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The Role of Omega-3 Polyunsaturated Fatty Acids in Blood Pressure Regulation and Secondhand Smoke-Induced Vascular Dysfunction

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THE ROLE OF OMEGA-3 POLYUNSATURATED FATTY ACIDS IN BLOOD PRESSURE REGULATION AND SECONDHAND SMOKE-INDUCED VASCULAR DYSFUNCTION

by

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B.A., Biochemistry, University Of Colorado Boulder, 2016

THESIS

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DEDICATION

I would like to dedicate this work to my husband, Patrick, who has provided me with undying support during one of the most challenging periods of my life. Your encouragement has been a place of refuge in the turmoil-filled days.

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I would like to thank my mentor, Dr. Mary Walker, as well as my committee members, Dr. Matthew Campen and Dr. Dawn Delfín, for their guidance and wisdom as I faced both success and failure in my time as a graduate student. I am a better scientist today because of it. Secondly, I would like to thank my lab mate, Mary Walsh-Wilcox. To put it simply, I could not have done it without you. Your companionship over the past couple of years has meant the world to me.

The Role of Omega-3 Polyunsaturated Fatty Acids in Blood Pressure Regulation and Secondhand Smoke-Induced Vascular Dysfunction

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ABSTRACT

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) may lower cardiovascular disease risk by reducing blood pressure (BP). We hypothesized that *fat-1* transgenic mice, which convert n-6 PUFAs to n-3 PUFAs, would have improved vascular function in BP regulation and following secondhand smoke (SHS) exposure, compared to wild type (WT) mice.

BP was recorded in mice fed an n-6 PUFA, or standard chow diet. On the n-6 PUFA diet *fat-1* mice had a lower mean arterial pressure and a greater decrease in diastolic BP when treated with an ACE inhibitor, compared to WT mice. No differences were observed on the chow diet. Next, *fat-1* mice on the n-6 PUFA or chow diet were exposed to SHS. SHS enhanced vasoconstriction on the n-6 PUFA diet, but not on chow, but did not affect vasorelaxation.

These data suggest n-3 PUFAs have a protective role in basal BP regulation and SHS-induced vascular dysfunction.

TABLE OF CONTENTS

TABLE OF ABBREVIATIONS

Name	Abbreviation
Acetylcholine	ACh
AhR Interacting Protein	AIP
AhR Nuclear Translocator	ARNT
Analysis of Variance	ANOVA
Angiotensin-Converting Enzyme	ACE
Angiotensin II	Ang II
Angiotensinogen	AGT
Arachidonic Acid	AA
Area Under the Curve	AUC
Aryl Hydrocarbon Receptor	AhR
Blood Pressure	BP
Calcium-Activated Potassium Channel	BK_{Ca}
Calmodulin	CaM
Cardiovascular Disease	CVD
Cyclooxygenase II	COX2
Cytochrome P450	CYP
Diastolic Blood Pressure	DBP
Dioxin-Response Element	DRE
Docosahexaenoic Acid	DHA
Docosapentaenoic Acid (n-3)	n-3 DPA
Docosapentaenoic Acid (n-6)	n-6 DPA
Eicosapentaenoic Acid	EPA
Endothelial Nitric Oxide Synthase	eNOS
Endothelium-Derived Hyperpolarizing Factor	EDHF
Environmental Tobacco Smoke	ETS
Epoxydocosapentaenoic Acid	EDP
Epoxyeicosatetraenoic Acid	EEQ
Epoxyeicosatrienoic Acid	EET
Flow-Mediated Dilation	FMD
Harmful or Potentially Harmful Constituent	HPHC
Heat Shock Protein 90	Hsp90
Heme Oxygenase 1	HMOX1
Human Coronary Artery Endothelial Cell	HCAEC
Human Umbilical Vein Endothelial Cell	HUVEC

Name	Abbreviation
Hypoxanthine-Guanine Phosphoribosyltransferase	HPRT
Linoleic Acid	LA
L-N^{G}-Nitroarginine Methyl Ester	L-NAME
L-N^{ω}-Nitro-L-arginine	L-NNA
Mean Arterial Pressure	MAP
Monounsaturated Fatty Acid	MUFA
NAD(P)H Quinone Dehydrogenase 1	NQO1
Nitric Oxide	NO
Nuclear Factor - Kappa B	NF-κB
Nuclear Factor Erythroid 2-Related Factor 2	Nrf2
Omega-3 Polyunsaturated Fatty Acid	n-3 PUFA
Omega-6 Polyunsaturated Fatty Acid	n-6 PUFA
Peripheral Vascular Resistance	PVR
Perivascular Adipose Tissue	PVAT
Peroxisome Proliferator-Activated Receptor γ	PPARγ
Phenylephrine	PE
Physiological Saline Solution	PSS
Polycyclic Aromatic Hydrocarbon	PAH
Reactive Nitrogen Species	RNS
Reactive Oxygen Species	ROS
Secondhand Smoke	SHS
S-nitroso-N-acetyl-D,L-penicillamine	SNAP
Soluble Epoxide Hydrolase	sEH
Soluble Guanylyl Cyclase	sGC
Spontaneously Hypertensive Rat	SHR
Systolic Blood Pressure	SBP
Tobacco-Specific Nitrosamine	TSNA
Total Antioxidant Capacity	TAC
Total Particulate Matter	TPM
Wild Type	WT
α-linolenic Acid	ALA

LIST OF FIGURES

CHAPTER 1

CHAPTER 3

CHAPTER 4

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 1 – INTRODUCTION

1.1. Omega-3 Polyunsaturated Fatty Acids (n-3 PUFAs) and Cardiovascular Disease

A. Structure and Dietary Sources of n-3 PUFAs

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) are distinguished from other fatty acids in that they contain more than two *cis* double bonds beginning at the third carbon from the omega (methyl) end (Calder and Yaqoob, 2009) (Fig. 1.1).

Eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are considered long-chain n-3 PUFAs and can be derived from the shorter chain n-3 PUFA, α-linolenic acid (ALA, 18:3n-3). Animals do not have the ability to synthesize ALA. As a result, ALA is considered an essential fatty acid. However, ALA can be further desaturated and elongated to EPA and ultimately DHA. In humans, these conversion processes are extremely inefficient (Calder and Yaqoob, 2009). This is due, in part, to competition between ALA and linoleic acid (LA, 18:2n-6) to be converted to EPA and arachidonic acid (AA, 20:4n-6), respectively, by a Δ6 desaturase enzyme. In the Western diet, the amount of LA consumed far exceeds ALA, so conversion of ALA to EPA is limited (Burdge and Calder, 2006). Therefore, EPA and DHA are almost exclusively taken in through diet.

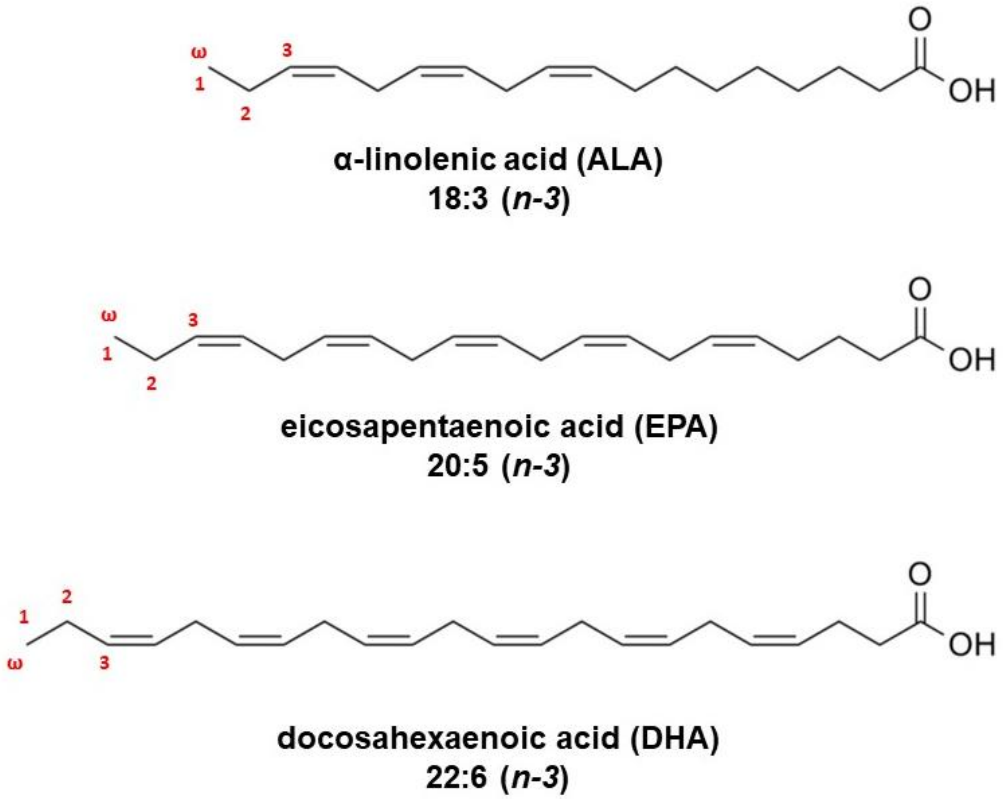


Fig. 1.1. Structure of the short-chain, plant-derived omega-3 polyunsaturated fatty acid (n-3 PUFA), α-linolenic acid (ALA) and the long-chain, marine-derived n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). n-3 PUFAs have a *cis* double bond beginning at the third carbon from the omega (ω) end.

ALA is plant-derived and can be found in leafy green vegetables, pea plants, and a wide variety of seed oils (Williams and Burdge, 2006). In contrast, EPA and DHA are largely derived from marine sources such as tuna, herring, mackerel, anchovies, sardines, salmon, krill, and microalgae (Rimm et al, 2018; Monroig et al., 2013).

The omega-3 index is defined as the erythrocyte content of EPA and DHA and is represented as a percentage of total fatty acids. The omega-3 index is a biomarker for n-3 PUFA consumption and is inversely correlated with cardiovascular health - a low omega-3 index is a cardiovascular disease (CVD) risk factor (von Schacky, 2014). Using previous epidemiological and prospective cohort studies examining the association between n-3 PUFAs and CVD risk, Harris and von Schacky determined that an omega-3 index of ≤4% is of high CVD risk, 4-8% is of intermediate CVD risk, and ≥8% is cardioprotective (Harris and von Schacky, 2004).

B. Protective Mechanisms of n-3 PUFAs

i. Effects of n-3 PUFAs on the Bioavailability of Nitric Oxide

Nitric oxide (NO) is potent signaling molecule produced in vascular endothelial cells that, when present, relaxes vascular smooth muscle cells, resulting in arterial and venous vasodilation. NO in vascular endothelial cells is produced as a product of the conversion of L-arginine to L-citrulline by endothelial nitric oxide synthase (eNOS) upon various stimuli, including shear stress and muscarinic and bradykinin receptor activation. Once produced, NO

diffuses across the cell membranes of vascular smooth muscle cells, in part, through aquaporin-1, and stimulates vasodilation via activation of soluble guanylyl cyclase (sGC) (Frei et al., 2009; Herrera and Garvin, 2007).

A number of studies have suggested that n-3 PUFAs, specifically EPA and DHA, increase NO-dependent vasodilation. Such alterations in NO-dependent vasodilation may be mediated by increases in the expression of two endothelial proteins, heat shock protein 90 (Hsp90) and Akt kinase, which are important participants in the post-transcriptional activation of eNOS (Takahasi and Mendelsohn, 2003). A study on human coronary artery endothelial cells (HCAECs) treated with DHA for 7 days demonstrated an increased activation of Akt kinase (increased phospho-Akt expression) at doses of 5 nM and 50 nM and an increased expression of Hsp90 at doses of 50 nM and 1 μM. Additionally, basal cGMP, a product of sGC-dependent vasodilation, was increased in HCAECs treated with 5 nM DHA (Stebbins et al., 2008).

In unstimulated endothelial cells, eNOS is located within membrane microdomains known as caveolae. Here, eNOS associates with caveolin, a scaffolding protein. Various stimuli promote the binding of eNOS to calmodulin (CaM) and a subsequent dissociation of eNOS from caveolin and the membrane, resulting in activation of eNOS. Another study on bovine endothelial cells *in situ* revealed that treatment of cells with 30 and 60 μM EPA significantly stimulated the production of NO. In addition, in human umbilical vein endothelial cells (HUVECs) treated with 30 μM EPA for 15 minutes, eNOS, to a large degree,

translocated from the plasma membrane to the cytosol, suggesting an activation of eNOS activity (Omura et al., 2001).

Animal studies have echoed the results seen in cell culture studies that n-3 PUFA treatment increases NO-dependent vasodilation. For example, *ex vivo* treatment of isolated pulmonary arteries from sheep with 30 μM EPA resulted in a large degree of vasodilation, which was prevented with L-N^G-nitroarginine methyl ester (L-NAME, NOS inhibitor) treatment or denuding of the endothelium, suggesting increased NOS-derived synthesis of NO (Singh et al., 2010). Similarly, EPA treatment improved acetylcholine (ACh)-induced vasodilation in aortic rings from Apo-E knockout mice, which was attenuated with genetic and/or pharmacologic inhibition of 5'-AMP-activated protein kinase and eNOS (Wu et al., 2012). Furthermore, aortic rings from rats fed a menhaden (fish) oil diet containing EPA and DHA showed no differences in PE constriction compared to rings of control rats fed a corn oil diet. However, when aortic rings were treated with Nω-Nitro-L-arginine (L-NNA, NOS inhibitor) in combination with PE, the fish oil diet resulted in a greater increase in the degree of constriction compared to rings from control rats fed a corn oil diet, indicating a higher basal NO level in fish oil-fed rats. Aortic rings from fish oil-fed rats also relaxed to a greater degree in response to ACh stimulation than did rings from corn oil-fed rats (López et al., 2001). In another study by the same group, it was revealed that fish oil supplementation increases both eNOS mRNA and protein expression as well as production of citrulline, NO, and cGMP (López et al., 2004).

Other studies have proposed that n-3 PUFAs are protective by reducing NO scavenging caused by oxidative stress such as occurs with exposure to cigarette smoke or environmental air pollution, or with cardiovascular diseases, such as hypertension and diabetes (Zuo et al., 2014; Lodovici and Bigagli, 2011; Grossman, 2008). These conditions alter the counterbalance between antioxidants and free radical species such that reactive oxygen and/or nitrogen species (ROS and RNS) concentrations are significantly increased. Free radicals can react with NO, thus reducing the bioavailability of NO. It has been shown that 100 μM EPA and 50 μM DHA treatment prevented doxorubucin-induced increases in ROS in H9C2 (cardiomyocyte) cells (Hsu et al., 2014). Similarly, n-3 PUFA treatment reduced ROS in HUVECs treated with 50 μg/mL fine particles ($PM_{2.5}$) (Bo et al., 2016). Taken together, these studies suggest that EPA and/or DHA treatment improve endothelial function through, in part, reduced scavenging of NO.

ii. Epoxide Metabolites of n-3 PUFAs as Vasodilators

Arachidonic acid (AA), an n-6 PUFA, is readily metabolized by cytochrome P450 epoxygenases, specifically CYP2C and 2J, to epoxide metabolites known as epoxyeicosatrienoic acids (EETs). EETs stimulate vasodilation and are hypothesized to be endothelium-derived hyperpolarizing factors (EDHF). The P450 metabolism of long-chain n-3 PUFAs also produces epoxides known as epoxyeicosatetraenoic acids (EEQs; EPA-derived) and epoxydocosapentaenoic acid (EDPs; DHA-derived). EEQs and EDPs are also vasodilators and may be

even more potent than EETs. For instance, *ex vivo* administration of EPA-derived epoxides to canine and porcine coronary microvessels resulted in a similar or greater degree of vasodilation and stimulation of calcium-activated potassium (BK_{Ca}) channels as compared to administration of AA-derived epoxides (Xhang et al., 2001). EETs, EEQs, and EDPs can be further metabolized by soluble epoxide hydrolase (sEH) to diols, which have considerably lower bioactivity.

There is some evidence to suggest that EEQs and EDPs are efficacious in lowering systolic blood pressure (SBP) in mouse models of angiotensin II (Ang II)-dependent hypertension. Mice infused with Ang II and concurrently treated with a n-3 PUFA-rich diet and an sEH inhibitor demonstrated decreases in SBP as compared to Ang II-infused mice fed a corn oil diet (Ulu et al., 2013). In a follow-up study, the efficacy of 19,20-EDP to lower SBP was assessed in relation to that of 14,15-EET, an epoxide metabolite of AA. In combination with a sEH inhibitor, treatment of mice with 19,20-EDP reduced blood pressure by a greater extent than did 14,15-EET (Ulu et al., 2014).

The mechanism through which epoxide metabolites of long-chain n-3 PUFAs act as vasodilators has not been fully elucidated. It has been proposed that EDPs and EEQs potently activate large-conductance calcium-activated potassium (BK_{Ca}) channels located within the cell membranes of vascular smooth muscle cells, causing hyperpolarization of the cell membrane and inactivation of voltage-dependent Ca^{2+} channels, thereby inducing relaxation (Ye et al., 2001; Morin et al., 2008; Ledoux et al., 2006). It has been reported that the BKα

channel subunit specifically is responsible for the vasodilatory effects of n-3 PUFA epoxide metabolites (Hercule et al., 2007).

iii. Keto-Metabolites of n-3 PUFAs as Antioxidants

Another pathway of n-3 PUFA metabolism is a two-step process involving cyclooxygenase-II (COX-2) and cellular dehydrogenases within activated macrophages, generating α,β-unsaturated keto-derivatives (Cipollina, 2015; Groeger et al., 2010). These electrophilic derivatives adduct nucleophilic protein residues - the electron-poor β-carbon of the electrophile accepts a pair of electrons from the electron-rich nucleophile (Cipollina, 2015). Treatment with n-3 PUFAs in healthy adults has been shown to increase the formation of α,β-unsaturated keto-derivatives such as 7-oxo-DHA and 5-oxo-EPA without modulating AA metabolites (Cipollina et al., 2014b). A number of studies suggest that the antioxidant interactions between n-3 PUFA electrophilic oxo-derivatives and nucleophiles mediate the cardiovascular protective effects seen n-3 PUFA treatment.

One mechanism through which keto-metabolites of n-3 PUFAs are protective is by serving as an antioxidant in conditions of oxidative stress. α,β-unsaturated keto-derivatives activate the cytoprotective and antioxidant nuclear factor erythroid 2-related factor 2 (Nrf2) pathway. 17-oxo-DHA, an oxo-derivative of DHA, has been shown to induce Nrf2, thereby leading to the transcriptional activation of cytoprotective enzymes such as heme oxygenase 1 (HMOX1) and

NAD(P)H quinone dehydrogenase 1 (NQO1) as well as increased production of glutathione (Cipollina et al., 2014a).

Another potential mechanism through which n-3 PUFA α,β-unsaturated keto-derivatives may be protective is by suppressing inflammation. For instance, treatment of peripheral blood mononuclear cells with 17-oxo-DHA reduced both the transcriptional activation and release of IL-1β and TNFα; these cytokines play a significant role in the initiation and persistence of inflammation (Cipollina et al., 2016). Secondly, α,β-unsaturated keto-derivatives serve as inhibitors of the pro-inflammatory nuclear factor-kappa B (NF-κB) pathway, thus leading to reduced inflammation. Electrophilic n-3 PUFA oxo-derivatives suppress p65, leading to an inability of the NF-κB/p65/p50 complex to bind DNA to activate transcription of inflammatory mediators (Cipollina 2015). Furthermore, α,β-unsaturated keto-derivatives of n-3 PUFAs serve as partial agonists in the peroxisome proliferator-activated receptor γ (PPARγ) pathway, which decreases the inflammatory response, especially within vascular endothelial cells (Cipollina et al., 2015; Hamblin et al., 2009).

iv. n-3 PUFAs and the Renin-Angiotensin System

The renin-angiotensin system (RAS) is a major contributor to basal blood pressure regulation and its ultimate product is Ang II, a vasoconstrictor. Recently, evidence has emerged, which suggests that n-3 PUFAs may be cardioprotective by decreasing the activation of the RAS pathway. In a randomized controlled trial of 126 hypertensive individuals, a 2 g/day dose of EPA and DHA over 90 days

significantly decreased SBP compared to individuals given corn oil. This reduction in SBP was accompanied by significantly lower plasma concentrations of Ang II compared to those treated with corn oil (Yang et al., 2019). This data suggests that n-3 PUFAs may be protective by inhibiting the formation of Ang II, thereby preventing Ang II-dependent vasoconstriction.

C. Effects of n-3 PUFAs on Blood Pressure

Hypertension is one of the most common heath conditions worldwide and often precedes many cardiovascular diseases, including ischemic heart disease, stroke, and premature death (Kearney et al., 2005; Kung et al., 2015). It has long been postulated that n-3 PUFAs may have a hypotensive effect due to their vasodilatory, oxidative stress reducing, and antioxidant potential.

Evidence suggests that n-3 PUFAs reduce blood pressure in both hypertensive and normotensive individuals. For example, a meta-analysis of 17 randomized controlled trials regarding the effects of fish oil supplementation on blood pressure found significant reductions in both systolic and diastolic blood pressure (DBP) in hypertensive individuals when placed on an n-3 PUFA intervention. In addition, there were non-significant reductions in systolic and DBP in normotensive individuals when placed on the same n-3 PUFA interventions (Campbell et al., 2012). Another meta-analysis examining fish consumption found that circulating levels of long-chain n-3 PUFA, which are highly correlated with increased consumption of EPA and DHA, were significantly associated with a decreased incidence of elevated blood pressure (Yang et al.,

2016). Furthermore, a recent cross-sectional study of young, normotensive adults reported a linear inverse association of n-3 PUFA consumption with systolic and DBP. Individuals in the highest omega-3 index quartile had systolic and DBPs that were 4 and 2 mmHg lower, respectively, as compared to individuals in the lowest quartile (Filipovic et al., 2018).

A limited number of animal studies have yielded some mechanistic details regarding the potential hypotensive effects of n-3 PUFAs. In a rat model of Ang II-dependent hypertension (Ren-2 rat), consumption of an n-3 PUFA-enriched diet significantly reduced SBP, indicating that n-3 PUFAs and/or their metabolites may protect against Ang II-mediated hypertension (Jayasooriya et al., 2007). Secondly, intravenous (IV) administration of DHA to wild type mice reduced arterial blood pressure; in contrast, IV administration of DHA to Slo1 (pore-forming subunit of large-conductance Ca^{2+} - and voltage-activated K^{+} (Slo1 BK) channels) knockout mice did not alter blood pressure. This suggests that n-3 PUFAs may reduce blood pressure by activating the pore-forming unit of BK channels, thus leading to hyperpolarization and vasodilation (Hoshi et al., 2013).

In another model, spontaneously hypertensive rats (SHRs) and normotensive Wistar Kyoto rats were fed a PUFA-enriched diet containing EPA, DHA, and γ-linolenic acid (18:3n-6) for 10 weeks. This diet was associated with a significant drop in blood pressure and an increase in red blood cell total antioxidant status in SHRs, but not the Wistar Kyoto controls (Frenoux et al., 2000). Similarly, SHRs fed a krill oil diet had a small, but significant reduction in SBP that was not seen in normotensive Sprague Dawley rats. The blood

pressure decrease in SHRs was accompanied by an increase in total serum NOS activity (Zhou et al., 2017). These animal studies suggest that n-3 PUFAs prevent hypertension by stimulating vasodilation, increasing antioxidant status, and increasing NO.

1.2. Secondhand Cigarette Smoke

A. Cigarette Smoke Composition

Tobacco cigarette smoke is a complex mixture of at least 5,000 chemicals consisting of droplets, particles, and gases (Talhout et al., 2011; Streibel et al., 2013). Upward of 100 of these chemicals are harmful or potentially harmful constituents (HPHCs) and many are mutagenic and/or carcinogenic (Margham et al., 2016). Most notably, cigarette smoke contains toxic compounds such as polycyclic aromatic hydrocarbons (PAHs), nicotine, tobacco-specific nitrosamines (TSNAs), aromatic amines, acetaldehyde, formaldehyde, benzene, and various toxic metals (US Department of Health and Human Services, 2006).

PAHs are generated from the incomplete combustion of organic material and are a prominent component of cigarette smoke. Cigarette smoke contains more than 500 PAHs; amongst these, benzo(a)pyrene being is recognized as the most potent procarcinogenic PAH (Vu et al., 2015). The toxic and carcinogenic effects of PAH exposure are mediated through activation of the aryl hydrocarbon receptor (AhR), a cytosolic ligand-activated transcription factor. In its unbound state, the AhR is complexed with a heat shock protein 90 dimer and AhR interacting protein (AIP). When present, PAHs diffuse across the cellular

membrane and bind the AhR, thereby causing a conformational change allowing the ligand-bound complex to be translocated into the nucleus. After dissociating from chaperone proteins, the AhR-ligand complex forms a heterodimer with the AhR nuclear translocator (ARNT), thus allowing the AhR to directly interact with dioxin-response elements (DREs) contained within DNA. Such interactions induce the transcription of several genes, including phase I metabolic enzymes cytochrome P450 (CYP) 1A1 and 1B1 (*Cyp1a1* and *Cyp1b1*). These enzymes bioactivate PAHs such as benzo(a)pyrene to more reactive compounds that form adducts within DNA, resulting in mutagenesis and possibly carcinogenesis (Moorthy et al., 2015).

B. Mainstream versus Sidestream Smoke

When a cigarette is ignited, two chemically distinct tobacco smokes are generated. Mainstream smoke refers to the smoke that is inhaled and subsequently exhaled by a smoker, while sidestream smoke refers to the smoke originating from the burning end of a cigarette. Secondhand smoke (SHS), also referred to as environmental tobacco smoke (ETS), is a combination of approximately 85 percent sidestream smoke and 15 percent exhaled mainstream smoke (US Department of Health and Human Services, 2006).

Qualitatively, mainstream and sidestream smoke share many similarities; however, their constituents differ in terms of quantity, thus resulting in different toxicological profiles (US Department of Health and Human Services, 2006; Streibel et al., 2013). The vast majority of cigarette smoke components are

present at a higher concentration in sidestream smoke than mainstream smoke, especially PAHs (Streibel et al., 2013). This is namely due to the fact that sidestream smoke is generated at lower temperatures than is mainstream smoke, which results in an incomplete combustion and a production of larger amounts of PAHs relative to mainstream smoke . Additionally, sidestream smoke becomes significantly diluted when exposed to room air, which often results in changes in the chemical and physical properties of the smoke (Streibel et al., 2013). Lastly, sidestream smoke is unfiltered, while mainstream smoke typically is filtered, thereby altering the composition of the smoke. All of these factors contribute to differences in both the concentrations and types of combustion products present within the smoke.

It has been reported that, at equal doses, sidestream smoke/SHS is more toxic than mainstream smoke. Based upon a series of unpublished studies performed on rats by the Philip Morris Tobacco Company in the 1980s, sidestream smoke was found to be four times more lethal than mainstream smoke per gram total particulate matter (Schick and Glantz, 2005).

1.3. Cardiovascular Disease in Passive Smokers

A. Health Impacts of Exposure to Secondhand Tobacco Smoke

Exposure to SHS is one of the most substantial causes of indoor air pollution worldwide with up to 40 percent of children, 35 percent of women, and 33 percent of men exposed on a regular basis (Öberg et al., 2011). This ultimately leads to an estimated 603,000 deaths worldwide (in the year 2004)

attributable to SHS exposure (Öberg et al., 2011). A majority of these deaths occur in third world and developing countries where there is still a large population of active smokers and/or few to no indoor smoking regulations (Stone and Zhou, 2016).

Exposure to SHS increases the risk of developing cardiovascular disease (CVD) by as much as 30 percent (US Department of Health and Human Services, 2006). CVD encompasses a large family of diseases of the cardiovascular system, including atherosclerosis, ischemic heart disease, coronary heart disease, peripheral vascular disease, hypertension, myocardial infarction, stroke, aortic aneurysm, and others (GBD 2013 Mortality and Causes of Death Collaborators, 2013; World Health Organization).

There are several mechanisms through which exposure to SHS negatively impacts the vasculature. SHS impairs endothelial dysfunction, promotes a pro-thrombotic environment in the vasculature, causes oxidative stress, and reduces heart rate variability (Celermajer et al., 1996; Otsuka et al., 2001; Kato et al., 2006; Pope et al., 2001). All of these manifestations of SHS exposure contribute to an increased CVD risk.

B. Mechanisms of Secondhand Smoke – Induced Vascular Disease

i. Endothelial Dysfunction

Endothelial dysfunction precedes clinical CVD and is characterized by impaired endothelium-dependent vasodilation resulting, in part, from a reduced bioavailability of NO. In dysfunctional endothelial cells, loss of NO can result from

decreased production and/or increased scavenging by reactive free radicals (Zanetti et al., 2015). Mechanisms involved in reduced production of NO include uncoupling of eNOS, posttranslational modifications to eNOS, inhibition of eNOS, and downregulation of eNOS (Förstermann and Münzel, 2006; Karbach et al., 2014). Increased scavenging largely occurs in conditions of oxidative stress in which there is an abundance of ROS and RNS.

A vast number of studies have reported that exposure to SHS causes endothelial dysfunction. Passive smokers have impaired flow-mediated dilation (FMD) of the brachial artery as compared to those not exposed to SHS, indicating the presence of endothelial dysfunction in passive smokers (Celermajer et al., 1996). Similarly, nonsmokers acutely exposed to SHS experience a significant reduction in coronary flow velocity nearly equal to the levels seen in active smokers (Otsuka et al., 2001). Additionally, using ultrasound, the diameter of epicardial coronary artery of female nonsmokers, passive smokers, and active smokers was monitored after administration of ACh. The arteries of passive and active smokers constricted, while the arteries of nonsmokers dilated (Sumida et al., 1998). Both of these studies suggest a similar degree of endothelial dysfunction in passive and active smokers.

Endothelial dysfunction is an important contributing factor to the development and progression of atherosclerosis. Normal, healthy endothelial cells, largely through the production of NO, promote vasodilation and inhibit the formation of atherosclerosis and thrombosis. However, dysfunctional endothelial cells promote vasoconstriction and support a pro-inflammatory and pro-

thrombotic environment (US Department of Health and Human Services, 2006). In conditions of endothelial dysfunction, endothelial cells lose their non-thrombogenic ability and platelets begin to adhere all the while releasing chemoattractants and mitogens, thereby encouraging proliferation of vascular smooth muscle cells (Ross, 1993). Atherosclerosis ultimately perpetuates many other cardiovascular diseases.

ii. Oxidative Stress

Oxidative stress is an overarching theme in the development of SHS-induced CVD. Oxidative stress refers to an imbalance between free radicals and antioxidant defense systems that leads to increased accumulation of ROS and RNS. Free radicals such as superoxide anion ($O_2^{\cdot -}$), nitric oxide ($NO^{\cdot}$), and hydroxyl radical ($H^{\cdot}$), and reactive compounds such as peroxynitrite ($ONOO^{-}$) are produced during normal biochemical processes such as aerobic respiration, oxidation of catecholamines, and activation of electrons in the AA cascade (Betteridge, 2000). However, at increased concentrations of ROS and RNS, cell and tissue damage results, which negatively affects the vasculature (Betteridge, 2000).

Importantly in CVD, under oxidative stress, NO can react with superoxide to form peroxynitrite (US Department of Health and Human Services, 2006). Not only does this process reduce NO bioavailability, but it also creates a highly toxic reactive compound. Uncoupling of eNOS, lipid peroxidation, protein peroxidation and nitration, and inactivation of various enzymes all promote formation of free

radicals and the perpetuation of oxidative stress (Förstermann and Münzel, 2006; Novo and Parola, 2008).

1.4. Limitations of Smoke-Free Legislation

Despite popular belief, designated smoking areas do not constitute an adequate means of preventing SHS exposure since ventilation systems within buildings and environmental conditions do not allow smoke to be contained (Hammond, 2002; Yamoto et al., 2013). However, the implementation of smoke-free regulations at bars, casinos, restaurants, workplaces, and other public spaces is an effective means of reducing exposure to SHS. Amongst those benefiting significantly from smoke-free legislation are children and pregnant women. In these populations, smoke-free legislation has been associated with fewer perinatal deaths, preterm births, and hospital visits for respiratory tract infections/asthma in children (Faber et al., 2016). Recent studies also suggest that smoke-free legislation may encourage smokers to quit and may discourage teenagers from becoming smokers (Katikireddi et al., 2016).

Despite its reported successes, more than 80 percent of the world's population remains unprotected by smoke-free legislation (WHO Report on the Global Tobacco Epidemic, 2015). Additionally, smoke-free legislation does not protect from SHS exposure in private places such as in vehicles and/or at home. An analysis of the 2016 National Youth Tobacco Survey of U.S. 6^{th} to 12^{th} graders suggested that, among the 20,675 participants, 21.4% are exposed to SHS in vehicles and 21.7% are exposed at home (Agaku et al., 2019). As a

result, smoking cessation is the ultimate goal and programs have been established by numerous organizations to promote cessation. However, there remain many individuals that cannot or choose not to quit and as many as 58 million individuals continue to be exposed to SHS. As a consequence, it is necessary to develop alternative strategies to counter the cardiovascular morbidity that is brought about by exposure to SHS.

1.5. Rationale for Research

A. Aims and Hypotheses

The benefit and efficacy of n-3 PUFAs in reducing blood pressure, especially in normotensive individuals, has been heavily debated and there is a lack of preclinical animal studies that have investigated the relationship between n-3 PUFAs and blood pressure regulation. Therefore, we aimed to investigate the role of n-3 PUFAs in the blood pressure regulation of *fat-1* transgenic mice and their wild type (WT) littermates. The *fat-1* transgenic mouse contains a fatty acid desaturase enzyme from *Caenorhabditis elegans* that allows for the efficient conversion of n-6 PUFAs to n-3 PUFAs (Kang et al., 2004). Since *fat-1* mice make their own n-3 PUFAs, they do not require supplementation through diet. We hypothesized that *fat-1* mice would have lower blood pressure as a result of decreased contribution of Ang II to blood pressure regulation, compared to WT mice on the same diet.

Epidemiological and animal studies have suggested that treatment with n-3 PUFAs reduces CVD risk. In contrast, exposure to SHS increases CVD risk by

up to 30 percent (US Department of Health and Human Services, 2006). This raises the question of whether treatment with n-3 PUFAs could counteract the vascular damage caused by exposure to SHS. We aimed to determine the effects of SHS exposure on the vascular function on *fat-1* mice fed an n-6 PUFA diet or a standard chow diet with the hypothesis that n-3 PUFAs would protect against SHS-induced endothelial dysfunction and oxidative stress by preserving NO bioavailability.

CHAPTER 2 – METHODS

2.1. The Role of Omega-3 Polyunsaturated Fatty Acids in Blood Pressure Regulation

A. Chemicals

Chemicals for physiological saline solution (PSS: NaCl, KCl, KH_2PO4, $MgSO_4$, $NaHCO_3$, dextrose, $CaNa_2EDTA$, and $CaCl_2$,), phenylephrine (PE), and acetylcholine (ACh) were purchased from Sigma-Aldrich (St. Louis, MO). Captopril was purchased from Fisher Scientific (Pittsburgh, PA).

B. Animals and Diet

Male *fat-1* transgenic mice and their WT littermates (hereafter referred to as WT) were placed on an n-6 PUFA-enriched diet at seven weeks of age (TD.110517, 15% safflower oil, Teklad Diets, Envigo, Indianapolis, IN) (Table 2.1). Mice were maintained on the n-6 PUFA-enriched diet for a total of eight weeks at which point mice were switched to a standard chow diet (2920X Rodent Diet, Teklad Diets, Envigo, Indianapolis, IN) for an additional four weeks. All animal protocols were approved by the University of New Mexico Animal Care and Use Committee.

TABLE 2.1. Fatty acid content of n-6 PUFA and standard chow diets.

	Total Fatty Acids (% of Diet)	Calories From Fat (%)	% of Total Fatty Acids			
			Saturated	MUFA[b]	PUFA[c]	
					n-6 PUFA	n-3 PUFA
n-6 PUFA	15.0[a]	32.6	8.9	13.1	78.0	0.0
Standard Chow	6.5	16.0	14.5	20.0	47.3	5.5

aValues estimated from data sheets provided by the manufacturer.
[b]MUFA: monounsaturated fatty acid.
[c]PUFA: polyunsaturated fatty acid.

C. Aortic Vasoreactivity

Mice were anesthetized with an intraperitoneal (i.p.) injection ketamine/xylazine (80/4 mg/kg) and were subsequently euthanized by exsanguination. The thoracic aorta was excised and placed in ice-cold PSS (130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO4, 1.17 mM $MgSO_4$, 14.9 mM $NaHCO_3$, 5.5 mM dextrose, 26 mM $CaNa_2EDTA$, 1.8 mM $CaCl_2$, pH 7.4). The aorta was cleaned of perivascular adipose tissue (PVAT), sectioned into two 3 mm rings, and mounted to a wire myograph (Radnoti Glass Technologies, Monrovia, CA) attached to a force transducer (Grass Technologies, West Warwick, RI). Aortic rings were contained in 37°C PSS bubbled with 20% O_2, 5% CO_2, and balance N_2. Aortic rings were allowed to equilibrate for 80 minutes, gradually increasing to a baseline tension of 1500 mg. The rings were then constricted with 100 mM KCl in order to confirm viability. To ensure an intact endothelium, aortic rings were constricted with a bolus dose of PE (10^{-5} M) and subsequently dilated with ACh (10^{-5} M). Dose-dependent constriction to PE (10^{-}

$^{7.5}$ – $10^{-5.0}$ M) was assessed and was immediately followed by a dose-dependent relaxation to ACh ($10^{-8.2}$ – $10^{-5.1}$ M).

D. Measurement of Blood Pressure, Heart Rate, and Activity

At 10 weeks of age, male WT and *fat-1* mice were surgically implanted with PA-C10 radiotelemeters (Data Sciences International, St. Paul, MN) to monitor mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), and activity. MAP was calculated as $MAP \cong DBP + \frac{1}{3}(SBP - DBP)$. Mice were allowed to recover for 3-4 weeks before baseline recordings began. Blood pressure was recorded for a 10-second period once every 15 minutes for a total of 6 days (3 days of baseline and 3 days of captopril treatment) while mice were fed an n-6 PUFA-enriched diet. This procedure was repeated when mice were switched to a standard chow diet.

E. Captopril Treatment

For each diet, after 3 days of baseline recording, WT and *fat-1* mice were administered captopril, an angiotensin-converting enzyme (ACE) inhibitor, in drinking water at a dose of 4 mg/kg/day for a 3-day duration. Before sacrifice, mice underwent a 3-day washout period.

F. Body and Tissue Weights

Body weights were determined twice weekly beginning at the date of surgery and continuing until mice were sacrificed. Mice were anesthetized with

an intraperitoneal (i.p.) injection of ketamine/xylazine (80/4 mg/kg) and then euthanized by exsanguination. Whole blood was collected by cardiac puncture and serum was obtained by centrifugation at 3000 g and 4°C for 15 minutes. The heart was collected and atria were removed to obtain a total heart weight. Kidney weights were recorded as well. Serum, heart, kidneys, and abdominal/thoracic aorta were collected, flash frozen, and stored at -80°C for future analyses.

G. Fatty Acid Analysis

Approximately 50 µL of whole blood was collected onto antioxidant-treated filter paper provided by OmegaQuant (Sioux Falls, SD) from mice on a standard chow diet, mice on an n-6 PUFA-enriched diet for eight weeks, and mice on an n-6 PUFA-enriched diet for eight weeks followed by chow diet for four weeks. These dried blood spots were sent to OmegaQuant (Sioux Falls, SD) for analysis of fatty acid content using a gas chromatographic method described previously (Harris and Polreis, 2016).

H. Gene Expression

Total RNA was isolated from lung, heart, and liver using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was isolated from adipose tissue using a combined TRIzol and miRNeasy kit (Qiagen, Hilden, Germany) method (Cirera, 2013). cDNA was generated using an iScript Reverse Transcription Supermix (BioRad Laboratories, Inc., Hercules, CA). qPCR analysis was performed using iTaq Universal SYBR Green Supermix (Bio-Rad), cDNA, and forward and

reverse primers for hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) or angiotensinogen (*Agt*) on the CFX Connect Real-Time PCR Detection System (Bio-Rad) (Table 2.2).

TABLE 2.2. Real-Time qPCR Primer Sequences

Gene	Forward Primer	Reverse Primer
Hprt	5' AGGGATTTGAATCAGTTTG	5' TTTACTGGCAACATCAACAG
Agt	5' TCCTGACTTGGATAGTGAAC	5' CTATTGAGAACCTCTCCCAC

I. Statistical Analysis

Fatty acid profiles of WT and *fat-1* mice on an n-6 PUFA diet and on a standard chow diet were compared using Two-Way Analysis of Variance (ANOVA). Body weight, MAP, SBP, DBP, pulse pressure, heart rate, and activity of WT and *fat-1* mice were compared to each other at baseline and during captopril treatment within each diet using t-test. ΔMAP, ΔSBP, ΔDBP, Δheart rate, and Δactivity (change from baseline) resulting from captopril treatment as well as *Agt* mRNA expression were compared in WT and *fat-1* mice using t-test.

2.2. The Role of Omega-3 Polyunsaturated Fatty Acids in Secondhand Smoke-Induced Vascular Dysfunction

A. Chemicals and Materials

3R4F research cigarettes were purchased from the University of Kentucky (Lexington, KY). Chemicals for PSS (NaCl, KCl, KH_2PO4, $MgSO_4$, $NaHCO_3$, dextrose, $CaNa_2EDTA$, and $CaCl_2$,), PE, ACh, and Nω-Nitro-L-arginine (L-NNA)

were purchased from Sigma-Aldrich (St. Louis, MO). U46619 and S-nitroso-N-acetyl-D,L-penicillamine (SNAP) were purchased from Cayman Chemical (Ann Arbor, MI).

B. Animals and Diet

Male *fat-1* transgenic mice were housed in a temperature-controlled environment and were fed a standard chow diet (2920X Rodent Diet, Teklad Diets, Envigo, Indianapolis, IN) until four weeks of age. At four weeks old, half of the mice were switched to an n-6 PUFA-enriched diet (TD.110517, 15% safflower oil, Teklad Diets, Envigo, Indianapolis, IN) for a total of eight weeks, while the other half of the mice remained on the chow diet until the conclusion of the study (see Table 2.1 on page 21).

C. Exposures to Secondhand Tobacco Smoke

Fat-1 mice were assigned to either air- or a secondhand smoke (SHS)-exposed groups according to their diet (four groups: n-6 PUFA/Air, n-6 PUFA/SHS, Chow/Air, Chow/SHS, n = 6-9 per group). Beginning at eight weeks of age, mice were exposed to the whole-body sidestream smoke of nine University of Kentucky 3R4F reference cigarettes (9.4 mg tar and 0.73 mg nicotine per cigarette). This process was repeated five days per week for a total of four weeks. Sidestream smoke was generated using the SCIREQ inExpose system (SCIREQ, Montreal, Canada). Mainstream smoke was produced and immediately evacuated by a two second, 35 mL puff per minute. A bias flow of

1.2 L/min from the burning end of the cigarette maintained air and sidestream smoke concentrations within the whole-body exposure chamber. Total particulate matter (TPM) was measured every two weeks and was maintained at an average of 115.4 mg/m^3. Mice were sacrificed 24 to 72 hours after the conclusion of the last SHS exposure.

D. Body and Tissue Weights

Body weights were determined twice daily for the eight week duration of the study. Urine was collected onto a sheet of parafilm upon removing mice from their cages and stored at -80°C. Mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine/xylazine (80/4 mg/kg) and were subsequently euthanized by exsanguination. Whole blood was collected by cardiac puncture and serum was obtained by centrifugation at 3000 g and 4°C for 15 minutes. The heart was collected and atria were removed to obtain a total heart weight. The right ventricle (RV) was dissected away to obtain right ventricle and left ventricle plus septal weights (LV+S). The LV+S section was divided into two pieces – one half fixed in 10% neutral buffered formalin and the other flash frozen and stored at -80°C. Additionally, lung, liver, and kidneys were weighed and these tissues plus abdominal adipose were flash frozen and stored at -80°C.

E. Fatty Acid Analysis

Approximately 50 µL of whole blood was collected onto antioxidant-treated filter paper cards supplied by OmegaQuant (Sioux Falls, SD) from mice on a

standard chow diet, mice on an n-6 PUFA-enriched diet for four weeks, and mice on an n-6 PUFA-enriched diet for eight weeks. Dried blood spots were sent to OmegaQuant for analysis of fatty acid content using a gas chromatographic method described previously (Harris and Polreis, 2016).

F. Aortic Vasoreactivity

After 4 weeks of SHS or air exposure, *fat-1* mice were euthanized as described above. The thoracic aorta was excised and placed in ice-cold PSS (130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO4, 1.17 mM $MgSO_4$, 14.9 mM $NaHCO_3$, 5.5 mM dextrose, 26 mM $CaNa_2EDTA$, 1.8 mM $CaCl_2$, pH 7.4). The aorta was cleaned of perivascular adipose tissue (PVAT), sectioned into two 3 mm rings, and mounted to a wire myograph (Radnoti Glass Technologies, Monrovia, CA) attached to a force transducer (Grass Technologies, West Warwick, RI). Aortic rings were contained in 37°C PSS bubbled with 20% O_2, 5% CO_2, and balance N_2. Aortic rings were allowed to equilibrate for 80 minutes, gradually increasing to a baseline tension of 1500 mg. The rings were then constricted with 100 mM KCl in order to confirm viability. To ensure an intact endothelium, aortic rings were constricted with a bolus dose of PE (10^{-5} M) and subsequently dilated with ACh (10^{-5} M). Afterward, dose-dependent constriction to either PE ($10^{-7.5}$ – $10^{-5.0}$ M) or U46619 ($10^{-9.0}$ – $10^{-8.3}$ M) was assessed and was immediately followed by a dose-dependent relaxation to either ACh ($10^{-9.0}$ – $10^{-8.3}$ M) or SNAP ($10^{-9.0}$ – $10^{-8.3}$ M). PE, U46619, and ACh dose responses were

completed in the absence and presence of L-NNA (10 μM, 30 minute pre-incubation), a NOS inhibitor.

G. Urinary Cotinine

Urine was collected from mice within 18 hours of the last exposure. Urinary cotinine, a metabolite of nicotine, was assessed as an index of exposure to SHS using a cotinine ELISA kit (MyBioSource, Inc., San Diego, CA).

H. Gene Expression

Total RNA was isolated from lung, heart, and liver using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was isolated from adipose tissue using a combined TRIzol and miRNeasy kit method (Cirera, 2013). cDNA was generated using an iScript Reverse Transcription Supermix (BioRad Laboratories, Inc., Hercules, CA). qPCR analysis was performed using iTaq Universal SYBR Green Supermix (Bio-Rad), cDNA, and forward and reverse primers for hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), Cytochrome P450 1A1 (*Cyp1a1*), Cytochrome P450 1B1 (*Cyp1b1*), NADP(H) quinone dehydrogenase 1 (*Nqo1*), or heme oxygenase 1 (*Hmox1*) on the CFX Connect Real-Time PCR Detection System (Bio-Rad) (Table 2.3).

TABLE 2.3. Real-Time qPCR Primer Sequences

Gene	Forward Primer	Reverse Primer
Hprt	5' AGGGATTTGAATCAGTTTG	5' TTTACTGGCAACATCAACAG
Cyp1a1	5' GGAACTAGACACAGTGATTG	5' TTGGGGATATAGAAGCCATTC
Cyp1b1	5' ACTATTACGGACATCTTCGG	5' ATCTGGTAAAGAGGATGAGC
Nqo1	5' CCTTTCCAGAATAAGAAGACC	5' AATGCTGTAAACCAGTTGAG
Hmox1	5' CATGAAGAACTTTCAGAAGGG	5' TAGATATGGTACAAGGAAGCC

I. Oxidative Stress Analysis

Total antioxidant capacity was assessed as an index of oxidative stress using a commercially available kit (Cayman Chemical, Ann Arbor, MI).

J. Histology

The left ventricle of each heart was fixed in 10% neutral buffered formalin (NBF) and stored at 4°C. Paraffin-embedded sections were stained using picrosirius red and visualized using light microscopy. The degree of fibrosis within the cardiac sections was assessed qualitatively.

K. Statistical Analysis

Diet and exposure comparisons of body and tissue weights, fatty acid profiles, total antioxidant capacity, and gene expression from *fat-1* mice were assessed using Two Way ANOVA. Constriction and relaxation dose response curves were compared between air- and SHS-exposed *fat-1* mice using Two-Way Repeated Measures (RM) ANOVA. Urinary cotinine concentrations and area under the curve (AUC) was analyzed by t-test.

CHAPTER 3 – RESULTS – The Role of Omega-3 Polyunsaturated Fatty Acids in Blood Pressure Regulation

3.1. Body Weight at Baseline Did Not Differ Between WT and Fat-1 Mice on Either Diet

Body weight at baseline did not differ significantly between WT and *fat-1* mice when fed an n-6 PUFA diet or a standard chow diet (Table 3.1). At baseline, WT mice fed a standard chow diet weighed significantly more than WT mice fed an n-6 PUFA diet. This effect was not seen in *fat-1* mice.

TABLE 3.1. Average body weight of WT and *fat-1* mice measured at baseline when fed an n-6 PUFA diet and a standard chow diet.

	Body Weight at Baseline (g)		**Average Age**
	WT	*Fat-1*	
n-6 PUFA	28.0 ± 0.6[a]	27.1 ± 0.8	15 Weeks
Standard Chow	32.9 ± 1.0*	39.5 ± 1.7	20 Weeks

[a]Values are expressed as mean ± SE (n=5). Data were analyzed by t-test.
*$p<0.05$ versus WT mice fed an n-6 PUFA diet.

3.2. Genotype and Diet Significantly Altered Red Blood Cell Fatty Acid Composition

To determine the effects of genotype and diet on fatty acid composition, levels of red blood cell fatty acids were assessed. When mice were fed an n-6 PUFA diet, levels of all n-3 PUFAs, including ALA, EPA, DHA, and docosapentaenoic acid (n-3 DPA), were significantly higher in *fat-1* mice compared to WT mice (Table 3.2). In contrast, levels of the n-6 PUFAs, AA, docosatetraenoic acid, and docosapentaenoic acid (n-6 DPA), were significantly

lower in *fat-1* mice compared to WT when fed an n-6 PUFA diet. When mice were fed a standard chow diet, levels of EPA, n-3 DPA, and DHA as well as omega-3 index (red blood cell percentage of EPA and DHA) were significantly higher in *fat-1* mice compared to WT. Levels of the n-6 PUFAs, LA, AA, docosatetraenoic acid, and n-6 DPA, were significantly lower in *fat-1* mice relative to WT.

When comparing between diets, both WT and *fat-1* mice fed a standard chow diet had significantly higher levels of all n-3 PUFAs and significantly lower levels of all n-6 PUFAs, except eicosatrienoic acid, compared to WT and *fat-1* mice fed the n-6 PUFA diet.

In addition, the omega-3 index was significantly higher in *fat-1* mice versus WT on both diets, and was significantly higher in mice fed the standard chow diet, compared to the n-6 PUFA diet (Fig. 3.1A). As the omega-3 index decreased, the n-6 PUFA/n-3 PUFA ratio tended to increase (Fig. 3.1B). WT and mice fed an n-6 PUFA diet had a significantly higher n-6 PUFA/n-3 PUFA ratio compared to WT and *fat-1* mice fed a standard chow diet. On each diet, WT mice had a significantly higher n-6 PUFA/n-3 PUFA ratio relative to their *fat-1* counterparts.

TABLE 3.2. Red blood cell fatty acid composition of WT and *fat-1* mice fed an n-6 PUFA diet for 8 weeks and a standard chow diet for 4 weeks.

	n-6 PUFA Diet		Standard Chow Diet	
Fatty Acid	WT	*Fat-1*	WT	*Fat-1*
n-3 PUFAs				
C18:3n3 (α-Linolenic, ALA)	0.03 ± 0.01[a]	**0.15 ± 0.01***	0.38 ± 0.03[#]	0.46 ± 0.07[#]
C20:5n3 (Eicosapentaenoic, EPA)	0.03 ± 0.01	**0.85 ± 0.04***	0.18 ± 0.01[#]	**1.14 ± 0.05 *[#]**
C22:5n3 (Docosapentaenoic, n-3 DPA)	0.08 ± 0.01	**1.06 ± 0.04***	0.45 ± 0.02[#]	**1.39 ± 0.03*[#]**
C22:6n3 (Docosahexaenoic, DHA)	1.08 ± 0.04	**5.27 ± 0.13***	4.12 ± 0.05[#]	**6.54 ± 0.19*[#]**
Omega-3 Index (EPA+DHA)	1.12 ± 0.05	**6.11 ± 0.16***	4.31 ± 0.05[#]	**7.68 ± 0.23*[#]**
Σn-3 PUFAs	1.23 ± 0.05	**7.42 ± 0.20***	5.14 ± 0.06[#]	**9.53 ± 0.23*[#]**
n-6 PUFAs				
C18:2n6 (Linoleic)	24.22 ± 0.59	25.02 ± 0.40	20.01 ± 0.54[#]	22.02 ± 0.89[#]
C18:3n6 (γ-Linolenic)	0.25 ± 0.02	0.28 ± 0.02	0.17 ± 0.02[#]	0.19 ± 0.01[#]
C20:2n6 (Eicosadienoic)	0.44 ± 0.03	0.43 ± 0.02	0.25 ± 0.02[#]	0.30 ± 0.02[#]
C20:3n6 (Eicosatrienoic)	1.40 ± 0.05	1.40 ± 0.03	1.44 ± 0.04	1.53 ± 0.08
C20:4n6 (Arachidonic)	19.82 ± 0.41	**16.18 ± 0.40***	17.47 ± 0.59[#]	**12.19 ± 0.94*[#]**
C22:4n6 (Docosatetraenoic)	1.78 ± 0.04	**0.99 ± 0.02***	1.33 ± 0.03[#]	**0.64 ± 0.03*[#]**
C22:5n6 (Docosapentaenoic, n-6 DPA)	3.49 ± 0.06	**0.92 ± 0.04***	1.54 ± 0.04[#]	**0.43 ± 0.03*[#]**
Σn-6 PUFAs	51.41 ± 0.56	**45.21 ± 0.41***	42.21 ± 0.32[#]	**37.30 ± 0.31*[#]**
Σn-6/n-3 PUFA ratio	42.05 ± 1.60	**6.13 ± 0.20***	8.21 ± 0.11[#]	**3.92 ± 0.08***

[a]Values are expressed as mean ± SE (n=5). Data were analyzed by Two-Way ANOVA with Holm-Sidak *post hoc* comparisons.
**p*<0.01 versus WT mice fed the same diet.
#*p*<0.01 versus mice of the same genotype fed an n-6 PUFA diet.

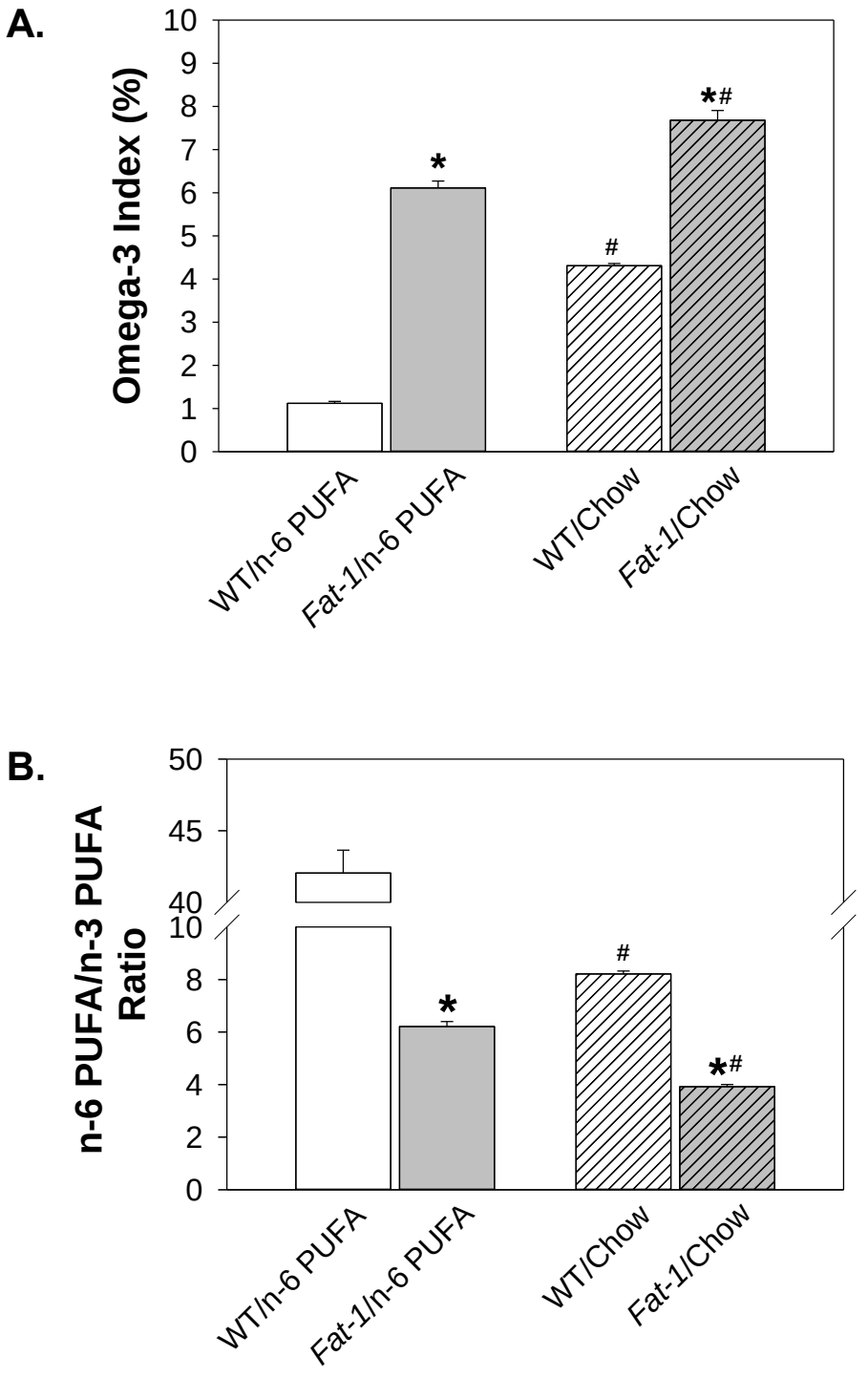


Fig. 3.1. Omega-3 index (A) and n-6 PUFA/n-3 PUFA ratio (B) in WT and *fat-1* mice fed an n-6 PUFA diet or standard chow diet. Data are presented as mean ± SE (n=5) and were analyzed by Two-Way ANOVA with Holm-Sidak *post-hoc* comparisons. *p<0.05 versus WT mice on the same diet, #p<0.05 versus mice of the same genotype fed an n-6 PUFA diet.

3.3. Aortic Vasoreactivity to Phenylephrine and Acetylcholine Was Not Affected By Genotype on Either Diet

Dose-dependent constriction and relaxation were performed on isolated aortic rings using PE and ACh, respectively, in order to assess the contribution of NO to the regulation of vascular tone. If the contribution of NO to blood pressure regulation were increased in *fat-1* mice, compared to WT, it would be expected that constriction to PE would be reduced and relaxation to ACh would be increased. However, aortic responsiveness to PE did not differ between WT and *fat-1* mice on either diet (Fig. 3.2A,B). In addition, relaxation to ACh was not different between WT and *fat-1* mice fed an n-6 PUFA diet (Fig. 3.2C); however, relaxation to ACh was significantly greater at the two highest doses in *fat-1* mice fed the standard chow diet, compared to WT (Fig. 3.2D). Taken together, these results suggest a limited role of NO in aortic vasoreactivity.

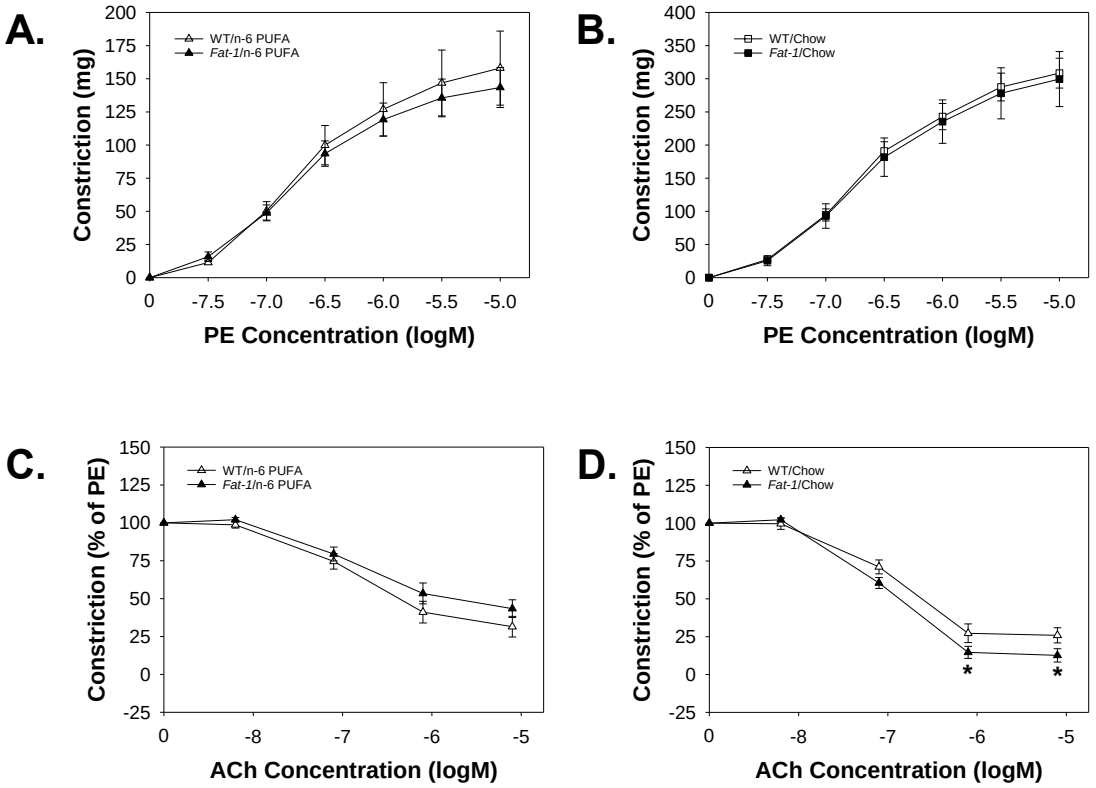


Fig. 3.2. Dose-dependent aortic constriction to PE (A,B) and relaxation to ACh (C,D). Data are presented as mean ± SE (n=7-9) and were analyzed by Two-Way RM ANOVA with Holm-Sidak *post-hoc* comparisons. *p<0.05 versus WT mice on the same diet

3.4. Blood Pressure was Lower in Fat-1 Mice Compared to WT When Fed an n-6 PUFA Diet

MAP, SBP, and DBP were measured over the course of two 3-day periods (3 days baseline followed by 3 days captopril treatment (4 mg/kg/day in drinking water)) in WT and *fat-1* mice first fed an n-6 PUFA diet for 8 weeks and then a standard chow diet for 4 weeks. When fed an n-6 PUFA diet, MAP was significantly lower in *fat-1* mice at baseline and on captopril, compared to WT mice (Fig. 3.3A). Further, there was a trend towards a larger decrease in MAP (ΔMAP) when *fat-1* mice were treated with captopril, compared to WT (p=0.09) (Fig. 3.3B).

SBP was not significantly different between WT and *fat-1* mice at baseline or on captopril, but there was a trend towards a lower SBP in *fat-1* mice both at baseline and on captopril (p=0.10 and p=0.09, respectively) (Fig. 3.3C). However, the decrease in SBP (ΔSBP) during captopril treatment was not different between WT and *fat-1* mice (Fig. 3.3D). In contrast to SBP, DBP was significantly lower in *fat-1* mice, compared to WT when on captopril (Fig. 3.3C), and showed a trend towards a lower DBP at baseline (p=0.07), compared to WT mice (Fig 3.3E). Notably, the decrease in DBP (ΔDBP) during captopril treatment was significantly greater in *fat-1* mice fed an n-6 PUFA, compared to WT mice fed the same diet (Fig. 3.3F).

When fed a standard chow diet, MAP, SBP, and DBP as well as ΔMAP, ΔSBP, and ΔDBP did not differ significantly between WT and *fat-1* mice at baseline or on captopril (Fig. 3.4A-F). Further, pulse pressure, the difference

between SBP and DBP, was not different between WT and *fat-1* mice at baseline or on captopril (Supplementary Fig. 3.6).

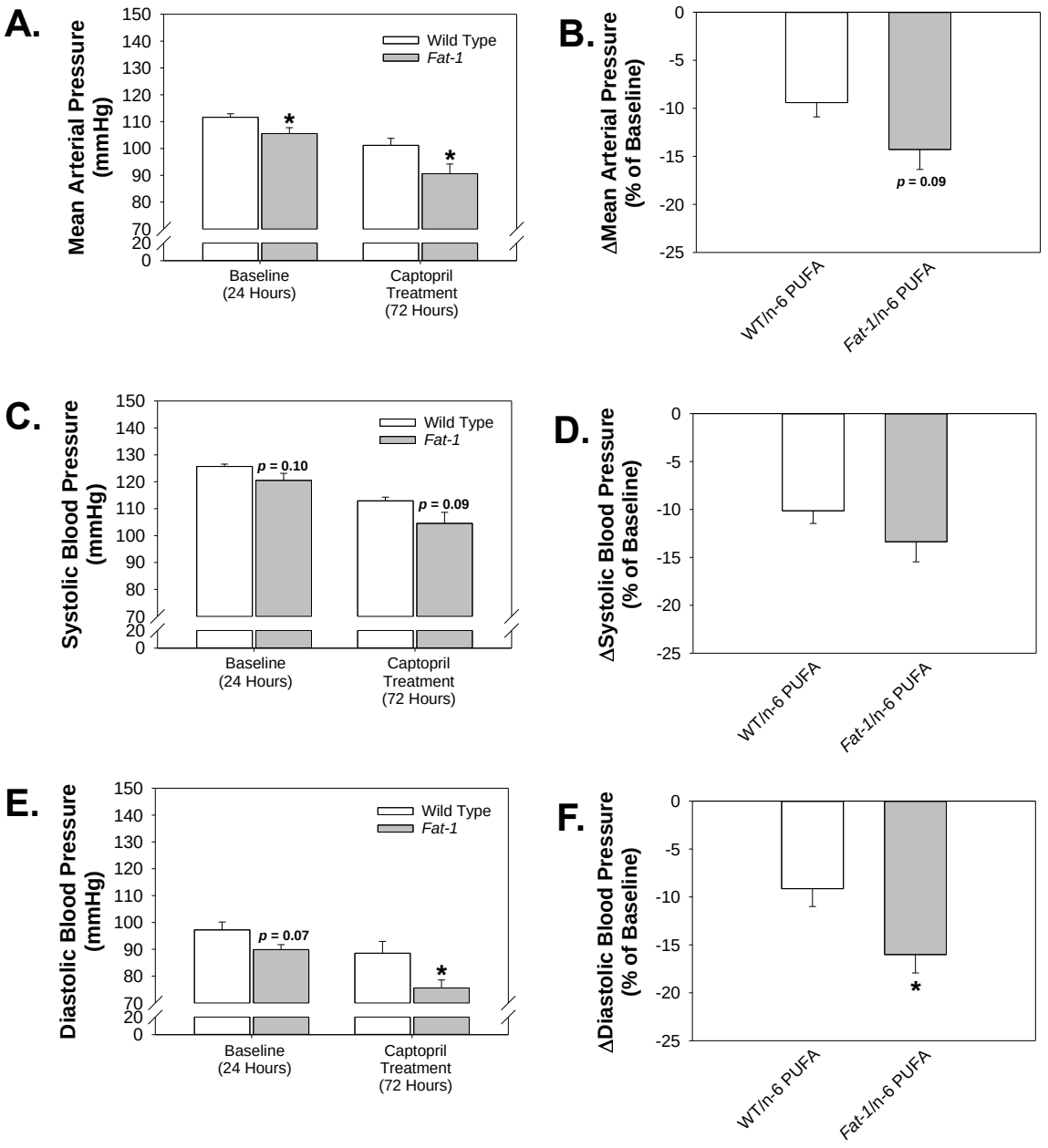


Fig. 3.3. MAP (A), SBP (C), and DBP (E), in WT and *fat-1* mice fed an n-6 PUFA diet at baseline and on captopril (4 mg/kg/day in drinking water on an n-6 PUFA diet. ΔMAP (B), ΔSBP (D), and ΔDBP (F) resulting from captopril treatment. Data are presented as mean ± SE (n=5) and were analyzed by t-test.

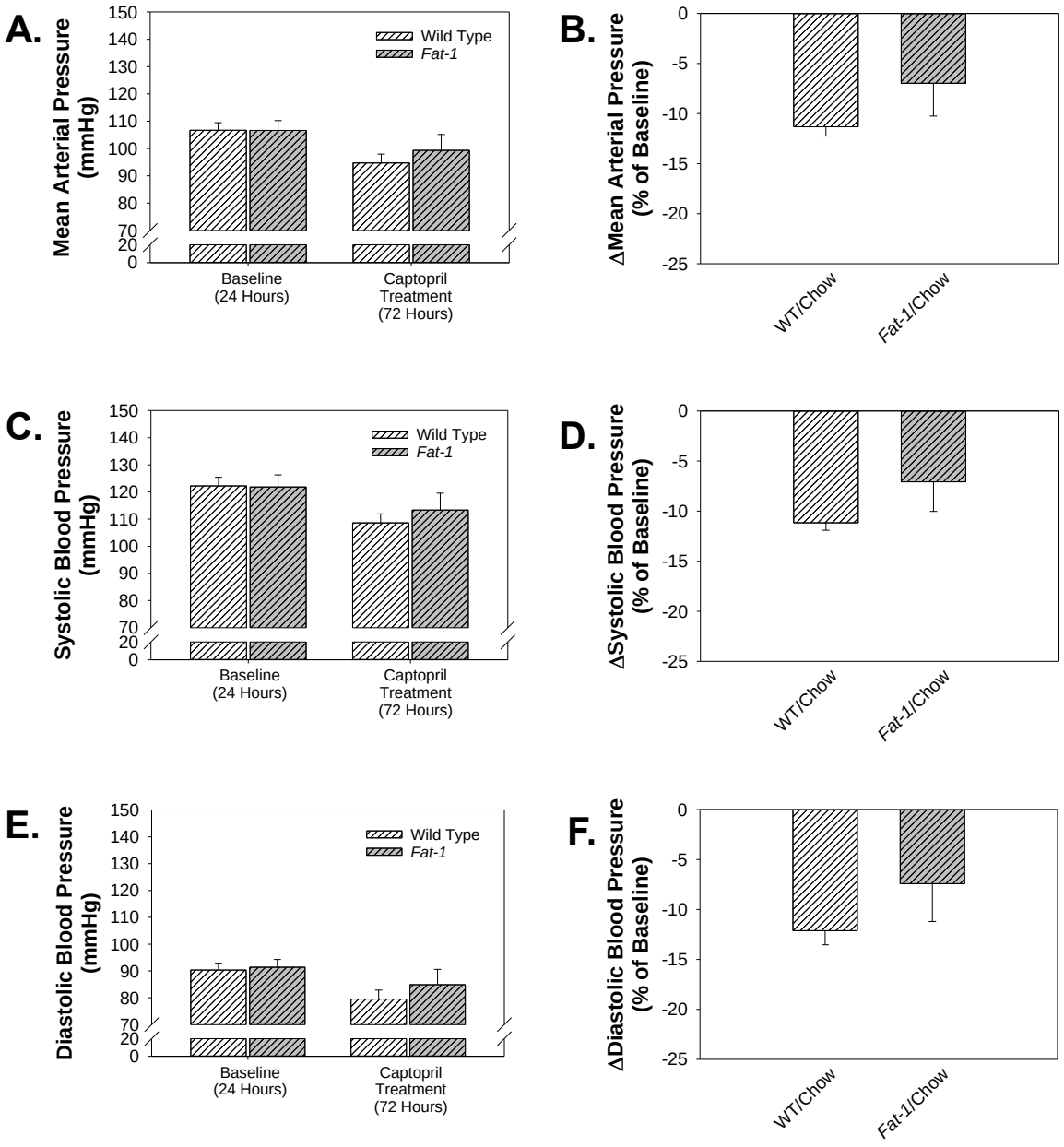


Fig. 3.4. MAP (A), SBP (C), and DBP (E), in WT and *fat-1* mice fed a standard chow diet at baseline and on captopril (4 mg/kg/day in drinking water on a standard chow diet. ΔMAP (B), ΔSBP (D), and ΔDBP (F) resulting from captopril treatment. Data are presented as mean ± SE (n=5) and were analyzed by t-test.

3.5. Heart Rate and Activity Were Not Significantly Different Between WT and Fat-1 Mice on Either Diet

In addition to the measurements of BP, surgically implanted radiotelemeters also recorded heart rate and activity of WT and *fat-1* mice over two 3-day periods (3 days baseline followed by 3 days captopril treatment). Regardless of diet, there were no significant differences in heart rate or activity between WT and *fat-1* mice at baseline or on captopril (Supplementary Fig. 3.7A-D and 3.8A-D). In addition, there were no significant differences between WT and *fat-1* mice in Δheart rate or Δactivity when treated with captopril.

3.6. Angiotensinogen (Agt) mRNA Expression Was Not Significantly Different Between WT and Fat-1 Mice Fed an n-6 PUFA Diet

To further examine the role of Ang II in the regulation of blood pressure in WT and *fat-1* mice, *Agt* mRNA expression, the substrate for the rate-limiting enzyme in Ang II generation, was measured in abdominal white adipose tissue, heart, liver, and lung. In adipose, there was a trend towards decreased *Agt* expression in *fat-1* mice compared to WT (p=0.06) (Fig. 3.4A). However, *Agt* mRNA expression did not differ between WT and *fat-1* mice fed an n-6 PUFA diet in heart, liver, or lung (Fig. 3.5B-D). Finally, *Agt* mRNA expression did not differ between WT and *fat-1* mice fed the standard chow diet (data not shown).

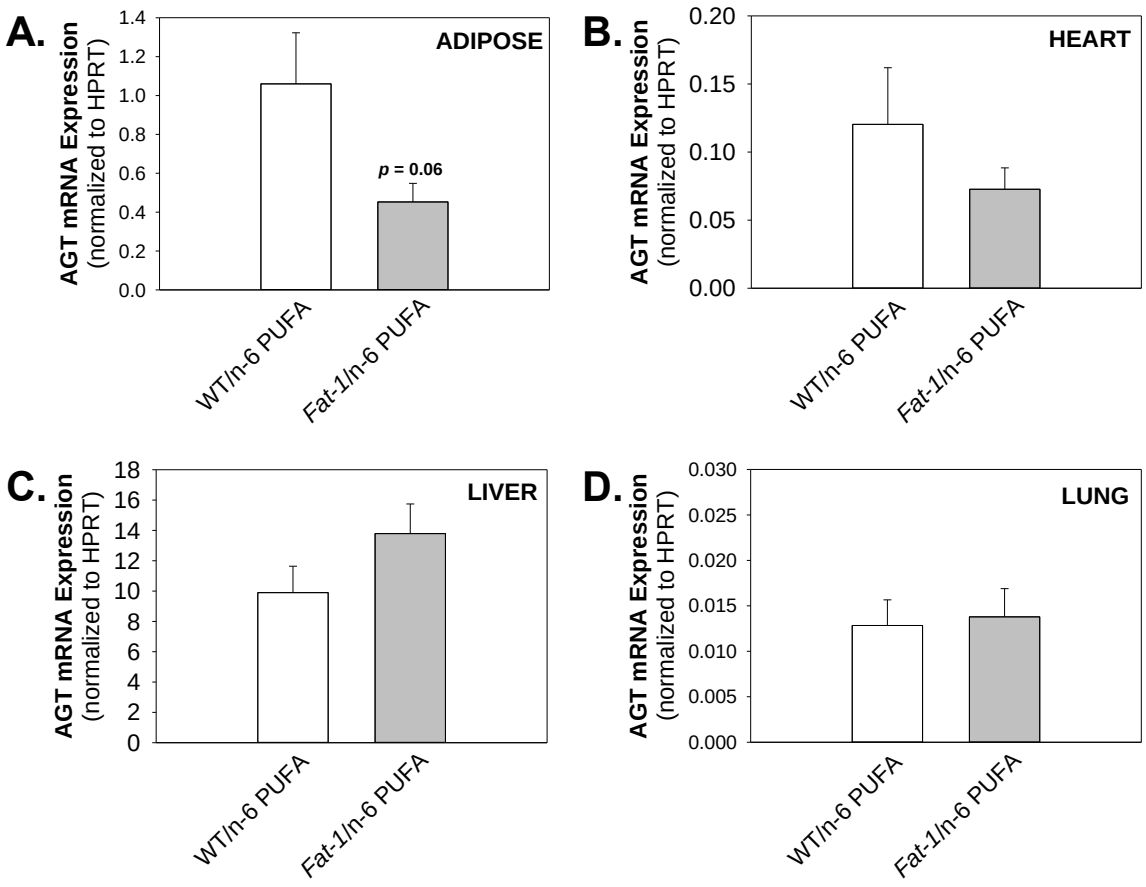


Fig. 3.5. *Agt* mRNA expression in abdominal adipose, heart, liver, and lung tissues from WT and *fat-1* mice fed an n-6 PUFA diet. Data were normalized to the housekeeping gene, *Hprt*. Data are presented as mean ± SE (n=5) and were analyzed by t-test. **p*<0.05 versus WT mice fed the same diet, #*p*<0.05 versus mice of the same genotype fed an n-6 PUFA diet.

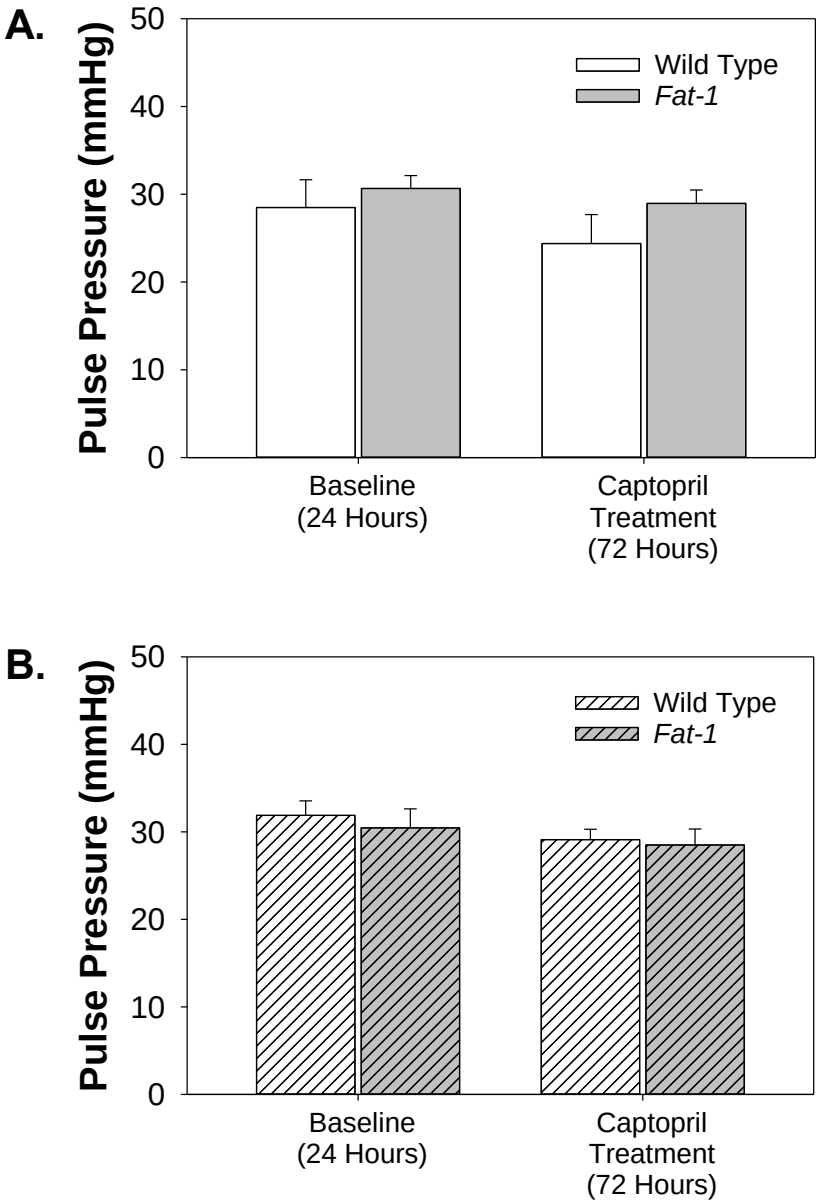
3.7. Summary

Vascular reactivity to PE and ACh were not different between WT and *fat-1* mice when fed the n-6 PUFA diet, while ACh showed an increased relaxation in *fat-1* mice on a standard chow diet. When mice were fed the n-6 PUFA diet, MAP was significantly lower in *fat-1* mice, compared to WT. When treated with captopril, MAP and DBP were reduced and ΔDBP was significantly greater in *fat-1* mice fed an n-6 PUFA diet, compared to WT mice fed the same diet. However, no significant differences were seen between WT and *fat-1* mice in MAP or SBP. When mice were fed a standard chow diet, MAP, SBP, and DBP were unaffected by genotype and diet both at baseline and on captopril treatment. Regardless of diet, there were no differences in heart rate or activity. There was a trend towards a decreased *Agt* mRNA expression in *fat-1* mice compared to WT only when mice were fed an n-6 PUFA diet.

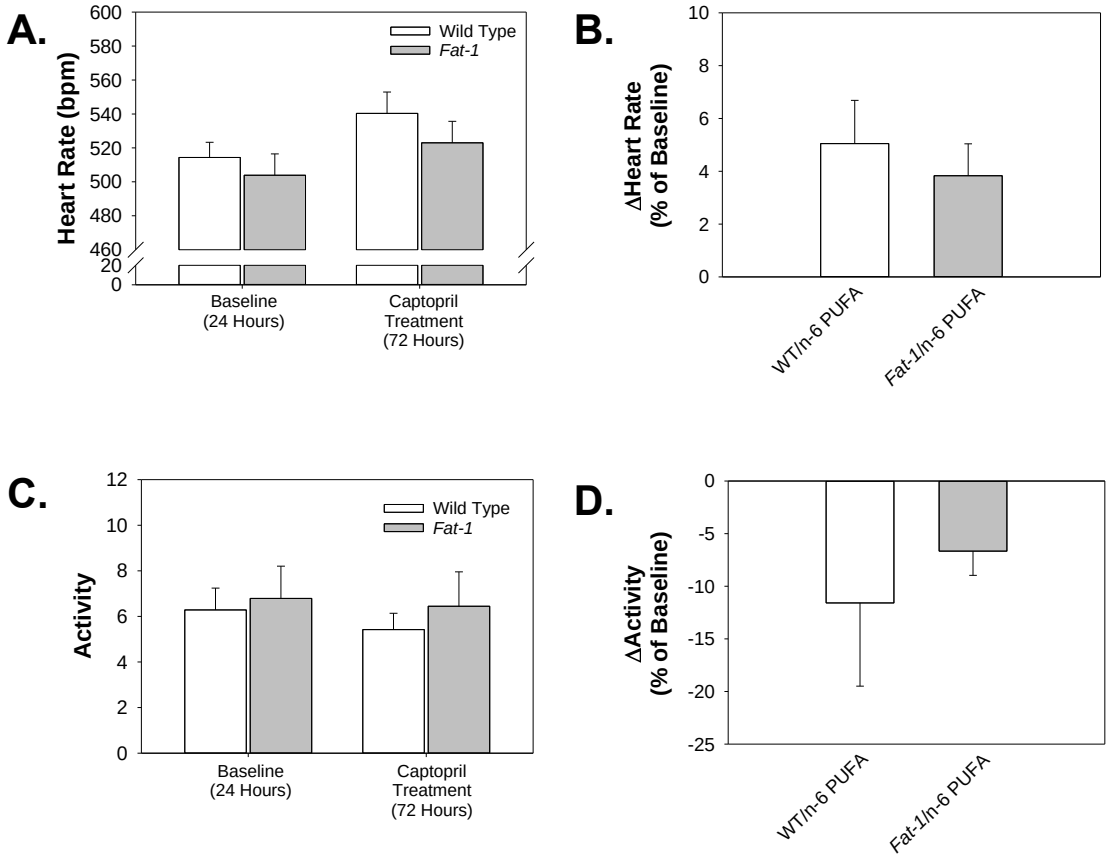
Taken together these data suggest that there may a small but detectable increase in NO bioavailability to vascular relaxation as the omega-3 index approaches a level defined as protective in humans (≥ 8%; *fat-1* mice on standard chow diet, 7.7 ± 0.2%). However, this increase in NO is not reflected by any changes in BP regulation, compared to WT mice with an omega-3 index in the intermediate risk range (4-8%; WT mice omega-3 index of 4.3 ± 0.2%). In contrast, however, an omega-3 index in the intermediate risk range observed in *fat-1* mice on an n-6 PUFA diet (6.1 ± 0.2%) results in significantly lower DBP and increased contribution of Ang II to BP regulation, compared to a very low and high risk omega-3 index (1.1 ± 0.1%). This suggests that the BP lowering

benefits of n-3 PUFAs occur at intermediate omega-3 index levels, compared to very low omega-3 index levels.

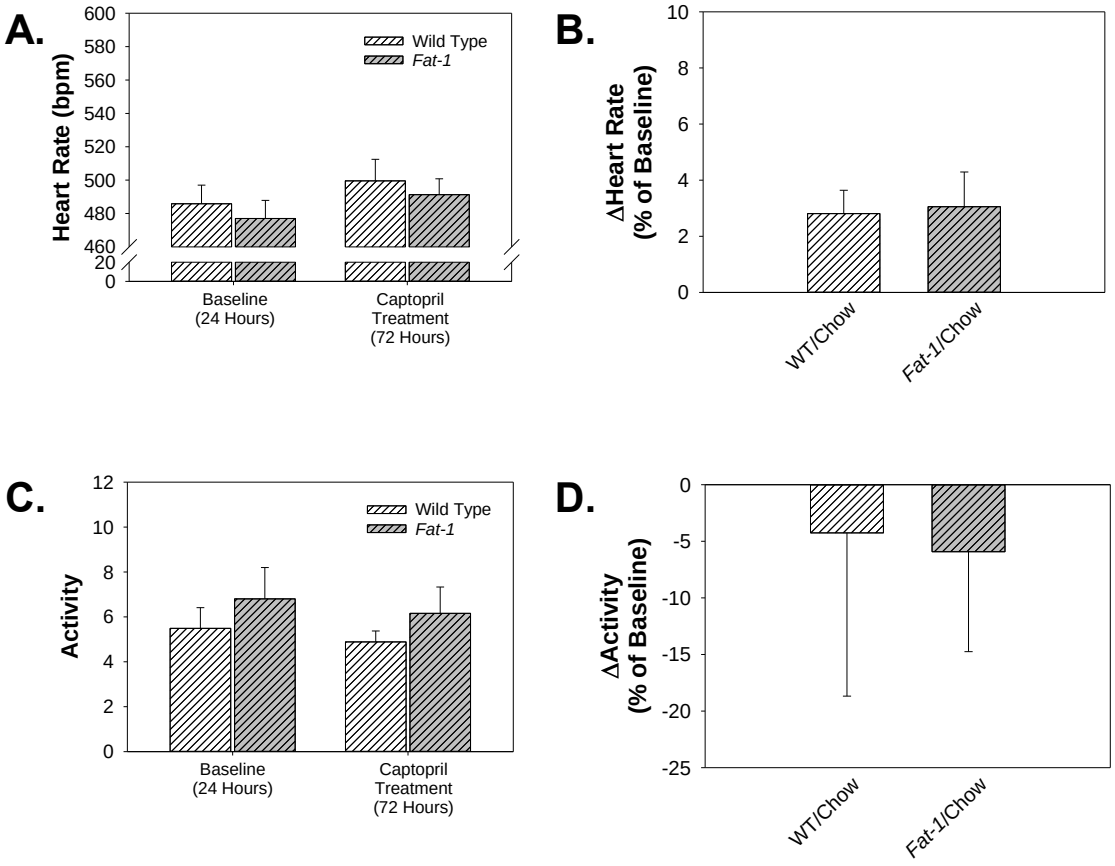
3.8. Supplementary Information



Supplementary Fig. 3.6. Pulse pressure in WT and *fat-1* mice fed an n-6 PUFA diet (A) or a standard chow diet (B) at baseline and on captopril (4 mg/kg/day in drinking water). Data are presented as mean ± SE (n=5) and were analyzed by t-test.



Supplementary Fig. 3.7. Heart rate (A) and activity (C) in WT and *fat-1* mice fed an n-6 PUFA diet at baseline and on captopril (4 mg/kg/day in drinking water). ΔHeart rate (B) and Δactivity (D) resulting from captopril treatment. Data are presented as mean ± SE (n=5) and were analyzed by t-test.



Supplementary Fig. 3.8. Heart rate (A) and activity (C) in WT and *fat-1* mice fed a standard chow diet at baseline and on captopril (4 mg/kg/day in drinking water). ΔHeart rate (B) and Δactivity (D) resulting from captopril treatment. Data are presented as mean ± SE (n=5) and were analyzed by t-test.

CHAPTER 4 – RESULTS – The Role of Omega-3 Polyunsaturated Fatty Acids in Secondhand Smoke-Induced Vascular Dysfunction

4.1. Total Particulate Matter

Total particulate matter (TPM) was measured within the whole-body exposure chamber every other week throughout the course of the study. Over 10 measurements, the average TPM was 115 mg/m^3. A representative time course of TPM is shown in Fig. 4.1.

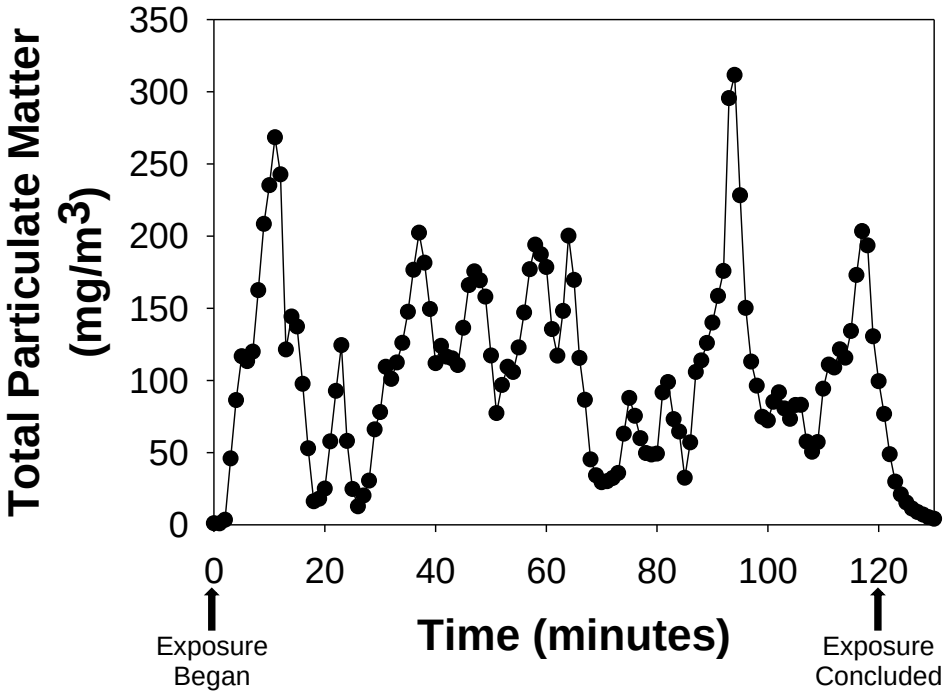


Fig. 4.1. TPM in the whole-body exposure chamber measured over a 120-minute time course. TPM was maintained at an average of 115 mg/m^3.

4.2. Urinary Cotinine was Elevated in SHS-Exposed Mice Compared to Air-Exposed Mice

Urinary cotinine concentrations were determined by ELISA at baseline and at four weeks post air or SHS exposure as an index of exposure to SHS (Table 4.1). At baseline, cotinine concentrations were not significantly different between air- and SHS-exposed mice. After 4 weeks of either air or SHS exposure, urinary cotinine levels were significantly higher in SHS-exposed mice compared to air exposed mice. The urinary cotinine concentrations seen in SHS-exposed mice are comparable to urinary cotinine concentrations seen in human nonsmokers exposed to SHS (Jarvis et al., 1987; Jung et al., 2012).

TABLE 4.1. Urinary cotinine concentrations at baseline and after 4 weeks of air or SHS exposure.

	Urinary Cotinine (ng/mL)[a]	
	Baseline	4 Weeks Exposure
Air	0.28 ± 0.03[b]	0.26 ± 0.03
SHS	0.27 ± 0.03	3.83 ± 0.92*#

[a]Limit of detection was 0.1 ng/mL.
[b]Data are presented as mean ± SE (n=7-9) and were analyzed by t-test.
**p*<0.05 versus air-exposed mice at the same time point.
#*p*<0.05 versus SHS-exposed mice at baseline.

4.3. Cyp1a1 and Cyp1b1 mRNA Expression

Cyp1a1 and *Cyp1b1* mRNA expression was assessed as an index of exposure to SHS. Adipose tissue, a storage depot for SHS-derived organic compounds, demonstrated increased *Cyp1a1* mRNA expression following SHS exposure in mice fed the standard chow diet, but not mice fed the n-6 PUFA diet (Fig. 4.2A). Additionally, both air- and SHS-exposed mice fed the standard chow diet had a significantly lower *Cyp1a1* mRNA expression, compared to mice fed the n-6 PUFA diet. In contrast, SHS exposure significantly increased *Cyp1b1* mRNA expression in adipose of mice fed an n-6 PUFA diet, but not in mice fed a standard chow diet (Fig. 4.2B). *Cyp1a1* and *Cyp1b1* mRNA expression in lung and heart tissue demonstrated no effects of diet or SHS exposure (Supplementary Fig. 4.10).

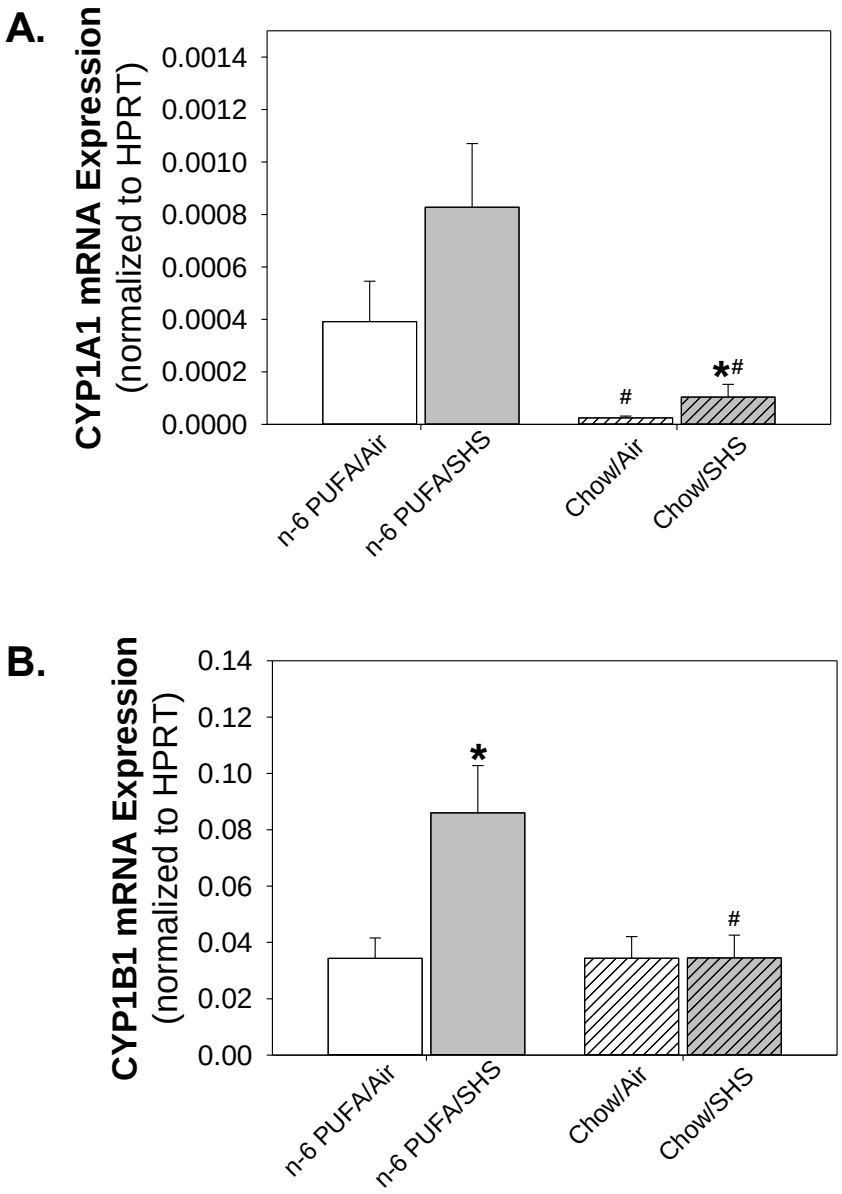


Fig. 4.2. *Cyp1a1* (A) and *Cyp1b1* (B) mRNA expression in abdominal adipose tissue from air- and SHS-exposed mice fed an n-6 PUFA diet or standard chow diet. Data were normalized to the housekeeping gene, *Hprt*. Data are presented as mean ± SE (n=5-6) and were analyzed by Two-Way ANOVA with Holm-Sidak *post hoc* comparisons. **p*<0.05 versus air-exposed mice fed the same diet, #*p*<0.05 versus mice of the same exposure group fed an n-6 PUFA diet.

4.4. SHS Increased Left Ventricle + Septum-To-Body Weight Ratio and Lung-To-Body Weight Ratio SHS-Exposed Mice Fed a Chow Diet

Body weights of *fat-1* mice were not significantly affected by diet or SHS exposure (Table 4.2). In addition, total heart-to-body weight, right ventricle-to-body weight, and kidneys-to-body weight ratios were not significantly different due to diet or SHS exposure. The left ventricle + septum-to-body weight ratio was significantly larger in SHS-exposed mice fed a standard chow diet, compared to air-exposed mice fed the same diet as well as SHS-exposed mice fed an n-6 PUFA diet. The lung-to-body weight ratio was increased significantly in SHS-exposed mice fed a standard chow diet, compared to air-exposed mice also fed a standard chow diet. Both air- and SHS-exposed mice fed the standard chow diet had a significantly higher liver-to-body weight ratio, compared to mice fed the n-6 PUFA diet.

TABLE 4.2. Body and tissue weights of air- and SHS-exposed *fat-1* mice fed an n-6 PUFA or standard chow diet.

	n-6 PUFA Diet		**Standard Chow Diet**	
	Air	SHS	Air	SHS
Body Weight Pre-Exposure (g)	24.58 ± 0.79	25.38 ± 0.51	25.47 ± 0.26	26.28 ± 0.83
Body Weight Post-Exposure (g)	28.62 ± 1.03	27.77 ± 0.78	29.46 ± 0.25	27.05 ± 1.47
Total Heart to Body Weight Ratio (x10^2)	0.40 ± 0.01	0.40 ± 0.01	0.41 ± 0.01	0.45 ± 0.03
Left Ventricle + Septum to Body Weight Ratio (x10^2)	0.31 ± 0.01	0.31 ± 0.01	0.32 ± 0.01	**0.38 ± 0.03*#**
Right Ventricle to Body Weight Ratio (x10^2)	0.09 ± 0.004	0.08 ± 0.007	0.09 ± 0.004	0.07 ± 0.004
Lung to Body Weight Ratio (x10^2)	0.54 ± 0.02	0.55 ± 0.01	0.52 ± 0.01	**0.62 ± 0.04***
Liver to Body Weight Ratio (x10^2)	4.68 ± 0.08	4.88 ± 0.15	5.35 ± 0.09#	5.99 ± 0.38#
Kidneys to Body Weight Ratio (x10^2)	1.01 ± 0.02	0.98 ± 0.02	1.00 ± 0.03	1.10 ± 0.07

[a]Values are expressed as mean ± SE (n=6-9). Data were analyzed by Two-Way ANOVA with Holm-Sidak *post hoc* comparisons.
**p*<0.05 versus air-exposed mice fed the same diet.
#*p*<0.05 versus mice of the same exposure group fed an n-6 PUFA diet.

4.5. SHS Exposure Reduced n-3 PUFAs in Mice Fed a Standard Chow Diet, but Not in Mice Fed an n-6 PUFA Diet

As observed in our previous study, n-3 PUFAs were higher and n-6 PUFAs were lower in mice fed the standard chow diet, compared to mice fed the n-6 PUFA diet (Table 4.3). Interestingly, however, SHS exposure significantly reduced the omega-3 index as well as the red blood cell DHA content in mice fed the standard chow diet, compared to air-exposed mice (Table 4.3, Fig. 4.3A). However, SHS exposure had no effect on the n-6 PUFA profile of mice fed the standard chow diet. SHS exposure also had no effect on the red blood cell fatty acid profile of mice fed the n-6 PUFA diet. Lastly, SHS exposure had no effect on the n-6/n-3 PUFA ratio (Fig. 4.3B).

TABLE 4.3. Red blood cell fatty acid composition of *fat-1* mice fed an n-6 PUFA diet or a standard chow diet.

	n-6 PUFA Diet		Standard Chow Diet	
Fatty Acid	Air	SHS	Air	SHS
n-3 PUFAs				
C18:3n3 (α-Linolenic, ALA)	0.15 ± 0.01	0.20 ± 0.03	0.49 ± 0.03#	0.57 ± 0.05#
C20:5n3 (Eicosapentaenoic, EPA)	0.85 ± 0.04	0.85 ± 0.05	1.52 ± 0.04#	1.33 ± 0.20#
C22:5n3 (Docosapentaenoic, DPA)	1.06 ± 0.04	1.01 ± 0.03	1.69 ± 0.05#	1.43 ± 0.18#
C22:6n3 (Docosahexaenoic, DHA)	5.27 ± 0.13	5.05 ± 0.14	8.11 ± 0.25#	**6.48 ± 0.36*#**
Omega-3 Index (EPA+DHA)	6.11 ± 0.16	5.90 ± 0.19	9.63 ± 0.26#	**7.81 ± 0.55*#**
Σn-3 PUFAs	7.33 ± 0.19	7.11 ± 0.23	11.81 ± 0.28#	**9.82 ± 0.75*#**
n-6 PUFAs				
C18:2n6 (Linoleic)	25.02 ± 0.40	25.03 ± 0.49	21.44 ± 0.42#	22.27 ± 0.61#
C18:3n6 (γ-Linolenic)	0.28 ± 0.02	1.69 ± 0.05	0.24 ± 0.01	0.24 ± 0.01
C20:2n6 (Eicosadienoic)	0.43 ± 0.02	0.45 ± 0.03	0.29 ± 0.01#	0.28 ± 0.01#
C20:3n6 (Eicosatrienoic)	1.40 ± 0.03	1.41 ± 0.03	1.69 ± 0.03#	1.62 ± 0.06#
C20:4n6 (Arachidonic)	16.18 ± 0.40	15.82 ± 0.41	10.83 ± 0.41#	9.81 ± 0.88#
C22:4n6 (Docosatetraenoic)	0.99 ± 0.02	0.99 ± 0.02	0.55 ± 0.04#	0.56 ± 0.10#
C22:5n6 (Docosapentaenoic)	0.92 ± 0.04	0.96 ± 0.06	0.31 ± 0.03#	0.33 ± 0.09#
Σn-6 PUFAs	45.23 ± 0.36	44.90 ± 0.34	35.36 ± 0.24#	35.11 ± 0.66#
Σn-6/n-3 PUFA ratio	6.21 ± 0.19	6.37 ± 0.24	3.01 ± 0.08#	3.76 ± 0.49#

[a]Values are expressed as mean ± SE (n=6-10). Data were analyzed by Two-Way ANOVA with Holm-Sidak *post hoc* comparisons.
*p<0.01 versus WT mice fed the same diet.
#p<0.01 versus mice of the same genotype fed an n-6 PUFA diet

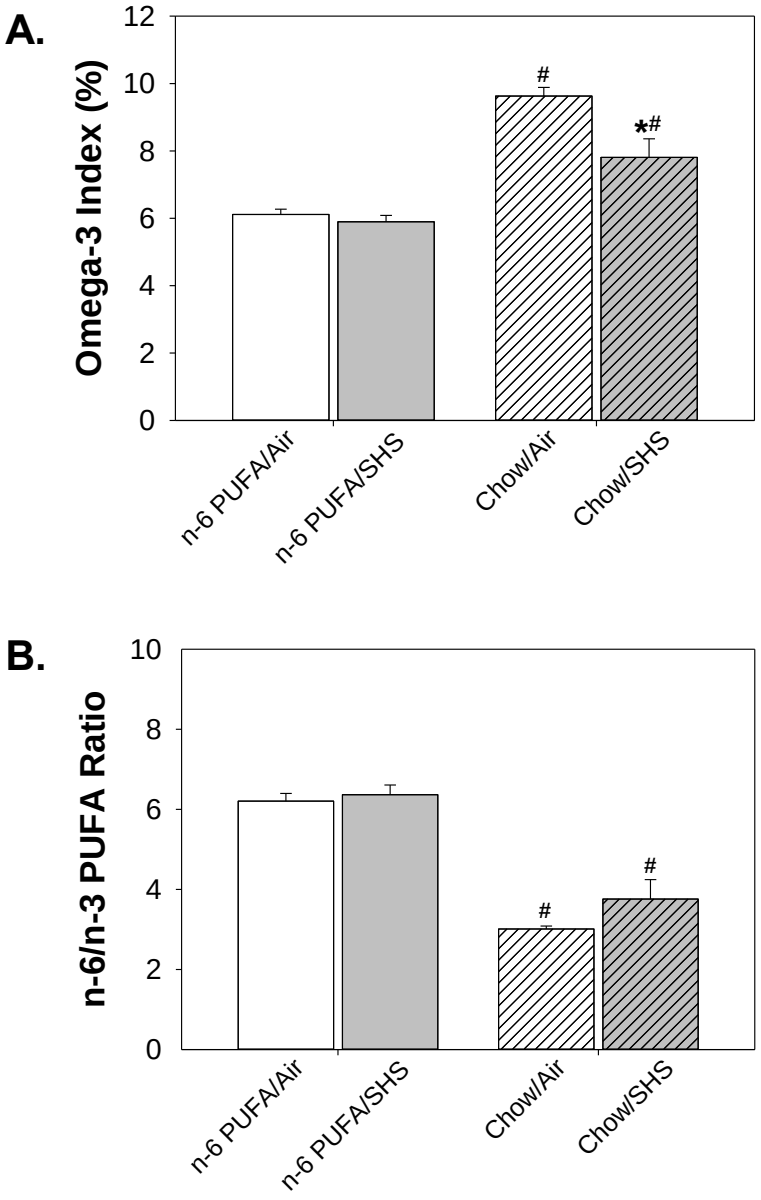


Fig. 4.3. Red blood cell omega-3 Index (A) and n-6/n-3 PUFA ratio (B) air- or SHS-exposed mice fed an n-6 PUFA diet or a standard chow diet. Data are presented as mean ± SE (n=6-10) and were analyzed by Two-Way ANOVA with Holm-Sidak *post hoc* comparisons. **p*<0.01 versus air-exposed mice fed the same diet, #*p*<0.01 versus mice of the same exposure group fed an n-6 PUFA diet.

4.6. Vasoconstrictor Responses Were Enhanced In SHS-Exposed Fat-1 Mice Fed an n-6 PUFA Diet

Aortic rings from air- and SHS-exposed mice fed an n-6 PUFA diet or a standard chow diet were constricted with either PE or U46619 and were immediately relaxed with either ACh or SNAP.

When dosed with PE, rings from SHS-exposed mice fed an n-6 PUFA diet had a significantly higher degree of constriction as well as area under the curve (AUC) than did rings from air-exposed mice fed the same diet (Fig. 4.4A and C). When rings were treated with PE following L-NNA pre-incubation, a NOS inhibitor, the rings from SHS-exposed mice fed an n-6 PUFA diet still constricted significantly more and trended toward a higher AUC than rings from the air-exposed mice fed the same diet (Fig. 4.4B and D). There were no differences in PE constriction or AUC between air- and SHS-exposed mice fed a standard chow diet (Fig. 4.5A-D).

Similar results were observed when rings were constricted with U46619, a thromboxane receptor agonist. Aortic rings from SHS-exposed mice fed an n-6 PUFA diet constricted significantly more at the highest dose than did air-exposed mice fed the same diet. (Fig. 4.6A). When the rings were dosed with U46619 following L-NNA, rings from SHS-exposed mice fed an n-6 PUFA diet constricted significantly more than air-exposed mice fed the same diet (Fig. 4.6B). Similar to the results with PE, there were no differences in U46619 constriction between air- and SHS-exposed mice fed the standard chow diet. significantly between air- and SHS-exposed mice fed either diet (Fig. 4.6C and D).

Regardless of the vasoconstrictor used to pre-constrict the aortic ring, ACh and SNAP relaxation did not differ between air- and SHS-exposed mice fed either an n-6 PUFA or standard chow diet (Supplementary Figs. 4.11-4.14).

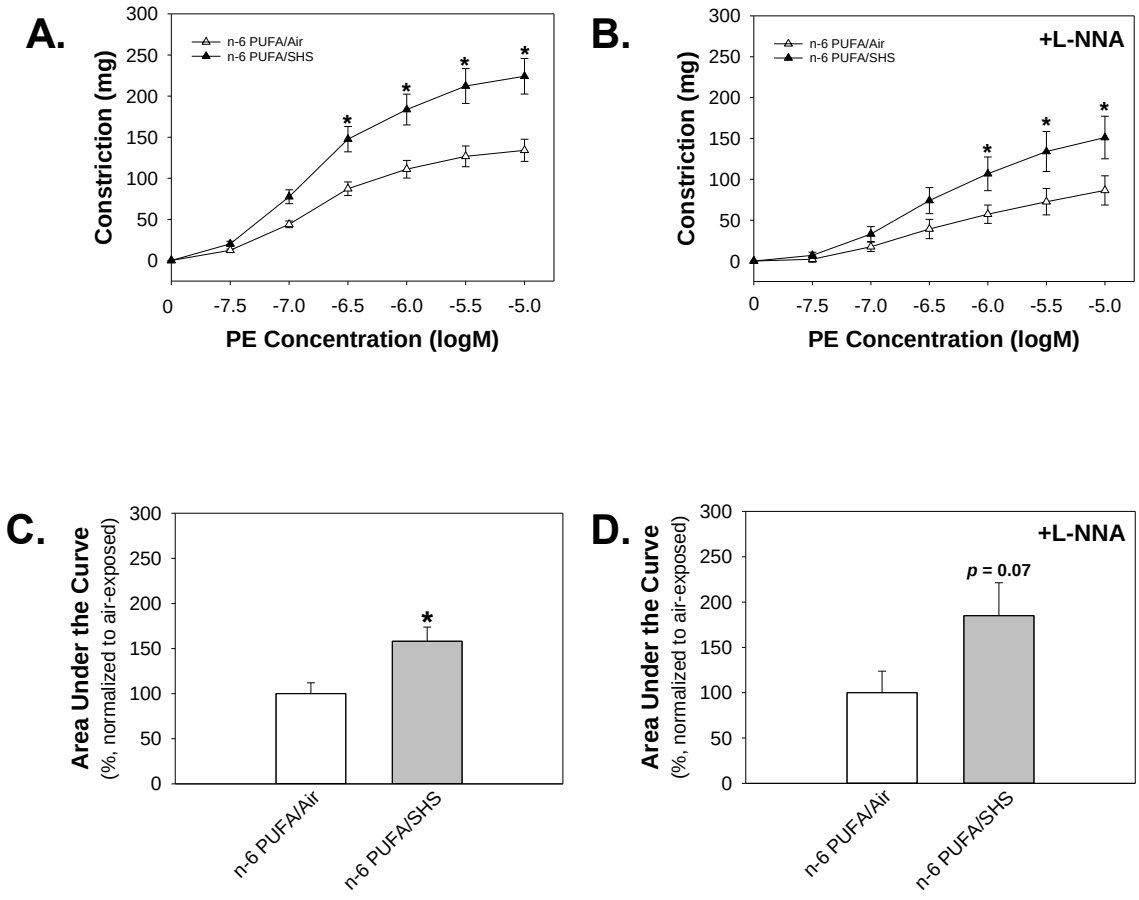


Fig. 4.4. PE constriction of aortic rings from air- and SHS-exposed mice fed an n-6 PUFA diet in the absence (A) and presence (B) of the NOS inhibitor, L-NNA. PE AUC of aortic rings from air- and SHS-exposed mice fed an n-6 PUFA diet in the absence (C) and presence (D) of L-NNA. Data are presented as mean ± SE (n=6-9) and were analyzed by Two-Way RM ANOVA with Holm-Sidak *post hoc* comparisons (A,B) or t-test (C,D). *p<0.05 versus air-exposed mice.

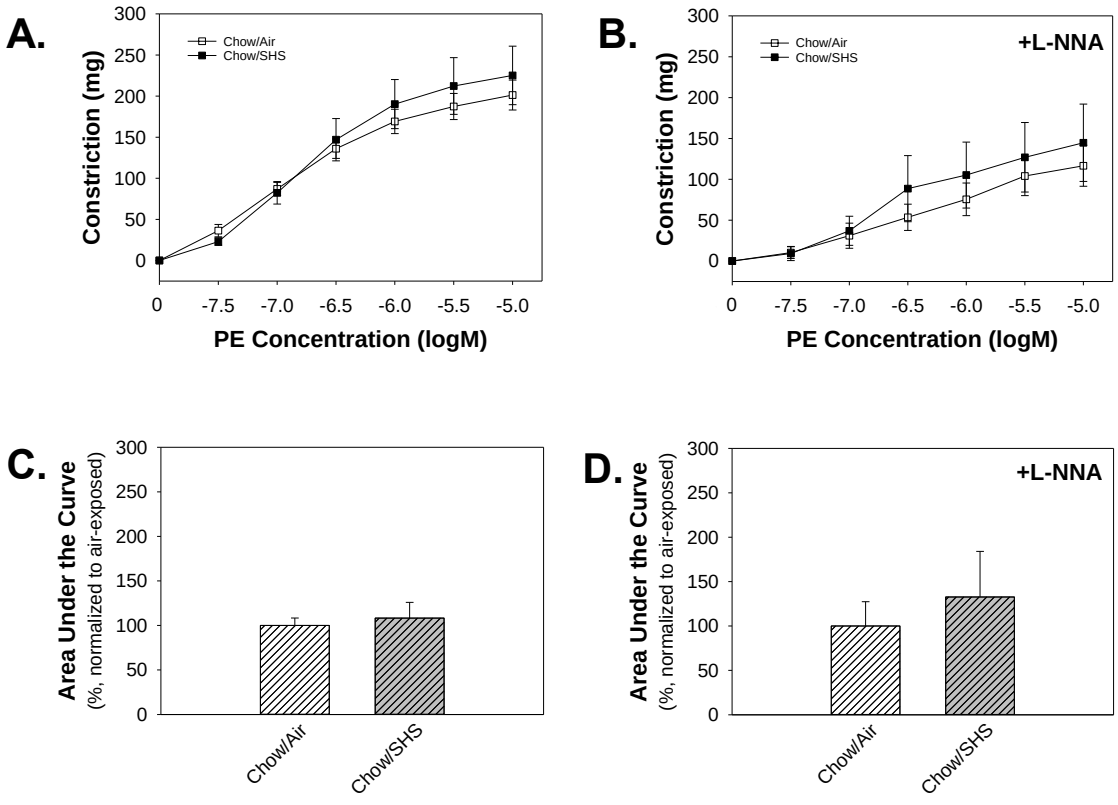


Fig. 4.5. PE constriction of aortic rings from air- and SHS-exposed mice fed a standard chow diet in the absence (A) and presence (B) of the NOS inhibitor, L-NNA. PE AUC of aortic rings from air- and SHS-exposed mice fed a standard chow diet in the absence (C) and presence (D) of L-NNA. Data are presented as mean ± SE (n=6-9) and were analyzed by Two-Way RM ANOVA with Holm-Sidak *post hoc* comparisons (A,B) or t-test (C,D). **p*<0.05 versus air-exposed mice.

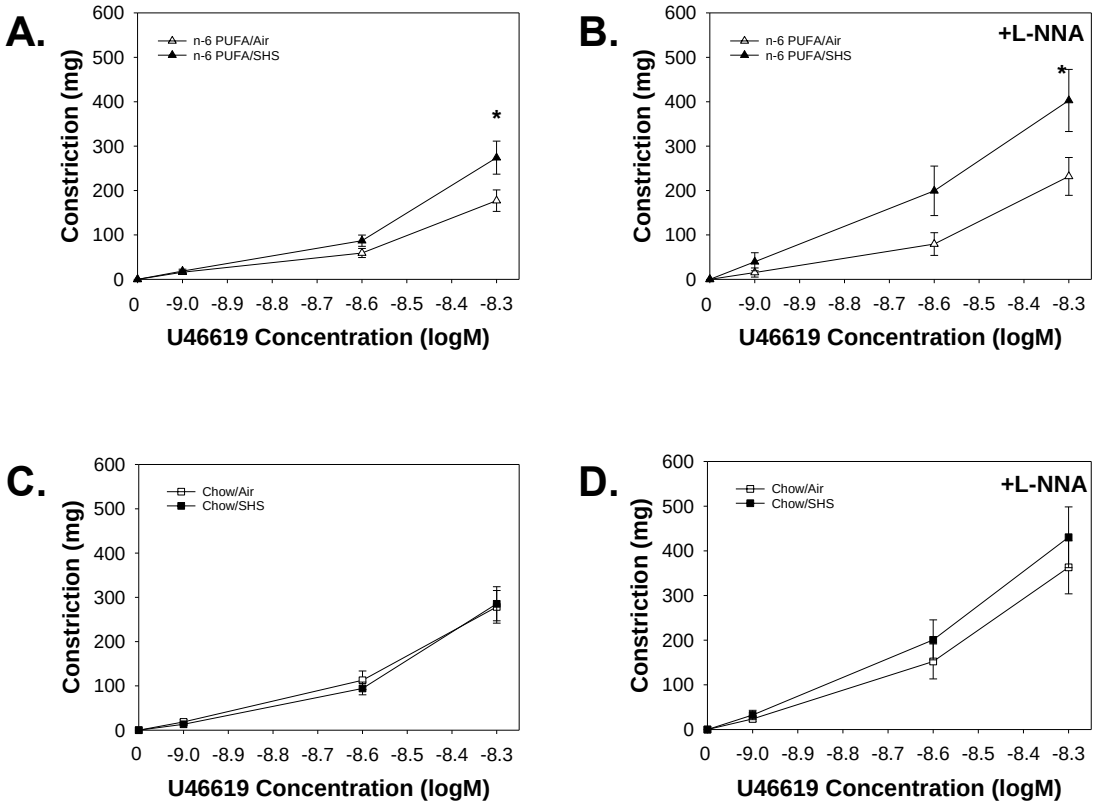


Fig. 4.6. U46619 constriction of aortic rings from air- and SHS-exposed mice fed an n-6 PUFA diet or a standard chow diet in the absence (A,C) and presence (B,D) of the NOS inhibitor, L-NNA. Data are presented as mean ± SE (n=6-9) and were analyzed by Two-Way RM ANOVA with Holm-Sidak *post hoc* comparisons. *p<0.05 versus air-exposed mice fed the same diet.

4.7. SHS Exposure Increased Adipose Hmox1 mRNA Expression in Fat-1 Mice Fed an n-6 PUFA Diet

In order to determine whether oxidative stress is involved in the SHS-induced enhancement of constriction when mice are fed an n-6 PUFA diet, mRNA expression of Nrf2-regulated antioxidant genes, *Hmox1* and *Nqo1*, were assessed in adipose, lung, and heart tissue. In adipose, SHS exposure significantly increased *Hmox1* mRNA expression in mice fed an n-6 PUFA diet, compared to air-exposed mice fed the same diet (Fig. 4.7A). In contrast, there was no change in *Hmox1* mRNA expression in adipose between SHS- and air-exposed mice fed the standard chow diet. Additionally, there were no other SHS-induced changes in *Hmox1* or *Nqo1* mRNA expression observed in adipose, lung, or heart (Fig. 4.7B-F).

4.8. SHS Exposure Increased Serum Total Antioxidant Capacity in Fat-1 Mice Fed an n-6 PUFA Diet

Total antioxidant capacity (TAC) was evaluated to further understand the role of oxidative stress in SHS-induced increases in constriction. SHS exposure significantly increased TAC in mice fed an n-6 PUFA diet, compared to air-exposed mice fed the same diet (Fig. 4.8). Notably, however, SHS had no effect on TAC in mice fed the standard chow diet.

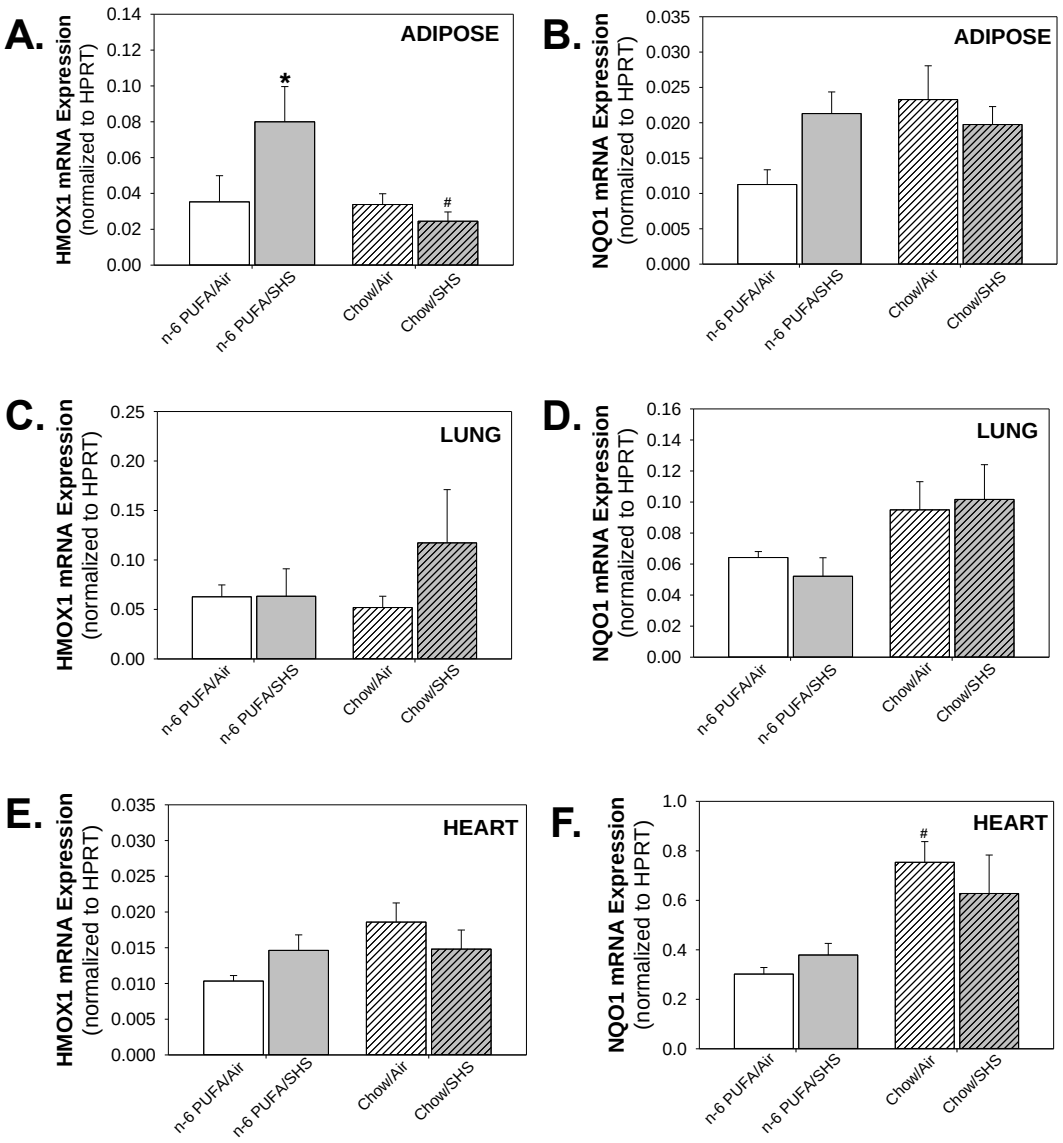


Fig. 4.7. mRNA expression of oxidative stress markers, *Hmox1* and *Nqo1*, in adipose (A,B), lung (C,D), and heart (E,F) tissues from air- and SHS-exposed mice fed an n-6 PUFA or standard chow diet. Data were normalized to the housekeeping gene, *Hprt*. Data are presented as mean ± SE (n=5-6) and were analyzed by Two-Way ANOVA with Holm-Sidak *post hoc* comparisons. **p*<0.05 versus air-exposed mice fed the same diet, #*p*<0.05 versus mice of the same exposure group fed an n-6 PUFA diet.

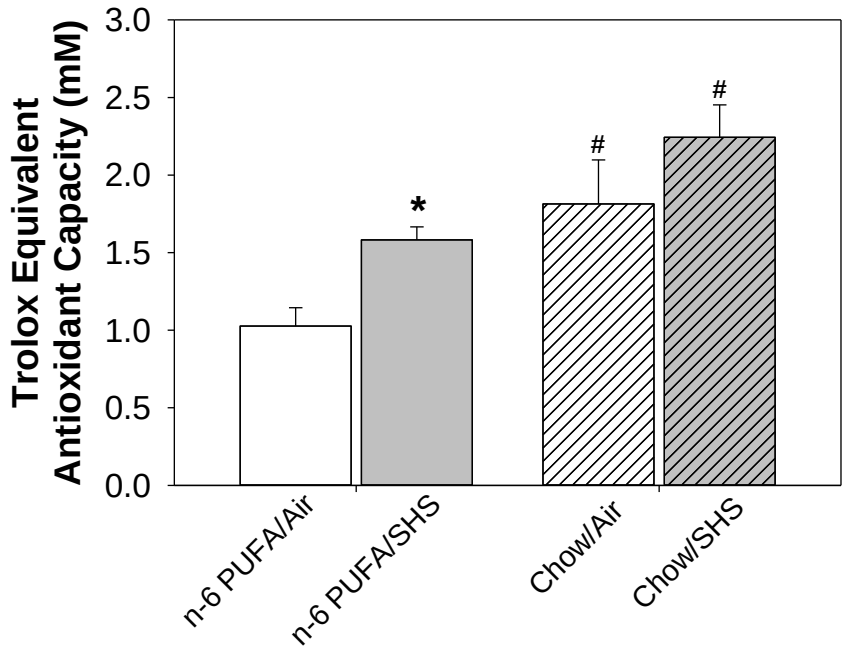


Fig. 4.8. Serum total antioxidant capacity (TAC) in *fat-1* air- and SHS-exposed mice fed an n-6 PUFA or standard chow diet. Data are presented as mean ± SE (n=6-9) and were analyzed by Two-Way ANOVA with Holm-Sidak *post hoc* comparisons. *p<0.05 versus air-exposed mice fed the same diet, #p<0.05 versus mice of the same exposure group fed an n-6 PUFA diet.

4.9. SHS Exposure Did Not Affect Collagen Staining

Cardiac sections (left ventricle) were stained using picro-sirius red to determine if the 4-week SHS exposure resulted in any fibrosis of the heart. Collagen staining was found predominantly in a perivascular location and qualitatively did not appear to differ between mice exposed to SHS or air (Fig. 4.9).

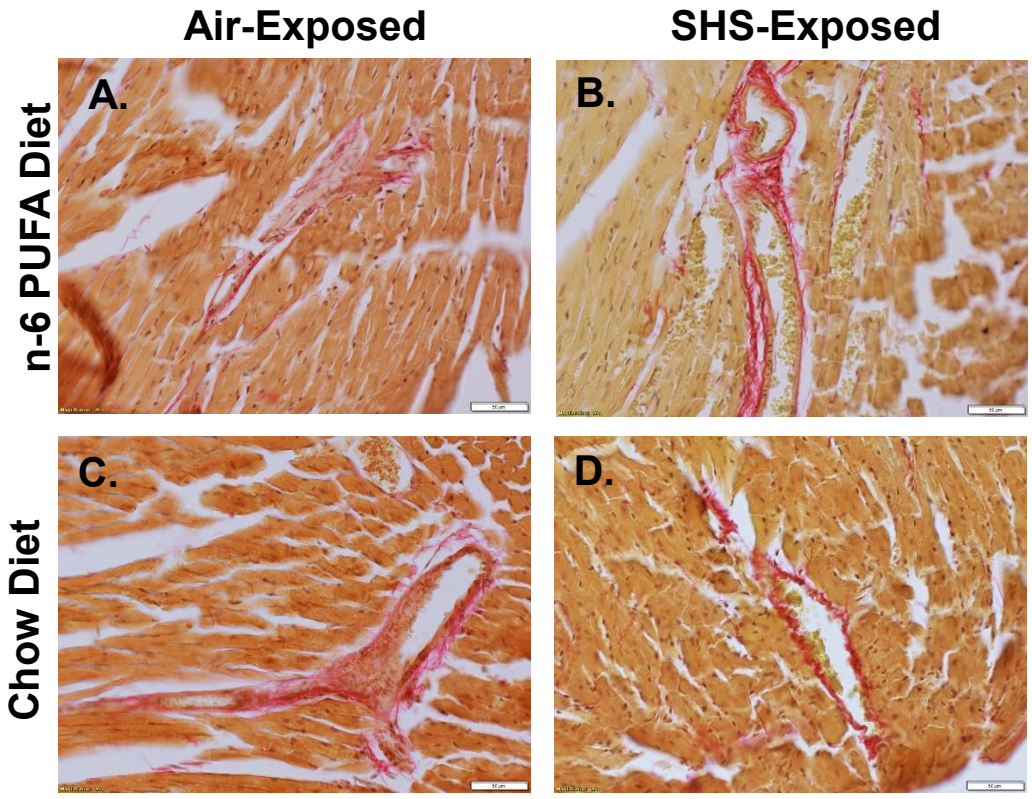


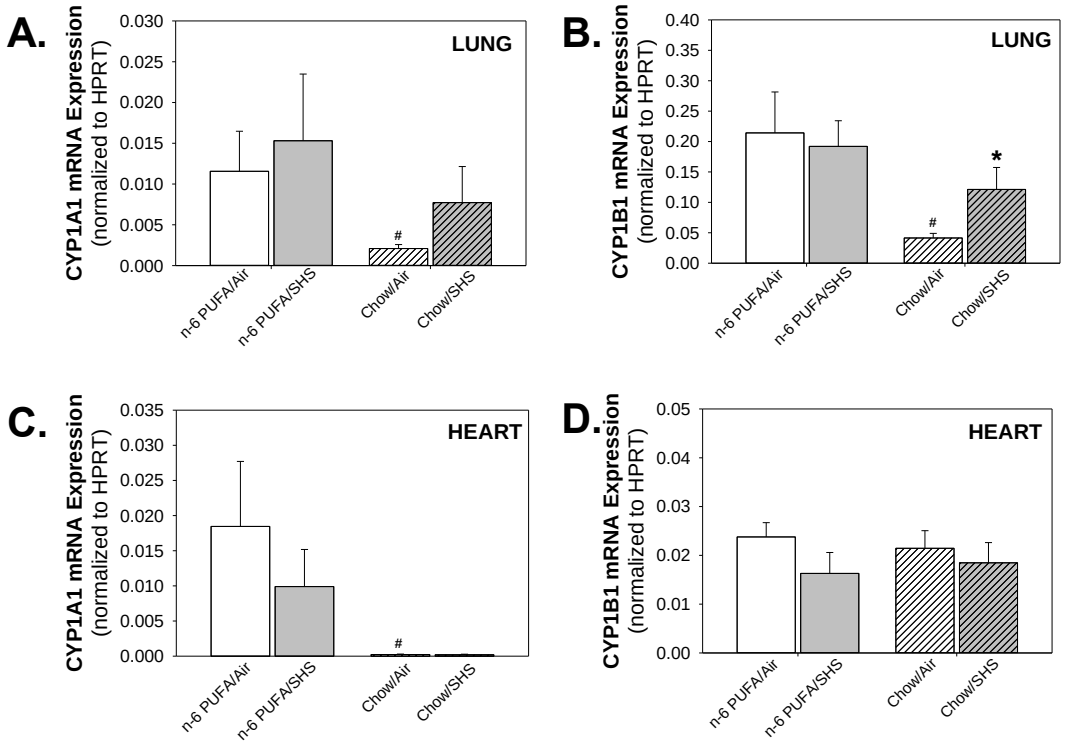
Fig. 4.9. Picro-sirius red staining of the left ventricle of the heart for fibrosis in air- and SHS-exposed mice fed an n-6 PUFA diet (A,B) or a standard chow diet (C,D).

4.10. Summary

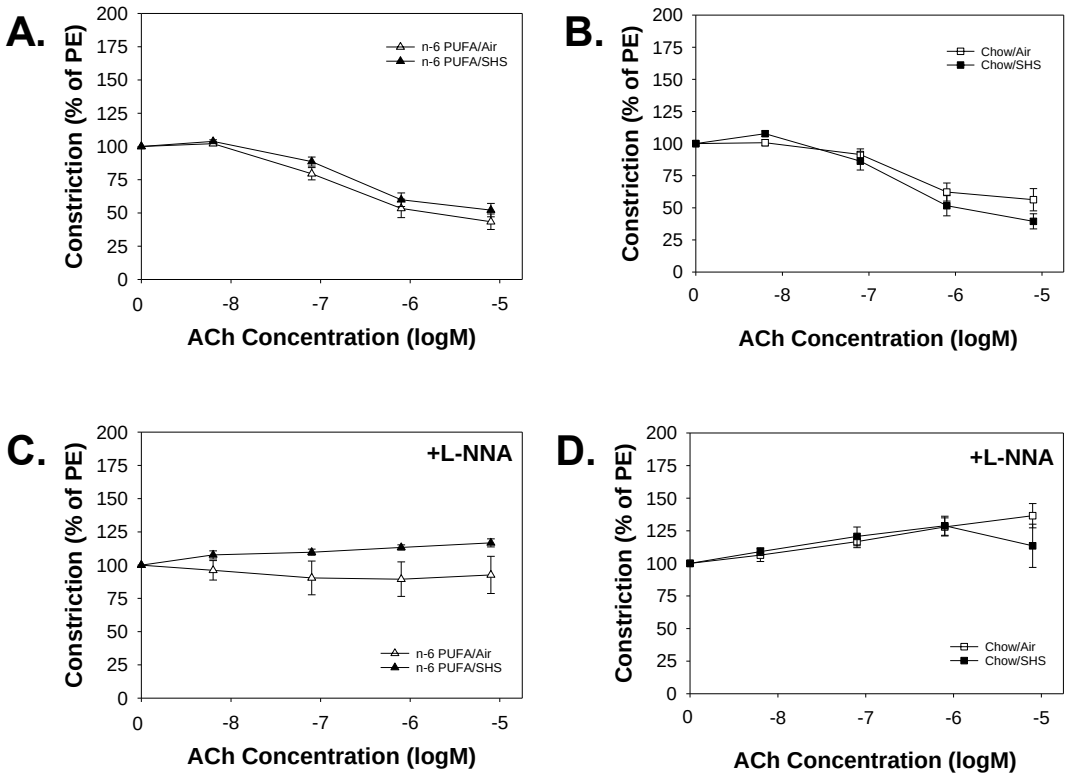
Constriction to PE and U46619 was enhanced in SHS-exposed mice fed the n-6 PUFA diet, compared to air-exposed mice fed the same diet. This SHS-induced enhancement of constriction was not evident in mice fed the standard chow diet. No differences in aortic vasoreactivity to ACh and SNAP were seen between air- and SHS-exposed mice fed either diet. TAC was increased in SHS-exposed mice fed the n-6 PUFA diet compared to air-exposed mice fed the same diet. Additionally, TAC tended to be higher in chow-fed mice than in n-6 PUFA-fed mice. Adipose mRNA expression of the Nrf2-regulated antioxidant enzyme, *Hmox1*, was significantly higher in SHS-exposed mice fed an n-6 PUFA diet, compared to air-exposed mice fed the same diet and SHS-exposed mice fed the standard chow diet. Lastly, qualitatively, collagen staining of cardiac sections did not differ between air- and SHS-exposed mice.

Taken together, these data suggest that SHS exposure of mice enhances vascular constriction and induces indices of an antioxidant response, compared to air-exposed controls, at an omega-3 index in the intermediate risk range (4-6%; *fat-1* mice on n-6 PUFA diet omega-3 index of 5.9-6.1%). In contrast, when the omega-3 index is high (9.3 ± 0.3%) and in the range considered protective of CVD in humans (≥ 8%), SHS exposure of mice significantly reduces omega-3 index by nearly 2% (7.8 ± 0.6). Nonetheless, these levels remain sufficiently high to prevent the enhancement in vasoconstriction and induction of antioxidant responses observed when the omega-3 index levels are in the intermediate risk range (4-8%).

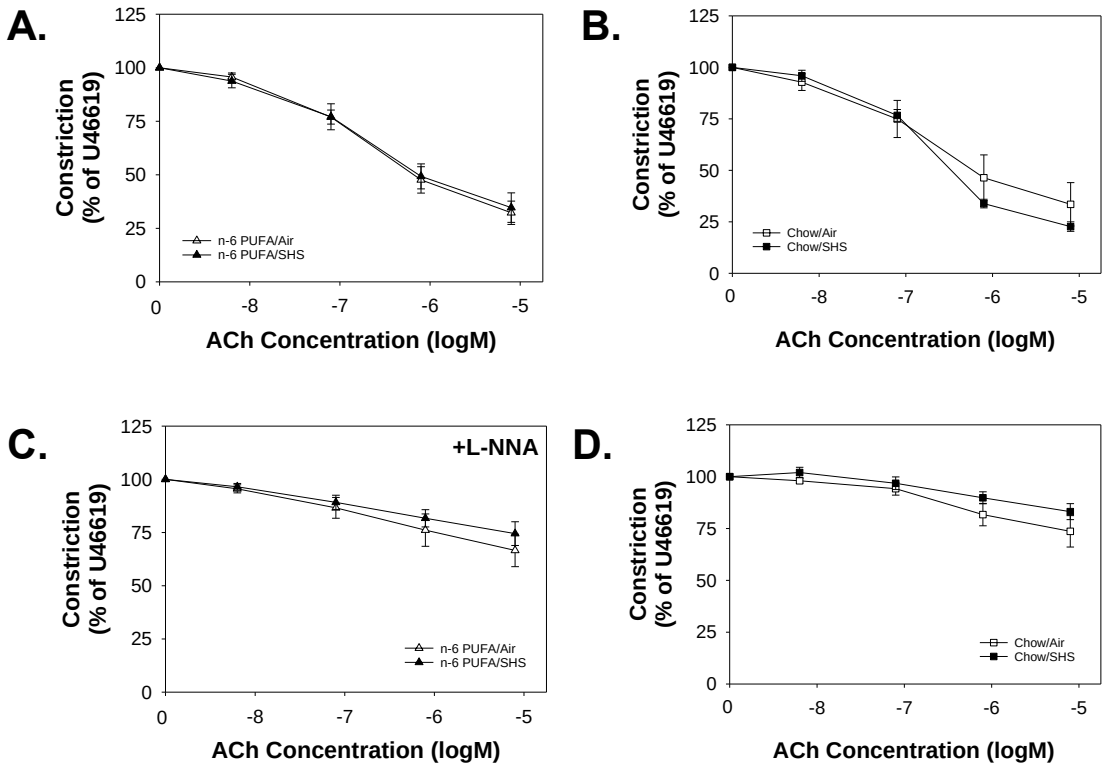
4.11. Supplementary Information



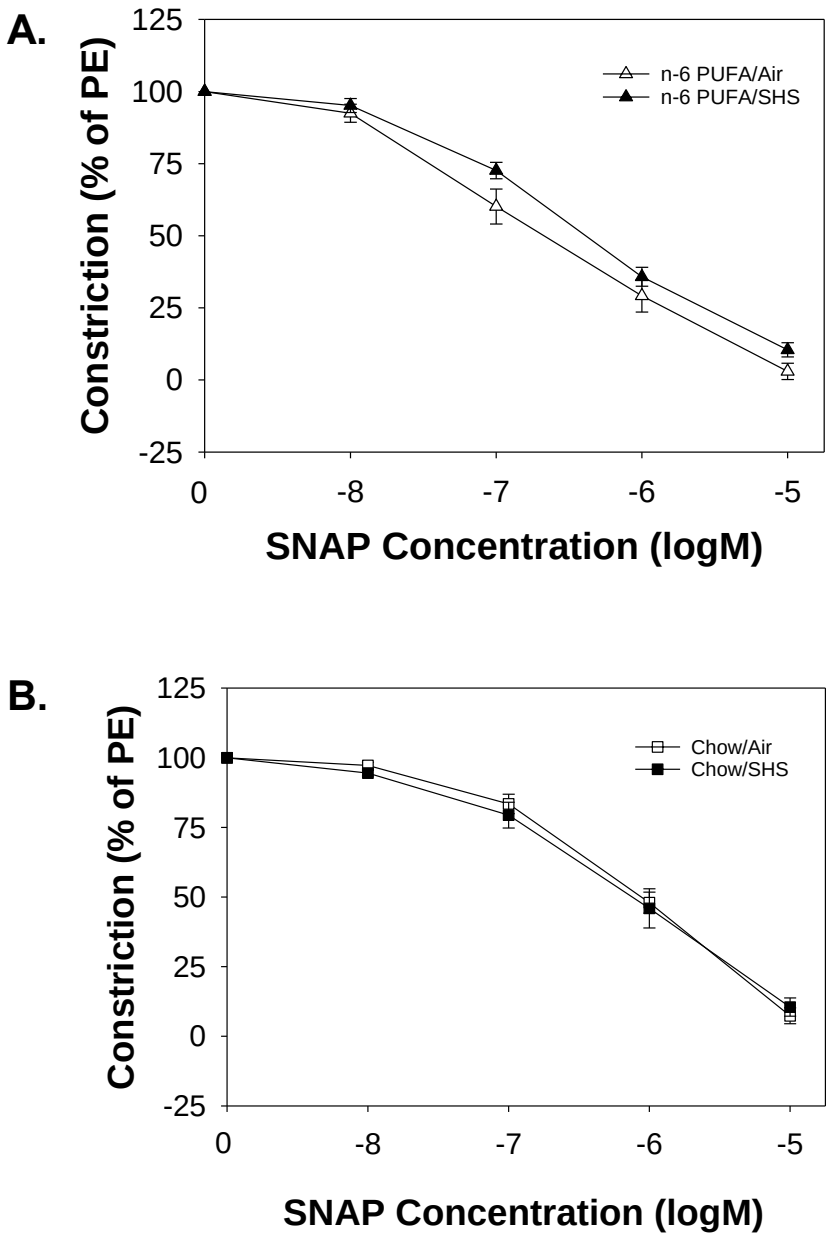
Supplementary Fig. 4.10. *Cyp1a1* and *Cyp1b1* mRNA expression in lung (A,B) and heart (C,D) tissues from air- and SHS-exposed mice fed an n-6 PUFA diet or standard chow diet. Data were normalized to the housekeeping gene, *Hprt*. Data are presented as mean ± SE (n=5-6) and were analyzed by Two-Way ANOVA with Holm-Sidak *post hoc* comparisons. **p*<0.05 versus air-exposed mice fed the same diet, #*p*<0.05 versus mice of the same exposure group fed an n-6 PUFA diet.



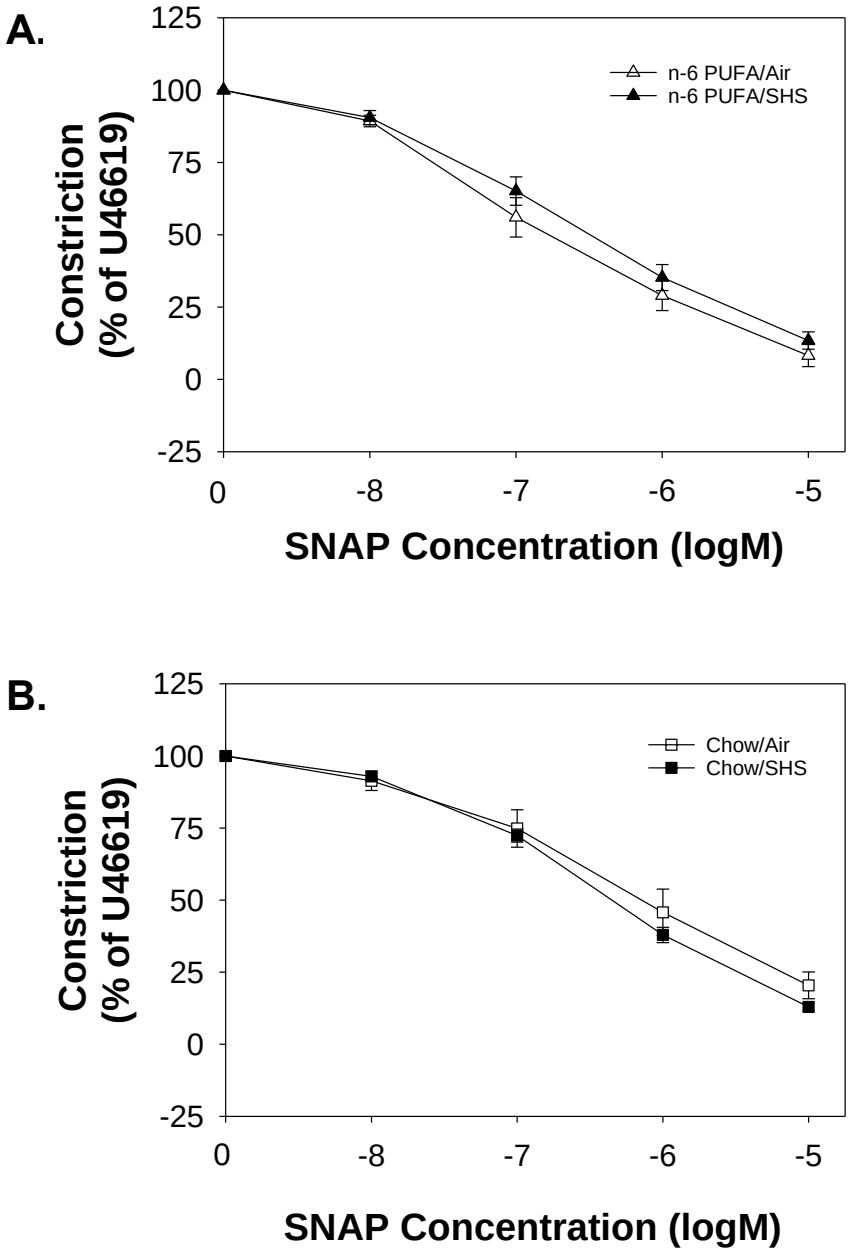
Supplementary Fig. 4.11. ACh relaxation of aortic rings pre-constricted with PE from air- and SHS-exposed mice fed an n-6 PUFA diet or standard chow diet in the absence (A,B) and presence (C,D) of L-NNA. Data are presented as mean ± SE (n=6-9) and were analyzed by Two-Way RM ANOVA with Holm-Sidak *post hoc* comparisons (A,B). **p*<0.05 versus air-exposed mice fed the same diet.



Supplementary Fig. 4.12. ACh relaxation of aortic rings pre-constricted with U46619 from air- and SHS-exposed mice fed an n-6 PUFA diet or standard chow diet in the absence (A,B) and presence (C,D) of L-NNA. Data are presented as mean ± SE (n=6-9) and were analyzed by Two-Way RM ANOVA with Holm-Sidak *post hoc* comparisons (A,B). *p<0.05 versus air-exposed mice fed the same diet.



Supplementary Fig. 4.13. SNAP relaxation of aortic rings pre-constricted with PE from air- and SHS-exposed mice fed an n-6 PUFA diet (A) or standard chow diet (B). Data are presented as mean ± SE (n=6-9) and were analyzed by Two-Way RM ANOVA with Holm-Sidak *post hoc* comparisons (A,B). *p<0.05 versus air-exposed mice fed the same diet.



Supplementary Fig. 4.14. SNAP relaxation of aortic rings pre-constricted with U46619 from air- and SHS-exposed mice fed an n-6 PUFA diet (A) or standard chow diet (B). Data are presented as mean ± SE (n=6-9) and were analyzed by Two-Way RM ANOVA with Holm-Sidak *post hoc* comparisons (A,B). *p<0.05 versus air-exposed mice fed the same diet.

CHAPTER 5 - DISCUSSION

5.1. The Role of Omega-3 Polyunsaturated Fatty Acids in Blood Pressure Regulation

Evidence suggests that n-3 PUFAs are efficacious in reducing blood pressure in both hypertensive and normotensive individuals (Campbell et al., 2012; Yang et al., 2016; Filipovic et al., 2018; Yang et al., 2019). However, there have been few mechanistic studies examining the hypotensive effects of n-3 PUFAs. n-3 PUFA metabolites, activation of voltage-gated potassium channels, and increases in total antioxidant status and NO bioavailabilty have been implicated as possible mechanisms (Jayasooriya et al., 2007; Hoshi et al., 2013; Zhou et al., 2017). In this study, we found that, when fed an n-6 PUFA diet, *fat-1* mice have lower MAP, compared to WT, and that this difference in blood pressure is driven by reductions in DBP.

Previous studies suggest that increased NO bioavailability is an important mechanism through which n-3 PUFAs are cardioprotective (Takahasi et al., 2013; Stebbins et al., 2008; Omura et al., 2001; Singh et al., 2010; Wu et al., 2012; López et al., 2001; López et al., 2004). However, in contrast to previous studies, we saw no differences in aortic vasoconstriction to PE and only a very small increase in ACh relaxationat the highest n-3 PUFA levels, suggesting that neither basal NO, nor stimulated NO, is meaningfully increased in *fat-1* mice, compared to WT mice.

Aside from NO, another major contributor to the regulation of basal blood pressure is Ang II. To investigate the contribution of Ang II to basal blood

pressure regulation in WT and *fat-1* mice, we treated WT and *fat-1* mice with captopril, an ACE inhibitor, for a period of 3 days and monitored blood pressure via radiotelemeter. The degree to which blood pressure is reduced under captopril treatment (Δ) is attributable to the contribution of Ang II to basal blood pressure regulation. In addition, evidence suggests that treatment with captopril unmasks the contibution of NO to blood pressure regulation (Bernátová et al., 1996; Bernátová et al., 1999; Török et al., 2002; Sanchez-Mendoza et al., 1998; Minami et al., 1995; Pechanova et al., 2006; Sládková et al., 2007). Therefore, we expected ΔMAP to be equal in WT and *fat-1* mice due to equal contributions of NO to blood pressure regulation as was established in our aortic vasoreactivity studies. However, when mice were fed the n-6 PUFA diet, there was a trend towards a significantly larger ΔMAP in *fat-1* mice compared to WT. Furthermore, ΔDBP was significantly higher in *fat-1* mice compared to WT. These data did not support our hypothesis and suggested that, when fed the n-6 PUFA diet, *fat-1* mice actually have a greater contribution of Ang II to blood pressure regulation than do WT mice despite the fact that the MAP of *fat-1* mice is significantly lower than WT at baseline. This conclusion is was not supported by *Agt* mRNA expression in adipose, a site of significant AGT production (Pahlavani et al., 2017), which trended towards a significant decrease (p=0.06) in *fat-1* mice, compared to WT mice fed an n-6 PUFA diet. However, *Agt* mRNA expression is a limited assessment of the contribution of Ang II to blood pressure regulation. Therefore, additional alalyses investigating the involvement of the RAS pathway must be performed.

The lower MAP seen in *fat-1* mice, compared to WT under basal conditions, is predominantly driven by reductions in DBP as opposed to SBP as was evidenced by lower DBP at baseline and higher ΔDBP under captopril treatment in *fat-1* mice, relative to WT mice when fed an n-6 PUFA diet. Additionally, we saw no changes in heart rate or pulse pressure, which are both reflective of SBP, once again suggesting that the lower MAP in *fat-1* mice on an n-6 PUFA diet is driven by DBP as opposed to SBP. DBP is largely a product of peripheral vascular resistance (PVR), so a reduction in DBP in *fat-1* mice, but not in WT mice, is suggestive that *fat-1* mice have a lower PVR than do WT mice when fed an n-6 PUFA diet. A larger ΔDBP under ACE inhibition by captopril in *fat-1* mice compared to WT suggests that removing Ang II reflects a bigger drop in PVR in *fat-1* mice than WT when fed the n-6 PUFA diet. Therefore, Ang II appears to contribute more to basal DBP and PVR in *fat-1* mice than in WT mice when fed the n-6 PUFA diet.

The fact that MAP at baseline is significanly lower in *fat-1* mice than in WT mice fed an n-6 PUFA diet, despite a greater contribution of Ang II, implies a contribution of another vasodilator mechanism other than NO. The lower blood pressure seen in *fat-1* mice at baseline could potentially be mediated by increases in bradykinin, a vasodilator peptide. Bradykinin has previously been established as playing a key role in the cardioprotective effects of ACE inhibition such as with captopril. However, previous studies have also suggested that kinins, including bradykinin, are not essential for the maintenance of basal blood pressure (Rhaleb et al, 1998). Furthermore, evidence suggests that ACE

inhibition by captopril does not increase circulating plasma bradykinin levels (Matthews et al., 1979, MacGregor et al, 1981). As a consequence, bradykinin is likely not responsible in total for the lower MAP and DBP seen in *fat-1* mice, compared to WT fed an n-6 PUFA diet under basal conditions.

Epoxide metabolites of EPA and DHA (EEQs and EDPs, respectively) may serve as another mechanism through which MAP and DBP are reduced in *fat-1* mice, compared to their WT littermates. EEQs and EDPs are potent vasodilators generated from metabolism of EPA and DHA by CYP2C and 2J (Zhang et al., 2001; Ye et al., 2001; Morin et al. 2008). Epoxide metabolites of n-3 PUFAs have been shown to be efficacious in lowering blood pressure. It is possible that, due to their ability to endogenously convert dietary n-6 PUFAs to n-3 PUFAs, *fat-1* mice may maintain higher plasma levels of the vasodilatory n-3 PUFA epoxide metabolites than do WT mice. To assess this possibility, an analysis of plasma and/or tissue fatty acid metabolites would need to be performed.

Using the *fat-1* mouse model as well as its WT littermates, we fed mice an n-6 PUFA diet and a standard chow diet to produce ranges of omega-3 index that are similar to that of individuals living in New Mexico (3.88 ± 0.15%) and in coastal regions such as in Alaskan Eskimos, who have a range of omega-3 index from 7.01 to 11.63% (Zehr et al., 2019; O'Brien et al., 2009; Parkinson et al., 1994; Ebbesson et al., 2010; Makhoul et al., 2011). However, the use of the omega-3 index as a biomarker of n-3 PUFA consumption may be artificial in that, by definition, the omega-3 index only accounts for red blood cell content of EPA

and DHA, the long-chain, marine-derived n-3 PUFAs. However, we found that the red blood cell content of ALA, a shorter-chain, plant-derived n-3 PUFA, is significantly increased in *fat-1* mice, compared to WT, when mice are fed an n-6 PUFA diet. Therefore, it is possible that the reductions seen in MAP and DBP in *fat-1* mice compared to WT when fed an n-6 PUFA diet could be attributable, at least in part, to ALA.

Many studies have found a similar hypotensive effect of ALA as has been seen with EPA and DHA. Most notably, a meta-analysis of 11 studies investigating the efficacy of flaxseed oil to lower blood pressure found reductions of -1.77 mmHg SBP and -1.58 mmHg DBP (Khalesi et al., 2015). Additionally, hypertensive individuals taking flaxseed oil supplements (630 mg four times per day for a total of 90 days) saw significant reductions in MAP, SBP, and DBP (Yang et al., 2019).

Moreover, in our *fat-1* mice, n-6 PUFAs are endogenously converted to n-3 PUFAs, so while n-3 PUFA levels increase, n-6 PUFAs also decrease. At this time, we cannot rule out the possibility that the reductions in blood pressure seen in *fat-1* mice fed an n-6 PUFA diet are actually attributable to decreased n-6 PUFA content. Thus, the n-6 PUFA to n-3 PUFA ratio may serve as a better index of n-3 PUFA consumption. Furthermore, the distribution of n-3 PUFAs may vary from tissue to tissue in WT and *fat-1* mice. For example, DHA accumulates more heavily in heart tissue than in red blood cells in WT mice fed an n-3 PUFA-enriched diet (Agbor et al., 2014). As a consequence, red blood cells may not be the optimal tissue for the determination of fatty acid profiles.

The lack of differences in MAP, SBP, and DBP between WT and *fat-1* mice fed a standard chow diet both in the absence and presence of captopril suggests that an omega-3 index of 4.3 ± 0.2% (measured in WT mice) serves as a threshold in which the blood pressure-reducing potential of n-3 PUFAs is maximally effective in mice.

An omega-3 index of less than 4%, as was seen in WT mice fed the n-6 PUFA diet (1.1 ± 0.1%), is considered to be of high cardiovascular risk (Harris and von Schacky, 2004), while an omega-3 index of greater than 8% is considered cardioprotective. Taken together, these data demonstrate that, as omega-3 index increases towards a more cardioprotective level, blood pressure tends to decrease. This decrease in blood pressure is driven by DBP, thereby suggesting an effect of n-3 PUFAs on PVR.

Our results highlight a large number of future studies that could further elucidate the underlying mechanism of n-3 PUFAs beneficial BP lowering effects. Future studies should evaluate the contribution of NO to basal BP directly by using NOS inhibitors and the potential contribution of n-3 PUFA epoxide metabolites to basal BP by using sEH inhibitors. Additionally, the apparent increased activation of the renin-angiotensin system should be confirmed by assessing plasma AGT levels and plasma renin activity, the rate limiting substrate and enzyme in Ang II formation, respectively.

5.2. The Role of Omega-3 Polyunsaturated Fatty Acids in SHS-Induced Vascular Dysfunction

In this study, we established an animal model of SHS exposure using the *fat-1* transgenic mouse fed either an n-6 PUFA-enriched diet or a standard chow diet, resulting in an omega-3 index ranging from 5.9 to 9.6%. We found that, when fed the standard chow diet (higher n-3 PUFA content and lower n-6 PUFA content), *fat-1* mice (omega-3 index 7.8 ± 0.6%) are protected from SHS-induced enhancement of aortic constriction as is seen when mice are fed the n-6 PUFA diet (omega-3 index 6.1 ± 0.1%). Importantly, our data show that n-3 PUFAs protect against SHS-induced vascular dysfunction in a manner that is independent of NO.

Dose-dependent responses of aortic rings to PE revealed a significantly larger degree of constriction of rings from SHS-exposed mice, compared to rings from air-exposed controls when mice were fed the n-6 PUFA diet. Notably, mice fed a standard chow diet were protected from SHS-induced increases in PE consticton. Regardless of diet, however, when the rings were treated with L-NNA, a NOS inhibitor, prior to PE, the enhancement of constriction by PE remained evident in SHS-exposed mice, suggesting that it did not result from a loss of NO. Dose-dependent constriction of aortic rings with U46619 yielded similar results. Taken together, this suggested that there was no difference in basal NO levels in SHS-exposed mice, compared to air-exposed mice, regardless of diet. Therefore, the differences in PE and U46619 constriction in mice fed an n-6 PUFA diet likely occurred independent of SHS-induced changes

in NO bioavailability, which is in contrast to previous reports that SHS exposure depletes NO (Förstermann and Münzel, 2006; Karbach et al., 2014, Zanetti et al., 2015).

Moreover, dose-dependent relaxation of aortic rings to ACh was not reduced by SHS exposure regardless of diet. In the presence of L-NNA, relaxation was completely inhibited across all groups with no significant differences between SHS- and air-exposed mice. This implies that SHS-exposure does not affect the ability of ACh to stimulate NO production. When aortic rings were treated with SNAP, an NO donor, relaxation was not impaired by SHS exposure regardless of diet, thereby indicating that there was also no effect of SHS in NO signaling downstream of NO generation. Taken together, the normal NO-dependent relaxation responses and enhanced PE and U46619 constriction, even in the absence of NO, suggest that the vascular effects of SHS occur independent of changes in NO.

One of the potential mechanisms through which SHS causes vascular dysfunction is through increases in oxidative stress. Several previous studies have reported associations between SHS exposure and indices of oxidative stress. For instance, a study of youth and adolescents found an inverse correlation between the number of smokers in the home and total antioxidant capacity (TAC), suggesting that SHS may lower TAC, perhaps through increases in oxidative stress (Groner et al., 2016). Similarly, another study reported that SHS-exposed infants and their mothers have a significantly lower TAC than nonsmoking infants and mothers (Aycicek et al., 2005). In addition, previous

studies have demonstrated robust increases in the mRNA and protein expression of *Hmox1*, an Nrf2-regulated antioxidant genes (Chang et al., 2017; Wiest et al., 2017). Therefore, we evaluated TAC and measured gene expression of Nrf2-regulated antioxidant genes, *Hmox1* and *Nqo1*, in air- and SHS-exposed mice fed the n-6 PUFA diet or the standard chow diet. In contrast to previous studies, TAC was increased in SHS-exposed mice fed the n-6 PUFA diet, compared to air-exposed mice fed the same diet. However, this suggests that SHS is inducing an antioxidant response, but that the exposure is likely not severe and/or chronic enough to completely deplete the antioxidant status. In addition, there was evidence of an induction of *Hmox1* mRNA expression in adipose, indicating an Nrf2-dependent antioxidant response to SHS, which has been seen in previous studies. Both of these factors suggest the formation of systemic oxidative stress with SHS exposure when mice were fed the n-6 PUFA diet.

A limited number of animal studies have suggested that SHS exposure may lead to fibrosis of the heart. For instance, in a Wistar rat model of chronic SHS exposure (6 months), fibrosis of the heart was evident by picro-sirius red staining for collagen, compared to unexposed controls (Boor et al., 2010). Therefore, we assessed the degree of cardiac fibrosis in air-and SHS-exposed mice fed the n-6 PUFA diet or standard chow diet. Qualitatively, collagen staining of cardiac sections demonstrated no evidence of fibrosis as a result of SHS exposure within the left ventricle of the heart. This finding was in contrast to previous studies suggesting that SHS exposure causes cardiac fibrosis. However, for such changes to be evident, a more chronic SHS exposure,

consisting of higher TPM concentrations and a longer duration of exposure, may be necessary. In addition, a more systematic and blinded quantitative approach must be delevoped in order to assess for fibrosis of the heart.

In this study, we found that, when fed a standard chow diet, the omega-3 index of SHS-exposed mice (7.8 ± 0.6%) is significantly lower than that of air-exposed mice (9.3 ± 0.3%). This finding is in accordance with studies on mainstream smoke exposure, which have found that smokers have a lower omega-3 index than nonsmokers (Wiest et al., 2015; Zehr et al., 2019).

In conclusion, we show that mice fed a standard chow diet are protected from SHS-induced increases in vascular constriction and that this protection appears to be independent of changes in NO. Further, our data suggest that there may be a role for systemic oxidative stress in the perpetuation of SHS-induced vascular dysfunction.

Future studies should further evaluate the degree of SHS-induced oxidative stress systemically and in vascular tissues and whether it contributes to the enhancement of vasoconstriction using antioxidant therapies such as N-acetyl cysteine. Additionally, studies could be conducted to determine whether the antioxidant properties of keto-metabolites of n-3 PUFAs contribute to the mechanism underlying the vasoprotective effect of n-3 PUFAs following SHS exposure.

CHAPTER 6 – GLOBAL IMPACT

Hypertension is one of the most common health conditions worldwide and often precedes clinical CVD (Kearney et al., 2005; Kung et al., 2015). Further, clinical trials show that reducing blood pressure reduces the risk of myocardial infarction by 20% - 25%, of stroke by 35%-40%, and of heart failure by 50% (Antonakoudis et al., 2007). As noted in the Introduction to this thesis, numerous interventional and observational studies show that n-3 PUFAs reduce blood pressure in both hypertensive and normotensive individuals. Our animal data are consistent with these human studies and demonstrate that blood pressure in normotensive mice is significantly lower when the omega-3 index is above 6%, compared to an omega-3 index of 1%.

These data are particularly relevant to individuals living in New Mexico. Our earlier research demonstrated that the omega-3 index in adults in New Mexico ranges from 1.8-7.4% and is inversely associated with both SBP and DBP (Zehr et al., 2019). Thus, our experimental animal data (1) establish a cause-and-effect relationship between the omega-3 index and BP lowering and (2) demonstrate this relationship over the same range of omega-3 indices measured in New Mexicans. This provides evidence supporting the concept that increased dietary n-3 PUFAs can lower BP and thus have the potential reduce CVD risk. Additionally, this evidence establishes the use of the *fat-1* mouse model to further investigate the mechanisms through which n-3 PUFAs mediate their beneficial BP lowering effects.

While hypertension is one of the most common health conditions, CVD is the leading cause of death in the United States and it is well established that SHS exposure increases CVD risk by up to 30% (US Department of Health and Human Services, 2006). Although recent meta-analyses of randomized clinical trials suggest that n-3 PUFAs may be of limited benefit in secondary prevention of CVD, observational studies provide a large body of evidence suggesting that dietary n-3 PUFAs have protective effects on primary prevention of CVD (Del Gobbo et al., 2016; Harris et al., 2017; Harris et al., 2018; Alexander et al., 2017; Pan et al., 2012; Wei et al., 2018; von Schacky, 2011; de Oliveira Otto et al., 2013) . Therefore, dietary consumption of n-3 PUFAs may represent a novel means through which the development or severity of CVD may be reduced or prevented following SHS exposure. Our data showing that higher n-3 PUFAs protect against SHS-induced vascular dysfunction provide evidence of how dietary n-3 PUFAs may reduce CVD risk following SHS exposure.

Individuals exposed to SHS are unique from mainstream smokers in that they smoke involuntarily. In addition, individuals exposed to SHS often make up a handful of especially at-risk groups, including pregnant women and children. Smoking cessation and smoke-free legislation are still some of the most powerful means of preventing SHS exposure, but SHS exposure also occurs at home and in other private places. Therefore, our research findings could be important in preventing SHS-induced vascular dysfunction in those who are unable to escape the reality of living with a smoker or working in a smoky environment, such as a casino.

Taken together, our studies provide evidence of a beneficial effect of n-3 PUFAs in reducing blood pressure and preventing SHS-induced vascular dysfunction, both of which could contribute to lowering CVD risk.

REFERENCES

Agaku, I. T., Odani, S., King, B. A., & Armour, B. S. (2019). Prevalence and correlates of secondhand smoke exposure in the home and in a vehicle among youth in the United States. Preventive Medicine, 126, 105745.

Agbor, L. N., Wiest, E. F., Rothe, M., Schunck, W.-H., & Walker, M. K. (2014). Role of CYP1A1 in Modulating the Vascular and Blood Pressure Benefits of Omega-3 Polyunsaturated Fatty Acids. *Journal of Pharmacology and Experimental Therapeutics*, *351*(3), 688.

Alexander, D. D., Miller, P. E., Van Elswyk, M. E., Kuratko, C. N., & Bylsma, L. C. (2017, January). A meta-analysis of randomized controlled trials and prospective cohort studies of eicosapentaenoic and docosahexaenoic long-chain omega-3 fatty acids and coronary heart disease risk. In *Mayo Clinic Proceedings* (Vol. 92, No. 1, pp. 15-29).

Antonakoudis, G., Poulimenos, I., Kifnidis, K., Zouras, C., & Antonakoudis, H. (2007). Blood pressure control and cardiovascular risk reduction. *Hippokratia*, *11*(3), 114.

Aycicek, A., Erel, O., & Kocyigit, A. (2005). Decreased total antioxidant capacity and increased oxidative stress in passive smoker infants and their mothers. *Pediatrics International*, *47*(6), 635-639.

Bernátová, I., Pecháňová, O., & Šimko, F. (1996). Captopril prevents NO-deficient hypertension and left ventricular hypertrophy without affecting nitric oxide synthase activity in rats. *Physiological research*, *45*, 311-316.

Bernátová, I., Pecháňová, O., & Šimko, F. (1999). Effect of Captopril in L-Name-Induced Hypertension on the Rat Myocardium, Aorta, Brain and Kidney. *Experimental Physiology*, *84*(6), 1095–1105.

Betteridge, D. J. (2000). What is oxidative stress? *Metabolism*, *49*(2, Supplement 1), 3–8.

Bo, L., Jiang, S., Xie, Y., Kan, H., Song, W., & Zhao, J. (2016). Effect of Vitamin E and Omega-3 Fatty Acids on Protecting Ambient PM2.5-Induced Inflammatory Response and Oxidative Stress in Vascular Endothelial Cells. PLOS ONE, 11(3), e0152216-.

Burdge, G. C., & Calder, P. C. (2006). Dietary α-linolenic acid and health-related outcomes: a metabolic perspective. *Nutrition Research Reviews*, *19*(1), 26–52.

Calder, P. C., & Yaqoob, P. (2009). Understanding Omega-3 Polyunsaturated Fatty Acids. *Postgraduate Medicine*, *121*(6), 148–157.

Campbell, F., Dickinson, H. O., Critchley, J. A., Ford, G. A., & Bradburn, M. (2012). A systematic review of fish-oil supplements for the prevention and treatment of hypertension. *European Journal of Preventive Cardiology*, *20*(1), 107–120.

Celermajer, D. S., Adams, M. R., Clarkson, P., Robinson, J., McCredie, R., Donald, A., & Deanfield, J. E. (1996). Passive Smoking and Impaired Endothelium-Dependent Arterial Dilatation in Healthy Young Adults. *New England Journal of Medicine*, *334*(3), 150–155.

Centers for Disease Control and Prevention (US), & U.S. Department of Health and Human Services. (2006). *The Health Consequences of Involuntary Exposure to Tobacco Smoke: A Report of the Surgeon General*. *Publications and Reports of the Surgeon General*.

Cipollina, C. (2015). Endogenous Generation and Signaling Actions of Omega-3 Fatty Acid Electrophilic Derivatives. *BioMed Research International*, *2015*, 501792.

Cipollina, C., Di Vincenzo, S., Gerbino, S., Siena, L., Gjomarkaj, M., & Pace, E. (2014). Dual anti-oxidant and anti-inflammatory actions of the electrophilic cyclooxygenase-2-derived 17-oxo-DHA in lipopolysaccharide- and cigarette smoke-induced inflammation. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1840*(7), 2299–2309.

Cipollina, C., Di Vincenzo, S., Siena, L., Di Sano, C., Gjomarkaj, M., & Pace, E. (2016). 17-oxo-DHA displays additive anti-inflammatory effects with fluticasone propionate and inhibits the NLRP3 inflammasome. *Scientific Reports*, *6*, 37625.

Cipollina, C., Salvatore, S. R., Muldoon, M. F., Freeman, B. A., & Schopfer, F. J. (2014). Generation and Dietary Modulation of Anti-Inflammatory Electrophilic Omega-3 Fatty Acid Derivatives. *PLOS ONE*, *9*(4), e94836-.

Cirera, S. (2013). Highly efficient method for isolation of total RNA from adipose tissue. *BMC Research Notes*, *6*(1), 472.

Chang, W. H., Thai, P., Xu, J., Yang, D., Wu, R., & Chen, C. H. (2017). Cigarette smoke regulates the competitive interactions between NRF2 and BACH1 for heme oxygenase-1 induction. *International journal of molecular sciences*, *18*(11), 2386.

Del Gobbo LC, Imamura F, Aslibekyan S, Marklund M, Virtanen JK, Wennberg M, Yakoob MY, Chiuve SE, Dela Cruz L, Frazier-Wood AC, Fretts AM, Guallar E, Matsumoto C, Prem K, Tanaka T, Wu JH, Zhou X, Helmer C, Ingelsson E, Yuan JM, Barberger-Gateau P, Campos H, Chaves PH, Djousse L, Giles GG, Gomez-Aracena J, Hodge AM, Hu FB, Jansson JH, Johansson I, Khaw KT, Koh WP, Lemaitre RN, Lind L, Luben RN, Rimm EB, Riserus U, Samieri C, Franks PW, Siscovick DS, Stampfer M, Steffen LM, Steffen BT, Tsai MY, van Dam RM, Voutilainen S, Willett WC, Woodward M, Mozaffarian D, Cohorts for H, Aging Research in Genomic Epidemiology Fatty A, and Outcomes Research C (2016) omega-3 Polyunsaturated Fatty Acid Biomarkers and Coronary Heart Disease: Pooling Project of 19 Cohort Studies. JAMA Intern Med 176 : 1155-1166

de Oliveira Otto, Marcia C., et al. "Circulating and dietary omega-3 and omega-6 polyunsaturated fatty acids and incidence of CVD in the Multi-Ethnic Study of Atherosclerosis." *Journal of the American Heart Association* 2.6 (2013): e000506.

Ebbesson, S. O. E., Devereux, R. B., Cole, S., Ebbesson, L. O. E., Fabsitz, R. R., Haack, K., … Comuzzie, A. G. (2010). Heart rate is associated with red blood cell fatty acid concentration: The Genetics of Coronary Artery Disease in Alaska Natives (GOCADAN) study. *American Heart Journal*, *159*(6), 1020–1025.

Faber, T., Been, J. V, Reiss, I. K., Mackenbach, J. P., & Sheikh, A. (2016). Smoke-free legislation and child health. *NPJ Primary Care Respiratory Medicine*, *26*, 16067.

Filipovic, M. G., Aeschbacher, S., Reiner, M. F., Stivala, S., Gobbato, S., Bonetti, N., … Beer, J. H. (2018). Whole blood omega-3 fatty acid concentrations are inversely associated with blood pressure in young, healthy adults. *Journal of Hypertension*, *36*(7).

Förstermann, U., & Münzel, T. (2006, April). Endothelial nitric oxide synthase in vascular disease: From marvel to menace. *Circulation*.

Frei, E., Huster, M., Smital, P., Schlossmann, J., Hofmann, F., & Wegener, J. W. (2009). Calcium-dependent and calcium-independent inhibition of contraction by cGMP/cGKI in intestinal smooth muscle. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *297*(4).

Frenoux, J.-M. R., Prost, E. D., Belleville, J. L., & Prost, J. L. (2001). A Polyunsaturated Fatty Acid Diet Lowers Blood Pressure and Improves Antioxidant Status in Spontaneously Hypertensive Rats. *The Journal of Nutrition*, *131*(1), 39–45.

Groeger, A. L., Cipollina, C., Cole, M. P., Woodcock, S. R., Bonacci, G., Rudolph, T. K., … Schopfer, F. J. (2010). Cyclooxygenase-2 generates anti-inflammatory mediators from omega-3 fatty acids. *Nature Chemical Biology*, *6*(6), 433–441.

Groner, J. A., Huang, H., Eastman, N., Lewis, L., Joshi, M. S., Schanbacher, B. L., … Bauer, J. A. (2016). Oxidative Stress in Youth and Adolescents With Elevated Body Mass Index Exposed to Secondhand Smoke. *Nicotine & Tobacco Research*, *18*(7), 1622–1627. https://doi.org/10.1093/ntr/ntw025

Grossman, E. (2008). Does Increased Oxidative Stress Cause Hypertension? *Diabetes Care*, *31*(Supplement 2), S185.

Hamblin, M., Chang, L., Fan, Y., Zhang, J., & Chen, Y. E. (2009). PPARs and the cardiovascular system. *Antioxidants & Redox Signaling*, *11*(6), 1415–1452.

Hammond, S. K. (n.d.). The Efficacy Of Strategies To Reduce Environmental Tobacco Smoke Concentrations In Homes, Workplaces, Restaurants, And Correctional Facilities.

Harris, W. S., Del Gobbo, L., & Tintle, N. L. (2017). The Omega-3 Index and relative risk for coronary heart disease mortality: Estimation from 10 cohort studies. *Atherosclerosis*, *262*, 51-54.

Harris, W. S., & Polreis, J. (2016). Measurement of the Omega-3 Index in Dried Blood Spots. *Ann Clin Lab Res*, *4*, 4.

Harris, W. S., Tintle, N. L., Etherton, M. R., & Vasan, R. S. (2018). Erythrocyte long-chain omega-3 fatty acid levels are inversely associated with mortality and with incident cardiovascular disease: The Framingham Heart Study. *Journal of clinical lipidology*, *12*(3), 718-727.

Harris, W. S., & von Schacky, C. (2004). The Omega-3 Index: a new risk factor for death from coronary heart disease? *Preventive Medicine*, *39*(1), 212–220.

Hercule, H. C., Salanova, B., Essin, K., Honeck, H., Falck, J. R., Sausbier, M., … Gollasch, M. (2007). The vasodilator 17,18-epoxyeicosatetraenoic acid targets the pore-forming BK α channel subunit in rodents. *Experimental Physiology*, *92*(6), 1067–1076.

Herrera, M., & Garvin, J. L. (2007). Novel role of AQP-1 in NO-dependent vasorelaxation. American Journal of Physiology-Renal Physiology, 292(5), F1443–F1451.

Hoshi, T., Wissuwa, B., Tian, Y., Tajima, N., Xu, R., Bauer, M., … Hou, S. (2013). Omega-3 fatty acids lower blood pressure by directly activating large-conductance Ca2+-dependent K+ channels. *Proceedings of the National Academy of Sciences*, *110*(12), 4816–4821.

Hsu, H.-C., Chen, C.-Y., & Chen, M.-F. (2014). N-3 polyunsaturated fatty acids decrease levels of doxorubicin-induced reactive oxygen species in cardiomyocytes -- involvement of uncoupling protein UCP2. Journal of Biomedical Science, 21(1), 101.

Jarvis, M. J., Tunstall-Pedoe, H., Feyerabend, C., Vesey, C., & Saloojee, Y. (1987). Comparison of tests used to distinguish smokers from nonsmokers. *American Journal of Public Health*, *77*(11), 1435–1438.

Jayasooriya, A. P., Begg, D. P., Chen, N., Mathai, M. L., Sinclair, A. J., Wilkinson-Berka, J., … Weisinger, R. S. (2008). Omega-3 polyunsaturated fatty acid supplementation reduces hypertension in TGR(mRen-2)27 rats. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *78*(1), 67–72.

Jung, S., Lee, I. S., Kim, S. B., Moon, C. S., Jung, J. Y., Kang, Y. A., … Kim, E. Y. (2012). Urine Cotinine for Assessing Tobacco Smoke Exposure in Korean: Analysis of the Korea National Health and Nutrition Examination Survey (KNHANES). *Tuberculosis and Respiratory Diseases*, *73*(4), 210–218.

Kang, J. X., Wang, J., Wu, L., & Kang, Z. B. (2004). Fat-1 mice convert n-6 to n-3 fatty acids. *Nature*, *427*(6974), 504.

Karbach, S., Wenzel, P., Waisman, A., & Daiber, T. M. and A. (2014). eNOS Uncoupling in Cardiovascular Diseases - the Role of Oxidative Stress and Inflammation. *Current Pharmaceutical Design*.

Katikireddi, S. V., Der, G., Roberts, C., & Haw, S. (2016). Has Childhood Smoking Reduced Following Smoke-Free Public Places Legislation? A Segmented Regression Analysis of Cross-Sectional UK School-Based Surveys. *Nicotine & Tobacco Research*, *18*(7), 1670–1674.

Kato, T., Inoue, T., Morooka, T., Yoshimoto, N., & Node, K. (2006). Short-term passive smoking causes endothelial dysfunction via oxidative stress in nonsmokers. *Canadian Journal of Physiology and Pharmacology*, *84*(5), 523–529.

Khalesi, S., Irwin, C., & Schubert, M. (2015). Flaxseed Consumption May Reduce Blood Pressure: A Systematic Review and Meta-Analysis of Controlled Trials. *The Journal of Nutrition*, *145*(4), 758–765.

Kung, H.-C., & Xu, J. (2015). Hypertension-related Mortality in the United States, 2000-2013. *NCHS Data Brief*, (193), 1–8.

Ledoux, J., Werner, M. E., Brayden, J. E., & Nelson, M. T. (2006). Calcium-activated potassium channels and the regulation of vascular tone. *Physiology*, *21*(1), 69-78.

Lodovici, M., & Bigagli, E. (2011). Oxidative stress and air pollution exposure. *Journal of Toxicology*, *2011*, 487074.

López, D., Caballero, C., Sánchez, J., Puig-Parellada, P., & Mitjavila, M. T. (2001). Free radical production in aortic rings from rats fed a fish oil-rich diet. *American Journal of Physiology-Heart and Circulatory Physiology*, *280*(6), H2929–H2935.

López, D., Orta, X., Casós, K., Sáiz, M. P., Puig-Parellada, P., Farriol, M., & Mitjavila, M. T. (2004). Upregulation of endothelial nitric oxide synthase in rat aorta after ingestion of fish oil-rich diet. *American Journal of Physiology-Heart and Circulatory Physiology*, *287*(2).

MacGregor, G. A., Markandu, N. D., Roulston, J. E., Jones, J. C., & Morton, J. J. (1981). Maintenance of blood pressure by the renin–angiotensin system in normal man. *Nature*, *291*(5813), 329–331.

Makhoul, Z., Kristal, A. R., Gulati, R., Luick, B., Bersamin, A., O'Brien, D., … Boyer, B. (2011). Associations of obesity with triglycerides and C-reactive protein are attenuated in adults with high red blood cell eicosapentaenoic and docosahexaenoic acids. *European Journal of Clinical Nutrition*, *65*(7), 808–817.

Margham, J., McAdam, K., Forster, M., Liu, C., Wright, C., Mariner, D., & Proctor, C. (2016). Chemical Composition of Aerosol from an E-Cigarette: A Quantitative Comparison with Cigarette Smoke. *Chemical Research in Toxicology*, *29*(10), 1662–1678.

Matthews, P. G., McGrath, B. P., & Johnston, C. I. (1979). Hormonal Changes with Long-Term Converting-Enzyme Inhibition by Captopril in Essential Hypertension. *Clinical Science*, *57*(s5), 135s-138s.

Minami, N., Imai, Y., Hashimoto, J.-I., & Abe, K. (1995). Contribution of Vascular Nitric Oxide to Basal Blood Pressure in Conscious Spontaneously Hypertensive Rats and Normotensive Wistar Kyoto Rats. *Clinical Science*, *89*(2), 177–182.

Moorthy, B., Chu, C., & Carlin, D. J. (2015). Polycyclic aromatic hydrocarbons: from metabolism to lung cancer. *Toxicological Sciences : An Official Journal of the Society of Toxicology*, *145*(1), 5–15.

Morin, C., Sirois, M., Echave, V., Rizcallah, E., & Rousseau, E. (2009). Relaxing effects of 17(18)-EpETE on arterial and airway smooth muscles in human lung. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *296*(1), L130–L139.

Naghavi, M., Wang, H., Lozano, R., Davis, A., Liang, X., Zhou, M., … Temesgen, A. M. (2015). Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: A systematic analysis for the Global Burden of Disease Study 2013. *The Lancet*, *385*(9963), 117–171.

Novo, E., & Parola, M. (2008). Redox mechanisms in hepatic chronic wound healing and fibrogenesis. *Fibrogenesis & Tissue Repair*, *1*(1), 5.

Öberg, M., Jaakkola, M. S., Woodward, A., Peruga, A., & Prüss-Ustün, A. (2011). Worldwide burden of disease from exposure to second-hand smoke: a retrospective analysis of data from 192 countries. *The Lancet*, *377*(9760), 139–146.

O'Brien, D. M., Kristal, A. R., Jeannet, M. A., Wilkinson, M. J., Bersamin, A., & Luick, B. (2009). Red blood cell δ15N: a novel biomarker of dietary eicosapentaenoic acid and docosahexaenoic acid intake. *The American Journal of Clinical Nutrition*, *89*(3), 913–919.

Omura, M., Kobayashi, S., Mizukami, Y., Mogami, K., Todoroki-Ikeda, N., Miyake, T., & Matsuzaki, M. (2001). Eicosapentaenoic acid (EPA) induces Ca2+-independent activation and translocation of endothelial nitric oxide synthase and endothelium-dependent vasorelaxation. FEBS Letters, 487(3), 361–366.

Otsuka, R., Watanabe, H., Hirata, K., Tokai, K., Muro, T., Yoshiyama, M., … Yoshikawa, J. (2001). Acute Effects of Passive Smoking on the Coronary Circulation in Healthy Young Adults. *JAMA*, *286*(4), 436–441.

Pahlavani, M., Kalupahana, N. S., Ramalingam, L., & Moustaid-Moussa, N. (2011). Regulation and functions of the renin-angiotensin system in white and brown adipose tissue. *Comprehensive Physiology*, *7*(4), 1137-1150.

Pan, A., Chen, M., Chowdhury, R., Wu, J. H., Sun, Q., Campos, H., ... & Hu, F. B. (2012). α-Linolenic acid and risk of cardiovascular disease: a systematic review and meta-analysis. *The American journal of clinical nutrition*, *96*(6), 1262-1273.

Parkinson, A. J., Cruz, A. L., Heyward, W. L., Bulkow, L. R., Hall, D., Barstaed, L., & Connor, W. E. (1994). Elevated concentrations of plasma ω-3 polyunsaturated fatty acids among Alaskan Eskimos. *The American Journal of Clinical Nutrition*, *59*(2), 384–388.

Pechnova, O., Matuskova, J., Capikova, D., Jendekova, L., Paulis, L., & Simko, F. (2006). Effect of spironolactone and captopril on nitric oxide and S-nitrosothiol formation in kidney of L-NAME-treated rats. *Kidney International*, *70*(1), 170–176.

Pope, C. A., Eatough, D. J., Gold, D. R., Pang, Y., Nielsen, K. R., Nath, P., … Kanner, R. E. (2001). Acute exposure to environmental tobacco smoke and heart rate variability. *Environmental Health Perspectives*, *109*(7), 711–716.

Rhaleb, Nour-Eddine, Hongmei, P., E, A. M., G, S. E., & A, C. O. (1999). Effect of ACE Inhibitor on DOCA-Salt– and Aortic Coarctation–Induced Hypertension in Mice. *Hypertension*, *33*(1), 329–334.

Rimm, E. B., Appel, L. J., Chiuve, S. E., Djoussé, L., Engler, M. B., M, Kris-Etherton, P. M, Mozaffarian, D., Siscovick, D. S., & Lichtenstein, A. (2018). Seafood Long-Chain n-3 Polyunsaturated Fatty Acids and Cardiovascular Disease: A Science Advisory From the American Heart Association. *Circulation*, *138*(1), e35–e47.

Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*, *362*(6423), 801–809.

Sánchez-Mendoza, A., Hong, E., & Escalante, B. (1998). The role of nitric oxide in angiotensin II-induced renal vasoconstriction in renovascular hypertension. *Journal of Hypertension*, *16*(5).

Schick, S., & Glantz, S. (2005). Philip Morris toxicological experiments with fresh sidestream smoke: more toxic than mainstream smoke. *Tobacco Control*, *14*(6), 396.

Singh, T. U., Kathirvel, K., Choudhury, S., Garg, S. K., & Mishra, S. K. (2010). Eicosapentaenoic acid-induced endothelium-dependent and -independent relaxation of sheep pulmonary artery. *European Journal of Pharmacology*, *636*(1–3), 108–113.

Sládková, M., Kojsová, S., Jendeková, L., & Pechánová, O. (2007). Chronic and acute effects of different antihypertensive drugs on femoral artery relaxation of L-NAME hypertensive rats. *Physiological research*, *56*(2), S85.

Stebbins, C. L., Stice, J. P., Hart, C. M., Mbai, F. N., & Knowlton, A. A. (2008). Effects of dietary decosahexaenoic acid (DHA) on eNOS in human coronary artery endothelial cells. *Journal of Cardiovascular Pharmacology and Therapeutics*, *13*(4), 261–268.

Stone, E. C. A., & Zhou, C. (2016). Slowing the Titanic: China's Epic Struggle with Tobacco. *Journal of Thoracic Oncology*, *11*(12), 2053–2065.

Streibel, T., Mitschke, S., Adam, T., & Zimmermann, R. (2013). Time-resolved analysis of the emission of sidestream smoke (SSS) from cigarettes during smoking by photo ionisation/time-of-flight mass spectrometry (PI-TOFMS): towards a better description of environmental tobacco smoke. *Analytical and Bioanalytical Chemistry*, *405*(22), 7071–7082.

Sumida, H., Watanabe, H., Kugiyama, K., Ohgushi, M., Matsumura, T., & Yasue, H. (1998). Does Passive Smoking Impair Endothelium-Dependent Coronary Artery Dilation in Women? 11This study was supported in part by Grant-in-Aid C09670730 from the Ministry of Education, Science, and Culture; by research grants for cardiovascular disease (7A-3, 9A. *Journal of the American College of Cardiology*, *31*(4), 811–815.

Talhout, R., Schulz, T., Florek, E., van Benthem, J., Wester, P., & Opperhuizen, A. (2011). Hazardous compounds in tobacco smoke. *International Journal of Environmental Research and Public Health*, *8*(2), 613–628.

Török, J., Babál, P., Matušková, J., Lupták, I., Klimeš, I., & Šimko, F. (2002). Impaired Endothelial Function of Thoracic Aorta in Hereditary Hypertriglyceridemic Rats. *Annals of the New York Academy of Sciences*, *967*(1), 469–475.

Ulu, A., Harris, T. R., Morisseau, C., Miyabe, C., Inoue, H., Schuster, G., … Hammock, B. D. (2013). Anti-inflammatory Effects of ω-3 Polyunsaturated Fatty Acids and Soluble Epoxide Hydrolase Inhibitors in Angiotensin-II–Dependent Hypertension. *Journal of Cardiovascular Pharmacology*, *62*(3).

Ulu, A., Stephen Lee, K. S., Miyabe, C., Yang, J., Hammock, B. G., Dong, H., & Hammock, B. D. (2014). An Omega-3 Epoxide of Docosahexaenoic Acid Lowers Blood Pressure in Angiotensin-II–Dependent Hypertension. *Journal of Cardiovascular Pharmacology*, *64*(1).

von Schacky, C. (2014). Omega-3 index and cardiovascular health. *Nutrients*, *6*(2), 799–814.

von Schacky, C. (2011). The Omega-3 Index as a risk factor for cardiovascular diseases. *Prostaglandins & other lipid mediators*, *96*(1-4), 94-98.

Vu, A. T., Taylor, K. M., Holman, M. R., Ding, Y. S., Hearn, B., & Watson, C. H. (2015). Polycyclic Aromatic Hydrocarbons in the Mainstream Smoke of Popular U.S. Cigarettes. *Chemical Research in Toxicology*, *28*(8), 1616–1626.

Wei, J., Hou, R., Xi, Y., Kowalski, A., Wang, T., Yu, Z., ... & Ali, M. K. (2018). The association and dose–response relationship between dietary intake of α-linolenic acid and risk of CHD: a systematic review and meta-analysis of cohort studies. *British Journal of Nutrition*, *119*(1), 83-89.

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. *Toxicology letters*, *232*(2), 422-428.

Wiest, E. F., Walsh-Wilcox, M. T., & Walker, M. K. (2017). Omega-3 Polyunsaturated Fatty Acids Protect Against Cigarette Smoke-Induced Oxidative Stress and Vascular Dysfunction. *Toxicological Sciences*, *156*(1), 300–310.

Williams, C. M., & Burdge, G. (2006). Long-chain n−3 PUFA: plant v. marine sources. *Proceedings of the Nutrition Society*, *65*(1), 42–50.

World Health Organization (2015). *WHO Report on the Global Tobacco Epidemic, 2015: Raising taxes on tobacco*. *World Health Organization*. Luxembourg.

Wu, Y., Zhang, C., Dong, Y., Wang, S., Song, P., Viollet, B., & Zou, M.-H. (2012). Activation of the AMP-Activated Protein Kinase by Eicosapentaenoic Acid (EPA, 20:5 n-3) Improves Endothelial Function In Vivo. *PLOS ONE*, *7*(4), e35508-.

Yamato, H., Mori, N., Horie, R., Garcon, L., Taniguchi, M., & Armada, F. (2013). *Designated Smoking Areas in Streets Where Outdoor Smoking is Banned*. *Kobe J. Med. Sci* (Vol. 59).

Yang, B., Shi, M.-Q., Li, Z.-H., Yang, J.-J., & Li, D. (2016). Fish, Long-Chain n-3 PUFA and Incidence of Elevated Blood Pressure: A Meta-Analysis of Prospective Cohort Studies. *Nutrients*, *8*(1).

Yang, B., Shi, L., Wang, A., Shi, M., Li, Z., Zhao, F., Li, D. (2019). Lowering Effects of n-3 Fatty Acid Supplements on Blood Pressure by Reducing Plasma Angiotensin II in Inner Mongolia Hypertensive Patients: A Double-Blind Randomized Controlled Trial. *Journal of Agricultural and Food Chemistry*, *67*(1), 184–192.

Ye, D., Zhang, D., Oltman, C., Dellsperger, K., Lee, H.-C., & VanRollins, M. (2002). Cytochrome P-450 Epoxygenase Metabolites of Docosahexaenoate Potently Dilate Coronary Arterioles by Activating Large-Conductance Calcium-Activated Potassium Channels. *Journal of Pharmacology and Experimental Therapeutics*, *303*(2), 768.

Zanetti, M., Grillo, A., Losurdo, P., Panizon, E., Mearelli, F., Cattin, L., … Carretta, R. (2015). Omega-3 polyunsaturated fatty acids: Structural and functional effects on the vascular wall. *BioMed Research International*. Hindawi Publishing Corporation.

Zehr, K. R., Segovia, A., Shah, M., Walsh-Wilcox, M. T., Brumbach, B. H., Anderson, J. R., & Walker, M. K. (2019). Associations of medium and long chain omega-3 polyunsaturated fatty acids with blood pressure in Hispanic and non-Hispanic smokers and nonsmokers. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *144*, 10–15.

Zhang, Y., Oltman, C. L., Lu, T., Lee, H.-C., Dellsperger, K. C., & VanRollins, M. (2001). EET homologs potently dilate coronary microvessels and activate BKCa channels. American Journal of Physiology-Heart and Circulatory Physiology, 280(6), H2430–H2440.

Zhou, D.-Y., Liu, Y.-X., Xu, Z.-L., Yin, F.-W., Song, L., Wan, X.-L., … Zhu, B.-W. (2017). Effects of long-term intake of Antarctic krill oils on artery blood pressure in spontaneously hypertensive rats. *Journal of the Science of Food and Agriculture*, *97*(4), 1143–1148.

Zuo, L., He, F., Sergakis, G. G., Koozehchian, M. S., Stimpfl, J. N., Rong, Y., … Best, T. M. (2014). Interrelated role of cigarette smoking, oxidative stress, and immune response in COPD and corresponding treatments. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *307*(3), L205–L218.