketones, of DHA that would activate antioxidant mechanisms and increase production of downstream antioxidants, thus decreasing a particular measure of oxidative stress. This work highlights that this particular non-enzymatically-controlled metabolite of DHA, 17-oxo-DHA, activates Nrf2 and increases its localization to the nucleus, while increasing

several of its downstream antioxidant pathways. This is consistent with previous literature showing increases in antioxidant markers with 17-oxo-DHA treatment (Cipollina et al. 2014; Cipollina et al. 2016; Groeger et al. 2011; Ceriello et al. 2014).

One caveat to our cell culture study is that the only commercially available ketone product of DHA is 17-oxo-DHA; assessing more DHA ketones in our cell culture model would allow us to observe if particular ketones activate Nrf2 more than other ketones. Future directions of our cell culture study involve purifying a range of DHA ketones, and treating cells with these ketones to assess Nrf2 activity. Another product of DHA non-enzymatic oxidation that appeared in the LC-MS chromatograms of our old DHA stock, peroxy-metabolites, were not further assessed and could be assessed for future investigation. Furthermore, precise determination of compounds present in the old stock of DHA from commercial supplier 1 could not be completed due to technical limitations. Peroxy-metabolites and ketones will be assessed in future studies to assess their ability to activate Nrf2 and its downstream antioxidant metabolites.

Future work could also assess DHA ketone effects *in vivo*. This could be done by inserting oxmotic mini pumps into CS- or air-exposed mice that would pump 17-oxo-DHA into the blood stream. Assessing antioxidant markers and indices of oxidative stress could determine more precise antioxidant mechanisms of 17-oxo-DHA in the tissues of these mice. Smokers also have increased risk of atherosclerotic CVD (Zehr and Walker 2017), so inserting osmotic mini pumps with 17-oxo-DHA into an atherosclerotic mouse model, such as the apolipoprotein null mice, could highlight anti-atherosclerotic effects, as well as oxidative stress mechanisms that could be decreased with 17-oxo-DHA treatment.

Further, 17-oxo-DHA is produced from 15-hydroxyprostaglandin dehydrogenase (15-PGDH) metabolism of 17-hydroxy-DHA. There is a 15-PGDH null mouse model that could be exposed to CS or a single toxicant such as benzo(a)pyrene and treated with 17-hydroxy-DHA, which would highlight whether the presence of 15-PGDH and its product, 17-oxo-DHA, is necessary for reducing CS-induced oxidative stress through increased Nrf2 and downstream antioxidant expression. Furthermore, 15-PGDH-null mice could be placed on an n-3 PUFA-rich diet and exposed to CS or a single toxicant such as benzo(a)pyrene, and lipid analysis should also be performed on the tissues of the mice to assess expression levels of ketone metabolites and their precursors, and relate these to oxidative stress endpoints, as well as Nrf2 and its downstream antioxidant expression. This could be expanded into human subjects by giving smokers a n-3 PUFA or 17-oxo-DHA supplement to assess endothelial function, oxidative stress and antioxidant markers before and after treatment. Metabolites of n-9, n-6 and n-3 PUFAs could be assessed of these human subjects to assess relationships between changes in metabolite profiles and endothelial function oxidative stress and antioxidant markers.

In our human subject study, our results suggest that n-3 PUFAs, as well as other fatty acids may be recommended for furthering the CV health of smokers. One strength of the human subject study was that assessing fatty acids and their metabolites, and relating them to oxidative stress markers, clinical measurements, antioxidant markers and FMD had never been shown before. This showed, to the best of our knowledge, the first evidence these relationships. In assessing oxidative stress, CLA was most predictive of MDA and the AA epoxide, 14,15-EET, was most predictive of 8-epi-$PGF_{2\alpha}$. Due to its negative association with MDA but simultaneously a positive association with 8-epi-

$PGF_{2\alpha}$ in our study. Interestingly, CLA has been shown to significantly reduces serum MDA in patients with atherosclerosis and rheumatoid arthritis, respectively (Aryaeian et al. 2014; Eftekhari et al. 2013). Further, EETs have been shown to protect against cigarette smoke-induced inflammation in cultured cells and *in vivo* (Smith et al. 2005; Li et al. 2017), and can lead to Nrf2 activation. In assessing FMD, one n-9 monounsaturated fatty acid (MUFA), oleic acid, and the omega-3 index, were most predictive for FMD. Increased omega-3 index associated with higher FMD is consistent with previous literature (Siasos et al. 2013), and that the omega-3 index could be used as a biomarker for CVD (Harris 2007; Harris 2008). Further, it is possible that a Mediterranean diet rich in oleic acid, which has been previously shown to improve endothelial function (Fuentes et al. 2001), may be recommended, alongside n-3 PUFAs, to improve FMD in those with CS-induced risk factors for CVD.

Although smoking cessation is heavily argued instead of therapy, only 4-7% of smokers are successful in the endeavor of quitting for at least one year (World Health Organization 2008; Fiore 2008). It is difficult for smokers to quit since nicotine is pharmacologically addictive (Office of Public Affairs 1998), and smoking-related illness in the United States costs over $300 billion each year (US Department of Health and Human Services 2014). Thus, finding pharmacological treatment such as these fatty acids or their metabolites is vital to lowering CVD risk in smokers. This work may also provide possible future therapeutic options to those affected by secondhand smoke, as secondhand smoke is associated with increased oxidative stress, remodeled vasculature, oxidative stress and endothelial dysfunction (Raupach et al. 2006; Kato et al. 2006). Due

to these associations, we predict that those who encounter secondhand smoke would have similar fatty acid and oxidative stress results to our smokers.

One limitation includes a comparison of a non-smoker group, which would be helpful for comparison of different fatty acids, their metabolites, and their associations with different antioxidant and oxidative stress markers. While funding for this work did not allow us to assess non-smokers and smokers simultaneously, we are currently recruiting non-smokers and comparing their measurements to our smoker group. Oxidative stress is increased in smokers, which stresses and depletes antioxidant systems, and thus will be of interest to compare to a non-smoker group (Cai and Harrison 2000; Neunteufl et al. 2000; Ozaki et al. 2010). It is possible that differences in these antioxidant systems between smokers and non-smokers will show differences in PUFA levels. Smokers have decreased serum EPA and DHA due, in part, to enzyme inhibition responsible for the conversion of ALA to EPA and DHA (Risé et al. 2009; Ghezzi et al. 2007; Marangoni et al. 2004). This could explain why we did not observe n-3 PUFA metabolites correlated with oxidative stress markers or antioxidant levels as we hypothesized.

It is possible that CS, as well as age (Csiszar 2009), stresses antioxidant mechanisms that cannot compensate for continuous oxidative damage over extended periods of time. This may be why we also did not find n-3 PUFAs correlate with oxidative stress. This oxidative damage that is induced by CS includes directly causing oxidative damage, increasing inflammation that leads to downstream oxidative damage and decreasing or exhausting antioxidant defenses (Bernhard and Wang, 2007). Thus, we hypothesize that n-3 PUFAs will be increased in non-smokers due to (1) lower oxidative

stress levels compared to smokers, and (2) uninterrupted n-3 PUFA metabolism that occurs in smokers. As a result, we expect that these increased n-3 PUFA levels in non-smokers will be associated with increased FMD and antioxidant markers, and lower oxidative stress compared to smokers. If increases in oleic acid concentration showed strong relationships with increases in FMD in non-smokers, then it should be further pursued as a possible biomarker for endothelial function. In relation to oxidative stress, it will be interesting to see how 14,15-EET relates to both MDA and 8-epi-$PGF_{2\alpha}$ in non-smokers, since it negatively associated with MDA but positively associated with 8-epi-$PGF_{2\alpha}$ in our smokers.

CHAPTER V.

APPENDICES

The following data sets in appendix A and B were produced as supplemental work for chapter II and chapter III of this work. The *in vitro* supplemental data for chapter II were produced in an effort to assess cell viability with all treatments, to make certain that observed results were due to direct effects on nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and not due to decreased cell viability. The supplemental data for chapter III included all statistical results of the human subject study. Appendix C is a review of omega 3 polyunsaturated fatty acid (n-3 PUFA) supplementation impact on those at risk for cardiovascular disease (CVD) that we published, and thus is considered a contribution to this body of work.

APPENDIX A

Supplemental data for chapter II

Table 5.1. Sense and antisense primers for PCR. Downstream antioxidant genes Hmox1, Nqo1 and Gclc were used and Gapdh was used as a positive control housekeeping gene.

Gene	Sense Primer	Antisense
hHmox1	CAACAAAGTGCAAGATTCTG	TGCATTCACATGGCATAAAG
hNqo1	AGTATCCACAATAGCTGACG	TTTGTGGGTCTGTAGAAATG
hGclc	TTATTAGAGACCCACTGACAC	TTCTCAAAATGGTCAGACTC
hGapdh	GGACCTGACCTGCCGTCTA	TAGCCCAGGATGCCCTTGAG

Abbreviations: Hmox1: Heme-oxygenase-1; Nqo1: NAD(P)H quinone dehydrogenase 1; Gclc: Glutamate-cysteine ligase catalytic subunit; Gapdh: Glyceraldehyde 3 phosphate dehydrogenase**.**

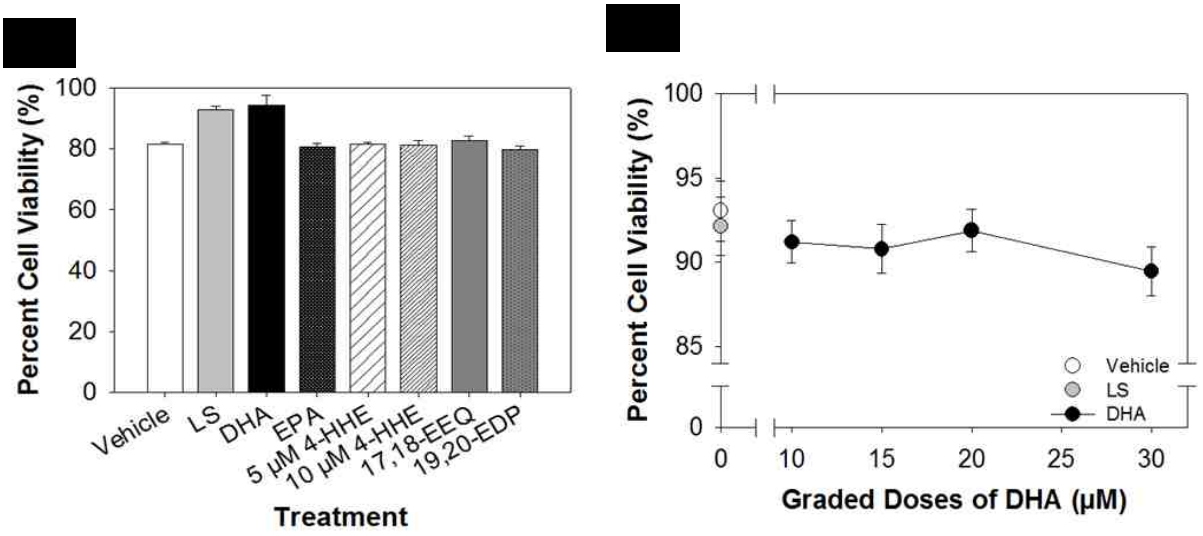

Figure 5.1. Nrf2-mediated luciferase expression in HepG2-ARE cells treated with A) DHA, EPA, positive control LS, DHA non-enzymatic oxidation product 4-HHE, EPA epoxide 17,18-EEQ and DHA epoxide 19,20- EDP and B) graded doses of DHA. Data were analyzed by standard one-way ANOVA for B and one-way ANOVA on ranks for C. No significant differences were found between groups; n=4 for all treatments. (Abbreviations: Nrf2: Nuclear factor (erythroid-derived 2)-like 2; HepG2-ARE: HepatocyteG2-antioxidant response element; n-3 PUFAs: Omega-3 polyunsaturated fatty acids; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; LS: L-sulphoraphane; 4-HHE: 4-hydroxyhexenal; 17,18-EEQ: 17,18- epoxyeicosatetraenoic acid; 19,20-EDP: 19,20- epoxydocosahexaenoic acid)

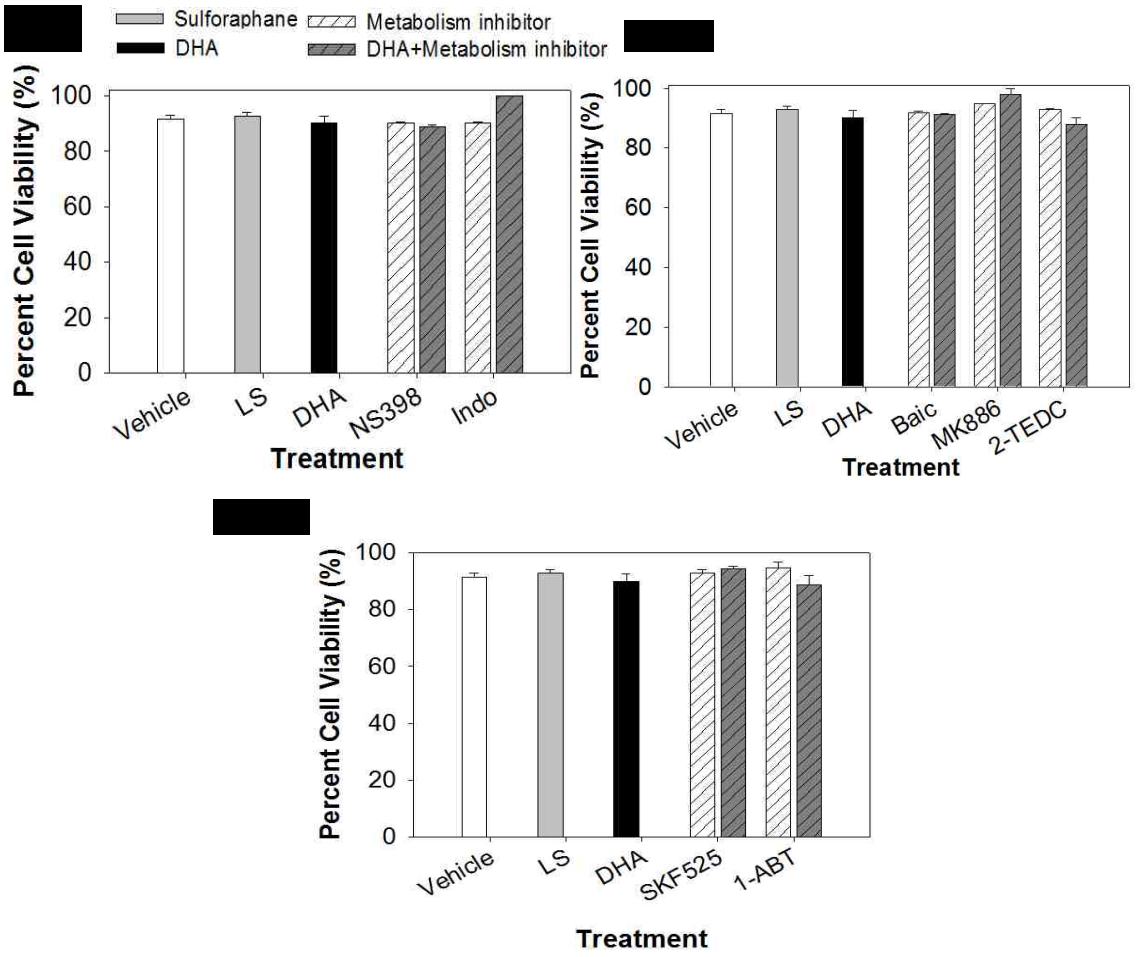

Figure 5.2. Cell viability assessed in HepG2-ARE cells treated with A) COX, B) LOX and C) P450 inhibitors with and without DHA. Data were analyzed by one-way ANOVA on ranks for B and C, and standard one-way ANOVA for C. No significant decreases in cell viability existed between groups; n=6 for DHA & n=8 for vehicle, n=3 for all other treatments. (Abbreviations: HepG2-ARE: HepatocyteG2-antioxidant response element; DHA: Docosahexaenoic acid; LS: L-sulphoraphane; COX: Cyclooxygenase; LOX: Lipoxygenase; P450: Cytochrome P450; NS398: N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide; Indo: Indomethacin; Baic: Baicalein; MK886: 1-[(4-chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]-α,α-dimethyl-5-(1-methylethyl)-1H-indole-2-propanoic acid; 2-TEDC: 2-(1-Thienyl)ethyl 3,4-dihydroxybenzylidenecyanoacetate; SKF525: β-diethylaminoethyl diphenylpropylacetate hydrochloride; 1-ABT: 1-aminobenzotriazole)

APPENDIX B

Supplemental data for chapter III

Table 5.2. Ethnicity and race of cigarette smokers.

Variable	Number	n
Ethnicity		67
Hispanic/Latino	28	
Non-Hispanic/Latino	27	
Unknown or not reported	11	
Race		67
Caucasian/White	37	
Unknown or not reported	13	
More than one race	7	
Black/African American	4	
American Indian/Alaska Native	4	
Native Hawaiian/Other Pacific Islander	1	
Asian	1	

Table 5.3. PUFA metabolites and metabolite groupings used for multivariate analysis with MDA, 8-epi-PGF2α and FMD.

Parent PUFA and Source of Metabolites	Metabolite Groups Used for Multivariate Analysis	Individual Metabolites in Each Group
Linoleic acid		
CYP-generated epoxide	Epoxyoctadecenoic acid (EpOME)	9,10- and 12,13-EpOME
LOX-generated monohydroxy	Hydroxyoctadecadienoic acid (HODE)	NA
sEH-generated dihydroxy	Dihydroxyoctadecenoic acid (DiHOME)	9,10- and 12,13-DiHOME
Arachidonic acid		
CYP-generated epoxide	Epoxyeicosatrienoic acid (EET)	5,6-, 8,9-, 11,12- and 14,15-EET
LOX- and autoxidation*-generated monohydroxy	Hydroxyeicosatetraenoic acid (HETE)	LOX: 5-, 8*-, 9*-, 11*-, 12-, and 15-HETE
LOX-generated dihydroxy	Leukotriene B4 (LTB4)	NA
sEH-generated dihydroxy	Dihydroxyeicosatrienoic acid (DHET)	5,6-, 8,9-, 11,12- and 14,15-DHET
LOX-generated trihydroxy	Lipoxin A4 (LXA4)	NA
Eicosapentanoic acid		
CYP-generated epoxide	Epoxyeicosatetraenoic acid (EEQ)	8,9-, 11,12-, 14,15- and 17,18-EEQ
LOX- , COX- and autoxidation*-generated monohydroxy	Hydroxyeicosapentaenoic acid (HEPE)	LOX: 5-, 8*-, 9*-, 12-, and 15-HEPE; COX: 18*-HEPE
sEH-generated dihydroxy	Dihydroxyeicosatetraenoic acid (DiHETE)	5,6-, 8,9-, 11,12-, 14,15- and 17,18-DiHETE
LOX-generated dihydroxy	Leukotriene B5 (LTB5)	NA
COX2-generated trihydroxy	Resolvin E1	NA
Docosahexanoic acid		
CYP-generated epoxide	Epoxydocosapentaenoic acid (EDP)	7,8-, 10,11-, 13,14-, 16,17- and 19,20-EDP
CYP-, LOX- and autoxidation*-generated monohydroxy	Hydroxydocosaexaenoic acid (HDHA)	LOX: 4-, 7-, 8*-, 10*-, 11*-, 13*-, 14-, 16*-, and 17-HDHA; 20*-HDHA; CYP: 21- and 22-HDHA
LOX-generated dihydroxy	Dihydroxydocosahexaenoic acid (DiHDHA)	NA
sEH-generated dihydroxy	Dihydroxydocosapentaenoic acid (DiHDPA)	7,8-, 10,11-, 13,14-, 16,17- and 19,20-DiHDPA

Parent PUFA and Source of Metabolites	Metabolite Groups Used for Multivariate Analysis	Individual Metabolites in Each Group
COX2-generated trihydroxy	Resolvin D1	NA

*Metabolites that can be generated via autoxidation in addition to enzymatically. These twelve (12) metabolites were also combined into a single metabolite group for multivariate analysis.
NA-Not analyzed as part of any metabolite group

Table 5.4. Antioxidant levels in plasma and RBCs from current cigarette smokers.

Compound	Mean ± SD	Range (min - max)	n
SOD (U/mL)	4.17 ± 1.75	1.03 - 10.41	65
CAT (nmol/min/mL)	37.52 ± 25.81	6.44 - 152.74	65
GSH (µM)	410.74 ± 228.38	25.36 - 979.90	64
GSSG (µM)	98.19 ± 64.24	3.23 - 220.22	64
GSH/GSSG	7.72 ± 9.49	0.84 - 46.55	64

Abbreviations: SOD: Superoxide dismutase; CAT: Catalase; GSH: Reduced glutathione; GSSG: Oxidized glutathione.

Table 5.5. Percentage of saturated, monunsaturated, and polyunsaturated fatty acids in red blood cells of current cigarette smokers.

Fatty Acid Name	Formula	Mean (%) ± SD	Range (min - max%)	n
Myristic acid	C14:0	0.27 ± 0.07	0.14 - 0.47	63
Palmitic acid	C16:0	22.03 ± 1.24	19.88 - 25.69	63
trans Palmitoleic acid	C16:1n7t	0.13 ± 0.03	0.07 - 0.21	63
Palmitoleic acid	C16:1n7	0.38 ± 0.23	0.09 - 1.27	63
Stearic acid	C18:0	17.53 ± 0.79	15.23 - 18.93	63
Elaidic acid	C18:1t	0.90 ± 0.29	0.46 - 1.96	63
Oleic acid	C18:1n9	14.46 ± 0.96	12.62 - 16.40	63
Conjugated Linoleic acid	C18:2n6t	0.25 ± 0.09	0.11 - 0.45	63
Linoleic acid (LA)	C18:2n6	13.10 ± 1.67	9.33 - 17.25	63
Arachidic acid	C20:0	0.14 ± 0.02	0.09 - 0.20	63
γ-Linolenic acid	C18:3n6	0.11 ± 0.05	0.03 - 0.24	63
Eicosenoic acid	C20:1n9	0.27 ± 0.04	0.19 - 0.35	63
α-Linolenic acid (ALA)	C18:3n3	0.16 ± 0.04	0.08 - 0.27	63
Eicosadienoic acid	C20:2n6	0.35 ± 0.05	0.25 - 0.46	63
Behenic acid	C22:0	0.16 ± 0.05	0.07 - 0.32	63
Eicosatrienoic acid	C20:3n6	2.03 ± 0.41	1.26 - 3.02	63
Arachidonic acid (AA)	C20:4n6	16.17 ± 1.39	12.44 - 19.03	63
Lignoceric acid	C24:0	0.39 ± 0.16	0.16 - 0.85	63
Eicosapentaenoic acid (EPA)	C20:5n3	0.37 ± 0.11	0.18 - 0.60	63
Nervonic acid	C24:1n9	0.37 ± 0.18	0.15 - 0.84	63
Docosatetraenoic acid	C22:4n6	4.35 ± 0.64	2.94 - 5.83	63
Docosapentaenoic acid n-6	C22:5n6	0.84 ± 0.18	0.47 - 1.29	63
Docosapentaenoic acid n-3	C22:5n3	2.37 ± 0.31	1.61 - 3.04	63
Docosahexanoic acid (DHA)	C22:6n3	2.86 ± 0.81	1.45 - 4.67	63
Σ Saturated fatty acids	Σ(CX:0)	40.52 ± 1.05	38.17 - 43.69	63
Σ Monounsaturated fatty acids	Σ(CX:1)	16.50 ± 1.10	14.68 - 19.52	63
Σ n-6 PUFA	Σ(CX:n6)	37.20 ± 1.30	33.92 - 39.78	63
Omega-3 Index	EPA+DHA	3.24 ± 0.87	1.78 - 5.16	63

Abbreviations: PUFA: Polyunsaturated fatty acid

Table 5.6. Concentration of PUFA metabolites in plasma of current cigarette smokers.

Parent PUFA	Metabolite*	Mean (ng/mL) ± SD	Range (min - max ng/mL)	n
LA	**13-HODE**	253.57 ± 102.2	101.21 - 623.57	62
LA	**9,10-EpOME**	10.55 ± 5.26	4.70 - 32.01	62
LA	**12,13-EpOME**	8.53 ± 5.95	3.39 - 45.12	62
LA	**9,10-DiHOME**	7.44 ± 3.2	3.52 - 20.09	62
LA	**12,13-DiHOME**	5.33 ± 3.66	2.05 - 28.65	62
AA	**5,6-EET**	1.90 ± 0.60	0.92 - 3.34	62
AA	**8,9-EET**	2.96 ± 1.43	1.47 - 10.17	62
AA	**11,12-EET**	1.62 ± 0.44	0.89 - 2.73	62
AA	**14,15-EET**	1.87 ± 0.53	1.02 - 3.23	62
AA	**5,6-DHET**	4.19 ± 2.36	1.19 - 13.64	62
AA	**8,9-DHET**	2.50 ± 1.63	0.90 - 9.60	62
AA	**11,12-DHET**	0.74 ± 0.49	0.30 - 4.01	62
AA	**14,15-DHET**	0.62 ± 0.30	0.33 - 2.22	62
AA	**LTB4**	0.52	0.41 - 0.64	2
AA	**LXA4**	ND	ND	62
AA	**5-HETE**	12.49 ± 4.26	5.05 - 24.09	62
AA	**8-HETE**	5.29 ± 1.77	2.27 - 10.36	62
AA	**9-HETE**	6.39 ± 2.20	2.70 - 12.78	62
AA	**11-HETE**	8.55 ± 2.90	3.46 - 17.86	62
AA	**12-HETE**	5.30 ± 1.80	2.57 - 11.62	62
AA	**15-HETE**	8.76 ± 3.25	4.42 - 18.06	62
AA	**19-HETE**	ND	ND	62
AA	**20-HETE**	1.02 ± 0.49	0.46 - 2.90	62
EPA	**8,9-EEQ**	0.16 ± 0.08	0.03 - 0.40	62
EPA	**11,12-EEQ**	0.04 ± 0.03	0.01 - 0.17	62
EPA	**14,15-EEQ**	0.10 ± 0.04	0.05 - 0.25	62
EPA	**17,18-EEQ**	0.17 ± 0.06	0.08 - 0.38	62
EPA	**5,6-DiHETE**	0.55 ± 0.47	0.13 - 2.28	62
EPA	**8,9-DiHETE**	0.29 ± 0.17	0.09 - 0.83	62
EPA	**11,12-DiHETE**	0.10 ± 0.06	0.00 - 0.41	58
EPA	**14,15-DiHETE**	0.16 ± 0.07	0.06 - 0.47	62
EPA	**17,18-DiHETE**	0.91 ± 0.32	0.30 - 1.90	62
EPA	**Resolvin E1**	ND	ND	62
EPA	**LTB5**	ND	ND	62
EPA	**5-HEPE**	0.94 ± 0.49	0.20 - 2.25	62
EPA	**8-HEPE**	0.26 ± 0.13	0.05 - 0.67	62
EPA	**9-HEPE**	0.42 ± 0.20	0.14 - 1.07	62
EPA	**12-HEPE**	0.31 ± 0.16	0.11 - 0.88	62

Parent PUFA	Metabolite*	Mean (ng/mL) ± SD	Range (min - max ng/mL)	n
EPA	**15-HEPE**	0.26 ± 0.11	0.10 - 0.65	62
EPA	**18-HEPE**	0.93 ± 0.53	0.29 - 3.00	62
EPA	**19-HEPE**	ND	ND	62
EPA	**20-HEPE**	ND	ND	62
DHA	**Resolvin D1**	ND	ND	62
DHA	**10,17-DiHDHA**	0.04 ± 0.03	0.01 - 0.16	62
DHA	**4-HDHA**	2.39 ± 1.09	0.78 - 5.60	62
DHA	**7-HDHA**	1.35 ± 0.56	0.54 - 2.86	62
DHA	**8-HDHA**	1.88 ± 0.87	0.69 - 4.25	62
DHA	**10-HDHA**	1.22 ± 0.58	0.46 - 2.73	62
DHA	**11-HDHA**	1.38 ± 0.60	0.54 - 2.91	62
DHA	**13-HDHA**	1.23 ± 0.58	0.47 - 2.68	62
DHA	**14-HDHA**	1.68 ± 0.85	0.08 - 4.08	62
DHA	**16-HDHA**	1.11 ± 0.52	0.43 - 2.41	62
DHA	**17-HDHA**	1.71 ± 1.10	0.24 - 4.78	62
DHA	**20-HDHA**	3.01 ± 1.38	1.09 - 6.92	62
DHA	**21-HDHA**	0.63 ± 0.29	0.14 - 1.38	62
DHA	**22-HDHA**	0.60 ± 0.35	0.04 - 1.64	62
DHA	**7,8-EDP**	0.82 ± 0.27	0.27 - 1.51	62
DHA	**10,11-EDP**	0.68 ± 0.22	0.26 - 1.19	62
DHA	**13,14-EDP**	0.43 ± 0.14	0.16 - 0.72	62
DHA	**16,17-EDP**	0.45 ± 0.15	0.18 - 0.81	62
DHA	**19,20-EDP**	0.53 ± 0.16	0.21 - 0.93	62
DHA	**7,8-DiHDPA**	0.44 ± 0.25	0.13 - 1.49	62
DHA	**10,11-DiHDPA**	0.07 ± 0.03	0.02 - 0.20	62
DHA	**13,14-DiHDPA**	0.03 ± 0.03	0.00 - 0.15	62
DHA	**16,17-DiHDPA**	0.26 ± 0.13	0.09 - 0.82	62
DHA	**19,20-DiHDPA**	0.49 ± 0.22	0.18 - 1.21	62

*Metabolite definitions can be found in Table 6.2

ND–Not detected

Table 5.7. Spearman correlation analysis with oxidative stress marker MDA and clinical characteristics, antioxidant markers, RBC fatty acids and plasma PUFA metabolites.

Variable	r	P	n
SOD	-0.103	0.414	65
CAT	0.365	**0.003**	65
GSH	-0.006	0.965	64
GSSG	0.034	0.794	63
GSH:GSSG	-0.019	0.881	63
8-epi-PGF2α	0.049	0.698	65
Heart rate	0.028	0.824	65
BMI	-0.098	0.436	65
SBP	0.112	0.373	65
DBP	0.184	0.142	65
Pack years	0.165	0.187	65
HbA1c	0.063	0.620	64
Total cholesterol	0.079	0.529	65
HDL	0.286	**0.021**	65
LDL	0.049	0.698	65
Triglycerides	-0.076	0.545	65
Framingham risk score	-0.127	0.312	65
Sex	0.132	0.294	65
Age	0.054	0.666	65
Race	0.101	0.423	65
Urine cotinine	-0.019	0.880	65
C14:0*	0.153	0.230	63
C16:0*	0.321	**0.010**	63
C16:1n7t*	-0.100	0.433	63
C16:1n7*	0.032	0.801	63
C18:0*	-0.076	0.554	63
C18:1t*	-0.133	0.298	63
C18:1n9*	-0.174	0.171	63
C18:2n6t*	-0.568	**1.45E-06**	63
C18:2n6*	0.034	0.790	63
C20:0*	-0.254	**0.044**	63
C18:3n6*	-0.010	0.940	63
C20:1n9*	-0.144	0.260	63
C18:3n3*	-0.046	0.718	63
C20:2n6*	-0.008	0.953	63
C22:0*	-0.282	**0.025**	63
C20:3n6*	-0.131	0.306	63

Variable	r	P	n
C20:4n6*	-0.131	0.306	63
C24:0*	-0.179	0.160	63
C20:5n3*	-0.017	0.896	63
C24:1n9*	-0.154	0.228	63
C22:4n6*	0.078	0.545	63
C22:5n6*	-0.036	0.779	63
C22:5n3*	-0.152	0.235	63
C22:6n3*	-0.043	0.739	63
Omega-3 index*	-0.041	0.750	63
13-HODE **	-0.323	**0.011**	62
9,10-EpOME **	-0.290	**0.023**	62
12,13-EpOME**	-0.360	**0.004**	62
9,10-DiHOME**	0.074	0.569	62
12,13-DiHOME**	-0.182	0.156	62
ΣDiHOME/ΣEpOME	0.337	**0.008**	62
5,6-EET**	0.145	0.261	62
8,9-EET**	-0.024	0.853	62
11,12-EET**	-0.159	0.216	62
14,15-EET**	-0.083	0.519	62
5,6-DHET**	0.120	0.350	62
8,9-DHET**	0.060	0.644	62
11,12-DHET**	0.100	0.440	62
14,15-DHET**	0.135	0.295	62
ΣDHET/ΣEET	0.223	0.082	62
8,9-EEQ**	0.142	0.270	62
11,12-EEQ**	-0.090	0.484	62
14,15-EEQ**	0.001	0.993	62
17,18-EEQ**	-0.095	0.461	62
5,6-DiHETE**	0.202	0.116	62
8,9-DiHETE**	0.162	0.207	62
11,12-DiHETE**	0.098	0.461	58
14,15-DiHETE**	0.268	**0.035**	62
17,18-DiHETE**	0.171	0.183	62
ΣDiHETE/ΣEEQ	0.230	0.072	62
7,8-EDP**	0.055	0.673	62
10,11-EDP**	0.051	0.691	62
13,14-EDP**	0.014	0.911	62
16,17-EDP**	0.016	0.903	62
19,20-EDP**	0.010	0.939	62
7,8-DiHDPA**	0.156	0.224	62
10,11-DiHDPA**	0.191	0.137	62
13,14-DiHDPA**	0.148	0.251	62

Variable	r	P	n
16,17-DiHDPA**	0.196	0.126	62
19,20-DiHDPA**	0.143	0.268	62
ΣDiHDPA/ΣEDP**	0.267	0.036	62
LTB4**	-1.000	1.000	2
LXA4**	ND	ND	
5-HETE**	-0.055	0.672	62
8-HETE**	-0.058	0.652	62
9-HETE**	-0.115	0.372	62
11-HETE**	-0.089	0.493	62
12-HETE**	-0.167	0.195	62
15-HETE**	-0.183	0.153	62
19-HETE**	ND	ND	
20-HETE**	0.011	0.931	62
Resolvin E1	ND	ND	
LTB5**	ND	ND	
5-HEPE**	-0.056	0.664	62
8-HEPE**	0.026	0.840	62
9-HEPE**	-0.060	0.642	62
12-HEPE**	-0.043	0.741	62
15-HEPE**	-0.041	0.748	62
18-HEPE**	-0.094	0.465	62
19-HEPE**	ND	ND	
20-HEPE**	ND	ND	
Resolvin D1	ND	ND	
10,17-DiHDHA**	-0.085	0.509	62
4-HDHA**	0.035	0.787	62
7-HDHA**	0.023	0.858	62
8-HDHA**	-0.040	0.760	62
10-HDHA**	-0.013	0.920	62
11-HDHA**	-0.049	0.702	62
13-HDHA**	-0.028	0.829	62
14-HDHA**	-0.149	0.245	62
16-HDHA**	-0.015	0.911	62
17-HDHA**	-0.139	0.279	62
20-HDHA**	-0.059	0.648	62
21-HDHA**	0.007	0.957	62
22-HDHA**	0.074	0.566	62

*Fatty acid definitions can be found in Table 6.4
**Metabolite definitions can be found in Table 6.2
ND–Not detected
Abbreviations: MDA: Malondialdehyde; PUFA: Polyunsaturated fatty acid.

Table 5.8. Linear regression analysis using those variables that significantly associated with MDA in Spearman correlation at $P < 0.05$.

Variable-noted as 'x' (independent) or 'y' (dependent)*	β ± SE	r	P	n
Catalase (x)	0.194 ± 0.080	0.292	**0.018**	65
HDL (y)	0.178 ± 0.073	0.294	**0.017**	65
C16:0 (x)**	2.345 ± 0.864	0.328	**0.009**	63
C18:2n6t (x)**	-0.505 ± 0.101	0.54	**<0.001**	63
C20:0 (x)**	-0.612 ± 0.301	0.252	**0.046**	63
C22:0 (x)**	-0.264 ± 0.159	0.208	0.102	63
13-HODE (x)$^{+}$	-0.337 ± 0.132	0.313	**0.013**	62
9,10-EpOME (x)$^{+}$	-0.235 ± 0.119	0.248	0.052	62
12,13-EpOME (x)$^{+}$	-0.253 ± 0.105	0.297	**0.019**	62
14,15-DiHETE (x)$^{+}$	0.267 ± 0.128	0.26	**0.041**	62
ΣDiHOME/ΣEpOME (x)$^{+}$	0.347 ± 0.127	0.333	**0.008**	62
ΣDiHDPA/ΣEDP (x)$^{+}$	0.324 ± 0.172	0.236	0.065	62

*Both the x- and y-variables were log10 transformed
**Fatty acid definitions can be found in Table 6.4
$^{+}$Metabolite definitions can be found in Table 6.2

Table 5.9. Spearman correlation analysis with oxidative stress marker 8-epi-PGF2α and clinical characteristics, antioxidant markers, RBC fatty acids and plasma PUFA metabolites.

Variable	r	P	n
SOD	0.145	0.249	65
CAT	-0.001	0.995	65
GSH	0.062	0.624	64
GSSG	0.165	0.194	63
GSH:GSSG	-0.072	0.576	63
MDA	0.049	0.698	65
Heart rate	0.210	0.092	65
BMI	-0.169	0.177	65
SBP	0.083	0.511	65
DBP	0.135	0.282	65
Pack years	-0.019	0.880	65
HbA1c	-0.048	0.708	64
Total cholesterol	0.257	**0.039**	65
HDL	0.062	0.624	65
LDL	0.207	0.098	65
Triglycerides	0.132	0.292	65
Framingham risk score	0.108	0.390	65
Sex	0.130	0.300	65
Age	0.037	0.770	65
Race	0.006	0.964	65
Urine cotinine	-0.021	0.871	65
C14:0*	0.089	0.486	63
C16:0*	0.239	0.059	63
C16:1n7t*	0.141	0.271	63
C16:1n7*	0.203	0.111	63
C18:0*	-0.112	0.381	63
C18:1t*	0.228	0.072	63
C18:1n9*	0.006	0.961	63
C18:2n6t*	0.249	**0.050**	63
C18:2n6*	-0.095	0.460	63
C20:0*	-0.052	0.682	63
C18:3n6*	-0.066	0.607	63
C20:1n9*	-0.136	0.287	63
C18:3n3*	-0.080	0.534	63
C20:2n6*	-0.141	0.269	63
C22:0*	-0.169	0.186	63
C20:3n6*	0.059	0.648	63

Variable	r	P	n
C20:4n6*	0.064	0.617	63
C24:0*	-0.172	0.177	63
C20:5n3*	0.021	0.871	63
C24:1n9*	-0.166	0.193	63
C22:4n6*	0.012	0.925	63
C22:5n6*	0.055	0.668	63
C22:5n3*	0.123	0.337	63
C22:6n3*	-0.108	0.396	63
Omega-3 index	-0.081	0.528	63
13-HODE**	0.124	0.335	62
9,10-EpOME**	0.110	0.395	62
12,13-EpOME**	0.076	0.556	62
9,10-DiHOME**	0.181	0.158	62
12,13-DiHOME**	0.135	0.295	62
ΣDiHOME/ΣEpOME	0.081	0.532	62
5,6-EET**	0.277	**0.029**	62
8,9-EET**	0.270	**0.034**	62
11,12-EET**	0.307	**0.016**	62
14,15-EET**	0.354	**0.005**	62
5,6-DHET**	0.277	**0.029**	62
8,9-DHET**	0.198	0.122	62
11,12-DHET**	0.218	0.088	62
14,15-DHET**	0.082	0.526	62
ΣDHET/ΣEET	0.053	0.681	62
8,9-EEQ**	0.016	0.900	62
11,12-EEQ**	0.089	0.490	62
14,15-EEQ **	0.100	0.437	62
17,18-EEQ**	0.126	0.328	62
5,6-DiHETE**	0.196	0.126	62
8,9-DiHETE**	0.063	0.627	62
11,12-DiHETE**	0.157	0.239	58
14,15-DiHETE**	0.023	0.858	62
17,18-DiHETE**	0.189	0.140	62
ΣDiHETE/ΣEEQ	0.172	0.180	62
7,8-EDP**	0.180	0.160	62
10,11-EDP**	0.044	0.735	62
13,14-EDP**	0.136	0.290	62
16,17-EDP**	0.147	0.254	62
19,20-EDP**	0.153	0.233	62
7,8-DiHDPA**	0.207	0.107	62
10,11-DiHDPA**	0.050	0.701	62
13,14-DiHDPA**	0.026	0.841	62

Variable	r	P	n
16,17-DiHDPA**	0.013	0.920	62
19,20-DiHDPA**	0.067	0.602	62
ΣDiHDPA/ΣEDP	0.035	0.784	62
LTB4**	1.000	1.000	2
LXA4**	ND	ND	
5-HETE**	0.197	0.124	62
8-HETE**	0.184	0.152	62
9-HETE**	0.212	0.098	62
11-HETE**	0.195	0.128	62
12-HETE**	0.165	0.198	62
15-HETE**	0.203	0.113	62
19-HETE**	ND	ND	
20-HETE**	0.172	0.181	62
Resolvin E1	ND	ND	
LTB5**	ND	ND	
5-HEPE**	0.128	0.320	62
8-HEPE**	0.102	0.430	62
9-HEPE**	0.133	0.302	62
12-HEPE**	0.135	0.293	62
15-HEPE**	0.081	0.529	62
18-HEPE**	0.081	0.533	62
19-HEPE**	ND	ND	
20-HEPE**	ND	ND	
Resolvin D1	ND	ND	
10,17-DiHDHA**	0.355	**0.005**	62
4-HDHA**	-0.019	0.886	62
7-HDHA**	0.042	0.743	62
8-HDHA**	0.042	0.743	62
10-HDHA**	0.005	0.970	62
11-HDHA**	0.062	0.633	62
13-HDHA**	-0.002	0.989	62
14-HDHA**	0.011	0.931	62
16-HDHA**	0.010	0.940	62
17-HDHA**	-0.037	0.776	62
20-HDHA**	0.002	0.988	62
21-HDHA**	0.046	0.720	62
22-HDHA**	0.161	0.209	62

*Fatty acid definitions can be found in Table 6.4
**Metabolite definitions can be found in Table 6.2
ND–Not detected
Abbreviations: 8-epi-PGF2α: 8-isoprostane; PUFA: Polyunsaturated fatty acid.

Table 5.10. Linear regression analysis using those variables that significantly associated with 8-epi-PGF2α in Spearman correlation at $P < 0.05$.

Variable-noted as 'x' (independent) or 'y' (dependent)*	β ± SE	r	P	n
Total cholesterol (y)	0.215 ± 0.167	0.16	0.204	65
C18:2n6t (x)**	0.424 ± 0.175	0.296	**0.019**	63
5,6-EET (x)[+]	0.414 ± 0.212	0.245	0.055	62
8,9-EET (x)[+]	0.244 ± 0.175	0.177	0.169	62
11,12-EET (x)[+]	0.531 ± 0.248	0.266	**0.037**	62
14,15-EET (x)[+]	0.588 ± 0.235	0.307	**0.015**	62
5,6-DHET (x)[+]	0.279 ± 0.133	0.261	**0.04**	62
10,17-DiHDHA (x)[+]	0.187 ± 0.070	0.325	**0.01**	62

*Both the x- and y-variables were log10 transformed

**Fatty acid definitions can be found in Table 6.4

[+]Metabolite definitions can be found in Table 6.2

Table 5.11. Spearman correlation analysis with FMD and clinical characteristics, oxidative stress markers, antioxidant markers, RBC fatty acids and plasma PUFA metabolites.

Variable	r	P	n
SOD	-0.154	0.248	58
CAT	-0.246	0.063	58
GSH	0.198	0.139	57
GSSG	0.030	0.826	56
GSH:GSSG	0.098	0.471	56
MDA	-0.197	0.138	58
8-epi-PGF2α	-0.025	0.850	58
Heart rate	-0.072	0.582	60
BMI	0.002	0.991	60
SBP	-0.136	0.299	60
DBP	-0.083	0.528	60
Pack years	0.079	0.548	60
HbA1c	-0.216	0.107	57
Total cholesterol	-0.033	0.805	58
HDL	-0.119	0.372	58
LDL	0.095	0.475	58
Triglycerides	-0.136	0.306	58
Framingham risk score	-0.105	0.430	58
Sex	0.121	0.356	60
Age	-0.048	0.712	60
Race	-0.157	0.230	60
Urine cotinine	0.112	0.394	60
C14:0*	0.038	0.783	56
C16:0*	-0.070	0.606	56
C16:1n7t*	-0.011	0.934	56
C16:1n7*	0.163	0.230	56
C18:0*	0.136	0.317	56
C18:1t*	-0.022	0.871	56
C18:1n9*	0.302	**0.024**	56
C18:2n6t*	0.158	0.244	56
C18:2n6*	-0.087	0.523	56
C20:0*	-0.063	0.645	56
C18:3n6*	-0.154	0.256	56
C20:1n9*	-0.290	**0.030**	56
C18:3n3*	-0.117	0.389	56
C20:2n6*	-0.251	0.062	56
C22:0*	-0.089	0.514	56
C20:3n6*	-0.120	0.379	56

Variable	r	P	n
C20:4n6*	0.048	0.726	56
C24:0*	-0.118	0.385	56
C20:5n3*	0.016	0.906	56
C24:1n9*	-0.071	0.600	56
C22:4n6*	-0.267	**0.047**	56
C22:5n6*	-0.034	0.800	56
C22:5n3*	-0.039	0.773	56
C22:6n3*	0.048	0.727	56
Omega-3 index	0.028	0.834	56
13-HODE**	0.145	0.288	55
9,10-EpOME**	-0.068	0.618	55
12,13-EpOME**	-0.028	0.838	55
9,10-DiHOME**	-0.113	0.412	55
12,13-DiHOME**	0.102	0.458	55
ΣDiHOME/ΣEpOME	-0.024	0.862	55
5,6-EET**	-0.015	0.915	55
8,9-EET**	0.147	0.284	55
11,12-EET**	0.203	0.137	55
14,15-EET**	0.169	0.215	55
5,6-DHET**	-0.018	0.898	55
8,9-DHET**	0.061	0.658	55
11,12-DHET**	0.101	0.463	55
14,15-DHET**	0.110	0.423	55
ΣDHET/ΣEET	-0.039	0.775	55
8,9-EEQ**	-0.009	0.945	55
11,12-EEQ**	-0.073	0.597	55
14,15-EEQ**	0.071	0.607	55
17,18-EEQ**	0.035	0.799	55
5,6-DiHETE**	-0.102	0.459	55
8,9-DiHETE**	-0.030	0.828	55
11,12-DiHETE**	-0.241	0.082	53
14,15-DiHETE**	-0.056	0.682	55
17,18-DiHETE**	0.136	0.321	55
ΣDiHETE/ΣEEQ	-0.029	0.821	55
7,8-EDP**	0.116	0.397	55
10,11-EDP**	0.149	0.277	55
13,14-EDP**	0.159	0.244	55
16,17-EDP**	0.216	0.112	55
19,20-EDP**	0.213	0.118	55
7,8-DiHDPA**	0.061	0.657	55
10,11-DiHDPA**	0.040	0.773	55

Variable	r	P	n
13,14-DiHDPA**	0.117	0.395	55
16,17-DiHDPA**	0.306	**0.023**	55
19,20-DiHDPA**	0.203	0.137	55
ΣDiHDPA/ΣEDP	0.053	0.699	56
LTB4**	1.000	1.000	2
LXA4**	ND	ND	
5-HETE**	0.192	0.160	55
8-HETE**	0.151	0.269	55
9-HETE**	0.160	0.243	55
11-HETE**	0.120	0.383	55
12-HETE**	0.164	0.231	55
15-HETE**	0.175	0.201	55
19-HETE**	ND	ND	
20-HETE**	0.048	0.729	55
Resolvin E1	ND	ND	
LTB5**	ND	ND	
5-HEPE**	0.120	0.382	55
8-HEPE**	0.049	0.721	55
9-HEPE**	0.046	0.735	55
12-HEPE**	0.048	0.726	55
15-HEPE**	0.054	0.695	55
18-HEPE**	0.007	0.961	55
19-HEPE**	ND	ND	
20-HEPE**	ND	ND	
Resolvin D1	ND	ND	
10,17-DiHDHA	-0.016	0.909	55
4-HDHA**	0.135	0.325	55
7-HDHA**	0.131	0.339	55
8-HDHA**	0.149	0.276	55
10-HDHA**	0.148	0.281	55
11-HDHA**	0.141	0.303	55
13-HDHA**	0.142	0.301	55
14-HDHA**	0.136	0.321	55
16-HDHA**	0.130	0.345	55
17-HDHA**	0.149	0.277	55
20-HDHA**	0.129	0.346	55
21-HDHA**	0.123	0.368	55
22-HDHA**	0.051	0.709	55

*Fatty acid definitions can be found in Table 6.4

**Metabolite definitions can be found in Table 6.2

ND–Not detected; Abbreviations: FMD: Flow-mediated dilation; PUFA: Polyunsaturated fatty acid.

Table 5.12. Linear regression analysis using those variables that significantly associated with 8-epi-PGF2α in Spearman correlation at $P < 0.05$.

Variable-noted as 'x' (independent)*	β ± SE	r	P	n
CAT (x)**	-0.229 ± 0.123	0.242	0.068	58
18:1n9 (x)**	0.305 ± 0.131	0.301	**0.024**	56
20:1n9 (x)**	-0.281 ± 0.124	0.294	**0.028**	56
22:4n6 (x)**	-0.259 ± 0.129	0.263	**0.05**	56
16,17-DiHDPA (x) [+]	0.296 ± 0.128	0.302	**0.025**	55

*The x-variable was log10 transformed and FMD values were ranked.
**Fatty acid definitions can be found in Table 6.4
[+]Metabolite definitions can be found in Table 6.2

APPENDIX C

Omega-3 Polyunsaturated Fatty Acids Improve Endothelial Function in Humans at Risk for Atherosclerosis

Kayla R. Zehr and Mary K. Walker. Omega-3 Polyunsaturated Fatty Acids Improve Endothelial Function in Humans at Risk for Atherosclerosis: A Review

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ABSTRACT

Epidemiology studies and clinical trials show that omega-3 polyunsaturated fatty acids (n-3 PUFAs) can prevent atherosclerotic morbidity and evidence suggests this may be mediated by improving endothelial dysfunction. Endothelial dysfunction is characterized by reduced vasodilation and a pro-inflammatory, pro-thrombotic state, and is an early pathological event in the development of atherosclerosis. Flow-mediated dilation (FMD), a gold standard for assessing endothelial dysfunction, is a predictor of future cardiovascular events and coronary heart disease risk. Notably, risk factors for endothelial dysfunction include classic risk factors for atherosclerosis: Elevated lipids, diabetes, hypertension, elevated BMI, cigarette smoking, and metabolic syndrome. In this paper, we review the ability of n-3 PUFAs to improve endothelial dysfunction in individuals with classic risk factors for atherosclerosis, but lacking diagnosed atherosclerotic disease, with the goal of identifying those individuals that might gain the most vasoprotection from n-3 PUFA supplements. We include trials using eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), or alpha-linolenic acid (ALA) alone, or EPA+DHA; and assessing endothelial function by FMD, forearm blood flow, or peripheral arterial tonometry. We found that n-3 PUFAs improved endothelial dysfunction in 16 of 17 studies in individuals with hyperlipidemia, elevated BMI, metabolic syndrome, or that smoked cigarettes, but only in 2 of 5 studies in diabetics. Further, these trials showed that use of EPA+DHA consistently improve endothelial dysfunction; ALA-enriched diets appear promising; but use of EPA or DHA alone requires further study. We conclude that individuals with hyperlipidemia, elevated BMI,

metabolic syndrome, or that smoke could derive vasoprotective benefits from EPA+DHA supplementation.

INTRODUCTION

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) prevent atherosclerotic disease development in humans based on epidemiology studies and clinical trials (Thies et al. 2003; von Schacky 1987; Dolecek 1992; Daviglus 1997). Atherosclerotic associated-diseases include peripheral artery disease, coronary artery disease, aneurysm, and stroke. Endothelial dysfunction, which is characterized by a loss of vasodilatory and anti-inflammatory factors and a gain of vasoconstrictive and pro-inflammatory factors, is an early pathological event in the development of atherosclerotic cardiovascular disease (CVD) (Hadi, Carr, and Al Suwaidi 2005). Risk factors for endothelial dysfunction include classic risk factors for atherosclerosis; elevated cholesterol, elevated triglycerides, diabetes, hypertension and cigarette smoking (Versari et al. 2009). Thus, n-3 PUFAs would be considered to be a form of primary prevention since the consumption of n-3 PUFAs decreases the incidence of atherosclerotic disease as individuals age.

Endothelial dysfunction is one of the earliest events in the pathological development of atherosclerotic diseases, and flow-mediated dilation (FMD) is the non-invasive, gold standard for measuring endothelial dysfunction in the clinic. Briefly, to assess FMD the baseline brachial artery diameter is recorded via ultrasound and a blood pressure cuff is placed around the forearm and inflated for 5 minutes, restricting blood flow. The cuff is then deflated, causing reactive hyperemia, and the vessel diameter is continually recorded for 2 minutes post-cuff deflation. This shear-stress induced production of endothelial-derived vasodilatory factors, primarily nitric oxide (NO),

causes the brachial artery to dilate and FMD is expressed as the percentage increase in artery diameter relative to baseline diameter (Raitakari and Celermajer 2000).

Prospective studies have shown that FMD is an independent predictor of CV events, such as heart attack or stroke, in individuals without clinical CVD or at low CVD risk. A prospective study of 2,264 women (54 ± 6 years) free of clinical CVD demonstrated that FMD is significantly associated with CV events in a 4 year follow-up and is independent of other classic risk factors (Rossi et al. 2008). Similarly, a prospective study of 435 individuals (54 ± 12 years) without apparent coronary heart disease (CHD) show that FMD is the best independent predictor of future CV events (Shechter et al. 2009). In an earlier meta-analysis of 211 publications, FMD is significantly predictive of the 10-year risk of CHD, but only in those individuals with low Framingham risk scores (Witte et al. 2005). Furthermore, studies have shown that improving FMD reduces the number of CV events in individuals with existing CVD. For example, patients with hypertension and persistently impaired FMD (7.1 ± 2.5%) exhibit 3.5 non-fatal CV events/100 person-years, which is significantly higher ($P < 0.0001$) than 0.51 CV events/100 person-years in patients with improved FMD (13.9 ± 2.6%) (Modena et al. 2002). Thus, these studies suggest that some individuals could benefit from therapies specifically targeted to improve endothelial dysfunction.

Evidence from multiple epidemiological, experimental and clinical studies suggests that n-3 PUFAs can decrease the risk of CVD, in part, by improving vascular function**.** Three n-3 PUFAs have been found to be vasoprotective; eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) enriched in fish oil, and alpha-linolenic acid (ALA, 18:3n-3) enriched in certain plants. Some of these vasoprotective

benefits include decreased arterial plaque buildup (Renier et al. 1993) increased anti-inflammatory properties (Calder 2006), improved endothelial-dependent vasodilation as measured by FMD (Wiest, Walsh-Wilcox, and Walker 2017; Goodfellow et al. 2000; Siasos et al. 2013), decreased blood pressure (Ulu et al. 2014; Hoshi et al. 2013; Miller, Van Elswyk, and Alexander 2014), and increased antioxidant capacity (Thorlaksdottir et al. 2006).

METHODS

In this article, we review the ability of n-3 PUFA supplementation to improve endothelial function in individuals with classic risk factors for atherosclerosis, including hyperlipidemia, hypertension, diabetes, smoking, elevated BMI, and metabolic syndrome with the goal of identifying those individuals that might gain the most vasoprotective benefit from n-3 PUFA supplements prior to the clinical evidence of atherosclerotic disease. In addition to FMD, we have included two other methods for assessing vascular function; peripheral arterial tonometry (PAT) and forearm blood flow (FBF). The PAT method measures blood flow in the index fingers using plethysmographic probes prior to, during, and after compression of blood flow in one upper arm (EndoPAT; Itamar Medical), and results are expressed as the reactive hyperemia index (RHI) (Axtell, Gomari, and Cooke 2010). The FBF is assessed using strain-gauge plethysmography prior to and following upper arm occlusion or prior to and following arterial infusion of an endothelial-dependent vasodilator. While all three methods are indices of endothelial function, FMD assesses the dilation occurring in a conduit artery, while FBF and PAT

assess the dilation occurring in resistance arterioles of the forearm and fingers, respectively.

To identify published articles on this intervention strategy, Pubmed was searched using the following terms: (endothelial dysfunction OR flow-mediated OR FMD) AND (omega-3) AND (hyperlipidemia OR hypertriglyceridemia OR cigarette OR smoking OR hypertension OR diabetes OR metabolic syndrome) between 1951-January 2017. We excluded articles in which individuals had diagnosed peripheral artery disease, coronary artery disease, stroke, angina, atherosclerosis, acute myocardial infarction, and studies with healthy individuals who had no classic risk factors for atherosclerotic cardiovascular disease. We also excluded articles in which n-3 PUFA intake was estimated from dietary consumption of fish and treatments < 2 weeks. Finally, we identified additional articles from the reference lists and cited-searches of the publications found in PubMed. A summary of each of the 22 articles is presented in Table 5.13, grouped by atherosclerosis CVD risk factor and then listed in chronological order. Additionally, we calculated a dose of n-3 PUFA consumed daily, based on the reported dose and the percentage of n-3 PUFA in the formulation.

RESULTS

Hyperlipidemia

Since elevated cholesterol and triglycerides are risk factors for endothelial dysfunction (Lundman et al. 2001) and atherosclerosis (Kannel et al. 1971), we reviewed those papers that assessed the ability of n-3 PUFA supplementation to improve

Table 5.13. Summary of study subject characteristics, study design, and outcomes on endothelial function and triglycerides (TG), following n-3 PUFA supplementation and grouped according to underlying atherosclerosis CVD risk factor.

Study	Description of Subjects	n-3 PUFA dose [Consumed daily dose][a]	Duration	Placebo control (Y/N)	Crossover design (Y/N)	Washout duration	Group size	Outcomes
Hyperlipidemia								
(Goodfellow et al. 2000)	Hypercholesterolemic 50-56 ± 13 years old (yo) 19 Male (M), 10 Female (F)	4 g/d - 85% EPA+DHA [3.4 g/d EPA+DHA]	17 wks	Y Corn oil	N	NA	Placebo: 15 n-3 PUFA: 13	□ FMD □TG
(Mori et al. 2000)	Hyperlipidemic & BMI 25-30 kg/m^3 Age- and BMI-matched 49-50 ± 2 yo 40 M	4 g/d - 96% EPA or 92% DHA [3.8 g/d EPA or 3.7 g/d DHA]	6 wks	Y Olive oil	N	NA	Placebo: 14 EPA: 13 DHA: 13	DHA only: □ FBF[b] DHA only: □ TG
(Okumura et al. 2002)	Hypertriglyceridemic 38-44 ± 5 yo 15 M	1.8 g/d - 98% EPA [1.8 g/d EPA]	12 wks	N	N	NA	Normolipidemic: 7 Hyperlipidemic: 8	□ FBF[b] □TG
(Ros et al. 2004)	Hypercholesterolemic 26-75 yo Sex NR	Mediterranean diet or isoenergetic diet plus 45-60 g/d walnuts [3.7-6 g/d ALA]	4 wks	N	Y	None	21	□ FMD ↔ TG
(Engler et al. 2004)	Hyperlipidemic 9-19 yo Sex NR	6 g/d - 20% DHA [1.2 g/d DHA]	6 wks	Y Corn/soy oil	Y	6 wks	20	□ FMD ↔ TG
(Mindrescu et al. 2008)	Hyperlipidemic 27-78 yo 23 M, 7 F	6 g/d - 43% EPA + 32% DHA and 10 mg/d rosuvastatin, or 10 mg/d rosuvastatin [4.53 g/d EPA+DHA]	4 wks	N	Y	None	30	□ FMD □ TG
(West et al. 2010)	Hypercholesterolemic 36-65 yo 20 M, 3 F (pm)[c]	American diet - 0.8% ALA[d] Linoleic acid diet - 3.6% ALA ALA diet - 6.5% ALA [15 g/d ALA]	6 wks	N	Y	≤ 3 wks	12 (subset of 23)	ALA diet: □ FMD □ TG[e]
(Skulas-Ray et al. 2011)	Hypertriglyceridemic 44 ± 10 yo (23-65) 23 M, 3 F (pm)[c]	1 or 4 g/d - 84% EPA+DHA [0.84 or 3.4 g/d EPA+DHA]	8 wks	Y Corn oil	Y	6 wks	26	↔ FMD ↔ RHI[f] □ TG (4 g/d)
(Koh et al. 2012)	Hypertriglyceridemic Age-, Sex- & BMI-matched 54-55 ± 1 yo 87 M, 58 F	2 g/d - 84% EPA+DHA or 160 mg/d fenofibrate [1.7 g/d EPA+DHA]	8 wks	Y NR	N	NA	Placebo: 49 n-3 PUFA: 50 Fenofibrate: 48	□ FMD ↔ TG

Study	Description of Subjects	n-3 PUFA dose [Consumed daily dose][a]	Duration	Placebo control (Y/N)	Crossover design (Y/N)	Washout duration	Group size	Outcomes
(Yamakawa et al. 2012)	Hyperlipidemic Age- and sex-matched, 61.6 ± 10.6 yo 13 M, 21 F	1.8 g/d - 98% EPA [1.8 g/d EPA]	12 wks	N	N	NA	Normolipidemic: 18 Hyperlipidemic: 16	□ FBF[g] ↔ TG
(Oh et al. 2014)	Hypertriglyceridemic 54-55 ± 9 yo 91 M, 82 F	1, 2 or 4 g/d - 84% EPA+DHA [0.84, 1.7, or 3.4 g/d EPA+DHA]	8 wks	Y NR	N	NA	Placebo: 42 1 g/d: 44 2 g/d: 43 4 g/d :44	□ FMD (1, 2, & 4 g/d)) □ TG (4 g/d)
(Casanova et al. 2016)	Hypertriglyceridemic & hypertensive 49-59 ± 1 yo 17 M, 12 F	1.8 g/d - 60% EPA + 40% DHA or 100 mg/d ciprofibrate [1.8 g/d EPA+DHA]	12 wks	N	Y	8 wks	Low CV-risk: 13 High CV-risk: 16	□ FMD ↔ RHI □ TG
Type 2 Diabetes Mellitus								
(McVeigh et al. 1993)	T2DM 45-61 yo 20 M, 3 F	10 g/d - 30% EPA+DHA [3 g/d EPA+DHA]	6 wks	Y Olive oil	Y	6 wks	23	↑ FBF[b] ↔ TG
(Woodman et al. 2003)	T2DM & hypertensive 61.2 ± 1.2 yo (40-75) 39 M, 12 F (pm)	4 g/d - 96% EPA or 92% DHA [3.8 g/d EPA or 3.7 g/d DHA]	6 wks	Y Olive oil	N	NA	Placebo: 16 EPA: 17 DHA: 18	↔ FMD
(Wong et al. 2010)	T2DM 59-61 ± 9 yo 43 M, 54 F	4 g/d - 67% EPA+DHA [2.7 g/d EPA+DHA]	12 wks	Y Olive oil	N	NA	Placebo: 48 n-3 PUFA: 49	↔ FMD ↓ TG
(Stirban et al. 2010)	T2DM 56.8 ± 8.3 yo (37-70) Sex NR	2 g/d - 84% EPA+DHA [1.7 g/d EPA+DHA]	6 wks	Y Olive oil	Y	6 wks	32	↔ Fasting FMD Prevent Postprandial ↓ FMD ↔ Fasting TG Prevent Postprandial ↑ TG
(Lobraico et al. 2015)	T2DM 64.8 ±8.8 yo 31 M, 16 F	1 g/d krill oil (% n-3 PUFA NR)[h]	4 wks	Y Olive oil	Y	2 wks	47	□ RHI ↔ TG
		1 g/d - krill oil	17wks	N	N	NA	34	□ RHI ↔ TG
Cigarette Smoking								
(Din et al. 2013)	Cigarette smoker 28 ± 2 yo 20 M	2 g/d - 84% EPA+DHA [1.7 g/d EPA+DHA]	6 wks	Y Olive oil	Y	4 wks	20	□ FBF[b] ↔ TG
(Siasos et al. 2013)	Cigarette smoker 27.6 ± 2.6 yo 13 M, 7 F	2 g/d - 84% EPA+DHA [1.7 g/d EPA+DHA]	12 wks	Y NR	Y	4 wks	20	□ FMD ↔ TG

Study	Description of Subjects	n-3 PUFA dose [Consumed daily dose][a]	Duration	Placebo control (Y/N)	Crossover design (Y/N)	Washout duration	Group size	Outcomes
Elevated BMI								
(Hill et al. 2009)	BMI > 25 kg/m^3 + ≥ 1 CV risk factor(s) 47-52 ± 2 yo 24 M, 41 F	6 g/d - 32% EPA+DHA [1.9 g/d EPA+DHA]	12 wks	Y Sunflower oil	N	NA	Placebo: 18 Placebo+Ex:14 n-3 PUFA: 17 n-3 PUFA+Ex: 16	□ FMD □ TG
(Dangardt et al. 2010)	BMI > 30 kg/m^3 16 ± 1 yo 11 M, 14 F	10 g/d - 9% EPA + 3% DHA [1.2 g/d EPA+DHA]	12 wks	Y Medium chain TG	Y	6 wks	25	□ RHI ↔ TG
Metabolic Syndrome								
(Tousoulis et al. 2014)	Metabolic Syndrome 44 ± 12 yo 15 M, 14 F	2 g/d - 46% EPA + 38% DHA [1.7 g/d EPA+DHA]	12 wks	Y NR	Y	8 wks	29	□ FMD □ TG

NR – Not reported; NA – Not applicable

[a] n-3 PUFA dose consumed daily is based on the weight of capsules consumed daily (g/d) multiplied by the percentage of n-3 PUFA in the formulation. For example, from (Goodfellow et al. 2000): 4 g/d x 84% EPA+DHA = n-3 PUFA dose consumed daily of 3.4 g/d EPA+DHA.

[b]FBF – Forearm blood flow was assessed following intra-arterial infusion of the endothelial-dependent vasodilator, acetylcholine.

[c]PM – All females in the cohort were postmenopausal.

[d]Percent energy in the diet derived from ALA.

[e]Reported in (Zhao et al. 2004)

[f]RHI – Reactive hyperemia index was assessed in the digital microcirculation using PAT.

[g]FBF – Forearm blood flow was assessed following reactive hyperemia.

[h]A consumed daily dose could not be calculated without information on the percentage of EPA and DHA in the capsule

endothelial dysfunction in individuals with hyperlipidemia. Additionally, since n-3 PUFAs are one prescription therapy approved for patients with very high triglycerides (≥ 500 mg/dL) (Jacobson et al. 2014) and the American Heart Association recommends that consuming 2-4 g/d EPA+DHA can achieve triglyceride-lowering effects (Kris-Etherton 2002), we also report the outcome of n-3 PUFA supplementation on triglyceride levels when available.

In a small study, triglycerides were lowered and FMD was improved in subjects with hypercholesterolemia (total cholesterol > 575 mg/dL), who were given placebo or EPA+DHA for 17 weeks (Goodfellow et al. 2000). The n-3 PUFA treatment significantly decreased triglycerides, compared to placebo (placebo: 202.67 ± 184.97 mg/dL; n-3 PUFA: 153.11 ± 84.08 mg/dL, $P < 0.05$) and significantly improved FMD (placebo: 0.04 ± 0.10 mm, n-3 PUFA: 0.12 ± 0.07 mm, $P < 0.05$).

In another study, DHA, but not EPA, improved FBF and significantly lowered triglycerides in overweight and mildly hyperlipidemic men (Mori et al. 2000). In this double-blind, placebo-controlled trial, men who were overweight (BMI, 25-30 kg/m^3) and hyperlipidemic (total cholesterol ≥ 232 mg/dL, triglycerides ≥ 159 mg/dL, or both) were randomly assigned to placebo, EPA or DHA. After 6 weeks, FBF, measured in response to infusion of acetylcholine, was significantly increased in the DHA treatment group, compare to baseline ($P < 0.047$) and compared placebo at 6 weeks ($P < 0.04$). In contrast, the EPA group showed no improvement in FBF. In addition, DHA reduced triglycerides by 20% post-intervention ($P < 0.006$), while EPA showed a non-significant reduction of 18.4% ($P < 0.068$).

In another small study of men with hypertriglyceridemia, EPA supplementation improved FBF and significantly reduced triglycerides (Okumura et al. 2002). Men with elevated triglycerides (150 – 500 mg/dL) were given EPA for 12 weeks. Acetylcholine-induced FBF was significantly lower at baseline, compared to men with normal triglycerides ($P < 0.034$), and was significantly increased, compared to baseline ($P < 0.011$). Additionally, EPA significantly reduced serum triglycerides, compared to baseline (baseline: 274 ± 27 mg/dL; 12 weeks: 188.9 ± 34.5 mg/dL; $P < 0.025$).

In another study, subjects with hypercholesterolemia treated with walnuts exhibited significant increases in FMD (Ros et al. 2004). Walnuts are highly enriched in the n-3 PUFA, ALA, the metabolic precursor to EPA and DHA. In this study, subjects with moderate hypercholesterolemia (LDL cholesterol > 130 mg/dL and triglycerides < 250 mg/dL) were randomized into a control Mediterranean-type diet or an isoenergetic diet plus walnuts for 4 weeks followed by crossover without washout. The walnut-enriched diet significantly improved FMD, compared to the control diet (control diet: 3.6 ± 3.3%, walnut diet: 5.9 ± 3.3%; $P = 0.043$). There was no significant difference in triglycerides between groups.

While the above studies were conducted in adults, the following study highlights that n-3 PUFA supplementation can improve FMD in children with possible hypercholesterolemia or hyperlipidemia hereditary factors (Engler et al. 2004). In a double-blind, randomized, placebo-controlled crossover study, offspring of family members with hypercholesterolemia or the phenotype of the familial combined hyperlipidemia were placed on a National Cholesterol Education Program Step II (NCEP-II) diet for 6 weeks. Subjects were then randomized into placebo or DHA groups

for 6 weeks, followed by washout, and subsequent cross over to the other treatment arm, while remaining on the NCEP-II diet throughout. There were no significant differences in serum triglycerides among any of the groups. While placebo + diet significantly improved FMD over baseline, only the DHA supplement + diet significantly improved FMD (7.9 ± 2.9%), compared to all other groups (baseline: 5.9 ± 2.3%, $P < 0.001$; diet only: 6.3 ± 2.6%, $P < 0.002$; placebo + diet: 6.8 ± 2.4%, $P < 0.012$; washout + diet: 6.5 ± 2.7, $P < 0.001$).

In another study, the combination of an n-3 PUFA supplement with a statin significantly improved endothelial function and decreased triglycerides, compared to the statin alone (Mindrescu et al. 2008). In this study, individuals with hyperlipidemia (LDL cholesterol >100 mg/dL and/or triglycerides >150 mg/dL) were treated with rosuvastatin plus EPA+DHA (Group 1) or rosuvastatin alone (Group 2). After 4 weeks, lipids and FMD were measured and the groups crossed over to the other treatment arm without washout. In Group 1, triglycerides were significantly decreased after 4 weeks, compared to baseline (baseline: 139 ± 57 mg/dL; rosuvastatin + EPA+DHA: 91 ± 40 mg/dL; $P < 0.05$). After n-3 PUFA supplementation was stopped, triglycerides significantly increased (99 ± 27 mg/dL; $P < 0.05$). In Group 2, triglycerides did not significantly change after 4 weeks, compared to baseline (baseline: 137 ± 71 mg/dL; rosuvastatin alone: 141 ± 71 mg/dL). However, after the n-3 PUFA supplementation was added, triglycerides significantly decreased (103 ± 36 mg/dL, $P < 0.05$). The FMD of Group 1 was significantly increased after 4 weeks of rosuvastatin + n-3 PUFA, compared to baseline (baseline: -1.42 ± 3.27%, 4 weeks: 11.36 ± 4.33%); however, after the n-3 PUFA supplement was discontinued, FMD decreased (0.59 ± 8.32%). The FMD of Group 2 did

not differ after 4 weeks of rosuvastatin alone; however, after n-3 PUFA supplement was added to the statin therapy, the FMD significantly increased, compared to baseline (14.73 ± 10.77%, $P < 0.05$).

In another study using a diet enriched in walnuts, walnut oil and flaxseed oil, subjects with elevated cholesterol exhibited a significant improvement in FMD (West et al. 2010). Participants with elevated cholesterol (200-240 mg/dL) were randomly assigned to a sequence of three diets (American, Linoleic acid, ALA) that were each consumed for 6 weeks, followed by washout and crossover to the next diet. Last, FMD was assessed in a subset (n=12) of individuals at the end of each diet period. Individuals consuming the ALA diet exhibited the highest FMD when all diets were compared (American diet: 6.1 ± 1.1%; Linoleic acid diet: 6.7 ± 1.0%; ALA diet: 8.2 ± 1.0%; $P < 0.02$ diet effect by ANOVA). Notably, in an earlier study using the same diets and experimental design, both the linoleic acid diet and ALA diet significantly reduced triglycerides (Zhao et al. 2004).

In one placebo-controlled study, n-3 PUFAs significantly reduced high triglycerides, but failed to improve FMD or RHI assessed by PAT (Skulas-Ray et al. 2011). In this study, subjects with high triglycerides (140-339 mg/dL) were enrolled in a 3-period crossover study of 8-week treatment periods with 6-week washout periods. The treatments included placebo or a low or high dose of EPA+DHA. Triglyceride levels in those treated with the high dose decreased significantly by 27% (173.7 ± 17.5 mg/dL, $P = 0.002$), compared to placebo (237.3 ± 17.5 mg/dL), while there was no change in triglyceride levels in the low dose group (215.3 ± 17.5 mg/dL). However, neither post-treatment FMD nor RHI differed among the three treatment groups.

In a randomized, single-blind, placebo-controlled study of hyperlipidemic subjects, comparison of n-3 PUFA supplementation versus fenofibrate showed that both treatments effectively improved FMD and reduced triglycerides (Koh et al. 2012). Subjects with high triglycerides (≥ 150 mg/dL) were treated for 8 weeks with placebo, EPA+DHA or fenofibrate alone. Both n-3 PUFAs and fenofibrate significantly improved FMD, compared to baseline (n-3 PUFA: baseline 4.72 ± 0.27% vs 8 weeks 7.28 ± 0.33%, $P < 0.001$; fenofibrate: baseline 4.63 ± 0.26% vs 8 weeks 6.97 ± 0.28%, $P < 0.001$). Additionally, both n-3 PUFAs and fenofibrate significantly reduced triglycerides, compared to baseline (n-3 PUFA: baseline 290 ± 12 mg/dL vs 8 weeks 226 ± 16 mg/dL, $P < 0.001$; fenofibrate: baseline 274 ± 19 mg/dL vs 8 weeks 174 ± 11 mg/dL, $P < 0.001$), but only fenofibrate reduced triglycerides, compared to placebo. In a subsequent trial of similar design with hyperlipidemic subjects, the combination of EPA+DHA plus fenofibrate did not result in significant improvements in FMD or triglycerides over EPA+DHA or fenofibrate alone (Koh et al. 2012).

In a small hyperlipidemic cohort with hypercholesterolemia (243 ± 29 mg/dL) and/or hypertriglyceridemia (209 ± 92 mg/dL), n-3 PUFA supplementation improved FBF, but failed to reduce triglyceride levels (Yamakawa et al. 2012). In this study, subjects were given EPA and serum lipids and FBF were measured at 0, 4 and 12 weeks of treatment. In the hyperlipidemic group there was no significant change in triglycerides after treatment, compared to baseline. Notably, FBF was significantly lower in the hyperlipidemic group at baseline, compared to the normolipidemic group (hyperlipidemic baseline: 15.4 ± 6.1 mL/min/100 g; normolipidemic: 22.8 ± 1.2 mL/min/100 g, $P < 0.01$).

However, after 12 weeks of EPA treatment FBF was significantly improved in the hyperlipidemic group, compared to baseline, (21.7 ± 4.4 mL/min/100 g; P = 0.046).

In one placebo-controlled study, n-3 PUFAs significantly reduced borderline high triglycerides and simultaneously improved FMD (Oh et al. 2014). In this study, subjects with hypertriglyceridemia were treated for 8 weeks with placebo, or 1, 2, or 4 g/d EPA+DHA. Triglycerides in the groups treated with 1 or 2 g/d were not different from placebo, but levels in individuals treated with 4 g/d were significantly decreased (191 ± 117 mg/dL, P < 0.05), compared to placebo (247 ± 102 mg/dL). However, all three groups treated with EPA+DHA had significantly improved FMD, compared to baseline (P < 0.001), and to the placebo group (placebo: 6.31 ± 1.56%, 1 g/d: 7.61 ± 1.68%, 2 g/d: 7.64 ± 1.74%, and 4 g/d: 8.37 ± 1.51%; P < 0.05).

In a recent randomized, crossover study, comparison of n-3 PUFA supplementation versus ciprofibrate in individuals with both hypertriglyceridemia and hypertension showed that both treatments effectively improved FMD and reduced triglycerides, but not RHI (Casanova et al. 2016). In this study, individuals taking hypertension medication and with high triglycerides (150-499 mg/dL) were classified as having low (< 7.5%, n=13) or high (≥ 7.5%, n=16) CV risk, based on their estimated 10-year risk for atherosclerotic CVD (ASCVD) (Stone et al. 2014), and randomized to receive EPA+DHA or ciprofibrate for 12 weeks. This was followed by washout and subsequent crossover to the other treatment. In the low-risk subjects, FMD was significantly improved with both n-3 PUFA supplementation and ciprofibrate treatment, compared to baseline (n-3 PUFA: baseline: 10.1 ± 1.5%; 12 weeks: 13.5 ± 1.2, P = 0.012; ciprofibrate: baseline: 8.7 ± 1.5%; 12 weeks: 14.0 ± 1.9, P = 0.036). There were

no significant changes in RHI in either treatment group as measured by PAT. Triglycerides were significantly decreased after n-3 PUFA supplementation and ciprofibrate treatment (n-3 PUFA: baseline: 259 ± 29 mg/dL; 12 weeks: 200 ± 26 mg/dL, P = 0.040; ciprofibrate: baseline: 250 ± 26 mg/dL; 12 weeks: 152 ± 28 mg/dL, P ≤ 0.001). In the high-risk subjects, FMD was significantly increased with n-3 PUFA supplementation, compared to baseline (n-3 PUFA: baseline: 11.1 ± 1.6%; 12 weeks: 13.5 ± 1.2, P = 0.010), but there was no improvement after ciprofibrate treatment alone. There were no significant changes in RHI in either treatment group. Triglycerides were significantly decreased after n-3 PUFA supplementation and ciprofibrate treatment (n-3 PUFA: baseline: 255 ± 20 mg/dL; 12 weeks: 199± 36 mg/dL, P = 0.020; ciprofibrate: baseline: 244 ± 16 mg/dL; 12 weeks: 149 ± 19 mg/dL, P ≤ 0.001).

Among the twelve studies enrolling hyperlipidemic subjects, eleven studies showed that n-3 PUFA supplementation, including formulations (EPA+DHA, EPA alone, DHA alone, and ALA alone), improved endothelial dysfunction as assessed by FMD (8 studies) or FBF (3 studies) and seven studies showed n-3 PUFA supplementation also reduced triglycerides. The reasons why one study failed to observe an improvement in FMD is not clear (Skulas-Ray et al. 2011). The study was a randomized, double-blind, placebo-controlled, crossover design with six weeks washout between treatment arms. The investigators used a high dose EPA+DHA for an eight-week duration and observed a significant decrease in triglycerides. However, while the enrolled group size was similar to other studies (n=26), the age range was large and disproportionately male. It has been reported that the age-related decline in endothelial function occurs significantly earlier in men (41 yo) than in women (58 yo) (David S. Celermajer et al. 1994). Thus, it is

possible that the age and sex characteristics of the enrolled cohort resulted in the inability of n-3 PUFA supplementation to improve FMD. Additionally, two studies using an EPA+DHA formulation also assessed endothelial dysfunction by PAT; however, neither study observed an improvement in RHI (Skulas-Ray et al. 2011; Casanova et al. 2016). Taken together these results showed that n-3 PUFA supplementation in hyperlipidemic individuals can improve endothelial dysfunction, particularly as assessed by FMD, and this improvement can occur even in the absence of a reduction in triglycerides.

Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is a pathological contributor to both endothelial dysfunction and atherosclerosis (Kannel and McGee 1979; Henry et al. 2004). Thus, we also reviewed those studies that assessed the ability of n-3 PUFA supplementation to improve endothelial dysfunction in individuals with T2DM. One study has shown that n-3 PUFA supplements cannot improve fasting FMD, but can improve postprandial FMD in subjects with T2DM (Stirban et al. 2010). In this study, subjects with controlled T2DM were randomly divided into placebo or EPA+DHA for 6 weeks followed by washout and crossover. Then, FMD was measured following a 12 hr fast and again at 2, 4 and 6 hrs after eating a high-fat meal. Fasting FMD did not differ between placebo and n-3 PUFA treatment groups. While postprandial FMD decreased in both groups with a maximal reduction at 4 hr, the decrease was only significant in the placebo group. In the placebo group, postprandial FMD was significantly lower at 4 hr, compared to fasting FMD, (FMD - fasting: $5.54 \pm 0.55\%$ vs 4 hr postprandial: $3.45 \pm 0.54\%$; -38%, $p < 0.05$). In contrast, in the n-3 PUFA group postprandial FMD at 4 hr did differ from fasting FMD

(FMD - fasting: 4.85 ± 0.6% vs 4 hr postprandial: 4.23 ± 0.48%; -13%). Further, the overall postprandial decrease in FMD (expressed as area under the curve, AUC% x hr) was significantly greater in the placebo group, compared to the n-3 PUFA group (placebo: -8.24 ± 2.20% x hr; n-3 PUFA: -2.31 ± 1.83% x hr, $P < 0.05$). While fasting triglycerides did not differ between placebo and n-3 PUFA treatment groups, the postprandial increase in triglycerides was significantly attenuated in the n-3 PUFA group at 4 hr (placebo: 233 ± 26 mg/dl; n-3 PUFA: 198 ± 28 mg/dL, $P < 0.05$) and when integrated across the 6 hr post prandial period (placebo: 587 ± 62 mg/dL x hr; n-3 PUFA: 518 ± 58 mg/dL x hr; $P < 0.05$).

Two additional studies in diabetic subjects also failed to show an improvement in fasting FMD following n-3 PUFA treatment. In one study individuals with controlled T2DM and diagnosed hypertension were randomly assigned to placebo, EPA or DHA for 6 weeks (Woodman et al. 2003). Interestingly, FMD, assessed at baseline and during the final 2 weeks of treatment, did not differ among the three groups. In a second study individuals with controlled T2DM were divided between placebo or EPA+DHA for 12 weeks (Wong et al. 2010). Placebo and n-3 PUFA groups did not have a significant difference in FMD ($P = 0.95$); however, triglycerides were significantly decreased in the n-3 PUFA group, compared to the placebo (placebo group: 141.6 ± 88.5 mg/dL; n-3 PUFA: 106.2 ± 44.25 mg/dL; $P = 0.01$).

Despite these negative outcomes, two studies showed an improvement in endothelial dysfunction. The first study assessed endothelial function by FBF following acetylcholine infusion (McVeigh et al. 1993). In a double-blind, placebo controlled, crossover study, individuals with T2DM were randomized into placebo or EPA+DHA for

6 weeks, followed by washout and then crossover. Following intra-arterial infusion of increasing doses of the endothelial-dependent vasodilator, acetylcholine, FBF was measured. Interestingly, FBF in response to acetylcholine was significantly increased at all doses when taking n-3 PUFA supplements, compared to baseline and to placebo ($P < 0.01$).

In another double-blind, placebo controlled, crossover study, endothelial function was significantly improved in individuals with T2DM consuming krill oil (percentage of EPA and DHA not specified) (Lobraico et al. 2015). Participants were randomized into placebo or krill oil for 4 weeks followed by washout and crossover, and then a subset of the same individuals (n=34) participated in an additional 17-week krill oil supplementation period. After 4 weeks of krill oil supplementation, RHI was significantly increased compared to placebo (placebo: 1.83 ± 0.56; krill oil: 2.04 ± 0.52; $P = 0.025$), and after 17 weeks of krill oil supplementation, compared to baseline (baseline: 1.90 ± 0.54; krill oil: 2.16 ± 0.73; $P = 0.041$).

Of the five studies reviewed in individuals with T2DM, all were blinded, randomized and placebo-controlled; four used an EPA+DHA combination and one did a direct comparison of EPA versus DHA. Two studies using an EPA+DHA combination observed an improvement of endothelial dysfunction. In contrast, two studies using an EPA+DHA combination and one study comparing EPA to DHA failed to observe an improvement. The one notable difference between the studies with positive and negative outcomes on endothelial dysfunction was the method used to assess endothelial function. The two studies with positive outcomes used FBF following acetylcholine infusion and PAT, respectively, whereas the three studies with negative outcomes used FMD.

Assessment of endothelial function via FMD of the brachial artery is primarily mediated by NO, while FBF and PAT of the forearm and digital microvasculature, respectively, likely involve a combination of NO, prostaglandins and endothelial-derived hyperpolarizing factors. Despite the fact that each method is differentially associated with CV risk factors, typically the three methods do not correlate with each other (Lind et al. 2005; Hamburg et al. 2011). These limited data suggest that n-3 PUFA supplementation in individuals with T2DM may be able to improve endothelial dysfunction in the microvasculature, but not in conduit vessels, such as the brachial artery.

Cigarette smoking

Cigarette smoking is a risk factor for endothelial dysfunction and is one of the single biggest independent risk factors for atherosclerosis (Ozaki et al. 2010; Freund et al. 1993; Celermajer et al. 1993). Thus, we reviewed those studies that assessed the ability of n-3 PUFA supplementation to improve endothelial dysfunction in current cigarette smokers. In a prospective, double-blind, crossover, placebo-controlled, randomized study, n-3 PUFAs were shown to improve FBF in response to endothelium dependent vasodilators (Din et al. 2013). Healthy male smokers (≥ 5 cigarettes/day), were placed on placebo or EPA+DHA for 6 weeks followed by washout and crossover. Dose-dependent responses in FBF were measured following infusion of two endothelium-dependent vasodilators; acetylcholine and substance P. Individuals taking n-3 PUFA supplements had increased FBF, compared to placebo, but only acetylcholine-induced vasodilation was significantly increased (acetylcholine: $P = 0.0032$; substance P: $P = 0.056$).

In another study n-3 PUFAs were shown to improve FMD and reduce triglycerides in a double-blind, crossover, placebo-controlled study in current cigarette smokers (> 20 cigarettes per day for > 5 years), lacking any other classic risk factors of CVD (Siasos et al. 2013). A baseline FMD was measured before randomly assigning each subject to receive placebo or EPA+DHA for 12 weeks followed by washout and crossover; FMD was measured again after 4 and 12 weeks. The n-3 PUFA supplement non-significantly increased FMD after 4 weeks, compared to baseline, (baseline: 7.27 ± 2.56%, 4 weeks n-3 PUFA: 8.53 ± 3.55%), but significantly improved it after 12 weeks (9.98 ± 5.30%, $P < 0.05$), compared to baseline. Additionally, compared to placebo, individuals taking the n-3 PUFA supplement exhibited significantly higher FMD at both 4 weeks ($P < 0.05$) and 12 weeks ($P < 0.001$).

Elevated Body Mass Index (BMI)

Elevated BMI has been shown to be a risk factor for endothelial dysfunction (Oflaz 2003; Williams et al. 2005) and for atherosclerosis (Hubert et al. 1983). We reviewed two studies that investigated the relationship between n-3 PUFA supplementation, elevated BMI and endothelial dysfunction. In the first study, individuals were recruited that were overweight or obese (BMI > 25 kg/m^2) and had one or more CV risk factors, including hypertension (140/90-160/100 mm Hg), increased plasma triglycerides (> 142 mg/dL) or elevated total cholesterol (> 212 mg/dL) (Hill et al. 2009). Subjects were randomly divided into four groups. Two groups consumed EPA+DHA and two groups consumed placebo for 12 weeks. One n-3 PUFA group and one placebo group were required to run or walk 3 times/week for 45 minutes. Individuals

taking the n-3 PUFA supplement exhibited a significant time-dependent improvement in FMD, compared to placebo, (treatment x time interaction, $P < 0.05$), and a significantly higher FMD, compared to the placebo group, after 12 weeks ($P < 0.01$). There was no effect of exercise status on this outcome. The n-3 PUFA supplement also significantly reduced triglycerides at both 6 and 12 weeks, compared to placebo ($P < 0.05$).

While the previous study assessed n-3 PUFA supplementation in overweight or obese adults, this trial assessed effects in adolescents. In a double-blind, placebo-controlled, crossover study obese adolescents (BMI > 30 kg/m^3) were randomly assigned to placebo or EPA+DHA for 12 weeks, followed by washout and crossover (Dangardt et al. 2010). Endothelial function, assessed by PAT, and serum triglycerides were measured at the end of each treatment period. However, n-3 PUFA supplementation had no effect on triglycerides, compared to placebo. Pair-wise comparison of RH response curves showed that n-3 PUFAs significantly improved endothelial dysfunction ($P = 0.01$).

Metabolic Syndrome

Metabolic syndrome is defined as a cluster of conditions that increase risk for atherosclerotic cardiovascular disease and diabetes. These conditions include: dyslipidemia, hypertension, impaired fasting glucose, and abdominal fat (McNeill et al. 2005; Walther et al. 2015). We reviewed one study where participants with diagnosed metabolic syndrome were enrolled in a double-blind, placebo-controlled, crossover trial (Tousoulis et al. 2014). Individuals were randomized into placebo or EPA+DHA for 12 weeks. This was followed by washout and then crossover. Interestingly, FMD was significantly improved after 12 weeks of n-3 PUFA supplementation, compared to baseline, and exhibited significant improved overtime (baseline: 3.67 ± 3.57%, 4 weeks:

5.13 ± 4.51%, 12 weeks: 7.72 ± 4.17%, $P < 0.05$ baseline vs 12 wks; $P < 0.001$ time-dependent trend). There were no significant differences observed in the placebo group. Supplementation with n-3 PUFA also significantly decreased triglycerides over time (baseline: 180 ± 22 mg/dL; 4 weeks: 175 ± 21 mg/dL; 12 weeks: 166 ± 17 mg/dL; $P < 0.001$ trend), in contrast to no changes in the placebo group over time.

DISCUSSION

The review of these published studies reveals that n-3 PUFA supplementation can successfully improve endothelial dysfunction in individuals with traditional risk factors for atherosclerotic CVD, including hyperlipidemia, cigarette smoking, elevated BMI, and metabolic syndrome. Of the 22 studies, n-3 PUFA supplementation improved endothelial dysfunction in 18 of them, including 11 of 12 studies of hyperlipidemic individuals, 2 of 2 studies of young cigarette smokers, 2 of 5 studies of individuals with T2DM, and 3 of 3 studies of individuals with elevated BMI or metabolic syndrome.

Since the n-3 PUFA dose, composition, and treatment duration used in these studies varied, we devised an approach to more easily compare the outcomes across all trials. First, we calculated an n-3 PUFA dose consumed daily by multiplying the capsule weight consumed per day (g/d) by the percentage of n-3 PUFA in the capsule (Table 5.13). We then calculated a cumulative dose consumed by multiplying the dose consumed daily (g/d) by 7 days/wk by the number of weeks of treatment, and then grouped the results by the composition of the formulation (Table 5.14). One study was excluded from this analysis due to incomplete information to complete the calculations (Lobraico et al. 2015). This approach illustrated that endothelial dysfunction was

Table 5.14. Summary of n-3 PUFA cumulative dose, outcomes on endothelial function and triglycerides (TG), and atherosclerosis CVD risk factor, grouped according to the n-3 PUFA formulation.

n-3 PUFA Formulation	Cumulative Dose (g)[a]	Outcomes[b]		Atherosclerosis CV Disease Risk Factor	Reference
		Improved Endothelial Dysfunction	Reduced TG		
EPA+DHA	47	- (FMD)	-	Hypertriglyceridemia	(Skulas-Ray et al. 2011)
	47	+ (FMD)	-	Hypertriglyceridemia	(Oh et al. 2014)
	71	+ (FBF)	-	Smoking	(Din et al. 2013)
	71	- (FMD)	-	T2DM	(Stirban et al. 2010)
	95	+ (FMD)	-	Hypertriglyceridemia	(Koh et al. 2012)
	95	+ (FMD)	-	Hypertriglyceridemia	(Oh et al. 2014)
	101	+ (RHI)	-	Elevated BMI	(Dangardt et al. 2010)
	126	+ (FBF)	-	T2DM	(McVeigh et al.1993)
	126	+ (FMD)	+	Hyperlipidemia	(Mindrescu et al. 2008)
	143	+ (FMD)	-	Smoking	(Siasos et al. 2013)
	143	+ (FMD)	+	Metabolic syndrome	(Tousoulis et al. 2014)
	151	+ (FMD)	+	Hypertriglyceridemia & hypertension	(Casanova et al. 2016)
	160	+ (FMD)	+	Elevated BMI	(Hill et al. 2009)
	190	- (FMD)	+	Hypertriglyceridemia	(Skulas-Ray et al. 2011)
	190	+ (FMD)	+	Hypertriglyceridemia	(Oh et al. 2014)
	227	- (FMD)	+	T2DM	(Wong et al. 2010)
	405	+ (FMD)	+	Hypercholesterolemia	(Goodfellow et al. 2000)
EPA	151	+ (FBF)	-	Hyperlipidemia	(Yamakawa et al. 2012)
	151	+ (FBF)	+	Hypertriglyceridemia	(Okumura et al. 2002)
	160	- (FMD)	-	Hyperlipidemia & elevated BMI	(Mori et al. 2000)
	160	- (FMD)	NR	T2DM	(Woodman et al. 2003)
DHA	50	+ (FMD)	-	Hyperlipidemia	(Engler et al. 2004)
	155	- (FMD)	NR	T2DM	(Woodman et al. 2003)
	155	+ (FMD)	+	Hyperlipidemia & elevated BMI	(Mori et al. 2000)
ALA	104-168	+ (FMD)	-	Hypercholesterolemia	(Ros et al. 2004)
	630[c]	+ (FMD)	+	Hypercholesterolemia	(West et al. 2010)

[a] Cumulative dose was calculated by multiplying the dose of n-3 PUFA consumed daily in g/d (Table 5.13) by 7 d/wk by the number of weeks of treatment. For example, from (Goodfellow et al. 2000): 3.4 g/d EPA+DHA x 7 d/wk x 17 wk = cumulative dose of 405 g.

[b] "+" indicates improved endothelial dysfunction and reduced TG, while "-" indicates no improvement in endothelial dysfunction and no reduction in TG.

[c] Based on the report of 3.3 g/d ALA consumed from walnuts, 1.5 g/d ALA consumed from walnut oil, and the assumption of 10.4 g/d of ALA consumed from flaxseed oil.

consistently improved by cumulative doses of ≥ 95 g EPA+DHA and that triglycerides were consistently reduced by cumulative doses of ≥ 151 g EPA+DHA. This suggests that higher doses of EPA+DHA may be required to reduce triglycerides than are needed to needed to improve endothelial dysfunction, which would be consistent with the approved prescription dose of 4 g/d (84% EPA+DHA, e.g. Lovaza®, Vacepa®) for reducing triglycerides in patients with very high triglycerides (≥ 500 mg/dL). However, these data also suggest that endothelial dysfunction can be improved in the absence of a reduction in triglycerides.

The data for formulations containing only EPA or DHA alone were limited with highly variable outcomes. Interestingly, EPA successfully improved endothelial dysfunction measured by FBF in two studies at a cumulative dose of 151 g (Okumura et al. 2002; Yamakawa et al. 2012), but failed to improve endothelial dysfunction measured by FMD in two studies at a cumulative dose of 160 g (Mori et al. 2000; Woodman et al. 2003). In contrast, DHA improved endothelial dysfunction as measured by FMD at cumulative doses of 50 and 155 g (Mori et al. 2000; Engler et al. 2004), but not at 160 g in diabetics (Woodman et al. 2003). Lastly, two studies of diets enriched in ALA suggested a low cumulative dose (100-200 g) improved endothelial dysfunction, but did not reduce triglycerides, while a much higher cumulative dose (630 g) improved both outcomes.

The one notable atherosclerosis CV disease risk factor for which n-3 PUFA supplementation did not consistently improve endothelial dysfunction was in individuals with T2DM. n-3 PUFA supplementation failed to improve endothelial dysfunction in three of the five studies conducted in diabetic subjects (Woodman et al. 2003; Wong et al.

2010; Stirban et al. 2010). All three of the studies with negative outcomes were placebo-controlled and assessed endothelial function using the gold standard, FMD. One study used a cumulative dose as high as 227 g EPA+DHA (Wong et al. 2010), which is higher than doses that improved endothelial dysfunction in ten other trials in non-diabetic subjects. Further, the lack of improvement in FMD occurred despite significant increases in platelet n-3 PUFAs in one study (Woodman et al. 2003) and significant decreases in triglycerides in another (Wong et al. 2010). It is noteworthy that the two studies in which EPA+DHA improved endothelial dysfunction in diabetic subjects assessed endothelial dysfunction in microvascular beds of the fingers and forearm (McVeigh et al. 1993; Lobraico et al. 2015). Thus, it is possible that n-3 PUFA supplementation only improves endothelial function in microvascular arterioles, but not conduit arteries in diabetic subjects. Potentially, this difference could reflect the mechanisms by which (1) T2DM induces endothelial dysfunction, (2) n-3 PUFAs improve endothelial dysfunction, and (3) endothelial-dependent vasodilation is regulated in micro- versus macro-vasculature.

Future studies of all these mechanisms are needed to understand the potential vasoprotective benefit of n-3 PUFA supplementation in individuals with T2DM.
The mechanisms underlying the improvement of endothelial dysfunction by n-3 PUFA supplementation in human subjects have not been fully elucidated. However, numerous studies suggest that n-3 PUFAs may improve endothelial function by increasing NO levels (Ander et al. 2003). For example, EPA increases NO in endothelial cells in situ and stimulates endothelial and NO-dependent dilation in bovine coronary arteries ex vivo (Omura et al. 2001). Further, both EPA and DHA activate endothelial nitric oxide synthase (eNOS) in cultured human endothelial cells (Omura et al. 2001; Stebbins et al.

2008) and dietary n-3 PUFAs significantly increase eNOS activation in the mouse aorta (Agbor et al. 2014). It has been shown that DHA also increases NO by increasing interleukin-1B-induced inducible nitric oxide synthase (iNOS) mRNA by activation of p44/42 mitogen-activated protein kinase signaling (Hirafuji et al. 2003). Furthermore, this allows for increased extracellular calcium release from vascular smooth muscle cells, which improves vasoreactivity (Hirafuji et al. 2003). *In vivo*, dietary n-3 PUFA supplementation normalizes endothelial dysfunction in mouse mesenteric arterioles that is induced by cigarette smoke exposure (Wiest, Walsh-Wilcox, and Walker 2017). Notably, however, the improvement in mesenteric arteriolar FMD is mediated by an increase in NO-independent dilation, suggesting that n-3 PUFAs also can increase the expression and/or activity of other endothelial-derived vasodilators, in addition to NO. This mechanism may account for differences in the vasodilatory benefits of n-3 PUFA supplementation between microvascular arterioles versus conduit arteries.

Another possible mechanism underlying the improvement of endothelial function by n-3 PUFAs includes decreasing reactive oxygen species (ROS). Vascular ROS can reduce NO bioavailability and increase endothelial-derived vasoconstrictors, thus impairing endothelial-dependent vasodilation. It has been shown that n-3 PUFAs decrease ROS in doxorubicin-treated cardiomyocytes and in endothelial cells exposed to environmental particulates in vitro (Hsu, Chen, and Chen 2014; Bo et al. 2016). In a mouse model, dietary n-3 PUFA supplementation significantly reduces cigarette smoke-induced increases in two markers of oxidative stress, 8-isoprostane and heme oxygenase-1 mRNA (Wiest, Walsh-Wilcox, and Walker 2017). In a menopausal rat model, n-3 PUFA supplementation is associated with decreased ROS production through modulation

of NADPH oxidase and iNOS (Gortan Cappellari et al. 2013). In one clinical study, n-3 PUFA intake is positively correlated with in total antioxidant capacity, but unfortunately endothelial function was not assessed in these participants (Thorlaksdottir et al. 2006).

Lastly, endothelial function also is impaired by endothelial activation, inflammation, and hypertension, which are all risk factors for the development of atherosclerosis. Studies show that n-3 PUFAs can inhibit endothelial activation, and are anti-inflammatory and antihypertensive. Endothelial activation is associated with increases in surface expression of adhesion molecules which promotes leukocyte adhesion and inflammation. Both EPA and DHA reduce adhesion molecule expression and leukocyte adhesion to endothelial cells in vitro (De Caterina et al. 1994; Huang, Sheu, and Chiang 2015; Elsen, Garssen, and Willemsen 2012). Further, clinical studies also show that n-3 PUFA supplementation reduces monocyte activation, markers of inflammation and hypertension, all of which can contribute to endothelial dysfunction (Miller, Van Elswyk, and Alexander 2014; Y. Yang, Lu, and Chen 2012; Ellulu et al. 2016).

CONCLUSIONS

Based on our review of the literature, we conclude that individuals with traditional risk factors for atherosclerotic CVD, including hyperlipidemia, cigarette smoking, elevated BMI, and metabolic syndrome, could derive vasoprotective benefits from n-3 PUFA supplementation, particularly from an EPA+DHA formulation resulting in cumulative dose ≥ 95 g over a minimum of 4 weeks. While results from ALA-enriched diets appear promising, currently the evidence is inadequate to conclude whether

formulations containing only ALA, EPA or DHA have similar benefit as formulations of EPA+DHA. Given that endothelial dysfunction, as measured by FMD, is an independent predictor of future CV events and an early predictor of CHD risk, evidence from these studies suggest that n-3 PUFA supplementation could serve as a primary prevention strategy for atherosclerotic disease for many individuals at risk. This conclusion is consistent with the recommendations of the American Heart Association, the World Health Organization, and other health agencies that individuals without CVD can derive CVD risk reduction by intake of n-3 PUFAs (Gebauer et al. 2006).

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