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THE ROLE OF FATTY ACIDS AND CIGARETTE SMOKE TOXICANTS IN CIGARETTE SMOKE-INDUCED CARDIOVASCULAR DISEASE

by

Elani F. Wiest

B.S., Chemistry, University of Kentucky, 2011

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY
BIOMEDICAL SCIENCES

The University of New Mexico
Albuquerque, New Mexico

DECEMBER, 2016

DEDICATION

I would like to dedicate this work to my parents, Marius and Leana Fourie, who have sacrificed so much to provide me with a good education and the opportunity to excel in life. You have always been role models who showed me that through hard work and dedication, anything can be achieved.

I equally dedicate this work to my husband, Nathaniel Wiest, who has tirelessly supported me over the last few years by spending late nights helping me in the lab, proofreading my work, and listening to me practicing oral talks. Your love, support, and encouragement mean the world to me.

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The Role of Fatty Acids and Cigarette Smoke Toxicants in Cigarette Smoke-Induced Cardiovascular disease.

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ABSTRACT

Young, healthy smokers have an increased risk of developing cardiovascular disease (CVD), but early identification of these individuals can prevent progression to more severe cardiovascular diseases like atherosclerosis, stroke, and heart attacks. However, methods developed to detect cardiovascular disease in its early stages are limited and very costly. The goal of this project is to identify biomarkers that can be tested in a single blood draw from smokers in order to both assess their risk of developing cardiovascular disease and identify possible therapeutic targets to prevent disease progression.

The long term goals of this project are to investigate the association between CVD risk, aryl hydrocarbon receptor (AHR) activity, and polyunsaturated fatty acids (PUFAs) in smokers, and to elucidate the mechanisms of their contribution to cigarette smoke (CS)-induced vascular dysfunction.

We have shown that AHR activity and α-linolenic acid (ALA, an n-3 PUFA) are potential biomarkers for CVD risk in young, healthy smokers. It is possible that the biomarkers identified in young, healthy smokers may serve not only as early identification of individuals most at risk of developing CVD, but also as biomarkers for future CVD risk in smokers with early stage CVD. As expected, the biomarkers

identified in young, healthy smokers are different from biomarkers identified in older smokers with hypertension, a risk factor for development of CVD.

In a second study conducted in subjects between 40 and 70 yrs old with physician-diagnosed hypertension. We found that ALA, which we previously demonstrated to predict flow-mediated dilation (FMD) in young, healthy smokers, is not associated with atherosclerotic cardiovascular disease (ASCVD) risk in smokers with preexisting hypertension. Nonetheless, we found that 13,14-dihydroxydocosapentaenoic acid (DiHDPA), along with 11- and 20-hydroxyeicosatetraenoic acid (HETE), predict ASCVD risk in smokers, whereas 14,15-epoxyeicosatetraenoic acid (EEQ), 13,14- and 16,17-epoxyeicosatetraenoic acid (EDP), and 16,17-DiHDPA predict ASCVD risk in non-smokers. These results suggest that the fatty acids identified in this study may be used to predict CVD risk in subjects with pre-existing CVD risk factors.

In order to investigate the specific mechanisms through which n-3 PUFAs protect against CS-induced vascular dysfunction in humans, we generated a mouse model of CS-exposure that resembles what is seen in young, healthy smokers. We made the novel observation that although CS impairs nitric oxide (NO)-dependent flow-mediated dilation (FMD), an n-3 PUFA diet restores FMD by increasing NO-independent dilation. We also found that the vasoprotective properties of n-3 PUFAs may be, at least in part, due to its antioxidant properties and reduction of Cyp1a1 expression.

These studies will contribute to the understanding of CVD progression in smokers and the mechanisms of how n-3 PUFAs protect against CS-induced CVD. In

addition, these findings will continue to contribute to the development of biomarkers to detect patients at high CVD risk while they are at early stages of the disease.

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CHAPTER I

Introduction

Cardiovascular disease in smokers

Cardiovascular health impacts of cigarette smoke

Cigarette smoke (CS) is a major independent risk factor for development of cardiovascular disease (CVD)(Donnell and Elosua, 2008). CS-induced CVD includes atherosclerosis, myocardial infarction, aortic aneurysms, and stroke (Kawachi et al., 1997; Naya et al., 2008; Shinton and Beevers, 1989; Wilmink et al., 1999).

Between 2004 and 2009, the annual mortality for CVD in cigarette smokers was estimated to be 151,000 in the United States (US Department of Health and Human Services, 2014) and even higher in developing countries (Mathers and Loncar, 2006). Although the prevalence of CS among adults in the U.S. decreased from 20.9% to 16.8% from 2005-2014 (US Department of Health and Human Services, 2014), it is on the rise in developing countries including China and India. It is reported that CS causes one million of all male deaths per year in China and this number will rise to 3 million by 2050 (Chen et al., 2015).

CS causes both structural and functional modifications in the vasculature. These changes include a loss of vasodilators, a loss of endothelial barrier function, and the formation of plaques and inflammation, all of which may lead to CVD (Messner and Bernhard, 2014). The pathophysiological results of these modifications are highly dependent on other factors, including the individual, the environment, family history of CVD and the type and amount of smoking (Leone, 2011). Smoking cessation is the best

treatment to reduce the risk of developing CS-induced CVD and many programs have been established to promote cessation.

Limitations of smoking cessation therapy

Although smoking cessation programs have been established, successful cessation is not achieved by everyone. According to the national health interview surveys between 2001 and 2010, approximately 68.8% of adult smokers in the USA show an interest in cessation, 52.4% make an attempt to quit, yet only 6.2% are successful in achieving smoking cessation for more than 6 months (Centers for Disease Control and Prevention, 2011; Yong et al., 2014).

This is largely due to the addictive properties of nicotine, which activates reward systems in the brain and prolongs reward sensitivity, as well as environmental stimuli including social pressure and habitual ambiance that reinforce the effects of nicotine (Kenny and Markou, 2005; Le Foll and Goldberg, 2005).

Limitation to current therapy is that many of the drugs approved to aid in smoking cessation may increase heart rate and mean arterial pressure; however, this is mostly seen in individuals without CVD and most of these studies are underpowered (Sobieraj et al., 2013). The three drugs approved for smoking cessation in the USA are Varenicline, Bupropion, and nicotine replacement therapy. The most common side effects of nicotine replacement therapies are neuropsychological events (suicidal thoughts and depression) and stimulation of the central nervous system (anxiety and insomnia)(Anthenelli et al., 2016).

Since smoking cessation is quite difficult, there is a need to find alternative treatments to prevent or delay the effects of toxic chemicals in CS on CVD.

Cigarette smoke

Composition of cigarette smoke

CS is a complex mixture of more than 8,000 chemicals (Rodgman and Perfetti, 2009). The specific chemicals and amounts of each differ by season and manufacturer. Many of these chemicals have been to be toxicants, carcinogens, and mutagens.

Some chemicals are derived from raw material found in the tobacco plant, including nicotine which not only gives CS its addictive properties, but also impairs vessel function (Neunteufl et al., 2002). The tobacco plant also absorbs chemicals, particularly heavy metals like cadmium, arsenic, and lead from the soil and fertilizers, which are then released when the cigarette is burned (Bozhinova, 2016). Cigarette companies also incorporate additives into cigarettes. These additives include ammonia, which improves nicotine bioavailability, and flavorings like cocoa and licorice. In addition, humectants are also added to preserve moisture and prolong shelf life. Examples of humectants include glycerol, glycol, triethylene glycol, and sorbitol. Combustion byproducts, which are chemicals released due to incomplete combustion, such as polycyclic aromatic hydrocarbons (PAHs), are also released upon burning of a cigarette. Other byproducts of manufacturing are also present in cigarette smoke. For example, nitrates in the tobacco leaf are microbially reduced to nitrites during the curing stage, which in turn react with secondary and tertiary amines in the leaf to form nitrosamines (Klus et al., 2012).

Over the years, alternative tobacco products have been developed and revised in an attempt to attenuate adverse health effects. Examples are nicotine-free cigarettes, smokeless tobacco, electronic nicotine delivery systems (electronic cigarettes), and hookah. Although some of these reduce secondhand smoke exposure, they are not completely safe, and may still caufer specific health risks (Boffetta and Straif, 2009; Lippi et al., 2013; Vardavas et al., 2012; Yatsuya and Folsom, 2010). More studies investigating long term effects of these alternative tobacco products need to be conducted.

CS can be characterized as mainstream smoke, which is smoke inhaled by the smoker from the mouthpiece of the cigarette, and side-stream smoke, known popularly as secondhand smoke, which is generated by the tip of the smoldering cigarette and exhaled mainstream smoke and is inhaled by both the smoker and others in close proximity. Qualitatively, side-stream smoke consists of the same chemicals as mainstream smoke but in lower concentrations. Importantly, side-stream smoke is associated with adverse health effects in humans. A meta-analysis of 20 studies consisting of 885,307 participants show that there is a strong, dose-dependent relationship between secondhand smoke exposure and risk of a stroke (Oono et al., 2011). In another study of 2,400 children between the age of 6 and 19, those exposed to secondhand smoke at home have significantly higher levels of PAH metabolites in their urine, including 2-hydroxyfluorene, 9-hydroxyfluorene, 2-hydroxyphenanthrene, and 1-hydroxypyrene (Jain, 2014). PAHs have been shown to activate transcription pathways by binding the aryl hydrocarbon receptor (AHR).

Polycyclic aromatic hydrocarbons and the aryl hydrocarbon receptor

The AHR is a ligand-activated transcription factor belonging to the basic helix-loop-helix/Per-ARNT-Sim family of DNA binding proteins. In the unbound state, AHR forms a complex with aryl hydrocarbon receptor-associated protein 9 (ARA-9) and two copies of heat shock protein 90 (HSP90), which act as chaperone proteins that help trafficking of the ligand-bound AHR-ARA-9 complex into the nucleus. When AHR ligands such as PAHs (Benzo[*a*]-pyrene (BaP), Benzo[a]-anthracene (BaA), and pyrene) bind the AHR, a conformational change occurs in the AHR to cause translocation of the AHR-ligand-chaperone complex into the nucleus where the AHR-ligand complex dissociates from the chaperone complex (Fig. 1.1). The free AHR-ligand complex then dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) to form a heterodimer that then interacts with dioxin-response elements (DREs). These DREs are 8-nucleotide 5' T/GNGCGTGA/C 3' elements. DREs regulate more than 20 genes, many of which are phase I metabolizing enzyme genes, including monoxygenase cytochrome P450 enzymes (CYP450) CYP1A1, CYP1A2, and CYP1B1 (Beischlag et al., 2008; Burchiel et al., 2007).

The P450 enzymes oxidize PAHs to form highly reactive and potentially mutagenic epoxides that are detoxified and made water soluble by phase II conjugation with glutathione and excreted in the urine (Shimada and Fujii-Kuriyama, 2004). PAHs have independently been associated with obstructive lung disease (bronchitis, emphysema, and asthma), cancer and CVD (Burstyn et al., 2003; Mastrangelo, 1996; Xu et al., 2010).

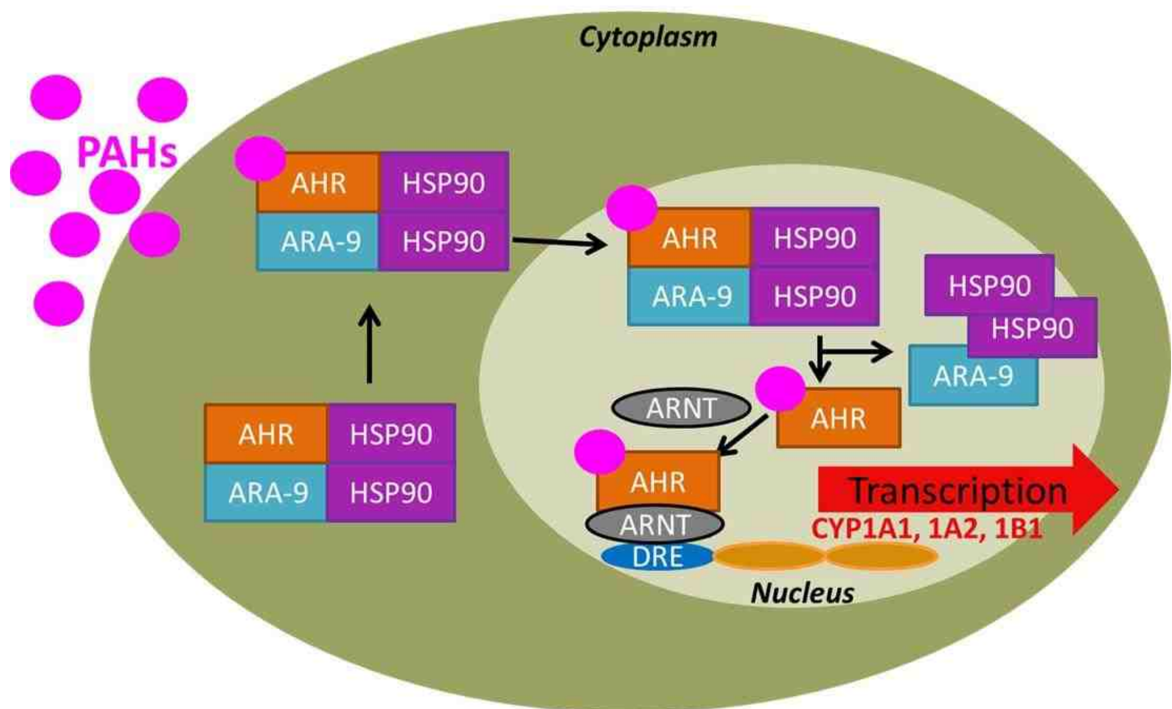

Figure 1.1 AHR signaling pathway. AHR forms a complex with chaperone molecules ARA-9 and 2 copies of HSP90. Upon binding to AHR ligands such as PAHs, the AHR translocates into the nucleus and dissociates from the chaperone molecules. The AHR-ligand complex dimerizes with ARNT, which in turn binds the DRE, leading to transcription of phase I metabolic enzymes CYP1A1, CYP1A2, and CYP1B1. (Abbreviations: AHR: aryl hydrocarbon receptor; ARA-9: aryl hydrocarbon receptor-associated protein 9; HSP90: heat shock protein 90; PAH: polycyclic aromatic hydrocarbons; ARNT: aryl hydrocarbon receptor nuclear translocator; DRE: dioxin response element; CYP1A1: cytochrome P450 1A1)

Mechanisms of cigarette smoke-induced cardiovascular disease

Vascular endothelium

The endothelium comprises the innermost lining of blood vessels and veins. Endothelial cells create an anti-thrombotic layer consisting of heparin sulfate and express ectonucleatidases and thrombomodulin to facilitate the smooth flow of blood through the circulatory system (Nilius and Droogmans, 2001). The endothelium plays a pivotal role in the regulation of vessel tone, which in turn regulates blood pressure and blood flow.

Endothelial cells are rich in membrane-bound receptors, which regulate the uptake of nutrients, endocrine factors, macromolecules, metabolites, and dissolved gasses (Nilius and Droogmans, 2001; Tang and Vanhoutte, 2010). Many of these receptors play a role in endothelium-dependent vasodilation and vasoconstriction. Examples of receptors playing a role in vasodilation include histamine binding the histaminergic receptor, ATP and ADP binding the purinergic receptors, and bradykinin binding the kinin receptor. Examples of receptors playing a role in vasoconstriction include endothelin-1 binding the endothelin-receptor, norepinephrine binding the α_2 adrenergic receptor, and angiotensin II binding the angiotensin II receptor (Tang and Vanhoutte, 2010). When healthy, endothelium-derived vasodilators and vasoconstrictors act in balance, and relay opposing effects to the underlying smooth muscle cells to maintain normophysiologic vascular tone. However, under conditions of chronic hypertension or chronic exposure to cigarette smoke, the ability of the endothelium to regulate vascular

tone and homeostasis is impaired. This leads to an increase in vasoconstrictors and a decrease in vasodilators. This process is known as endothelial dysfunction, which is an early event leading to CVD (Tang and Vanhoutte, 2010).

Measurement of flow-mediated dilation (FMD) in the brachial artery is often used to assess endothelial function. This method relies on a sudden increase in blood flow in a certain region of the brachial artery, termed reactive hyperemia, which leads to an increase in shear stress exerted by the blood cells on the endothelial cells, which leads to dilation (Mathers and Loncar, 2006; Münzel et al., 2008).

There are multiple mechanisms by which shear stress cause vasodilation. Studies in transient receptor potential cation channel subfamily V member 4 (TRPV4) knock-out mice demonstrate impaired shear-stress induced vasodilation in carotid and mesenteric arterioles, thus showing that TRPV4 channels are critical for flow-mediated dilation (Hartmannsgruber et al., 2007; Mendoza et al., 2010). Additionally, shear stress activates dissociation of endothelial nitric oxide synthase (eNOS) from caveolin, which then associates with calmodulin, leading to nitric oxide (NO)-synthesis from L-arginine by eNOS. Furthermore, shear stress upregulates eNOS expression and eNOS mRNA stability (Balligand et al., 2009; Rizzo et al., 1998).

Nitric oxide

NO is a potent vasodilator. NO is generated physiologically by the enzyme nitric oxide synthase (NOS). There are 3 NOS isoforms: inducible NOS (iNOS or NOS I), neuronal NOS (nNOS or NOS II), and endothelial NOS (eNOS or NOS III). eNOS is

highly expressed in vascular endothelial cells and is responsible, in part, for regulating systemic blood pressure (Shesely et al., 1996). NOS enzymes are synthesized as monomers each containing an oxygenase and reductase domain. These monomers are able to transfer electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to flavins. The monomers bind calmodulin which stimulates electron transfer within the reductase domain. Two inactive monomers bind heme to form active NOS dimers that enable NOS to bind cofactor tetrahydrobiopterin (BH4) and substrate L-arginine (Förstermann and Münzel, 2006). Various stimuli, such as shear stress, acetylcholine (ACh) receptor activation, and bradykinin receptor activation can lead to a transient increase in intracellular calcium, which binds calmodulin to activate eNOS dimers and stimulates production of NO.

Phosphorylation of eNOS by AKT and PKA at the Ser 1177 and Ser 615 positions have been shown to augment NO production. In addition, PKA-mediated Ser 635- phosphorylation by PKA also promotes NO production. In the contrary, AMP-activated kinase (AMPK) and cyclin-dependent kinase 5 (CDK5) - mediated phosphorylation of Ser 114 and AMPK-mediated phosphorylation of Thr 495 inhibit eNOS enzyme activity (Fulton et al., 1999; Heiss and Dirsch, 2014).

NO is a gas that diffuses to the smooth muscle through aquaporin-1 (Herrera et al., 2006). In the smooth muscle, NO activates soluble guanylate cyclase (sGC) to convert guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which in turn activates protein kinase G (PKG). PKG activate 1) the calcium pump SERCA, which pumps calcium into the sarcoplasmic reticulum, and 2) activates large conductance calcium-dependent potassium channels (BK channels), thereby decreasing intracellular

calcium concentrations. This decrease in intracellular calcium results in smooth muscle relaxation (Frei et al., 2009)(Fig. 1.2).

eNOS expression is increased in cigarette smokers, yet NO production and activity of eNOS are decreased, suggesting that CS may inhibit NO production, but may not affect eNOS expression (Barua et al., 2001).

Oxidative stress

Under physiological conditions, endothelial cells produce basal amounts of reactive oxygen species (ROS) that act as cell signaling molecules to mediate responses such as gene expression and proliferation. At increased concentrations, ROS can cause cell damage and apoptosis that may negatively impact
vessel function (Frey et al., 2009).

NO is a reactive gas that interacts readily with ROS. Volatile free radicals in the gas-phase of cigarette smoke play a role in decreasing NO bioavailability. Free radicals like the ROS, superoxide anion react with NO to form reactive peroxynitrite ($ONOO^-$) (figure 1.2), which further leads to protein nitration (Förstermann and Münzel, 2006).

In one study investigating the effect of superoxide dismutase (SOD, an endogenous free radical scavenger) and tetrahydrobiopterin (BH4) on NO levels in human coronary artery endothelial cells (HCAECs) following treatment with serum from smokers and non-smokers. In the same study, NO levels are significantly reduced in HCAECs treated with serum from smokers compared to non-smokers. Furthermore, the addition of either superoxide dismutase (SOD) or BH4 could restore NO levels and eNOS activity, thereby suggesting that the CS-induced loss of NO is due, at least in part,

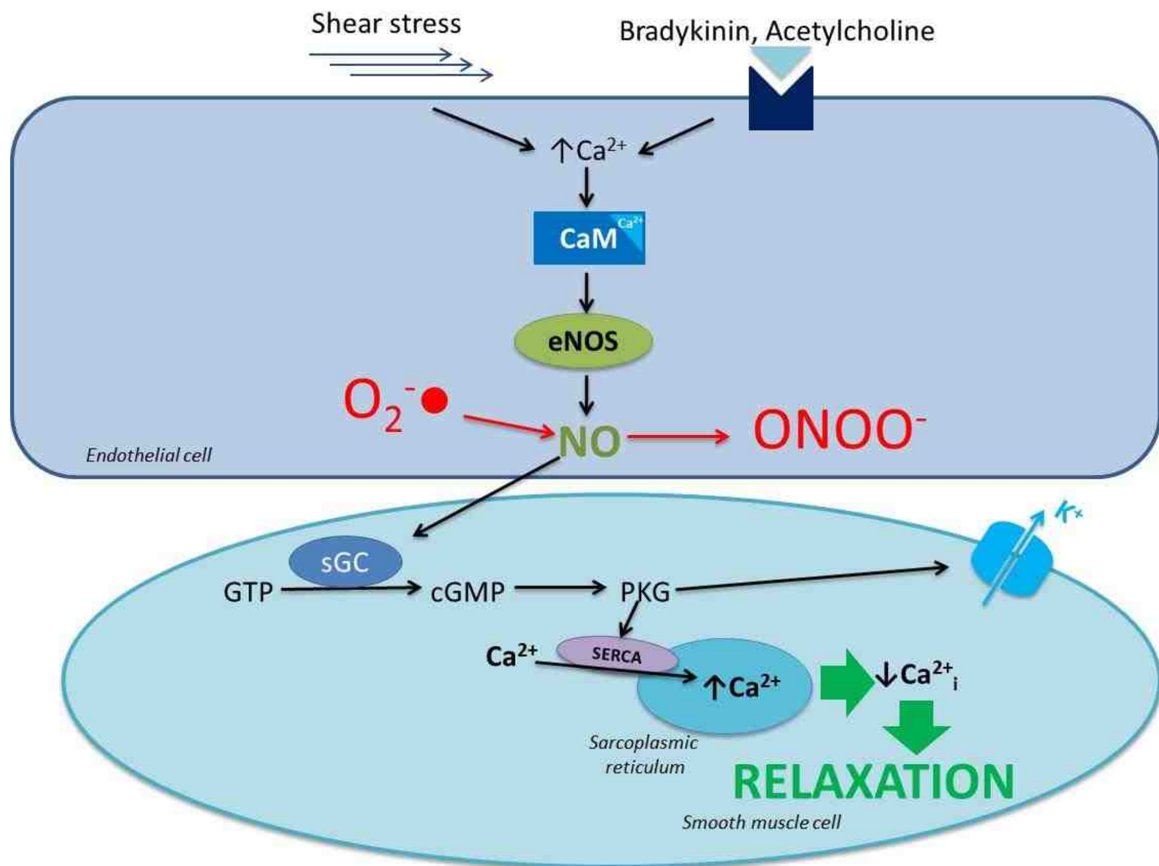

Figure 1.2 NO-dependent dilation. Stimuli such as shear stress, bradykinin, and acetylcholine increase intracellular calcium in the vascular endothelial cell. Calcium binds CaM, which activates eNOS to release NO. NO diffuses to the smooth muscle cell where it activates sGC, which converts GTP to cGMP and subsequently activates PKG PKG activate SERCA, which pumps calcium into the sarcoplasmic reticulum as well as calcium activated potassium channels (BK channels). This leads to a decrease in intracellular calcium and results in smooth muscle relaxation. When there is an increase in superoxide anion (O_2^-•), as occurs in response to cigarette smoke exposure, O_2^-• can react with NO to form peroxynitrite ($ONOO^-$) which leads to decreased NO bioavailability. Abbreviations: NO: nitric oxide; CaM: calmodulin; eNOS: endothelial nitric oxide synthase; sGC: soluble guanylate cyclase; GTP: guanosine triphosphate; cGMP: cyclic guanosine monophosphate; PKG: protein kinase G; SERCA: sarcoplasmic endoplasmic reticulum calcium ATPases;

to ROS (Barua et al., 2003). In addition to the effect of CS-induced oxidative stress on NO-bioavailability, oxidative stress can contribute to vascular dysfunction via other mechanisms.

In addition to short-lived ROS present in the gas phase of CS, CS also contains more stable chemicals in the particulate phase that produce ROS indirectly as a result of metabolism. Examples of such stable chemicals include acrolein and crotonaldehyde. These chemicals increase superoxide anion in endothelial cell culture models originating from bovine pulmonary arteries, human pulmonary arteries, and rat pulmonary arteries.

CS-induced ROS also activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), leading to the expression of a variety of pro-inflammatory cytokines and adhesion molecules, which promotes plaque adhesion at the site of inflammation to form a plug that can later develop into an atherosclerotic lesion (Cacciola et al., 2007; Collins, 1993; Csordas and Bernhard, 2013).

In another study, $ApoE^{-/-}$ mouse (a mouse model of hyperlipidemia and atherosclerosis) aortic explants containing atherosclerotic lesions were exposed *ex vivo* to acrolein (an aldehyde found in CS). Aortic explants exposed to acrolein show a 10-fold increase in matrix metalloproteinase (MMP) activity, which is significant because upregulation of MMPs can cause extensive damage to the endothelium. They further show that acrolein generates ROS by activation of xanthine oxidase, thus showing an association between acrolein-mediated MMP activation and ROS (O'Toole et al., 2009).

Inhaled CS causes injury to the lung, which results in systemic arteriolar endotheial inflammation. One mechanism is by activation of alveolar macrophages through phagosynthesis of inhaled particles, which leads to oxidant production and

release of inflammatory mediators such as TNFα (Lohmann-Matthes et al., 1994; Sinden and Stockley et al., 2010). This results in increases in circulating cytokines, systemic inflammation (increases in circulating leukocytes and interleukin-6) and ultimately lead to endothelial dysfunction (Tamagawa et al., 2008).

In summary, CS generates ROS through a variety of direct and indirect mechanisms that can lead to endothelial dysfunction and CVD, highlighting the importance of finding therapies to reduce levels of oxidative stress, which could reduce the risk of developing CVD.

Endothelial dysfunction

CS causes inflammation and oxidative stress that contribute to endothelial dysfunction. Endothelial dysfunction is an early event that precedes the development of CVD and is defined as a loss of endothelium-dependent vasodilators within the vasculature and an increase in endothelium-dependent vasoconstrictors. The literature suggests that endothelial dysfunction, as measured by flow-mediated dilation (FMD), precedes myocardial infarction, coronary artery disease, peripheral artery disease, hypertension, and stroke (Mathers and Loncar, 2006; Münzel et al., 2008; Perticone et al., 2001).

FMD is a non-invasive, low-risk tool often used in studies for early identification of patients at risk for developing CVD. Brachial artery FMD is measured as follows: Ischemia is induced in the distal forearm by inflating a cuff in the proximal forearm. When the cuff is released, reactive hyperemia causes dilation in the distal brachial artery

of the forearm. The diameter of the brachial artery at baseline and after release of the cuff is monitored by Doppler ultrasound (Harris et al., 2010).

It is well documented that FMD is impaired in smokers. In a study of 200 healthy subjects, FMD is impaired in current smokers. This impairment in FMD is dose dependent and negatively correlates with pack-years (subjects with less pack-years have better FMD). Furthermore, former smokers (who quit for at least 3 months) show improved FMD (Celermajer et al., 1993). In a follow-up study of 78 healthy subjects, FMD is impaired in active and passive smokers compared to never-smokers. Administration of exogenous NO in the form of nitroglycerin restores FMD, suggesting that CS impairs FMD by impairing NO-dependent dilation (Celermajer et al., 1996)

Smokers with diminished FMD can reduce their risk of developing CVD by changing their lifestyle, specifically by incorporating more exercise, dietary changes, and consuming omega-3 (n-3) polyunsaturated fatty acid (PUFA) supplements (Siasos et al., 2013; Wang and Widlansky, 2009). n-3 PUFAs have been shown to be vascular-beneficial however, the mechanism in which n-3 PUFAs protect against cardiovascular disease in smokers is still unclear. In summary, it has been shown that impaired FMD is generally due to the loss of NO and that by increasing NO levels, FMD may be improved or restored.

Omega-3 polyunsaturated fatty acids and cardiovascular disease

Structure and sources of omega-3 polyunsaturated fatty acids

n-3s are PUFAs, meaning that they contain more than one *cis* double bond. Structurally, n-3 PUFAs consist of a carboxylic acid group on the alpha-end and a methyl

group at the terminal (omega) end. The first double bond is 3 carbons in from the methyl (omega) end (Bradbury, 2011).

Docosahexanoic acid (DHA 22:6 n-3) and eicosapentanoic acid (EPA 20:5 n-3) are n-3 PUFAs derived from a shorter chain n-3 PUFA precursor, α-linolenic acid (ALA 18:3 n-3) (Fig. 1.3). n-3 PUFAs are essential fatty acids that are not synthesized effectively by the human body. This is, in part, due to substrate competition between ALA and the n-6 PUFA, linoleic acid (LA 18:2 n-6) for Δ6 desaturase needed for conversion of ALA to EPA and DHA as well as LA conversion to arachidonic acid (AA 20:4 n-6). Additionally, studies using C^{13} or H^2-labeled ALA show that ALA is rapidly catabolized to carbon dioxide and less than 1% is converted into DHA (Arterburn et al., 2006). The elongase and desaturase genes needed for conversion of ALA to DHA and EPA are highly polymorphic (Koletzko et al., 2008; Konkel and Schunck, 2011) and influenced by sex hormones, accounting for large individual differences in conversion efficiencies (Konkel and Schunck, 2011).

It is therefore critical to obtain these essential fatty acids through diet. Plant-derived n-3 PUFAs are limited to ALA and can be found in leafy green vegetables, soy, walnuts, and flaxseed oil. Some species of seafood, such as salmon and shellfish, are rich in ALA, EPA and DHA (Williams and Burdge, 2006). These fatty acids are essential constituents of phospholipid membranes (Konkel and Schunck, 2011).

Marine microalgae, heterotrophic protists, and bacteria can synthesize PUFAs *de novo* due to the presence of desaturases that introduce a double bond at the n-3 and n-6 positions. Some species of fish and invertebrates on the other hand are unable to synthesize ALA, but are able to convert ALA into longer fatty acids such as EPA and

DHA. Fish therefore, occupying higher trophic levels, can contribute to trophic upgrading by converting ALA into EPA and DHA and are therefore an excellent source of n-3 PUFAs for humans (Monroig et al., 2013).

Omega-3 polyunsaturated fatty acid metabolism

Cytochrome P450s (CYP450s) are a superfamily of heme-containing mono-oxygenases. They play a significant role in phase I metabolism where they use heme iron to oxidize substrates like n-3 PUFAs to make them more water soluble. The specific reactions that will be covered in this section are hydroxylation and epoxygenation: During hydroxylation, an alcohol (-OH) group is added and during epoxygenation, an epoxy (=O) group is added (McDonnell and Dang, 2013).

Arachidonic acid (AA) competes with EPA and DHA as a substrate for CYP450s. ω-Hydroxylases shown to effectively metabolize the n-3 PUFAs EPA and DHA include mouse Cyp4a12, rat CYP4A1, and human CYP4A11, CYP4F2, CYP4F3A, and CYP4F3B, CYP4F8, CYP4F12, CYP4A1, Cyp4A12, CYP1A1, CYP1A2, and CYP2E1 (Konkel and Schunck, 2011). While CYP4A1 and CYP4A12 act exclusively as AA hydroxylases, they also act predominantly as an epoxygenase towards EPA to yield 17,18-epoxyeicosatetraenoic acid (EEQ) (Lauterbach et al., 2002; Muller et al., 2007). DHA is preferred as a substrate over EPA for CYP4F2, CYP4F3A, and CYP4F3B. EPA and DHA can also undergo epoxygenation by CYP4F8 and CYP4F12 to yield 17, 18-EEQ and 19,20 epoxydocosapentaenoic acid (EDP) respectively (Fer et al., 2008a).

CYP1A1 and CYP1A2 are both AA hydroxylases but they act as stereoselective epoxygenases with EPA and DHA as substrates, yielding 17(R), 18(S)-EEQ and 19(R),

20(S)-EDP respectively (Schwarz et al., 2004). There are well studied CYP1A1 polymorphisms in humans. The CYP1A1 Ile to Val change at position 462 polymorphism is very common and results in a 5-fold increase in EPA hydroxylation and 2-fold increase in epoxygenation compared to wild-type (Schwarz et al., 2005). It is important to note that since PAHs in CS can induce CYP1A1, this may affect the CYP1A1-mediated metabolism of PUFAs.

AA epoxygenases also play an important role in EPA and DHA metabolism. These epoxygenases include rat CYP2C11 and CYP2C23 and human CYP2C8, CYP2C9, CYP2C18, CYP2C19, and CYP2J2 (Konkel and Schunck, 2011). CYP2C8 and CYP2J2 metabolize EPA and DHA to 17, 18-EEQ and 19, 20-EDP respectively (Lauterbach et al., 2002). CYP2C23 metabolizes EPA to 8,9-, 11,12-, 14,15-, and 17-18-EEQ (Barbosa-Sicard et al., 2005). Interestingly, CYP2J2 and all CYP2C isoforms with the exception of CYP1C8 show high stereoselectivity in favor of the 17(R), 18(S)-EEQ and 19(R), 20(S)-EDP enantiomers (Fer et al., 2008; Konkel and Schunck, 2011).

In addition to P450s, EPA and DHA can also be metabolized by lipoxygenases (LOX) and cycloxygenases (COX), though to a lesser extent than CYPs. EPA can be converted to 18-hydroxyperoxy-hydroxyeicosapentaenoic acid (HEPE) by CYP450s or by aspirin-acetylated COX-2 (ASA:COX-2), followed by 5-LOX conversion to yield resolvin E (RvEs). Similarly, DHA can be converted to 17(R)-

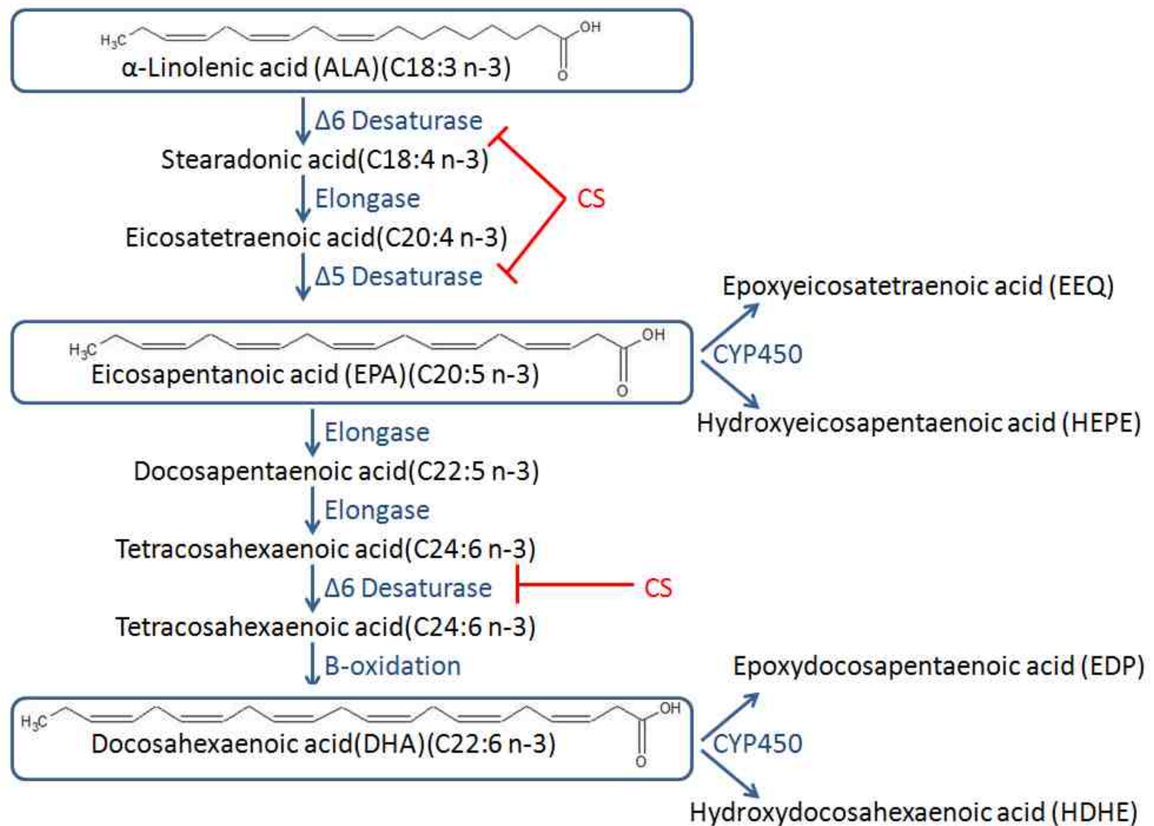

Figure 1.3 n-3 PUFA metabolism. Δ6 desaturase converts ALA into stearadonic acid by adding a double bond. Stearadonic acid is then elongated by elongase to eicosatetraenoic acid followed by addition of another double bond by Δ5 desaturase to form EPA. EPA is elongated to docosapentaenoic acid and then tetracosohexaenoic acid. With addition of a double bond by Δ 6 desaturase, tetracosahexaenoic acid is generated. DHA is generated by β-oxidation of tetracosahexaenoic acid. EPA can further be metabolized by CYP450s to EEQs and HEPEs. Similarly, DHA can be metabolized by CYP450s to EDPs and HDHEs.

hydroxyperoxy-hydroxydocosahexaenoic acid (HDHE), by CYP450s or by ASA:COX-2 followed by 15-LOX conversion to aspirin-triggered resolvin D (AT-RvD). Alternatively, DHA can be converted to 17(S)HDHE by 15-LOX, followed by 5-LOX conversion to RvDs (Chen, 2010; Kohli and Levy, 2009).

Importantly, EPA, DHA, and its epoxy-metabolites provide health benefits to patients, especially in relation to cardiovascular health.

Health benefits of omega-3 polyunsaturated fatty acids

Though the mechanism of action of n-3 PUFAs is still not fully understood and is an area of active study, studies show that they are anti-inflammatory, anti-thrombotic, and vasodilatory agents (Abeywardena and Patten, 2011; Kris-Etherton et al., 2002). Multiple lines of evidence suggest that the P450 epoxygenase-derived n-3 PUFA metabolites contribute to cardiovascular health (Agbor et al., 2014; Ye et al., 2002). Currently, three n-3 PUFA formulations have been approved by the FDA for treating very high triglycerides (TG; > 500 mg/dL). These are Lovaza (EPA and DHA ethyl esters), Vascepa (EPA ethyl ester), and Epanova (DHA and EPA free fatty acids) (Bradberry and Hilleman, 2013).

It has long been suggested that n-3 PUFAs are cardioprotective. In a study in which pigs were fed a high dose of fish-oil for 4 weeks, fish-oil treatment enhanced vasodilation in coronary arteries compared to control fed pigs. Furthermore, when the endothelial was removed, vasodilation was abolished, demonstrating that this response is dependent on an intact endothelium (Shimokawa et al., 1987). In a follow-up study, the authors show that in pigs fed a high fat diet or a atherosclerotic diet, those fed

concurrently with a high dose of fish-oil had improved vasodilation (Shimokawa and Vanhoutte, 1988).

Many studies suggest that n-3 PUFAs improve vasodilation by increasing NO mediated dilation. For example, in a study measuring FMD in patients with coronary artery disease at baseline and after 3 months of EPA treatment (1,800 mg/day), FMD was significantly improved following EPA treatment. In the presence of a NO-donor, sodium nitroprusside, there was no difference, showing that n-3 PUFAs improve endothelium-dependent vasodilation (Tagawa et al., 2002). When human umbilical vein endothelial cells (HUVECs) were treated with DHA or EPA, eNOS activity was increased. Moreover, when caveolae were disrupted, eNOS activity decreased, showing that DHA and EPA, at least in HUVECs, increase caveolae-dependent eNOS activity (Li et al., 2007; Omura et al., 2001).

In addition, n-3 PUFAs has also been shown to improve NO-independent FMD by shear stress-mediated activation of phospholipase A2 (PLA_2), which releases membrane-bound fatty acids, including EPA and DHA (Pearce et al., 1996; Rosa and Rapoport, 2009). EPA and DHA are then metabolized by P450s to the vasodilatory epoxides, EEQs and EDPs (Lauterbach et al., 2002). EEQs and EDPs cause vasodilation through opening of large conductance, calcium-dependent potassium channels (BK channels) and ATP-sensitive potassium channels (Agbor et al., 2012; Hoshi et al., 2013; Walker et al., submitted), and thus act as endothelium-dependent hyperpolarizing factors (EDHFs) (Fig. 1.4).

In a meta-analysis of 39,044 patients with CVD, n-3 PUFA supplementation (1.8 ± 1.2 g/day) reduced the risk of cardiovascular deaths, sudden cardiac death, all cause

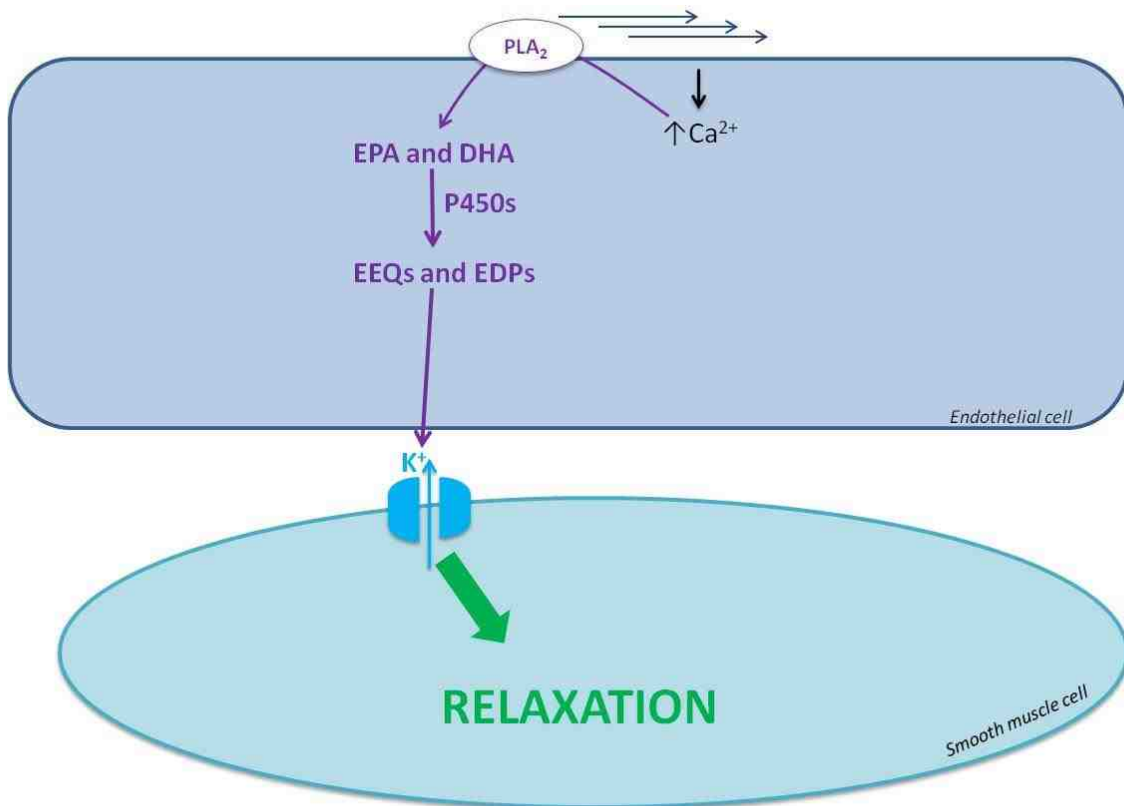

Figure 1.4 NO-independent dilation. Shear stress activates PLA_{2}, which releases membrane bound n-3 PUFAs, EPA and DHA. EPA and DHA is metabolized by CYP450s to its vasodilatory epoxides, EEQs and EDPs, which cause vasodilation through opening of BK- and ATP sensitive potassium channels. Abbreviations: PLA_2: phospholipase A2; EPA: eicosapentanoic acid; DHA: docosahesaenoic acid; EEQ: epoxyeicosatetraenoic acid; EDP: epoxyeicosatetraenoic acid; BK-channel: large conductance calcium sensitive potassium channel;

mortality, and non-fatal cardiovascular events (Marik and Varon, 2009). In another study with 173 patients, the effect of placebo or Lovaza (DHA and EPA ethyl esters; dose: 1 g, 2 g or 4 g/day) on TG levels and FMD in patients with hypertriglyceridemia were investigated. Lovaza improved TG levels and FMD in a dose-dependent manner (Oh et al., 2014). n-3 PUFA supplementation also improve cardiovascular health in healthy smokers, by improving FMD (Siasos et al., 2011).

Polyunsaturated fatty acids in smokers

Smokers have a decrease in red blood cell (RBC) DHA and EPA (Block et al., 2008). In a placebo controlled, double-blind study of 20 smokers, smokers treated with n-3 PUFA supplements have improved FMD compared to smokers treated with placebo in as little as 4 weeks (Siasos et al., 2013). Studies in human monocytic cells (THP-1) and human mammary gland cells (MCR-10A) exposed to CS-extract (generated by bubbling CS through serum added to cell culture media) show that CS decreases Δ5- and Δ6 desaturase activity in a dose dependent fashion. Furthermore, ALA conversion is reduced in CS-exposed cells in a dose-dependent manner (Ghezzi et al., 2007; Marangoni et al., 2004). These studies suggest that the decreases in EPA and DHA in smokers are partly due to the inhibition of enzymes responsible for the conversion of ALA to EPA and DHA.

Reduction of n-3 PUFAs in smokers, likely leads to decreased P450-mediated epoxide vasodilatory metabolites. P450 epoxygenases are induced in smokers, partly due to the presence of PAHs and other AHR ligands in CS. This induction of P450s and decrease in n-3 PUFA substrates may lead to P450-mediated metabolism of the n-6

PUFA AA into pro-inflammatory, pro-arrhythmic, pro-thrombotic vasoconstrictors (Abeywardena and Patten, 2011).

Rationale for research

Healthcare costs associated with mainstream and secondhand smoke exceed $170 billion annually in the U.S. alone and lost productivity attributed to death from cigarette smoking is estimated to top $150 million per year (U.S. Department of Health and Human Services, 2014). The World Health Organization estimates that cardiovascular disease (CVD) will contribute to 30% of tobacco-related deaths by 2015. Endothelial dysfunction, which can be detected by FMD, precedes many CVDs in smokers. n-3 PUFA metabolites are cardio-protective. Previous studies have shown a decrease in n-3 PUFAs in smokers and that n-3 PUFA supplementation improves FMD in smokers. A decrease in n-3 PUFAs led to decreased cytochrome P450 (P450)-derived cardiovascular protective epoxides metabolites. Our preliminary data suggest that lower n-3 PUFAs in smokers associated with endothelial dysfunction. In addition to the risk presented by lower n-3 PUFAs, PAHs found in CS can also contribute to endothelial dysfunction in smokers. PAHs activate the AHR, inducing various P450s involved in PAH metabolism. During PAH metabolism, ROS are generated due to enzymatic uncoupling of for example, eNOS, which can contribute to endothelial dysfunction. n-3 PUFA cardioprotective metabolites and PAH metabolism have not been investigated in terms of their contribution to endothelial dysfunction and CVD in cigarette smokers. Our objective is to investigate the association between CVD risk, AHR activity, and PUFAs in smokers and elucidate mechanistically how they contribute to CS-induced vascular dysfunction.

Central hypothesis: Decreased n-3 PUFAs and increased AHR activity increase vascular dysfunction and CVD risk following CS-exposure.

Aim 1: Determine the association between AHR activation, n-3 PUFAs, and FMD in healthy cigarette smokers.

Rationale: Early identification of young, healthy smokers with endothelial dysfunction allows for an opportunity for treatment that may prevent onset of CVD. FMD is a well established procedure to identify smokers with endothelial dysfunction. This procedure however is expensive, time consuming and technically difficult. There is thus a need for a cost effective and easy to conduct blood-biomarker test to identify patients with endothelial dysfunction to prevent early onset of CVD.

Approach: Our working hypothesis is that there will be a negative correlation between AHR activity and FMD and a positive correlation between n-3 PUFAs and FMD. To test this hypothesis, we recruited healthy, young (19-50 yr. old) Hispanic never (n=16) and current (n=16) smokers. We assessed FMD and other CVD risk factors and calculated the 10 year risk of developing CVD (Framingham risk score). n-3 and n-6 PUFAs were also measured by GC-MS/MS and their P450-derived epoxide metabolites were measured by LC-MS/MS. We used simple and multivariate regression analysis to assess correlation between AHR activity, PUFAs, and FMD.

Aim 2: Determine the association between n-3 PUFAs, their epoxide and diol metabolites and the 10-year atherosclerotic cardiovascular disease risk (ASCVD) in cigarette smokers with hypertension.

Rationale: Biomarkers identified in young, healthy smokers may serve not only as early identification of smokers with vascular dysfunction but also as biomarkers for future CVD risk in smokers with hypertension.

Approach: Our working hypothesis is that EPA, DHA and their epoxide metabolites would be negatively associated with ASCVD risk, while ALA and the diol metabolites of EPA and DHA would be positively associated with ASCVD risk. To test this hypothesis, we recruited individuals between 40 and 70 years of age with physician-diagnosed hypertension. CVD risk factors and n-3 PUFAs were assessed as in aim 1. Additionally, we also calculated ASCVD risk. We used simple and multivariate regression analysis to assess correlation between n-3 PUFAs, their epoxide and diol metabolites and ASCVD risk.

Aim 3: Elucidate the mechanism by which n-3 PUFAs protect against CS-induced vascular dysfunction.

Rationale: There is no mouse model that resembles CS-induced endothelial dysfunction seen in humans. Additionally, the mechanism of how n-3 PUFAs improve vascular function in smokers is unknown.

Approach: Our working hypothesis is that n-3 PUFAs protect against CS-induced vascular dysfunction by increasing NO-bioavailability and decreasing oxidative stress. To test this hypothesis, we fed C57BL/6 mice an n-3 PUFA-enriched diet or standard chow diet for 8 weeks and then exposed the mice to mainstream CS for 5 days. We conducted FMD vascular reactivity studies ± Nω-(Nitroamidino)-L-2,5-diaminopentanoic acid (LNNA, NOS inhibitor) and ± 4-hydroxy TEMPO (TEMPOL, SOD mimetic) in mesenteric arterioles. n-3 and n-6 PUFAs and markers of oxidative stress were also measured.

CHAPTER II

Association of Serum Aryl Hydrocarbon Receptor Activity and RBC Omega-3 Polyunsaturated Fatty Acids with Flow-Mediated Dilation in Healthy, Young Hispanic Cigarette Smokers

Wiest, E.F., Warneke, A., Walsh, M.T., Langsfeld, M., Anderson, J., Walker, M.K., 2015. Association of serum aryl hydrocarbon receptor activity and RBC omega-3 polyunsaturated fatty acids with flow-mediated dilation in healthy, young Hispanic cigarette smokers. *Toxicol Lett.* **232**, 423–428.

ABSTRACT

Impaired flow-mediated dilation (FMD) occurs prior to clinical disease in young cigarette smokers. We investigated two potential biomarkers of FMD: serum aryl hydrocarbon receptor (AHR) activity and RBC omega-3 polyunsaturated fatty acids in healthy young Hispanic cigarette smokers. We recruited never (n=16) and current (n=16) Hispanic smokers (32 ± 7 years old), excluding individuals with clinical cardiovascular disease. We measured FMD with duplex ultrasound, RBC fatty acids and serum AHR activity using a luciferase reporter assay. FMD was significantly impaired in smokers (5.8 ± 4%) versus never smokers (12.3 ± 7.4%, $p=0.001$). Serum AHR activity was significantly increased in smokers (1,467 ± 358 relative light units (RLU)) versus never smokers (689 ± 251 RLU, $p<0.001$), and correlated positively with FMD only in smokers ($r=0.691$, $p<0.004$). RBC percentage of α-linolenic acid (ALA%) was significantly increased in smokers (0.14 ± 0.03%) versus never smokers (0.11 ± 0.03%, $p=0.018$), and correlated inversely with FMD only in smokers ($r=-0.538$, $p=0.03$). The combination of serum AHR activity, ALA%, and systolic blood pressure significantly correlated with FMD in a multivariable regression model ($r=0.802$, $p<0.008$). These results suggest that serum AHR activity and RBC ALA% could serve as biomarkers of FMD in healthy, young Hispanic cigarette smokers.

INTRODUCTION

Cigarette smoking is a major independent risk factor for atherosclerosis and cardiovascular diseases, including myocardial infarction, stroke, aortic aneurysm, and peripheral artery disease (Ockene and Miller, 1997; Selvin and Erlinger, 2004). Although the pathophysiology of cigarette smoke-induced cardiovascular disease is not fully understood, changes in the structure and function of blood vessels are major contributing factors (Rahman and Laher, 2007). One early event in cigarette smoke-induced vascular injury is endothelial dysfunction, which results from the loss of endothelial-derived vasodilators. Endothelial dysfunction is a pathological precursor to many vascular diseases (Munzel et al., 2008) and has been shown to occur prior to symptoms of overt clinical disease in young cigarette smokers (Neunteufl et al., 2000; Ozaki et al., 2010).

Flow-mediated dilation (FMD) is frequently used to measure endothelial dysfunction. Studies have shown that impaired FMD is an independent predictor of future cardiovascular events in individuals without clinical cardiovascular disease or in individuals with low Framingham risk scores (Rossi et al., 2008; Shechter et al., 2009). However, FMD cannot be employed in a cost-effective manner on a wide scale basis, because equipment is costly and image acquisition is technically difficult. Thus, there is a need for a simple, reproducible and less expensive biomarker of endothelial dysfunction that could be used to identify those individuals at greatest risk.

Cigarette smoke-induced endothelial dysfunction is mediated, in part, by oxidative stress. Cigarette smoke is a source of free radicals, including superoxide anion and peroxynitrite (Pryor and Stone, 1993). In addition, cigarette smoke contains high

levels of polycyclic aromatic hydrocarbons (PAHs) (Ding et al., 2005), leading to activation of the aryl hydrocarbon receptor (AHR) (Denison and Nagy, 2003; Gebremichael et al., 1996; Kasai et al., 2006; Kitamura and Kasai, 2007) and to induction of various cytochrome CYPs, including CYP1A1 and 1B1 in the liver and extra hepatic tissues (Granberg et al., 2003; Kiyohara and Hirohata, 1997). These CYPs can generate reactive oxygen species directly by the inefficient coupling of NADPH consumption to substrate oxidation (Kopf and Walker, 2010; Morel et al., 1999; Schlezinger et al., 2006; Zangar et al., 2004) and indirectly by the metabolic activation of PAHs to highly reactive diol-epoxides and dihydrodiols (Bolton et al., 2000; Shimada, 2006). Furthermore, Hispanics commonly carry the CYP1A1*2A polymorphism (Swinney et al., 2011), which results in higher levels of CYP1A1 activity, and this increased CYP1A1 activity may put them at higher risk of cigarette smoke-induced oxidative stress. Thus, the degree to which the AHR is activated and the presence of the CYP1A1*2A polymorphism could be potential predictors of endothelial dysfunction in cigarette smokers.

In addition, studies have shown that cigarette smokers have lower levels of omega-3 polyunsaturated fatty acids (PUFAs), specifically eicosapentanoic acid (EPA, 20:5n-3) and docosahexanoic acid (DHA, 22:6n-3) (Ionescu et al., 2013; Simon et al., 1996). These fatty acids are metabolized by CYPs to potent vasodilators that contribute to endothelial-mediated dilation (Wang et al., 2011). Thus, the levels of omega-3 PUFAs also could be potential biomarkers of endothelial dysfunction in cigarette smokers.

The purpose of this study was to determine the degree to which serum AHR activity and RBC omega-3 PUFAs were associated with FMD in healthy, young Hispanic current and never cigarette smokers.

METHODS

Study Subjects

We recruited age-and sex-matched, healthy never (n=16) and current smokers (n=16) of self-described Hispanic ethnicity. Age was restricted to between 19-50 years and current smokers were defined as having smoked > 0.5 pk/d for at least the past year. Patients excluded from the study included those who were pregnant, those who had the atherosclerosis risk factors of hypertension or diabetes, and those who had a history of ischemic heart disease, stroke, or heart failure. All subjects were questioned regarding their consumption of fish and their use of dietary supplements, including fish oil. This study was approved by the University of New Mexico Institutional Review Board (HRRC: 12-168) and written informed consent was obtained from all participants.

Study design

Subjects were asked to fast and refrain from exercise, smoking, or drinking caffeinated beverages for 10 hours prior to their appointment for assessment of flow-mediated dilation (FMD). FMD was conducted between 7-10 am, in the same room, at a set temperature of 70-72° F and was performed by the same Registered Vascular Technologist. Subjects were placed in a supine position and ECG leads were placed. Blood pressure was measured in both arms; subjects rested quietly for 15 minutes with the room darkened, then brachial artery diameter was imaged using a 15-7 linear array transducer in the arm with the highest systolic blood pressure. The ECG was monitored continuously and directly connected to the scanner to allow for post-exam 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 mm Hg above the subject's systolic blood pressure. The cuff was inflated for 5 minutes and continuous scanning of the brachial artery was performed from 30 seconds prior to cuff release until 2 minutes after cuff release. Longitudinal view diameter measurements were recorded and the peak percent change in brachial artery diameter from baseline to the point of greatest diameter during the hyperemic phase was used as a measure of FMD. Measurements were conducted by a vascular surgeon that was blinded to the patient group.

Following assessment of FMD, blood samples were taken to measure cotinine, HbA1c, lipids (VAP test, AtheroTech, Birmingham, AL), AHR activation potential in serum, RBC omega-3 and omega-6 PUFAs (OmegaQuant, Souix Falls, SD), and cytochrome P4501A1 (CYP1A1) and CYP1B1 mRNA and presence of CYP1A1*2A polymorphism. To assess serum AHR activation potential, we used a recombinant cell-based bioassay, termed CALUX (chemically activated luciferase expression), to detect the ability of serum to induce a luciferase reporter construct requiring AHR activation (He et al., 2008; Ziccardi et al., 2000). Human hepatoma cells (HepG2) stably transfected with an AHR-luciferase reporter were treated with benzo(a)pyrene (10^{-8} M) as a positive control or 25% serum from never or current smokers for 24 hr, and luciferase expression measured (Promega, Madison, WI). To assess CYP1A1 and 1B1 mRNA expression, RNA was isolated from whole blood (PAXgene RNA kit, Qiagen) and analyzed by qPCR and normalized to RNA polymerase 2 as described previously (Kopf and Walker, 2010). To determine the CYP1A1*2A genotype, DNA was isolated from whole blood (PAXgene kit, Qiagen) and analyzed by a PCR/restriction digest method as described (Jarvis et al., 2010). The wild type genotype was identified by a single 340 bp

band; the heterozygous genotype was identified by the presence of a 340 bp band (wild type allele) and two bands of 200 and 140 bp resulting from MspI digestion (variant allele); and the homozygous variant identified by presence of only the 200 and 140 bp bands.

Statistical analysis

We expected that serum AHR activity and n-3 PUFAs would correlate with FMD in all subjects and thus our objective was to have adequate power to detect a relationship in multivariate regression analyses. We estimated needing a sample size of 36 to achieve 80% power to detect a correlation (r=0.35) in multivariable regression model with three predictors and alpha=0.05. Data are expressed as mean ± standard deviation (SD) and were compared between never and current smokers using a paired t-test when the data were normally distributed and a Wilcoxon Signed Rank test when the data were not normally distributed. In addition, we tested categorical variables with a chi square test. Based on our hypothesis that serum AHR activity and n-3 PUFAs would be predictive of FMD, we conducted correlation analysis of FMD versus serum AHR activity, CYP1B1 mRNA, ALA%, DHA%, or EPA%, using the Pearson correlation test. We also conducted correlation analysis of FMD versus the individual factors used to calculate the Framingham risk score, including age, total cholesterol, high density lipoprotein, and systolic blood pressure, using the Pearson correlation test. Since we found that correlation of FMD with serum AHR activity and ALA% was statistically significant for each variable alone, we conducted multivariate regression analysis for FMD versus both serum AHR activity and ALA% (Model #1). Finally, we added individual factors used to

calculate the Framingham risk score to the multivariate regression Model #1 to assess the degree to which they improved prediction of FMD. $P < 0.05$ was considered statistically significant.

RESULTS

The cigarette smokers recruited for this study were in good cardiovascular health, failing to exhibit any significant changes in classic cardiovascular disease risk factors, including blood pressure, triglycerides (TG), and low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol (Table 2.1). In addition, total cholesterol was significantly lower in smokers than never smokers. The Framingham risk score, an estimate of the 10-year risk of developing coronary heart disease, was significantly elevated in smokers solely as a result of their smoking. However, the score of 6.8% represented a low risk of developing coronary heart disease in the next ten years.

Cigarette smokers exhibited significantly impaired FMD, compared to never smokers (Fig. 2.1, $p = 0.001$), while baseline diameter of the brachial artery prior to reactive hyperemia did not differ between groups (never smokers, 3.78 ± 0.12; smokers, 3.98 ± 0.12; $p > 0.2$).

We also assessed AHR-dependent luciferase expression from serum and CYP1A1 and 1B1 mRNA expression from WBCs, representing indices of exposure to PAHs. Cigarette smokers exhibited significantly higher levels of AHR-dependent luciferase expression when serum was applied to the CALUX assay ($p < 0.001$, Fig. 2.2A). Smokers also exhibited significantly higher levels of CYP1B1 mRNA in WBCs (p <

0.017, Fig. 2.2B). CYP1A1 mRNA was detectable from only 1 of 17 smokers and none of the never smokers (data not shown). Smokers tended to have a higher frequency of carrying one CYP1A1*2A variant allele (never smokers, 20%; current smokers, 60%), but this was not significantly different, and no individuals were identified with two CYP1A1*2A variant alleles. Further, the presence of the CYP1A1*2A polymorphism did not correlate with serum AHR activation potential in the CALUX assay.

Cigarette smokers also exhibited significant differences in the levels of two omega-3 PUFAs; α-linolenic acid (ALA, 18:3n-3) and DHA, expressed as a percentage of total RBC fatty acids. ALA% was significantly increased in smokers ($p < 0.018$), while DHA% was significantly decreased ($p < 0.018$, Fig. 2.3A and B). There was no significant difference in the percentage of EPA in RBCs (Fig. 2.3C). We also failed to find any differences in the percentages of omega-6 PUFAs, including linoleic acid (18:2n-6) and arachidonic acid (20:4n-6), and failed to find any differences in fish meals consumed per week or fish oil supplement usage. In correlation analysis, we failed to find any association between FMD and RBC fatty acids, CYP1B1 mRNA expression or serum AHR-activated luciferase expression when all subjects were included. However, we did find significant associations when considering smokers alone. We found that AHR-dependent luciferase expression correlated positively and significantly with FMD in smokers ($r = 0.691$, $p < 0.004$), but showed no relationship to FMD in never smokers ($r = -0.184$, $p = 0.530$; Fig. 2.4A and B). In addition, we found that ALA% in RBCs correlated inversely and significantly with FMD in smokers ($r = -0.538$, $p = 0.03$), but showed no relationship to FMD in never smokers ($r = 0.09$, $p = 0.75$; Fig. 2.5A and B). There was no association between FMD and other omega-3 PUFAs, including EPA and

Table 2.1. Characteristics of study subjects.

Characteristic	Never smoker	Current smoker	*P*-value
Male/female, *n*	8/8	8/8	1.000
Age (yr)	30.6 ± 6.6	32.1 ± 6.8	0.066
Cotinine (mg/dL)	1.1 ± 0.4	100 ± 62	<0.001
HbA1c (%)	5.3 ± 0.2	5.3 ± 0.2	0.520
Systolic BP (mmHg)	122 ± 11	125 ± 16	0.555
Diastolic BP (mmHg)	78 ± 10	83 ± 12	0.303
BMI (kg/m^2)	31 ± 11	27 ± 4	0.250
Total cholesterol (mg/dL)	198 ± 28	173 ± 31	0.014
LDL (mg/dL)	117 ± 31	100 ± 31	0.088
HDL (mg/dL)	58 ± 23	50 ± 12	0.194
TG (mg/dL)	120 ± 44	136 ± 93	0.575
Framingham risk score (%)[a]	0.6 ± 4.1	6.5 ± 3.9	<0.001

[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.

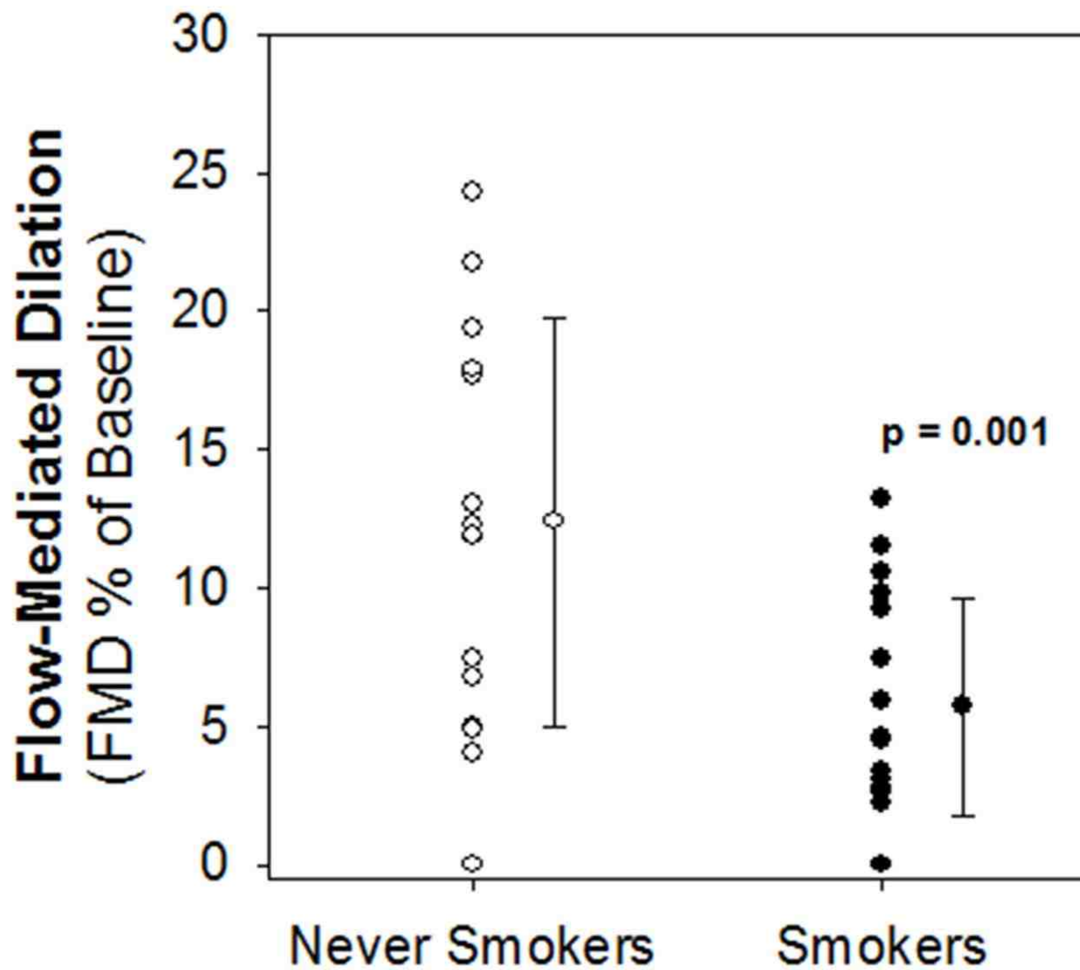

Figure 2.1. Change in brachial artery diameter as a percent of baseline resulting from flow-mediated dilation (FMD). Cigarette smokers exhibited significantly impaired FMD. Data are expressed as mean ± SD of never smokers and current smokers (n=16/group).

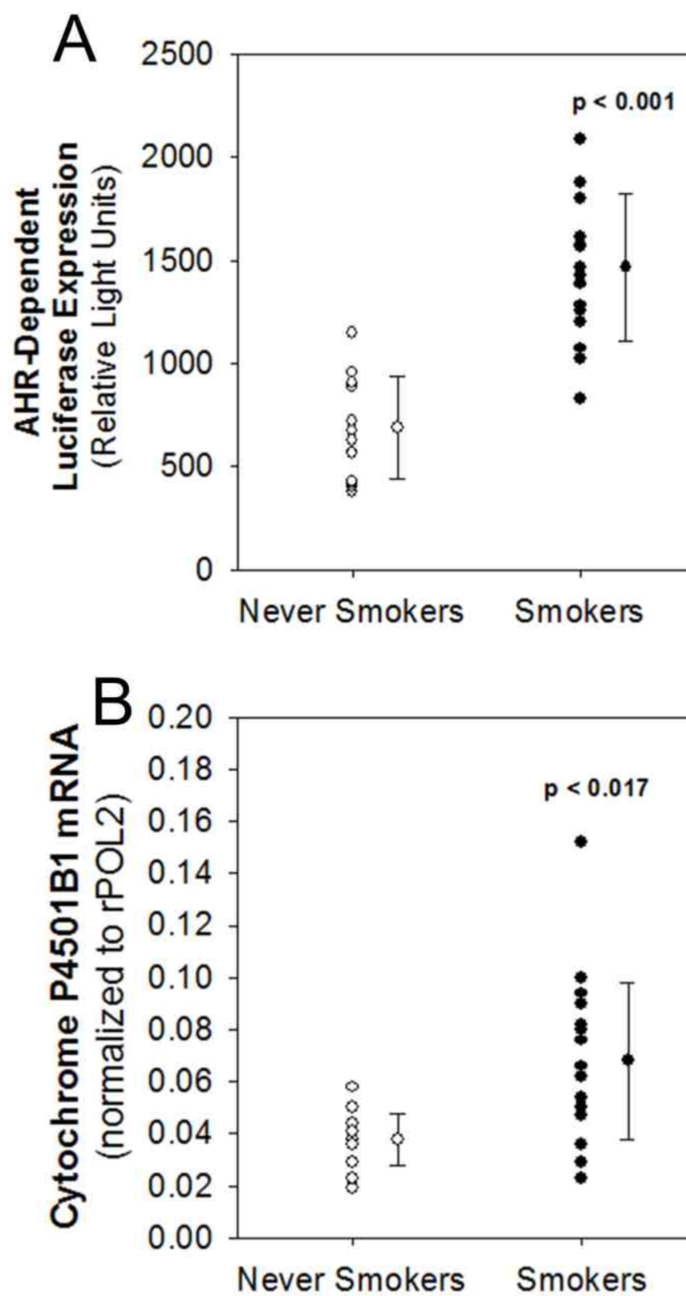

Figure 2.2. Serum AHR activation potential and WBC CYP1B1 mRNA expression, representing indices of PAH exposure. Current smokers exhibited significantly higher (A) AHR-dependent luciferase expression resulting from serum applied to CALUX assay and (B) CYP1B1 mRNA expression. Data are expressed as mean ± SD of never smokers and current smokers (n=16/group). rPOL2, RNA polymerase 2.

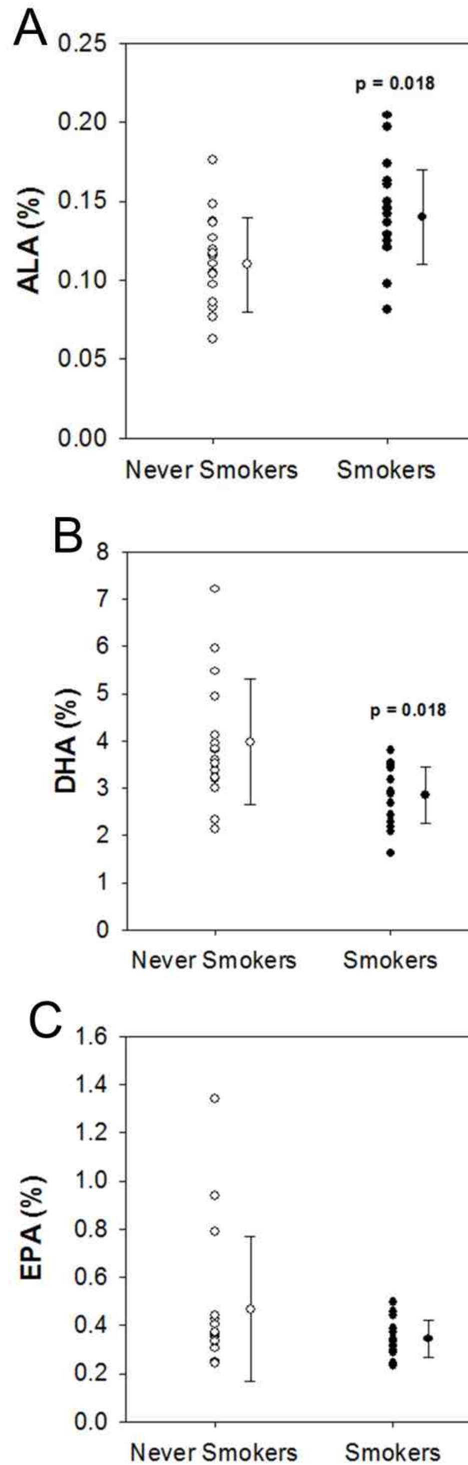

Figure 2.3. Omega-3 PUFAs expressed as a percentage of total fatty acids in RBCs. Cigarette smokers exhibited significantly (A) higher RBC percentage of ALA and (B) lower RBC percentage of DHA. There was no difference in RBC percentage of EPA between groups. Data are expressed as mean ± SD of never smokers and current smokers (n=16/group).

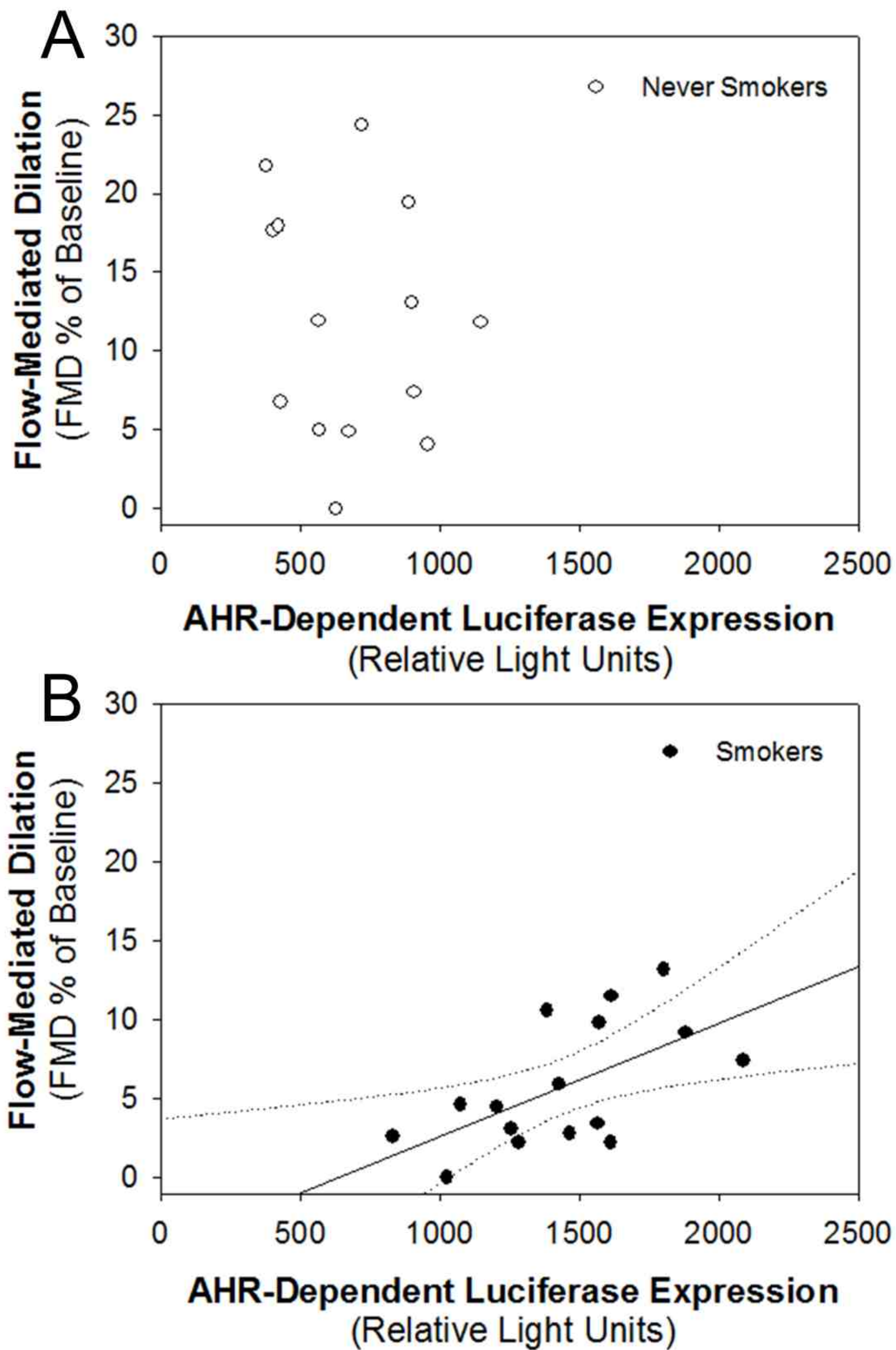

Figure 2.4. Relationship between AHR-dependent luciferase expression and FMD. There was no correlation between AHR-dependent luciferase expression and FMD in never smokers (A, $r = -0.184$, $p = 0.530$). In contrast, there was a significant positive correlation between AHR-dependent luciferase expression and FMD in current smokers (B, $r = 0.691$, $p < 0.004$). Dotted lines represent 95% confidence interval around the regression line.

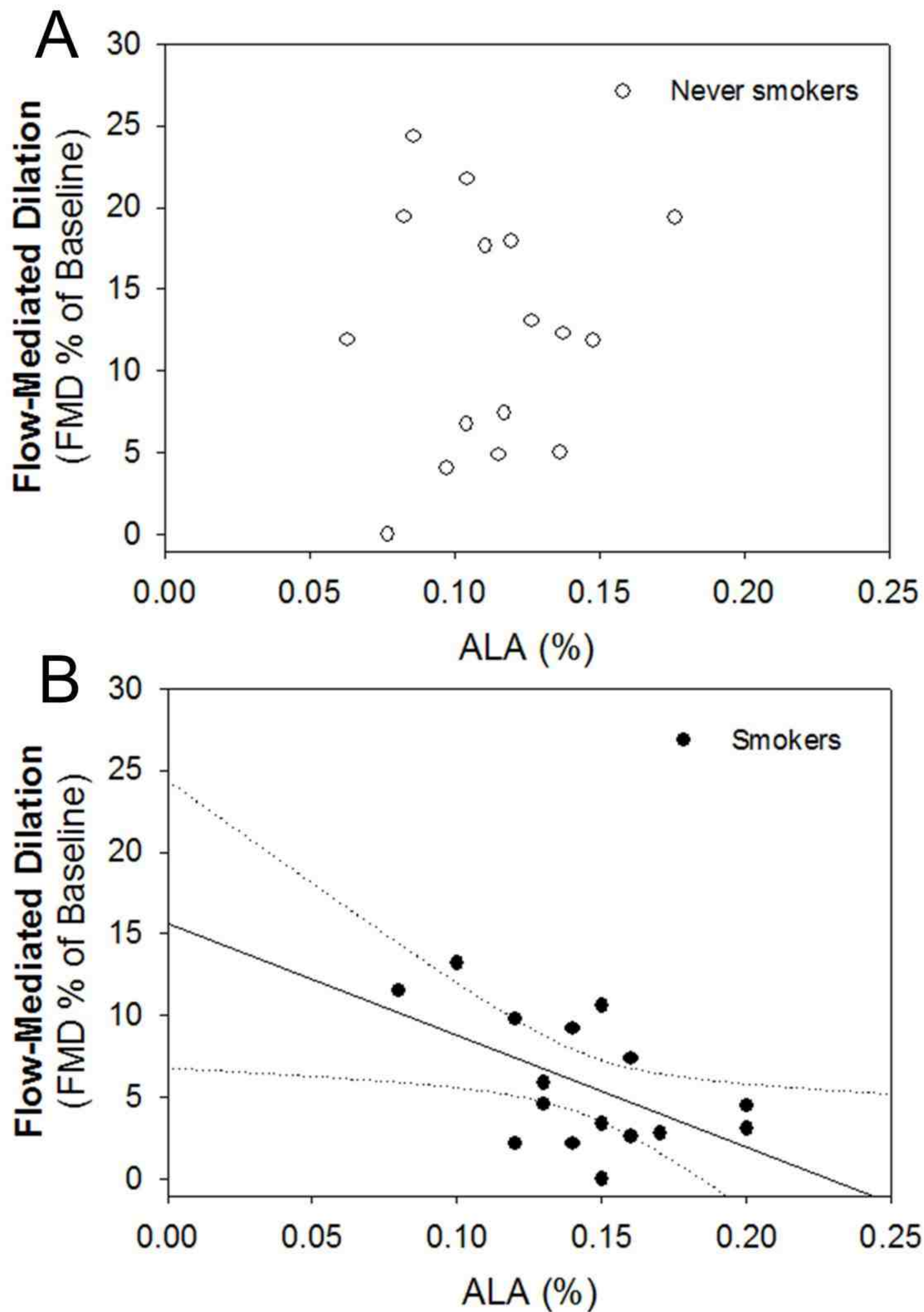

Figure 2.5. Relationship between RBC percentage of ALA and FMD. There was no correlation between ALA% and FMD in never smokers (A, r = 0.09, p = 0.75). In contrast, there was a significantly inverse correlation between ALA% and FMD in current smokers (B, r = -0.538, p = 0.03). Dotted lines represent 95% confidence interval around the regression line.

Table 2.2. Multiple regression models for FMD in current cigarette smokers.

Model	B coefficient ± SE	*P*-value	*R*, adjusted R^2 values
Model #1		0.013	
Intercept	4.1 ± 6.0	0.681	R = 0.718, adj R^2 = 0.434
Serum AHR activity	0.006 ± 0.002	0.036	
ALA%	−4621 ± 2573	0.098	
Model #2		0.008	
Intercept	−5.7 ± 7.3	0.451	
Serum AHR activity	0.006 ± 0.002	0.021	R = 0.802, adj R^2 = 0.545
ALA%	−6455 ± 2486	0.025	
Systolic BP	0.10 ± 0.05	0.073	

DHA, nor was there a correlation between ALA and AHR-dependent luciferase expression. Finally, none of the individual factors used to calculate the Framingham risk score showed a significant correlation with FMD. Multivariate regression analysis was used to develop a model to predict FMD. There was no significant association found when all factors used to calculate the Framingham risk score were included in the model (data not shown). However, a significant association was found when using AHR-dependent luciferase expression and ALA% as independent variables (Table 2.2). Furthermore, the addition of systolic blood pressure (model #2) was the only Framingham risk score variable that increased both R and adjusted R^2 values.

DISCUSSION

In this study we found that FMD was significantly impaired in healthy, young Hispanic male and female cigarette smokers, compared to never smokers, confirming numerous previous reports of impaired FMD in healthy young smokers of other ethnicities (Celermajer et al., 1993; Neunteufl et al., 2000; Ozaki et al., 2010; Siasos et al.). However, our data showed for the first time that serum AHR-dependent luciferase expression and RBC ALA% were independent predictors of FMD in healthy, young Hispanic cigarette smokers. Furthermore, the best prediction of FMD in smokers was observed when serum AHR activity, ALA%, and systolic blood pressure were used as independent variables in a multivariable regression model.

Cigarette smoking is the primary route of exposure to PAHs, which are formed from the incomplete combustion of organic material (Ding et al., 2005; Suwan-ampai et al., 2009). Thus, it is not surprising that current smokers have significantly higher serum

AHR activity than never smokers. The serum AHR activity measured from never smokers likely results from the “background” exposure to AHR active chemicals from outdoor air pollution and dietary sources. Interestingly, while serum AHR activity was not associated with FMD in never smokers, it was positively associated with FMD in current smokers, suggesting that smokers with the worst FMD responses had lower serum AHR activity. This finding was unexpected, but would make sense if PAH metabolism is considered. PAHs bind the AHR with high affinity, resulting in its nuclear translocation and activation of gene expression via binding to dioxin response elements (core sequence: 5'-GCGTG-3') in the promoters of gene transcriptionally regulated by the AHR, including CYP1A1 and CYP1B1. These P450s subsequently metabolize PAHs to highly reactive products, leading to the formation of reactive oxygen species and vascular injury (Bolton et al., 2000; Ramos and Moorthy, 2005; Shimada, 2006) . Thus, it has been shown that PAH toxicity occurs secondary to metabolism and the levels of unmetabolized parent PAHs are inversely related to P450 activity (Uno et al., 2006; Uppstad et al.). Consequently, the AHR-dependent luciferase expression in a recombinant cell-based bioassay would represent as a surrogate index of unmetabolized PAHs from the serum of smokers (Han et al., 2004; He et al., 2008; Ziccardi et al., 2000). Thus, we speculate that smokers with the worst FMD responses have higher rates of PAH metabolism and lower levels of parent PAHs in the serum.

Cigarette smoking also has been shown to significantly reduce long chain omega-3 PUFAs, EPA and DHA, and DHA specifically has been shown to correlate with FMD in healthy young cigarette smokers (Leeson et al., 2002). Further, omega-3 PUFA supplements containing EPA and DHA have been shown to significantly improve

endothelial function in healthy young cigarette smokers (Din et al.; Siasos et al.). We observed a significant decrease in RBC DHA% in current smokers consistent with previous studies; however, we did not find an association between DHA% and FMD. The reason for this is not clear. While the levels of DHA can be influenced by dietary intake and use of fish oil supplements, we did not find that dietary intake or supplement use differed in our cohort of smokers. It is possible that our small sample size precluded detecting a relationship between these two variables.

In addition to the decrease in RBC DHA%, we found a significant increase in RBC ALA% in smokers. Furthermore, we observed that RBC ALA% was significantly and inversely associated with FMD. ALA is a medium chain omega-3 PUFA that can be converted to longer chain omega-3 PUFAs, EPA and DHA, via desaturase and elongase enzymes, although this conversion is inefficient in humans. One previous study has reported that ALA% is increased in smokers (Simon et al., 1996) and a number of studies have reported that cigarette smoke inhibits the activities of the desaturase enzymes, slowing the conversion of ALA to EPA and DHA (Ghezzi et al., 2007; Marangoni et al., 2004). Our data show that individuals with the worst FMD responses have a higher percentage of RBC ALA. This would be consistent with an inhibition of ALA conversion to longer chain omega-3 PUFAs and subsequently lower levels of DHA. The relationship between ALA and cardiovascular disease risk is mixed, however. High dietary linolenic acid intake (combination of both ALA and γ-linolenic acid, 18:3n-6) has been reported to have cardiovascular protective effects, including being associated with a lower prevalence of coronary artery disease, lower prevalence of carotid plaques, and lower plasma TG (Djousse et al., 2003a; Djousse et al., 2003b; Djousse et al., 2001). In

contrast, higher RBC ALA% was associated with a significantly higher risk of sudden myocardial infarction (Lemaitre et al., 2009). The reasons for this disparity are not known. It is possible that the higher incorporation of ALA into cell membranes may reflect poor conversion into longer chain omega-3 PUFAs, while higher dietary intake may reflect better bioavailability of omega-3 PUFAs in general.

Study Limitations

The cross-sectional design of this study prevents us from establishing a cause-and-effect relationship between these potential blood-based biomarkers and impaired FMD. In addition, we only measured FMD, which represents a combination of the release of vasodilators from the endothelium and the response of the smooth muscle to these vasodilators. Thus, we did not establish that impaired FMD in our cohort of cigarette smokers resulted from endothelial dysfunction. Nonetheless, many previous studies in healthy young cigarette smokers have established that impaired FMD results from endothelial dysfunction and is independent of smooth muscle responses. Additionally, we used serum AHR-dependent luciferase expression as an index of unmetabolized PAHs in the systemic circulation, but we did not directly measure PAHs, PAH metabolites or P450-dependent activity. Lastly, our data are limited to a small group of healthy, young Hispanic cigarette smokers without clinical cardiovascular disease. It remains unknown whether these potential biomarkers of FMD are correlated with FMD in individuals with other cardiovascular disease risk factors, such as hypertension and hyperlipidemia.

CONCLUSIONS

FMD is an independent predictor of future cardiovascular events in individuals without clinical cardiovascular disease. Our results suggest that the ability of serum to activate the AHR and the percentage of ALA in RBC have the potential to serve as independent biomarkers of impaired FMD in healthy cigarette smokers. These biomarkers could be used by regulatory agencies to screen the potential cardiovascular disease risk posed by use of other tobacco products or possibly by occupational or environmental exposure to PAHs. Additional studies are warranted to confirm these associations in larger population samples and in multiple ethnic groups as well as to determine if these represent potential biomarkers of cardiovascular risk in individuals with clinical cardiovascular disease.

CHAPTER III

n-3 and n-6 Polyunsaturated Fatty Acids and their Metabolites as Predictors of Cardiovascular Disease Risk in Smokers and Non-smokers with Pre-existing Hypertension

Wiest, E.F., Walsh, M.T., Achrekar, A., Roth, M., Schunck, W.-H., Anderson, J., and Walker, M.K., 2016. N-3 and n-6 Polyunsaturated Fatty Acids as Predictors of Cardiovascular Disease Risk in Smokers and Non-smokers with Pre-existing Hypertension**.** Manuscript in preparation to be submitted to *Prostaglandins, Leukotrienes and Essential Fatty Acids.*

ABSTRACT

Introduction: The omega-3 (n-3) polyunsaturated fatty acids (PUFAs), α-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid, have been shown to predict brachial flow-mediated-dilation in young, healthy smokers. We sought to investigate if n-3 and n-6 PUFAs and their metabolites could also serve as predictors of 10-year atherosclerotic cardiovascular disease (ASCVD) risk in smokers and non-smokers with pre-existing hypertension.

Subjects and methods: We recruited 40-70 year old, current- and never-smokers with physician-diagnosed hypertension; determined 10-year ASCVD risk and measured n-3 and n-6 PUFAs and their metabolites from peripheral blood. Individual fatty acids were correlated with ASCVD risk and then multiple regression models were developed to predict ASCVD risk.

Results: ASCVD risk did not differ between smokers and non-smokers (smokers: 8.8, 95% CI [5.4, 12.2]; non-smokers: 7.4, 95% CI [4.3, 10.4]) and there were no significant differences in individual concentrations of PUFAs or their metabolites. Nonetheless, the association between fatty acid metabolites and ASCVD risk differed between smokers and non-smokers in multiple regression. In non-smokers, three n-3 PUFA epoxide and one diol metabolite were most predictive of ASCVD risk ($R = 0.876$, $p < 0.05$). In contrast, in smokers one diol and one P450-derived hydroxylase metabolite, and one auto-oxidation product were most predictive ($R = 0.636$, $p < 0.05$).

Conclusions: Taken together, these data suggest that smoking may contribute to ASCVD risk by shifting the overall pattern of n-3 and n-6 PUFA metabolism and responsiveness

to these metabolites. Further, these results demonstrate that use of fatty acid metabolites as potential biomarkers of ASCVD risk will be dependent on smoking status.

INTRODUCTION

Cigarette smoke (CS) remains the largest preventable cause of death in the U.S. and is a major risk factor for development of cardiovascular disease (CVD) (US Department of Health and Human Services, 2014). Brachial flow-mediated dilation (FMD), an index of vascular endothelial function) is impaired even in otherwise young and healthy cigarette smokers (Celermajer et al., 1993; Wiest et al., 2015). The literature further suggests that endothelial dysfunction as measured by FMD precedes myocardial infarction, coronary artery disease, peripheral artery disease, hypertension, and stroke (Mathers and Loncar, 2006; Münzel et al., 2008; Perticone et al., 2001). Smoking cessation is the best treatment; however, it is very difficult to achieve (Yong et al., 2014). There is therefore a need to develop biomarkers to identify smokers at greatest risk of developing CVD as well as effective interventions to prevent early onset of CVD in those at risk.

We have previously shown that FMD correlates negatively with the omega-3 (n-3) polyunsaturated fatty acid (PUFA) precursor, α-linolenic acid (ALA; 18:3n-3), in otherwise young and healthy smokers (Wiest et al., 2015). Subjects with the most impaired FMD had the highest levels of ALA. In addition, we found that smokers have significantly lower levels of the n-3 PUFAs, docosahexanoic acid (DHA 22:6 n-3) and eicosapentanoic acid (EPA 20:5 n-3), and significantly higher levels of ALA, compared to age- and sex-matched non-smokers, consistent with previous studies (Block et al., 2008;). It has been shown that ALA conversion to longer chain EPA and DHA is

reduced in CS-exposed cells in a dose-dependent manner (Ghezzi et al., 2007; Marangoni et al., 2004). Furthermore, studies in human monocytic cells (THP-1) and human mammary gland cells (MCR-10A) exposed to CS-extract show that CS decreases the activity of the Δ5- and Δ6-desaturase enzymes that are necessary to convert ALA to EPA and DHA. Thus, these studies suggest that the decreases in EPA and DHA in smokers may be due to the inhibition of enzymes responsible for the conversion of ALA to EPA and DHA. In addition, the elongase and desaturase genes needed for conversion of ALA to EPA and DHA are highly polymorphic (Koletzko et al., 2008; Konkel and Schunck, 2011) and influenced by sex hormones, accounting for large individual differences in conversion efficiencies (Konkel and Schunck, 2011). Taken together, these studies suggest that n-3 PUFA metabolism may be altered by both CS exposure and genetic factors.

n-3 PUFAs, especially EPA, DHA and their cytochrome P450-derived epoxide metabolites, epoxyeicosatetraenoic acid (EEQ) and epoxydocosapentaenoic acid (EDP), have been shown to be cardioprotective (Lauterbach et al., 2002). EEQs and EDPs cause vasodilation through opening of large conductance, calcium-dependent potassium channels (BK channels) and ATP-sensitive potassium channels (Agbor et al., 2012; Hoshi et al., 2013). EEQs and EDPs can further be metabolized into inactive diol compounds by soluble epoxide hydrolase (sEH).

The goal of this study was to determine if PUFAs and PUFA metabolites could function as biomarkers of atherosclerotic CVD (ASCVD) risk in smokers with pre-existing hypertension. It is possible that the biomarkers of vascular dysfunction identified in smokers without CVD risk factors may also serve as biomarkers for future CVD risk in

smokers with early onset CVD. We therefore hypothesized that EPA, DHA and their epoxide metabolites would be negatively associated with CVD risk as predicted by the new atherosclerotic CVD (ASCVD) risk estimator, while ALA and the diol metabolites of EPA and DHA would be positively associated with ASCVD risk.

METHODS

Study subjects

We recruited never (n=22) and current (n=25) smokers with physician-diagnosed hypertension. Age was restricted to between 40 – 70 years old. Subjects excluded from the study included those who were pregnant, had a history of heart failure, or were diagnosed with cancer, renal failure requiring dialysis, or pulmonary hypertension. This study was approved by the University of New Mexico Institutional Review Board (HRRC: 14-220) and written informed consent was obtained from all participants.

Study design

Subjects were asked to fast and refrain from smoking for 10 h prior to their appointment. Blood pressure was measured following American Heart Association (AHA) guidelines and the same blood pressure device was used for all study precipitants. Blood pressure was measured in both arms and repeated in the arm with the highest systolic blood pressure. Height, weight, and heart rate (HR) were also recorded. Blood samples were collected to measure serum cotinine, HbA1c, lipids, high sensitivity C-reactive protein (hs-CRP), basic metabolic panel, RBC n-3 and n-6 PUFAs (OmegaQuant, Souix Falls, SD), and n-3 and n-6 PUFA metabolites (Arnold et al 2010).

ASCVD risk calculation

The ASCVD risk is an estimate of the 10-year risk of atherosclerotic cardiovascular disease, defined as coronary death or nonfatal myocardial infarction, or fatal or nonfatal stroke (Stone et al., 2013). The information needed to calculate ASCVD risk includes systolic BP, HDL cholesterol, total cholesterol, age, race, sex, diabetes, hypertension treatment, and smoking status. An ASCVD risk of $\geq 7.5\%$ is considered high risk. We classified diabetics as subjects with HbA1c $\geq 6.5\%$ and fasting glucose $\geq$ 120 mg/dL. The ASCVD estimator used can be found at http://tools.acc.org/ASCVD-Risk-Estimator/.

HPLC detection of 2-naphthol

An Agilent 1200 series HPLC system, consisting of a quaternary pump with degasser, autosampler with infinity thermostat, thermost column compartment, diode array detector (UV), and fluorescence detector, was used. The column was 250 mm in length and had an inner diameter of 4.6 mm (J'sphere ODS-H80 reverse Phase, YMC, Wilmington, NC). Urinary 2-naphthol, a validated index of polycyclic aromatic hydrocarbon (PAH) metabolism, was measured as described previously (Kim et al., 1999). Briefly, a 3 mL aliquot of urine was aliquoted and buffered with sodium acetate followed by overnight incubation in a shaking water bath at 37^{0}C with β-glucuronidase (3,261 units) and aryl sulfatase (278 units) in order to remove phase II conjugates. Following hydrolysis, 5 mL acetonitrile was added to each sample followed by centrifugation. We fortified blank urine samples (used from the same batch of non-smoker urine) with 0.5-40 mg/L 2-naphthol to generate a standard curve. Urinary

creatinine was measured with a creatinine colorimetric assay kit (Cayman Chemical). Results were expressed as µM 1-2-naphthol/mol creatinine.

Serum cotinine

Serum cotinine was measured using an ELISA kit (Abnova, Taipei City, Taiwan) and quantified by using a cotinine standard curve. Pooled serum from never smokers collected previously served as a negative control and pooled serum from known current smokers served as positive control.

n-3 and n-6 PUFAs and their CYP450-derived epoxide metabolites, sEH-derived diol metabolites, lipoxygenase-derived metabolites and auto-oxidation products.

n-3 and n-6 PUFA metabolites and auto-oxidation products in plasma were measured 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 chemicals) in methanol. Each reaction underwent alkaline hydrolysis followed by neutralization to pH 6.0 with acetic acid. Solid-phase extractions were performed using Varian Bond Elut Certify II columns. HPLC measurements were performed using an Agilent 1200 HPLC system equipped with a Phenomenex Kinetex-C18 column. The HPLC was coupled with an Agilent 6460 triplequad mass spectrometer with an electrospray ionization source.

n-3 and n-6 PUFAs

Packed RBCs obtained from EDTA-treated whole blood were analyzed for PUFA content by OmegaQuant as described previously (Harris et al., 2013). Briefly, samples were heated with methanol and boron trifluoride to generate fatty acid methyl esters. These methyl esters were extracted with hexane and water and analyzed using a GC2010 (Shimadzu Corporation, Columbia, MD) gas chromatographer equipped with a 30 mm capillary column (Omegawax 250, Supelco, Bellefonte, PA). PUFAs were identified by comparison to standard PUFA methyl esters.

Statistical analysis

Normally distributed continuous variables were described using mean and standard deviation (SD). Categorical variables were described using frequency and percentages. Two sample t-test was used to compare normally distributed continuous variables between smokers and non-smokers, while Wilcoxon Mann-Whitney test was used to compare non-normally distributed continuous variables between smokers and non-smokers. Bonfferoni correction using $p < 0.01$ was applied when comparing the fatty acid metabolites between smokers and non-smokers since more than 100 comparisons were made. Chi-square test was used to compare categorical variables between smokers and non-smokers. Pearson or Spearman correlation was used to determine the association between 10-year ASCVD risk and individual continuous variables. Multivariable regression models were developed to identify the factors predictive of 10-year ASCVD risk. Variables that were significantly correlated at $p < 0.2$ were added to the multiple regression model. A simplified multiple regression model was then generated using stepwise variable selection requiring $p < 0.2$ to stay in the model. Analyses were

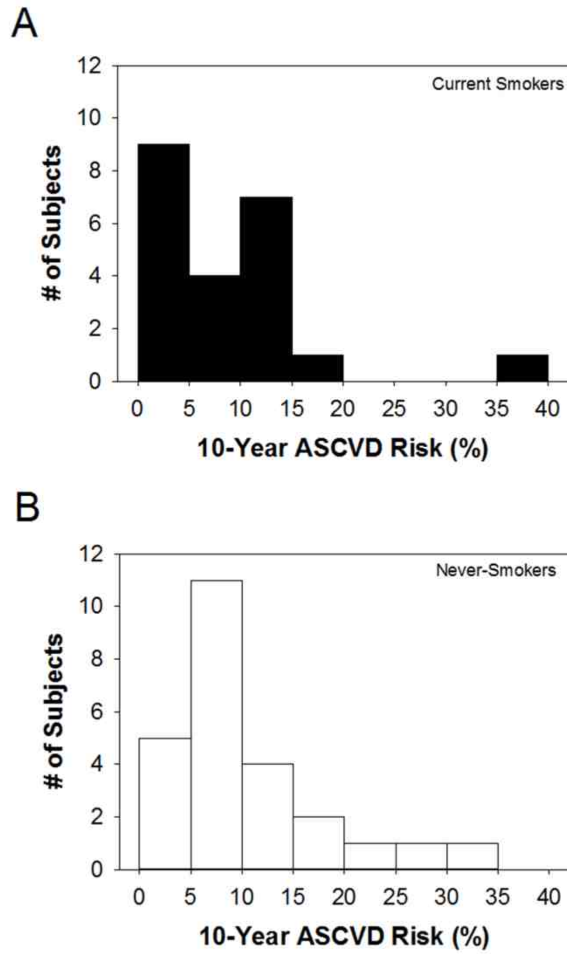

Figure 3.1. Range of 10-year ASCVD risk in (A) current smokers (n=25; median: 8.8 95% CI [5.44,12.16]) and (B) never-smokers (n=22; median: 7.4 95% CI [4.3,10.4])

Table 3.1. Characteristics of current and never-smokers

Characteristic	Never Smoker	Current Smoker	p-value
ASCVD risk (%)[b]	9.3 ± 7.9[a]	10.9 ± 7.6	0.472
ASCVD risk calculators			
Male (%)	63.8	64	0.471
Age (yr)	53.6 ± 8.6	53.2 ± 7.3	0.863
Race (% African American)	18	24	0.541
Systolic BP (mmHg)	136 ± 18	128 ± 22	0.179
HbA1c (%)	5.7 ± 0.4	5.8 ± 1.6	0.075
Fasting glucose (mg/dL)	106.0 ± 13.8	110.6 ± 43.6	0.331
Total cholesterol (mg/dL)	191 ± 33	188 ± 48	0.793
HDL cholesterol (mg/dL)	50 ± 17	54 ± 15	0.420
Other CVD risk factors			
Diastolic BP (mmHg)	88 ± 10	81 ± 13	0.055
Hs-CRP (mg/L)	4.6 ± 7.8	4.5 ± 6.1	0.890
LDL cholesterol (mg/dL)	112 ± 32	107 ± 34	0.616
Triglycerides (mg/dL)	140 ± 70	133 ± 75	0.751
BMI (kg/m²)	32 ± 4	29± 7	0.065
Indices of CS-exposure			
Years smoked	27.1 ± 13.4	NA	NA
Cotinine (ng/mL)	0.1 ± 0.6	32.9 ± 18.2	<0.0001*
Urinary 2-naphthol (ng/mL)	2.3 ± 2.7	2.8 ± 1.4	0.020*

[a] Data expressed as mean ± SD, n = 22-25. Two sample t-test was used to compare continuous variables between smokers and non-smokers, and chi-square was used to compare categorical data between smokers and non-smokers. *$p < 0.05$
[b] Estimate of the 10 – year atherosclerotic cardiovascular disease risk (ASCVD) taking into account age, sex, total cholesterol, HDL cholesterol, systolic blood pressure, treatment for hypertension, smoking, race and diabetes. A value ≤ 7.5 % is considered low risk.

conducted using SigmaPlot 13 and SAS 9.4 software.

RESULTS

Study demographics

The distribution of the ASCVD risk was skewed in both smokers and non-smokers (Fig. 3.1A and B), but there was no difference in ASCVD risk between these two groups (Table 3.1). Further, there were no differences in the CVD risk factors used to calculate ASCVD risk between smokers and non-smokers, including systolic BP, HbA1c, total cholesterol, HDL cholesterol, age or sex, and there were no differences in other CVD risk factors, including hs-CRP, BMI, LDL cholesterol or TG. As expected, smokers had significantly higher levels of markers of CS-exposure as measured by urinary 2-naphthol and serum cotinine, reflecting an average of 27.1 ± 13.4 years of smoking. Lastly, there were no differences in variables measured as part of the basic metabolic panel (Table 3.2).

n-3 and n-6 PUFAs and their CYP450-derived epoxide metabolites, sEH-derived diol metabolites, lipoxygenase-derived metabolites and auto-oxidation products.

There were no differences in individual n-3 and n-6 PUFAs between smokers and non-smokers, including ALA, EPA, DHA, LA, and AA, nor in the n-3 or n-6 PUFA indices, representing the sum of ALA, EPA and DHA; and LA and AA, respectively (Table 3.3). Furthermore, there were also no differences in levels of the individual n-3 and n-6 PUFA P450-derived epoxide metabolites, including all the regioisomers of the EETs, EEQs, and EDPs.

Table 3.2. Metabolic panel of current and never-smokers

Variable	Never smoker[a]	Current smoker	P-value
Years smoked	NA	27.1 ± 13.4	NA
Blood urea nitrogen (mg/dL)	14.5 ± 4.3	13.7 ± 3.1	0.362
Calcium (mg/dL)	9.6 ± 0.4	9.7 ± 0.5	0.740
Carbon dioxide (mmol/L)	23.7 ± 2.6	24.5 ± 2.6	0.269
Chloride (mmol/L)	106.9 ± 3.3	105.4 ± 3.2	0.114
Creatinine (mg/dL)	0.9 ± 0.2	0.9 ± 0.2	0.374
Potassium (mmol/L)	4.2 ± 0.3	4.2 ± 0.4	0.785
Sodium (mmol/L)	141.1 ± 2.9	139.8 ± 2.8	0.134

[a] Data expressed as mean ± SD, n = 22-25. Two sample *t*-test was used to compare variables between smokers and non-smokers.

Table 3.3 Fatty acid profile of current and never-smokers

Variable[a,b]	Never smoker	Current smoker	P-value
n-6 PUFAs			
LA[a]	12.62 ± 1.95	12.29 ± 1.47	0.767
AA	16.50 ± 1.91	16.64 ± 1.31	0.956
n-6 Index	29.12 ± 1.66	28.93 ± 1.65	0.696
AA Products			
Individual EETs			
5,6-EET	0.85 ± 1.14	1.11 ± 1.20	0.487
8,9-EET	2.62 ± 1.18	3.01 ± 1.13	0.255
11,12-EET	1.97 ± 0.75	1.97 ± 0.71	0.973
14,15-EET	3.29 ± 1.35	3.74 ± 1.44	0.283
Individual DHET			
5,6-DHET	2.27 ± 0.76	2.62 ± 0.90	0.167
8,9-DHET	1.25 ± 0.41	1.29 ± 0.51	0.799
11,12-DHET	0.72 ± 0.64	0.53 ± 0.16	0.328
14,15-DHET	0.28 ± 0.13	0.30 ± 0.14	0.545
P450 epoxygenase activity index			
5,6-EET+5,6-DHET	3.12 ± 1.64	3.73 ± 1.28	0.166
8,9-EET+8,9-DHET	3.87 ± 1.23	4.3 ± 1.45	0.291
11,12-EET+11,12-DHET	2.68 ± 0.95	2.50 ± 0.77	0.480
14,15-EET+14,15-DHET	3.58 ± 1.32	4.04 ± 1.46	0.265
sEH activity index			
5,6-EET/5,6-DHET	0.32 ± 0.42	0.50 ± 0.52	0.198
8,9-EET/8,9-DHET	0.70 ± 0.98	0.46 ± 0.18	0.517
11,12-EET/11,12-DHET	3.62 ± 1.95	3.99 ± 1.84	0.684
14,15-EET/14,15-DHET	13.81 ± 6.89	14.23 ± 6.81	0.836
Total sEH activity	18.46 ± 7.64	18.41 ± 9.05	0.986
n-3 PUFAs			
ALA	0.22 ± 0.05	0.20 ± 0.04	0.180
EPA	0.48 ± 0.20	0.49 ± 0.23	0.834
DHA	3.24 ± 0.89	3.19 ± 0.98	0.860
n-3 Index	3.72 ± 1.02	3.69 ± 1.12	0.922
(EPA+DHA)/ALA	18.32 ± 8.25	19.82 ± 8.44	0.489
EPA Products			
Individual EEQs			
5,6-EEQ	1.13 ± 1.70	0.89 ± 1.29	1.000
8,9-EEQ	0.20 ± 0.14	0.19 ± 0.16	0.846
11,12-EEQ	0.20 ± 0.13	0.15 ± 0.13	0.088
14,15-EEQ	0.14 ± 0.11	0.10 ± 0.08	0.261
17,18-EEQ	0.34 ± 0.38	0.34 ± 0.24	0.528

Variable[a,b]	Never smoker	Current smoker	P-value
Individual DiHETEs			
5,6-DiHETE	0.32 ± 0.28	0.37 ± 0.20	0.198
8,9-DiHETE	0.48 ± 0.47	0.46 ± 0.32	0.835
11,12-DiHETE	0.45 ± 0.38	0.39 ± 0.25	0.506
14,15-DiHETE	0.31 ± 0.24	0.27 ± 0.18	0.559
17,18-DiHETE	4.14 ± 3.42	3.60 ± 2.34	0.537
P450 epoxygenase activity index			
5,6-EEQ+5,6-DiHETE	1.45 ± 1.90	1.26 ± 1.36	0.684
8,9-EEQ+8,9-DiHETE	0.68 ± 0.53	0.64 ± 0.41	0.956
11,12-EEQ+11,12-DiHETE	0.66 ± 0.40	0.54 ± 0.34	0.283
14,15-EEQ+14,15-DiHETE	0.45 ± 0.31	0.37 ± 0.21	0.361
17,18-EEQ+17,18-DiHETE	4.48 ± 3.47	3.94 ± 2.39	0.540
sEH activity index			
5,6-EEQ/5,6-DiHETE	3.73 ± 5.13	2.43 ± 3.00	0.306
8,9-EEQ/8,9-DiHETE	0.48 ± 0.51	0.53 ± 0.49	0.710
11,12-EEQ/11,12-DiHETE	0.72 ± 0.8	0.34 ± 0.26	0.047
14,15-EEQ/14,15-DiHETE	0.47 ± 0.58	0.39 ± 0.57	0.361
17,18-EEQ/17,18-DiHETE	0.13 ± 0.19	0.12 ± 0.09	0.273
Total sEH activity	5.53 ± 5.14	3.66 ± 3.40	0.224
DHA Products			
Individual EDPs			
7,8-EDP	0.90 ± 0.57	0.92 ± 0.58	0.888
10,11-EDP	0.61 ± 0.43	0.63 ± 0.37	0.621
13,14-EDP	0.17 ± 0.16	0.15 ± 0.10	0.866
16,17-EDP	0.33 ± 0.32	0.35 ± 0.31	0.791
19,20-EDP	0.42 ± 0.30	0.38 ± 0.24	0.696
Individual DiHDPAs			
7,8-DiHDPA	0.10 ± 0.07	0.07 ± 0.05	0.063
10,11-DiHDPA	0.10 ± 0.18	0.04 ± 0.02	0.138
13,14-DiHDPA	0.01 ± 0.01	0.01 ± 0.01	0.165
16,17-DiHDPA	0.04 ± 0.03	0.04 ± 0.02	0.830
19,20-DiHDPA	0.25 ± 0.20	0.18 ± 0.11	0.385
P450 epoxygenase activity index			
7,8-EDP+7,8-DiHDPA	1.00 ± 0.57	0.99 ± 0.60	0.962
10,11-EDP+10,11-DiHDPA	0.71 ± 0.50	0.67 ± 0.38	0.785
13,14-EDP+13,14-DiHDPA	0.19 ± 0.16	0.15 ± 0.11	0.878
16,17-EDP+16,17-DiHDPA	0.37 ± 0.32	0.39 ± 0.31	0.807
19,20-EDP+19,20-DiHDPA	0.66 ± 0.41	0.57 ± 0.24	0.356

Variable[a,b]	Never smoker	Current smoker	P-value
sEH activity index			
10,11-EDP/10,11-DiHDPA	12.93 ± 10.28	17.38 ± 9.50	0.134
13,14-EDP/13,14-DiHDPA	10.69 ± 21.86	9.12 ± 14.35	0.860
16,17-EDP/16,17-DiHDPA	11.32 ± 11.79	9.71 ± 14.79	0.463
19,20-EDP/19,20-DiHDPA	2.30 ± 2.20	3.47 ± 4.31	0.582
Total sEH activity	54.35 ± 35.68	59.80 ± 38.01	0.873
Autoxidation products			
8-HETE	9.05 ± 2.94	10.32 ± 2.98	0.155
9-HETE	15.93 ± 5.33	17.19 ± 4.69	0.400
11-HETE	15.10 ± 5.38	16.00 ± 4.08	0.527
8-HEPE	1.32 ± 0.77	1.37 ± 0.81	0.803
9-HEPE	7.93 ± 5.25	7.93 ± 4.45	1.000
18-HEPE	13.04 ± 8.16	12.46 ± 7.06	0.800
8-HDHA	8.51 ± 3.67	8.76 ± 3.53	0.819
10-HDHA	3.18 ± 1.29	3.00 ± 1.24	0.835
11-HDHA	4.34 ± 1.57	4.22 ± 1.69	0.886
13-HDHA	4.03 ± 1.4	4.03 ± 1.69	0.999
16-HDHA	4.06 ± 1.34	3.90 ± 1.82	0.734
20-HDHA	31.44 ± 12.54	31.42 ± 12.93	0.974
Total auto oxidation products	100.05 ± 34.45	101.68 ± 31.22	0.867
5-LOX derived metabolites			
5-HEPE	4.31 ± 2.69	3.65 ± 2.00	0.346
5-HETE	18.79 ± 6.00	20.16 ± 5.88	0.436
4-HDHA	5.29 ± 2.40	5.32 ± 2.67	0.956
7-HDHA	2.97 ± 1.25	2.89 ± 1.19	0.824
Total 5-LOX products	31.36 ± 9.13	32.03 ± 9.74	0.811
12-LOX derived metabolites			
12-HEPE	1.72 ± 1.03	1.71 ± 1.00	0.992
12-HETE	10.91 ± 3.24	12.74 ± 3.25	0.062
14-HDHA	5.95 ± 2.36	5.94 ± 2.56	0.994
12-LOX products	22.91 ± 6.00	24.62 ± 6.77	0.371
15-LOX derived metabolites			
15-HEPE	5.37 ± 3.50	5.57 ± 3.20	0.800
15-HETE	81.73 ± 27.88	90.47 ± 26.30	0.280
10-HDHA	3.18 ± 1.29	3.00 ± 1.24	0.835
17-HDHA	30.51 ± 12.63	30.99 ± 11.22	0.893
15-LOX products	125.78 ± 38.09	136.14 ± 38.17	0.362
CYP4A12 products	1.38 ± 0.69	1.13 ± 0.62	0.214
CYP1A1 AA products	3.29 ± 1.35	3.74 ± 1.44	0.283
CYP1A1 EPA products	0.34 ± 0.38	0.34 ± 0.24	0.528
CYP1A1 EDP products	31.68 ± 12.53	31.60 ± 12.98	1.000

Variable[a,b]	Never smoker	Current smoker	P-value
19-HEPE	ND	ND	NA
20-HEPE	0.23 ± 0.18	0.19 ± 0.21	0.150
19-HETE	ND	ND	NA
20-HETE	1.15 ± 0.65	0.94 ± 0.60	0.275

Abbreviations: α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), linoleic acid (LA), arachidonic acid (AA), epoxyeicosatrienoic acid (EET), dihydroxyeicosatrienoic acid (DHET), epoxyeicosatetraenoic acid (EEQ), dihydroxyeicosatetraenoic acid (DiHETE), epoxyeicosatetraenoic acid (EDP), dihydroxydocosapentaenoic acid (DiHDPA), hydroxyeicosatetraenoic acid (HETE), hydroxyeicosapentaenoic acid (HEPE), hydroxydocosahexaenoic acid (HDHA).

[a] Values are expressed as % of total fatty acids.

[b] Data expressed as mean ± SD, n = 22-25. Two sample t-test was used to compare variables between smokers and non-smokers. $*p < 0.01$ was considered significant after applying Bonferroni correction

To assess P450 epoxygenase activity for AA, EPA, and DHA as substrates, the concentration of individual regioisomer epoxides was added to the concentration of their corresponding diols (e.g. 5,6-EET + 5,6-DHET). To assess sEH activity for epoxides as substrates, the concentration of individual regioisomer epoxides was divided by the concentration of their corresponding diols (e.g. 5,6-EET/5,6-DHET). There were no differences observed in P450 epoxygenase activity for AA, EPA, or DHA and there were no differences observed for sEH activity for epoxide substrates (Table 3.3).

Correlation of fatty acids with ASCVD risk

Correlation analysis was conducted between individual PUFAs, PUFA metabolites, or auto-oxidation products and ASCVD risk (Tables 3.4 and 3.5). In non-smokers, four PUFA metabolites correlated with ASCVD risk at $p < 0.05$, while an additional 11 correlated at $p < 0.2$. The four that correlated at $p < 0.05$ included an index of sEH activity, 14,15-EET/14,15-DHET, and three sEH-derived diol metabolites (Fig. 3.2A-D). For smokers, only one PUFA metabolite correlated at $p < 0.05$, an index of sEH activity 11,12-EET/11,12-DHET (Fig. 3.3), and an additional seven correlated at $p < 0.2$, including four sEH-derived diol metabolites.

To develop a model to predict ASCVD risk in smokers and non-smokers, multiple regression analysis was conducted using those PUFAs and PUFA metabolites that were individually correlated with ASCVD risk at $p < 0.2$. Then, a simplified model was generated retaining only those PUFAs and PUFA metabolites that exhibited a $p < 0.2$ in the full model. In non-smokers, the simplified model retained eight of the original 15 variables. Of these, the EH-derived diol metabolite of DHA, 16,17-

Table 3.4. Multiple regression models in never-smokers

Variable	Correlation[a]	Multiple Regression Model[b]		Simplified Model[c]	
	P-value	B coefficient	P-Value	B coefficient	P-Value
14,15-EET/ 14,15-DHET	0.033	-0.577	0.120	-0.294	0.175
17,18-EEQ/ 17,18-DiHETE	0.060	-11.201	0.283		
16,17-EDP / 16,17-DiHDPA	0.133	0.218	0.174	0.150	0.338
ALA	0.136	16.310	0.557		
14,15-EEQ	0.178	58.667	0.018	33.059	0.027
17,18-EEQ	0.155	-3.083	0.612		
13,14-EDP	0.197	-17.811	0.042	-19.830	0.032
16,17-EDP	0.060	-19.998	0.034	-14.943	0.009
5,6-DHET	0.121	3.161	0.101	1.528	0.394
14,15-DHET	0.002	-24.740	0.351		
11,12-DiHETE	0.016	6.377	0.228		
17,18-DiHETE	0.023	-0.880	0.117	-0.265	0.606
16,17-DiHDPA	0.008	166.917	0.109	151.967	0.020
15-HETE	0.138	0.191	0.327		
15-LOX products	0.126	-0.136	0.296		

[a]Pearson correlation (for normally distributed data) or spearman correlation (for non-normally distributed data) were used to determine the association between 10-year ASCVD risk and individual continuous variables.
[b]Multivariable regression models were developed to identify the factors predictive of 10-year ASCVD risk. Variables that were significantly correlated at $p < 0.2$ were used in the multiple regression model.
[c]A simplified multiple regression model was then generated using stepwise variable selection requiring $p < 0.2$ to stay in the model. Analyses were conducted using SigmaPlot 13 and all the analysis was done using SAS 9.4 statistical software. Multiple regression model: R=0.971; adjusted R^2=0.802, simplified model: R=0.876; adjusted R^2=0.625. $^*p<0.05$

Table 3.5. Multiple regression models in current smokers

Variable	Correlation[a]	Multiple Regression Model[b]		Simplified Model[c]	
	P-value	B coefficient	P-Value	B coefficient	P-Value
11,12-EET/ 11,12-DHET	0.031	1.475	0.166	1.496	0.070
14,15-DHET	0.190	11.945	0.429		
5,6-DiHETE	0.177	-5.255	0.755		
8,9-DiHETE	0.133	0.868	0.934		
13,14-DiHDPA	0.061	-245.576	0.084	-204.092	0.087
11-HETE	0.181	-1.095	0.039	-1.037	0.020
20-HETE	0.103	0.311	0.045	0.323	0.022
CYP450 4A12 Activity	0.087	1.690	0.549		

[a]Pearson correlation (for normally distributed data) or spearman correlation (for non-normally distributed data) were used to determine the association between 10-year ASCVD risk and individual continuous variables.
[b]Multivariable regression models were developed to identify the factors predictive of 10-year ASCVD risk. Variables that were significantly correlated at $p < 0.2$ were used in the multiple regression model.
[c]A simplified multiple regression model was then generated using stepwise variable selection requiring $p < 0.2$ to stay in the model. Analyses were conducted using SigmaPlot 13 and all the analysis was done using SAS 9.4 statistical software. Multiple regression model: R=0.709; adjusted R^2=0.237, simplified model: R=0.636; adjusted R^2=0.279. *p<0.05

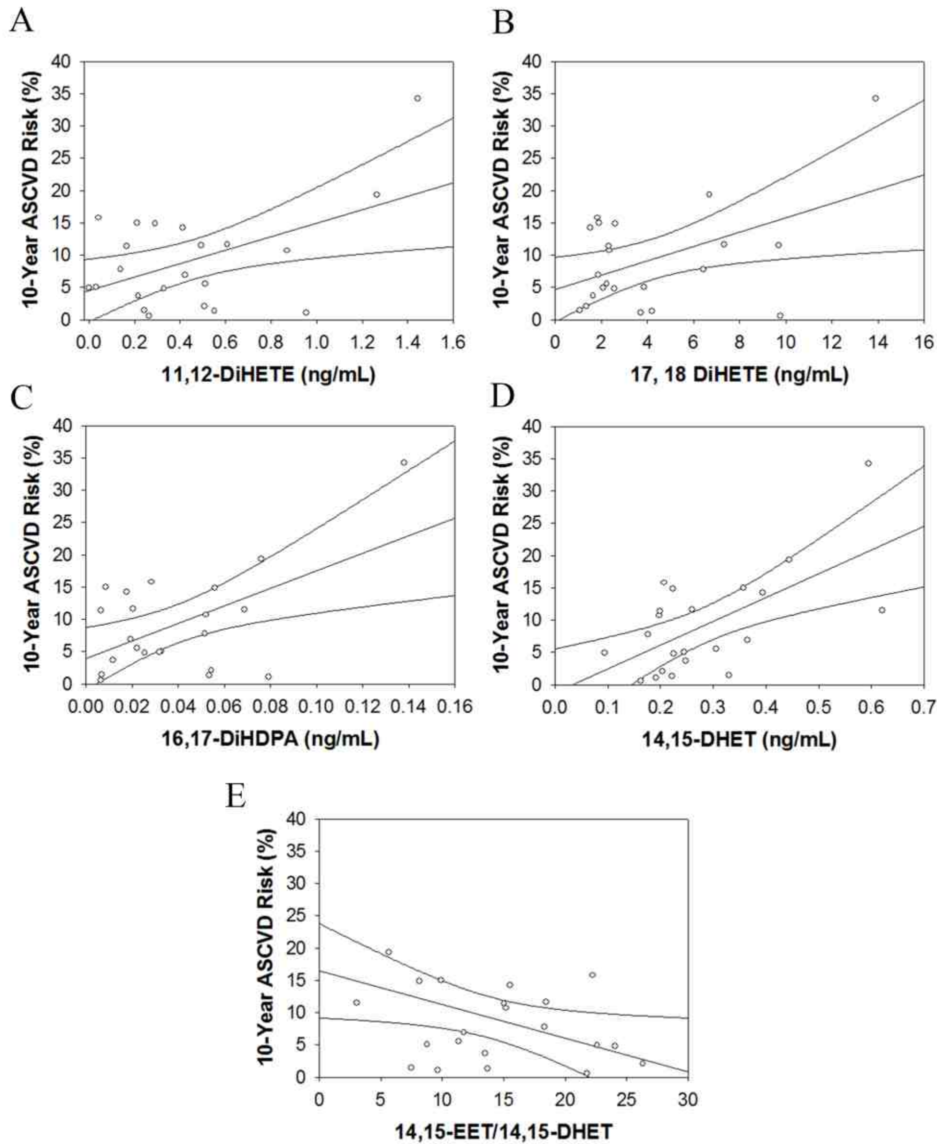

Figure 3.2. Relationship between 14,15-EET/14,15-DHET, 17,18-DiHETE, 14,15-DHET and 16,17-DiHDPA and 10-year ASCVD risk in never smokers. There were significant positive correlations between n-3 PUFA diol-metabolites: (A) 11,12-DiHETE, (B) 17,18-DiHETE, (C) 16,17-DiHDPA and n-6 PUFA diol-metabolite, (D) 14,15-DHET and 10-year ASCVD risk. In addition, there was a significant negative correlation between 14,15-EET/14,15-DHET and 10-year ASCVD risk. Pearson correlations with 95% confidence intervals around regression line.

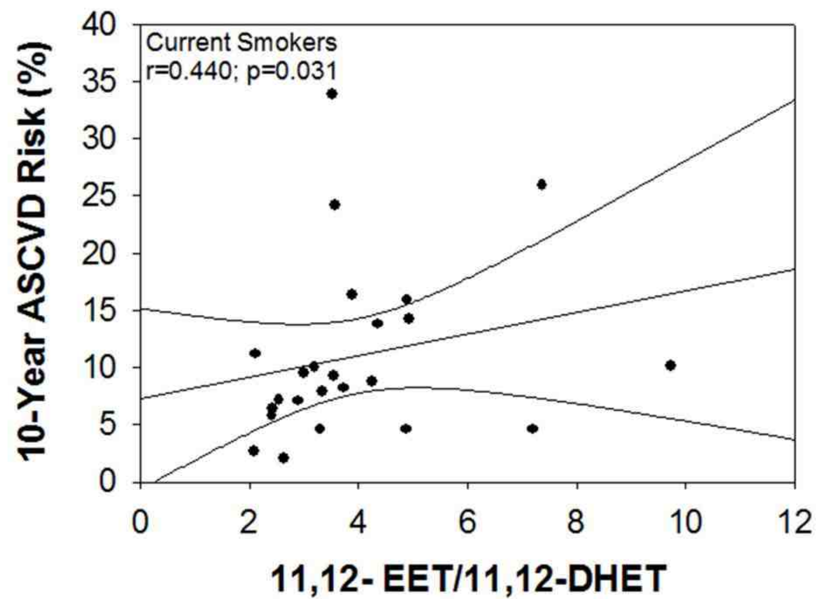

Figure 3.3. Relationship between 11,12-EET/11,12-DHET and 10-year ASCVD risk in current smokers. There was a significant positive correlation between 11,12-EET/11,12-DHET and 10-year ASCVD risk. Spearman correlation with 95% confidence intervals around regression line.

dihydroxydocosapentaenoic acid (DiHDPA) correlated positively with ASCVD risk with the largest effect size (beta coefficient = 151.967), while the P450-derived epoxide metabolite of EPA, 14,15-EEQ, also correlated positively (beta coefficient = 33.059). Two other P450-derived epoxides of DHA were negatively correlated with ASVD risk, 13,14- and 16,17-EDP. The remaining four variables retained in the simplified model all exhibited small effect sizes (beta-coefficients < 2) and $p < 0.175$; however, their removal from the model resulted in loss of overall significance.

In smokers, the simplified model retained four of the initial eight variables. Of these, the sEH-derived diol metabolite of DHA, 13,14-DiHDPA, correlated negatively with ASCVD risk with the largest effect size (beta coefficient = -204.092). The remaining three variables exhibited much smaller effect sizes (beta coefficients < 2), but all showed $p < 0.07$. Notably, the P450-derived hydroxylase metabolite of AA and pro-hypertensive product, 20-HETE, significantly correlated with ASCVD risk at $p < 0.02$.

DISCUSSION AND CONCLUSIONS

Discussion

This study is the first conducted to assess the associations between n-3 and n-6 PUFAs and their metabolites and ASCVD risk in smokers and non-smokers. Surprisingly, there were no significant differences in individual concentrations of PUFAs or their metabolites between smokers and non-smokers. Despite the lack of differences, the correlative association between fatty acid metabolites and ASCVD risk was considerably different between smokers and non-smokers. In non-smokers, three n-3 PUFA epoxide metabolites and one diol metabolite were most predictive of ASCVD risk.

In contrast, in smokers one sEH-derived diol, one P450-derived hydroxylase metabolite, and one auto-oxidation product were most predictive. Taken together, these data suggest that smoking may contribute to ASCVD risk by shifting the overall pattern of n-3 and n-6 PUFA metabolism.

Previously, we have shown that levels of n-3 PUFAs, EPA and DHA, are significantly lower in smokers and that ALA positively correlates with vascular dysfunction (Wiest et al. 2015). Thus, we hypothesized that EPA, DHA and their vasoprotective epoxide metabolites would be negatively associated with ASCVD risk in smokers with pre-existing hypertension. Surprisingly, there are no correlations between the n-3 PUFAs, ALA, EPA, or DHA and ASCVD risk in smokers. It is possible that increases in oxidative stress and/or inflammation associated with hypertension and smoking in this cohort may mask the potential predictive association between n-3 PUFAs and ASCVD risk.

Despite the lack of association between n-3 PUFA parent fatty acids and ASCVD risk, we did find significant but unique associations between PUFA metabolites and ASCVD risk in smokers and non-smokers. In non-smokers, the n-3 PUFA epoxides 14,15-EEQ, 13,14-EDP and 16,17-EDP correlate with ASCVD risk. The DHA-derived P450 epoxides, EDPs, which have been shown to be vasodilatory compounds (Ye et al., 2002), correlate negatively with ASCVD risk, indicating that increased levels of 13,14- and 16,17-EDPs predict a lower ASCVD risk. Consistent with this observation, 16,17-DiHDPA, generated by sEH-dependent metabolism of 16,17-EDP to the inactive diol, correlates positively with ASCVD risk, indicating that increased levels of 16,17-DiHDPA, predicts a higher ASCVD risk. Furthermore, the concentration of 16,17-DiHDPA is the

most predictive of ASCVD risk in multiple regression model with beta coefficient > 150. These data suggest that the metabolism of vasodilatory 16,17-EDP into its inactive diol 16,17-DiHDPA is associated with a higher ASCVD risk. Surprisingly, the EPA-derived P450 epoxide, 14,15-EEQ is positively associated with ASCVD risk, indicating that increased levels of 14,15-EEQ predicts a higher risk. This is unexpected given that both EEQs and EDPs are considered to be vasoprotective, although acting through slightly different mechanisms to cause vasodilation. While both EEQs and EDPs cause vasodilation through opening BK-channels, the mechanism of EEQ-mediated dilation is not calcium dependent, whereas EDP-mediated dilation is dependent on increases in intracellular calcium (Hercule et al., 2007; Wang et al., 2011).

Similar to our regression model in non-smokers, in smokers we found that an sEH-derived diol metabolite of DHA, 13,14-DiHDPA, is the single most predictive variable of ASCVD risk with a large effect size, beta coefficient > -200. In contrast to a positive association in non-smokers, however, 13,14-DiHDPA correlates negatively with ASCVD risk in smokers, indicating that increased levels of 13,14-DiHDPA predict a lower ASCVD risk in smokers. CS extract induces sEH gene expression in endothelial cells (Maresh et al. 2005) and thus CS exposure would be expected to increase PUFA diol metabolites. However, the association of a diol metabolite with lower ASCVD risk is counterintuitive to the published studies that reducing diol metabolites, using sEH inhibitors, results in anti-hypertensive and anti-inflammatory responses (Davis et al. 2011; Imig et al. 2002). Thus, the explanation for this strong negative association remains to be elucidated.

In smokers we also found that P450-derived hydroxylase metabolite of AA, 20-HETE, and the auto-oxidation product of AA, 11-HETE correlate with ASCVD risk. Smokers with the highest levels of 20-HETE predict a higher ASCVD risk. This is consistent with literature showing that 20-HETE is associated with vasoconstriction and increased risk of myocardial infarction (Han et al., 2013; Toth et al., 2013). 20-HETE levels were not different between smokers and non-smokers, suggesting that smokers may be more responsive to 20-HETE as a result of increased CS-induced oxidative stress and reduced NO. Surprisingly, smokers with the lowest levels of 11-HETE exhibit the highest degree of ASCVD risk. This contradicts other studies that demonstrate human subjects (>60 years) with higher levels of 11-HETE exhibit more severe CVD outcomes, including more severe atherosclerotic lesions and increased incidence of major adverse cardiovascular events (Waddington et al., 2003; Zu et al., 2016). One possible reason for this observation may be that the subjects in our study are at an early stage of CVD and have not yet developed advanced CVD.

Limitations

We are continuing to expand our statistical analyses of these data using more sophisticated approaches, including analysis of co-variance and principal component analysis. While we have successfully identified novel fatty acid metabolites that are strongly predictive of ASCVD risk in smokers and non-smokers, the strength of our results could be improved by a larger sample size Additionally, although all subjects were prescribed medication for hypertension, their self-reported compliance varied, which could influence their actual ASCVD risk. Contra-indications, including use of non-steroidal anti-inflammatory drugs (NSAIDs) and aspirin could also affect results. Lastly,

all subjects had a very low omega-3 index (< 4%) which has been associated with an increased risk of myocardial infarction and sudden cardiac death (Harris and von Schacky 2004). Thus, it would be beneficial to conduct a follow-up intervention study where subjects received varying dosages of n-3 PUFA supplements to determine if specific PUFA metabolites remained predictive of ASCVD risk.

Conclusions

We found that ALA, which we previously demonstrated to predict FMD in young, healthy smokers (Wiest et al. 2015), is not associated with ASCVD risk in smokers with preexisting hypertension. Nonetheless, we did identify novel predictors of ASCVD risk in smokers and non-smokers, including 13,14-DiHDPAand 11- and 20-HETE in smokers, and 14,15-EEQ, 13,14- and 16,17-EDPs, and 16,17-DiHDPA in non-smokers. Future studies with a larger cohort, a broader range of ASCVD risk, and broader distribution of omega-3 index would help to confirm that these n-3 and n-6 PUFA metabolites represent potential biomarkers of ASCVD risk in smokers and non-smokers.

CHAPTER IV

Omega-3 Polyunsaturated Fatty Acids Protect against Cigarette Smoke-Induced Oxidative Stress and Vascular Dysfunction

Wiest, E.F., Walsh-Wilcox, M.T., and Walker, Mary K. Omega-3 polyunsaturated fatty acids protect against cigarette smoke-induced oxidative stress and vascular dysfunction. Submitted to *Toxicological Sciences*

ABSTRACT

In cigarette smokers endothelial dysfunction, measured by flow-mediated dilation (FMD), precedes cardiovascular disease and can be improved by supplementation with n-3 polyunsaturated fatty acids (PUFAs). We developed a mouse model of cigarette smoke (CS)-induced endothelial dysfunction that resembles impaired FMD observed in human cigarette smokers and investigated the mechanism by which n-3 PUFAs mediate vasoprotection. We hypothesized that loss of nitric oxide (NO)-dependent vasodilation in CS-exposed mice would be prevented by dietary n-3 PUFAs via a decrease in oxidative stress. C57BL/6 mice were fed a chow or n-3 PUFA diet for 8 weeks and then exposed to mainstream CS or filtered air for 5 days, 2 hours/day. Mesenteric arterioles were pre-constricted with U46619 and dilated by stepwise increases in pressure (0-40mmHg), resulting in increases in flow, ± inhibitor of NO production or antioxidant, tempol. Markers of oxidative stress were measured in lung and heart. CS-exposed mice on a chow diet had impaired FMD, resulting from loss of NO-dependent dilation, compared to air exposed mice. Tempol restored FMD by normalizing NO-dependent dilation and increasing NO–independent dilation. CS-exposed mice on the n-3 PUFA diet had normal FMD, resulting from a significant increase in NO-independent dilation, compared to CS-exposed mice on a chow diet. Furthermore, n-3 PUFAs decreased two CS-induced markers of oxidative stress, 8-epiprostaglandin-F2α levels and heme oxygenase-1 mRNA, and significantly attenuated CS-induced cytochrome P4501A1 mRNA expression. These data demonstrate that dietary n-3 PUFAs can protect against CS-induced vascular dysfunction via multiple mechanisms, including increasing NO-independent vasodilation and decreasing oxidative stress.

INTRODUCTION

Cigarette smoke (CS) is a major independent risk factor for development of cardiovascular disease (CVD) (O' Donnell and Elosua, 2008), including atherosclerosis, myocardial infarction, aortic aneurysms and stroke (Kawachi et al., 1997; Naya et al., 2008; Shinton and Beevers, 1989; Wilmink et al., 1999). Endothelial dysfunction, which is the loss of vasodilators within the vasculature, is the earliest pathological event in the development of atherosclerosis. We and others have shown that flow-mediated dilation (FMD), a clinical assessment of endothelial dysfunction, is impaired in smokers (Celermajer et al., 1996, 1993; Ozaki et al., 2010; Taylor et al., 2016; Wiest et al., 2015), that impaired FMD is a predictor of future cardiovascular events (Schecter et al., 2009; Witte et al., 2005), and that smoking cessation can significantly improve impaired FMD (Celermajer et al., 1993; Wang and Widlansky, 2009; Johnson et al., 2010).

Nonetheless, although smoking cessation programs have been established, successful cessation is difficult to achieve. According to the national health interview surveys between 2001 and 2010, nearly 70% of adult smokers in the U.S. showed an interest in cessation, about 50% made an attempt to quit, and yet only 6% successfully stopped smoking for more than 6 months (Centers for Disease Control and Prevention, 2011; Yong et al., 2014). Thus, the cardiovascular benefits derived from successful cessation could take years to achieve, highlighting the need for therapeutic strategies that help to reduce the development and progression of CS-induced cardiovascular disease.

CS is a source of reactive oxygen species (ROS), such as superoxide anion and peroxynitrite. ROS present in CS can increase the levels of lipid peroxidation, as measured by thiobarbituric acid reactive substances (TBARS), (Miller et al., 1997;

Morrow et al., 1995) as well as increases in other biomarkers of oxidative stress, such as 8-epi prostaglandin F2α (8-isoprostane) and 8-hydroxydeoxyguanosine (8-OHdG) (Seet et al., 2011). Additionally, ROS present in CS can cause endothelial dysfunction by decreasing nitric oxide (NO) bioavailability (Barua et al., 2003). Studies have shown that impaired FMD assessed in conduit vessels is, at least in part, due to loss of NO-mediated dilation (Green et al., 2014; Kooijman et al., 2008).

One potential therapy for preventing CS-induced impaired FMD is supplements containing omega-3 polyunsaturated fatty acids (n-3 PUFAs). The n-3 PUFAs, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 20:6n-3), are derived from the shorter chain precursor, α-linolenic acid (ALA, 18:3n-3), and have been shown to be cardioprotective (Marik and Varon, 2009; Oh et al., 2014; Shimokawa and Vanhoutte, 1988). Although the mechanism of action of n-3 PUFAs are not fully understood, they have been shown to be anti-inflammatory, anti-thrombotic, and vasodilatory agents (Abeywardena and Patten, 2011; Kris-Etherton et al., 2002). Furthermore, studies specifically in smokers show that consuming n-3 PUFA supplements significantly improves FMD (Siasos et al., 2013).

The aim of this study was to develop a mouse model of CS-induced endothelial dysfunction that resembled impaired FMD observed in human cigarette smokers. This would enable us to mechanistically study the beneficial vascular properties of n-3 PUFAs and further elucidate the therapeutic potential of n-3-PUFAs to delay or prevent CS-induced CVD in smokers. We hypothesized that n-3 PUFAs protect against CS-induced vascular dysfunction by increasing NO-bioavailability and decreasing oxidative stress. To test this hypothesis, we fed C57BL/6 mice an n-3 PUFA-enriched diet or standard chow

diet and then exposed the mice to mainstream CS or filtered air. We assessed markers of oxidative stress, the contribution of NO and ROS to FMD and the ability of n-3 PUFAs to improve oxidative stress and FMD in CS-exposed mice.

MATERIALS AND METHODS

Chemicals.

Nω-nitro-L-arginine (LNNA) and 4-hydroxy-TEMPO (Tempol) and all components of physiological saline solution (PSS) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-PSS were purchased from Sigma-Aldrich (St Louis, MO). U46619 was purchased from Cayman Chemical Company (Ann Arbor, MI) and ionomycin was purchased from EMD Millipore (Billerico, MA).

Animals and diet.

Seven-week-old male C57Bl/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in a temperature-controlled environment, fed standard chow (2020X rodent diet, Teklad Diets, Envigo, Indianapolis, IN) and provided water ad libitum. After one week of acclimation, mice were randomly divided into two groups. One group continued to be fed the chow diet, while the second group was placed on an n-3 PUFA-enriched diet (TD.110516; 150 g oil/kg diet) (Teklad Diets) for 8 weeks.

Cigarette smoke exposure.

Mice from each diet were randomly assigned to either CS-exposed or air-exposed

groups (n=4-7/group). Mice were exposed to whole-body mainstream cigarette smoke generated from University of Kentucky 3R4F reference cigarettes (9.4mg tar, 0.73 mg nicotine per cigarette) in the SCIREQ InExpose system (SCIREQ, Montreal, Canada). Up to 5 mice were concurrently exposed to CS for 2 hrs/day (9 cigarettes total) for 5 consecutive days. Standard parameters, as set by the International Standard Organization (ISO 1991), were a 35 mL puff that lasted for 2 sec followed by 58 sec of fresh airflow at 6 mL/sec. Carbon monoxide levels were 300 ppm. Immediately prior to sacrifice, the spontaneous void of urine was collected on a sheet of parafilm when the mice were removed from their cage. Mice were sacrificed 4 hrs following the last CS-exposure. Mice were administered an intraperitoneal (ip) injection of sodium heparin, anesthetized with ip injection of ketamine/xylazine (80/4 mg/kg) and euthanized by exsanguination. Blood was collected by cardiac puncture, using syringes containing EDTA. Plasma and packed RBCs were collected from whole blood by centrifugation. The heart was harvested, atria were dissected and total left ventricle was weighed and recorded. Liver, lung and kidney weights were also recorded. Intestine was excised and immediately placed in ice cold HEPES-PSS (0.13 M NaCl, 0.006 M KCl, 0.001 M $MgCl_2$, 0.002 M $CaCl_2$, 0.01 M HEPES, 2.6×10^{-5} M EDTA, and 0.01 M glucose, pH 7.5). Tissue, urine, RBCs, and plasma were stored at -80°C. All animal protocols were approved by the University of New Mexico Animal Care and Use Committee (IACUC protocol 15-200331-HSC) and the investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Urinary cotinine:

Urinary cotinine was measured using an ELISA kit (Abnova, Taipei City, Taiwan) and analyzed by using a cotinine standard curve.

Gene expression analysis.

Total RNA was isolated from heart and lung tissue, using the RNeasy kit (Qiagen, GmbH, Germany). cDNA was synthesized using iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The reaction mixture used for the qPCR amplification consisted of iQ SYBR Green Supermix, 250 pg cDNA/µL and 500 nM
cytochrome P4501A1 (Cyp1a1), heme oxygenase 1 (Hmox1), NAD(P)H quinone dehydrogenase 1 (Nqo1) or DNA polymerase 2 (Pol2) sense and antisense primers (Table 4.1). An iCycler (Bio-Rad) was used to perform the qPCR amplification. Cycle threshold data for both the target gene of interest and control normalization gene, Pol2, were used to calculate mean normalized expression.

RBC n-3 and n-6 PUFAs.

Packed RBCs obtained from EDTA-treated whole blood were analyzed for fatty acid content by OmegaQuant (Sioux Falls, SD) as previously described (Harris et al., 2013). Briefly, samples were heated with methanol and boron trifluoride to generate fatty acid methyl esters. These methyl esters were extracted with hexane and water and analyzed using a GC2010 (Shimadzu Corporation, Columbia, MD) gas chromatographer equipped with a 30 m capillary column (Omegawax 250, Supelco, Bellefonte, PA). Fatty acids were identified by comparison to standard fatty acid methyl esters.

TABLE 4.1. Real Time PCR primer sequences

Gene	Sense Primer	Antisense Primer
Cyp1a1	5’ GGAACTAGACACAGTGATTG 3’	5’ TTGGGGATATAGAAGCCATTC 3’
Nqo1	5’ CCTTTCCAGAATAAGAAGACC 3’	5’ AATGCTGTAAACCAGTTGAG 3’
Hmox1	5’ CATGAAGAACTTTCAGAAGGA 3’	5’ TAGATATGGTACAAGGAAGCC 3’
Pol2	5’ TGACTCACAAACTGGCTGACATT 3’	5’ TACATCTTCTGCTATGACATGGG 3’

Biomarkers of oxidative stress.

All analyses were conducted in heart and lung tissue homogenates. Lipid peroxidation was investigated by measuring TBARS, a byproduct formed during decomposition of unstable peroxides derived from fatty acids, and expressed as malondialdehyde (MDA), based on a standard curve. Furthermore, the 8- isoprostane EIA kit (Cayman Chemical) was used to measure 8-*epi* $PGF_{2\alpha}$. Lastly, a glutathione assay kit was utilized to measure the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG).

Mesenteric vasoreactivity analysis.

First order mesenteric arterioles were cleaned of fat and connective tissue and a leak-free segment was mounted in a pressure myograph chamber (DMT-110 systems, Danish Myo Technology, Ann Arbor, MI), containing PSS at 37°C (0.13 M NaCl, 0.0047 M KCl, 0.0012 M KH_2PO_4, 0.0012 M $MgSO_4$, 0.015 M $NaHCO_3$, 2.6 x 10^{-5} M $CaNa_2$-EDTA, 0.0055 M glucose, and 0.0018 M $CaCl_2$, pH 7.4) and bubbled with 21% O_2, 6% CO_2 and balanced N_2. Arterioles were equilibrated from 5 mmHg to 75 mmHg by increasing pressure in 10 mmHg increments every 5 min followed by 30 min at 75 mmHg. Baseline internal diameter was measured using edge detection software (MyoView acquisition software, DMTVAS 6.2.0.59 Danish Myo Technology). Arterioles were deemed viable if they constricted to a minimum of 30% of baseline internal diameter following KCl (0.06 M) treatment. For FMD experiments, arterioles were constricted to 45% of baseline diameter with U46619 (10^{-8} M) and then subjected to increases in flow by stepwise increases in pressure from 0 – 40 mmHg between inflow and outflow, while keeping mean pressure at 75 mmHg. The study was then repeated

following 15 min incubation with LNNA (10^{-4} M). At the end of each study, passive internal diameter was determined using the calcium ionophore, ionomycin (10^{-5} M). There were no differences in passive internal diameter among groups (chow: Air 185.6 ± 7.3; CS: 190.8 ± 6.8, n-3 PUFA: Air: 177.3 ± 6.1; CS: 184.4 ± 5.1; Chow+ Tempol: Air: 172.1 ± 6.8, CS: 176.0 ±7.3). In addition, FMD experiments were conducted from air- and CS-exposed mice on a chow diet in the presence of Tempol (1 mM).

Statistical analysis.

All data were analyzed by SigmaPlot 13.0. Data are expressed as mean ± SEM, unless stated otherwise. FMD vascular reactivity dose response data was analyzed by two-way repeated measures analysis of variance (ANOVA) with post hoc Holm-Sidak comparisons. Total area under the curve for vascular reactivity and all other data were analyzed by two-way ANOVA with post hoc Holm-Sidak comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

CS Exposure Increased Urinary Cotinine Levels in Mice

Urinary cotinine (a metabolite of nicotine) was measured as an index of CS exposure. Mice exposed to CS exhibited increased urinary cotinine, compared to mice exposed to air on both a chow- and an n-3 PUFA diet (chow diet: Air: 0.082 ± 0.01 μg/mL, CS: 2.69 ± 0.14 μg/mL, $P < 0.05$; n-3 PUFA diet: Air: 0.23 ± 0.03 μg/mL, CS: 2.63 ± 0.14 μg/mL, $P < 0.05$).

TABLE 4.2 Comparison of body weights of 4 month old C57BL/6 male mice on a chow or n-3 PUFA diet and exposed to cigarette smoke or air for 5 days

	Chow diet[a]		n-3 PUFA diet	
Weight (g)	Air (n=10)	Cigarette smoke (n=10)	Air (n=15)	Cigarette smoke (n=15)
Body	26.9 ± 0.6	28.9 ± 0.6	30.6 ± 0.7	27.7 ± 0.7
Heart	0.108 ± 0.003 (0.40 ± 0.01)[b]	0.121 ± 0.003 (0.42 ± 0.01)	0.116 ± 0.003 (0.38 ± 0.01)	0.108 ± 0.003 (0.39 ± 0.01)
Kidney	0.280 ± 0.008 (1.04 ± 0.02)	0.292 ± 0.008 (1.01 ± 0.02)	0.325 ± 0.009[#] (1.07 ± 0.03)	0.305 ± 0.009 (1.11 ± 0.03)[#]
Lung	0.145 ± 0.004 (0.54 ± 0.02)	0.146 ± 0.004 (0.51 ± 0.02)	0.150 ± 0.004 (0.50± 0.02)	0.140 ± 0.004 (0.51 ± 0.02)
Liver	1.41 ± 0.06 (5.23 ± 0.10)	1.28 ± 0.06 (4.63 ± 0.10)*	1.408 ± 0.06 (4.58 ± 0.11)[#]	1.19 ± 0.06* (4.27 ± 0.11)[#]

[a]Values are expressed as mean ± SEM. Data were analyzed by two-way ANOVA with Holm-Sidak post-hoc analysis

[b](Organ weight (g)/body weight (g)) x 100

*****P < 0.05 versus air group fed the same diet

[#]P < 0.05 versus same treatment group fed the chow diet

CS Exposure Decreased Liver-to-Body Weight Ratio and the n-3 PUFA Diet Attenuated This Decrease

Body and organ weights, and organ-to-body weight ratios were analyzed to determine if CS had any overt adverse effects and whether these effects were altered by an n-3 PUFA diet. Body weights were not altered by CS on either diet (Table 4.2). However, CS

significantly decreased liver-to-body weight ratio in mice fed a chow diet. While CS significantly decreased liver weight in mice fed the n-3 PUFA diet, it failed to decrease liver-to-body weight ratio. An n-3 PUFA diet decreased liver-to-body weight ratio in both CS- and air-exposed mice. Lastly, the n-3 PUFA diet increased kidney weight in the air-exposed group and kidney-to-body weight ratio in the CS-exposed group, compared to mice on the chow diet.

CS and an n-3 PUFA Diet Significantly Altered RBC Fatty Acid Composition

To interpret physiological changes associated with CS exposure and the n-3 PUFA diet, levels of fatty acids in RBCs of mice were analyzed. CS exposure decreased ALA levels and increased arachidonic acid (AA) levels in mice on a chow diet, compared to the air exposed group. Notably, both these CS-induced changes were prevented by the n-3 PUFA diet (Table 4.3). Additionally, mice fed the n-3 PUFA diet exhibited significantly higher percentages of EPA and DHA, and significantly lower percentages of linolenic acid (LA) and AA, compared to mice fed a chow diet. Since the n-3 PUFA diet contained less of the n-3 PUFA precursor, ALA, than the chow diet, mice fed the n-3 PUFA diet exhibited significantly lower percentages of ALA.

TABLE 4.3. RBC fatty acid profile of 4 month old C57BL/6 male mice on a chow or n-3 PUFA diet and exposed to cigarette smoke or air for 5 days.

	Chow diet[b]		n-3 PUFA diet	
Fatty acids (%)	Air (n=10)	Cigarette smoke (n=10)	Air (n=15)	Cigarette smoke (n=15)
n-3 PUFAs[a]				
ALA	0.110 ± 0.004	0.092 ± 0.004*	0.040 ± 0.003#	0.039 ± 0.003#
EPA	0.228 ± 0.167	0.225 ± 0.167	10.1 ± 0.136#	10.4 ± 0.136#
DHA	6.64 ± 0.16	7.04 ± 0.16	14.00 ± 0.13#	13.80 ± 0.13#
n-6 PUFAs				
LA	12.5 ± 0.2	12.2 ± 0.1	2.3 ± 0.1#	2.2 ± 0.1#
AA	16.5 ± 0.1	17.1 ± 0.1*	8.4 ± 0.1#	8.2± 0.1#

[a]Abbreviations: α-linolenic acid (ALA, 18:3n3), eicosapentaenoic acid (EPA, 20:5n3), docosahexaenoic acid (DHA, 22:6n3), linoleic acid (LA, 18:2n6), arachidonic acid (AA, 20:4n6)
[b]Values are expressed as mean ± SE, n= 10-15. Data was analyzed by 2-way ANOVA with Holm-Sidak post hoc comparisons.

***** $P < 0.05$ versus chow air

# $P < 0.05$ versus same treatment group fed the chow diet

CS Induced, and the n-3 PUFA Diet Attenuated, Cyp1a1 Expression in the Heart and Lung

To further characterize the CS exposure and the influence of the n-3 PUFA diet, the degree to which a 5-day CS exposure induced Cyp1a1 mRNA was investigated. As expected, CS significantly induced Cyp1a1 in both the heart and lung of mice on the chow and n-3 PUFA diets (Fig. 4.1A and B). However, the n-3 PUFA diet significantly reduced both the basal and CS-induced levels of Cyp1a1 in the heart and reduced the basal levels of Cyp1a1 in the lung.

CS Induced One Biomarker of Oxidative Stress and the n-3 PUFA Diet Prevented this Induction

To assess if oxidative stress biomarkers were induced by CS and influenced by the n-3 PUFA diet, we measured the GSH/GSSG ratio, MDA and 8-isoprostane levels in the heart and lung. Neither CS nor the n-3 PUFA diet affected GSH/GSSG levels in the heart or lung (Fig. 4.2A and B). In addition, CS had no effect on MDA levels in the heart or lung, while the n-3 PUFA diet significantly increased MDA levels in the heart (Fig. 4.2C and D). Finally, CS significantly induced 8-isoprostane levels in the heart and this induction was prevented in mice fed the n-3 PUFA diet (Fig. 4.2E). Although CS failed to induce 8-isoprostane levels in the lung on the chow diet, 8-isoprostane levels were significantly lower in the CS exposure group on the n-3 PUFA diet (Fig. 4.2F).

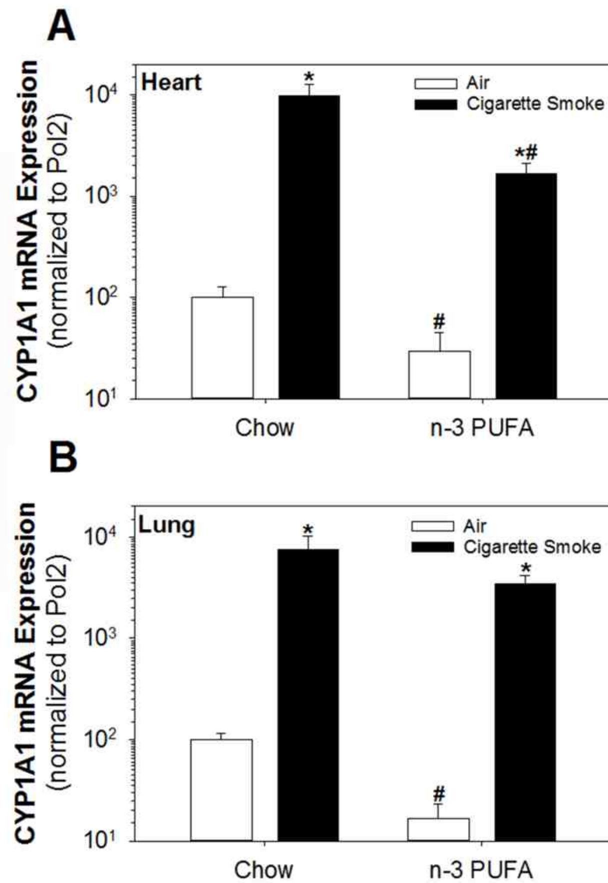

Figure 4.1. Effects of CS and an n-3 PUFA diet on mRNA expression of Cyp1a1. A-B, Heart and lung mRNA expression of Cyp1a1, normalized to the housekeeping gene, Pol2, from air- and CS-exposed mice fed a chow or n-3 PUFA diet. Data are shown as mean ± SE, n = 5-6 /group and analyzed by two-way ANOVA with post-hoc Holm-Sidak comparisons. *$P < 0.05$ versus air within the same diet; $^{\#}P < 0.05$ versus chow within the same exposure.

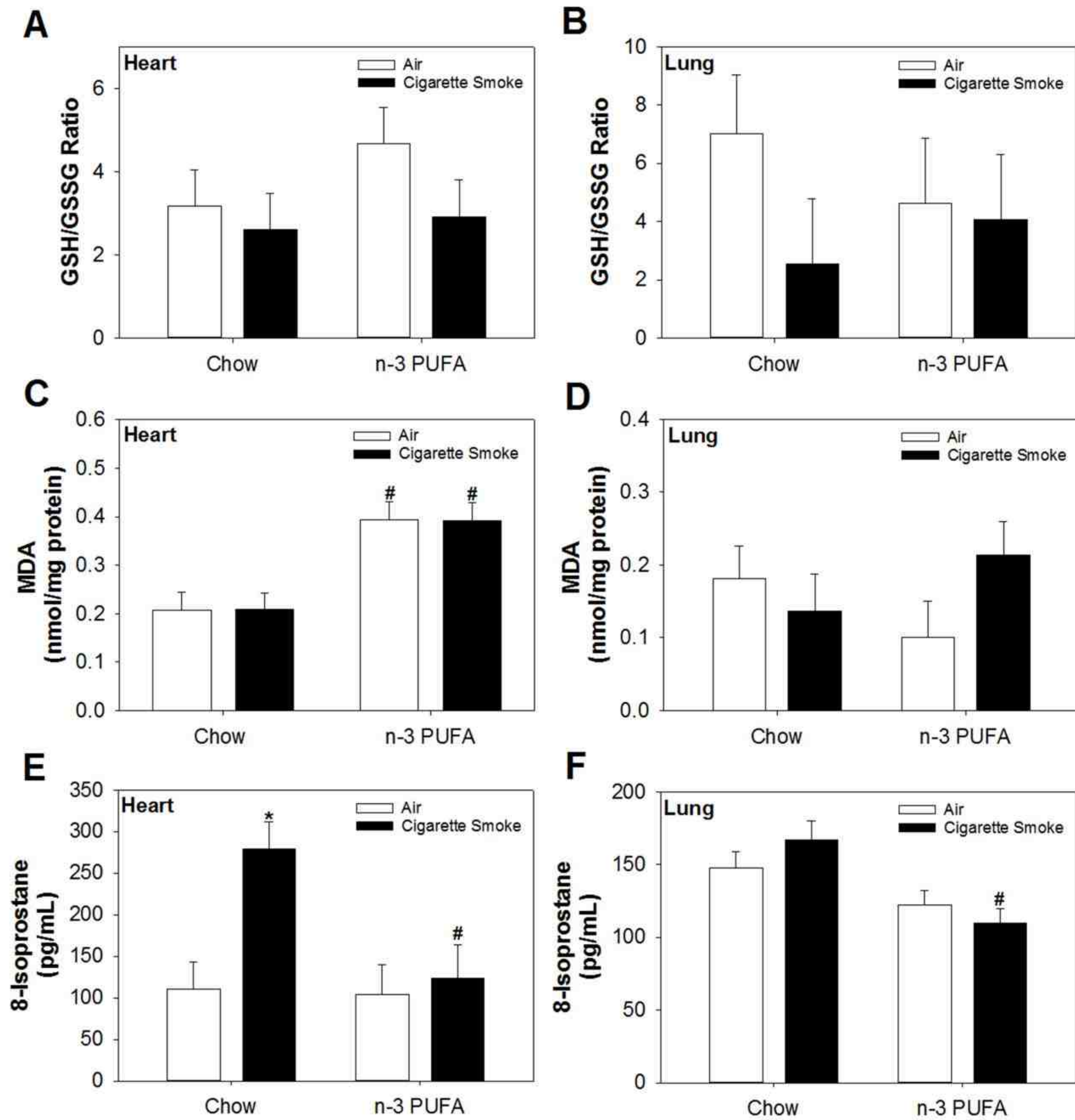

Figure 4.2. Effects of CS and n-3 PUFA diet on oxidative stress biomarkers. Heart and lung levels of A-B, GSH/GSSG ratio, C-D, MDA levels, and E-F, 8-isoprostane levels from air- and CS-exposed mice fed a chow or n-3 PUFA diet. Data are shown as mean ± SEM, n = 4-5/group and analyzed by two-way ANOVA with post-hoc Holm-Sidak comparisons. *$P < 0.05$ versus air, within the same diet; $^{\#}P < 0.05$ versus chow within the same exposure.

CS and the n-3 PUFA Diet Differentially Regulated Antioxidant Gene Expression in the Heart and Lung

To investigate the effect of CS and an n-3 PUFA diet on antioxidant gene expression, mRNA expression of Hmox1 and Nqo1 were measured in the heart and lung. CS significantly induced Hmox1 in the heart and lung from mice fed the chow diet, and this induction was normalized to the air exposure levels in mice fed the n-3 PUFA diet (Fig. 4.3A and B). In contrast, CS significantly induced Nqo1 mRNA expression in the lung, but not the heart, and while this induction was attenuated by the n-3 PUFA diet it was not restored to the air exposure levels (Fig. 4.3C and D).

CS Impaired FMD and the Antioxidant, Tempol, Prevented this Impairment by Increasing NO-independent Dilation

To determine the degree to which this acute 5-day CS exposure impaired FMD and establish the contribution of oxidative stress and NO to this impairment, FMD studies were conducted in pressurized mesenteric arterioles from air- and CS-exposed mice fed a chow diet in the presence or absence of the free radical scavenger, Tempol, and the NO synthase inhibitor, LNNA. CS exposure significantly impaired total FMD, compared to air-exposed mice, and this impairment was completely prevented by Tempol (Fig. 4.4A and C). By adding LNNA, the NO-dependent and –independent contribution to the FMD response could be determined from area-under-the-curve analysis. These analyses showed that the CS-induced impairment of FMD resulted from a significant reduction in NO-dependent dilation (Fig. 4.4B and D). Interestingly, while Tempol tended to normalize the NO-dependent dilation response ($p = 0.095$), compared to no Tempol, it

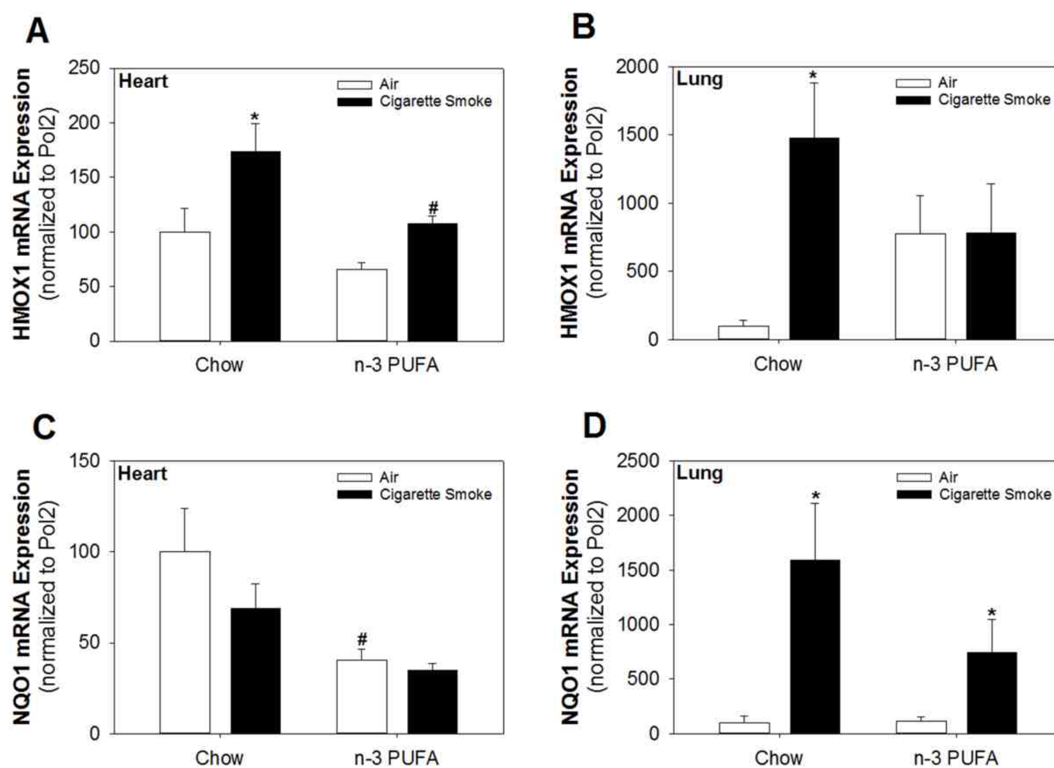

Figure 4.3. Effects of CS and an n-3 PUFA diet on mRNA expression of Hmox1 and Nqo1. Heart and lung mRNA expression of A-B, Hmox1, normalized to the housekeeping gene, Pol2; and C-D, Nqo1, normalized to the housekeeping gene, Pol2, from air- and CS-exposed mice fed a chow or n-3 PUFA diet. Data are shown as mean ± SE, n = 6-9/group and analyzed by two-way ANOVA with post-hoc Holm-Sidak comparisons. *$P < 0.05$ versus air, within the same diet; $^{\#}P < 0.05$ versus chow within the same exposure.

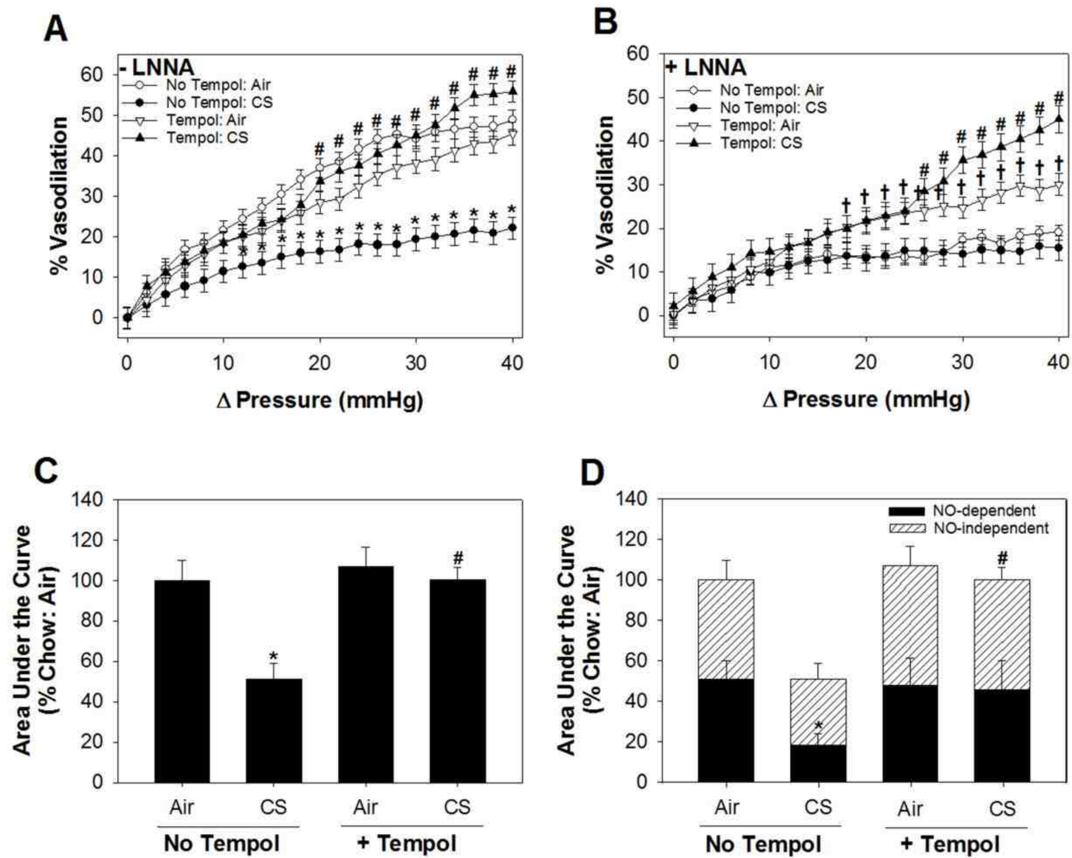

Figure 4.4. Effects of CS and Tempol on FMD. A-B, Dose response for FMD-mediated dilation in pressurized mesenteric arterioles of air- and CS-exposed mice fed a chow diet with and without Tempol and in the presence and absence of LNNA, respectively. C, Area-under-the-curve analysis of the dose-response curve shown in panel A. D, Area-under-the-curve analysis of the dose-response curves shown in panel A and B, showing NO-dependent and NO-independent components of the FMD response. Data are shown as mean ± SEM, n = 4-7/group. Panel A-B: analyzed by two-way repeated measures ANOVA with post-hoc Holm-Sidak comparisons. *P < 0.05 versus no Tempol, air; $^{\#}$P <0.05 versus no Tempol, CS. †P < 0.05 versus no Tempol, air. Panel C-D: analyzed by two-way ANOVA with post-hoc Holm-Sidak comparisons. *P < 0.05 versus air, no Tempol; $^{\#}$P < 0.05 versus no Tempol, CS.

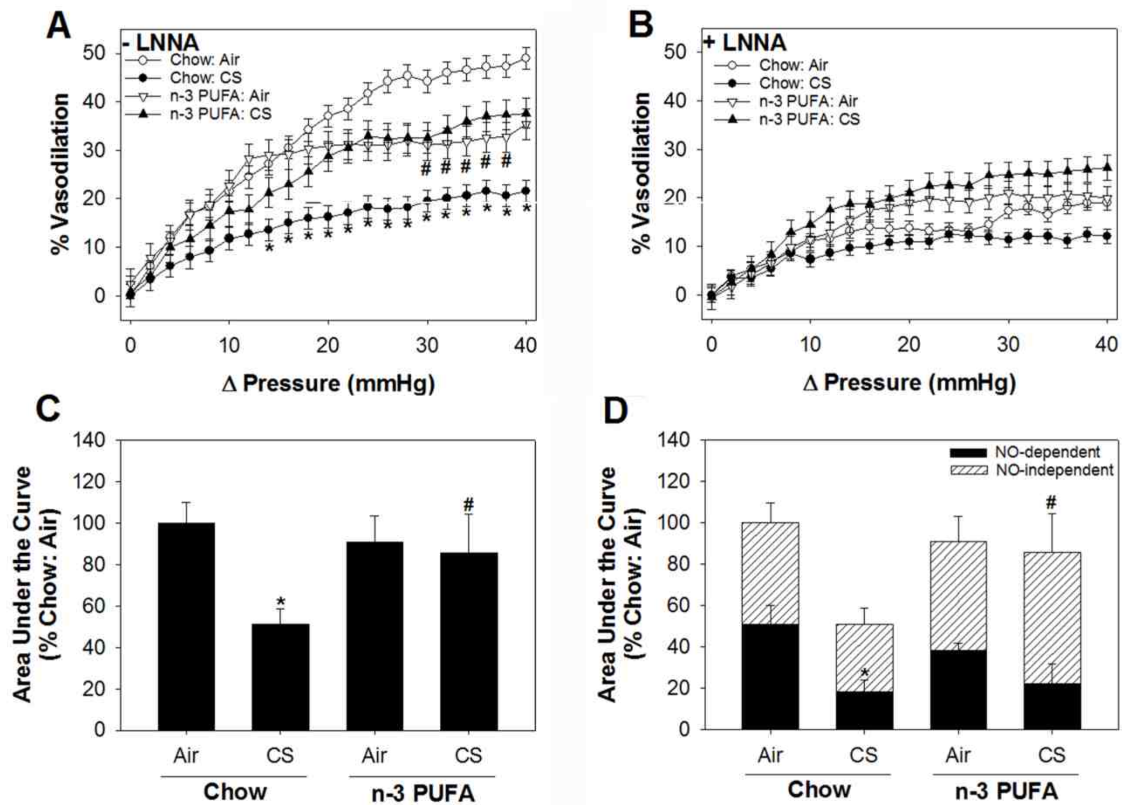

Figure 4.5. Effects of CS and an n-3 PUFA diet on FMD. A-B, Dose response for FMD-mediated dilation in pressurized mesenteric arterioles of air- and CS-exposed mice fed a chow or n-3 PUFA diet in the presence and absence of LNNA, respectively. C, Area-under-the-curve analysis of the dose-response curve shown in panel A. D, Area-under-the-curve analysis of the dose response curves shown in panel A and B, showing NO-dependent and NO-independent components of the FMD response. Data are shown as mean ± SEM, n = 6-7/group. Panel A-B: analyzed by two-way repeated measures ANOVA with post-hoc Holm-Sidak comparisons. *$P < 0.05$ versus chow, air; #$P < 0.05$ versus chow, air. Panel C-D: analyzed by two-way ANOVA with post-hoc Holm-Sidak comparisons. *$P < 0.05$ versus chow, air; #$P < 0.05$ versus chow, CS.

improved CS-impaired FMD by significantly increasing NO-independent dilation (Fig. 4.4D).

n-3 PUFA Diet Prevented CS Impairment of FMD by Increasing NO-independent Dilation

To determine the degree to which an n-3 PUFA diet could improve CS-impaired FMD and dissect the contribution of NO to this response, FMD studies were conducted in pressurized mesenteric arterioles from air- and CS-exposed mice fed an n-3 PUFA diet in the presence or absence of LNNA. The impairment of the total FMD response induced by CS on a chow diet was completely prevented in mice fed an n-3 PUFA diet (Fig. 4.5A and B). Surprisingly, area-under-the-curve analysis in the presence and absence of LNNA revealed that the n-3 PUFA diet prevented CS impaired FMD by significantly increasing NO-independent dilation without affecting NO-dependent dilation (Fig. 4.5B and D).

DISCUSSION

In this study we established a flow-mediated dilation model that mimics CS-impaired FMD in humans (i.e. loss of total dilation and loss of NO-mediated dilation) and found that a diet enriched in n-3 PUFAs can prevent CS-induced vascular dysfunction. However, the n-3 PUFA diet appears to have multiple mechanisms of potential benefit. First, the n-3 PUFA diet increases the contribution of NO-independent pathways to the dilation response with little-to-no effect on NO bioavailability. Second, the n-3 PUFA diet acts an antioxidant as is evidenced by decreasing a specific marker of oxidative stress and the gene expression of a specific ROS-induced antioxidant enzyme. Finally, the n-3 PUFA diet reduces CS-induced expression of Cyp1a1, which has been associated with

vascular dysfunction in earlier studies. Taken together these results demonstrate that an n-3 PUFA-enriched diet can protect against CS-induced vascular dysfunction via multiple mechanisms, including NO-independent vasodilation and antioxidant effects.

The most novel finding of our study is that dietary n-3 PUFAs protect against CS impaired FMD by improving NO-independent dilation. This result is unexpected since numerous earlier studies show that n-3 PUFAs increase NO and NO bioavailability (Agbor et al., 2014; Li et al., 2007; Stebbins et al., 2008; Tagawa et al., 2002; Wu et al., 2012). For example, in a study measuring FMD in patients with coronary artery disease at baseline and after 3 months of EPA treatment (1,800 mg/day), FMD is significantly improved following EPA treatment. In the presence of a NO-donor, sodium nitroprusside, there is no difference, showing that n-3 PUFAs improve dilation, at least in part, by improving NO-mediated dilation (Tagawa et al., 2002). In another study in bovine aortic endothelial cells, EPA (25µM) increases eNOS phosphorylation and NO production in a AMP-activated protein kinase (AMPK) dependent manner. Furthermore, acetylcholine-mediated dilation in eNOS$^{-/-}$ mouse aortic rings treated with EPA, is significantly impaired compared to ApoE$^{-/-}$ mice treated with EPA (Wu et al., 2012).

In our study, NO contributes approximately 50% to the FMD response in control air exposed mice and 18% in CS-exposed mice, while on an n-3 PUFA diet, the NO contribution is 38 and 22%, respectively. In contrast, the NO-independent component contributes 50% to FMD response in control air exposed mice and 32% in CS-exposed mice and is increased to 52 and 64%, respectively, on an n-3 PUFA diet. Thus, the NO-independent contribution doubles in CS-exposed mice on an n-3 PUFA diet.

NO-independent dilation is primarily mediated by endothelial-derived hyperpolarizing factors (EDHFs). Although the exact mechanism of EDHF-mediated dilation is still unknown, P450-dependent metabolites of AA, DHA and EPA as well as potassium ions are proposed as possible EDHFs (Chen and Cheung, 1996; Edwards et al., 1998). In our study the ability of the n-3 PUFAs to improve FMD may result from (1) shear stress-mediated activation of phospholipase A2 (PLA_2), which releases membrane-bound fatty acids, including EPA and DHA (Pearce et al., 1996; Rosa and Rapoport, 2009), and (2) the subsequent metabolism of EPA and DHA by P450s to vasodilatory epoxides, epoxyeicosatetraenoic acids (EEQs) and epoxydocosapentaenoic acids (EDPs) (Lauterbach et al., 2002). EEQs and EDPs cause vasodilation through opening of large conductance, calcium-dependent potassium channels (BK channels) and ATP-sensitive potassium channels (Agbor et al., 2012; Hoshi et al., 2013; Walker et al., submitted), and thus act as EDHFs.

It is also notable and unexpected that the antioxidant Tempol similarly increases NO-independent dilation significantly. Tempol increases the NO-independent component of FMD from 33 to 55% in mice exposed to CS. While FMD is largely thought to be NO-dependent (Rizzo et al., 1998; Tagawa et al., 2002), the mechanism of the FMD response may depend on vessel size where NO is the primary dilator in larger conduit vessels, while NO and EDHFs both contribute to FMD in smaller resistance vessels, like mesenteric arterioles. Nonetheless, our data suggest that oxidative stress may play a role in reducing NO-independent dilation. It is known that ROS can inhibit potassium channel function in the vasculature (Gutterman et al. 2005) and this is consistent with data

showing that EDHF-mediated dilation is impaired in a rat model, in part, due to an increase in ROS (Leo et al., 2011).

A second major finding of our study is that the 5-day acute exposure to CS induces two specific markers of oxidative stress and these are attenuated by the n-3 PUFA diet. It is well established that cigarette smokers have increased ROS, leading to an increase in markers of oxidative stress, such as GSH/GSSG ratio, MDA, and 8-isoprostanes (Jaimes et al., 2004; Seet et al., 2011). In our study we observe an increase in two specific markers of oxidative stress, 8-isoprostane and mRNA expression of the antioxidant enzyme, Hmox1, following CS exposure. 8-Isoprostane is produced by the non-enzymatic oxidation of AA in membrane phospholipids and our data are consistent with studies showing elevated levels of plasma 8-isoprostane in heavy smokers (Morrow et al., 1995) and in non-smokers exposed to secondhand smoke (Kato et al., 2006). Hmox1 is a gene induced by ROS via activation of the transcription factor, nuclear factor (erythroid-derived 2)-like 2 (nrf-2) and we also found that Hmox1 mRNA is increased in mice exposed to CS on a chow diet, consistent with other CS-exposed animal models (Wei et al., 2015; Yang et al., 2015). Importantly, we found that CS-induced increases in both 8-isoprostane levels and Hmox1 mRNA expression are reduced in mice fed an n-3 PUFA diet, compared to those fed a chow diet. While we did not observe any changes in the GSH/GSSG ratio or MDA levels in the heart and lung as a result of CS exposure, it is possible that GSH could be taken up from the plasma (Bai et al., 1994), while the lack of changes in MDA may result from nonspecific binding of thiobarbituric acid to other compounds in addition to lipids (Knight et al., 1988). While we did observe that mice on the n-3 PUFA diet exhibit increases in MDA in the heart, regardless of CS exposure, this

is consistent with the findings of others (Gonzalez et al., 1992; Kawachi et al., 1997), and may result from the accumulation of DHA in the heart (Agbor et al., 2014; Arnold et al., 2010) and the increased susceptibility of the heart to oxidation (Santos et al., 2011).

The third major finding of our work is that the CS induction of Cyp1a1 is significantly attenuated by the n-3 PUFA diet. Previous work shows that CYP1A1 is a risk factor for vascular dysfunction and hypertension in mice treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Kopf et al., 2010). Further, TCDD-induced increases in ROS is downstream of Cyp1a1 induction (Kopf and Walker, 2010a). Thus, CS-induced Cyp1a1 may contribute to the CS-induced increases in oxidative stress, while the attenuation of Cyp1a1 induction by the n-3 PUFA diet may contribute to decreases in oxidative stress. The ability of the n-3 PUFA diet to attenuate Cyp1a1 induction is consistent with previous studies of mice and rats treated with TCDD (Palaniswamy et al., 2014; Wiest et al., 2016); however, the mechanism underlying this attenuation remains to be investigated.

There are some potential limitations to this work. The 5-day CS exposure cannot be extrapolated to fully encompass the vascular injury and dysfunction that occurs following chronic CS exposure in humans. Nonetheless, our model of acute CS exposure allows us to mechanistically investigate the early events that contribute to impaired FMD prior to vascular remodeling, inflammation, and development of other CVD risk factors. Additionally, chronic cigarette smokers exhibit reduced levels of RBC EPA and DHA (Wiest et al., 2015), which likely contribute to impaired FMD. Thus, although the 5-day CS exposure is too short of an exposure time to reduce the levels of these n-3 PUFAs, this limitation could be overcome by putting mice on a diet low in n-3 PUFAs.

In summary, we show that loss of FMD in mesenteric arterioles of mice exposed to CS is prevented by an n-3 PUFA diet, specifically by increasing NO-independent dilation and this protection is associated with decreases in Cyp1a1 induction and two specific markers of oxidative stress. These data provide preclinical mechanistic evidence that support clinical data that n-3 PUFA supplements can improve impaired FMD in human cigarette smokers (Din et al., 2013; Siasos et al., 2013). Future studies are needed to elucidate the NO-independent mechanism by which n-3 PUFAs afford their vasoprotection following CS exposure.

CHAPTER V

Summary, Conclusions, and Future Directions

Summary

The overall goal of this project was to identify biomarkers of early vascular dysfunction in smokers and develop a mouse model to investigate the mechanisms by which these biomarkers provide protection. We studied two cohorts: healthy, young current- and never-smokers and older current-and never smokers with hypertension. Studying these cohorts enabled us to determine the limitations of our potential biomarkers. The biomarkers of interest were omega-3 (n-3) polyunsaturated fatty acids (PUFAs) and their epoxides metabolites.

In our first population of Hispanic healthy current- and never smokers, we measured brachial flow-mediated dilation (FMD) as a primary outcome. We also measured RBC n-3 PUFAs and AHR-dependent luciferase expression and correlated these measurements to FMD. Consistent with what others have found, we found that FMD is impaired in smokers (Celermajer et al., 1996, 1993). Furthermore we found that α-linolenic acid is increased in smokers; however, the longer-chain n-3 PUFA, docosahexanoic acid (DHA, 22:6), was decreased. This is consistent with literature showing that smokers have decreased ALA conversion to EPA and DHA (Ghezzi et al., 2007; Marangoni et al., 2004).

Interestingly, AHR-dependent luciferase expression, which is an index of polycyclic aromatic hydrocarbon (PAH) exposure, was increased in smokers, and Cyp1B1 mRNA expression was also increased in smokers, indicating that PAHs in cigarette smoke activate the AHR leading to transcription of Cyp1b1. Furthermore, FMD

correlated positively with AHR-dependent luciferase expression only in smokers. In other words, smokers with the worse FMD responses have the lowest serum AHR activity. One explanation of this may be related to the rate of PAH metabolism. Smokers with the worst FMD may have increased PAH metabolism and thus lower levels of parent PAHs and higher levels of PAH metabolites in the serum.

Lastly, we found that FMD correlated negatively with ALA. In other words, smokers with the worst FMD had the highest levels of ALA. A possible explanation may be that ALA is not being converted to the longer chain fatty acids, EPA and DHA. This is consistent with what others have shown, that CS impairs enzymes involved in conversion of ALA to DHA and EPA (Ghezzi et al., 2007; Marangoni et al., 2004).

In summary, our results suggest that the ability of serum to activate the AHR and the percentage of ALA in RBCs can serve as independent biomarkers of impaired FMD in healthy cigarette smokers. In addition, these biomarkers may be used by regulatory agencies when screening new tobacco products for cardiovascular disease (CVD) risk.

One limitation of this study is that biomarkers identified in young, healthy smokers may also be biomarkers of ASCVD risk in smokers with pre-existing CVD risk factors. We therefore conducted a second study in older smokers with hypertension, a risk factor for the development of CVD.

This limitation led to a second study in a population with risk factors of cardiovascular disease. We measured the 10-year atherosclerotic cardiovascular disease risk (ASCVD) as primary outcome in subjects 40 – 70 years old with hypertension. Furthermore, we correlated n-3 and n-6 PUFAs and their epoxides metabolites with 10-year ASCVD risk as possible biomarkers of CVD in subjects with a risk factor for CVD.

We found that ALA, which was demonstrated to predict FMD in young, healthy smokers, is not associated with ASCVD risk in smokers with preexisting hypertension. Nonetheless, we found that 13,14-DiHDPA, along with 11- and 20-HETE, predict ASCVD risk in smokers, whereas 14,15-EEQ, 13,14- and 16,17-EDPs, and 16,17-DiHDPA predict ASCVD risk in non-smokers. These results suggest that the fatty acids identified in this study may be used to predict CVD risk in subjects with pre-existing CVD risk factors.

In order to mechanistically determine how n-3 PUFAs protect against endothelial dysfunction in smokers, we developed a mouse model of CS-exposure. This model of acute CS-exposure enables us to mechanistically investigate the early events that lead to endothelial dysfunction in smokers, prior to development of CVD. We were able to examine the effect of cigarette smoke on NO-dependent and NO-independent FMD in mouse mesenteric resistance arterioles. CS-induced impairment of FMD occurs prior to vascular remodeling, inflammation, or development of other CVD risk factors. Thus, to study the mechanisms underlying this impairment and investigate potential therapeutic strategies to prevent it, we chose a short term (5 days, 2 hr/d) exposure of mice to mainstream cigarette smoke. This model allows us to elucidate the mechanisms associated with impaired FMD without confounding changes in vascular structure that results from chronic smoke exposure. Consistent with what we have observed in young, healthy smokers, we found that this short-term CS exposure impaired total FMD.

We found that an n-3 PUFA diet offers multiple mechanisms of benefit. n-3 PUFAs reduce CS-induced oxidative stress as measured by a very specific biomarker, 8-isoprostane and the gene expression of a specific ROS-induced antioxidant enzyme,

Hmox1. Furthermore, we also found that n-3 PUFAs reduce CYP1A1 induction, which has been associated with endothelial dysfunction. The observation that n-3 PUFAs prevent CYP1A1 induction therefore suggests that n-3 PUFAs prevent CS-induced endothelial dysfunction, in part, by reducing CYP1A1 induction. We also made the novel observation that n-3 PUFAs prevent CS-impaired FMD by increasing NO-independent dilation and without altering NO-dependent dilation. This suggests that n-3 PUFAs may act as endothelial derived hyperpolarizing factors (EDHFs). In our study the ability of the n-3 PUFAs to improve FMD may result from (1) shear stress-mediated activation of phospholipase A2 (PLA_2), which releases membrane-bound fatty acids, including EPA and DHA (Pearce et al., 1996; Rosa and Rapoport, 2009), and (2) the subsequent metabolism of EPA and DHA by P450s to vasodilatory epoxides, epoxyeicosatetraenoic acids (EEQs) and epoxydocosapentaenoic acids (EDPs) (Lauterbach et al., 2002). EEQs and EDPs cause vasodilation through opening of large conductance, calcium-dependent potassium channels (BK channels) and ATP-sensitive potassium channels (Agbor et al., 2012; Hoshi et al., 2013; Walker et al., submitted), and thus act as EDHFs.

In order to determine the effects of CS-induced oxidative stress on vascular function, we conducted vascular reactivity studies in mice on a chow diet in the absence or presence of the free radical scavenger, Tempol. Similar to what was seen in mice on an n-3 PUFA diet, we found that Tempol restores total FMD by increasing NO-independent dilation and normalizing NO-dependent dilation. This suggests that the vasoprotective benefits of n-3 PUFAs are due, at least in part, by reduction of CS-induced ROS.

There are some limitations concerning this study. One limitation is the short CS exposure. Although we focused on generating an animal model of early events leading to

endothelial dysfunction, some conflicting observations were made due to this short exposure. One of which is the CS-dependent change in RBC PUFAs. Although we have shown an increase in n-3 PUFAs and a decrease in n-6 PUFAs in mice on an n-3 PUFA diet for only 2 months, a 5 day CS exposure may be too short to alter these PUFAs. Nonetheless, a 5-day CS-exposure is sufficient to see changes in the vasculature. Another limitation could be the n-3 PUFA diet. This diet is extremely high in n-3 PUFAs, and is not relevant to most human diets.

Despite these limitations, we have shown that loss of FMD in mesenteric arterioles of mice can be restored by an n-3 PUFA diet, specifically by increasing the NO-independent dilation. One mechanism in which n-3 PUFAs may restore vessel function is by decreasing CYP1A1 induction and reducing oxidative stress.

Lifestyle changes and n-3 PUFA supplementation may reverse endothelial dysfunction and delay development of CVD. It is therefore of clinical importance to identify biomarkers and methods to treat these patients at an early stage.

Conclusions and future directions

Taken together, our results suggest that RBC ALA % and serum AHR-activity could be an alternative to measuring FMD to identify young, healthy smokers at risk of developing CVD. The reason for this correlation only in smokers may be due to the abundance of CS-induced oxidative stress. Therefore, it appears that n-3 PUFAs are protective in subjects with increased oxidative stress that may lead to premature development of CVD. One strength of our study is that it was unbiased in design. We recruited individuals with a variety of CVD risks and diverse backgrounds. These

conclusions, however, are limited to otherwise young, healthy subjects. In older subjects with pre-existing risk factors for development of ASCVD, the oxidative stress burden and inflammation may overshadow the beneficial effects of n-3 PUFA supplementation. It is possible that increases in oxidative stress and inflammation due to hypertension, smoking, and age puts strains on antioxidant mechanisms in the body. Increasing n-3 PUFA intake or other antioxidants like vitamin E or vitamin C may be more effective in decreasing oxidative stress and improving CVD risk. Also important to note is that this is a relatively small population and expanded studies (multiple locations and recruitment subjects from many different ethnic and socio-economic groups) will need to be conducted to confirm that these biomarkers correlate with CVD outcomes.

Our study in healthy smokers has shown that there is a correlation between FMD and percentage of ALA in RBCs, but there is also a large variance in their n-3 PUFA index. A possible explanation is that ALA is not being converted to its longer chain fatty acids, EPA and DHA. In addition, New Mexico is land-locked and fresh seafood is limited and culturally it is not a major part of the diet. This was also reflected in the food frequency questionnaires filled out by study subjects: the majority of our study subjects do not eat seafood on a regular basis. It is therefore understandable that this cohort of subjects have a large variance in n-3 index. By placing subjects on an n-3 PUFA supplement, or placebo, we would be able to reduce the variance in n-3 index. We would also be able to investigate the effects of an increase in DHA and eicosapentaenoic acid (EPA, 20:5) on FMD. A 4-month intervention study would allow adequate time for both EPA and DHA to reach steady state in RBCs. Measurements of oxidative stress markers, CVD risk factors, FMD, and n-3 PUFAs and their long chain metabolites pre- and post

treatment with n-3 PUFA supplements or placebo should be measured. This would enable us to determine if this increase in EPA and DHA could reduce young, healthy smoker's risk of developing CVD and if this can serve as a therapeutic approach to delay onset of CVD in young, healthy smokers.

Studies investigating n-3 PUFAs have been conducted, but these studies are conducted in subjects with different ethnicities, genders, and pathological conditions. Only a few studies have been conducted in healthy smokers and most studies are underpowered. Conducting a larger and longer study in healthy cigarette smokers looking at the effect of n-3 PUFAs on CVD risk factors will advance this field of study.

Additionally, studies to determine the mechanism in which n-3 PUFAs protect against oxidative stress in smokers could be another future direction. In numerous trials, antioxidants improve CVD endpoints in smokers, but only for a limited time (Neunteufl et al., 2000; Raitakari et al., 2000). This may be due to the variety of mechanisms in which CS causes oxidative damage. CS-mediated oxidative damage is induced by 1) free radicals in CS that cause oxidative damage directly, 2) agents in CS that lead to increased inflammation that indirectly leads to oxidative damage, 3) agents that down-regulate biological anti-oxidant defense mechanisms at the transcriptional and translational levels, and 4) agents that exhaust anti-oxidant defense systems like glutathione pools (Bernhard and Wang, 2007). By conducting a thorough study to determine which of these mechanisms are targeted by n-3 PUFA supplementations, a combination therapy to decrease global sources of oxidative stress and augment antioxidant defense mechanisms could serve as a potential therapeutic to treat oxidative damage in smokers long-term.

Mechanistically, we have demonstrated that CS-induced loss of NO-dependent dilation is restored by an n-3 PUFA diet by increasing NO-independent dilation. Additionally, we have also shown that n-3 PUFAs reduce oxidative stress and CYP1A1 mRNA expression that may also allude to the mechanism by which n-3 PUFAs protect against CS-induced vascular dysfunction. Additionally, we found that mice on an n-3 PUFA diet for 2 months had increased RBC n-3 PUFA index and a decrease in RBC n-6 PUFA index. CS-exposure however, did not have an effect on DHA and EPA in mice on a chow diet or an n-3 PUFA diet. In contrast to what is seen in human smokers, ALA was decreased in mice on a chow diet exposed to CS, compared with those exposed to air. It is possible that a 5-day CS exposure is too short to elicit changes in fatty acid composition within cell membranes. We found that vascular compensation occurs after an 8-week exposure to CS and impaired dilation is not detected. Therefore, longer exposure (8 months – 12 months) would lead to impaired vascular dilation and may be long enough to observe a change in RBC fatty acid composition. This would enable us to better assess the effect of chronic CS on RBC fatty acid profiles.

In addition, we found that an n-3 PUFA diet significantly increases n-3 PUFA index above physiological levels found in humans. This is a limitation of the n-3 PUFA diets, as the oils used to prepare the diets differ in percentage fatty acids other than n-3 PUFAs (Agbor et al., 2014). In order to mimic the n-3 PUFA levels in humans, the fat-1 transgenic mouse model may be used. The fat-1 transgenic mouse expresses n-3 fatty acid desaturase-1 (fat-1) from *Ceanorhabditis elegans*, allowing endogenous conversion of n-6 PUFAs to n-3 PUFAs (Kang et al., 2004). This more moderate increase in n-3

PUFAs may also be more readily affected by CS, which would enable us to observe small changes in n-3 PUFA levels.

We have shown that n-3 PUFAs restore CS-induced impairment of FMD by increasing NO-independent dilation. A possible mechanism in which n-3 PUFAs improve FMD is through shear stress-mediated activation of PLA_2, which has been shown to release fatty acids, including EPA and DHA, from the cell membrane (Pearce et al., 1996; Rosa and Rapoport, 2009). EPA and DHA are in turn is metabolized by CYP450s to vascular-beneficial epoxyeicosatetraenoic acids (EEQs) and epoxydocosapentaenoic acids (EDPs) (Lauterbach et al., 2002). Furthermore, EEQs and EDPs have been shown to cause vasodilation through opening of large conductance calcium-dependent potassium channels (BK channels) (Agbor et al., 2012; Hoshi et al., 2013). However, this has not been established in the case of FMD-mediated dilation. Conducting vessel myography studies in mesenteric studies in the absence and presence of specific potassium-channel inhibitors would test the hypothesis that n-3 PUFAs restore dilation by activation of potassium channels. Furthermore, by conducting this experiment in the presence of soluble epoxide hydrolase (sEH) inhibitors, it would prevent rapid metabolism of EEQs into its inactive diol compounds. This would allow the study of the effects of EEQs directly, before conversion into its diol compounds.

Lastly, we made the novel observation that n-3 PUFAs reduce CYP1A1 mRNA expression. This may be one of the mechanisms by which n-3 PUFAs protect against CS-induced vascular dysfunction. The mechanism by which n-3 PUFAs reduce CYP1A1 induction is not known. We have shown that it is not by increasing AHR protein levels.

Possible mechanisms may include up-regulation of repressor genes or preventing induction of AHR-dependent transcription genes. In addition, preventing AHR binding to the DRE is another possible mechanism that has not been investigated. It is also possible that the increased uptake of n-3 PUFAs, which serve as substrates for CYP1A1, may induce a negative feedback loop, inhibiting induction of CYP1A1 by one (or more) of the above mentioned mechanisms. A possible experimental set-up would be using CYP1A1 KO and WT mice on an n-3 PUFA or a chow diet (4 groups). In order to investigate the exact mechanism, conducting microarray studies and specifically looking at up-regulation of genes involved in fatty acid metabolism and AHR trafficking and binding to the DRE would give more insight into the pathways affected by n-3 PUFAs. This approach will be followed by sub cellular fractioning and quantification of AHR and protein levels in the cytoplasm vs. the nucleus to determine if n-3 PUFAs effect the translocation of AHR into the nucleus. ARNT levels in the nucleus could also be quantified. Next, binding of ARNT to the DRE could be determined by conducting chromatin immunoprecipitation (ChIP). As a third approach, transcription repression can be investigated by measuring histone acetylation and methylation of the CYP1A1 promoter. DNA methylation, another mechanism of gene repression, can be examined by bisulfate sequencing. Together, these experiments would provide novel information about the effect of n-3 PUFA supplementation on the nuclear translocation of AHR, the ability of AHR to bind DREs, and gene repression mechanisms.

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